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COLUMBIA UNIVERSITY

College of Physicians and Surgeons
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Dir., David J. Brenner, PhD, DSc; Assoc. Dir. Gerhard Randers-Pehrson, PhD; Mnger., Stephen A. Marino, MS

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Individuals from the following institutions collaborated with the Center’s faculty and staff in the research reports included in this year’s publication (for individual attributions see specific reports):

**Collaborating Columbia University Departments:**
- College of Dental Medicine, Division of Oral & Maxillofacial Pathology
- Department of Biomedical Engineering
- Department of Environmental Heath Sciences, Mailman School of Public Health
- Department of Epidemiology
- Department of Mechanical Engineering
- Department of Medicine
- Department of Neurology
- Department of Obstetrics and Gynecology
- Department of Radiation Oncology
- Department of Radiology

**Collaborating Institutions:**
- Center for Applied NanoBioscience, Arizona State University
- Computational Biology Division, Translational Genomics Research Institute, Phoenix, Arizona
- Department of Mathematics and Physics, University of California, Berkeley, CA
- Department of Biomedical Engineering, Tufts School of Medicine, Boston, MA
- Division of Physics and Biophysics, University of Salzburg, Salzburg, Austria
- Institute for Advanced Research, Tarapaca University, Arica, Chile
- Lombardi Comprehensive Cancer Center, Georgetown University, Washington, DC
- School of Radiology on Medicine and public health, Soochow University, Suzhou, China
- Sionex Corporation, Bedford, MA
- Space Technologies, Canadian Space Agency, Canada
- University of Bern, Switzerland
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  - National Institutes of Health, Bethesda, MD
    - Laboratory of Metabolism, Center for Cancer Research, National Cancer Institute

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- New York State Department of Health, Health Research Science Board

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- Department of Defense
  - Defense Threat Reduction Agency
- Department of Energy
  - Office of International Health Programs
  - Office of Science, Office of Biological and Environmental Research [Low Dose Radiation Research Program]
- Department of Health and Human Services
  - National Institutes of Health:
    - National Cancer Institute [Program Project (PO1) & Individual Research Grants (RO1s)]
    - National Center for Research Resources
    - National Institute of Biomedical Imaging and Bioengineering (P41)
    - National Institute of Allergy and Infectious Disease (U19)
    - National Institute of Environmental Health and Safety (RO1s)
    - National Institute of General Medical Sciences (RO1)
- National Aeronautics and Space Administration

Web Sites

- Center for Radiological Research ................................................................. http://crr-cu.org
- Radiological Research Accelerator Facility ..................................................... http://www.raraf.org
- Center for High-Throughput Minimally-Invasive Radiation Biodosimetry ....... http://www.cmcu.columbia.edu
- Mechanism of Bystander Effects ................................................................. http://www.radiation-bystander.columbia.edu
- Web-Rad-Train ......................................................................................... http://www.web-rad-train.org
- Department of Radiation Oncology ............................................................. http://cpmcnet.columbia.edu/dept/radoncology
- Radiation Safety Office ............................................................................... http://cpmcnet.columbia.edu/dept/radsafety
Introduction

This Introduction is designed to provide a brief overview of the recent life and times of our Center for Radiological Research, and especially to give a flavor of the principal research initiatives and academic activities.

A landmark event at our Center which occurred at the end of 2007 is that Dr Eric Hall has stepped down as Director of the Center, and Charles Geard stepped down as Associate Director. David Brenner became our Center Director and Higgins Professor of Radiation Biophysics, with Tom Hei moving into the role of Associate Director.

Dr. Hall would like to take this opportunity to thank the many faculty and staff, past and present, at the Center, some of whom he appointed many years ago as post docs, for their friendship, their loyalty, and their professional support. The “new guard” of David Brenner and Tom Hei will have the enthusiastic support of Drs. Hall and Geard, who will still be active in their own research programs at the Center.

As always, this report reflects the activities of a multi-disciplinary Center. Reports cover the fields of radiation physics, radiation biology, radiation oncology and molecular biology. Research activities of the Center for Radiological Research address diverse but inter-related goals:

- To probe the mechanisms of the biological effects of radiation at the molecular, cytogenetic and tissue levels.
- Radiation protection and the effects of low doses of ionizing radiation, of concern to the U.S. Department of Energy.
- Planning for the aftermath of a large-scale radiological event, either accidental or terrorist related.
- Research of relevance to Radiation Oncology, especially altered fractionation patterns and the impact of radiation-induced second cancers.
- The biological effects of high-energy heavy ions, unique to the space environment, that are of concern to NASA.
- Understanding the mechanism of other environmental carcinogens, at the molecular and tissue levels.

Dr. David Brenner directs both our Radiological Research Accelerator Facility (RARAF) and the Columbia Center for High-Throughput Minimally-Invasive Radiation Biodosimetry. These two programs, together with our NIH Program Project directed by Tom Hei, represent the three largest programs within our Center.


Our RARAF facility has had a very successful year, with the introduction of multi-photon imaging into the microbeam system, as well as a steady reduction in our microbeam spot size, which is now at 1 micrometer, with sub-micrometer beam spots expected in early 2008.

International collaboration and training remain an integral part of the Center activities, and are in line with the increasing emphasis on global activities of Columbia University as a whole. Dr. Tom Hei has coordinated many training opportunities for medical and doctoral students from both Japan and China in the past year.

Dr Charles Geard continues research into bystander responses, using our single-cell microbeam facility at RARAF, along with adaptive response and space related studies.

During the previous year, Dr. Howard Lieberman’s laboratory discovered that Rad9 is functionally linked to prostate cancer, and can act as an oncogene in that context. Studies in his lab are underway to exploit this finding, with the goals of possibly establishing Rad9 protein as a diagnostic marker, prognostic tool and/or novel therapeutic agent or target to combat this highly prevalent disease.

Dr. Sally Amundson is involved in the growing effort to integrate systems biology into radiobiology studies. She was an invited speaker at the first International Systems Radiation Biology Workshop, which was held in Munich in February, and was a member of the organizing committee for the second meeting, held in Washington, DC in 2008.

Dr. Yuxin Yin continues to study the molecular basis of carcinogenesis, and has provided strong evidence that PTEN has a prominent role as a guardian of the genome. Dr. Yin gave an oral presentation on this subject at the AACR Centennial Meeting in Los Angeles.

Dr. Lubomir Smilenov continued his work on individual genetic susceptibility. Additionally he started work on a new NASA funded project aiming to clarify the role of miRNA in radiation response.

Dr. Yongliang Zhao’s group continues to define the molecular mechanism of Big-h3 downregulation in human tumor progression using in vitro and in vivo model systems.

The productivity of the Center continues at a high level, as evidenced by a steady stream of scientific papers in peer-reviewed high-profile journals. Members of the staff are regularly invited to participate in national and international meetings, and to serve as consultants and reviewers.

The Center’s teaching activities include radiation biology and radiation physics for undergraduates, medical students...
During 2007, the Education Core of our Center for Minimally-Invasive Radiation Biodosimetry organized and taught two one-day courses entitled “Radiological Science in the Context of Radiological Terrorism”. They were taught in June at Columbia University Medical Center, and in December at Georgetown University Medical Center. On both occasions there was a registered audience of about 100, including first responders, health physicists and emergency room physicians.

A major event for the Center at the end of 2007 was the “changing of the guard”. Eric Hall and Charles Geard stepped down as, respectively, Director and Associate Director. Eric Hall was the Center Director for 23 years, following Gioachino Failla (1915 to 1960) and Harald Rossi (1960 to 1984). Our new Director and Associate Director are, respectively, Drs. David Brenner and Tom Hei, both of whom were hired by Eric Hall over 20 years ago. Both Eric Hall and Charles Geard remain at the Center, as PIs of their various grants.

Dr. Eric Hall received the prestigious Gray Medal of the International Commission on Radiation Units and Measurements (ICRU). The medal was awarded at the International Congress of Radiation Research at San Francisco in June 2007. In 1985 Harald Rossi, Eric Hall’s predecessor, received the Gray Medal, making Columbia the only Institution to have two individuals who received the Gray Medal!

- The hypofractionated prostate cancer radiotherapy protocols designed by Drs Brenner and Hall are now in Phase 3 trials, worldwide.
- Dr Brenner is now Chairperson of the Medical Center Joint Radiation Safety Committee and Radioactive Drug Research Committee, in addition to being Chair of the Columbia University Radiation Safety Committee. He received a brief 15 minutes of fame on all the major TV networks, on the subject of CT usage.
- Dr. Tom K. Hei continues to serve as an ad hoc member of the NIH cancer etiology study section and as chairman of several special emphasis panels. He has been appointed an Adjunct Professor in the School of Radiation Medicine and Public Health of Soochoo University. Dr. Hei’s work on the genotoxic mechanism of asbestos fibers was featured in the Superfund Basic Research Program report in October, 2007.
- Dr. Howard Lieberman served as an ad hoc reviewer for the NIH Radiation Therapeutics and Biology Study Section, and a member of several PO1 grant review panels. He received the Distinguished Alumnus Award from the Lay Advocate Program of Brooklyn College, for outstanding contribution in the field of science.
- Dr. Sally Amundson served as a member of the NIAID Project Bioshield study section. Dr. Amundson also served as a member of a National Academies of Science committee evaluating radiation shielding needs for a Moon base or a manned Mars mission.

Dr. Eric Hall received the Gray Medal from Dr Paul DeLuca, the President of the International Commission on Radiological Units.

- Dr. Yuxin Yin was invited to write a review in Oncogene on the role of PTEN as a new guardian of the genome. Dr. Yin also gave an oral presentation in the AACR Centennial Meeting in Los Angeles, April, 2007 on this topic.
- Ms. Bingyan Li, a doctoral student from Soochoo University, spent nine months in Dr. Hei’s laboratory studying the role of integrin and the BigH3 gene in neoplastic transformation of mesotheliomas as part of a graduate student exchange program.
- Dr. Geoff Johnson, senior resident in radiation oncology, spent six months in Dr. Hei’s lab studying the mechanism of radiation induced apoptosis in human melanoma cells.
- Dr. Sunirmal Paul, an Associate Research Scientist in Dr. Amundson’s laboratory, won a travel award to attend the International Congress of Radiation Research, and presented his work on “Gene expression profiles for radiation biodosimetry with a fully integrated biochip.”
- Dr. Alexandre Mezentsev, an Associate Research Scientist in Dr. Amundson’s laboratory, was selected to attend the three week intensive NASA Space Radiation Summer School at Brookhaven National Laboratory.
- Dr Kenichi Tanaka of Hiroshima University, is spending a sabbatical with us at RARAF.

We will miss a number of staff members who have left...
the Center in the past year, for new adventures in other places, and we wish them all the very best:

Ms. Gloria Jenkins-Baker, a mainstay of the RARAF biology group and a friend to all, retired and returned to her home state of South Carolina. She had been at Columbia for 34 years, of which 20 years were spent at the Medical Center and the last 14 years at RARAF.

Several Post-Doctoral Research Scientists, Drs. Aparajita Dutta, An Xu, Cindy Liu, Guangming Zhou, Yanrong Su, Yu-chen Lien and Ye Zhang, have left the Center for new positions elsewhere.

Ms. Heidy Hernandez, who had been on the staff as an Accountant in the Center has left for a new position in Columbia administration. Also leaving in 2007 were Staff Associate Jaeyong Ahn and Technician Dimitar Zlatev.

The Center is pleased to welcome new staff members:

Dr. Helen Turner has joined the Center as an Associate Research Scientist in Dr. David Brenner’s group. New Post-Doctoral Research Scientists are Dr. Chuanxin Huang in Dr. Yin’s lab, Dr. Alex Kofman in Dr. Geard’s lab, Dr. Mei Hong in Dr. Hei’s lab, and Drs. Yanping Xu and Andrew Harken at RARAF.

Jing Nie and Ling Han have joined the Center as Staff Associates, and Jinshuang Lu joined the Center as a Junior Programmer. Jennifer Maerki in Dr. Smilenov’s lab, Benjamin Yaghoubian in Dr. Amundson’s lab, and Bharat Patel and Abel Bencosme at RARAF, have joined our team as Technicians. Instrument makers Robert Morton, and Dennis Keaveney also joined us during this year.

The Center is delighted to welcome Ms. Yvette Acevedo as Administrative Assistant, and Margaret Zhu as Administrative Coordinator.

Dr. Helen Turner has joined the Center as an Associate Research Scientist in Dr. David Brenner’s group. New Post-Doctoral Research Scientists are Dr. Chuanxin Huang in Dr. Yin’s lab, Dr. Alex Kofman in Dr. Geard’s lab, Dr. Mei Hong in Dr. Hei’s lab, and Drs. Yanping Xu and Andrew Harken at RARAF.

Jing Nie and Ling Han have joined the Center as Staff Associates, and Jinshuang Lu joined the Center as a Junior Programmer. Jennifer Maerki in Dr. Smilenov’s lab, Benjamin Yaghoubian in Dr. Amundson’s lab, and Bharat Patel and Abel Bencosme at RARAF, have joined our team as Technicians. Instrument makers Robert Morton, and Dennis Keaveney also joined us during this year.

The Center is delighted to welcome Ms. Yvette Acevedo as Administrative Assistant, and Margaret Zhu as Administrative Coordinator.

Columbia Colloquium and Laboratory Seminars

At regular intervals during the year the Center for Radiological Research is pleased to welcome accomplished specialists from around the world to present formal seminars and/or spend time discussing ongoing research. The seminars are attended by Center and RARAF professional staff, senior technical staff and graduate students, as well as doctors and scientists from other departments of the College of Physicians & Surgeons interested in collaborative research. Attention has focused on recent findings and future plans, with special emphasis on the interdisciplinary nature of our research effort.

The 2007 sessions included the following guest speakers (listed alphabetically):

- Dr. Susan M. Bailey, Colorado State University: “Telomeres and DNA double-strand break: Ever the twain shall meet?”
- Dr. Walter E. Berdon, Department of Radiology, Columbia University, College of Physicians & Surgeons: “Proximal Symphalangism: The past and the present as seen by an interested clinical pediatric radiologist.”
- Dr. Vilhelm A. Bohr, Chief, Laboratory of Molecular Gerontology, National Institutes of Health, National Institute on Aging, Gerontology Research Center, Baltimore, MD: “Werner Syndrome and Other Human Premature Aging Conditions Are Deficient In DNA Repair.”
- Dr. Marcus Cooper, Department of Cancer Biology, Dana-Farber Cancer Institute, Boston, MA: “Defects in energy homeostasis in Leigh syndrome French Canadian variant through PGC-1alpha/LRP130 complex.”
- Dr. Kenichi Masumura, Division of Genetics and Mutagenesis, National Institute of Health Sciences, Tokyo, Japan: “gpt Delta Transgenic Mouse for In Vivo genotoxicity assays: Molecular Characterization of Mutants Induced by Environmental Mutagens.”
- Dr. Peter B. Schiff, Department of Radiation Oncology, Columbia University, College of Physicians & Surgeons: “Radiation Oncology in Developing Countries: Ocean Road Cancer Institute, Tanzania.”

Seminars were also conducted by professionals from our own Center staff:

- Dr. Yuxin Yin: “PTEN as a new guardian of genome.”
- Dr. Vladimir Ivanov: “Sodium arsenite treatment accelerates TRAIL-mediated apoptosis in melanoma cells.”
- Dr. Sally A. Amundson: “An integrated approach for applying gene expression profiling to radiation biodosimetry.”
Faculty:

ERIC J. HALL, D.Phil., D.Sc., FACR, FRCR, FASTRO, FSRP
— Director
Higgins Professor of Radiation Biophysics
Professor of Radiology and Radiation Oncology
CHARLES R. GEARD, Ph.D.
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Professor of Clinical Radiation Oncology
DAVID J. BRENNER, Ph.D., D.Sc.
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Professor of Environmental Health Science
Chairman, Joint Radiation Safety Committee
Chairman, Radioactive Drug Research Committee
TOM K. HEI, Ph.D.
Professor of Radiation Oncology
Professor of Environmental Health Sciences
HOWARD B. LIEBERMAN, Ph.D.
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YUXIN YIN, M.D., Ph.D.
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JAELYONG AHN, M.S.
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JING NIE, B.S.
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SHANAZ GHANDHI, Ph.D.
ANDREW HARKEN, Ph.D.
MEI HONG, Ph.D.
BURONG HU, Ph.D.
CHUANXIN HUANG, Ph.D.
ALEXANDER KOFMAN, Ph.D.
CORINNE LELOUP, Ph.D.
YU-CHIN LIEN, Ph.D.
CYNTHIA LIU, Ph.D.
JARAH MEADOR, Ph.D.
WENHONG SHEN, Ph.D.
YANRONG SU, Ph.D.
KENICHI TANAKA, Ph.D.
GENYUN WEN, Ph.D.
AN XU, Ph.D.
YANPING XU, Ph.D.
GUANGMING ZHOU, Ph.D.

Visiting Research Scientists:

LING HAN, M.D.
YE ZHANG, Ph.D.

Design & Instrument Shop:

GARY W. JOHNSON, A.A.S., Senior Staff Associate
— Design & Instrument Shop Director
DAVID CUNIBERTI, B.A., Instrument Maker
DENNIS KEAVENY, Instrument Maker
ROBERT C. MORTON, Instrument Maker

Technical Staff:

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XIAOJIAN WANG, M.S., Research Worker
JENNIFER MAERKI, B.S., Technician B
BHARAT PATEL, B.S., Technician B
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4th row: Mr. Stephen Marino, Dr. Adayabalam Balajee, Mr. Dennis Keaveney, Dr. Alan Bigelow, Mr. Benjamin Yaghoubian, Dr. Sunirmal Paul, Dr. Guy Garty, Dr. Peter Grabham, Dr. Yanping Xu, Mr. Yunfei Chai.

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Not pictured: Dr. Gloria Calaf, Dr. Wenhong Shen, Dr. Jarah Meador, Ms. Sarah Huang, Ms. Sasha Lyulko.
The Permanent Magnet Microbeam at Columbia University

Guy Garty, Andrew Harken, Gerhard Randers-Pehrson and David J. Brenner

Introduction

The permanent magnet microbeam (PMM), under development at RARAF, presents an alternative approach to microbeam design. Instead of focusing the ion beam using electromagnetic or electrostatic lenses, this system uses permanent magnets, which require no power supplies or water cooling and yet provides comparable if not better optical properties.

PMM layout

4He ions from the accelerator are focused using a compound magnetic lens consisting of two quadrupole triplets. The first triplet is placed 2m above the object aperture, with a second (identical) triplet placed 2m above the focal plane of the first. Each triplet consists of 3 sets of 4 permanent magnets on micrometric screws. The magnets can be extended or retracted in a steel yoke, forming an adjustable quadrupole. Since each triplet does not have identical demagnifications in the x and y axes, the two lenses are rotated by 90° in the x-y plane so that a circular beam spot is obtained (Russian Symmetry).

Limiting apertures are placed before the first triplet and inside the second triplet to reject ions which have very large aberrations.

In order to homogenize the beam, preventing spot size instability due to beam fluctuations, the object aperture was initially covered with a 1.8 µm-thick Al scattering foil. The foil had been removed and a magnetic phase space sweeper has been installed, just before the object aperture. The phase space sweeper traces the beam in a Lissajous figure over the area of the first limiting aperture just below the first triplet. By directing the beam through all possible transmission angles through the object aperture, the object appears to the focusing system to be an isotropic source.

The cells to be irradiated are placed at the image plane of the compound lens. The PMM is mounted on the original microbeam endstation consisting of a microscope with a particle detector mounted on one of the objective lenses and a stepping motor stage. Details of the microbeam end-station are given elsewhere.

Beam tests

Just prior to the decommissioning of the RARAF Van de Graaff accelerator in June 2005, we had attained a beam spot size of 20µm, two times larger than the theoretical prediction.

In 2006, the magnetic quadrupole system had to be removed for the construction of the laboratories on the third floor and was reassembled in early 2007. After realignment of the system, and without adjusting the magnets, we measured a spot size of 20 microns, demonstrating the robustness of this design.

Following the replacement of the scattering foil with the phase space sweeper and the installation of a smaller object aperture (0.5mm), we have obtained a beam spot approximately 8µm in diameter (a demagnification of x60, compared to the theoretically attainable x100) and the process is continuing with the aim of achieving a beam spot diameter of 6µm or less.

Once fully optimized the PMM will provide a useful secondary microbeam facility at RARAF and will enable biology to be performed in parallel with developments on the electrostatic microbeam.

References

Multiphoton Microscopy: Available Imaging Technique at RARAF

Alan W. Bigelow, Gerhard Randers-Pehrson and David J. Brenner

Multiphoton microscopy, a laser-based, 3D imaging technique is now integrated into the Microbeam II endstation at RARAF. The multiphoton microscope was custom-designed around the Nikon Eclipse E600-FN research fluorescence microscope at the endstation and is intended for imaging cell dynamics in tissue and cell-culture samples following irradiation. Now operational, this multiphoton microscope is the first of its kind to be assembled onto a microbeam cell-irradiation platform.

Multiphoton excitation is a phenomenon where multiple photons, coincident in time and space, can act like a single photon having a superposition of energies from the incident photons. With two photon excitation, for example, two incident photons at a laser focal point can act like one photon of half the wavelength. A Chameleon (Coherent Inc.) tunable Titanium:Sapphire laser with a 705-950nm wavelength range is the light source for the multiphoton microscope at RARAF. These infrared wavelengths are less photo-damaging and have greater penetration depth than single-photon excitation. These characteristics support exciting fluorophores at the laser focal plane, and when the imaging technique is coupled to a precision xyz-stage, optical sectioning in 3D tissue samples is possible.

Pictured in Figure 1, the setup of the multiphoton microscope at RARAF includes a scanning laser source that is built into the endstation microscope. The laser path starts at the Ti:S laser, which is bolted to the optics table. The light path proceeds through a variable attenuator and a fast shutter before being directed vertically along the rotation axis of the microscope’s pivot mount, which is used to switch between online and offline positions. A beam expander increases the beam to a size that will over-fill the back aperture of the objective lens. The platform attached to the microscope mount accommodates the scanning optics. These optics components point along an axis into the side of a custom-built extension tube for the CCD camera that is used during fluorescence microscopy. A pivoting mirror inside the extension tube selects between fluorescence microscopy or multiphoton microscopy. For multiphoton microscopy, this mirror guides the laser vertically down through both the microscope tube lens and objective lens.

Fluorophores in a sample absorb the laser light through multiphoton excitation at the laser focal point. As these markers fluoresce, signal light entering the objective is deflected towards a photomultiplier detector (H5783, Hamamatsu). Shown in Figure 2, signal detection is accomplished with a photomultiplier tube that is mounted to the side of a specially-designed dichroic and filter holder. Control software monitors the correlation between the laser scan position and signal at the photomultiplier tube to produce the images.

An emission filter placed before the photomultiplier tube...
designates the wavelength range (window) of detected signal. With multiple fluorophores, color images can be developed by changing emission filters per fluorophore. In Figure 3, the image of bovine pulmonary artery endothelial cells (FluoCells prepared slide #1, Molecular Probes) is an example of a color image produced by switching emission filters. The cells in this image were stained with BODIPY FL phallacidin to label the filamentous actin (F-actin) and were counterstained with DAPI to label the nucleus. The objective lens used for imaging these cells was a Nikon CFI LU Epi Plan Fluor 50X objective, with 0.80 numerical aperture and 1.0mm working distance. To develop this image, 20 to 30 single frames were acquired per color at a rate of 2 seconds/frame. A routine written in MATLAB® subsequently summed and combined the images into a 3D matrix for color display.

For imaging two colors simultaneously, a holder for two photomultiplier tubes, which are optically separated by a dichroic mirror, is being constructed as an attachment to the Microbeam II endstation microscope. With two photomultiplier tubes, experiments using fluorescence resonance energy transfer (FRET) will be possible. FRET optics sets are on hand for the following transitions: BFP > GFP, CFP > YFP, and Alexa 488 > Alexa 594. FRET experiments concurrent with cell irradiation are examples of monitoring post cell-irradiation dynamics using the multiphoton microscope at RARAF.

References
improve the attainable spot size since it contains no material within the beam path, and therefore does not induce scattering. It will also obviate the current need for removing medium from cells pre-irradiation.

**LD² structure**

The LD² detector, shown in Figure 1, consists of a 1 m long string of 250 cylindrical pickup electrodes. Each projectile particle passing through a pickup electrode induces a mirror charge, identical in magnitude and opposite in polarity to its own, on the inside of the pickup electrode. The pickup electrodes are connected by surface mount inductors and capacitively coupled to ground, forming a lumped delay line with a time constant that can be matched to the velocity of the projectile. Thus, the signals from all pickup electrodes add in phase, generating a detectable signal.

Tuning the LD² time constant is done, as shown in Figure 1, by pivoting a grounded electrode around the pickup electrodes, thus changing their capacitance to ground and hence the delay line time constant.

**Results**

An LD2 prototype, containing 47 electrodes, 100 nH inductors and using Rexolite as a dielectric was used to test the variation in capacitance as a function of the angle $\alpha$, shown in Figure 1. This was done by setting the angle $\alpha$ in 2º steps, measuring the delay time and subtracting the delay due to the cables, obtained as the intercept of the graph of delay vs. # of segments (see for example Figure 2 in 1) and finally converting to capacitance using $\delta t = \sqrt{LC}$. The results are shown in Figure 2. This figure also plots the results of a finite element analysis calculation for the same geometry. The agreement is rather good.

Following these measurements we have assembled a full length LD² using 250 segments, 330nH inductors and a Rexolite dielectric.

Figure 3 shows the pulse delay as a function of the number of segments. This corresponds to a pulse propagation velocity of $1.5 \times 10^7$ m/sec, which is expected from 4.6 MeV helium nuclei. Based on the dynamic range observed in Figure 2, we conclude that the full length LD² will be able to detect 4.6-9 MeV helium nuclei and 1.2-2.4 MeV protons. Tests using the RARAF accelerator are expected in early 2008.

**Fig. 2.** The measured and simulated tuning range of a short LD² prototype. The symbols are the Rexolite prototype solid, line is the FEA simulation.

**Fig. 3.** The delay vs. number of segments in the full length LD².

**References**

Introduction
The development of the soft x-ray microbeam for low-LET ionization of single cells and sub-cellular organelles is moving forward. The design of the x-ray microbeam has been significantly changed from the previously reported arrangement. The largest change is switching from a transmission x-ray source (x-rays emitted from the opposite side of a target foil) to a reflection x-ray source (x-rays emitted from the proton entrance face), as seen in Figure 1.

Proton target development
The new proton target design overcomes three concerns from the previous approach. First, the change in target orientation will place the x-ray microbeam on a horizontal beam line eliminating the possibility of protons transmitting through the target and zone plate into the irradiation specimens, which was a concern with the transmission source assembly. Second, the heating caused by the stopping of the proton beam will be countered by the copper mount which will provide enough thermal mass to effectively cool the titanium and will have a water cooling option also available. This will allow larger current densities to achieve greater x-ray production in a shorter time increasing the expected dose rates on the specimen. Third, the switching mechanism previously proposed to change between the x-ray beam and the particle beam would require unachievable focusing of the proton beam by the microbeam lenses at two different extreme locations.

The new target will consist of a round titanium plug embedded in a copper cooling block. The titanium will be aligned with the proton beam and will have a polished face that is set to give a proton incidence angle of 20°. This target will generate titanium–characteristic Kα x-rays (4.5 keV).

The proton beam in the new design will be focused onto the titanium target using the electrostatic quadrupole quadruplet that was previously used as a particle microbeam lens (prototype for the quadrupole double triplet currently used for the charged particle microbeam work). The use of the electrostatic lens will allow the beam to be focused to approximately 20µm, which, in combination with the proton incidence angle, will provide an apparent x-ray source size of 20µm in the vertical direction.

X-Ray focusing
The x-rays will be focused onto the irradiation samples using a Fresnel zone plate. A Fresnel zone plate is a circular diffraction grating that has decreasing ring width with increasing radii giving greater diffraction angles at the edges. When constructed properly, the diffracted photons will focus to a single point from all parts of the zone plate. The zone plate that has been chosen has a diameter of 120µm and an outermost ring width of 50nm. Calculations of the emitted x-rays and the transmission of the selected zone plate predict a demagnification of ~11, giving a final spot size of 1 to 2µm. An electron micrograph of a zone plate is shown in Figure 2.

The x-ray microbeam will deliver dose rates in the range of 1 to several mGy/second at the low end, and we are looking into developing capabilities to provide up to 1 Gy/second.

Conclusions
The design of the x-ray microbeam is near completion. The assembly and testing should begin early this year, and we expect to be using the x-ray microbeam for biological experiments later in the year.
Dr and Mrs Hall are greeted by Sir David Manning, the British Ambassador to the United States, at a dinner in the British Embassy in Washington following a symposium on “The Special Relationship”.

Professor Tom K. Hei received the certificate of adjunct professorship and Ph.D. student mentor appointment in the School of Radiation Medicine and Public Health of Suzhou University from the dean of the faulty, Professor Lun Bai.

Visitors to the Center for Radiological Research, Professor Marco Durante of the University of Naples Federico II, Italy with his two students. (L-r): Miss Patrizia Paome, Professor Marco Durante, Professor Tom K. Hei and Dr. Danielle Trani.

Dr. Guangming Zhou’s Farewell Party. (L-r): Dr. Eric Hall, Mrs. Jing Nie, Dr. Guangming Zhou, Dr. Lubomir Smilenov and Dimitar Zlatev.

At the dinner after Professor Eric Hall delivered the Sieman’s lecture in the Department of Radiology on October 24, 2007.

The retiring Director, Dr. Eric Hall (right) with Gary Johnson (left), head of the Design & Instrument Shop in our Center, the only member of the Center to have served longer than Dr. Hall’s 39 years.
Mitochondrial Function and NF-κB Mediated Signaling in Radiation-Induced Bystander Effects

Hongning Zhou, Vladimir N. Ivanov, Yu-Chin Lien, Mercy Davidsona and Tom K. Hei

Introduction
Radiation-induced bystander effect is defined as the induction of biological effects in cells that are not directly traversed by a charged particle, but are receiving signals from the irradiated cells that are in close proximity to them. Although the bystander effects have been well described over the past decade, the precise mechanisms of the process remain unclear. In sub-confluent cultures, there is evidence that reactive oxygen species (ROS), nitric oxide (NO), and cytokines such as transforming growth factor β (TGFβ) are involved in mediating the process. On the other hand, gap junction-mediated cell-cell communications have been shown to be critical for bystander effects in confluent cultures of either human or rodent cells. It is likely that a combination of pathways involving both primary and secondary signaling processes is involved in producing a bystander effect.

Using primary human fibroblasts, we showed previously that the cyclooxygenase-2 (COX-2) signaling cascade, including the activation of mitogen activated protein kinase (MARK) pathways, plays an essential role in the bystander process. Since COX-2 is often induced after treatment with growth factors and cytokines such as TNFα, we further demonstrated that treatment of bystander cells with anti-TNFα monoclonal antibody (mAb) partially suppressed the NF-κB and MAPK pathways. The observations that extracellularly applied antioxidant enzymes such as superoxide dismutase and catalase can inhibit medium-mediated bystander response suggest a role of reactive radical species in the bystander process. Since mitochondria are the main source of energy production as well as generators of free radicals, we hypothesized that the bystander process involves mitochondrial dysfunction.

Fig. 1. Characterization of mitochondrial DNA depleted human skin fibroblasts (ρ0) and their parental cells (ρ+). A. PCR amplification of genomic DNA from ρ0 and ρ+ cells using two different segments of mitochondrial DNA as primers. PCR products for the two primer sets: primer A (251 bp) and primer B (261 bp). B. Assessment of mitochondrial membrane potential (ΔΨm) in ρ0 and ρ+ cells using DiOC6 staining (20 nM) and flow cytometry. ρ0 cells show lower membrane potential compared with ρ+ cells. C. Assessment of superoxide production in ρ0 and ρ+ cells using hydroethidine (2 μM) and flow cytometry. ρ0 cells show lower superoxide production compared with ρ+ cells. D. Rates of oxygen consumption for ρ+ and ρ0 cells using an oxygen electrode unit. Average of duplicate determinants from two or three independent clones from each line; bars represent ± SD.
radicals in cells, especially in pathological and stressful conditions, the present studies were conducted to define the contribution of mitochondria to the bystander response using mutagenesis as an endpoint. There is recent evidence that point mutations in the mitochondrial genome are induced among either directly irradiated cells with a 5Gy dose of gamma-rays or by exposure to bystander factor(s) obtained from such cells. To better understand the role of mitochondria in the radiation-induced bystander effect, mitochondrial DNA depleted human skin fibroblasts ($\rho^0$) and their parental mitochondrial functional cells ($\rho^+$) were used in conjunction with the Columbia University charged particle microbeam and track segment irradiation system. Since TNF$\alpha$ and ROS may induce the inhibitor nuclear factor $\kappa$B kinase (IKK)-NF-$\kappa$B pathways, we further examined the link between the NF-$\kappa$B signaling pathway and the bystander response.

Results and Discussion

Characterization of mitochondrial DNA depleted ($\rho^0$) cells and their parental ($\rho^+$) cells

A series of experiments were conducted to characterize the differences between $\rho^0$ cells and their parental $\rho^+$ cells. In the presence of uridine, $\rho^0$ cells grew very well in culture. Addition of uridine to culture medium did not affect the radiosensitivity of HSF and the dose response survival curves of $\rho^0$ and $\rho^+$ HSF culture were similar (data not shown). Compared with $\rho^+$ cells, $\rho^0$ cells showed only a slight decrease in saturation density as the culture approached confluency (data not shown). Using two different primer sets that corresponded to different segments of the mitochondrial DNA, we detected no mitochondrial DNA band in $\rho^0$ cells; in contrast, a clear band was detected in $\rho^+$ cells with either of the primer pairs (Fig. 1A). When the cells were stained with the mitochondrial membrane potential probe, DiOC6, $\rho^0$ cells showed a decrease in fluorescence intensity (mean intensity 95.1 arbitrary units, A.U.) relative to control $\rho^+$ cells (mean intensity 226 A.U. and Fig. 1B). Likewise, mitochondrial DNA depleted cells showed a decrease in intracellular superoxide content (mean intensity 8.2 A.U.) when compared with wild type cells (mean intensity 30.0 A.U. and Fig. 1C). To further confirm that $\rho^0$ cells had compromised mitochondrial function, we showed that these cells had an oxygen consumption level that was 20 times less than wild type cells (Fig. 1D). These data clearly indicate that $\rho^0$ cells have dysfunctional mitochondria, and that this cell line is a good model to investigate the role of this organelle in radiation-induced bystander responses.

Alpha-irradiation induces a higher bystander mutagenic rate in $\rho^0$ cells than in $\rho^+$ cells

**Fig. 2.** HPRT$^-$ mutation of bystander cells in mixed cultures of $\rho^+$ and $\rho^0$ cells irradiated with the Columbia microbeam. A. $\rho^+$ cells were used as the bystander cells when 10% of $\rho^0$ or $\rho^+$ cells were irradiated with 20 alpha particles each. B. $\rho^0$ cells were used as the bystander cells when 10% of $\rho^+$ or $\rho^0$ cells were irradiated with 20 alpha particles each. C. HPRT$^-$ mutation of bystander and directly irradiated $\rho^+$ cells exposed to a 0.5Gy dose of alpha particles using the specially designed strip dishes. D. Same as C with $\rho^0$ cells. Data are pooled from 3-5 independent experiments. Bars represent ± SD.
To explore the role of mitochondria in the radiation-induced bystander effect, a microbeam was used to lethally irradiate either \( \rho^0 \) or \( \rho^- \) cells with 20 alpha particles each in a mixed, confluent culture, and the bystander response was determined in the non-irradiated fraction. We found that \( \rho^0 \) cells, when compared with \( \rho^- \) cells, showed a higher bystander \( HPRT^- \) mutagenic response (15.2 vs 5.4 per 10^6 survivors, \( p<0.05 \)) in confluent monolayer when 10% of the same population were lethally irradiated (Fig. 2A, 2B). It should be noted that the background mutant fraction of \( \rho^0 \) and \( \rho^- \) cells was similar, being \( \sim 1.0 \) per 10^6 survivors. However, using mixed cultures of \( \rho^0 \) and \( \rho^- \) cells and targeting only one population of cells with a lethal dose of alpha particles, a decreased bystander mutagenesis was uniformly found with both cell types. For example, when 10% of the CTO-stained \( \rho^0 \) cells (in culture with 90% of non-stained \( \rho^0 \) cells) were irradiated with 20 alpha particles, the mutant fraction among the non-hit, bystander \( \rho^0 \) cells was \( \sim 15.2 \) per 10^6 survivors. However, when a similar number of \( \rho^0 \) cells were irradiated among 90% of wild type cells, the resultant bystander mutant fraction was only 3.1 per 10^6 surviving \( \rho^- \) cells (\( p<0.05 \)). Similarly, when 10% CTO-stained \( \rho^- \) cells were lethally irradiated with alpha particles, the mutant yield was about 5.4 in 10^6 surviving bystander \( \rho^- \) cells, but only 1.8 per 10^6 surviving bystander \( \rho^0 \) cells (\( p<0.05 \)). These results indicate that mitochondrial deficient cells cannot effectively communicate the bystander signals with wild type cells; or alternatively, signals from one cell type can modulate expression of the bystander response in another cell type.

**Track segment irradiation confirms bystander mutagenesis in \( \rho^- \) and \( \rho^0 \) cells**

Since the microbeam can only irradiate a limited number of cells, to generate sufficient bystander cells for mechanistic studies we used the specially designed strip mylar dishes and track segment irradiation as described. Since cells that were seeded on the thicker mylar (38µm) would not be traversed by alpha particles but would be in the vicinity of those seeded on thinner mylar (6µm) that would, we had, effectively, a pure population of bystander cells. Exposure of \( \rho^- \) cells to a dose of 0.5Gy alpha particles increased the bystander \( HPRT^- \) mutant yield to a level 2.6 times higher than the background incidence. However, under similar irradiation conditions, \( \rho^0 \) cells had a bystander mutant fraction that was 7.1 fold higher than non-irradiated \( \rho^0 \) cells (Fig. 2C & 2D). These results are consistent with the data generated from microbeam irradiation showing that mitochondrial deficient cells have a higher mutation frequency in both directly irradiated and bystander cells. Comparing with the data generated using the microbeam, the bystander mutagenesis obtained using the broad, track segment beam for both the \( \rho^- \) and \( \rho^0 \) HSFs was significantly reduced (\( p<0.05 \)).

**Fig. 3.** A. Effect of the nitric oxide scavenger, c-PTIO (20µM, 2 hr before and maintained overnight after irradiation) on \( HPRT^- \) mutant fractions of \( \rho^- \) and \( \rho^0 \) cells. B. Effect of the NF-\( \kappa \)B inhibitor, Bay 11-7082 (1µM, 2 hr before and maintained overnight after irradiation) on \( HPRT^- \) mutant fractions of \( \rho^- \) and \( \rho^0 \) cells. Data are from 3-4 independent experiments. Error bars represent ± SD. C. Characterization of NF-\( \kappa \)B DNA binding activities of control, bystander cells and directly irradiated (0.5Gy dose of alpha particles) \( \rho^- \) and \( \rho^0 \) cells using EMSA. FP: free labeled oligonucleotide probe. D. Western blot analyse of COX-2 and iNOS protein levels in bystander and directly irradiated (0.5Gy dose of alpha particles) \( \rho^- \) and \( \rho^0 \) cells. \( \beta \)-Actin was used as loading controls.
Effect of c-PTIO on bystander mutagenesis

To determine if nitric oxide is linked to mitochondrial function in mediating the bystander response, we treated cells with 20µM c-PTIO, a NO scavenger, 2 hours before and maintained overnight after irradiation. As shown in Figure 3A, treatment with c-PTIO significantly reduced the bystander mutagenesis in both \( \rho^0 \) and \( \rho^+ \) cell lines (p<0.05). However, the effect of c-PTIO on the bystander response in \( \rho^+ \) cells was more pronounced than in \( \rho^0 \) cells. The induced mutation frequency was reduced from 1.90 to 0.37 per 10^6 survivors (5.1 fold) in wild type cells compared with a reduction from 4.19 to 2.05 per 10^6 survivors (2.0 fold) in \( \rho^0 \) cells. These results indicated that, in addition to NO, other signaling molecules might play a role in modulating the bystander effects in mitochondrial deficient cells.

Role of NF-\( \kappa \)B in the bystander response

Expression of the iNOS gene is controlled by the transcription factor NF-\( \kappa \)B. To define the function of NF-\( \kappa \)B in radiation-induced bystander effects, cells were treated with 1 \( \mu \)M Bay 11-7082, a pharmacological inhibitor of IKK-NF-\( \kappa \)B activation, 2 hours before irradiation, and maintained overnight after irradiation. The dose of Bay 11-7082 used was non-toxic, non-mutagenic in both \( \rho^0 \) and \( \rho^+ \) cell lines. Treatment of both cell types with Bay 11-7082 resulted in a significant reduction of the bystander mutagenesis (p<0.05, Fig. 3B). The inhibition by Bay 11-7082 on radiation induced bystander effects was similar to that of c-PTIO: being more effective in \( \rho^+ \) (3.0 folds, p<0.01) than in \( \rho^0 \) cells (1.5 folds, p<0.05). Consistent with these observations, we found that the basal and inducible (both directly irradiated and bystander) levels of nuclear NF-\( \kappa \)B DNA-binding activity were notably higher in \( \rho^+ \) compare to \( \rho^0 \) cells (Fig. 3C). Consequently, expression levels of NF-\( \kappa \)B-dependent proteins such as iNOS and COX-2 were notably lower in \( \rho^0 \) cells (Fig. 3D).

We previously reported that the COX-2/PGE2 signaling pathway, which is a hallmark of inflammation and ROS production, was critically linked to radiation bystander phenomenon in normal human fibroblasts. In the present study, 3 and 6 fold increases in COX-2 expression level were found in directly irradiated and bystander \( \rho^+ \) cells, respectively (Fig. 3D). However, COX-2 expression increased only slightly in directly irradiated and bystander \( \rho^0 \) cells (Fig. 3D). Taken together, these results indicated that inducible (but not basal) expression of COX-2, which was substan-

![Fig. 4](image-url)
tially lower in mitochondria-deficient cells, plays a critical role in regulating mechanisms of bystander effects, which is consistent with our previous findings. However, these results also pointed out that other mechanisms of radiation-induced bystander effect might be different in mitochondrial deficient cells. Furthermore, we can not exclude that signaling pathways, other than the COX-2/PGE2 and iNOS/NO pathways, might be activated by NF-κB and can induce bystander mutagenesis.

**Role of NF-κB in alpha particle-induced bystander effects in normal human lung fibroblasts (NHLF)**

Since NF-κB is an important transcription factor for many signaling genes including COX-2, we used NHLF cultures, which were used previously to document COX-2 activities in bystander signaling, to exam the role of NF-κB in the bystander response. Alpha particle irradiation upregulated NF-κB binding activity in both directly irradiated and bystander cells, while Bay 11-7082, a pharmacological inhibitor of the IKK/NF-κB, efficiently suppressed this up-regulation and also reduced levels below basal amount (Fig. 4A). This inhibitor of NF-κB activity also efficiently downregulated COX-2 and iNOS-expression levels in both directly irradiated and bystander fibroblasts (Fig. 4B). Micronuclei formation was readily detected in directly irradiated and bystander cells, while both Bay 11-7082 (Fig. 4C) and c-PTIO (Fig. 4D) effectively decreased this level in bystander cells (p<0.05).

**Effects of cytokines on the bystander effects**

TNFα might be an excellent candidate in mediating bystander effects in NHLF. Exogenous TNFα in concert with IL1β directly controls COX-2 expression in NHLF (Fig. 5A). Both TNFα and IL1β could be induced following α-irradiation of NHLF. The inhibitory mAb against TNFα, which was introduced into the cell media, substantially decreased levels of NF-κB (Fig 5B) and JNK (data not shown) that was accompanied by a well pronounced decrease in the COX-2 expression level in both irradiated and, especially, in bystander NHLF (Fig. 5C). Simultaneously, the negative effect of anti-TNF mAb on ERK activity (phospho-ERK protein level) was relatively modest (data not shown). As an additional physiological test, we determined clonogenic survival of NHLF during a partial suppression of TNF levels by the inhibitory effect of anti-TNF mAb. We found a significant increase in cell survival of bystander cells treated with anti-TNF mAb (p<0.05, Fig. 5D).

In the present study, we found that ρ0 cells were more sensitive to alpha particle induced bystander mutagenesis. In addition, NF-κB activity and both NF-κB-dependent COX-2 and iNOS expression levels were lower in bystander ρ0 cells compared with bystander ρ+ cells. Furthermore, we found that Bay 11-7082, a pharmacological inhibitor of NF-κB activation, and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO), a scavenger of nitric oxide (NO), significantly decreased the mutation frequency in both bystander ρ0 and ρ+ cells. Our results indicated that mi-

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**Fig. 5.** Effects of TNFα on the bystander response. A. Combined treatment of NHLF with TNFα (20ng/ml) and IL1β (2ng/ml) induced COX-2 expression as determined by Western blot analysis. B. EMSA of NF-κB DNA-binding activity in control and bystander cells, with or without anti-TNF mAb (5µg/ml) in the medium. C. Effects of treatment with the inhibitory anti-TNF mAb (5µg/ml) on COX-2 levels in control, α-irradiated and bystander cells. D. Clonogenic survival assay of NHLF after indicated treatment with and without inhibitory anti-TNF mAb (5µg/ml). Data are pooled from three independent experiments. Bars represent ± SD.
tochondria played an important role in radiation-induced bystander effect, partially via mitochondria-dependent regulation of iNOS and COX2 signaling pathways. A unifying model of the signaling pathways involved in radiation-induced bystander effects is shown as Figure 6.

**Acknowledgments**

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**Improved Isolation of RNA for Microarray Analysis from Thin Slices of Human Skin Tissue**

Sunirmal Paul and Sally A. Amundson

**Introduction**

The MatTek EPI-200 reconstituted human skin model is being used in radiation bystander studies as a model of a 3-dimensional human tissue with intact signaling and differentiation. The tissue is constructed from neonatal foreskin-derived epidermis which is a multilayered, differentiated tissue consisting of basal, spinal and cornified layers resembling human epidermis. We are interested in understanding how signaling between irradiated cells and un-irradiated bystanders may differ between a standard 2D tissue culture model and a 3D tissue model. To this end, our major aim has been to conduct gene expression profiling experiments using
these 3D tissues. Although protocols and reagents for preparation of RNA from tissues are available from a number of commercial sources, quality and yield of total RNA have been generally inadequate for successful microarray experiments. Our lab previously optimized a standard method for slicing unfixed tissues for bystander work and reported a technique to isolate RNA from sliced 3D tissues using Tri-reagent (RNAlater®, Ambion) at -80°C slicing of tissues and subsequent preservation of slices in Sample preparation and purification of RNA for 24 hours to allow for gene expression.

We assessed the quality of RNA extracted from 3D tissues by the above-described protocol using a NanoDrop spectrophotometer and the Agilent 2100 Bioanalyzer. Important elements of RNA quality determinants are listed in Table 1. RNA yield per sample (from approximately 1/5 of a tissue) ranged from 3.8–5.13µg. Purity assessment based on ratios of 260/280 and 260/230 indicated reproducibly high purity of RNA free from DNA contamination. RNA sample integrity was determined by Agilent 2100 bioanalyzer and RNA 6000 nano-Reagent/Chip®. The RNA integrity number (RIN) value and ribosomal RNA ratios (28S:18S) observed were highly reproducible and matched with highly pure reference RNA for all the samples tested. An electropherogram of a typical sample detailing the regions that are indicative of RNA quality is shown in Figure 1.

DNA contamination in RNA preparation is a major concern for microarray hybridization when the input material must be amplified prior to labeling. In our initial studies, we performed DNase treatment in the harvested RNA followed

<table>
<thead>
<tr>
<th># of samples</th>
<th>260/280</th>
<th>260/230</th>
<th>Yield/Sample (µg)</th>
<th>28S:18S</th>
<th>RIN</th>
</tr>
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<tr>
<td>0Gy</td>
<td>23</td>
<td>2.09±0.05</td>
<td>1.73±0.36</td>
<td>5.13±1.77</td>
<td>1.58±0.51</td>
</tr>
<tr>
<td>0.5Gy</td>
<td>8</td>
<td>2.13±0.05</td>
<td>1.54±0.35</td>
<td>4.29±1.26</td>
<td>1.85±0.10</td>
</tr>
<tr>
<td>2.5Gy</td>
<td>14</td>
<td>2.07±0.04</td>
<td>1.67±0.34</td>
<td>3.80±1.36</td>
<td>1.43±0.17</td>
</tr>
<tr>
<td>8Gy</td>
<td>8</td>
<td>2.13±0.06</td>
<td>1.65±0.38</td>
<td>5.08±2.87</td>
<td>1.66±0.19</td>
</tr>
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Note: Quality and quantity (yield) of RNA prepared from 3D tissues. The ratios of 260/280 and 260/230 provide a gauge of protein and carbohydrate contamination of a RNA sample. RIN and 28S:18S ratio are the determinants for integrity of RNA. RIN of 0.5Gy samples was not determined (ND).
by inactivation of DNase by a proprietary reagent as recommended in the RNAquous instructions. To avoid potential interference of any chemical residue in downstream labeling reactions, we have switched to DNase treatment on the column prior to elution (as described above). In order to determine if column DNase treatment was sufficient to eliminate genomic DNA from the RNA preparation, we checked for DNA contamination in RNA prepared by the two methods using PCR with DNA-specific primers and no reverse transcription step. Interestingly, PCR analysis demonstrated a much-reduced level of genomic DNA contamination in the RNA when DNase treatment was performed on columns compared to DNase treatment in solution (Figure 2).

We next assessed the performance of this RNA in microarray hybridization using Agilent’s low RNA input fluorescent linear amplification kit. Both amplification and specific activity values of Cy3 labeled cRNA were robust, and within target values. Hybridization of the labeled cRNA to microarrays also resulted in quality control reports within target parameters, indicating successful experiments. A scanned image of a typical one color microarray is shown in Figure 3. In summary, our current protocol for RNA extraction from 3D tissue provides improvements in both yield and quality over the previous approach. It will allow recovery of sufficient high quality RNA from pooled 250µm tissue slices to enable microarray profiling in bystander tissue.
Ionizing Radiation-Induced Bystander Effect Activates Protein Kinase C-ε Expression

Burong Hu, Yanrong Su, Peter Grabham, Adayabalam S. Balajee and Charles R. Geard

Over the past decade, several studies have illustrated the existence of a biological phenomenon of radiation-induced bystander effect, in which the non-irradiated bystander cells show a similar stress/DNA damage response to that of irradiated cells for some of the biological endpoints studied. These include mutation, micronuclei formation and clonogenicity.1,2 Understanding the mechanism(s) for bystander effect is important not only for radiation risk assessment but also for minimizing the harmful effects of radiation to normal adjacent cells during cancer treatment.

Ionizing radiation (IR) induces cellular response is complex, involving the activation of multiple signal transduction pathways. Among them, members of protein kinase C (PKC), a family of phospholipid-dependent serine/threonine kinase, have been widely implicated in the regulation of various biological processes such as cellular proliferation and differentiation. An earlier study has demonstrated isoform-specific activation of protein kinase C (p-PKC-α/β, θ, βII) specifically in bystander cells.3 PKC-ε, another member of novel PKCs, has been shown to be activated in multiple cell types by second messengers such as diacylglycerol (DAG), fatty acids, and phosphatidylinositol 3,4,5-triphosphate (PIP3). The activated kinase translocates from the cytoplasm to the membrane or cytoskeleton in response to DAG or PIP3, whereas binding of certain fatty acids causes translocation to the Golgi network.4 It is believed to function as an antiapoptotic protein and is the only PKC isozyme that has been associated with oncogenesis.5 It remains unknown whether PKC-epsilon is activated in radiation induced bystander cells.

In the present study, normal human lung fibroblasts (NHDF) were collected from the bottom side (3h after sham irradiation or irradiation with 5Gy alpha particles) and upper side (associated bystander) of a special double sided Mylar film dish.6 Our initial studies with signal transduction pathway finder gene specific array (Gene Arrays, Bethesda, MD) showed increased expression of a few genes (Table 1) in bystander NHDF cells after α-particles irradiation. Among them, PKC-ε was increased by 2-3 fold in comparison to that of sham treated control cells. To verify the increased expression of PKC-ε at the mRNA level in bystander cells, quantitative real time PCR was performed (Fig. 1). For this purpose, cDNA was synthesized from total cellular RNA isolated 3 h after treatment from sham treated, irradiated and bystander NHDF cells. PKC-ε expression was normalized to internal β-actin control. Triplicate samples were analyzed for each treatment.

The PKC pathway has demonstrated roles in the control of

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<th>Table 1.</th>
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<td>Gene name</td>
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<tr>
<td>Irradiated cells</td>
</tr>
<tr>
<td>p21/Waf1/Cip1</td>
</tr>
<tr>
<td>PKC-ε</td>
</tr>
<tr>
<td>PKC-α</td>
</tr>
<tr>
<td>PKC-β</td>
</tr>
<tr>
<td>iNOS</td>
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<tr>
<td>COX-2</td>
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Note: Up/down-regulation of gene expression in irradiated and bystander cells after treatment with 5Gy alpha particles.
of cell growth and differentiation. In an earlier study, we have shown the increased proliferation of bystander cells in medium transfer experiments. Consistent with the increased proliferation potential, increased cellular transformation events have also been reported. Enhanced expression of PKC isoforms observed in human bystander fibroblasts after low LET radiation is consistent with the rapid proliferative advantage of bystander cells. These results tend to suggest that the bystander cells receive growth stimulatory signals from irradiated cells which is mediated by the PKC pathway. If PKC-ε is important for mediating the bystander effects, its inhibition is expected to abolish some of the biological endpoints that are often studied for assessing the bystander response. For competitive inhibition of PKC, we have used Bisindolylmaleimide IX (Ro 31-8220), which is a selective and ATP-competitive PKC inhibitor with an IC50 of 10nM, whereas the IC50 for the inhibition of PKA is 900nM. Although the IC50 values for the inhibition of the group of PKCs range from 5-27nM and that for PKC-ε it is 24nM, our western-blotting analysis results showed that the optimum concentration required for complete inhibition of PKC-ε protein level was 1μM (Fig. 2). Data not shown for treatment with 100nM Ro 31-8220. For assessing the effects of PKC inhibition on bystander response, medium transfer experiments were carried out. The procedure was essentially the same as described before. Cells were or were not treated with 1μM Ro 31-8220 and incubated for 1.5h. The conditioned media collected from the sham irradiated and irradiated cells (donor) 1h post-irradiation were transferred to the receptor cells (bystander) and incubated for an additional 1h. Cells were subsequently trypsinized and replated for micronuclei analysis. Cells were harvested 36h later and the micronuclei were scored in acridine orange stained cells. Data are pooled from 3 independent experiments. Error bars represent ±SD.
We were interested in the levels of DNA damage as a response, micronuclei were scored in sham treated, irradiated or bystander cells. Inhibition of PKC suppressed the induction of micronuclei frequency both in irradiated and bystander cells. In addition to micronuclei suppression, a modest effect of PKC inhibitor on cell proliferation was noticed in bystander cells (Fig. 3 and 4). However, it is hard to explain the increased proliferation of both irradiated and bystander cells after PKC inhibition. It is possible that PKC inhibition modulates the cell cycle checkpoints through inhibition of Chk1 and Chk12 kinases. These preliminary results suggest the radiation-induced bystander effect involves the PKC pathway. As the inhibitor Ro 31-8220 is also known to specifically inhibit protein kinase A, further experiments are in progress using lower concentrations of Ro 31-8220 (100nM) to confirm the role of PKC-ε in the inhibition of bystander effects.

References
total bi-nucleate cells for statistically significant measures of MN index (Fig. 1). Fewer than 500 binucleate cells were obtained at any of the earlier time points, which were therefore judged to be inadequate for counting micronucleus index. The presence of the mylar growth surface in the stained mounts did not interfere with visualization of the cells, and micronuclei could be easily scored (Fig. 1).

Conclusions

In this study we used variations of the standard cytogenetic micronucleus assay protocol and timings to determine the best method of measuring chromosomal damage as an indicator of the bystander response in small airway epithelial cells. Due to the limited number of population doublings that these cells undergo before they senesce in culture, and our observation of their inability to form a cell monolayer on mylar surfaces, we chose to irradiate, culture and fix the cells on mylar directly. This method allowed us to gather important information regarding the response of bystanders to irradiated cells as a prelude to the functional genomics study that is the main focus of our project. In conclusion, we found that directly irradiated SAEC cells showed a marked MN formation response (Table 1). Bystander cells did show a response at higher levels than controls though certainly not as robust as the directly irradiated cells.

References


Improved Preparation of Keratinocytes from 250μm-Thin Tissue Slices for Binucleate Micronucleus Analysis

Alexandre V. Mezentsev and Sally A. Amundson

In collaboration with other members of the Center for Radiological Research (CRR), we are pursuing the extension of studies of bystander effects from standard 2-dimensional culture to 3-dimensional tissue models. The transition to 3-dimensional models required us to redesign previously used protocols and adopt them to the specific characteristics of the new experimental models. Here, we describe a new method that can be used for isolation of undifferentiated human keratinocytes from thin sections of artificial tissues, EPI-200 (Mat-Tek, Ashland, MA), precisely imitates the structure of the epidermis. It is comprised of about 20 layers of cells, with each layer representing keratinocytes at a certain step of differentiation. For most endpoints our interests are focused on the lowest cell layer since this is the only cell layer in the whole tissue where cells can divide. Irradiation of this basal layer allows us to observe effects in both the undifferentiated keratinocytes (the epidermal stem cells) that remained attached to the supporting membrane and their descendant cells through the terminal differentiation program. These tissues are cultivated in Epi-NMM-100 medium by using a

<table>
<thead>
<tr>
<th>Sample</th>
<th>MN index (± SD)</th>
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<tr>
<td>1. SAEC control</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td>2. SAEC irradiated</td>
<td>5.5 ± 1.9</td>
</tr>
<tr>
<td>3. SAEC bystander</td>
<td>1.6 ± 1.0</td>
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technique with air–liquid interface with the upper tissue surface facing the air for better stimulation of keratogenesis.

For some of our endpoints of interest, it is necessary to isolate keratinocytes from the 3-D tissues, for instance, in order to prepare high quality metaphase spreads to enable cytogenetic studies, or to enhance scoring of micronuclei in binucleate cells. Isolation of keratinocytes from the skin usually involves a partial digestion of the epidermal sheets with trypsin or collagenase. The isolated cells can be cultured in a monolayer or used to recreate structures similar to their parental organ. The ability of keratinocytes to participate in both cell and organ culture studies makes them an attractive model for comparison of both approaches and validation of experimental data. About two years ago, Curren and co-workers published the first protocol for isolation of keratinocytes from EPI-200 and used the obtained cells in the micronucleus assay. Around the same time, Dr. Brenner’s group reported a similar method that takes only a few hours to prepare and fix cells.

Despite their advantages, neither procedure recovered sufficient numbers of cells for use in bystander studies. For our bystander studies, we need to be able to recover sufficient numbers of dividing cells from narrow strips of the tissue. Previously, we developed a method for isolation of total RNA from 250 μm tissue strips. In the present paper, we describe isolation of cells from tissue strips of the same size.

While our method (Fig. 1) is based on the traditional approach (dissociation of undifferentiated keratinocytes from the supporting membrane by trypsin), we made several changes to adopt it to shape and size of our samples. Since a single 250μm strip is about 2.65% of the tissue, we found it necessary to pool six pairs of fragments cut at the same distance from the irradiation site in order to obtain a realistic number of cells. Taken together, these tissue strips comprise ~52.6% of a full EPI-200 tissue.

Our initial application of this approach was to improve scoring of the binucleate micronucleus assay. After proton irradiation of a single plane across the tissue diameter, tissues were incubated with cytochalasin B for 48h to collect binucleated cells. The tissues were cut using the departmental tissue chopper (Fig. 2) previously developed in the CRR Instrumental Shop for the EPI-200 bystander studies. Cut strips were collected on ice in Aedesta preservation medium (Cell Preservation Solutions, Columbia SC). Aedesta preservation medium was used to protect the tissues from overdrying. Moreover, Aedesta kept tissues intact for a prolonged period of time. According to the manufacturer’s report this period can be as long as 5 days without a sufficient change in cell morphology and contents. Samples were then washed in 1ml PBS/sample for 10 minutes in order to swell the cells and remove any remaining nutrients from tissue surfaces and membranes. After washing, the tissues were treated with 0.1% EDTA in HBSS (350 μl/sample; 15 min; room temperature) to facilitate cell dissociation from the supporting membrane and to minimize the concentration of protease inhibitors. Incubation with EDTA was also used to reduce calcium contents in the medium. It is well documented that calcium plays a crucial role in the keratinocyte metabolism improving intracellular contacts and orchestrating their terminal differentiation. Incubation in EDTA helps to preserve cells in an undifferentiated state.

Tissues were then treated with trypsin-EDTA (350μl/samples; 2 x 30 min; room temperature). While trypsinization is the main part of the other procedures, we improved it by gently pipetting the tissues through a cut pipette tip. Pipetting facilitates disassociation due to a disruption of loosened intracellular contacts and removal of the cells remaining on the supporting membrane. After reincubation in fresh trypsin, washing solutions were pooled together and trypsin was neutralized by an equal volume of keratinocyte serum free medium, KSFM, (Invitrogen, Carls-
Neutralization in KSFM should be considered an important part of this protocol. A failure to neutralize trypsin will lead to extra cell damages and reduce the yield. Furthermore, FBS, commercial neutralizers of trypsin, and even Mat-Tek growth medium were not considered good alternatives to KSFM due to their high calcium contents. High calcium causes an aggregation of cells that would interfere with later imaging analysis. Unlike other neutralizers, KSFM is calcium free, while it has similar buffering capacity as the regular growth medium. To separate cells from debris we filtered neutralized solutions through 70μm mesh and centrifuged the filtrates (1000rpm; 10min +18°C). Filtering the samples is a common practice during the isolation of keratinocytes from skin. However, it was not required for EPI-200 except for isolation of keratinocytes from thin tissue strips.

In order to fix cells immediately for scoring in the micronucleus (MN) assay, the cell pellets were washed in 10 volumes of 0.075M KCl, which was added in several small increments to each tube. The cells were centrifuged again and gently resuspended in 300 μl of freshly made methanol-acetic acid (3:1) and kept at 4°C in vertical position for 72h. Unlike the express method that involves a relatively short fixation (3h), we found a sufficiently longer period of fixation improved recovery of cells. Indeed, fixation in methanol: acetic acid generally makes cells harder to recover by centrifugation. Storing the tubes in a vertical position for 72h promotes settling of the cells by gravity and improves their recovery. Subsequent centrifugation (500rpm; 10min; 4°C) was performed to remove remaining fixative. According to our observation, faster centrifugation led to greater cell loss, possibly due to fragility of the fixed cells. Thus, fixed cell pellets should be resuspended very gently in the remaining liquid (about 20μl/pellet), before applying them on glass slides. The slides are then air-dried for at least 30min prior to staining and mounting.

Quantification of cells (Fig. 3) suggested that our method recovers cells in numbers sufficient for the binucleate MN assay. Interestingly, the express method failed to recover cells from the tissue strips and led to about 10-fold lower recovery from the whole tissue than the current protocol. Our method allowed us to obtain about 2000 binucleate cells from a whole tissue and 865±240 binucleate cells from the strips. These numbers were proportional to the amount of the starting material (100% and ~52.6%, respectively). Unfortunately, we do not know the exact factor(s) responsible for lowering the cell yield.

The isolated cells were stained with DAPI (Fig. 4) and showed an increased frequency of MN compared to non-irradiated controls. Previously, we observed a similar bystander effect after irradiation with α-particles. The elevated MN response extended approximately the same distance from the plane of irradiation with either protons or α-particles (750 and 600μm, respectively). The relative fold-induction of MN in the bystander tissue compared to controls was similar in both studies, despite the different LET and relative biological effectiveness of the initiating particles. However, the control levels of MN were about 4-fold lower in the experiment with α-particles. Several factors may account for this. We counted MN in cells isolated from the tissue while Belyakov and co-workers counted MN in situ in paraffin sections. The in situ approach may be hindered by steric difficulties that appear after sectioning the tissues. The appearance of MN in each section depends on how the blade traversed the tissue. A MN may be hidden behind the nucleus. Thus, using an in situ approach may potentially decrease the number of MN scored. This view is supported by earlier studies of MN formation in EPI-200 following topical applications of sodium chloride or organic solvents in which a protocol similar to ours was used. Although the authors of that study did not discuss their choice of method, they widely used paraffin sections in the same paper to score binucleated cells, suggesting potential limitations of in situ MN counting.

Compared to the MN assay in paraffin sections, our protocol has several advantages. Firstly, isolated keratinocytes predominantly represent lower cell layers comprised of proliferating cells or cells at the early stages of differentiation. This improves chances to discover binucleated cells. Secondly, a typical in situ experiment presumes scoring of 1,500-10,000 cells, at each distance from the irradiation site.
Bystander Responses of p53 and c-JUN Proteins in Artificial Epithelial Tissues

Bharat Patel, Brian Ponnaiya, Stephen Marino and Charles R. Geard

Bystander studies have pointed to the role of distinct cellular pathways and phosphorylation of specific proteins in propagating the bystander response in non-irradiated cells following ionizing radiation exposure. Here we look at two proteins of interest- p53 and c-JUN. The p53 tumor suppressor protein plays a major role in the cellular response to DNA damage and other genomic aberrations. Exposure to ionizing radiation has been shown to result in the phosphorylation of p53 at Serine 15. P53 is also phosphorylated through the SAPK/JNK signaling pathway, thought to be one of the pathways through which the bystander response is propagated. It is through this pathway also that phosphorylation of the transcription factor component c-JUN is stimulated at Serine 63/73.

Fig. 1. H&E staining of section of EpiDerm tissue.
Artificial human epidermal tissue, Epi-200 from MatTek, was used for this study. This tissue consists of fully differentiated human-derived epidermal keratinocytes very similar to normal human epidermis (Fig. 1). These tissues were irradiated with 1Gy alpha particles using the track segment facility at RARAF. Control tissue samples were run along with the irradiated tissues. Tissues were then fixed at 15, 30, 45, and 60 minutes post irradiation in ten percent Neutral Buffered Formalin, paraffin-embedded, and sectioned at the Core Histology Facility of Columbia University Medical Center.

In this study, we utilized the Tyramide Signal Amplification Kits from Molecular Probes to assay phosphorylated p53 and c-JUN levels. This kit uses an enzyme mediated detection method utilizing the catalytic activity of horseradish peroxidase (HRP) to generate high-density labeling of a target protein. The first step in this method involves the binding of a probe to the target via immunoaffinity. In this case the probes were p53 and c-JUN antibodies obtained from GenScript and CellSignalling respectively. The second step involves secondary detection of the probe with a HRP-labeled antibody, followed by activation of multiple copies of dye-labeled tyramide derivative by HRP. An Alexa-Fluor 488 dye-labeled tyramide was used for this purpose. The final step involves covalent coupling of the resulting highly reactive short-lived tyramide radicals to the protein of interest. Following this staining procedure, the tissue sections were counterstained with DAPI in ProLong Gold Mounting Media from Invitrogen. Subsequently, tissue sections were imaged and the individual nuclei analyzed for phosphorylation levels using Image Pro Plus imaging software.

As seen in Figure 2, irradiated tissue sections display greater intensities of phosphorylated p53 protein at all time points compared to control tissue sections. The graphs show the frequency distribution of fluorescence intensity as a function of probability for p53 and c-JUN proteins at 15, 45, and 60 minutes post irradiation (PI).

**Fig. 2.** Phosphorylation intensities for irradiated (red curve) and control (black curve) tissue sections of p53 and c-JUN proteins at 15, 45, and 60 minutes post irradiation (PI) as a function of probability. P53 protein is represented in A (15 minutes PI), B (45 minutes PI), and C (60 minutes PI), whereas c-JUN protein is represented in D (15 minutes PI), E (45 minutes PI), and F (60 minutes PI).
points compared to corresponding controls. Intensities are plotted as a function of frequency distribution (a cumulative frequency distribution expressed as a cumulative percentage of cells), where the right-shift of a line indicates higher intensity of signal, corresponding to more of the protein being phosphorylated. Differences in phosphorylation between irradiated and control sections are seen beginning at 15 minutes post irradiation, peaking at 45 minutes post irradiation, and decreasing at 60 minutes post irradiation. The irradiated curve displays the phosphorylation levels of both irradiated and bystander nuclei. Since alpha particles of 1Gy can only traverse about 25µm into irradiated tissue (Fig. 1), only one tenth of the cells in the irradiated tissue section and thus the irradiated curve are actually considered irradiated, while the rest of the cells are considered non-irradiated or bystander. Looking at Figure 2, since greater than one tenth of the irradiated cell curve is of higher intensity than the control curve at 15, 45, and 60 minutes post irradiation for p53, we can conclude that bystander cells are clearly playing a role in the increased expression of phosphorylated p53 at all time points.

As seen in Figure 2, irradiated tissue sections display higher levels of phosphorylated c-JUN protein 15 minutes post irradiation. At 45 minutes post irradiation however, the irradiated sections display a lower level of phosphorylation compared to the control sections. After this time point, c-JUN phosphorylation levels for irradiated tissue sections settle back to their corresponding control levels at 60 minutes post irradiation. As discussed earlier, since greater than one tenth of the irradiation curve is of higher intensity than the control curve at 15 minutes post irradiation, we can conclude that bystander cells are contributing to the increased phosphorylation levels seen at that time point. Other mechanisms may be involved in bringing down c-JUN phosphorylation levels at later time points; however more studies would be needed to investigate these mechanisms further. Additional studies would involve looking at other proteins of interest along the cellular pathways associated with the bystander effect.

### References


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**Biophysical Model of Spatial Patterns of Radiation-Induced Bystander Effects in Three-Dimensional Tissues**

Igor Shuryak, Rainer K. Sachs and David J. Brenner

There is considerable evidence that ionizing radiation affects cells that are located near the site of irradiation but are not themselves traversed by any particle or photon tracks. Such non-targeted radiation effects are diverse and cell type specific. They include increased mutagenesis and genomic instability, differentiation, micronucleus formation, and either decreased or increased response as regards plating efficiency, apoptosis or proliferation. These phenomena, which have been termed radiation-induced bystander effects, were observed not only in cell culture but also in vivo. They are presumably caused by intercellular signaling. The molecular identities of the relevant signals have not yet been determined, but it is known that their propagation can involve diffusion through the extracellular medium, migration of molecules directly between cells through gap junctions, or both.

The main goal of the present model is to translate the currently available conceptual understanding of the bystander effect, such as the role of oxidative stress as a likely mediator for this effect, into a quantitative mathematical formalism, which focuses specifically on the spatial patterns of the bystander effect. Although describing the details of biochemical signaling pathways is beyond the scope of the model, the formalism is intended to be as mechanistic as possible without undue complexity.

Quantitative modeling of the spatial aspect of bystander effects is particularly relevant in situations where most cells have only a small probability of suffering any ionizing “hits”—as would be the case at low radiation doses and/or low dose rates and/or high LET. Under such conditions, it is important to predict how many bystander cells (i.e. what volume of tissue) will be affected per each cell directly “hit” by an ionizing track. The model developed here can provide
such estimates and thus may have important implications for radiation protection, radiotherapy and understanding radiation-induced carcinogenesis at low doses/dose rates.

The data set of Belyakov et al.\(^1\) for bystander responses in an artificial human three-dimensional skin system is modeled. In this experiment, only a thin plane of cells was irradiated with an \(\alpha\)-particle microbeam, and bystander effect end points (apoptosis and micronucleus yield) were measured in parallel planes located at various distances from the irradiated one. This particular data set was selected because it provides rather detailed information on spatial propagation of the bystander response. In addition, it measures the bystander effect in a tissue surrogate instead of in a monolayer culture and therefore may be a better approximation of processes occurring in vivo.

The main assumptions of the model are based on experimental studies suggesting the existence of at least one (and probably many) rapidly spreading signal, which is produced by irradiated cells and induces long-term alterations of the redox balance (activation) in multiple neighboring cells, leading to accumulation of extra DNA damage and modification of cell behavior. The initial signal itself may be oxidative or non-oxidative in nature.

The model describes the selected data sets adequately (Fig. 1). Only two parameter combinations are required to describe the shape of the bystander response as a function of distance away from the irradiated plane, and two others act as proportionality constants used for normalization of the data. As new information about the identity of bystander signals and their properties accumulates, increasingly rigorous testing of the model can be performed.

**References**

Methylation Screening of *Betaig-h3* Promoter in Human Lung and Prostate Cancer by Methylation-specific PCR Method

Jinesh N. Shah, Genze Shao, Tom K. Hei and Yongliang Zhao

Both lung and prostate cancer are the leading causes of cancer death in the United States.\(^1\) For lung cancer, almost 50% of the patients already developed metastasis at the time of diagnosis, which results in less than 15% overall survival rate.\(^1,2\) The persistent poor survival for lung cancer patients is largely attributed to undetectable tumor micrometastasis at the time of surgery, which is responsible for the later relapse with the development of nodal and/or visceral metastasis.\(^3\) Similarly, there is no consistently effective curative therapy for advanced or hormone-refractory prostate cancer.\(^4\) Thus, a better understanding of the molecular mechanisms associated with lung and prostate cancer progression may contribute to improved diagnosis, clinical management and outcome prediction. In particular, the discovery of methylation biomarkers related to cancer invasiveness and metastasis would help to identify patients with relatively early-stage localized lung and prostate cancer who are more likely to experience regional or distant cancer spread, which would guide clinicians to provide more aggressive local cancer treatment(s) for such patients in order to decrease the likelihood of such tumor progression. In the present study, the MSP method for screening *Betaig-h3* methylation was established. By using this approach, the methylation profile of the *Betaig-h3* promoter was investigated in 100 cases of lung and prostate cancer specimens. A dense methylation detected by P5/P6 primer pair was found to correlate with the invasive/metastatic phenotype in lung and prostate cancer. Furthermore, *Betaig-h3* promoter hypermethylation was causally linked to the gene silencing in human lung and prostate tumor cell lines.

**Design of methylation-specific primers for the MSP method**

A 620-bp length of a CpG island, spanning the proximal promoter and the first exon of the *Betaig-h3* gene, was identified by screening the entire *Betaig-h3* genomic sequence using CpG plot prediction analysis (http://www.ebi.ac.uk/emboss/cpgplot). Our previous work had defined the methylation status of a total of 49 CpGs across 0.6 kb of the *Betaig-h3* promoter in human tumor cell lines, and we had demonstrated a dense methylation pattern in lung, kidney and DU145 prostate tumor cell lines. Based on these data, we have designed three pairs of methylation-specific PCR primers (P1-P6, Fig. 1 and Table 1). Nucleotide positions were numbered relative to the translation start site (TSS) (+1). Each primer was designed to bind to a different stretch of the *Betaig-h3* promoter with each of these distinct stretches containing 2-3 possible methylated sites (bold in Table 1). The nucleotide C in non-CpG sites and unmethylated CpG sites would be modified to T (sense strand) or A (antisense strand) by bisulfite treatment, whereas C would remain as C in methylated CpG sites. Thus, for example, PCR amplification with primer set P1-P2 on a particular sample would yield a PCR product only if the *Betaig-h3* region in this sample was actually methylated at two CpG sites encompassed by primer P1 and three CpG sites encompassed by primer P2. As a control, primers U1/U2 were designed to amplify unmethylated DNA, in which case the nucleotide C in CpG sites would be modified to T or A by bisulfite treatment.

![Fig. 1](image-url)

**Fig. 1.** Predicted CpG island and methylated CpG site profiles of the *Betaig-h3* promoter in human tumor cell lines. Location of methylation-specific primers is marked as “−”.

Molecular Studies
Validation of methylation–specific primers by MSP

To ensure the accurate and efficient amplification of methylated Betaig-h3 DNA by methylation-specific primers, we optimized the annealing temperature for these primers using 293T and H522 tumor cells as positive controls, and normal human bronchial epithelial (NHBE) cells and human mammary epithelial cells (HMEC) as negative controls. Our previous bisulfite sequencing studies demonstrated that 293T and H522 are densely methylated at the Betaig-h3 promoter region, whereas NHBE and HMEC cells are unmethylated. MSP was performed in a final reaction volume of 25μl, including 1μl of diluted PCR products (1:200) from the M3/M4 amplification as the template and 1.25μl of primer set P1/P2, P3/P4 or P5/P6. The FailSafe™ PCR System (2 X PreMix D buffer, Epicentre Biotechnologies, Madison, WI) was used for all of the PCRs, and the PCR conditions were as follows: a hot start at 94°C for 2 minutes followed by 35 cycles of PCR (94°C for 30 seconds, annealing for 30 seconds, and 72°C for 30 seconds) with a final extension for 5 minutes at 72°C. All PCR products were analyzed by 2% agarose gel electrophoresis and ethidium bromide staining. The optimized annealing temperature for each primer set and the length of PCR products are shown in Table 1. Dense methylation of the Betaig-h3 promoter has been demonstrated in 293T and H522 cells but not in NHBE and HMEC cells, PCR products were identified in 293T and H522 cell lines but not in the NHBE and HMEC negative control cells (Fig. 2). Primer set U1/U2 was used to amplify unmethylated DNA; thus, no PCR products were obtained for 293T and H522 cells but products were obtained for NHBE and HMEC cells.

**Table 1.** Primer sequences and optimized annealing temperatures for each pair of primers

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
<th>CpG sites</th>
<th>Location</th>
<th>Length (bp)</th>
<th>Annealing Temp. (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>5’TATGTAGGATCGAGTTTTC3’</td>
<td>2</td>
<td>−405 −64</td>
<td>341</td>
<td>60-62</td>
</tr>
<tr>
<td>P2</td>
<td>5’AAAAACGCCTCCGGCG3’</td>
<td>3</td>
<td>+26 −51</td>
<td>180</td>
<td>52-57</td>
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<td>P3</td>
<td>5’GGGTAGTGCCGGAGC3’</td>
<td>2</td>
<td>−235 +22</td>
<td>257</td>
<td>65-67</td>
</tr>
<tr>
<td>P4</td>
<td>5’CAACCCGAGAAAAACGC3’</td>
<td>3</td>
<td>−180 −26</td>
<td>155</td>
<td>64.5</td>
</tr>
<tr>
<td>P5</td>
<td>5’GAGGGTTAGGGCGGTTTC3’</td>
<td>3</td>
<td>+341 60-62</td>
<td>64-71</td>
<td>52-57</td>
</tr>
<tr>
<td>P6</td>
<td>5’AATCAAGGCGACGACAC3’</td>
<td>3</td>
<td>−180 +71</td>
<td>252</td>
<td>62</td>
</tr>
<tr>
<td>U1</td>
<td>5’GAGGGTTAGGGCGGTTTC3’</td>
<td>3</td>
<td>−180 +71</td>
<td>252</td>
<td>62</td>
</tr>
<tr>
<td>U2</td>
<td>5’CCCACCCAAATCAACAC3’</td>
<td>3</td>
<td>−180 +71</td>
<td>252</td>
<td>62</td>
</tr>
</tbody>
</table>

**MSP-based methylation screening of the Betaig-h3 promoter in lung tumor samples**

Fifty lung cancer samples were screened for Betaig-h3 methylation by MSP. 293T and HMEC cell lines were used as positive and negative controls, respectively. As is shown in Figure 3a, 21 of 47 samples (44.7%) were detected to harbor methylated sites at different levels in the Betaig-h3 promoter. Methylation frequencies detected by primer set P1/P2, P3/P4, and P5/P6 in primary lung cancers are 39.5%, 31.6% and 10.5%, respectively. Since all those samples harboring Betaigh3 methylation at P5/P6 loci are detected to have methylation at both P1/P2 and P3/P4 loci, methylation detected by P5/P6 primer set represents a dense methylation of Betaig-h3 promoter ($\geq$16 CpG sites).

**MSP-based methylation screening of the Betaig-h3 promoter in prostate tumor samples**

Fifty prostate cancer patient samples were screened for Betaig-h3 methylation by MSP. H522 and HMEC cell lines were used as positive and negative controls, respectively. P1-P6 primers bound to a total of 16 potential sites of CpG methylation (Table 1). The results from MSP reactions are shown in Fig. 3b. Overall, the methylation frequencies of the Betaig-h3 promoter detected by the primer sets P1/P2, P3/P4 and P5/P6 were 80% (40/50), 54% (27/50), and 28% (14/52), respectively. Consistent with the findings in lung cancer, most tumor samples (26/27 or 96%) that harbored methylated CpG sites detected by P3/P4 were identified to have CpG methylation at P1/P2 loci. Furthermore, 100% (14/14) of cancer samples with methylated CpG sites detected by P5/P6 also had CpG methylation at both P1/P2 and P3/P4 regions. Therefore, the presence of CpG methylation detected by P5/P6 was an indication of a highly dense level of CpG methylation in the Betaig-h3 promoter region.

**Correlation of the Betaig-h3 methylation with pathological features**

To determine whether the Betaig-h3 methylation status of the patient samples was correlated with clinical pathological features of lung cancer patients, an univariable analysis was carried out to correlate the methylation frequency of the Betaig-h3 promoter with clinical pathological parameters including sex, pathological stages, differentiation, tumor size...
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and metastatic phenotype (lymph node and remote metastasis). However, only dense methylation of the Betaig-h3 promoter was found to correlate with metastatic phenotype, with a significantly higher frequency of dense methylation in metastatic tumors (50%, 6/12) than in primary tumors (10.5%, 4/38) (Table 2, p<0.01). These results suggest a relationship between methylation of the Betaig-h3 promoter and lymph node/remote metastasis.

Meanwhile, Betaig-h3 methylation status of prostate cancer material was correlated with pathological profiles in the patients’ prostatectomy specimens including extracapsular extension of tumor, seminal vesicle involvement by tumor, margin positivity with tumor, and/or regional lymph node tumor involvement. As for lung cancer, a much higher frequency of dense methylation profiles was identified in prostate cancer specimens with the feature of locoregional invasiveness. For this clinicopathological phenotype, a dense methylation of the Betaig-h3 promoter was present in 41.2% (7/17) of prostate cancers with locoregional invasiveness vs. 18.6% (6/32) of prostate cancers without locoregional invasiveness (Table 3, p<0.05). Therefore, dense methylation of the Betaig-h3 promoter was associated with a higher probability of invasiveness and metastasis in both lung and prostate cancer patients.

Table 2. Betaig-h3 methylation in lung tumors metastasized to lymph nodes and remote sites vs. primary lung tumors

<table>
<thead>
<tr>
<th></th>
<th>Number of methylated CpG sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung cancer</td>
<td>0 5-10 16</td>
</tr>
<tr>
<td>Metastasized</td>
<td></td>
</tr>
<tr>
<td>(n=12)</td>
<td>4 (33.3%) 2 (16.7%) 6 (50%)*</td>
</tr>
<tr>
<td>Primary cancer</td>
<td></td>
</tr>
<tr>
<td>(n=38)</td>
<td>23 (60.5%) 11 (28.9%) 4 (10.5%)</td>
</tr>
</tbody>
</table>

* P<0.01

Fig. 3. Methylation status of the Betaig-h3 promoter in lung cancer tissue samples (A) and prostate cancer tissue samples (B) using the MSP method. “M”: DNA 2-log marker; “+”: H522 positive methylated control; “−”: HMEC negative unmethylated control. Numbers 1-50 correspond to each of the 50 lung cancer patients.
In summary, we have designed methylation-specific primers and optimized the MSP conditions to examine the methylation status of the Betaig-h3 promoter in a large number of human lung and prostate cancer specimens. The present method demonstrated that the Betaig-h3 promoter was densely methylated (at least 16 methylated CpG sites) in 16% of lung cancer and a relative higher percentage (28%) of prostate cancer specimens, which correlates with invasive and metastatic phenotypes. In addition, Betaig-h3 promoter hypermethylation was associated with the gene silencing in human lung and prostate tumor cell lines. Thus, Betaig-h3 promoter hypermethylation may represent a valuable prognostic biomarker in both lung and prostate cancer patients.

Acknowledgements
This work was supported by NASA funding (NAG2-1637) and NIH grant ES 11804.

References

Table 3. Methylation status of the Betaig-h3 promoter in prostate cancer with or without locoregional invasiveness

<table>
<thead>
<tr>
<th>Prostate cancer</th>
<th>Number of methylated CpG sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Locoregional invasiveness (n=17)</td>
<td>0 (5.9%)</td>
</tr>
<tr>
<td>Primary cancer (n=32)</td>
<td>8 (25%)</td>
</tr>
<tr>
<td>* P&lt;0.05</td>
<td></td>
</tr>
</tbody>
</table>

HRAD9 is Associated with Tumor Formation in Nude Mice
Aiping Zhu, Xiangyuan Wang and Howard B. Lieberman

HRAD9 is a checkpoint protein that acts as a DNA damage sensor. It forms a PCNA-like heterotrimeric complex with HRAD1 and HHUS1, possessing similar yet also distinct functions compared to PCNA. HRAD9 protein can induce apoptosis, maintain genomic stability, and be phosphorylated by ATM in response to DNA damage. It has 3’ to 5’ exonuclease activity, can bind p53 consensus DNA binding sequences and upregulate transcription of p21 as well as other downstream genes. Recently, it was reported that expression of HRAD9 was increased in non-small cell lung carcinoma and breast cancer. We also found that HRAD9 had aberrantly high expression in prostate cancer cell lines PC-3, LNCaP, DU145 and CWR22, in comparison to noncancerous prostate cells (PrEC). Human prostate cancer tissue also had high levels of HRAD9 protein, compared to normal prostate tissue, as determined by immunostaining. Therefore, it is clear that HRAD9 is involved in tumorigenesis. In order to study HRAD9’s role in human prostate cancer, mouse tumor models were used in this study. The strategy involved knocking down HRAD9 expression levels in prostate tumor cells using siRNA and overexpressing HRAD9 in normal prostate cells using the pcDNA3-HRAD9 construct. We wanted to test whether knocking down HRAD9 expression will reduce or eliminate tumor formation, and whether overexpression of HRAD9 in noncancerous cells injected into nude mice will result in tumor formation.

HRAD9 siRNA reduced tumor formation in nude mice
The HRAD9 siRNA target sequence (AGGCCCCGCAUCUUCACCA) was designed by Oligoengine Inc. The pSUPER.retro.puro siRNA expression vector (Oligoengine Inc.) was used to construct HRAD9/siRNA. The pSUPER.retro.puro HRAD9 siRNA plasmid was transfected with retrovirus package phi-NX into a cell line using lipofectamine (Invitrogen Inc.) to produce recombinant virus for infection of prostate cancer cells. After PC-3, DU145, and CWR22 cells were infected by recombinant virus for 48 hours, puromycin (1µg/ml) was added to select stable HRAD9 siRNA clones from the prostate cancer cell populations. Western-blotting was used to check positive clones. The siRNA was most effective in reducing levels of HRAD9 protein in DU145 cells, but levels were also
markedly reduced in PC-3 cells (Fig. 1). The siRNA was least effective in CWR22. Densitometric scanning of HRAD9 and beta-actin (control) bands indicated that HRAD9 abundance level was reduced by 86% for DU145, 76% for PC-3 and only by 34% for CWR22, relative to untransfected or insertless vector controls.

HRAD9 siRNA positive clones and insertless vector control cells (6X10^6 cells per mouse in 0.2 ml of PBS) were injected into the back of nude mice subcutis (4 to 6 weeks male Nu/Nu nude mice were used. They are from Harlan Sprague Dawley, Inc.). Tumors formed after 2 to 3 weeks post-injection. Tumor size was measured every 5 days. When tumor size reached sufficient dimensions and showed evidence of necroses, mice were sacrificed and tumor tissue samples were saved for analysis. Mice injected with DU145 cells containing insertless vector (Fig. 2A) formed detectable tumors starting at day 20, which continued to grow until day 35 (last day of monitoring). However, sites injected with siRNA-containing DU145 cells had no tumor growths. These sites were checked for 5 months, and still no tumors formed. Sites injected with PC-3 cancer cells bearing insertless vector developed tumors that grew progressively during the 35 days post-injection. Interestingly, sites containing the same parental cells but with pSUPER.retro.puro HRAD9 siRNA, which reduced levels of the protein significantly but not as dramatically as for DU145, developed small tumors by day 20, but they stopped growing shortly thereafter and remained approximately the same size through day 35 (Fig. 2B). In contrast, for CWR22 cells with insertless vector, which has high human Rad9 levels, tumors grew so aggressively that by day 25 the experiment was terminated. siRNA was not very effective in reducing Rad9 protein levels in CWR22, and for most independent siRNA transfectants tumors grew aggressively at injection sites (Fig. 2C). Some variability in tumor size and growth rate was observed for all injections of similar cells, and this could reflect differences in mice, in exact numbers of cells injected, internal growth of tumors not easily measurable in vivo, or changes in Rad9 levels post-injection. Nevertheless, it is clear that in general the more Rad9 protein present in prostate cancer cells the more aggressively they form tumors when injected into nude mice, indicating a functional relationship between Rad9 abundance and prostate cancer.

Fig. 1. Western-blot showed that Rad9 siRNA reduced the expression level of HRAD9 differently in three prostate cancer cell lines.

Fig. 2. Tumor formation in DU145, PC-3 and CWR22 prostate cancer cells transfected with Rad9 siRNA or empty vector. The Rad9 siRNA in DU145 was most effective at reducing Rad9 protein levels, followed by Rad9 siRNA in PC-3. The Rad9 siRNA in CWR22 cells was least effective to suppress tumor formation.
Immortalized human prostate cells (PWR-1E), with HRAD9 overexpressed, demonstrated tumor formation in Nude mice

The pcDNA3/HRAD9 plasmid and pcDNA3 empty vector were transfected into PWR-1E cells by electroporation. The cells were challenged with G418 (200ug/ml) 24 hours after electroporation. Culture medium was changed every 3 to 4 days until clones formed, picked and amplified. Western-blotting was used to identify positive clones (Fig. 3). The pcDNA3 empty vector stable clone and overexpression HRAD9 stable clones 14 and 15 were used for injection. Cells (1.2X10^7 per site; ref. 1) in 0.2 ml of PBS were injected into the back of nude mice subcutis. Tumors were formed 2 to 3 weeks after injection. Tumor size was measured every 5 days. A total of 14 sites were injected with cells overexpressing HRAD9. Ten of fourteen sites formed tumors; the percentage of tumor formation is 71.4%. In contrast, 15 sites were injected with pcDNA3 vector alone-transfected PWR-1E cells. Only 1 of 15 sites formed a small tumor that regressed after 25 days (table 1).

HRAD9 has many functions associated with tumorigenesis, such as promoting resistance to DNA damage, cell cycle checkpoint control, DNA repair, and apoptosis. We first demonstrate here that HRAD9 expression level is directly associated with tumor formation in prostate cancer cells. Knockdown of HRAD9 gene expression can reduce tumor size, and the tumor size depends on the level of HRAD9 expression in prostate cancer cells. The less expression of HRAD9 in cancer cells, the less tumor formation in nude mice. Overexpression of HRAD9 in non-tumorigenic non-cancerous prostate cells can induce tumor formation. So, it is clear that HRAD9 plays an important role in tumor forma-

![Image: Western-blot showed overexpression HRAD9 in PWR-1E cells, compare to PWR-1E cells only and empty vector alone.](image)

### Table 1. Tumor formation in nude mice by subcutis injection of PWR-1E stable clone cells bearing an HRAD9 expression plasmid or empty vector.

<table>
<thead>
<tr>
<th>Injection Cells</th>
<th>Injection sites</th>
<th>Tumor Volume(mm³)</th>
<th>Percent for Tumor formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>PWR1E/HRAD9 cells</td>
<td>1</td>
<td>32.45</td>
<td>71.4%</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>7.13</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2.88</td>
<td></td>
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<tr>
<td></td>
<td>4</td>
<td>25.2</td>
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</tr>
<tr>
<td></td>
<td>5</td>
<td>31.9</td>
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</tr>
<tr>
<td></td>
<td>6</td>
<td>45.25</td>
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<td></td>
<td>7</td>
<td>39.7</td>
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<td></td>
<td>8</td>
<td>75.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>21</td>
<td></td>
</tr>
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<td></td>
<td>11</td>
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<tr>
<td></td>
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<td></td>
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<tr>
<td></td>
<td>13</td>
<td>0</td>
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<tr>
<td></td>
<td>14</td>
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<tr>
<td><strong>Total</strong></td>
<td><strong>14</strong></td>
<td><strong>Tumor form 10</strong></td>
<td><strong>71.4%</strong></td>
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<td>PWR1E/pcDNA3 cells</td>
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<td>0</td>
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<td><strong>Total</strong></td>
<td><strong>15</strong></td>
<td><strong>Tumor form 1</strong></td>
<td><strong>6.6%</strong></td>
</tr>
</tbody>
</table>
tion in prostate cells. What mechanism is responsible for HRAD9 promoting prostate tumorigenesis? So far it is not clear but is being investigated.

References


Comparison between Mrad9 and Mrad9B

Corinne Leloup, Kevin M. Hopkins, Xiangyuan Wang, Aiping Zhu, Debra J. Wolgemuth and Howard B. Lieberman

Mrad9B was discovered as a paralog of Mrad9. The proteins encoded by those genes are 50% similar and 35% identical at the amino acid level. Mrad9B is expressed predominantly in the testis and Mrad9 is expressed more universally in different tissues. Nevertheless, we expected that these proteins have related functions since they are structurally similar. Based on known Rad9 functions, we started to investigate Rad9B involvement in DNA repair, cell cycle checkpoint control and development. For this purpose, we generated Mrad9B−/− mouse embryonic stem (ES) cells and Mrad9B−/− mice.

Mrad9 or Mrad9B deletion cause embryonic lethality but their inactivation causes different phenotypes.

As seen in table 1, Mrad9B−/− embryo resorption started between days E7.5 and E8.5. It was not possible to obtain any knock out embryos starting on day E9.5, which is also the time when heterozygous embryos start to display an open brain phenotype.

Mrad9−/− embryos die later, between E9.5 and E12.5. They start to display gross morphological abnormalities on day E7.5. The Mrad9−/− embryos did not display morphological abnormalities and they were viable. Therefore Mrad9 and Mrad9B are both essential for embryogenesis but at a different stage of development.

Mrad9 and Mrad9B do not complement each other for promoting survival after exposure to DNA damage inducing agents.

Table 1. Number of embryos from timed Mrad9B+/− x Mrad9B+/− matings

<table>
<thead>
<tr>
<th>Stage (Day)</th>
<th>9B+/−</th>
<th>9B+/−</th>
<th>9B−/−</th>
<th># litters</th>
</tr>
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<tbody>
<tr>
<td>7.5</td>
<td>3</td>
<td>0/6</td>
<td>0/10</td>
<td>3</td>
</tr>
<tr>
<td>8.5</td>
<td>6</td>
<td>0/10</td>
<td>4/7</td>
<td>3</td>
</tr>
<tr>
<td>9.5</td>
<td>7</td>
<td>4/13</td>
<td>6/6</td>
<td>3</td>
</tr>
<tr>
<td>10.5 *</td>
<td>9</td>
<td>5/16</td>
<td>6/6</td>
<td>4</td>
</tr>
<tr>
<td>12.5 **</td>
<td>3</td>
<td>0/4</td>
<td>1/1</td>
<td>2</td>
</tr>
</tbody>
</table>

Note: Numbers represent the sum of all embryos derived from the indicated number of litters. In the Mrad9B−/− column, results are presented as number of embryos with an open brain phenotype out of the total number of embryos examined. In the Mrad9B−/− column, results are presented as number of resorbed embryos out of the total number of embryos. * and ** : additionally there were respectively 1 and 4 completely absorbed embryos whose genotyping was impossible.

We have shown that Mrad9B−/− cells display more sensitivity to UV, gamma-rays and mitomycin C induced DNA damage than WT cells, but are not more sensitive to hydroxyurea, cisplatin or ethyl methane-sulfonate (data not shown). This differs from profiles of Mrad9 mutant sensitivity. Mrad9−/− cells are extremely sensitive to all of the above treatments (ref. 1 and KH, unpublished results).

Furthermore the proteins seem to operate at different steps of the damage response because Mrad9 highly expressed in Mrad9B−/− cells does not restore survival to WT levels after treatment with gamma-rays or hydroxyurea, and vice versa (Fig. 1).

Mrad9B, unlike Mrad9, is not necessary to maintain genomic stability.

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Mrad9 protects cells from spontaneous development of chromosome aberrations. For example, the percentage of metaphases with spontaneous chromosomal aberration is 14% for Mrad9−/− cells while it is only 2% for WT cells. No such difference was found between WT cells and Mrad9B−/− cells. Similarly, Mrad9−/− cells display high spontaneous hprt mutation frequencies while Mrad9B−/− cells do not (KH, unpublished data).

Mrad9−/− cells display a higher number of sister chromatid exchanges (Fig. 2A) as well as more micronuclei (Fig. 2B) than WT cells. Mrad9B−/− cells however behave like WT cells. While Mrad9 is essential for maintaining genomic integrity, Mrad9B does not to play any significant role in the process.

Mrad9−/− cells, unlike those containing Mrad9B−/−, do not maintain the radiation-induced G2 checkpoint cell cycle delay.

It has been reported that Mrad9−/− ES cells do not sustain the G2 checkpoint block as long as WT cells. Our data presented in figure 3 and table 2 show that Mrad9B−/− cells behave the same as WT cells in this regard. Therefore, Mrad9B does not play an essential role in G2/M checkpoint control.

**Conclusion**

Although based on structural overlap it was expected that Mrad9 and Mrad9B would have similar functions, possibly in different tissues, our studies indicate that the genes are non-redundant with respect to activity. Mrad9 is a central player for genome maintenance, being involved in DNA repair, checkpoint control and stabilizing the genome. It is also essential for mid-gestation development. Thus far, it seems that Mrad9B plays a marginal role in protecting against DNA damage but a major one in early embryonic development.
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Fig. 2. Genomic stability measurements. A: Spontaneous sister chromatid exchange (SCE) in WT, Mrad9/− and Mrad9B/− ES cells. B: Spontaneous micronuclei in WT, Mrad9/− and Mrad9B/− ES cells. Cell division was blocked after one nuclear division and micronuclei were scored in binucleated cells.

Fig. 3. Distribution of WT and Mrad9B/− cells at different stages of the cell cycle. Cells were examined after mock treatment or after exposure to 8Gy of gamma-rays in the absence or presence of nocodazole. Histograms are from one experiment. Regions of the profiles corresponding to G1, S and G2/M are defined below the graphs.

Table 2. Percentage of WT and Mrad9B/− cells mock treated or irradiated with 8Gy of gamma-rays at different stages of the cell cycle.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Time (h) Postirradiation</th>
<th>% of population in cell cycle phase:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>G1</td>
</tr>
<tr>
<td>Mrad9B+/+</td>
<td>0</td>
<td>35.1±4.01</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>6.8±3.15</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>3.8±0.32</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>9.2±0.67</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>21.1±4.26</td>
</tr>
<tr>
<td></td>
<td>12 + Noc</td>
<td>0.7±0.23</td>
</tr>
<tr>
<td>Mrad9B−/−</td>
<td>0</td>
<td>30.8±5.60</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>5.7±1.70</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>2.6±3.00</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>7.4±3.13</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>14.7±7.31</td>
</tr>
<tr>
<td></td>
<td>12 + Noc</td>
<td>1.1±0.67</td>
</tr>
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</table>

Note: The results are presented as percent of cells in each stage and are the average ± standard deviation for 2 independent experiments.

References


Alterations in Human Rad9 Protein that Modulate Gamma-Ray and Hydroxyurea Resistance

Kevin M. Hopkins, Xiaojian Wang and Howard B. Lieberman

Introduction

Mouse embryonic stem cells that have been deleted for Mrad9 are sensitive to gamma-rays and hydroxyurea.1 To define the functional domains within the Rad9 protein that promote radiation resistance and hydroxyurea resistance, mouse Mrad9-/- ES cells containing HRAD9 mutant cDNA plasmids were assessed for survival after exposure to these two DNA damaging agents.

The regions of the HRAD9 protein chosen for this study, which are illustrated in Figure 1, are evolutionarily conserved sites (S2, S4, and S5) or are regions with a known function (16-30 and 52-92).2 S2, S4, and S5 are evolutionarily conserved among four organisms (Homo sapiens, Mus musculus, S. pombe and S. octosporus). Sites 16-30 and 52-92 are respectively an apoptosis-related BH3 domain and a region demonstrated to encode 3’ to 5’ exonuclease activity.4 These five regions were changed by site directed mutagenesis. The conserved sites of S2, S4, and S5 were changed to alanine whereas the 16-30 and 52-92 regions were completely deleted.

Results

As shown in Figure 2 (A, B, and C), 4 of the 5 mutant cDNAs affected radioresistance. Mrad9+/- mouse ES cells expressing HRAD9-S5 showed wild-type levels of radioresistance. Expression of HRAD9-S2 and HRAD9-S4, on the other hand, made the mutant cells gamma-ray sensitive, relative to Mrad9+/- cells at 4Gy and 8Gy. Cells containing HRAD9-16-30 or HRAD9-52-92 were very sensitive to gamma-rays, relative to Mrad9+/- cells.

To define regions of the protein important for providing resistance to hydroxyurea, cells containing HRAD9 mutant cDNAs were transferred to medium containing hydroxyurea. As shown in Figure 2D, all 5 mutant cDNAs were sensitive to hydroxyurea, relative to Mrad9+/- cells.

Fig. 1. Comparison of amino acid sequence of human, mouse, S. pombe, and S. octosporus rad9 proteins.2 S2, S4, and S5 indicate sites which are evolutionarily conserved. Dashed lines: sites changed to alanine. Red boxes: sites deleted.
cDNA plasmids were assessed for sensitivity to this chemical, as shown in Figure 3 (A, B, and C). HRAD9-S5 conferred wild-type levels of hydroxyurea resistance upon mouse Mrad9-/- ES cells. In contrast, cells expressing HRAD9-S2 or HRAD9-S4 demonstrated hydroxyurea sensitivity at a level equivalent to Mrad9-/- cells. Cells containing HRAD9-16-30 or HRAD9-52-92 were as sensitive to hydroxyurea as Mrad9-/- cells.

Summary
HRAD9-S5 conferred wild-type level survival to Mrad9-/- ES cells after exposure to gamma-rays or hydroxyurea. This suggests that the altered HRAD9-S5 site is not important for promoting radiosensitivity or hydroxyurea resistance. HRAD9-S2 and HRAD9-S4 conferred an intermediate level of survival upon the Mrad9-/- ES cells for gamma-rays when compared to Mrad9+/+ and Mrad9-/- ES cells not bearing any HRAD9 plasmids. Cells expressing HRAD9-S2 or HRAD9-S4 are sensitive to hydroxyurea at a level similar to Mrad9-/- cells. This suggests that the S2 and S4 sites have different roles in promoting resistance to gamma-rays compared to hydroxyurea. HRAD9-16-30 and HRAD9-52-92 conferred mutant levels of survival to Mrad9-/- ES cells for both gamma-rays and hydroxyurea treatment. This indicates that 4 of the 5 targeted groups of amino acids play a role in promoting resistance to both radiation and hydroxyurea, but some have a more critical function than others.

References
Human RAD9 Binds In Vitro and In Vivo to a Palindrome Motif in the Cox-2 Promoter

Xiaojian Wang, Kevin M. Hopkins, Yuxin Yin and Howard B. Lieberman

Introduction
Mammalian Rad9, i.e. human hRAD9 and mouse Mrad9, participates in multiple biological processes. The protein plays important roles in cell cycle checkpoint control, DNA damage repair and the maintenance of genomic integrity. In addition, human and mouse Rad9 bear a BH3-like domain and serve as mediators of apoptosis.

Studies of hRAD9 demonstrated that the protein can bind to the p53 consensus DNA-binding sequence in the p21 promoter, and acts as a transcription activator of the gene, just like p53. It is well known that p53 has many downstream target genes in addition to p21, and Cox-2 is one of them. p53 upregulates Cox-2 in esophageal and colon cell lines. Interestingly, a recent study examined by PCR with primer pairs spanning the palindrome sequences used were:

Wildtype: CTTCTTTTCTGGTTGTATATATATATATATATAG
Mutation 1 (M1): CTTCTTTTCTGGTTGTATATATATATATATAG
Mutation 2 (M2): CTTCTTTTCTGGTTGTATATATATATATATAG
Mutation 3 (M3): CTTCTTTTCTGGTTGTATATATATATATATAG

Chromatin Immunoprecipitation (ChIP)
ChIP assays were performed according to the manufacturer’s protocol with some modifications (Upstate, now part of Millipore Corporation). H1299 cells (2x10^6) were fixed with 0.5% formaldehyde at room temperature for 10 minutes, followed by washing twice with cold PBS containing protease inhibitors. As a result, DNA and protein were cross-linked. After nuclear protein extract was prepared, DNA was sheared to 200-1000bp fragments by sonication. Sample in each tube was then split into two. The first half of sheared chromatin complex was treated to reverse the cross-linking. DNA was purified by phenol/chloroform extraction and used as input DNA. The second half of the sample was diluted 10-fold with ChIP dilution buffer and then pre-cleared with salmon sperm DNA/protein A/G agarose beads (Upstate, 50% protein A, 50% protein G, pre-blocked with 5% BSA/ChIP buffer) for 1.5 hours. Sample was then incubated overnight at 4°C with monoclonal Rad9 antibody (BD Transduction Laboratory) or normal rat IgG (as a negative control). Mock ChIP sample did not undergo this last step. The next day, immunoprecipitated chromatin was purified from the chromatin/antibody mixtures by incubating with the same agarose beads for one hour, and the beads were subsequently washed several times. The DNA was then eluted. After reversing the crosslink at 65°C overnight and removal of proteins by proteinase K digestion, DNA was purified and examined by PCR with primer pairs spanning the palindrome sequences in the Cox-2 promoter region. The primer sequences used were:

F 5’-GAGGAGAAAGGCTTCTAGATGAG-3’
R 5’-ATCTATCATGGTGATGCTCAGG-3’

Results
Palindromic sequences in the Cox-2 promoter region
The entire Cox-2 promoter is 1824 bps long. Analyses of the region revealed two overlapping palindromes (Fig. 1).

hRAD9 binds to the Cox-2 palindrome sequence in vitro and in vivo.
To determine whether hRad9 binds to the palindromic sequences in vitro, we performed EMSA by using U937 cell nuclear extract (source of hRad9) and 32bp probes containing the first palindrome motif derived from the Cox-2 promoter region. As shown in Fig. 2A, two shifted bands were visualized after incubation of the extract (lane 2). The reason that there were multiple shifted bands was described previously, i.e. the two shifted bands may contain different phosphorylated forms of hRAD9-DNA complexes or the binding of hRAD9 and other proteins in the extract. Nevertheless, these two shifted bands were eliminated by the addi-
tion of excessive unlabeled wild type oligonucleotide as competitor (lane 3), indicating the specificity of protein-probe binding. Furthermore, a super-shifted band was detected by adding hRAD9 monoclonal antibody into the binding mixture (lane 4), indicating the band represents a DNA/hRAD9 complex. To further characterize which part of the palindrome is critical for binding, we generated three mutated probes with M1 not carrying the first 4 base pairs, M2 not having the middle 4 base pairs, and M3 not having the last 4 base pairs of the palindrome. To our surprise, M1 and M2 showed the same binding pattern (two shifted bands) as wild type probe while M3 only showed the top shifted band (lanes 5-7). We then did the cold competition assays. This time, we used excessive unlabeled mutation probes as competitors. As expected, M1 and M2 competed in similar ways as the cold wild type probe. However, M3 could not compete as well as the cold wild type probe. Cold M3 was not able to compete for the bottom-shifted band (lanes 8-10). Overall, this indicated that M3 is lacking the tail 4 base pairs that are essential for binding.

Because hRad9 binds to the palindrome sequence in vitro, it is predicted that the protein can physically interact with the Cox-2 promoter in vivo. To test this, we performed a ChIP assay with antibody against hRad9 in the cell line H1299. As shown in Fig. 2B, the Cox-2 promoter containing the palindrome motif was amplified by PCR from input genomic DNA. The hRAD9 antibody pulled down the Cox-2 promoter. However, the Cox-2 promoter could not be precipitated by IgG antibody and the ChIP background was negative from the mock procedure. These results suggest that, in vivo, hRad9 binds to the Cox-2 promoter specifically.

Discussion

In this report, we show that hRad9 can bind both in vitro and in vivo to a palindromic motif in the Cox-2 promoter. Thus, Cox-2 may be a potential target of hRad9 for regulating transcription. Dr. Hainaut’s group detected p53 binding to the Cox-2 promoter in vivo by ChIP assays6. However, preliminary experiments failed to detect p53 binding to the palindrome motif in vitro, indicating that p53 may bind somewhere else in the Cox-2 promoter region and coordinate Cox-2 transcription from a different location.

Fig. 1. Two overlapping palindromes are found in the Cox-2 promoter region. The diagram shows the hairpin structures. Numbers correspond to those from the NCBI website. Start codon is numbered 1825.

Fig. 2. Physical interaction of hRad9 with the Cox-2 promoter in vitro and in vivo. A. Testing the binding of hRad9 protein to oligonucleotides bearing the palindrome motif. Arrows, shifted, super-shifted and free probe bands. EMSA was performed using U937 cell nuclear extract and 32P labeled double stranded probes as indicated in the panel. Competition and super-shift reactions were performed as described in Materials and Methods. B. Testing the physical interaction of hRad9 with chromatin containing the Cox-2 promoter by the ChIP assay. PCR using primers flanking the Cox-2 promoter region amplified both genomic DNA not undergoing immunoprecipitation (input) and the ChIP products. Gel electrophoresis was used to analyze the PCR products.

Acknowledgments

We thank Dr. Yuxin C. Liu for technical guidance related to the EMSA experiments.

References


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**TP53-Dependent Radiation Responses in the NCI60 Cell Lines**

*Sally A. Amundson*

NCI60 is a panel of human tumor cell lines representing nine tumor types (breast, CNS, colon, leukemia, lung, melanoma, ovarian, prostate and renal). The panel was established by the National Cancer Institute in the 1980’s as a tool for *in vitro* drug screening, but has grown to be much more. The NCI60 now represents the most extensively molecularly and toxicologically characterized *in vitro* cancer cell model. Well over 100,000 potential chemotherapy agents have been tested in these cell lines, and molecular information is available for the mutation status of many oncogenes as well as global profiles of mRNA, miRNA and protein expression, comparative genomic hybridization and global DNA methylation studies.

Despite the extensive toxicological information available on the NCI60 cell lines, there has been relatively little study of their radiation responses. In an early study characterizing the TP53 tumor suppressor pathway in these cell lines, however, several aspects of radiation-induced cell cycle delay and the relative radiation-induction of three known p53-regulated genes were reported. The same study also determined the TP53 status of each of the cell lines using sequencing and a functional assay in yeast. We have recently completed a study that measured clonogenic survival after radiation exposure and profiled gene expression response to 8Gy gamma-rays in these cell lines.

The genes showing altered expression in at least 10% of the cell lines were analyzed further in BRB-Array Tools. A random variance t-test was used to identify genes with radiation response ratios that were significantly different in cell lines with wild-type TP53 compared to those with mutant TP53. 31 features on the array, corresponding to 27 unique genes, were found with responses to gamma-rays that differed significantly (p<0.005) as a function of cellular TP53 status (Table 1). The false discovery rate (FDR) was also calculated and found to be <10% for all genes. Many of the genes identified by this analysis, such as *CDKN1A*, *BTG2* and *DDB2*, are known to be regulated by TP53. Others, such as *PHLDA3* and *ST14*, had not been previously reported as either radiation responsive or TP53 regulated.

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<th>p-value</th>
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<td>PPMD1</td>
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<td>ST14</td>
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<tr>
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</tr>
<tr>
<td>CHML</td>
<td>Hs.654545</td>
<td>0.0028</td>
<td>0.0928</td>
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*Note:* Genes with radiation response ratios significantly different in TP53 wild-type and mutant cancer cell lines. UniGene cluster ID’s are from Build 209. Parametric p-values and FDR were calculated in BRB tools.
The distribution of radiation response ratios among TP53 wild-type and mutant cell lines is illustrated for several of these genes in Figure 1. Although responses varied considerably among the cell lines, these genes were relatively unresponsive in all mutant cell lines, with a median expression ratio close to 1. In contrast, expression of these genes in the TP53 wild-type cell lines showed much greater variability. Not all wild-type cell lines showed up-regulation of every gene, but the median response ratios were all above 1, and significantly different from those in the mutant lines (unpaired t-test with Welch's correction for unequal variance: $BTG2 \ p=0.0004$, $DDB2 \ p<0.0001$, $XPC \ p=0.0018$, $PHLDA3 \ p=0.0007$, $ST14 \ p<0.0001$, $PLK3 \ p=0.001$). The tumor cell lines of the NCI60 are quite heterogeneous, and it is likely that other abnormalities in the TP53 wild-type cell lines can also affect regulation of specific genes in response to ionizing radiation stress, such as preventing increased gene expression that would otherwise occur in the presence of activated TP53.

We have used the TP53 wild-type TK6 cell line and its TP53 knock-out derivative, NH32, in order to confirm the TP53 regulation of several of the genes identified by this analysis (Fig. 2). Expression of $PHLDA3$ and $PLK3$ increases with increasing dose in the presence of wild-type TP53. However, in the same genetic background lacking only TP53, only minimal elevations above control levels are seen even after high doses.

There is still some question whether TP53 deficiency makes tumors resistant to ionizing radiation, or if it may actually sensitize cells to killing. It was therefore also of interest to compare clonogenic survival of the NCI60 cell lines with and without functional TP53 (Fig. 3). The overall distributions of surviving fractions were similar among wild-type and mutant cell lines at all doses tested, and the mean surviving fractions did not differ as a function of TP53 status.
Histone H2AX is a Critical Factor for Cellular Protection against Genotoxic Agents

Jarah A. Meador, Munan Zhao, Yanrong Su, Charles R. Geard and Adayabalam S. Balajee

Histone H2A variant H2AX is a dosage dependent suppressor of oncogenic chromosome translocations. H2AX plays a critical role in DNA double strand break repair but its participation in other DNA repair pathways is not known. To evaluate the role of H2AX in repair pathways other than DSB, a clonogenic survival assay was performed in H2AX proficient and deficient ES cells following treatment with two monofunctional alkylating agents: Methyl methane sulfonate (MMS) and N-methyl-N’-nitro-N-nitosoguanidine (MNNG). Mouse embryonic stem (ES) cells differing in the functional status of H2AX [wild type (+/+), heterozygous (+/-) and null (-/-)] were generously provided by Drs. C.H. Bassing and F.W. Alt (Department of Genetics, The CBR Institute for Biomedical Research, The Children’s Hospital, Harvard Medical School, Boston, MA 02115, USA). Clonogenic survival was performed after irradiation with γ-rays to characterize and confirm the genotype of the cells used in the present study. In accordance with previously published reports,1, 2 H2AX null cells were found to be 6 and 8 fold more sensitive than H2AX heterozygous and wild type cells respectively at the highest dose (4Gy) of γ-rays (Fig.1A) employed in this study. The sensitivity of H2AX proficient and deficient cells to monofunctional alkylating agents was next analyzed by a clonogenic survival assay. Cells in exponential growth phase were treated with different concentrations of MMS and MNNG for 90 min, followed by incubation in drug free complete medium for 7-10 days. Cells deficient in H2AX were found to be markedly sensitive to different concentrations of both MMS and MNNG (Fig.1B and C). The LC50 value (the concentration that kills 50% of the cells in clonogenic survival assay) for MMS and MNNG was found to be 0.25mM and 1.25µM respectively for H2AX null cells as compared to 1mM and 3.1µM for H2AX proficient wild type cells. In addition, the effect of MNNG on cell proliferation was determined using the CyQuant assay (Fig.1D). Treatment of H2AX proficient cells with 5µM and 10µM of MNNG resulted in a reduction of proliferation by 18.5% and 21.9% respectively. In contrast, proliferation was greatly reduced by MNNG at both concentrations in H2AX null cells (49.7% for 5µM and 66.4% for 10µM). H2AX heterozygous cells showed an intermediate response in proliferation after 5µM (35.1%) and 10µM (46.4%) of MNNG treatment. The results showed a clear dosage dependent effect of H2AX on the proliferation potential of cells after treatment with 5 and 10µM of MNNG.

In contrast to wild type cells, H2AX deficient cells also displayed extensive apoptotic death due to a lack of cell cycle arrest at the G2/M phase. Lack of the G2/M checkpoint correlated well with increased mitotic irregularities involving anaphase bridges and gross chromosomal instability observed in H2AX null cells. The increased mitotic irregularities observed in H2AX deficient cells illustrate an important protective role for H2AX in mitotic cell integrity against alkylation DNA damage. Observation of elevated PARP-1 cleavage mediated by activation of caspases 3 and 7 further suggests that MNNG-induced apoptosis in H2AX deficient cells.

References
cells occurs by PARP-1 dependent manner. Consistent with this, increased activities of PARP and poly (ADP) ribose polymer (PAR) synthesis detected in both H2AX heterozygous and null cells suggest a role for H2AX in the regulation of PARP-1 activity. This interesting molecular interplay between H2AX and PARP-1 is currently under investigation.

Our study has also unraveled a novel molecular link between the H2AX and MAPK pathways. H2AX deficient cells showed impaired activation and persistence of two of the MAPK family members, ERK1 and ERK2. Phosphorylated levels of both ERK1 and ERK2 proteins were greatly reduced with increasing concentrations of MNNG in both heterozygous and null cells at both recovery times (3hr and 24hr). However, the total cellular levels of unmodified ERK1 and ERK2 proteins did not show any alterations in all the three cell lines. This observation clearly indicates that H2AX is critical for the optimal activation and persistence of the MAPK pathway after alkylation DNA damage. Interestingly, a clear H2AX dosage dependent effect was observed in the phosphorylated level of ERK1 and ERK2 even in undamaged cells. Our study highlights the importance of H2AX in the regulation of the MAPK signal transduction pathway. Elucidation of the precise molecular link between MAPK and H2AX awaits further investigations.

In addition to G2/M checkpoint deficiency, an in vitro base excision repair assay demonstrated the reduced ability of H2AX null cells to excise some of the oxidatively modified base lesions. Collectively, our novel study demonstrates that H2AX, similar to PARP-1, confers cellular protection against alkylation induced DNA damage through regulation of both cell cycle checkpoint and base excision repair activities. Therefore, targeting either PARP-1 or histone H2AX may provide an effective way of maximizing the chemotherapeutic value of alkylating agents for cancer treatment.

References


Environmental Mutagens Induced Transversions but not Transitions in the Regulatory Region of Mitochondrial DNA

Michael A. Partridge, Sarah Huang, Muhammad G. Kibriya, a Habibul Ahsan, a,b Mercy M. Davidson c and Tom K. Hei

Introduction

A major goal of our research has been to identify the mechanisms by which a variety of known carcinogens cause nuclear mutations in order to better understand the process of oncogenic development. 1-5 Mitochondria, the energy generating organelles in the cell, have been proposed as a potential mediator of nuclear mutations, in part due to the high level of ROS generated during oxidative phosphorylation. 6 In support of this view, a number of studies have identified mitochondrial DNA (mtDNA) point mutations in cancer cells or cultured cells exposed to known nuclear mutagens. 7-10 Importantly, there has recently been criticism of some previous findings of mtDNA mutations in diseased cells on methodological grounds. In addition, the hypothesis that point mutations in the regulatory region of mtDNA is causative or predictive of cancer, has also been questioned. 11-14 Consequently, our goal was to analyze, using a simple sequencing method designed to reveal heteroplasmic and homoplasmic mtDNA mutations, whether exposing cells to known nuclear mutagens that in most cases also cause increases in ROS, would increase the background level of point mutations in mtDNA. The agents tested included γ-radiation, UV-radiation, asbestos and arsenic. We used a variety of normal cell lines, both human and animal and we also obtained mtDNA from a human population naturally exposed to arsenic through drinking water in Bangladesh. 15

Results

We first determined whether there was an increase in the number of heteroplasmic base changes in mtDNA extracted from cell cultures exposed to various known nuclear mutagens. By sequencing heteroplasmic segments of mtDNA directly we were able to reduce the number of potential errors identified in previous studies. 14 Electropherograms of homoplasmic and heteroplasmic sequences were examined where they differed from the reference sequence and all bona fide base changes were compared with known

Table 1. Incidence of Heteroplasmic mtDNA Base Changes in Cultured Cells Exposed to Known Nuclear Mutagens.

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<th>Samples</th>
<th>Base Pairs</th>
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<tr>
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<tr>
<td>Arsenic</td>
<td>54</td>
<td>31428</td>
<td>36 (6)</td>
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<tr>
<td>UV Radiation</td>
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<td>4284</td>
<td>4 (0)</td>
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<td><strong>CHO Coding Region</strong></td>
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<tr>
<td>Untreated</td>
<td>11</td>
<td>8250</td>
<td>10 (0)</td>
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<tr>
<td>Ionizing Radiation</td>
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<td><strong>SAEC Regulatory Region</strong></td>
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<tr>
<td>Asbestos - 4ug/cm²</td>
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</table>

*Number of transversions are given in parentheses. Genomic DNA was isolated from cells and the regulatory region of human and hamster mtDNA amplified (25 cycles) using a high fidelity polymerase (Ex Taq, TaKaRa, Japan) with the following primers: human, 15938 Sense CCTTTTTCCAAGGACAAAATCAGAG and 541 antisense GTATGGGGTTAGCAGCGGTGTGT; hamster, regulatory region -15775 Sense CCAGGCATCTGGTTCTTTC and 120 antisense GCATGTGTAACCATACCTTC, coding region - 2644 Sense CAAGATAAGGGTTTATTAGGGTG and 3499 antisense GTTGCTTAAGTATAAAATTAGTAG. The PCR products were purified and cloned into a TA-cloning vector (Invitrogen). Colonies were screened for insertion of the mtDNA fragment by PCR and the resultant product was sequenced using an ABI 3100 capillary sequencer. Sequence data was analyzed using ChromasPro software (Technelysium, Australia). Cells were exposed to the following mutagens: 1 µg/mL arsenic for 5 days; 10 Gray γ radiation from a GammaCell 40 137Cs irradiator (dose rate, 0.82Gy/min) of Columbia University; 10-J/m² UV-C light; crocidolite (µg/cm²) asbestos for 3 days.
polymorphisms. Using this method, we were readily able to definitively identify numerous heteroplasmic base changes in cells treated with agents such as γ-radiation, UV-radiation, asbestos and arsenic. However, when we compared the number of mutations in exposed groups to the unexposed controls, there was no statistically significant difference (by Chi squared tests) in the number of base changes, either transitions (purine → purine; pyrimidine → pyrimidine) or transversions (purine ↔ pyrimidine), for any of the environmental agents tested (Table 1).

Secondly, we wanted to determine whether changes in proliferation rate of cells in culture or the longer exposure times in human studies affected the mutation rate of mtDNA. Using lymphocyte DNA samples from a human population exposed to arsenic from drinking water, we determined the homoplasmic sequence of the regulatory region from 47 samples (~1100 bp each). Sixty eight unique homoplasmic mutations were identified among the 47 individuals tested. However, there was no correlation between the number of mutations (either transitions or transversions) in each individual and the level of arsenic exposure. Furthermore, all but three of the 68 base changes (C330T, T466C and T504C) have been previously reported as polymorphisms in the human mtDNA control region. We also sequenced a small protein coding region of mtDNA in lymphocyte samples from the Bangladesh study population. Analysis of a ~520 bp region from 11 individuals encoding the tRNA-Leu and NADH1 gene revealed only one C-T transition at nt3645, a silent polymorphism that has been reported previously.

In contrast, when we compared mtDNA point mutations to smoking status among the arsenic exposed subjects, transversions, but not transitions, were significantly more common amongst smokers (Table 2). These results suggested that base substitutions per se were not an indicator of an increased rate of mutation. Only changes in the number of rare base transversions may be indicative of mutagenesis, at least in the regulatory region. It should be noted that although the observed transversions were all known polymorphisms, the difference between smokers and non-smokers was highly significant (p=0.001), and given the number of reported mtDNA base changes in the regulatory region, it is perhaps not surprising that identification of new base changes is rare.

Discussion
Recent research has questioned whether mtDNA mutations in the regulatory region are causative or predictive of cancer. In our studies using a direct sequencing approach, we were unable to detect an increase in heteroplastic base changes in cells exposed to a variety of known nuclear mutagens. In addition, we did not find any affect of arsenic exposure on homoplastic point mutations in a human population. However, our study revealed an increase in rare transversions in smokers from the arsenic-exposed population. It should be noted that the smokers were, on average, five years older than the non-smokers, and it is known that an increase in mtDNA mutations is correlated with age. However, the number of mutations did not differ by age groups and because of small numbers we could not make formal age-adjusted analyses. Regardless of whether the increase in mtDNA mutations was related to age or smoking status or a combination of both, transversions were the only changes that were significantly different between the two groups (smokers vs. non-smokers).

One possible reason that total mtDNA base changes were not correlated with exposure to mutagenic agents is that a high rate of spontaneous base transitions in mtDNA would obscure any increase that could be attributed to external mutagens. Interestingly, studies that have measured base substitutions in mtDNA show that there is an overwhelming bias favoring transitions over transversions, despite the naïve mathematical prediction that if all 12 possible base changes were equally probable, transversions would be twice as common as transitions. Our finding that only rare base transversions were associated with exposure to cigarette smoke or age (both risk factors for cancer) would be consistent with this and suggests that only transversions may be a reliable marker of increased point mutations in mtDNA in cancer.

In addition to point mutations, the role of mtDNA deletions in cancer is also unclear. Although it is well known that the incidence of deletions correlates with age, as does cancer, and that exposure to a range of known nuclear mutagens increases the incidence of mtDNA deletions in cultured cells and in human studies, the role of deletions in carcinogenesis is unknown. Interestingly, data suggests that although normal tissues show an increased incidence of mtDNA deletions after exposure to mutagenic agents, cancer cells may actually have a reduction in deletions compared to surrounding tissues. Therefore, the precise role of mtDNA mutations, both point mutations and deletions, in cancer is still not understood. However, there is increasing evidence that the total number of point mutations in mtDNA (transversions plus transitions), is not predictive or causative of carcinogenesis.

### Table 2. Incidence of Homoplasmic Transitions and Transversions in Regulatory Region mtDNA of Smokers Versus Non-Smokers in an Arsenic Exposed Human Population.

<table>
<thead>
<tr>
<th></th>
<th>Smokers</th>
<th>Non-Smokers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transversions</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Total Base Changes</td>
<td>19</td>
<td>49</td>
</tr>
<tr>
<td>Number of Subjects</td>
<td>13</td>
<td>34</td>
</tr>
<tr>
<td>Average Age (± SD)</td>
<td>36.2 ± 6.0</td>
<td>30.9 ± 5.7</td>
</tr>
<tr>
<td>Average Urinary Arsenic</td>
<td>275 ± 202</td>
<td>376 ± 325</td>
</tr>
</tbody>
</table>

1 Chi squared p<0.001.
2 Student’s t-test p<0.012.
3 Student’s t-test p<0.22.

Blood samples were obtained and processed as described previously. DNA was isolated using the QIAamp 96 DNA Blood Kit (Qiagen) according to the manufacturer’s instructions. The regulatory region of human mtDNA was amplified with the following primers: 15938 Sense CCTTTTTCCAAGGACAATCAGAG and 541 antisense GTATGGGGTTAGCAGCGGTGTGT. The PCR products were sequenced and the data were analyzed using ChromasPro software (Technelysium, Australia).
References


BigH3 Protein Expression as a Marker for Breast Cancer

Gloria M. Calaf, a C. Echiburú-Chau, a Yongliang Zhao and Tom K. Hei

The current hypothesis of tumorigenesis in humans suggests that cancer cells acquire their hallmarks of malignancy through accumulation of advantageous gene activation and inactivation over long periods of time.1 In breast cancer development, this multistep process may manifest itself as a sequence of pathologically defined stages. It is accepted that breast cancer initiates as the premalignant stage of atypical ductal hyperplasia, progresses into the preinvasive stage of ductal carcinoma in situ, and culminates in invasive ductal carcinoma. Tumor grade has been a highly valuable prognostic factor for breast cancer. High-grade ductal carcinoma in situ lesions are associated with poor clinical outcome. BigH3 was first detected in a human lung adenocarcinoma cell line after stimulation by TGF-Beta,2 then in human tissues such as cornea, skin, lung, bone, bladder, and kidney (schor) as well as involved in certain human diseases such as corneal dystrophies,3,4 melorheostosis, osteogenesis,5 diabetic angiopathy, athero-thrombosis and restenosis.6 For breast cancer development, this multistep process may manifest itself as a sequence of pathologically defined stages. Tumor grade has been a highly valuable prognostic factor for breast cancer, as poorly differentiated, high-grade ductal carcinoma in situ lesions are associated with significantly poorer clinical outcome.7-10

The aim of this work was to investigate BigH3 protein expression changes in relation to breast cancer progression, in comparison to benign specimens by tissue microarray technology, to assess its potential as a marker for breast cancer. A commercially available slide with tissue microarray sections was used to evaluate 192 patients with breast cancer undergoing primary surgery. Tissues were classified for histological types and tumor grading according to the World Health Organization classification. Images were computerized and data generated were analyzed by using an Olympus CX31 binocular microscope (40X) connected with a Motic MCCamera (2.0 megapixel; MC2001 interface). Motic Image Plus 2.0 ML software was used. Paint Shop® Pro® was used to measure BigH3 protein expression level. Female breast cancer patients had a mean age of 63.98 years, disease stages ranged from 0 to I, II, III and IV, and pathological characteristics of breast tissues ranged from benign lesions to breast cancer. Results indicated a significant (P<0.05) decrease in BigH3 protein expression from benign tissues to in situ ductal carcinoma, lobular carcinoma, infiltrating ductal carcinomas, carcinomas, scirrhous carcinoma, adenocarcinomas to infiltrating colloid carcinomas. Figure 1 shows (A), the tissue microarray, (B) the BigH3 protein expression and (C) the computerized images. The intensity of BigH3 protein expression in infiltrating ductal carcinomas was decreasing from stage I to stage IV. Figures 2 and 3 correspond to the average and standard error of BigH3 protein expression from representative images of the breast samples. These studies confirmed the effect of BigH3 gene expressed in other tissues and cell lines11,12 and its role as a marker for breast cancer progression.

Fig. 1. A. Immunohistochemical images that represent BigH3 protein expression in tissue microarray. B. Immunohistochemical images of BigH3 protein expression present in the array of the slide. C. Computerized images of BigH3 protein expression.

Fig. 2. The bars represent the average and standard error of an arbitrary unit of relative luminescence of BigH3 protein in benign lesions and breast tumors found in the tissue microarray. Abbreviations: b, benign tissues; ic, in situ ductal carcinoma; lc, lobular carcinoma; idc, infiltrating ductal adenocarcinoma; c, carcinoma; sc, scirrhous carcinoma; a, adenocarcinoma; icd, infiltrating colloid carcinoma.

* Institute for Advanced Research, Tarapaca University, Arica, Chile
The ability of cells and organisms to adapt to stressful situations has been acknowledged for many years. An adaptive response for ionizing radiation was demonstrated by Wolff and colleagues using cytogenetic endpoints. Other studies have examined mutation and oncogenic transformation. Some studies supported the data for an adaptive response, while others demonstrated that the adaptive response was not universal. That is, some individuals were not capable of showing such a response. The variable finding of radio-adaptive responsiveness can perhaps be related to individual genetic make-up in the ability of genes for DNA damage signaling and response pathways to recognize and respond to stress. This would be particularly pertinent for genes in a heterozygous state.

To address this issue for some genes known to be involved in tumorigenic phenotype in asbestos treated immortalized human bronchial epithelial cells, mouse embryo fibroblasts were obtained from both wild type mice as well as those heterozygous for the atm gene. These cells were analysed for initial and delayed chromosomal damage following exposure to low doses of low LET X-rays followed by treatment with pro-mutagens. It was found that the ability of these cells to adaptively respond to radiation was dependent on their genetic background, with wild type mice showing a greater ability to adaptively respond compared to the heterozygous atm mice. This suggests that the variable finding of radio-adaptive responsiveness may be related to individual genetic make-up in the ability of genes for DNA damage signaling and response pathways to recognize and respond to stress. This would be particularly pertinent for genes in a heterozygous state.
by higher doses of high LET track segment alpha-particles. Frequencies of chromosomal changes were assessed using PCC induction and Giemsa staining at different times post irradiation from near-immediate (24 hours post irradiation), to 7 days post irradiation.

The data for chromatid and chromosome type aberrations at 24 hours post irradiation are presented in Tables 1 and 2. As can be seen, there were no significant differences between cells that received the low LET adaptive dose followed by the alpha-particle dose and the populations that were exposed to only alpha-particles.

Frequencies of chromatid and chromosome type aberrations observed at 7 days post irradiation are presented in Tables 3 and 4. As can be seen from Table 3, while there were no differences in chromatid aberrations in populations that received only X-rays, there seemed to be some decrease in the yields of aberrations in the cells that received the adaptive dose followed by the alpha-particle dose. At 7 days, 21% of the cells that were irradiated with only alpha-particles demonstrated chromatid aberrations. These frequencies were reduced to almost half in the populations that received both X-rays and alpha-particles. This reduction did not appear to be dependent on the X-ray dose delivered. In addition, there appeared to be a slight decrease in the chromosome aberration frequencies in the 0.03Gy X-ray + 1Gy alpha-particle population when compared to the other two populations.

Taken together, the data presented here suggest that while the adaptive response may not contribute to the reduction of chromosomal damage in atm heterozygous populations for damage induced immediately following the irradiation, the phenomenon may play a role in the development of chromosomal instability (as seen by the decreased yields of chromatid aberrations) at later times.

**Table 1:** Chromatid-type aberrations in atm heterozygote cells at 24 hours post-irradiation

<table>
<thead>
<tr>
<th>X-rays</th>
<th>0 Gy</th>
<th>1 Gy</th>
<th>X-rays</th>
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<tr>
<td>0 Gy</td>
<td>0.13</td>
<td>0.4</td>
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<td>17</td>
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<td>0.03 Gy</td>
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**Table 2:** Chromosome-type aberrations in atm heterozygote cells at 24 hours post-irradiation

<table>
<thead>
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<th>X-rays</th>
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<tr>
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<td>2.9</td>
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**Table 3:** Chromatid-type aberrations in atm heterozygote cells at 7 days post-irradiation

<table>
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<tr>
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<td>0.15</td>
<td>0.12</td>
<td>0.1 Gy</td>
<td>15</td>
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**Table 4:** Chromosome-type aberrations in atm heterozygote cells at 7 days post-irradiation

<table>
<thead>
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<th>Aberrations/Cell</th>
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<tr>
<td><strong>X-rays</strong></td>
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<td>1 Gy</td>
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<tr>
<td><strong>α-particles</strong></td>
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<td>1 Gy</td>
<td>0.66</td>
</tr>
<tr>
<td><strong>X-rays</strong></td>
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</tr>
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</tr>
<tr>
<td><strong>α-particles</strong></td>
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<td>0.1 Gy</td>
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<tr>
<td>1 Gy</td>
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**References**


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**High Sensitivity Antigen Detection using Luminescence Substrates in ELISA**

*Michael A. Partridge and Tom K. Hei*

**Introduction**

After a number of years of use, the old plate reader in room 223 in the Center for Radiological Research was replaced in July 2007 with a new BioTek Synergy™ HT Multi-Detection Microplate Reader. In addition to having greatly improved capabilities for fluorescence and absorbance measurements (and a vastly improved software interface), the new machine also has the capacity to read luminescence signals.

Of the three detection techniques, measuring absorbance of colorimetric substrates is probably the most commonly used, and in some respects the most limiting. Fluorescent substrates allow for a greater dynamic range, but do not confer a significant improvement in sensitivity compared to colorimetric substrates. Luminescence detection, however, has been reported to provide an order of magnitude improvement in sensitivity over absorbance-based detection, potentially a tremendous advantage for assaying molecules that are present at very low concentrations in biological samples.
Results and discussion

In order to test the level of sensitivity obtained with luminescent substrates using the new BioTek plate reader, preliminary experiments were performed using standards provided in commercially available ELISA kits. Using white plates to eliminate leaching of luminescence from adjacent wells, we were successfully able to generate a standard curve with IL1-α as the antigen simply by substituting standard chemiluminescence reagent used for western blotting as the substrate in the ELISA (Figure 1A).

However, the measurements generated in these experiments with luminescent substrates displayed a high variability among replicates (Figure 2A). This was likely explained by the substantial row-to-row variation that was observed when using luminescence assays. Interestingly, the readings in alternate rows were uniformly higher or lower than those obtained in adjacent rows (Table 1). This suggested that the method by which the machine read the plates, in a back and forth, bi-directional, format (A1-A12, B12-A1, etc), may have resulted in alternatively higher or lower measurements in each row, a result that was particularly apparent at very low antigen concentrations.

In order to fix this problem, the machine had to be programmed to read only in one direction (A1-A12, B1-B12, etc). However, the reader is configured to read an entire plate in a bi-directional pattern. Fortunately, the software was flexible enough so that the machine could be directed to perform 8 discrete measurements all in the same direction: Read #1, A1-A12; Read #2, B1-B12, etc. Microsoft Excel could then present the data from the 8 individual reads as 1 complete plate. Using this approach, the standard deviation for replicate measurements was dramatically reduced, in some cases by an order of magnitude (Figure 2B).

When measurements obtained using colorimetric substrates were compared back-to-back with luminescence measurements acquired with the unidirectional reading protocol, luminescence did provide a substantially greater degree of sensitivity. For colorimetric ELISA readings, the first measurable change in average absorbance was between 0.457 and 1.37 pg/mL IL1-α antigen concentration (Figure 1B). Readings below 1.37pg/mL were not statistically different from zero antigen controls. In contrast, there was a small (17%) but significant (p<0.05) increase in ELISA signal between 0.051 and 0.152pg/mL IL1-α antigen using chemiluminescent substrates obtained with a unidirectional reading protocol, an antigen concentration 9-fold lower than could be discriminated between when using colorimetric substrates (Figure 1B and 2B).

When using a bi-directional reading protocol, the average...
The luminescence ELISA response did increase for each data point with increasing concentration of IL1-α from 0.051 pg/mL. However, because of the high variation between replicates observed with this reading method, the change in ELISA signal obtained at the lowest antigen concentration (0.051 pg/mL) was not statistically different from the signal obtained from an IL1-α concentration 27-fold higher (1.37 pg/mL) (Figure 2A). These data clearly demonstrate not only the advantage of using the unidirectional reading protocol, but also emphasize the necessity to reduce the noise in an assay, as the variability observed with the bidirectional reading method rendered the luminescence ELISA less sensitive than the colorimetric assay. Assay noise can be generated by both variability in replicates and by background signal, usually defined as the response obtained with zero antigen. The increased sensitivity of detection obtained when using luminescence substrates in ELISA with the unidirectional reading protocol may provide a critical advantage where the sample size is limiting, such as with biological materials, or where the concentration of the target antigen in the sample is very low.

Materials and methods
Antibody pairs used in sandwich ELISAs for this study were from Biorad (Camarillo, Ca). Clear (colorimetric) or white (luminescence) microwell plates (Maxisorp, Nunc) were coated with 100 µL of capture antibody overnight at 4°C. For blocking, 200 µL of 3% bovine serum albumin (BSA) in PBS was added to wells (1 h at 20°C). Antigen was diluted in 2% BSA in PBS containing 0.05% Tween 20 (PBST) and 100 µL added to the wells followed immediately by addition of biotin-conjugated detection antibody, diluted in 50 µL of 2% BSA-PBST. After a 90 minute incubation at 20°C with shaking, plates were washed (4x PBST) and streptavidin-HRP was added, diluted in 100 µL of 2% BSA-PBST. Following 1h incubation at 20°C, microwells were washed, and 100 µL of either tetramethylbenzidine (TMB, Pierce) for colorimetric measurements, or chemiluminescent substrate (Pierce) for luminescence measurements was added. Colorimetric reactions were incubated for 15-30 mins at 20°C, then stopped by addition of 0.9 M H2SO4 (50 µL) and product absorbance was determined at 450 nm. Glow luminescence was determined using a photomultiplier tube detector (BioTek, Vermont) after 10-15 minute incubation.

References

Nuclear PTEN is a New Guardian of the Genome

Wenhong Shen, Adayabalam S. Balajee, Jianli Wang and Yuxin Yin

PTEN PTEN is one of the most frequently mutated tumor suppressor genes in human cancers. PTEN is a lipid phosphatase that inactivates the PI3-kinase/AKT pathway in the cytoplasm. We have revealed a novel role of PTEN in maintaining chromosome integrity via multiple mechanisms in the nucleus. PTEN deficiency leads to spontaneous chromosomal instability. The most remarkable feature of PTEN null metaphases is centromeric breakage. We found that PTEN is located at centromeres and physically associated with a centromere protein, CENP-C, an integral component of the kinetochore that forms a functional centromere. Interestingly, the phosphatase activity of PTEN is dispensable but the C-terminus of PTEN is necessary for its association with centromeres. We observed that C-terminal PTEN mutants derived from Cowden syndrome lose their association with CENP-C, although they retain an intact phosphatase domain (Figure 1A and 1B). As a result, Cowden-derived lymphoblastoid cells exhibit severe chromosomal aberrations, including premature centromere separation (PCS) and centromeric breakage (Figure 1C-1F). The frequency of chromosomal aberrations is much higher in PTEN null breast cancer cells compared to normal human mammary epithelial cells (Figure 2A-2D). In addition, introduction of cancer-prone PTEN mutants into normal cells disrupts the physical association of PTEN with centromeres and causes massive centromeric breakage (Figure 2E-2J), suggesting that these C-terminal mutants act in a dominant-negative fashion and interrupt the normal function of wild-type PTEN in centromere stability. Our results demonstrate that PTEN plays an essential role in maintaining centromere stability through physical interaction with CENP-C. Another new aspect of PTEN function is that PTEN controls DNA repair. We show that disruption of PTEN reduces Rad51 expression and results in spontaneous accumulation of DNA double strand breaks.
breaks (DSBs). Introduction of PTEN into PTEN null cells elevates the levels of Rad51 transcripts. We show that PTEN acts on chromatin and regulates transcription of Rad51. Ectopic expression of either PTEN or Rad51 in PTEN null cells suppresses the incidence of spontaneous DSBs, suggesting that the PTEN-Rad51 signaling cascade constitutes an essential DSB repair pathway. Our findings establish the fundamental role of nuclear PTEN in the maintenance of chromosomal stability through multiple mechanisms.

**MOLECULAR STUDIES**

**Fig. 1.** Loss of PTEN-centromere association in lymphoblastoid cells from Cowden syndrome. Lymphoblastoid cells from a patient with Cowden syndrome containing a PTEN exon 6 skipping mutation (IVS6+1 G>T) and a PTEN wild-type normal control (CCF00189) were employed for analyses of PTEN expression (A), PTEN-centromere association (B) and chromosome aberrations (C-F). (A) Cell lysates were subjected to Western blotting using a polyclonal PTEN antibody. Positions of wild-type and truncated PTEN are indicated. (B) Truncated PTEN in Cowden lymphoblastic cells disrupts PTEN association with centromere. Equal amounts of protein (500 μg) from the above described cell lysates were used for immunoprecipitation with a PTEN monoclonal antibody and subsequently for immuno-detection of CENP-C by Western blotting. (C) Cowden syndrome lymphoblastic cells exhibit centromeric aberrations. Cenomeric and telomeric FISH was performed with the normal and Cowden lymphoblastoid cells. Centromeric fragments (CF, d3/f3) and premature centromeric separation (PCS, d1/f1) were observed in PTEN mutated Cowden lymphoblastic cells. Additional insertion of the centromeric sequence was also observed in some chromosomes (d2, arrows).

**Fig. 2.** Relationship between PTEN dysfunction and centromere instability in human cells. Upper panel (A-D): Metaphase chromosome spreads prepared from normal breast epithelial cells (MCF-10A) and breast cancer cells (MB468) with a mutant PTEN were hybridized to fluorescein labeled human centromeric peptide nucleic acid probe to determine centromere associated chromosomal instability. Metaphase spreads stained with DAPI (shown in grey scale) and the combined images of DAPI and fluorescein are shown below respectively. Arrows indicate the chromosomes with centromeric abnormalities. Note the terminal location of additional centromeric DNA in two of the chromosomes (b3, b4) probably resulting from breakage and mis-rejoining of broken centromeric regions. One of the chromosomes in the metaphase spread displays the insertion or amplification of centromeric DNA (b1). Lower panels (E-J): Human normal airway epithelial cells (AEF) were transiently transfected with either U6 control vector (E), U6/PTENsiRNA vector (F), or pcDNA3/HisPTEN189 (G), respectively. Metaphase spreads from these groups of cells were hybridized with fluorescein labeled human centromeric peptide nucleic acid probe and stained with DAPI (shown in grey scale). Centromeric abnormalities are indicated with arrows (CF) and brackets (PCS).
Center for Radiological Research 2007 Departmental Pic- 
nic. (L-r): Dr. Eric J. Hall, Dr. Mei Hong and Dr. Hongning 
Zhou.

Center for Radiological Research 2007 Departmental Pic- 
nic. (L-r): Dr. Eric J. Hall, Dr. Mei Hong and Dr. Hongning 
Zhou.

Center for Radiological Research 2007 Departmental Pic- 
nic. (L-r): Dr. Eric J. Hall, Dr. Mei Hong and Dr. Hongning 
Zhou.

Center for Radiological Research 2007 Departmental Pic- 
nic. Dr. Lubomir Smilenov (left) and Dr. David J. Brenner 
(right) made special mixed wines with fruits.

Center for Radiological Research 2007 Departmental Pic- 
nic. Dr. Yanping Xu (left) and Dr. Burong Hu (right).

Center for Radiological Research 2007 Departmental Pic- 
nic. CRR members played soccer game.

Center for Radiological Research 2007 Departmental Pic- 
nic. Dr. Corinne Leloup (left) and Sarah Huang (right).

Center for Radiological Research 2007 Departmental Pic- 
nic. Anne Sutthoff (left) and Dr. Sally Amundson (right).
Ionizing radiation therapy and chemotherapy are predominantly used approaches for antineoplastic treatment. The cytotoxic effects of most usual anti-cancer drugs and ionizing radiation are mediated through pleiotropic mechanisms, which include DNA damage and the subsequent activation of DNA damage induced cell signaling pathways (both p53-dependent and p53-independent). It results in cell cycle arrest and/or in the induction of cell death by apoptosis, necrosis, autophagy or mitotic catastrophe. In this scenario, Bcl-2 family members, such as Bcl-xL, play a crucial role by neutralizing the function of p53-dependent BH3-only protein BID to transmit an apoptotic signal to the executor proteins BAX and BAK, which negatively regulate mitochondrial function. However, alternative therapies, which have been suggested for induction of apoptosis in cancer cells, are based on the direct activation of the extrinsic death signaling pathways using recombinant death ligands of the TNF superfamily, such as Fas Ligand and TRAIL. Targeting TRAIL-receptor mediated signaling pathways for induction of apoptosis is currently evaluated in multiple clinical trials for several types of cancer. Finally, combined modality treatments, which include γ-irradiation and stimulation of the TRAIL-R/Fas-mediated

**Fig. 1.** Radiation resistance of melanoma cell lines. (A, B) Clonogenic survival assay of human WM35, WM9, LU1205, HHMSX and mouse SW1 melanoma lines 12 days after γ-irradiation at doses of 1.25-5 Gy. (C) Western blot analysis of indicated proteins 6 h after irradiation. (D) NF-κB DNA-binding activity was determined by EMSA 6 h after irradiation. (E) Cell cycle-apoptosis analysis of human melanoma lines WM35 and WM9 48 h after γ-irradiation using PI staining DNA and flow cytometry. Gamma-irradiation induces the G2/M arrest of the cell cycle, while levels of apoptosis are relatively low.
Fig. 2. Inhibition of COX-2 and PI3K--AKT decreased WM35 melanoma cell survival following γ-irradiation. (A) Effects of γ-irradiation, COX2 inhibitor NS398 (50 µM) and PI3K-AKT inhibitor LY294002 (50 µM) alone or in combination on the cell cycle of WM35 melanoma. Cells were stained with PI and analyzed by flow cytometry. (B) Effects of γ-radiation and COX2 inhibitor NS398 (50 µM) alone or in combination on TRAIL-R2/DR5 and Fas surface expression. Immunostaining with anti-DR5-PE and anti-Fas-PE mAb and FACS analysis were used. (C, D) Clonogenic survival assay of WM35 and WM9 cells 12 days after γ-irradiation at doses of 1.25-5 Gy alone or in combination with either NS398 or LY294002 or recombinant TRAIL.
pathways with recombinant ligands or agonistic antibodies, appear to be important procedures to control and suppress cancer development.\textsuperscript{7,8}

Melanoma, the most aggressive form of skin cancer, is known to be highly resistant to radio- and chemo-therapeutic treatment. In the USA approximately 60,000 new cases were diagnosed and 8,100 deaths occurred in 2007.\textsuperscript{9} Numerous observations indicate that the incidence of melanoma has significantly increased over the last twenty years in the USA and worldwide. However, only limited therapies for metastatic stage of the disease are currently available. Various attempts have been made to restore high levels of apoptosis in response to treatment for this type of cancer. The main purpose of this study is to substantially increase radiosensitivity of melanoma cells by inhibiting activity/expression levels of the main regulators of cell survival, COX-2\textsuperscript{10} and AKT.\textsuperscript{11}

Effects of suppression of COX-2 and PI3K--AKT on radiosensitivity of melanoma cells have been investigated. Results of clonogenic survival assays demonstrated that melanoma cell lines used in this study exhibit differential radiation resistance, which was relatively high for SW1 mouse and HHSMS human metastatic cells, average for LU1205 human metastatic and WM35 human radial growth phase melanoma cells, and low for WM9 human metastatic melanoma cells (Fig. 1A and B). Most melanomas possess non-mutated wild-type p53, which functions as an important transcription factor controlling TRAIL-R2, FAS, BAX, BID, PUMA, NOXA and p21-WAF gene expression and mediating the cell cycle arrest and/or apoptosis.\textsuperscript{12,13} However, p53 appears not be involved in the quick executive regulation of apoptosis via p53-dependent BH3-only proteins (PUMA, NOXA) in many melanoma lines due to endogenous protective mechanisms, such as cancer-specific suppression of mitochondrial function and the mitochondrial death pathway (Ivanov and Partridge, unpublished observations). Hence, direct $\gamma$-irradiation of melanoma cells was accompanied by Ser20 phosphorylation of p53 (Fig. 1C), followed by the G2/M arrest of the cell cycle rather than pronounced p53-mediated apoptosis (Fig. 1E). Correspondingly, no procaspase-3 activation was detected (Fig. 1C). The early response to $\gamma$-radiation includes, besides p53-Ser20 phosphorylation (Fig. 1C), upregulation of NF-$\kappa$B p65-p50 DNA-binding activity (Fig. 1D), probably via the AKT-IKK pathway. NF-$\kappa$B activation is a common protective reaction of cells to stress conditions via the regulation of transcription of numerous genes controlling cell survival.

However, the apoptotic machinery of the extrinsic death

![Fig. 3. Inhibition of COX-2 and PI3K--AKT decreased LU1205 melanoma cell survival following $\gamma$-irradiation. (A). Dose-dependent effect of $\gamma$-irradiation on kinetics of LU1205 growth in cell culture. (B) Typical results of clonogenic survival assay for LU1205 cells. (C) Effects of $\gamma$-radiation on TRAIL-R2/DR5 and Fas surface expression. Immunostaining with anti-DR5-PE and anti-Fas-PE mAb and FACS analysis were used. (D) Clonogenic survival assay of LU1205 cells 12 days after $\gamma$-irradiation at doses of 1.25-5 Gy alone or in combination with either NS398 or LY294002 or recombinant TRAIL (50ng/ml).](image-url)
pathway in WM35 early phase and, especially, in WM9 metastatic melanoma cells was perfectly active based on results of recombinant TRAIL or FasL stimulation of death receptors that was accompanied by apoptosis. While WM9 cells were relatively radiosensitive, WM35 and LU1205 cells exhibit only average levels of radiosensitivity (Fig. 1A and 3A). Low radioresistance of WM9 cells correlates with low levels of active AKT, compared to WM35 (Fig.1C) or LU1205 cells (data not shown). To decrease radioresistance of WM35 and LU1205 cells, we used inhibitors of enzymatic activity of COX-2 and AKT, which were permanently active in WM35, LU1205 and WM9 cells (Fig. 1C and data

**Fig. 4.** RNAi-mediated knockdown of COX-2 expression substantially increased levels of the G2/M arrested metastatic melanoma cells and decreased cancer cell survival. (A) Suppression COX-2 expression levels by specific RNAi affect p53 (P-Ser20) basal levels in LU1205 melanoma cells. (B) Effects of COX-2 knockdown on the cell cycle in LU1205 and WM9 melanoma cells. PI staining and FACS analysis were used. (C) Effects of γ-irradiation on surface TRAIL-R2/DR5 levels in the control and COX-2 knockdown LU1205 cells. (D, E) Clonogenic survival assay of the control and COX-2 knockdown LU1205 and WM9 cells after γ-irradiation.
not shown) in combination with γ-irradiation. Inhibitors of COX2 (NS398, 50μM) or PI3K--AKT (LY294002, 50μM) were added to WM35 and LU1205 cell cultures immediately after γ-irradiation. Both inhibitors have substantial effects on increasing the G2/M arrest of irradiated WM35 (Fig. 2A) and LU1205 cells (data not shown). NS398 also had some additional effects for increasing apoptosis in WM35 cells, while LY294002, which targets PI3K-related kinases, ATM, ATR and DNA-PK, was more efficient for increasing the % G2/M arrested cells (Fig. 2A). Surface expression of death receptors was not changed for irradiated cells in the presence of NS398 (Fig. 2B) that allowed us to exclude effects of upregulation in death receptor levels on cell death. However, cell survival was substantially decreased in the presence of NS398 or LY294002, indicating probably that mixed type of slow cell death by both necrosis and apoptosis was involved (Fig. 2C). Although WM9 cells were already radiosensitive, both inhibitors notably accelerated cell death of irradiated cells (Fig. 2D). Based on our previous observation, we induced TRAIL-mediated apoptosis of irradiated melanoma cells that was very effective for WM35 cells, while TRAIL-sensitive WM9 cells exhibit only small additional benefits for induction of TRAIL-mediated apoptosis after irradiation (Fig. 2D). Results obtained with LU1205 were relatively similar when tested with WM35 cells (Fig. 3). Indeed, both cell lines possess an average level of radiosensitivity that could be further increased by combined treatment with γ-irradiation and COX-2 or PI3K-AKT inhibitors (Fig. 3A, B and C). Irradiation also increases surface expression of DR5 and FAS (Fig. 3C) and decreased cell survival after recombinant TRAIL treatment (Fig. 3C).

To obtain additional proof of the effects of COX-2 suppression on activation of p53 via the ATM-Chk2-p53(P-Ser20) pathway and the G2/M arrest, we established LU1205 and WM9 mass culture with partial suppression of COX-2 expression levels by specific RNAi (Fig. 4A and data not shown). Basal levels of total p53 and p53 (P-Ser20) were substantially increased after COX-2 knockdown (Fig. 4A). Levels of the G2/M arrest were correspondently higher in cells with COX-2 knockdown (Fig. 4B). On the other hand, clonogenic survival analysis demonstrated a substantial decrease in survival of cells with suppressed COX-2 after irradiation (Fig. 4D and E). No additional effects were detected for upregulation of surface TRAIL-R2/DR5 (Fig. 4C) or Fas expression. Similarly with results for chemical inhibition of COX-2 activity, COX-2 knockdown increased levels of mixed, mainly non-apoptotic death of melanoma cells. Hence, data obtained demonstrated pronounced effects of inhibition of COX-2 and PI3K--AKT on upregulation of killing of irradiated melanoma cells.

Although inhibitors of cell survival and recombinant TRAIL relatively similarly increased radiosensitivity of melanoma cells, there is a critical difference for cancer cells to die by necrosis or apoptosis in vivo. How will it be possible to connect γ-irradiation with predominant induction of apoptosis in melanoma cells? Is there an alternative for usage of exogenous recombinant TRAIL (Fig. 2C, D and 3D)? Our recent results demonstrate that resveratrol (RSV), a polyphenolic phytoalexin, may accelerate TRAIL-mediated death in many melanomas and, for some melanoma lines, such as SW1 and LOX, RSV by itself may serve as an efficient inducer of apoptotic death (Ivanov et al., 2008, in press).

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Increased Susceptibility of Human Small Airway Epithelial Cells to Apoptosis after Long Term Arsenic Treatment

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Introduction

Arsenic is a naturally occurring metalloid and a known human carcinogen. Millions of people are exposed to arsenic through contaminated drinking water and food. Studies have shown that long term exposure to inorganic arsenic results in an increased risk of developing skin, lung, liver, kidney, and bladder cancers.

Apoptosis, or programmed cell death, is associated with a wide variety of fundamental biological processes, including cell development, differentiation and response to injury. It has been known for some time that short term arsenic treatment can induce apoptosis in a variety of cell types, including cancer cells that are resistant to apoptosis. It remains unknown how a specific type of cells, e.g., SAE cells in this study, when treated with arsenic in such a long period, respond to apoptotic agents.

There are two principal means by which apoptosis can be initiated; via intrinsic signals mediated by the mitochondria, probably activated by oxidative stress, or via extrinsic signals mediated by receptor-ligand interactions. Signaling for both pathways converge with a group of cysteinyl-aspartate-directed proteases called caspases. These proteases degrade multiple substrates within the cell, including regulatory proteins that contribute to the progression of cell death. Caspases play a central role in apoptotic signaling and execution. Caspases are grouped into two categories: ‘initiator’ caspases (e.g. caspase-2, -8 and -9) that cleave and thereby activate various enzymes and proteins, including other caspases, and ‘effector’ caspases (e.g. caspase-3, -6 and -7) that degrade many cellular targets during apoptosis. Activation of caspase 3 is often considered as the point-of-no-return in the apoptotic signaling cascade.

One example of an extrinsic apoptosis signal is the initiation of cell death by the trans-membrane protein and death receptor Fas. Fas is expressed by a variety of cell types in the lung, including bronchiolar and alveolar epithelial cells, fibroblasts and alveolar macrophages, and has been reported to be involved in activation of various tyrosine kinases. The TNF death receptor super family induced signaling pathways were largely affected upon short term arsenic treatment. However, the detailed cellular mechanism of Fas-mediated apoptosis in long term arsenic exposed cells remains to be elucidated.

It is well known that direct exposure of cells to \( \text{H}_2\text{O}_2 \) leads to apoptosis, ROS acts as an intrinsic mediator in the process of apoptosis. We wanted to know the different effects on apoptosis caused by ROS in long term arsenic-exposed human airway epithelial cells.

PARP is involved in the maintenance of genome integrity by inhibition of cell cycle progression or the induction of apoptosis. PARP has been demonstrated to play a key role in determining whether DNA injury leads to cell recovery by repairing, or surrender and apoptosis. There is also growing evidence that PARP is required for the rapid accumulation of...
and activation of p53. All of these results imply that PARP might be an important regulator of apoptosis in bronchial and alveolar epithelial cells.

The mitogen-activated protein kinase (MAPK) pathways regulate a vast array of cellular responses, including cell proliferation, differentiation and cell death. Among the MAPK family, the signaling cascade of p38 MAPK is particularly well-known as an important mediator of apoptosis. Blocking of p38 MAPK pathways has been used in certain cell types to prevent apoptosis.

The human small airway epithelial (SAE) cells used in this study have been continuously exposed to arsenic at 0.5 µg/mL for 28 weeks and acquired prominent transformation characteristics, such as increased plating efficiency, anchorage-independent growth and genomic instability, etc. The aim of this study was to determine whether a cell that has undergone such a long term exposure to arsenic has the capacity to resist apoptosis, as exhibited by other transformed cells. A better appreciation of the process of apoptosis induction in long-term arsenic treated cells will help us to understand the mechanisms by which arsenic causes cancer, and may provide ideas for therapeutic approaches to increase apoptosis levels in arsenic induced tumors.

Results

Increased apoptosis in arsenic treated cells

The primary goal of this study was to understand how the long-term arsenic exposed/transformed small airway epithelial cells respond to apoptotic stimulation. To do this, we examined the effect of graded doses of Fas ligand (Fas L) or H2O2 on the arsenic treated (designated as SAEC-A0.5) and non-treated control SAE cells. Cells were treated with Fas L at doses between 0 to 100ng/mL to determine the survival incidence indicated by PDA-PI staining (Fig. 1A). At low doses of Fas L (20ng/mL), the survival incidence was similar in both arsenic treated and control cells. However, at high doses, arsenic treated SAE cells exhibited increased sensitivity to Fas L treatment in comparison to control SAE cells. At a dose of 50ng/mL, SAEC-A0.5 had a survival incidence that was two fold lower than the control cells.

Fig. 2. Effect of Fas ligand, H2O2 and SB 203580 on DNA content by flow cytometry. Fas ligand and H2O2 treatment dependency: Confluent SAEC and SAEC-A0.5 cells were treated with either Fas ligand (50ng/ml) or H2O2 (0.2 mM) alone for 24h, or after 30 minutes pretreatment with the p38 kinase inhibitor, SB 203580 (SB) (10µM). Cells were harvested at the indicated times and DNA content was analyzed by flow cytometry. Vertical axis: number of cells; horizontal axis: DNA content. Apoptosis levels were determined as percentage of cells with hypodiploid DNA in the pre-G0-G1 region. Shown is a representation of two separate experiments, both with similar results.
(P<0.01). Similar results were obtained when cells were exposed to graded doses of H$_2$O$_2$ (Fig. 1B). To avoid the use of cytotoxic doses of Fas L or H$_2$O$_2$ in subsequent experiments, we chose intermediate doses (Fas L at 50 ng/ml, H$_2$O$_2$ at 0.2 mM) to characterize the specific intracellular events in the pro or anti-apoptotic process.

Analysis of DNA content by flow cytometry

Having established that arsenic-exposed cells had a lower surviving incidence after exposure to H$_2$O$_2$ or Fas L, we next wanted to determine whether the cells were undergoing apoptosis. To do this, we treated cells with apoptosis inducing agents and measured the DNA content by flow cytometry. Interestingly, arsenic-exposed SAE cells (SAEC-A0.5) were more susceptible to apoptosis after Fas L or H$_2$O$_2$ treatment when compared with arsenic non-treated SAE cells, as estimated by the appearance of cells in sub G1 phase (Fig. 2). The percentage of apoptotic cells in arsenic exposed cultures were 14.7 and 33.1 upon treatment with H$_2$O$_2$ or Fas L, respectively. The corresponding levels in similarly non treated control SAE cells were 0.23 and 2.22, respectively. However, this increase in apoptosis was largely reduced by concurrent treatment with the p38 kinase inhibitor, SB 203580 (Fig. 2). Similarly, in the presence of SB 203580, the increase in apoptosis induced by H$_2$O$_2$ treatment was reduced from 14.7 to 3.6%, and from 33.1 to 18.1% for Fas L, a reduction of 4-fold and almost 2 fold, respectively.

PARP and Caspase-3 in apoptosis cleaved in SAEC and SAEC-A 0.5

Caspases (cysteine-aspartate proteases) are known to play a central role in apoptotic signaling. Cleavage of caspase 3 to its active form, and subsequent cleavage of PARP are traditional indicators of apoptosis. To ensure that the increase in cell death in arsenic exposed cells after treatment with H$_2$O$_2$ or Fas L was mediated by caspases, we examined cleaved PARP and cleaved Caspase-3 by Western-blot and by immunfluorescence microscopy. As shown in Figure 3A, after treatment with Fas L or H$_2$O$_2$, the cleaved form of PARP was readily detected spanning from 6 hours till 24 hours in arsenic exposed cells; meanwhile, the cleaved caspase 3 fluorescence images were readily observed in the

![Figure 3](image)

Fig. 3. Treatment of arsenic exposed cells with Fas L or H$_2$O$_2$ resulted in cleavage of PARP and Caspase 3. (A) SAE cells and cells exposed to arsenic for 28 weeks were treated with Fas ligand (50ng/ml) (left panel) or H$_2$O$_2$ (0.2mM) (right panel) in the indicated time points, cleaved PARP were examined by Western blot with antibodies specific for cleaved PARP. β-actin was probed as loading controls. (B) Arsenic exposed SAE cells as well as controls were plated on a glass chamber slide and treated with Fas ligand or H$_2$O$_2$, fixed, stained with antibodies to cleaved Caspase-3, and images were captured on a scanning confocal microscope.
nucleus of these apoptotic cells (Fig. 3B), a phenotype not observed in the controls.

**Induced p38 phosphorylation in arsenite treated cells upon Fas L or H2O2**

The Fas signalling pathway is involved in maintaining homeostasis in various cellular processes. A variety of cellular stresses, including oxidative stress, heat shock, and UV exposure activate p38 signaling cascades and thereby activate the apoptotic pathways. Therefore we tested whether p38 was activated after treatment with Fas L or H2O2 in arsenic-exposed cells versus control cells. Interestingly, phosphorylation of p38 was substantially induced upon Fas L stimulation in SAEC-A 0.5 cells after 10 and 20 min, and then returned to lower levels as shown in Figure 4A. At 120 min after treatment, another higher band appeared, presumably a different isoform of p38 became phosphorylated. However, this effect was not seen in control SAE cells. Analysis of p38 protein expression indicated that there was no difference between control and arsenic treated cells over the same time course.

A number of studies have demonstrated that ROS such as H2O2 plays a critical role in many disease processes as well as in the regulation of many important biologic processes. It is also well-known that cellular injury triggered by arsenic is mediated through reactive oxygen species (ROS). Therefore, we examined whether H2O2 could also induce phosphorylation of p38 MAPK in arsenic exposed cells. As shown in Figure 4B, treatment of arsenic-exposed cells with H2O2 (0.2mM) induced the phosphorylation of p38 as soon as 10 minutes post treatment. The phospho-p38 level reached a peak at 20 minutes post treatment and then decreased quickly over time. However, this effect was not seen in control SAE cells. Analysis of p38 protein showed no difference in expression level between control and arsenic treated cells.

**Discussion**

The purpose of this study was to investigate the ability of arsenic exposed human small airway epithelial (SAE) cells to respond to apoptotic agents, Fas L and H2O2, and whether they induced biological processes characteristic of apoptosis, such as cleavage of PARP and Caspasps. A large amount of evidence indicates that arsenic induces apoptosis in many cell types; this has been shown related to its disruption of DNA methylation and repairing systems. More specifically, arsenic-induced apoptosis is provoked through increased oxidative stress, caspase activation, and changes in mitochondrial membrane potential and Bcl-2 expression.

The SAE cell line used in this study was immortalized by H-TERT incorporation into the genome, which has minimal effect on the cell’s genomic integrity, and is thus an ideal model for transformation and toxicology studies. After extensive and continuous arsenic (0.5ug/ml) treatment, the cells exhibited characteristics indicative of transformation.

It has been reported that DNA repair largely depends on enzymes such as PARP, and this enzyme is inactivated through cleavage by proteases like Caspase 3, a canonical indicator of apoptotic cell death as distinct from necrosis; as anticipated, the long-term arsenic exposed SAE cells, either responding to Fas ligand or to H2O2 stimulation, cleaved PARP and Caspase 3 significantly increased compared with the controls.

JNK and p38 MAP kinases are preferentially activated by cytotoxic stresses, such as X-ray/UV irradiation, heat/osmotic shock and oxidative/nitrosative stress, as well as by pro-inflammatory cytokines such as tumour necrosis factor α (TNFα) and interleukin-1 (IL-1). Interestingly, we observed an increased activation of p38 MAPK, as shown by an enhanced p38 phosphorylation after Fas L or H2O2 treatment in arsenic treated SAE cells, as opposed to the controls; inhibition of this kinase resulted in a decrease in apoptosis, implicating that in long term arsenic-exposed cells, p38 derived signals play an important role in mediating apoptosis upon treatment with both extrinsic and intrinsic stimuli, whereas we were not able to detect any significant changes in JNK (data not shown).

One potential mechanism by which long term arsenic exposure could cause cancer is to degrade the cells’ ability to respond to apoptotic stimuli. Thus, cells incur DNA damage but do not undergo apoptosis and therefore accumulate mutations. Interestingly, other researchers have suggested just such a mechanism in human bronchial epithelial cells when exposed to cigarette smoke. Importantly, our study indicates that cells exposed to arsenic do not have a reduced response to apoptotic stimuli. On the contrary, this report suggests that long term arsenic exposure renders the cell more sensitive to both extrinsic and intrinsic apoptosis triggers. Further work needs to be done to determine whether these results are specific to the h-TERT immortalized human SAE cells. Additionally, it would be interesting to determine whether the cells would lose their sensitivity to apoptosis triggers if arsenic treatment was resumed.
TGFBI Suppresses Malignant Mesothelioma and Breast Cancer by Prolonging G1-S Transition and De-regulating Cell Invasion

Bingyan Li, Genyun Wen, Jian Tong and Tom K. Hei

Introduction
TGFBI (transforming growth factor-β induced, also named Betaig-h3) can be induced by transforming growth factor-β in multiple cell types, including human epithelial cells, keratinocytes, lung fibroblast and melanoma cells. TGFBI consists of 683 amino acids. The nascent TGFBI protein contains an N-terminal secretory signal sequence (resides 1–23), four homologous internal domains (FAS1 domain), and a cell attachment site (C-terminal RGD motif). After existing from the Golgi apparatus, TGFBI is secreted into the extracellular matrix. TGFBI has been implicated in the regulation of cellular growth, differentiation, adhesion, migration, and angiogenesis. More recently, TGFBI was found to induce microtubule stabilization, potentially affecting metabolisms of certain drugs.

Malignant mesothelioma arises from mesothelial cells in superficial layer of the serosal membranes lining the cavities of the thorax; it is characterized by a long latency and aggressive invasiveness; it is resistant to traditional chemotherapy and radiation therapy. Breast cancer, the second most common type of cancer and the fifth most common cause of cancer death worldwide, threatens peoples life in a variety of ways. Any progress in research and clinical therapy of these two different type of malignances would produce tremendous impact on the general public. The present study was focused on how TGFBI affects progression of these two types of cancer, both in vitro and in vivo, by ectopically expressing TGFBI in malignant pleural mesothelioma cell line H28 and breast cancer cell line MDA-MB-231.

Results
TGFBI inhibits tumor cell growth in vitro
The down-regulation of TGFBI in a series of cell lines derived from lung, mammary and prostate cancer led us to hypothesize that TGFBI may function as a tumor suppressor. To test this idea, we incorporated TGFBI expressing constructs into different cell lines and established multiple TGFBI highly expressing breast cancer cell clones (T-8, T-9, and T-13) and mesothelioma cell clones (T-4, T-6 and T-7). Firstly, we investigated the effect of TGFBI expression on the growth of these cells, controlled by their parental breast cancer cell line MDA-MB-231 and mesothelioma cell line H28, which have modest TGFBI expression (Fig. 1A). Growth kinetics showed the cell population doubling time dramatically decreased by at least 1.89 fold over the duration of 3 to 7 days with expression of TGFBI (Fig. 1B). Consistently, TGFBI drastically decreased relative plating effi-
efficiency (PE) from 98.00% to 29.71% in mesothelioma cells (Fig. 1C). In breast cancer cells, the PE was not significantly affected by TGFBI; however, the relative area or size of colonies was reduced from 98.82% to 67.76% (P<0.05). Separately, we demonstrated that TGFBI resulted in a significant inhibition of anchorage-independent growth in contrast with controls, by reducing growth 10.98-fold in breast cancer cells and 1.98-fold in mesothelioma cells, respectively, (Figure 1D). Taken together, these results reveal an important role for TGFBI in the suppression of tumor cell proliferation and transformation.

TGFBI overexpression results in G1 phase arrest and S phase entry delay

Our data and previous studies from our group\(^{10,11}\) have established that TGFBI inhibits cell growth and proliferation. To determine whether the changes are accompanied by the alteration in cell cycle progression, we examined cell cycle profiles of TGFBI-expression MDA-MB-231 and H28 cells along with their parental tumor cells by using flow cytometry analysis. Cells were synchronized in G0/G1 phase by culturing in serum-free medium for 48h and then stimulated by adding 10% serum. The percentage for G1 phase arrest was 66.29% and 68.57% in parental and TGFBI-expressing breast cancer cells; whereas it was 60.27% and 57.99% in parental and TGFBI-expressing mesothelioma cells. However, the G1 phase percentage of TGFBI-expressing tumor cells is substantially higher than that of the...
parental tumor cells at 4h post serum stimulation (Fig. 2B). At 12h after serum stimulation, 64.20% of parental breast cancer cells were in G1, whereas 78.78% of TGFBI-expression breast cancer cells were in G1. TGFBI-expressing cells have been shown to be more refractory to enter the cell cycle, as opposed to the parental tumor cells

**Fig. 2.** TGFBI results in G1 phase arrest and S phase delay. Cells were cultured in serum-free medium for 48h, and subsequently stimulated with serum to reenter the cell cycle. 

A. Cell proliferation was assessed by CyQUANT NF proliferation kit at indicated times after stimulation. Proliferation rate is expressed as increased percentage = [(Fluorescence at nh) – (Fluorescence at 0h)] / (Fluorescence at 0h).

B. Flow cytometry analysis of cell cycle profiles. Cells collected at different times after stimulation were fixed, labeled with propidium iodide, and assessed by flow cytometry. Line graph and plot illustrate the distribution of cells in the G1 and S phases over a time period of 32h.

C. Temporal expression of p21 and p53 in response to serum stimulation. Cells were synchronized in quiescence by serum starvation and induced to reenter the cell cycle by the addition of serum. Expression of p21 and p53 was assessed by western blotting of whole-cell lysates from cells harvested at indicated times after stimulation.
between 4h and 12h after serum stimulation. This effect implies that TGFBI prolonged the G1-S transition and delayed entry into S phase.

To investigate if p21 and p53, tumor suppressors known as regulators of cell cycle progression, are associated with TGFBI’s cell cycle suppression, we compared the expression of p21 and p53 in TGFBI-expressing and control cells upon serum stimulation. As shown in Figure 2C, TGFBI-expressing MDA-MB-231 and H28 cells exhibited elevated p21 and p53 levels between 12h and 24h after serum stimulation; by contrast, parental MDA-MB-231 and H28 exhibited minimal changes after stimulation. Therefore, the upregulated p21 and p53 may be responsible for the TGFBI delayed S-phase entry described above. Consistent with the cell-cycle progression, when TGFBI expressed, the cell proliferation rate of H28 decreased from 45.39% to 20.58% and MDA-MB-231 from 64.44% to 39.62% at 24h post serum stimulation, as assessed by the CyQUANT NF Cell Proliferation Assay (Fig. 2A).

**TGFBI negatively regulates cell invasion**

TGFBI is an extracellular matrix protein and has been demonstrated to regulate a variety of integrins associated signaling transduction. We therefore speculated that it might be also involved in tumor cell motility and dissemination. For testing this hypothesis, an *in vitro* cell invasion system (a barrier containing an 8 micron pore size PET membrane coated with a thin layer of matrigel basement membrane matrix) was used to measure the tumor cells invasion ability. Surprisingly, with TGFBI’s expression, tumor cell invasion

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**Fig. 3.** TGFBI negatively regulates invasion. **A.** Invasion ability was measured by BD Matrigel Invasion Chambers. Data are expressed as the percentage invasion = (OD₅₉⁵ in invasion chamber) / (OD₅₉⁵ in control chamber), compared with parental tumor cells, p < 0.01. Representative photomicrographs are shown. **B.** Cell adhesion to fibronectin and TGFBI protein was assessed. 96-well plates were coated with increasing concentration of fibronectin containing 5µg/ml of TGFBI protein for overnight and incubated with H28 cells at 37°C for 0.5h. The cells were rinsed with PBS, were fixed with 75% ethanol, and stained with crystal violet. Data is expressed as [OD₅₉⁵(sample)] / OD₅₉⁵max. **C.** Migration through transwells was measured using Falcon HTS 24-Multiwell Insert System. Data are expressed as OD₅₉⁵ of trespassed experimental groups of cells, compared with those of controlled parental tumor cells, p<0.05. Representative photomicrographs are shown. **D.** Relative activity of MMP2 in conditioned media of TGFBI-expression or parental tumor cells. Data are shown as ΔOD₄₅₀ = (OD₄₅₀max[PBS] - OD₄₅₀max[MMP sample]), compared with parental tumor cells, p<0.01. Relative activity of a dilution series of MMP-2 positive control samples is shown on the upper panel; supernatant samples of tumor cells cultured for 24h on the lower panel.
was markedly impaired (p<0.01, Fig. 3A).

Invasiveness is a complex cellular process coordinated by several distinct cellular processes such as adhesion, motility, detachment, and extracellular matrix (ECM) proteolysis. For clarifying how TGFBI interfered with tumor cell invasion, we examined the above features separately. As shown in Figure 3B, TGFBI increased the adhesion activity of the tumor cells (fibronectin concentration: 0.01, 0.05, 0.1, 0.5, 1 and 5µg/ml), while it inhibited mesothelioma cell migration. But in breast cancer cells, these effects seemed did not show significance (Fig. 3C).

The Matrix metalloproteinases (MMPs) comprise a family of secreted or membrane-associated zinc-extracellular endopeptidases that enhance invasion and metastases. To determine if decreased invasion of TGFBI-expressing tumor cells was associated with concomitant change of MMP gelatinase activity, MMP-2 and MMP-9 combined activity was measured in the cell culture supernatant. Indeed, the enzyme activity was significantly attenuated in the supernatant of TGFBI-expressing tumor cells, compared with the parental tumor cells (p<0.01, Fig. 3D).

In summery, our results suggest that TGFBI exhibits a tumor suppressor function in breast cancer as well as in mesothelioma, by de-regulating the cell cycle and cell invasion. Further experiments need to be done to extend promoting these findings.

References


Human Endothelial Cells in 3D Model Vessel Systems; Differential Effects of High and Low LET Space Radiations

Peter Grabham, Burong Hu, Gloria Jenkins and Charles R. Geard

Introduction

Travel into space will result in exposure to both low LET (proton) ionizing irradiation and high LET (e.g. iron ion [Fe]) ionizing irradiation. Very little is known about the effects of these types of radiation on the human body. In addition to the primary concern of the carcinogenic potential, there is more recent concern of non-carcinogenic effects, which can be comparable to those seen for cancer. Among these risks are diseases of the vasculature such as heart disease and stroke. The human vasculature represents a prime target for ionizing radiation. There is an estimated 60,000 miles of vessels that network the entire body. The present study is aimed at understanding the non-cancer effects of space ionizing radiation on the formation and maintenance of blood vessels. To date, studies on the effects of irradiation on endothelial cells in vitro have been in 2-dimensional monolayers, although data on space radiations are scarce.

Studies we carried out on proliferating monolayers showed that human umbilical cord endothelial cells (HUVEC) respond to protons and Fe ions much the same as fibroblasts. Low and high LET irradiation induced chromosome damage, repair, and long-term chromosome aberrations such as reciprocal translocations suggest that in vitro endothelial stem cells could be transformed into cancer cells.

Proliferating endothelial stem cells are a relatively minor population in the body and endothelial cancers are not common. For non-cancerous effects of space radiation we wanted to assess the impact on more typical endothelial cells in the body, that is, post mitotic endothelial cells arranged in vessel like tubes. When the cells are exposed to growth factors such as VEGF and FGF and embedded in collagen gels, they become post-mitotic and fully differentiate and assemble into capillary tubes. The cytoskeleton changes dramatically, cell to cell contacts are more developed and the cells express a different profile of genes.

2-Dimensional studies

First, we wanted to investigate the cytotoxic effect of space radiation on proliferating cells versus post mitotic cells. For this, we differentiated 2D monolayers of endothelial cells with the same growth factors but without the collagen gel and compared them to proliferating 2D monolayers. After irradiation, cultures were assessed for abnormal nuclei (Fig. 1). As expected, a dose of 3.2Gy Fe ions (1GeV) induced a level of micronuclei, chromosome bridges and fragmented nuclei (late stage apoptosis) in proliferating HUVEC’s 5-fold over controls. Differentiated post-mitotic HUVEC’s showed no increase of abnormal nuclei over controls. Even late stage apoptosis was not induced at this dose. Similar results were found for low LET protons (1GeV) and low LET gamma irradiation.

3-Dimensional studies

The effect of space radiations on mature vessels was examined. Fully formed vessels were exposed to doses of protons (1GeV) and Fe ions (1GeV) up to 3.2Gy. 24 and 48 hours after irradiation the vessel cultures were fixed and cells visualized by a total protein stain (DTAF) and propidium iodide. 3-dimensional images were collected using a confocal microscope. This allowed us to assay for cell death (late stage apoptosis) by counting fragmented nuclei and to assay for the structural integrity of the vessels. Vessel structure is an arrangement of endothelial cells formed in a tube that surrounds a central lumen. For an accurate measure of structure we determined the length of lumen per cell.

At a dose of 3.2Gy, which is high enough to produce a 5-fold increase in cell death in dividing monolayers, no increase in cell death was seen in vessel cells exposed to Fe ions, protons or gamma irradiation. Furthermore, no increase in micronuclei was observed. However, the effects of each particle on the integrity of the vessel structure were very different. Fe ions caused a breakdown of vessel structure at doses above 40cGy. At least 50% of tubular structures became narrow and spindly without a discernable lumen when exposed to 3.2Gy (Fig. 2 and 4). Protons up to the same dose appeared to have no effect on vessel structure. Control vessel cultures were indistinguishable from vessels exposed to 3.2Gy (Fig. 2 and 3). This decreased sensitivity was also seen in similar experiments using gamma rays. Doses up to

Fig. 1. Abnormal nuclei produced by Fe ion irradiation in proliferating cells but not in post-mitotic differentiated 2-D cultures. Each type of culture was exposed to 1GeV Fe ion irradiation. 24h later cultures were fixed, stained with propidium iodide and scored for abnormal nuclei. Proliferating HUVEC’s are sensitive to Fe irradiation. Differentiated HUVEC’s show no increase over controls up to 3.2Gy. Error bars = sem. B. Chromosome bridge seen in proliferating HUVEC’s exposed to 3.2Gy Fe ions. Bridges and micronuclei were not seen in differentiated cultures.
5Gy showed no breakdown of vessel structure. The effect of Fe ions on fully formed vessels was in direct contrast to protons and gamma irradiation and was supported by experiments that assessed the effect of Fe ions on vessel formation. When nascent vessel cultures (1 day old) were exposed to Fe ion (1GeV) doses above 40cGy vessel formation was inhibited.

Thus, differentiated 3D vessel cultures are more resistant to protons than Fe ions. Recent experiments using gamma irradiation show that 3D vessel cells are not entirely unresponsive to lower doses. Examination of DNA repair mechanisms by visualization of p53 binding protein 1 (53BP-1) in nuclei showed that doses below 0.5Gy are sufficient to induce foci much the same as in dividing monolayers. Foci formed as a bystander response can even be detected in vessel culture cells after medium transfer from irradiated human fibroblasts.

These results demonstrate that space radiations have different effects on human vascular tissue depending on LET. Although cell death is only induced by high doses of any type of irradiation in differentiated post mitotic 3D vessel cells, high LET particles appear to be deleterious to vascular tissue at lower doses whereas low LET particles are not. DNA repair responses, however, appear to be induced at low doses of gamma irradiation in both dividing cells and differentiated 3D vessel cells similarly. Future experiments will include assessing the extent of DNA repair mechanisms in response to lower doses >2Gy of Fe ions and protons.

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**Publications**

Breast-conserving surgery followed by radiotherapy is the standard of care for most women with early-stage breast cancer. However, the rate of second breast cancers is significant; for example, the average ipsilateral second-cancer rate from four long-term studies is 13% after 15 years. Rates in the contralateral breast are typically only slightly lower. We focus here on the rate of second cancers, which are genetically independent of the primary (i.e., not recurrences); this rate is much higher than could be explained from the natural background rate of breast cancer in the general population, for both breasts.

We have analyzed a data set in which second ipsilateral cancers were classified as either in the same or a different quadrant from that of the initial tumor. The data were from the Fox Chase Cancer Center (Philadelphia, PA) reported by Freedman et al. on 1,990 women with stage 0-II breast cancers who were treated with lumpectomy and ipsilateral whole-breast irradiation, with recurrence rates reported up to 20 years after treatment. Predictions of radiogenic cancer risks as a function of dose were obtained for each breast, using a quantitative mechanistic cell initiation/inactivation/proliferation model.

Our analysis suggests that in the contralateral breast, which receives a comparatively small radiation dose, most of the second cancer risk must be from background genetically independent cancers. However, virtually all of the risk of a genetically independent second cancer in the ipsilateral breast (which receives a large fractionated radiation dose of ~46Gy) can be attributed to the radiation exposure – thus, surprisingly, there appears to be essentially no contribution to second cancer risks in the ipsilateral breast from background genetically independent cancers. The likely explanation is that the approximately 46Gy fractionated dose administered to the ipsilateral breast has killed essentially all of the genetically independent premalignant cells in that breast that were present before the radiotherapy, and thus effectively eliminated the background cancer risk in the ipsilateral breast.

There is much recent interest in the notion of accelerated partial-breast radiotherapy, in which only the tumor bed plus a margin in the ipsilateral breast is targeted. The potential benefits of partial-breast irradiation in terms of cardiac and pulmonary sequelae, as well as breast cosmesis, are clearly attractive; however, such a strategy may well be associated with an increased long-term risk of genetically independent cancers in the nontargeted parts of the ipsilateral breast. If such an analysis were correct, a more efficacious approach to partial-breast irradiation would be to administer the full dose to the tumor bed and margin, as is now done, but also to administer a uniform prophylactic lower dose to the remainder of the ipsilateral breast to clear it of premalignant cells. By analogy with prophylactic cranial irradiation (PCI), designed to reduce brain metastases from small-cell lung cancer, we term this potential procedure prophylactic mammary irradiation (PMI).

An estimate of an effective PMI dose requires the number of premalignant cells present, a number that is not well established. Assuming that a very short treatment time, perhaps with the addition of neoadjuvant/concomitant tamoxifen, essentially prevents repopulation during the course of partial-breast radiotherapy, Figure 1 shows estimates of the PMI doses that would be required to kill the premalignant cells in the remainder of the ipsilateral breast, as a function of the total number of such cells in the breast.

Women with breast cancer have a much higher than average risk of developing a genetically independent cancer in the contralateral breast. Based on our analysis, adjuvant radiotherapy to the ipsilateral breast might be accompanied by low-dose PMI to the contralateral breast. In fact, a conceptually similar approach has been used successfully to treat carcinoma in situ in the contralateral testicle of men with unilateral testicular germ cell cancer.

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Stochastic Mechanistic Model of Second Cancers after Fractionated Radiotherapy

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When ionizing radiation is used in cancer therapy it can induce second cancers in nearby organs. Mainly due to longer patient survival times, these second cancers have become of increasing concern. Estimating the risk of solid second cancers involves modeling: because of long latency times, available data are usually for older, obsolescent treatment regimens. Moreover, modeling second cancers gives unique insights into human carcinogenesis, since the therapy involves administering well-characterized doses of a well-studied carcinogen, followed by long-term monitoring.

In addition to radiation initiation that produces premalignant cells, inactivation (i.e. cell killing), and cell repopulation by proliferation, can be important at the doses relevant to second cancer situations. A recent initiation/inactivation/proliferation (IIP) model characterized quantitatively the observed occurrence of second breast and lung cancers, using a deterministic cell population dynamics approach. To analyze if radiation-initiated pre-malignant clones become extinct before full repopulation can occur, we give a stochastic version of this IIP model. Combining Monte-Carlo simulations with standard solutions for time-inhomogeneous birth–death equations, we show that repeated cycles of inactivation and repopulation, as occur during fractionated radiation therapy, can lead to distributions of pre-malignant cells per patient with variance much greater than the mean, even when pre-malignant clones are Poisson-distributed. Thus fewer patients would be affected, but with a higher probability, than a deterministic model, tracking average pre-malignant cell numbers, would predict.

Our results are applied to data on breast cancers after radiotherapy for Hodgkin’s disease. An important new conclusion from the stochastic IIP model is that a growth advantage for initiated pre-malignant cells (relative to normal cells) is compatible with cancer excess relative risks (ERRs) that increase less rapidly than linearly at high doses (Fig. 1). By contrast, the deterministic IIP model predicted that initiated cells do not have a growth advantage over their normal counterparts in the short-term, whatever may happen on a longer-time scale. This result from the deterministic model was somewhat puzzling. That initiated, pre-malignant cells do have a growth advantage even on short time scales following radiation inactivation was found with parameter estimates using the two-stage clonal expansion model, and seems plausible since on long time scales hyperplasia is a common feature of pre-malignant cells. We found that with the stochastic model this puzzling feature of the IIP model is removed.


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Fig. 1. Comparing different models with data on second breast cancers in patients treated with radiotherapy for Hodgkin’s disease (points): the deterministic IIP model (dotted curve), the stochastic IIP model (solid curve), a simplified linear model (dashed straight line). All predictions use 25 daily acute dose-fractions, with weekend gaps. For all three curves, the slope at the origin is determined using data on Japanese atomic bomb survivors.
This and some other features of the stochastic model correspond to overdispersion, where the number of pre-malignant cells per patient has a variance much larger than its mean (although the number of clones per patient is Poisson-distributed). This overdispersion result is consistent with models, incorporating cell migration, applicable to second cancers that are leukemias. The low values of the inactivation parameters needed to bring about a fit between the stochastic IIP model and the data were a surprise. This result may point to extra radiore-sistance on the part of breast stem cells, as has indeed been directly observed. Possibly however, the result points to the fact that the current model, where even one radiation-initiated pre-malignant cell ultimately leads to cancer, is only a limiting case where stochasticity has maximum influence. The deterministic model gives an acceptable fit for more typical values of the inactivation parameters, so a model intermediate between the limiting case and deterministic models would not necessarily require small values.

References

Immersion Mirau Interferometry
Oleksandra Lyulko, Gerhard Randers-Pehrson and David J. Brenner

In cell irradiation experiments it is important to separate radiation-induced effects from damage done by introducing fluorescent stain. Immersion Mirau Interferometry (IMI) is a non-stain imaging technique that is currently under development at RARAF. The technique utilizes the Mirau interferometric approach. Several (normally 4) interference images are acquired at subwavelength distances near the focal plane which provide enough information to reconstruct the topological image of the sample. To visualize samples exposed to air, a commercial Leica interferometric attachment has been used. However, it is desirable that the cells stay in the cell medium during the course of the experiment. This maintains cell viability and allows for more accurate results. Using medium gives rise to the immersion version of Mirau Interferometry when the optics is submerged under cell me-dium. To ensure that the microscope objective and interferometric optics are protected against the medium and that there is no leakage, a specially designed attachment was needed.

A new attachment was built in 2007 in the CRR machine shop by Gary Johnson. The apparatus consists of the body that can be screwed onto the microscope objective (we use a threaded Leica objective) and an optics holder containing a beamsplitter and a spot mirror (Fig. 1, 2). The beamsplitter divides the incoming light into two waves. One of them is

![Fig. 1. Design drawing of the custom interferometric attachment: 1 – attachment body, 2 – optics holder, 3 – beamsplitter, 4 – spot mirror, 5 – adjustment screw.]

![Fig. 2. Custom-build interferometric attachment. A small spot mirror can be seen in the middle of the top window inside the device. Two adjustment screws regulate the inclination of the optics with respect to the sample. Next to the attachment is one of the additional optics holders with different reflectance value.]

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reflected off the sample and another – off the spot mirror. They combine in the image plane to form an interference pattern.

The space between the beamsplitter and the spot mirror is filled with water, to allow for equal optical pathways.

To enable interferometry, high precision optics is needed. We used fused silica windows of high surface quality with flatness 1/20 of the wavelength (540nm). The windows were custom manufactured by The Optical Corporation (Oxnard, CA). Coating was done by Navitar Coating Labs (Newport Beach, CA). Each beamsplitter has a protection layer of SiO₂ to prevent interaction of the coating with cell medium. Four interchangeable optics holders were built with 5%, 25%, 50% and 85% reflectance beamsplitters. Each unit can be disassembled for cleaning and re-filled with liquid. Figure 2 features the custom attachment and one of the optics holders.

The new attachment has been tested under different conditions. The acquired images are comparable to those that can be obtained using the commercial attachment.

Recent experiments revealed that vibrations are present in the building and introduce substantial motion of the microscope stage in the z-direction. This lowers the image quality since interferometry is very sensitive to even fraction-of-a-wavelength shifts in the vertical direction. Work is currently being done to develop vibration-insensitive imaging software that would extract additional information necessary to reconstruct phase images using interference patterns acquired in the presence of vibrations.

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**A Novel, Rapid Method to Characterize the Human Lymphocyte Concentration Based on Quantitative Light Absorption Analysis**

*Yanping Xu, Helen C. Turner, Guy Garty, Gerhard Randers-Pehrson and David J. Brenner*

**Introduction**

At the Center for High-Throughput Minimally Invasive Radiation Biodosimetry, Radiological Research Accelerator Facility (RARAF), Columbia University, we have conducted a set of light absorption imaging analyses to reproducibly measure lymphocyte concentration in a 30μl whole blood sample. We have developed a reproducible method to isolate human lymphocytes from small volumes of whole blood, such as those obtained from minimally-invasive procedures. The collected blood samples are transferred into plastic capillaries coated with lithium heparin as an anticoagulant. The lymphocyte band is separated from the RBC pellet by centrifugation (Fig. 1). To image and quantify the lymphocyte band in the capillary tube, we have developed a novel, rapid quantitative light absorption analysis (QLAA) method to characterize lymphocyte concentration instead of quantitative buffy coat analysis (QBCA).³

**Light scattering measurements**

Peripheral human blood samples (1-2ml) were collected in heparinized vacutainer tubes from healthy donors between 24 and 55 years.

For lymphocyte separation, 30ul of whole blood was layered over 50µl of lymphocyte separating medium (Histopaque-1083; Invitrogen) with density 1.083g/ml contained in a PVC capillary tube (Safe-T-Fill; RAM Scientific) and centrifuged at a speed of 4000 rpm for roughly five minutes (adjustable centrifuge Legend Micro 17; Thermo Scientific). The lymphocytes separated out from whole blood forming a compact cloud of roughly 100% purity (i.e. no RBC contamination) as shown in Figure 1.

The setup for light transmission measurements is presented in Figure 2. The light source is a Spectra Master multiple wavelength optical fluorimeter (Olympus America, Life Science Resource Ltd) where the incident light beam is well collimated by a set of customized collimators on an optical table. Right after transmitting through the sample, light will be collimated by another narrow collimator which will narrow the scattered light and only allow the transmittance light to pass. A Hamamatsu Orca 100 10-Bit digital CCD camera was used to image the tube and measure the light intensity. The camera has a resolution 1.3 million pixels with a frame rate of 18 frames per second. In analysis, we selected an average light intensity in an area with the width about 1/5 of the capillary diameter and the same height as the capillary.

Our experiments tested the light absorption intensity calibration curves with different lymphocyte cells of whole
blood. After blood separation, lymphocyte cells form a very compact, high concentration band in capillary tube. In such a case, the light absorption analysis method will be very useful to characterize the lymphocyte concentration. The wavelength of light we used was 360 nm and 494 nm respectively. To determine absolute lymphocyte cell number in the lymphocyte band and calibrate our system, we used a standard hemocytometer. The calibration curves in Figure 3 had been extracted out based on the 30 µl whole blood and 3000 lymphocyte cells per µl (compared to the 3000–10000 cells/µl is average range in fresh human blood).

Conclusion

We have developed a novel method to measure lymphocyte concentration that relies on quantitative light absorption analysis. Potentially, this method might provide a quick test for lymphocyte counting in a clinical setting.

Fig. 2. Light scattering measurement setup.

Fig. 3. Lymphocyte calibration curves of light absorption.

References


Investigation of Oxygen/Nitrogen Resonance Explosive Detector (ONRED) for Screening Baggage: Calculational Approach to its Characteristics and Feasibility

Kenichi Tanaka, Gehard Randers-Pehrson, Yanping Xu, Alan W. Bigelow, Stephen A. Marino and David J. Brenner

Introduction

Oxygen/Nitrogen Resonance Explosive Detector (ONRED) was proposed as a promising way of screening baggage for explosives, for usage in the security check, etc. The simulation calculations have been conducted to investigate the characteristics and feasibility of the ONRED system. The preliminary results are shown in this report.

Feasibility of ONRED concept

In order to observe the characteristics of the ONRED system, the yield of the reaction between neutron and nitrogen in the N\textsubscript{2} gas proportional counter has been calculated by MCNP-4C. The calculation geometry is shown in Fig 1. Li target of 1 cm in diameter was set as a neutron source. The neutron-nitrogen reaction yield in N\textsubscript{2} hexahedrons (2.5 cm square – 5 cm square x 100 cm, 1 atm), which corresponds to the number of pulse, was estimated using F4 tally with tally multiplier ‘FM’. Three N\textsubscript{2} hexahedrons were set in contact to each other, 100 cm away from the Li target. They are at 43.6, 45 and 46.4 degrees with respect to incident proton beam direction. The materials to simulate the content of the baggage were taken from a previous study, and the results were compared to TNT as an explosive.

The neutron production was calculated with the calculation code in RARAF. In this code, the neutron yield is evaluated by linear interpolation of \(^7\)Li(p,n) cross section by Liskien and Paulsen (1975) for proton energies dependent on the depth in Li calculated with stopping power. The neutron energy is calculated from kinematics for its emission angles. The calculation using the same basis was verified using Au activation in a H\textsubscript{2}O phantom.

The assumed Li target is of 0.5 \(\mu\)m thickness and the incident proton energy is 2.265 MeV. These parameters were chosen to obtain a desirable condition that the neutron energy spectrum at the N\textsubscript{2} detector at 45 degrees will have its peak at around 433 keV, similarly to nitrogen total cross section and the energy spread below the \(^{14}\)N(n,n) resonance width of 10 keV, as shown in Fig. 2. Here, neutron yield is \(8.1\times10^8\) (n/sec/mA). To reduce the calculation time, the neutron emission was limited to angles from 17.5 to 50.5 degrees, with the yield of \(3.1\times10^8\) (n/sec/mA). The influence of limiting angle was verified to be below the statistical error in the calculations.

In the ONRED system, the reduction ratio of neutron-nitrogen reaction yield by locating material in front of the detector will be measured for various angles, \(i.e.\) for various neutron energies. The reduction ratio is shown in Fig. 3. In order to focus on the change of the reduction ratio by angle, the plotted values are \((R_{\text{material}}/R_{\text{air}})_{\text{angle}}/(R_{\text{material}}/R_{\text{air}})_{45}\)-degree. Here, \(R_{\text{material}}\) denotes the neutron-nitrogen reaction yield in the geometry where the baggage simulator is set for each material, and \(R_{\text{air}}\) the material of air which corresponds to the geometry without baggage. The error bar shows the standard deviation of the MCNP calculations of around 0.5-5 %.

In combining detectors at 45 and 46.4 degrees, from Fig. 3(a), the materials for which the reduction ratio agrees to that for TNT of 1.4 within error are only canned tuna and toothpaste. For other materials, the reduction ratio is around 1.0-1.2, \(i.e.\) 15-30 % different from TNT. From Fig. 3(b), the combination of 45 and 43.6 degrees gives a closer reduction ratio between TNT and other materials than those for 45-46.4 degrees. However, in this case, canned tuna and toothpaste is able to be distinguished with discrepancy larger than statistical error. In this case, precise measurement will be
required with standard deviation of a few percent. These results suggest the feasibility of the ONRED concept in screening baggage. Other explosive materials and multilayer of explosives and non-explosives should be investigated.

**Proton current and pulse shape of N2 gas counter**

Among the aforementioned geometries, the neutron-nitrogen reaction yield \((R)\) ranges from \(6.5 \times 10^{-30}\) to \(2.5 \times 10^{-28}\) \((\text{reaction/cm}^3/\text{source-neutron})/(\text{N atom/cm}^3)\). Here, 99.8% of the neutron-nitrogen reaction is the elastic scattering.

The necessary proton current \((I_p)\) has been estimated following the equations (1) and (2),

\[
N_{\text{rec}} = R \times N_N \times V_N \times Y_n \quad (1),
\]

\[
I_p = C / N_{\text{rec}} / E_f / T \quad (2).
\]

\(Y_n\) is neutron yield in the Li target per proton current of \(3.0 \times 10^9\) (n/sec/mA) for emission angle from 17.5 to 50.5 degrees, \(N_N\) nitrogen atomic density of \(5.38 \times 10^{19}\) (N atom/cm\(^3\)) for 1 atmosphere pressure, \(V_N\) the volume of N\(_2\) hexahedron of 1458.3 (cm\(^3\)). Then the number of neutron-nitrogen reaction \((N_{\text{rec}})\) is \(1.5 \times 10^3\) to \(5.8 \times 10^4\) (reaction/sec/mA).

In estimating \(I_p\), 80% of neutron-nitrogen reaction was assumed to be detected as electric pulse, i.e. \(E_f\) is set at 0.8. This assumption is based on a calculation for detector pulse height estimation which was conducted using the geometry in Fig. 1 by replacing all the materials except for N\(_2\) hexahedron at 45 degrees with the vacuum, and using a monoenergetic neutron source of 433 keV as an example. The energy spectra were evaluated separately for neutrons entering N\(_2\) and those getting out of N\(_2\) using surface current (F1) tally. By subtracting the former from the latter, the recoiled nitrogen energy was estimated and shown in Fig. 4. Assuming recoiled nitrogen higher than 10keV can be detected experimentally, which corresponds to LLD of 10keV, \(E_f\) is around 0.8 from the integrating energy spectrum in Fig. 4.

Assuming the measurement time \((T)\) of 1 sec, necessary count \((C)\) of 1000 which gives standard deviation of 3 %, the resultant \(I_p\) is from 31 µA to 1.2 mA. Though this is a necessary current range for the geometry assumed here, this method can be applied to other geometries. The value of \(E_f\) should be determined by calculations considering neutron energy spread at N\(_2\) detector as well as verifying experiments in the future.

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**Dosimetry using ATOM Phantoms in the Diagnostic and Therapeutic Regimes**

*Carl D. Elliston, Edward L. Nickoloff,*\(^a\) *Cheng-Shie Wuu,*\(^b\) *Andrew J. Einstein,*\(^c\) *Sandra Russo*\(^b\) and *David J. Brenner*

Due in large part to this Center’s own Drs. Hall and Brenner,\(^1,2\) there has been increasing interest in more accurately quantifying radiation dose and subsequent radiation risks from both diagnostic and therapeutic procedures. Theirs and the works of others have already resulted in efforts by the medical community and CT industry to reduce radiation doses in children by offering pediatric-specific CT protocols. We have and are undertaking a series of studies to investigate and compare dose from procedures in both the diagnostic (primarily CT) and therapeutic regimes. Ultimately, our goal is to calculate cancer incidence and mortality risks for organs both inside and outside the irradiated volume and to use our understanding to facilitate discussion leading to ALARA (As Low As Reasonably Achievable) doses.

For dosimetry studies we possess three ATOM phantoms (CIRS, Norfolk, VA) modeling an adult male 173 cm tall and weighing 73 kg (model 701-D), a 10 year old 140 cm tall and weighing 32 kg (model 706-D), and a 1 year old 75 cm tall and weighing 10 kg (model 704-D) (Fig. 1). This family of phantoms provides a powerful tool for dose estimation using MOSFET dosimeters. All three phantoms include head, torso, upper femur, and genitalia. The adult phantom has four sets of breasts (large supine, large prone, medium supine, and medium prone) that can be attached for study of techniques, such as accelerated partial breast irradiation, requiring a female phantom. Additionally, the 1 year old pediatric phantom includes arms and legs.

Each phantom is made of tissue-equivalent (TE) plastics that simulate soft tissue, spinal cord, spinal disks, lung, brain, and bone. The composition of bone differs in the three phantoms, reflecting the increase in bone density with increasing age. The phantoms are sectional, consisting of a series of 25 mm-thick contiguous slices. In each section 5 mm diameter through holes are located at positions optimized for dosimetry in one of 20 internal organs. Tissue equivalent holders are used for MOSFET detector placement within these holes. Up to 20 MOSFET dosimeters can be placed and read in a phantom simultaneously.

Dose measurements were obtained using a Thomson & Nielsen AutoSense Reader (TN-RD-15, Thomson-Nielsen, Ottawa, Canada) with 4 bias supplies (TN-RD-22, Thomson-Nielsen, Ottawa, Canada) set to high sensitivity, and attached to 20, 5 per bias supply, MOSFET dosimeters (TN-1002RD for CT studies and TN-502RD for RT studies, Thomson-Nielsen, Ottawa, Canada). MOSFET calibration is tricky, particularly for organs far outside the treatment volume. The calibration factor for the dosimeters changes as a function of energy, considerably at lower energies. Therefore special care must be taken to understand these changes.

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In this study, all scans were performed from the dome of the CT scans on children using pediatric-specific protocols. Germany) to quantify the risk reduction offered by performing CT scans with pediatric-specific settings, cancer mortality risk is reduced by the amount in red.

As an example, we have performed experiments on a 16-channel MDCT scanner (Sensation 16; Siemens, Forchheim, Germany) to quantify the risk reduction offered by performing CT scans on children using pediatric-specific protocols. In this study, all scans were performed from the dome of the diaphragm to the symphysis pubis, using standard adult and pediatric CT protocols used in our institution. The adult phantom was scanned using standard adult CT settings but with CARE dose (a dose reduction technique) off. The 1- and 10-year old phantoms were each scanned using two different protocols: the first protocol used the same standard adult CT settings as in the adult phantom, and the other protocol was a pediatric, age-specific protocol.

For all CT scans a collimation of 16 × 1.5 mm, gantry rotation time of 500 ms, and tube voltage of 120 kV were used. Scans using adult settings were performed with an effective mAs of 120 mAs with CARE dose off and using a table feed of 48 mm/rot. The 10- and 1-year old pediatric CT scans were performed using the settings typically used at our institution for a 10-year old, 32 kg pediatric patient and a 1-year old pediatric patient, respectively. The 10-year old CT settings use an effective mAs of 55 mAs and a table feed of 24 mm/rot. The 1-year old CT settings use an effective mAs of 20 mAs and a table feed of 24 mm/rot.

Estimated lifetime excess cancer incidence and mortality risk per unit dose for females and males as a function of age at time of a single acute exposure were taken from BEIR VII (Biological Effects of Ionizing Radiations). Risks were multiplied by the measured dose at various organ sites for which both risk estimates and dose measurements existed such as stomach, colon, liver, lung breast (female), bladder, and thyroid (incidence only).

Figure 2 shows the estimated lifetime, organ-specific cancer mortality risks from a single CT scan performed at ages 1 and 10, respectively, for both male and female patients. Using typical adult CT settings results in mortality (and incidence) risks far greater than the risk incurred when patients are scanned with pediatric-specific CT settings. For example, a female one year old who undergoes a CT scan using an adult protocol has a .012% (1 in 8,600) risk of dying of stomach cancer. This risk is decreased by nearly a factor of 6 to .002% (1 in 49,000) simply by using a pediatric-specific CT protocol instead.

Several more in depth studies are under way. Presently, we are investigating the change in effective dose, which is presently used in risk estimation, that results in going from ICRP 60 to ICRP 103 in a variety of common CT techniques including head, abdominal, and cardiac CT. We are also investigating the increase relative to an adult in effective dose a pediatric patient receives for a given CT technique, using the new ICRP 103 definition. We further plan to look at dose reduction techniques such as tube current modulation, gating (in cardiac CT), and breast shielding as methods of sparing dose while retaining sufficient information to make a diagnosis. In all cases we take dose estimates from MOSFET dosimeters and use these estimates to calculate lifetime risks for cancer incidence and mortality.

In the therapeutic regime, we have designed a prostate cancer treatment plan for our adult phantom. Using our MOSFET dosimeters we are measuring the dose to specific points within the phantom. These doses are being compared to the dose at the same location predicted by the treatment planning system. These data will be evaluated by AAPM task group 158 (Measurements and calculations of doses outside the treatment volume from External Beam Radiation Therapy).

Further risk estimation experiments in accelerated partial breast irradiation studies, in collaboration with NYU, are also planned for the coming year.

References

The winners of raffle in 2007 Christmas celebration (L-r): Dr. Gloria Calaf, Sarah Huang, Yunfei Chai and Dr. Yongliang Zhao.

Center for Radiological Research 2007 Christmas Party. (L-r): Dr. Guy Garty, Dr. Shanaz Ghandhi and Dr. Helen Turner.

Center for Radiological Research 2007 Christmas Party. (L-r): Gloria Calaf, Monique Rey, Margaret Zhu, Yvette Acevedo, Angela Lugo, Anne Sutthoff and Marisol Cruz.

Christmas group lunch. (L-r) Dr. Aiping Zhu, Dr. Corinne Leloup and Dr. Gloria Calaf.

Center for Radiological Research 2007 Christmas Party. (L-r): Dr. Corinne Leloup, Sasha Lyulko and Dr. Helen Turner.
**Early Radiation-Induced Gene Expression in Human PBL**

Sally A. Amundson and Sunirmal Paul

One approach toward radiation biodosimetry being developed by the Center for High-Throughput Minimally Invasive Biodosimetry led by the CRR at Columbia is the development of a self-contained biochip capable of rapidly measuring gene expression signatures to define radiation exposure, dose and injury. The main goal of this biodosimetry project is to provide radiological triage to identify those individuals who will benefit from medical intervention, and those who will not, at an early stage following a large-scale radiological event.

Toward this goal, we have established an *ex vivo* irradiation model using human peripheral blood lymphocytes (PBL) drawn from healthy volunteers. We report here the identification of a large number of genes that are differentially expressed 6 hours after exposure to ionizing radiation.

**Irradiation and culture**

Peripheral blood from healthy volunteers (2 male and 3 female donors) was obtained with informed consent from all subjects and was drawn into 0.105 M sodium citrate vacutainer tubes (Becton Dickinson and Company, Franklin Lakes, NJ). The blood was divided into 3ml aliquots and exposed at a rate of 0.82Gy per minute to 0, 0.5, 2, 5 or 8Gy γ-rays using a Gamma-cell-40 137Cs irradiator (AECL, Ontario, Canada). After irradiation, blood samples were diluted 1:1 with RPMI 1640 medium (Mediatech Inc., Herndon, VA) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT) as described\(^1\) and were incubated for 6 hours at 37°C in a humidified incubator with 5% CO\(_2\). All experiments involving human subjects were approved by the Columbia University Medical Center Institutional Review Board IRB #3, and were conducted according to the principles expressed in the Declaration of Helsinki.

**Purification of RNA**

RNA was prepared using the Versagene™ Blood RNA Purification kit (Gentra Systems, Minneapolis, MN) following the manufacturer’s recommendations. This protocol differentially lyses red and white blood cells in whole blood. The red blood cells are lysed first and the nucleic acids released are washed away prior to lysis of the white blood cells for purification of RNA and on-column DNase treatment. This procedure depletes globin mRNA, but relatively high levels were still detected by semi-quantitative RT-PCR with β-globin specific primers (data not shown). As high amounts of globin message may affect detection of gene expression signatures derived from whole blood,\(^2\) globin mRNA was further reduced using GLOBINclear™ (Ambion Inc., Austin, TX) to specifically remove both α- and β-globin. RNA was quantified using a NanoDrop 1000 spectrophotometer and quality was monitored with the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA).

**Microarray hybridization and data extraction**

Cy3 labeled cRNA was prepared from 0.5 mg RNA using the One-Color Low RNA Input Linear Amplification PLUS kit (Agilent) according to the manufacturer’s instructions, followed by RNAeasy column purification (QIAGEN, Valencia, CA). Dye incorporation and cRNA yield were checked with the NanoDrop ND-1000 Spectrophotometer. 1.5mg of cRNA with incorporation of >10 pmol Cy3 per μg cRNA was fragmented and hybridized to Agilent Whole Human Genome Oligo Microarrays (G4112A) using the Gene Expression Hybridization Kit as recommended by Agilent. After hybridization with rotation for 17 hours at 65°C, microarrays were washed 1 minute at room temperature with GE Wash Buffer 1 (Agilent) and 1 minute with 37°C GE Wash buffer 2 (Agilent). Slides were scanned immediately using the Agilent DNA Microarray Scanner (G2404B). The images were analyzed with Feature Extraction Software 9.1 (Agilent) using default parameters for background correction, and flagging of non-uniform features. Background corrected hybridization intensities were imported into BRB-ArrayTools, Version 3.5log2-transformed and median normalized. Non-uniform outliers or features not significantly above background intensity in 25% or more of the hybridizations were filtered out. A further filter requiring a minimum 1.5-fold change in at least 10% of the hybridizations was also applied yielding a final set of 17313 features that were used in subsequent analyses. Class comparisons were conducted using BRB-ArrayTools to identify genes that were differentially expressed between the five radiation doses using a random-variance t-test.

We identified many genes that were expressed at levels significantly different from controls following exposure to different doses of ionizing radiation (Table 1). Overall, the number of genes responding increased with increasing dose up to 5Gy, then leveled off or declined at 8Gy. The majority

<table>
<thead>
<tr>
<th>Dose (Gy)</th>
<th>Up</th>
<th>Down</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>54</td>
<td>23</td>
</tr>
<tr>
<td>2</td>
<td>138</td>
<td>34</td>
</tr>
<tr>
<td>5</td>
<td>335</td>
<td>310</td>
</tr>
<tr>
<td>8</td>
<td>315</td>
<td>159</td>
</tr>
</tbody>
</table>

**Table 1.** Number of genes significantly regulated by different radiation doses (p<0.001).

<table>
<thead>
<tr>
<th>Dose (Gy)</th>
<th>2</th>
<th>5</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>41</td>
<td>61</td>
<td>56</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>94</td>
<td>104</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>-</td>
<td>216</td>
</tr>
</tbody>
</table>

**Table 2.** Number of genes significantly regulated in common by two doses of radiation.
of genes were up-regulated, although many genes also decreased expression in response to radiation exposure. Many genes were significantly regulated in response to multiple doses (Table 2), although only 39 genes were significantly regulated at all exposure levels studied.

An examination of the Gene Ontology (GO) functions of the genes responding to ionizing radiation showed many familiar processes involved in the response, such as apoptosis, cell cycle regulation, and DNA repair (Table 3). The genes that were significantly responsive to all four doses generally showed a trend of increasing response with increasing dose. The dose-response for relative fold-change over control levels for several of these genes is shown in Table 4. Although the dose-response relationship of these genes is not linear throughout the dose range of interest, these genes nonetheless provide strong candidates for radiation exposure biomarkers, or for building a classification algorithm for predicting exposure dose.

### References


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**Table 3.**

<table>
<thead>
<tr>
<th>GO ID</th>
<th>Biological Process</th>
<th>50R</th>
<th>200R</th>
<th>500R</th>
<th>800R</th>
</tr>
</thead>
<tbody>
<tr>
<td>45786</td>
<td>negative regulation of progression through cell cycle</td>
<td>10.51</td>
<td>6.51</td>
<td>2.13</td>
<td>2.62</td>
</tr>
<tr>
<td>7050</td>
<td>cell cycle arrest</td>
<td>18.44</td>
<td>10.18</td>
<td>3.05</td>
<td>4.68</td>
</tr>
<tr>
<td>8632</td>
<td>apoptotic program</td>
<td>N</td>
<td>14.69</td>
<td>4.4</td>
<td>5.91</td>
</tr>
<tr>
<td>8637</td>
<td>apoptotic mitochondrial changes</td>
<td>N</td>
<td>40.14</td>
<td>12.02</td>
<td>13.46</td>
</tr>
<tr>
<td>7005</td>
<td>mitochondrion organization and biogenesis</td>
<td>N</td>
<td>21.13</td>
<td>6.33</td>
<td>7.08</td>
</tr>
<tr>
<td>9966</td>
<td>regulation of signal transduction</td>
<td>N</td>
<td>2.39</td>
<td>2.58</td>
<td>2.5</td>
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<tr>
<td>6281</td>
<td>DNA repair</td>
<td>6.44</td>
<td>2.96</td>
<td>N</td>
<td>2.04</td>
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<tr>
<td>6974</td>
<td>response to DNA damage stimulus</td>
<td>7.8</td>
<td>3.85</td>
<td>N</td>
<td>2.32</td>
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<tr>
<td>45859</td>
<td>regulation of protein kinase activity</td>
<td>7.85</td>
<td>3.72</td>
<td>2.04</td>
<td>N</td>
</tr>
<tr>
<td>51338</td>
<td>regulation of transferase activity</td>
<td>7.85</td>
<td>3.72</td>
<td>2.04</td>
<td>N</td>
</tr>
</tbody>
</table>

Biological processes regulated at the RNA level by ionizing radiation exposure in PBL. GO ID: Gene Ontology category identifier. The numbers are the relative enrichment of responding genes at each dose observed for each category compared to the number expected by random chance.

**Table 4.**

<table>
<thead>
<tr>
<th>Symbol</th>
<th>GO process</th>
<th>50R</th>
<th>200R</th>
<th>500R</th>
<th>800R</th>
</tr>
</thead>
<tbody>
<tr>
<td>BBC3</td>
<td>Apoptosis</td>
<td>1.9</td>
<td>3.3</td>
<td>3.9</td>
<td>4.4</td>
</tr>
<tr>
<td>TNFRSF10B</td>
<td>Apoptosis</td>
<td>2</td>
<td>2.9</td>
<td>3.2</td>
<td>3.4</td>
</tr>
<tr>
<td>BAX</td>
<td>Apoptosis</td>
<td>2.8</td>
<td>3.8</td>
<td>4.1</td>
<td>4.2</td>
</tr>
<tr>
<td>TNFSF7</td>
<td>Apoptosis</td>
<td>4.5</td>
<td>11.4</td>
<td>12.2</td>
<td>16.7</td>
</tr>
<tr>
<td>MYC</td>
<td>Cell cycle</td>
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<td>0.55</td>
<td>0.36</td>
<td>0.34</td>
</tr>
<tr>
<td>PKL3</td>
<td>Cell cycle</td>
<td>1.3</td>
<td>1.7</td>
<td>2</td>
<td>2.1</td>
</tr>
<tr>
<td>CDKN1A</td>
<td>Cell cycle</td>
<td>2</td>
<td>3.5</td>
<td>4.9</td>
<td>5.7</td>
</tr>
<tr>
<td>SESN1</td>
<td>Cell cycle</td>
<td>2</td>
<td>3.2</td>
<td>3.3</td>
<td>4.1</td>
</tr>
<tr>
<td>GADD45A</td>
<td>Cell cycle</td>
<td>2.2</td>
<td>4.3</td>
<td>5.6</td>
<td>5.5</td>
</tr>
<tr>
<td>LIG1</td>
<td>DNA repair</td>
<td>1.3</td>
<td>1.9</td>
<td>2.2</td>
<td>2.3</td>
</tr>
<tr>
<td>XPC</td>
<td>DNA repair</td>
<td>1.8</td>
<td>2.8</td>
<td>3.2</td>
<td>3.6</td>
</tr>
<tr>
<td>PCNA</td>
<td>DNA repair</td>
<td>2.6</td>
<td>6.5</td>
<td>7.6</td>
<td>11.2</td>
</tr>
<tr>
<td>DDB2</td>
<td>DNA repair</td>
<td>3.8</td>
<td>7.9</td>
<td>8.5</td>
<td>11</td>
</tr>
<tr>
<td>FDXR</td>
<td>Electron transport</td>
<td>8.8</td>
<td>34.5</td>
<td>30.3</td>
<td>55.6</td>
</tr>
<tr>
<td>PHPT1</td>
<td>Phosphatase</td>
<td>2.6</td>
<td>5.5</td>
<td>4.8</td>
<td>6.5</td>
</tr>
</tbody>
</table>

Expression of genes 6 hours after exposure to various radiation doses. The numbers are average fold-induction in five donors relative to control levels normalized to 1.0. GO process: the major gene ontology category with which a gene is associated. Most genes participate in multiple processes and have multiple GO annotations.
Introduction

The automated high throughput imaging system under development for the RABIT (Rapid Automated Biodosimetry Tool) makes use of several novel techniques for speeding up the imaging process. Namely, we make use of multiple simultaneous light paths, light steering, rather than sample motion and single step auto-focusing. During 2007 we developed and tested the hardware implementation of these methods and are currently in the process of integrating them into a complete imaging system.

Multiple light paths

In order to reliably correlate nuclei and micronuclei with their parent cells (or γ-H2AX foci to their parent nucleus), we must analyze a two color image, corresponding to a nuclear and cytoplasmic stain (or nuclear and γ-H2AX stain). To efficiently do this we have assembled the imaging system shown in Figure 1. Fluorescent light emitted from the sample is split by a series of dichroic mirrors between three cameras, corresponding to blue (DAPI – nuclear stain), orange (cytoplasmic stain) and red (beads used for focusing system, see below). Figure 2a shows an example of images taken with the red and blue cameras, of the same test pattern (Fig. 2b). The image from the blue camera (left half of Fig. 2a) is flipped due to reflection in the dichroic mirror.

Sample manipulation

Commercial microscope stages such as the one used by the Metafer system are rather slow (70mm/sec). This is partially due to the fact that the main bottleneck in those systems is the generalized image acquisition and quasi-offline analysis and partially because of the desire to limit the accelerations experienced by living cells.

In our system both requirements are non-existent and so a much faster stage can be used. As in the microbeam, the motion of the sample is separated into two components. A slower coarse motion and a rapid fine motion. The coarse motion is performed by a high speed stage (Parker motion) capable of few-g accelerations. This motion is used to move between adjacent samples (9mm in 50msec). The fine motion between fields of view within a single sample is performed, not by moving the sample but rather by steering light, using fast galvanometric mirrors. Typical transit times between adjacent 80X fields of view were measured at 50µsec. Figure 3 shows an image of the USAF test pattern (Fig. 2b) stitched from multiple 80x fields of view.

Focusing

A major rate limiting step in an automated imaging system is focusing. In order to get good image quality, typically microscope objective lenses have a rather small depth of field and are sensitive to the roughness of the sample being imaged. The simple solution to this is to take several images...
at different object-lens distances, quantify “fuzziness” and search for the best setting. This process is very time consuming and therefore unacceptable. Our solution is to place a weak cylindrical lens in the optics path. The effect of this is shown in Figure 4. At the same plane (I) we will now have an image which is sharp in X and fuzzy in Y. At plane (II) we will get an image that is slightly fuzzy but symmetric, and at plane (III) we will get an image that is fuzzy in X and sharp in Y. By inserting a cylindrical lens before the red camera in Figure 1 and moving it to plane (II), we will get an image that is equally fuzzy in X and Y when the image in the other cameras will be in focus.

First tests of this are shown in Figure 5. Pictures of a 4µm fluorescent bead were taken at 1µm intervals from 70µm below to 30µm above the focal plane. The images were shifted by a given number of pixels in X or Y and a correlation computed. The aspect ratio is the ratio of the X-shifted correlation to the Y-shifted one and can serve as a quantifier of focus, in this case, up to 20µm away from focus. Larger ranges can be obtained by varying the strength of the cylindrical lens.

Fig. 5. Aspect ratio as function of distance from focus for different correlation length.

Automated Robotic System for High-Throughput Radiation Biodosimetry

Guy Garty, Anubha Bhatla, Jian Zhang, Alessio Salerno, Nabil Simaan, Lawrence Y. Yao and David J. Brenner

Introduction
The goal of this project is to develop a high-throughput radiation biodosimetry workstation, using robotic devices and advanced high-speed automated image acquisition. This document focuses on the design and implementation of robotic devices necessary to reach the desired throughput of 30,000 samples/day.

System layout
The RABIT (Rapid Automated Blodosimetry Tool) con-
sists of four main modules: centrifuge, cell harvesting system, liquid/plate handling robot and dedicated image acquisition/processing system.

The system layout, depicted in Figure 1, is currently under advanced stages of assembly as shown in the photo.

The layout shown in Figure 1 includes an automated centrifuge, a cell harvesting workstation, a liquid handling system, a robotic incubator and a dedicated imaging workstation. A SCARA robot is responsible for automating the sample transfer operations among these modules.

**Robotic centrifugation module**

The automated processing begins with loading blood samples, contained in bar-coded PVC capillaries, loaded in the field with 30-50µl each of separation medium and whole blood, into a centrifuge that will isolate the lymphocytes. The centrifuge (shown in figure 1 at right) is an Eppendorf 5810R, which has been equipped with a custom computer control board, an automated brake and lid opening mechanism. The rotor has been modified to accept 12 holders (Fig. 2) containing 44 capillaries each.

In addition, a bucket gripper arm was integrated into the service robot control scheme. An optical edge detector was used to allow picking of buckets using the robot at any given orientation of the centrifuge rotor. The program for this step was completed on the robot controller side.

Optimization of the centrifugation protocol is described elsewhere.2

**Cell harvesting module**

After centrifugation, the capillary holders are transferred from the centrifuge to the cell harvesting module where the lymphocytes are transferred to multiwell plates. A custom designed capillary gripper (Fig. 1, top left) removes the capillary from its holder and presents it to the imaging system, which locates the RBC/separation medium boundary (Fig. 3, right) and presents the capillary to a cutting laser (Fig. 3, left), while rotating it, to allow for even distribution of the laser-delivered power along the circumference of the capillary. Plasma and lymphocytes are then flushed, by application of air pressure through the gripper, into a well in a 96 well plate.

In parallel, a barcode, laser etched on the capillary, is read and the sample number associated with the corresponding multiwall plate and well number.

Laser cutting, bar-coding and reading, using the existing barcode reader was accomplished using the existing capillary gripper held by the service robot in front of the laser. Integrated segmentation and image acquisition/edge detection was carried out to command the service robot to locate the capillary in front of the laser. Dispensing the liquid from the severed capillaries using the capillary gripper in Figure 1 was also tested.

**Liquid handling and incubation**

The 96-well plates are then transferred by the SCARA robot to a dedicated, off the shelf, liquid handling system (Sciclone ALH3000, Caliper Life Science). We have programmed the liquid handling system to perform the first few stages of the cytokinesis blocked micronucleus assay. For the incubation steps we have acquired a fully automated robotic incubator (Liconic STX220).
The incubator was programmed and tested for its interaction with the sciclone and the robot controller. Currently the robot can move a sample from the centrifuge to the sciclone and from the sciclone to the incubator and back.

**Current status**

Over the past year we have acquired all necessary equipment for assembling the RABIT system and manufactured the many custom components required (grippers, control electronics etc.). We have also finalized the sample handling protocol and begun integrating the various components. The hardware for the master controller has been completed in order to allow it to control all components of the system. As described in the text, we have individual steps of the sample handling protocol programmed, but not fully and autonomously integrated. We are currently in the stages of writing a complete main program that runs the RABIT through an autonomous cycle without human intervention.

**References**


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**Automation of Biodosimetry Assays: Micronuclei and γ-H2AX Foci Formation in Human Lymphocytes**

*Helen C. Turner, Guy Garty, Aparajita Dutta and David J. Brenner*

**Introduction**

Measurement of micronucleus (MN) frequency in peripheral blood lymphocytes (PBL) is extensively used in molecular epidemiology and cytogenetics to evaluate the presence and the extent of chromosomal damage in human populations exposed to genotoxic agents or bearing a susceptible genetic profile. The *in vitro* cytokinesis-block micronucleus (CBMN) assay is used as an important component of genetic toxicity screening, wherein cultured cells are treated and scored for micronucleus induction using an image detection system. The use of this assay in biomonitoring studies has greatly increased in the last 15 years, and recent international efforts such as HUMN (human micronucleus) project (http://www.humn.org) have greatly contributed to improving the reliability of this assay, providing technical guidelines and analyzing major sources of variability.

Furthermore, immunofluorescence studies have revealed that H2AX is phosphorylated at the sites of DNA double-strand breaks induced by ionizing radiation and is required for recruitment of repair factors into nuclear foci after DNA damage. The number of γ-H2AX foci can therefore also be used as a sensitive radiation biodosimeter. The purpose of this study is to characterize the effect of radiation induced DNA damage in human peripheral blood lymphocytes by clearly quantifying micronuclei (MNi) and γ-H2AX foci formation. Thus far, in the characterization of MNi and γ-H2AX formation, we have adapted and optimized standard protocols for detection of these assays in our robotic, multiwell-plate-based biodosimetry workstation.

**Lymphocyte separation**

We have modified the lymphocyte separation protocol reported previously to work in plastic capillary tubes rather than glass ones. The advantage of plastic tubes is that they are safer to handle and are easily laser etched, allowing for...

**Fig. 1.** Frequency of micronucleus formation in human lymphocytes exposed to different doses of gamma radiation. The results show a linear increase in MNi frequency with increased exposure to γ-rays. Also presented, are PI-stained binucleate lymphocyte cells; a control, non-irradiated cell (0Gy) and the induction of MNi formation with 2 and 8Gy prior to culturing.
bar-coding and rapid cutting, both of which are requirements for use in our robotic system. Recently we have optimized the use of heparin-coated PVC capillaries (Safe-T-Fill capillaries; RAM Scientific) to reproducibly separate lymphocytes from 10 to 50µl of whole blood loaded onto 30 to 50µl lymphocyte separation media (Histopaque-1083; Invitrogen). Optimum separation was achieved when samples were spun at 4000 rpm for ~6 minutes. After testing a variety of lancets that offered a full range of blade depths and needle gauges to optimize blood flow with minimal pain, the Microtainer® Contact-Activated Genie™ Lancets from BD Diagnostic Systems have proven to be the most reliable in providing a 50-100µl finger drop of blood that we need for capillary blood collection.

**Micronuclei formation in human lymphocytes**

In order to reduce handling time and to facilitate medium exchange and the addition/removal of reagents without needing to pellet out the lymphocytes by centrifugation at each step, we have previously designed a system for culturing lymphocytes in 96 micro-well plates (HTS Solubility Filter Plates; Millipore) that contain non-fluorescent polycarbonate filters with a pore size of 0.4µm for this purpose. Although we have already modified the MN assay for a multi-well format, during the past year, we have continued to test MN assay parameters for temperature control and immunofluorescent detection. To this end, we have to be able to determine that the application of KCl is effective at room temperature and, for fixation, the methanol/acetic acid mix must be ice-cold when initially applied to the cells. For the clear detection of binucleate cells on the filter membranes, we have tested various combinations of cytoplasmic/nuclear stains.7

To examine micronucleus formation in cytokinesis-blocked lymphocytes in vitro, peripheral whole blood samples were irradiated with γ-rays (0 to 8Gy). MNi were scored according to the guidelines outlined by HUMN criteria.4 Briefly, MNi should be round in shape and clearly separated from the main nucleus; exhibit similar staining characteristics as the main nucleus, and found to be within the same cytoplasm. Cells undergoing degenerative processes (karyolysis, pycnosis, nuclear fragmentation) should also not be included in the evaluation. At least 1000 propidium iodide (PI) stained binucleated lymphocytes per dose were manually and systematically scored for micronuclei. The dose-response curve revealed a linear increase in MNi frequency with increased exposure to γ-rays (Fig. 1). To complete this study, the effect of 5 and 6Gy on MNi yields remains to be determined.

**γ-H2AX foci formation in human lymphocytes in response to different doses of γ-rays**

To induce γ-H2AX foci formation in human lymphocytes, peripheral (1-2ml) whole blood samples were irradiated with 0 to 4Gy and allowed 1-2 hours at 37°C for foci formation. Isolated lymphocytes were fixed for 5 mins in ice-cold methanol:acetone (1:1 mix) and transferred to +/+ glass microscope slides for immunolabeling. The cells were blocked with 2% BSA for 20 mins and incubated with an anti-human γ-H2AX monoclonal antibody (1:400 dilution; Abcam) for 30 mins. After two washes with phosphate buffered saline (PBS), the cells were visualized following the addition of goat anti-mouse Alexa 488 secondary antibody (1:300 dilution; Molecular Probes) for 20 mins. Cells were mounted in Vectashield mounting media containing 1.5µg/ml DAPI. Fluorescent microscopy identified the presence of concentrated γ-H2AX at the DNA double strand breaks, which appear as foci when immunolabeled with the γ-H2AX antibody (Fig. 2). The results show that there is a clear distinction between foci and DAPI-labeled nuclei as the frequency of foci increased with increasing doses of γ-ray irradiation. At higher doses of 4Gy, the detection of individual foci proved more difficult as the increased number of foci began to merge with each other and cause an overall increase in total fluorescence. In order to use the γ-H2AX assay as a rapid and sensitive screening tool, the protocol described above demonstrates that by changing the blocking agent and significantly reducing overall incubation times we have been able to reduce the γ-H2AX assay time by more than 50% without losing foci resolution.

**Summary**

In summary, the present report shows that we have used and adapted established biological assays to identify MN and γ-H2AX foci formation in gamma-irradiated peripheral human lymphocytes. The results show that increasing the dose of radiation treatments revealed a linear increase in MN
and γ-H2AX foci formation. At the Center for High-Throughput Minimally Invasive Radiation Biodosimetry, we continue to progress in the optimization, calibration and testing of these assays for their use as high throughput diagnostic markers for biodosimetry.

References


Optimization of Lymphocyte Imaging in Filter Bottomed Plates

Oleksandra Lyulko, Helen Turner, Guy Garty, Gerhard Randers-Pehrson and David J. Brenner

Sample preparation for imaging

The Micronucleus assay is performed in 96 well plates with filter bottoms. The plates then proceed to an imaging unit for analysis where stained lymphocytes are visualized on filter membranes.

As filter membranes are not a typical substrate for fluorescent imaging, information about their fluorescent properties is not readily available. Most filter materials have high fluorescent background, due to reflection and/or autofluorescence. Besides, only a few filter plate types that are compatible with high-throughput systems allow for membrane transfer (see below). Our objective was to optimize image quality by selecting plates with proper filter material and developing a compatible staining protocol.

During the past year we have tested numerous filtration plates from Millipore, Corning, Whatman, Pall, Nunc, Harvard Apparatus and Phenix Attic with different membrane material (PVDF, polycarbonate, polyester, polypropylene, nylon, fiber glass, mixed cellulose etc.)

We have tested quality of the images acquired on the membranes of various plates, using different combinations of sealing material and dye (see, for example, Fig. 1). To examine the membrane materials further, we measured their reflectance, which varied from a 2-10% for un laminated plates to as high as 30% for plates laminated with Abgene plate sealer (see “Sample transfer” section below). Following these tests we came to the conclusion that Millipore MultiscreenHTS Solubility Filter Plates with polycarbonate membranes have the lowest fluorescent background and are the most suitable for automated membrane transfer.

To facilitate high-throughput imaging, cell staining needs to be bright enough for the cells to be visualized with short exposure times, and stable enough to sustain sample storage. In addition, the excitation and emission spectra of nuclear and cytoplasmic probes should be well separated, to allow simultaneous illumination and imaging.

Previously, DAPI was selected as the nuclear stain and in 2007 we tested a number of stains for cytoplasm, plasma membrane, cytoskeleton, as well as whole cells: acridine orange, CellTracker™ Orange, propidium iodide, HCS CellMask™ Red, HCS CellMask™ Deep Red, CellMask™ Orange, phalloidene rhodamine, CellTracker™ CM-Dil, Red Counterstain C, Alexa Fluor® 488 and Alexa Fluor® 555 targeting various cell organelles. Amongst these, CellMask Orange plasma membrane stain appears to satisfy all of the above conditions and provide more uniform staining across the cell and sharper image of cell boundaries compared to other stains. The staining of one 96-well plate takes approximately 15 minutes.

Sample transfer

To accommodate the short working distance of the imaging optics as well as for generating compact, sealed archival specimens, the membrane bottoms of multi-well filtration plates have to be transferred to a flat, rigid substrate. We tested two different approaches for membrane transfer:
1. Transfer to a substrate covered with re-wettable adhesive and simultaneous sealing the membranes with...
polyvinyl acetate (PVA) solution;
2. Transfer to adhesive surface in conjunction with a punching system, followed by sealing of the samples with PVA or plate sealer.

The first procedure is done by filtrating 10% solution of PVA while the 96-well plate is pressed against the substrate. After the glue has dried, the well walls are removed, leaving the filters attached to the substrate.

In the second case we use ELISA plate sealers (adhesive strips used for covering plates during incubations) as substrates. We use a punching mechanism (MVS Pacific) to detach the membranes from the plates and to keep them intact. The same plate sealers can be used to seal the sample. Another option is to cover the exposed membranes and adhesive surface of the film with 10% PVA solution. Lamination of the samples is also possible and was tested, but since the adhesive film is not rigid, this method introduces some curvature.

The second method has proved to be more reliable in terms of transfer quality (100% of the membranes). The procedure can be performed instantaneously, which is crucial for a high-throughput system, whereas using re-wettable adhesive requires time for drying. Image quality is comparable, although less noise and higher contrast is observed when samples are covered with plate sealing film versus a layer of glue. Different brands of plate sealers were tested for light transmission (Corning, Fisher, Abgene). All of them proved not to reduce the image quality significantly compared to unsealed samples and the material from Corning is slightly preferable over the other two.

Dr. David Brenner became our Center Director at the end of 2007.
Radiological Science in the Context of Radiological Terrorism

Carl Elliston, David J. Brenner and Eric J. Hall

Particularly in the context of the potential for a large-scale radiological event, there is a growing necessity to train health care professionals at all levels in the radiological sciences. Correspondingly, there is increasing demand from health care professionals, most of whom have no background in the radiation sciences, for such information in an accessible format.

This year, as a part of our goal to provide regularly scheduled courses in a variety of locations, we held the 3rd and 4th training courses entitled Radiological Science in the Context of Radiological Terrorism. These courses were held at Columbia University on June 15 and Georgetown University on Dec. 17, respectively. Registration was free, and CME accreditation was available to qualified students, sponsored by Columbia University Medical Center. The one-day course was attended by over 80 physicians, nurses, hospital administrators, medical and health physicists, graduate students, and science communicators from Taiwan, California, North Carolina, Texas, Maryland, Pennsylvania, Virginia, Georgia, Florida, Wisconsin, and Washington, D.C.

This course covers a broad spectrum of topics to help participants understand 1) the nature of ionizing radiation; 2) how radiation is damaging to people; 3) how we know what we know about radiation risks; 4) potential radiological terrorist scenarios; and 5) emergency preparedness for a radiological event.

The course is designed around a series of lectures. As an example, the faculty and topics for the most recent course were:

- **The Nature of Radiological Terrorism**, David Brenner (Columbia University)
  - This topic describes a) the physical nature of radiation and b) the various possible scenarios, including “dirty bombs” that may be involved in a radiological incident.
- **Basics of Radiation Biology**, Sally Amundson (Columbia University)
  - This topic discusses the types of damage induced in DNA and other cellular compartments by ionizing radiation, how cells process this damage, and how to detect this resulting damage.
- **Acute Somatic Effects of Radiation**, Eric Hall (Columbia University)
  - This topic describes radiation-induced damage to the blood forming organs and gastrointestinal tract that can lead to serious injury or death at sufficiently high doses.
- **Radiation Epidemiology**, Elaine Ron (National Cancer Institute)
  - This topic surveys the information on radiation-induced cancer in human populations from high and low doses and describes techniques to obtain risk estimates for radiation-induced cancer from population studies.
- **Long-term Radiation Effects**, Eric Hall (Columbia University)
  - This topic discusses the long-term effects of radiation. Effects to be discussed include carcinogenesis, hereditary effects, cataractogenesis, and consequences to the developing embryo and fetus.
- **Anticipated Psychological Impact of Radiological Terrorism**, Ann Norwood (Uniformed Services University)
  - Psychological trauma probably is one of the greatest risks for individuals in a "dirty bomb" scenario. This topic highlights the symptoms and treatment modalities in the context of a radiological terrorism event.
- **Emergency Preparedness**, Stephen Morse (Columbia University)
  - This topic surveys the general aspects of preparedness for unexpected emergencies, based on the “all-hazards” approach. The relationship between healthcare providers and public health, planning considerations, inter-organizational relationships, and the concept of incident command/incident management are discussed.

Additionally, we have created a series of podcasts based on the lectures from all previous Radiological Science in the Context of Radiological Terrorism courses. Each podcast consists of about 40 minutes of audio, playable on any iPod or similar player, as well as a PDF of the PowerPoint slides used in the lecture. PDF handouts giving an outline of the key topics covered and optional podcasts with synched video of the accompanying PowerPoint slides are provided for some of the lectures and are in the process of being made for the remainder.

A web page has been created to allow users to subscribe to the material in a simple, straightforward manner. The URL is: http://cmcr.columbia.edu/podcasts/

Supported by NIAID grant U19 AI067773
High Throughput Biodosimetry using Gene Expression

Muriel Brengues

The center for Applied Nanobioscience (ANBC) is developing a completely self-contained radiation biodosimeter suitable for large scale screening. This portable device will be able to rapidly measure expression levels of a specific gene set that will define radiation exposure, dose and injury. A set of 14 genes that were discovered by our collaborators at Columbia University and Tgen, has been selected based on their differential expression level in response to radiation. One housekeeping gene, GAPDH, and one negative control, ANT, were added to complete the 16 gene radiation array. A diagram of the radiation array gene organization is shown in the figure. Gene expression is analyzed through direct signal amplification or quantitative Nuclease Protection Assay (qNPA) developed by our collaborators at High Throughput Genomics (HTG). Such an assay platform was developed for small volume analysis of whole blood that resulted in low coefficient of variation and quick result that is suitable for automation into a microfluidic cartridge. We are currently working on the conversion of this assay from its microplate format to a handheld detection device to determine the gene expression levels of individuals who may be exposed to radiation. We have fabricated a cartridge containing a front end module for the sample preparation of the nuclease protection assay and a back-end corresponding to the microarray chemiluminescent detection (see Figure). For the actuation and control of these cartridges, a printed circuit board and a computer controlled test-station including Labview software user interface were designed and fabricated. This universal test station (UTS) was made to accommodate testing open and closing valves, incubation of chambers at different temperatures, transfer of sample from one chamber to another, mixing and sample recovery. Once the assay is complete, the analysis is performed with a CCD-based chemiluminescence array reader (FireflyTM) modified to accommodate these integrated microfluidic cartridges (see Figure).

Although qNPA assay technology had been successfully used to detect well expressed genes in mouse blood and for expression profiling of isolated leukocytes, qNPA application to human whole blood always resulted in high background, S1 failures or no signal. However and recently, we were able to successfully adapt the quantitative Nuclease Protection Assay in order to measure gene expression levels from human whole blood samples. These preliminary data

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*a Center for Applied NanoBioscience, Arizona State University*
are shown in the figure. The array scanner gives a composite image of the chemiluminescence emission captured at the position of the array elements. A graphic analysis of the data extracted from the digital image is shown on the figure. The difference in the signal intensity reflects the difference in the gene expression level for the set of genes indicated from an unirradiated human whole blood sample.

We are currently testing the front and the back end cartridge and the adaptation of the assay protocol to the microfluidic platform. Next, the qNPA will be performed on the cartridges using whole blood. Once the qNPA will be validated on the cartridge, the test will be run using irradiated human whole blood. These experiments will be done in collaboration with Scottsdale Health Care that will provide us with state-of-the-art radio-oncology equipment and experts to study a large number of patient blood samples (~200x). These experiments will allow validating the gene panel and qNPA assay with the discovery assay and panel used with the Agilent microarray technology. These data will serve as a basis for a pending pre-IDE submission to the FDA. An integrated system could also be used for personalized monitoring of future radiotherapy treatment received by patients.

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**DMS-Mass Spec Detection of Biomarkers for Radiation Exposure**

*Erkinjon Nazarov*

Following the Year 2 successes with sensitive detection of 5 biomarker candidates for radiation exposure using DMS-prefiltered mass spectrometry, work at Sionex has been extended in Year 3 to include tests on biological samples and on dilute mixtures of identified biomarkers. In addition, plans have been developed for improvement in detection levels by changes in sample handling and ionization methods, and for reduction in MS system cost by use of miniature MS technology.

Two biomarkers are currently identified by Project 3 as most reliable. These molecules are hexanoylglycine and thymidine. Using urine samples from treated and untreated mice provided by Kris Krausz and Jeff Idle, Sionex has found that hexanoylglycine is easily separated and detected by ESI-DMS-MS in solutions buffered to neutral pH, as are several other masses appearing in the treated samples. Thymidine was not detected in these samples at Sionex. To determine the reason for this, we first reviewed the NCS LC-MS data for the treated and untreated cases. Thymidine appears at levels 50 times lower than hexanoylglycine in the LC-MS spectra received from NCI, so further work may be necessary to lower our thymidine detection limit.

A series of experiments was undertaken in order to determine the relative sensitivity of ESI-DMS-MS to hexanoylglycine and thymidine with urine samples and in mixtures at low concentrations. Analysis of thymidine mixed in urine samples with our current protocol reduces thymidine...
intensities by about a factor of 6. Mixing hexanoylglycine with thymidine leads to only a modest reduction in thymidine intensity. Additional work on sample conditions and ionization techniques for the biosamples should improve the detection limit for thymidine.

On the basis of these systematic experiments and analysis, we propose further work on sample handling, ionization efficiency and ionization techniques.

- In the ionization area, variation of pH into the pKa 10-12 range of thymine / thymidine may allow the enhancement of positive ion signals from H+, Na+ or K+ adducts, or to the enhancement of negative ion signals. The use of a short column (~1m.) might provide sufficient separation to prevent loss of sensitivity to thymidine in the biomatix.
- The recently-developed DART and DESI ionization methods may also provide better ionization efficiency for biosamples and mixtures. DART may be especially promising, and is significantly simpler than ESI, involving direct ionization from samples.

Separate testing on synthetic mixtures of 5 isobaric compounds is a striking demonstration that DMS is able to isolate ions that would not be resolved in a low or moderate resolution mass spectrometer. This indicates that current development on miniature MS instruments may be useful in this application. Sionex is working with several major groups on this technology, and has begun an internal development effort.

References

1. SionexCMCRYear2Report.pdf by Sionex Corporation, July 3, 2007. This report was previously distributed, but can be obtained on request.

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**Radiation Biodosimetry through Metabolomics**

*Jeffrey R. Idle,*a* Frank J. Gonzalez,*b* Erkinjon Nazarov*c* and Albert J. Fornace Jr.*d*

Metabolomics is the study of the small molecule complement of cells, tissues, organs, and organisms. The global profiling of metabolites in a biological matrix can facilitate the identification of altered metabolic processes, thus making it an ideal method for identifying biomarkers of exposure and disease. Project 3 of the Center for High-Throughput Minimally-Invasive Radiation Biodosimetry combines the expertise of its members in metabolomics, stress-signaling, and sensor-chip technology to develop instrumentation for rapid noninvasive assessment of radiation exposure and injury using metabolic markers. It also interacts with other projects to integrate metabolomic and expression profiling at the cellular level as well as in mouse and radiotherapy patient samples. We have successfully used metabolomics to develop ionizing radiation biodosimetry in the mouse1 as well as in a human lymphoid and a human fibroblast cell line.2 Self-organizing map algorithms using the Gene Expression Dynamics Inspector (GEDI) have been useful for providing global views of changing metabolome (urinary and cellular) after exposure to ionizing radiation.

Our major focus has been to demonstrate that metabolic markers for ionizing radiation exposure can be identified in mouse urine, and we are now extending our investigation to other biofluids such as saliva, blood, and sweat. In mouse urine, robust responses have been seen after high doses, such as 8Gy, and significant responses have been seen at lower doses, such as 1 and 3Gy.1 Results to date have demonstrated that a variety of metabolites are significantly altered by ionizing radiation exposure.

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Our current strategy is to carry out the discovery and product development components concurrently so current candidate markers have already been sent to team members at Sionex for development of in-field instrumentation. Sionex’s efforts center on several instrumentation developments. These include improvements in their microDMx technology, which is being optimized for in-field biodosimetry. Their new miniaturized implementation of GC-microDMx allows portable, sensitive and selective analyses of liquid and vapor samples after chromatographic separation of complex mixtures containing analytes at trace levels. Sionex’s nano-electrospray-coupled microDMx integrates a nanospray ion source directly to the microDMx sensor, which will allow for very fast screening of low volatility species in fluids and digests. Efforts are also underway to extend this to analysis of biofluids from patients undergoing total body irradiation.

During 2007 efforts were concentrated in two areas, analysis of the data being accrued on human blood subjected to irradiation ex vivo, and to identify the critical steps in the assay being produced for use in the analytical cartridge intended to provide measurements of radiation exposure based on changes in gene expression in peripheral blood lymphocytes.

Expression data from human blood, drawn and then exposed to varying doses of irradiation, were examined by a variety of analytic methods ranging from distributional tests to methods that identify particular patterns of expression in the presence of other, confounding expression activities. All the methods identified overlapping sets of genes whose expression patterns showed very clear changes in the irradiated versus un-irradiated controls. A number of genes showed differences in expression that differentiated higher expression doses, 5 and 8 Gy, from lower doses, 0.5 and 2 Gy, though the differences in these cases were less distinctive. One of the key difficulties in analysis of these data is the need to normalize across the individuals. Both differences in individuals, and differences in the biochemical analysis used to evaluate changes in expression patterns can change the scale of the changes observed, making it difficult to obtain a single threshold value that could be used across all individuals measured to accurately identify exposure and degrees of exposure. Currently in expression studies this problem is most accurately dealt with by the use of a common control, which is evaluated simultaneously with the case in question.

This adds a very great deal of operational complexity to the problem, requiring an assay that can be simultaneously evaluated for two input analytes, and the production and storage of the analyte itself. As a way of avoiding this added complexity, we have developed a method of identifying species of RNA in the sample that can serve as an internal control for variances in the amounts and quality of RNA going into the assay and for variances in the efficiency of detection of the RNA in the assay. We have used a method of exhaustive computational analysis of possible pairs of exposure detector RNAs and “normalizer” RNAs to find species that give excellent internal normalization. These pairs have been scrutinized for the quality of separation that can be achieved by two methods, typical bolstering, and strong feature analysis. In bolstering, a shell of further data points that fills out the area indicated by the available data points is added to the analysis of separation to see whether the separation distance between the classes is large or small. In strong features analysis, Gaussian shells of further points at increasing distances from the existing data are added to determine which separations are the most tolerant of noise. Good classifiers based on these have been found, and a test with much more data has been proposed as a validation of these.

A key requirement for the dosimeter device is the ability to work with very small amounts of blood, on the level of 50 microliters, which can be obtained through a simple finger-prick, as is used in blood monitoring for diabetes. The ability to convert whole blood to a lysate that can be used in the proposed qNPA assay for RNA has been problematic. Whole blood tends to form a gelatinous mass using the lysis monoclonal antibodies for RNA isolated from a single peripheral blood mononuclear cell to work with very small amounts of blood, on the level of 50 microliters, which can be obtained through a simple finger-prick, as is used in blood monitoring for diabetes. The ability to convert whole blood to a lysate that can be used in the proposed qNPA assay for RNA has been problematic. Whole blood tends to form a gelatinous mass using the lysis monoclonal antibodies for RNA isolated from a single peripheral blood mononuclear cell.

### References


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**Informatics, Biostatistics and Data Management**

Michael Bittner*  

*Computational Biology Division, Translational Genomics Research Institute, Phoenix, Arizona*
added noise and suppressed signal in the assay. Concerted efforts by the HTG, ASU and TGen teams to review possibilities and thoroughly analyze alternatives have produced a likely way to deal with this problem that can be instantiated in a cartridge.

Non-Invasive, High Throughput Cytogenetic Biodosimetry for Ionizing Radiation Exposure

Angela J. Yoon,a Jing Shen,b Hui-Chen Wu,b Regina Santellab

Background

A large segment of a population may be exposed to ionizing radiation during an accident or in a nuclear bioterrorist event. Such potential events demand an efficient method to conduct mass scale screening within a few days after the exposure. Our goal is to devise a high-throughput assay that measures expression of the signature biomarkers for radiation exposure in human tissue samples obtained by non-invasive methods. We hypothesize that γH2AX and pChk2, DNA damage response molecules, and 15-F₂t-IsoP, a marker of oxidative stress, are produced in irradiated cells and may serve as signature molecules of exposure.

Methods

A total of 100 healthy individuals undergoing routine dental radiographic examination (23.4 mGy) were included in the study. The exfoliated oral epithelial cells were collected using a mouthwash, before and 20 minutes after the radiographs were taken. The epithelial cells were cytospun onto glass slides for immunohistochemical analysis for γH2AX and pChk2. The intensity of nuclear staining in 50 randomly selected cells was analyzed using a software system (Becton Dickinson). The analysis by competitive ELISA is pending.

Results

Both biomarkers showed a statistically significant increased level of expression after the radiation exposure. The mean intensity for pChk2 before the radiation was 0.114 (SD=0.035) and after the radiation was 0.139 (SD=0.038). For γH2AX, the mean intensity before the radiation was 0.105 (SD=0.033) and after the radiation was 0.125 (SD=0.052). A paired t-test showed p< 0.001 for pChk2 and p< 0.001 for γH2AX. (Fig. 1 and 2).

Conclusion

The two biomarkers, pChk2 and γH2AX, may be sensitive markers for identification of individuals exposed to radiation.

Fig. 1. γH2AX and pChk2 positivity. Photomicrographs showing positive brown nuclear staining for γH2AX and pChk2 in the exfoliative oral epithelial cells.

Fig. 2. γH2AX and pChk2 expression intensity before and after radiation exposure. Box-plot of biomarker expression level comparison before and after radiation exposures: X axis, status of radiation exposure; Y axis, median of biomarker expression level; Boxes, inter-quartile range of the distribution (25th -75th percentile); horizontal line within the box, median; horizontal lines outside the box, 5th and 95th percentiles; Black dots, outliers.

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b Department of Environmental Health Sciences, Mailman School of Public Health, Columbia University, NY
High-Throughput Antibody-Based Assays to Detect Radiation-Induced Changes in Protein Levels

Michael A. Partridge and Tom K. Hei

Introduction

There is an urgent need for a rapid and high-throughput screening tool to identify individuals exposed to radiation in the event of a “dirty bomb” incident. After radiation exposure, the LD50 (dose causing 50% mortality) for humans is estimated to be between 3 and 4 Gray (Gy). However, isolation of patients and antibiotic treatment to prevent infection, blood transfusions and, as a final resort, bone marrow transplants, can raise the LD50 to ~7Gy. Consequently, a rapid and accurate test to detect individuals exposed to between 2 and 7Gy could save a substantial number of lives.

It is well established that exposure of cells to radiation changes the level of a variety of cellular proteins. These include a number of DNA repair enzymes as well as a number of paracrine signaling molecules, such as cytokines, their receptors and downstream cell adhesion molecules. A number of these proteins are either secreted or are expressed on the cell surface. These antigens are an appealing target for high-throughput assays because alterations in extracellular levels may be detected without lysing the cells, and ease of sample preparation is a critical consideration for high-throughput assays. By assaying changes in extracellular protein-level, potentially, blood from a finger prick or cells from a mouth swab could be assayed directly, without time consuming preparative steps.

Antibody-based assays are an attractive option for diagnostic tests because they are relatively cheap, non-toxic and yet highly specific. Furthermore, the enzyme-linked immunosorbent assay (ELISA), a test performed in a 96- or 384-well microplate, can be fully automated, allowing for high sample throughput while still maintaining the sensitivity required for accurate analysis. As a result, antibody-based assays are among the most commonly chosen techniques for diagnostic tests. The technology underpins a variety of assays that have both commercial and industrial applications as diverse as screening for specific protein expression in plant breeding programs, to detecting viral infections in livestock, to testing in humans for bladder cancer or pregnancy. Given this range of successful applications of antibody-based diagnostics, and the level of technical expertise available, we wanted to assess high-throughput immunoassays, such as ELISA, for developing a radiation biodosimeter. Using cultured human cells, we were able to detect changes in protein levels for two prominent cytokines, IL6 and TGFβ. Furthermore, these changes persisted for 48 or 72 hours and were observed in multiple cell lines, crucial criteria that must be met before further developing these assays for use in whole organisms.

Materials and methods

Cell lines

The following human cell lines were used in this study: HEK, 293T and WI38 from ATCC; Human Cardiac Fibroblasts (HCF) from ScienCell Research Laboratories (San Diego, Ca); HUVEC and NHFL cells obtained from Lonza Group (Switzerland); MRC5 cells from Coriell Cell Repositories (NJ); BEP2D and SAE cells.

Irradiation Procedure

Cells were plated in flasks and exposed to radiation from a GammaCell 40 Cs irradiator (dose rate, 0.82Gy/min) at Columbia University. Medium was changed every 24 hours and assayed by ELISA. TGF-β content was determined after acid treatment of supernatants to release the molecule from its latent complexes as described by the ELISA kit manufacturer.

Antibodies

Antibody pairs used in sandwich ELISAs for this study were all commercially available. Kits to detect EGF, ICAM-1, IL1-α, IL1-β, IL-4, IL-6, IL-10, TGF-β, TNF-α and VEGF were from Biosource (Camarillo, Ca). Antibody pairs for Fas and MMP-8 detection were from R&D Systems (Minneapolis, MN).

ELISA

Microwell plates (Maxisorp, Nunc) were coated with 100 µL of capture antibody overnight at 4°C. For blocking, 200 µL of 3% bovine serum albumin (BSA) in PBS was added to wells (1h at 20 °C). Antigen was diluted in 2% BSA in PBS containing 0.05% Tween 20 (PBST) and 100µL added to the wells followed immediately by addition of biotin-conjugated detection antibody, diluted in 50µL of 2% BSA-PBST. After 90 minute incubation at 20°C with shaking, plates were washed (4x PBST) and streptavidin-HRP was added, diluted in 100µL of 2% BSA-PBST. Following 1 h incubation at 20°C, microwells were washed and 100µL of tetramethylbenzidine (TMB, Pierce) added for 15-30 mins at 20°C. Reactions were stopped by addition of 0.9M H2SO4 (50µL) and product absorbance was determined at 450nm.

Results

Screening Human Cell Lines for Cytokine Expression after Irradiation

More than 260 proteins have been reported to be responsive to radiation, ostensibly providing a large pool of potential candidates for a protein-based radiation biodosimeter. However, the experiments from which these findings were derived were performed in literally hundreds of different laboratories using different systems (in vitro, in vivo), differ-
ent cell types (human, animal, immortalized, tumor), employing different detection systems (western, flow cytometry, ELISA, 2D-gels, etc) with different dose ranges and time of detection. In addition, in many cases the findings have been reported only once. We wanted to perform a more systematic examination of the most promising of the candidate antigens. We used a range of cultured human cells to detect changes in expression of a variety of secreted proteins and used ELISA as the detection system. Subsequently, we assessed whether the cells increased the expression of these proteins after irradiation, or if cells that were initially negative could be induced to express the protein above the threshold of detection in our assay. We selected human cell lines that were readily available in our laboratory and chose normal cell lines in preference to tumor cell lines. We then chose antigens whose protein expression had been reported to change after exposure to radiation, and in most cases selected proteins for which changes had been reported in multiple independent laboratories. In addition, we limited our testing to antigens for which antibody ELISA kits were commercially available. The results from this initial screen of cell culture medium from 9 cell lines for expression of a dozen different antigens are given in Table 1. Two results were obvious from this preliminary analysis: First, most antigens were not expressed in the media from any of the cell lines tested; Second, those antigens that were clearly positive in ELISA were usually expressed in multiple cell lines. Of the 12 antigens screened only 3 gave at least a moderate response in ELISA in multiple cell types; IL-6, TGFβ and VEGF. Importantly, both IL6 and TGFβ have been previously identified as promising target antigens for radiation biodosimetry. Interestingly, at least three of the cell lines which were positive for IL6 and TGFβ (HCF, HUVEC and NHLF) were primary cell lines. This was important because primary cells may be more similar to materials obtained from human subjects in the field than cell lines that have been immortalized (eg. 293T or SAEC).

In addition to the test for basal expression, we also wanted to determine if any of these cells could be induced to express the antigens after irradiation, even if they were initially negative. All cell lines were exposed to moderate to high levels of γ-radiation (2, 5 or 10Gy) and the culture media tested for secreted proteins. However, none of the cell lines negative for the antigens tested was positive in ELISA after irradiation (not shown), indicating that the antigens were not expressed de novo after irradiation, at least at the level of detection for colorimetric ELISA.

**IL6 and TGFβ Expression Increases after Irradiation**

Interestingly, some cell lines that were positive for either IL-6 or TGFβ antigens (and, to a small degree, Fas - not shown) did display a consistent increase in concentration in the culture media after irradiation. For example, Figure 1 shows two cell lines, WI38 and NHLF, in which TGFβ was detected at increased levels in the media after irradiation. Importantly, the observed change in TGFβ clearly persisted for at least 48 hours after irradiation, as the media sampled after 2 days from both cell lines had increased TGFβ levels. For this antigen, the optimum level of detection was within 50-150pg/mL, corresponding to a moderate ELISA response. Within this range the ELISA is highly sensitive, detecting up to a 4-fold change in TGFβ concentration after irradiation. However, outside this range the assay is less sensitive. For example, NHLF cells (Fig. 1A) have a greater than 3.5-fold increase in ELISA response after 24 hours. However, after 48 hours when basal levels are above 200pg/mL, the ELISA detects only a 1.4-fold increase in TGFβ in the NHLF cell supernatant, despite displaying an overall dose dependent change. It is likely that for NHLF cells at 48 hours, the antigen expression in the media in controls is already high due to increased cell density, so that increases in TGFβ levels in the media after irradiation confer only a small proportional increase. For WI38 cells, media sampled 48 hours after irradiation displayed an almost 4-fold change in ELISA signal (Fig. 1B). However, at the 24 hour time point WI38 cells show no change in TGFβ concentration in the media (Fig. 1B). For WI38 cells at 24 hours, either there was no increase in secreted TGFβ, or the levels

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ELISA Response (450nm): = zero; ± = 0.1; + = 0.1 - 0.3; ++ = 0.3 - 1.0; +++ = >1.0
n.d. = Not determined
were so low at that time point that they are below the detection threshold for the ELISA. These data illustrate the importance of accurately determining the basal antigen expression per cell in order to optimize the assay conditions.

In addition to TGFβ, we also observed, in multiple cell lines, increased levels of IL6 after irradiation. In fact, the increase in IL6 detected in the media after irradiation was much greater, and much longer lasting, than for TGFβ in the same cell type. In NHLF cells, there was a small but significant change in IL6 levels 24 hours after irradiation, even at doses as low as 1 Gy. Interestingly, at time points later than 24 hours, secreted IL6 in NHLF cultures increased much more dramatically in a time- and dose-dependent manner (Fig. 2A). Forty-eight hours after exposure, there was a 2-fold increase in IL6 levels in cultures exposed to 5 Gy, and an almost 3-fold increase after 72 hours. In cells exposed to 10 Gy, IL6 levels increased more than 6-fold (1604 pg/mL) compared to controls (260 pg/mL) after 72 hours. Even cells exposed to much lower doses displayed an almost 2-fold increase in IL6 detected in the media after irradiation (2 Gy, 72 hours, 502 pg/mL) compared to controls. This was important because for most antibody-based diagnostic applications an approximately 2-fold change is necessary for discriminating between samples. Importantly, even at doses as low as 1 Gy, there was a greater than 50% increase in IL6 detected after 48 and 72 hours in NHLF cells compared to controls (Fig. 2A). It should be noted that although there was a statistically significant difference (p<0.05) in response between controls and irradiated cultures after 24 hours (1, 2 and 5 Gy, Fig. 2A), this may not be sufficient for discriminating between samples in the field where there is a greater inherent variability.

In addition to NHLF cells, we also observed an increase in IL6 levels in SAE cell media after irradiation. When SAE cells were exposed to 5 Gy there was a greater than 2 fold increase in IL6 expression (107 pg/mL) after 48 hours compared to controls (46 pg/mL). Even at doses as low as 2 Gy, there was an almost 1.8-fold increase in IL6 detected after 48 hours (82 pg/mL). For the 48 hour time point, these results were remarkably consistent with radiation-induced lev-
outcomes are routinely used elsewhere and the technology to dramatically increasing the amount of information that can simultaneously detect different antigens in the same assay, a suite of antibodies labeled with different fluorophores could be particularly valuable for first responders to quickly identify those individuals, the majority of the population, that were unaffected by the radiological dispersion device. This would help achieve one of the main goals of developing a high-throughput biodosimeter: reassuring the vast majority of the population that is unharmed after terrorist attack and therefore helping to instill calm.

The present study, a systematic investigation of the change in cytokine levels in a range of normal cell lines after exposure to different radiation doses 24-72 hours after irradiation, serves to underscore the potential for IL6 and TGFβ as potential markers for a biodosimeter. It will be important to assess the efficacy of this and other proteins in immunoassays to discriminate between samples obtained from whole organisms exposed to radiation.

Discussion

The antigen-antibody combinations identified in this project need to be validated for their utility in other assay formats. The immunochromatography test (ICT) card format is widely used in situations where the user is untrained, such as the home pregnancy test. Another alternative is to combine multiple probes (IL6 and TGFβ, for example) in the same ELISA assay. Potentially, a suite of antibodies labeled with different fluorophores could simultaneously detect different antigens in the same assay, dramatically increasing the amount of information that can be obtained from a sample in one experiment, and potentially improving the level of sensitivity. Both these potential outcomes are routinely used elsewhere and the technology to develop these tests is both highly advanced and accessible.

It should be noted that there is a distinct difference between an elevated cytokine level in response to a specific “stressor” and a generalized stress response. The stress response, or fight-flight response, involves the rapid release of adrenal corticosteroid hormones that increase awareness and arousal, a process that is rapid and unrelated to a paracrine or inflammatory response. This reaction is also distinct from an acute stress disorder, otherwise known as shock, which is a psychological condition. Both the fight-flight response and shock are likely to occur in individuals in a population center that had been subjected to a terrorist attack. However, an elevated cytokine response would only occur after exposure to a specific agent, such as radiation.

One complication with assaying protein markers indicative of a radiation-induced cytokine response is that other stimuli can induce a similar reaction, such as acute infection and physical or chemotoxic trauma. However, it is important to note that additional potential markers of radiation exposure, such as induction of micronuclei and the phosphorylation of H2AX, are also induced by environmental stressors other than radiation. This highlights an important aspect of developing markers of radiation exposure; multiple biomarkers will likely be needed to unambiguously identify any exposed individuals. Given that terrorists would likely target a busy metropolitan area, the vast majority of potential victims would be healthy individuals engaged in typical daily activities and hence would not otherwise exhibit upregulated cytokine levels. As a result, evidence of an elevated cytokine response would strongly suggest the individual had been irradiated, and this could provide an initial marker of exposure to identify individuals that require further testing. In other words, the ELISA may be most useful for eliminating those individuals, the majority of the population, that were unaffected by the radiological dispersion device. This would help achieve one of the main goals of developing a high-throughput biodosimeter: reassuring the vast majority of the population that is unharmed after terrorist attack and therefore helping to instill calm.

References

8. Evermann JF and Jackson MK. Laboratory diagnostic...


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**Personal TLD Dosimeter in the Form of a Button**

Stephen A. Marino, Gary W. Johnson, David J. Brenner

In the event of a radiological incident, such as the triggering of a Radiological Dispersal Device (RDD or “dirty bomb”) or the release of radioactivity from a nuclear power plant, large numbers of people might receive significant doses of radiation. The dose received would depend on many parameters, such as proximity, time, and shielding by vehicles or structures, and could range widely. Because it is difficult, time-consuming and imprecise to calculate doses on an individual basis, exposures might have to be assessed by biological dosimetry in order to determine potential risk and possible treatment. If the general public wore personal dosimeters, the determination of exposure could be made more quickly, precisely and probably less expensively since it would require less sophisticated equipment. To this end we are investigating the design of a personal thermoluminescent dosimeter (TLD) in the form of a clothing button which would be inexpensive enough that it could be attached to every garment, either during manufacture or at home.

A prototype button made from polyethylene and heat sealed is shown in Figure 1. It is approximately 11 mm in diameter and 3.2 mm thick, about the size of a standard mens’ shirt button. The interior space is actually much larger than is needed for the amount of thermoluminescent (TL) powder that is required to determine the dose received. Two such buttons containing salt were attached to a shirt and have been laundered at home and at a commercial laundry and also dry cleaned. The salt inside the buttons has remained dry. Similar tests will be performed for other candidate materials before inserting TL material.

Two candidate TLD materials are under consideration at the moment—LiF:Mg,Ti (TLD-100) and LiF:Mg,Cu,P (TLD-100H). As a preliminary test of the TLD system, bare chips (3.2 x 3.2 x 0.89 mm) of both materials were irradiated with 250 kVp X-rays over the dose ranges of 0.6 to 46 rad and 0.3 to 11 rad for the LiF:Mg,Ti and LiF:Mg,Cu,P respectively.
They were read out with a manual TLD reader (Harshaw model 3500) using the time-temperature protocols recommended by the manufacturer. Results are quite linear, as shown in Figure 2. The linear least squares fits have small zero offsets, equivalent to about 20 mrad. As can be seen from the slopes of the curves, the LiF:Mg,Cu,P chips are almost 18 times more sensitive than the LiF:Mg,Ti chips, however this is not necessarily a great advantage since we are more interested in much larger doses than standard personnel dosimetry normally is.

Other candidate button materials, such as polycarbonate, may need to be sealed by gluing. Measurements for sealed buttons, both before and after laundering, dry cleaning and ironing are being undertaken to determine if the sealing process or routine cleaning, especially the heat from drying and ironing, affect the radiation response of the TLDs.

Written consent was obtained from healthy donors and blood was drawn into heparinized tubes. Half of each sample was irradiated with 1Gy γ-rays (Cs source, 0.8Gy/min) while the other half served as unirradiated controls. Control and irradiated samples were assayed for phospho-proteins using BD™ Cytometric Bead arrays at 1, 3 and 24 hours post irradiation. 0.5ml of each sample was transferred to a microfuge tube, spun down at 15000 g for 10 mins and the supernatant (plasma) discarded. The cell pellet was resuspended in 0.5 ml of 1X denaturing solution (with 2X protease and phosphatase inhibitors), and the tubes were placed in a boiling

**Protein Phosphorylation Based Assays as Biomarkers for Exposure to Ionizing Radiation**

Brian Ponnaiya

In the event of a large scale radiological event, estimation of exposures to individuals in the population will require biodosimeters that are rapid, accurate, have a high-throughput and are minimally invasive. We have proposed to develop biodosimeters that combine the ease of obtaining small blood samples with the high-throughput assays that analyze protein phosphorylation profiles in whole blood.

The most prevalent cell type in blood are, by far, erythrocytes. While the lack of nuclei preclude these cells from DNA based assays, they do contain signaling pathways identical to those seen in other cell types. For example, it has been demonstrated that MAP kinase cascades are activated in erythrocytes. In addition, erythrocytes have demonstrated apoptotic markers including colocalization of Fas with the raft marker proteins Galpha(s) and CD59; the existence of Fas-associated FasL, FADD and caspase 8; and caspase 8 and caspase 3 activity. Therefore these cells can potentially contribute to assays for phosphoprotein profiling following exposure to ionizing radiation.

Written consent was obtained from healthy donors and blood was drawn into heparinized tubes. Half of each sample was irradiated with 1Gy γ-rays (Cs source, 0.8Gy/min) while the other half served as unirradiated controls. Control and irradiated samples were assayed for phospho-proteins using BD™ Cytometric Bead arrays at 1, 3 and 24 hours post irradiation. 0.5ml of each sample was transferred to a microfuge tube, spun down at 15000 g for 10 mins and the supernatant (plasma) discarded. The cell pellet was resuspended in 0.5 ml of 1X denaturing solution (with 2X protease and phosphatase inhibitors), and the tubes were placed in a boiling

**Fig. 1.** Levels of phospho-Erk1/2 in control and irradiated whole blood at 1, 3 and 24 hours post irradiation.

**Fig. 2.** The response to X-rays for two types of LiF dosimeters.
The tubes were then centrifuged at 150g for 10 mins and the pellet containing the cellular debris was discarded. 12.5µl of each supernatant was then used in the Cytometric Bead arrays. Each sample was assayed for phosphorylated forms of Erk1/2, Rsk, Stat1, Jnk1/2 and p38 simultaneously according to the protocol. Standards for each of the phospho proteins were run concurrently. Data was obtained on the FACSCalibur flow cytometer and analyzed using FCAP array software.

As can be seen in Figure 1, irradiated blood from both donors had elevated levels of phospho-Erk1/2 over controls at 1and 3 hours post irradiation. There was some individual variability at 24 hours, when levels of irradiated samples from donor A were comparable to controls but were higher than controls in samples from donor B.

Figure 2 presents the alterations in levels of phospho-p38 in response to radiation in whole blood from donors A and B. At one hour post irradiation, there were 3-4 fold increases in phospho-p38 in blood from both donors that were reduced to control levels by 3 hours.

In conclusion, these data demonstrate that profiling of phosphorylation of specific proteins in whole blood has the potential to be sensitive biomarkers of exposure to ionizing radiation. The assays described here meet the requirements of being minimally invasive as well as high throughput. These features are critical for screening large numbers of people in the event of a large scale radiological event.

References


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**Radiation-Induced Mitochondrial DNA Damage: A Dosimeter for Radiation Exposure**

*Hongning Zhou, Yu-Chin Lien, Michael Partridge, Sarah Huang and Tom K. Hei*

There is an urgent need to develop a high-throughput screening method for detecting radiation exposure in the event of a radiological terrorist incident. One of the best characterized consequences of radiation exposure is damage to DNA. In addition to nuclear DNA, cells also contain DNA in their mitochondria (mtDNA) which is also known to be damaged by radiation exposure. Specifically, radiation exposure has been shown to induce a unique large deletion in the mtDNA sequence in a dose dependent manner. Additionally, radiation has been shown to change the copy number of mtDNA. Importantly, radiation-induced deletions as well as changes in the amount of mtDNA can be detected using a PCR-based assay, a technique which can be easily automated and is therefore capable of analyzing large numbers of samples. Consequently, measuring the damage to mtDNA after radiation exposure could potentially be adapted for use as a high-throughput radiation biodosimeter. Assaying mtDNA damage has some important advantages over other techniques, such as measuring changes in nuclear RNA expression levels. First, unlike nuclear DNA, mtDNA does not have any protective packaging proteins like histones. Therefore, analysis of mtDNA can be performed us-
ing simple and rapid sample preparations, which is vitally important in any high-throughput diagnostic device. Second, one of the most important, and yet most difficult, aspects of developing a diagnostic assay for humans, is effectively validating the technique in a whole organism. Because all mammals have mtDNA that is identical in gene organization and function to humans, there are convenient whole-animal models available to test the efficacy of measuring mtDNA damage in response to radiation.

Our first goal was to establish that whole cell suspensions could be used as a template in the PCR reaction without employing time-consuming DNA extraction protocols. AL cell pellets were briefly treated with light alkali solution, neutralized, and the resulting solution used directly as a template in the PCR. As shown in Figure 1, a 629bp fragment was amplified from the D-loop region of the hamster mtDNA sequence even when as few as 10 cells were added to the PCR as template. Significantly, using the same method we have also been able to amplify both nuclear and mitochondrial DNA fragments from mouse blood as well as human cells in culture (not shown). It will be important to optimize this procedure further to make the sample preparation as rapid as possible. Next we wanted to determine whether we could detect changes in the incidence of common deletion (CD) after irradiation of human cell cultures using real time PCR. Finally, in an attempt to more faithfully mimic testing biological material obtained from humans after a radiological incident, we wanted to establish whether we could use real-time PCR to detect changes in mtDNA from samples obtained from radiation exposed animals. This work is ongoing.

### Integrated Microfluidic Visualization on a Microchip for Ultrahigh-Throughput Low-Cost Radiation Biodosimetry

Daniel Attinger, Chee Wei Wong and Samuel K. Sia

In micro-biofluidic assays, a key limitation to high throughput visualization and scalability is that of the detection technology. For example, large microscopes are used to perform the microchip assay, which is a paradox with respect to the miniaturization of the microfluidic chip.

The purpose of the pilot project is to integrate a portable microscope with a flow-through microfluidic chip to do visualization of cells in flow. The whole setup is to be actuated from a notebook computer, communicating through USB ports.

A GpA-fluorescence tagging method will allow biodosimetry of red blood cells (Fig. 1). Glycophorin A (GpA) is a human glycoprotein on the surface of erythrocytes that determine the M and N blood group variant. γ-radiations induce mutations in the stem cells which can be detected by the failure of an erythrocyte to express an allelic form of GpA (MØ or NØ). Studies have shown that the mutation frequency can be related to the dose of radiation received. Limitations for this method are that only MN variant blood group is eligible for the assay (50% of the population), that

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*a Department of Mechanical Engineering, Columbia University, NY  
*b Department of Biomedical Engineering, Columbia University, NY
The optical part (Fig. 2) is made of a focalized blue high power LED (5W), a bandpass blue filter, a 20x microscope objective (N.A. 0.4), a bandpass green filter and a CCD Peltier cooled camera. Filters are needed to separate the FITC excitation light (peak at 490nm) from the FITC emission light (peak 518nm).

Injection of the samples is performed using a standard syringe. Flow in the microfluidic chip is driven by gravity, which is reliable and does not require power. A simple washing procedure using water and a syringe has been integrated to make the device re-usable. A PDMS microfluidic chip where cells flow through under the microscope has been manufactured using a photolithography method in the Columbia cleanroom. A critical requirement is to have an identical exposure time for all the cells flowing in the channel, which means to have an identical velocity. This has been achieved using the same pressure in both channels and designing a low height/width cross section ratio of the channels, according to the study. Experiments have shown a very good flow control correlation between simulations and experiments.

A software has been developed using Matlab which allows computer LED and camera control, automatic particles detection and tracking along the channel, automatic suppression of uninteresting particles (e.g. stuck to chip walls) and fluorescence intensity detection.

Results with the prototype in Figure 3 have shown that the fluorescent detection performance of the prototype is on the same order as an IX71 Olympus fluorescence microscope. A cooled CCD camera (Lumenera 3-1M) is used. Due to the low fluorescence intensity of the FITC tagged cell, the exposure time needed to obtain a usable image for detection (signal/noise \(\geq 2\)) is 7 s with the prototype (and 2s with the fluorescence microscope), which makes it the current bottleneck of the performance of the flow-through device.

References


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Phosphoproteome Profiling of Gamma Ray Exposed Lymphocytes

Yongliang Zhao, Gengyun Wen and Tom K Hei

Protein phosphorylations are among the most abundant post translational modifications involved in the regulation of almost all cellular processes, e.g. modulating protein folding, activity, interaction, targeting and degradation. A series of biochemical changes would occur when an individual is exposed to radiation, including phosphorylation of different types and amounts of proteins. A specific and comprehensive phosphoproteomic profile is hypothesized to characterize functional protein networks and their dynamic alterations after irradiation. To assess radiation exposure level expeditiously in a large number of individuals, rapid and reliable assays based on well-defined biomarkers are particularly needed. Profiling of phosphoproteomes with a minimal amount of tissue/cells is a promising way to achieve this.
We propose that phosphoproteomic changes after irradiation are closely related to doses and duration of exposure. Therefore, we should screen specific phosphoproteomes which are presumably underpinning radiation induced biological responses, in a dose and time-dependent manner. Ultimately, an anti-phosphoprotein antibody array will be designed based on the aberrant phosphoproteomes. This array can be used to recognize “signature” changes caused by radiation, estimate severity and monitor prognosis.

To achieve this goal, human lymphoblast cells IM-9 have been adopted as the radiation subject and irradiated with gamma ray at the dose of 0.5Gy, 2.0Gy and 5.0Gy. Various changes were observed at 4h and 24h post irradiation. Phosphoproteomes of human Immuno-receptors and MAPKs along with human cytokine array were used to characterize gamma-ray induced biochemical and biological responses. In addition, cell proliferation and cell-cycle changes were documented simultaneously.

### Table 1. Gamma radiation resulted in changes of cytokine production

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### Table 2. Gamma radiation resulted in changes of MAPKs phosphorylation

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### Table 3. Gamma radiation resulted in changes of immuno-receptors phosphorylation

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<td>1.7</td>
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<td>4.7</td>
<td>3.3</td>
<td>2.9</td>
<td>4.7</td>
<td>5.5</td>
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</tbody>
</table>

We propose that phosphoproteomic changes after irradiation are closely related to doses and duration of exposure. Therefore, we should screen specific phosphoproteomes which are presumably underpinning radiation induced biological responses, in a dose and time-dependent manner. Ultimately, an anti-phosphoprotein antibody array will be designed based on the aberrant phosphoproteomes. This array can be used to recognize “signature” changes caused by radiation, estimate severity and monitor prognosis.

To achieve this goal, human lymphoblast cells IM-9 have been adopted as the radiation subject and irradiated with gamma ray at the dose of 0.5Gy, 2.0Gy and 5.0Gy. Various changes were observed at 4h and 24h post irradiation. Phosphoproteomes of human Immuno-receptors and MAPKs along with human cytokine array were used to characterize gamma-ray induced biochemical and biological responses. In addition, cell proliferation and cell-cycle changes were documented simultaneously.

Tables 1, 2 and 3 summarize the aberrant phosphoproteome and associated altered cytokine production, which will potentially be used to characterize gamma radiation caused biological damage. Red numbers indicate an increase more than 2 fold, blue numbers indicate a decrease more than 2 fold. Other numbers indicate no significant changes.
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Andrew D. Harken, Ph.D. – Post-Doctoral Research Scientist
Kenichi Tanaka, Ph.D. – Post-Doctoral Research Scientist
Yanping Xu, Ph.D. – Post-Doctoral Research Scientist
Oleksandra Lyulko, – Pre-Doctoral Research Scientist
Bharat Patel, B.S., B.A. – Technician B
Introduction

The construction activity of the past two years is behind us. The Singletron, which replaced our aged Van de Graaff, is now over two years old and generally running well. The third floor laboratories are essentially complete. All the new third floor labs are in use and half the desks are presently occupied.

Research using RARAF

The main focus of the biological experiments at RARAF for the past several years has been the “bystander” effect, in which cells that are not irradiated show a response to radiation when in close contact with or even only in the presence of irradiated cells. Every biology experiment run this year examined this effect. The emphasis of the present experiments is to determine the mechanism(s) by which the effect is transmitted and whether the mechanisms are different for direct gap junction communication through cell membrane contact and indirect, long-range communication through the cell media. Both the microbeam and the track segment facilities continue to be utilized in various investigations of this phenomenon. The single-particle Microbeam Facility provides precise control of the number and location of particles so that irradiated and bystander cells may be distinguished but is somewhat limited in the number of cells that can be irradiated. The Track Segment Facility provides broad beam irradiation that has a random pattern of charged particles but allows large numbers of cells to be irradiated and multiple users in a single day.

Two special types of track segment dishes are being used to investigate the bystander effect using the Track Segment Facility: double-sided dishes and “strip” dishes. Double-sided dishes have thin (6-μm) Mylar foils glued on both sides of a stainless steel ring, 1 cm apart, with cells plated on the inside surfaces of both foils. The interior is completely filled with medium. This type of dish is used for investigation of the non-contact, long-range bystander effect since the cells on the two surfaces are not in direct contact, can only communicate through the culture medium, and only the cells on one surface are irradiated. “Strip” dishes consist of a stainless steel ring with thin (6-μm) Mylar foil glued to one side in which a second dish is inserted. The Mylar foil glued to the inner dish has alternate strips of the Mylar removed. Cells are plated over the combined surface and are in contact. The Mylar on the inner dish is thick enough (38μm) to stop the charged particles (4He ions) and the cells plated on it are not irradiated. These dishes are used for bystander experiments involving cell-to-cell communication.

Interest in irradiation of 3-D systems continued this past year, with tissue samples irradiated using either helium ions or protons. Imaging systems for the Microbeam Facility are being developed to enable observation and targeting of cells that are not in monolayers; in the interim, cultured human tissue samples are being irradiated using the Track Segment Facility. The tissue samples are on membranes on the end of cylindrical plastic holders. Plastic discs have been constructed that fit in the dish openings in the irradiation wheel and have small holes to provide precise alignment of the feet that are around the bottom edges of the tissue holders. A hole in the middle of each disc is fitted with two stainless steel half-discs that have a precise .001 inch (25μm) space between them. The tissue membrane is in contact with the stainless steel, which is thick enough to stop the charged particles. This provides a narrow line of irradiation across the center of the entire sample. The tissue samples are later sectioned, either parallel or crosswise to the line of irradiation, to observe bystander effects as a function of distance from the line of irradiation.

The experiments performed at RARAF from January 1 through December 31, 2007 and the number of days each was run in this period are listed in Table 1. Fractional days are assigned when experimental time is shared among several users (e.g., track segment experiments) or experiments run for more or less than a shift. Use of the accelerator for experiments was 49% of the regularly scheduled time (40 hours per week), 15% lower than last year (which was the highest use we have attained at Nevis Labs) but about average for the last 5 years. Ten different experiments were run during this period. Six experiments were undertaken by members of the CRR, supported by grants from the National Institutes of Health (NIH), the National Aeronautics and Space Administration (NASA), and the Department of Energy (DoE). Four experiments were performed by outside users, supported by grants and awards from the NIH, the NSF, and the National Natural Science Foundation of China (NSFC). Brief descriptions of these experiments follow.

Gerhard Randers-Pehrson, Alan Bigelow and Yanping Xu of the CRR continued development of a method to detect explosives in baggage (Exp. 82). They have been assisted this year by Kenichi Tanaka, a visiting scientist from Hiroshima University. The detection system is based on resonant elastic scattering of 0.43 MeV neutrons by nitrogen and oxygen, which are present in higher percentages in most explosive materials. Measurements of the neutron transmission through sample materials are made using neutrons produced in a very thin target by the 7Li(p,n) reaction. A high voltage is applied to the target and scanned slowly up and down across the proton energy required to produce neutrons.
at the resonance energy. The neutron transmission can then be measured over the range of 20 keV under identical target and focusing conditions to observe the ratio of transmission at the resonance relative to off the resonance energy. They are presently working on a new target design to extend the life of the thin lithium layer used to produce the neutrons.

Dr. Tanaka has worked on the design of a liquid nitrogen cold trap (Fig. 1) having a passage for the proton beam. The liquid nitrogen trap is extremely effective in removing water vapor from the system, which is a major cause of target deterioration. He has also been doing Monte Carlo neutron calculations using the MCNP code to assess the ability of the detection system to distinguish explosives from ordinary materials.

Studies of the bystander effect, examining the relationship between the radiation-induced bystander response and genomic instability (Exp. 103), were continued by Burong Hu and Charles Geard of the CRR. Normal human lung fibroblasts were cultured in double-sided Mylar dishes (see above) and one side was irradiated with 0.1 to 5 Gy of He ions using the Track Segment Facility. The range of the helium ions is very much shorter than the space between the two Mylar layers so that the cells on the other side of the dish were then bystanders, which could only be influenced by signal transfer through the medium. For microbeam studies, 20% of the nuclei of nearly confluent (in contact) fibroblasts were irradiated with 30 He ions each, which ensures that only non-hit bystander cells can survive over many cell generations. In both scenarios cells were harvested at 3 h and 24 h post-irradiation and after 5, 10, 15, 20 and 25 population doublings. Elevated levels of chromosomal damage in bystander cells were observed after G2-PCC, reflecting signal

![Liquid N2 Reservoir](image1)

**Fig. 1.** Design of the liquid nitrogen trap for the oxygen/nitrogen resonance explosives detector.

**Table 1. Experiments Run at RARAF, January 1 - December 31, 2007**

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Experimenter</th>
<th>Institution</th>
<th>Exp. Type</th>
<th>Experiment Title</th>
<th>No. of Days Run</th>
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<td>82</td>
<td>G Randers-Pehrson</td>
<td>CRR</td>
<td>Physics</td>
<td>Detection of explosives</td>
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<td>103</td>
<td>B. Hu C. R. Geard</td>
<td>CRR</td>
<td>Biology</td>
<td>Damage induction and characterization in known hit versus non-hit human cells</td>
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<td>106</td>
<td>B. Ponnaiya C. R. Geard</td>
<td>CRR</td>
<td>Biology</td>
<td>Track segment α-particles, cell co-cultures and the bystander effect</td>
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<td>110</td>
<td>H. Zhou Y-C. Lien M. Hong T. K. Hei</td>
<td>CRR</td>
<td>Biology</td>
<td>Identification of molecular signals of α-particle-induced bystander mutagenesis</td>
<td>31.1</td>
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<tr>
<td>112</td>
<td>Y. Horowitz A. Horowitz (S. A. Marino) Ben Gurion Univ., Nuclear Research Ctr., Beersheva</td>
<td></td>
<td>Physics</td>
<td>HCP and neutron irradiation of LiF:Mg, Ti TLD chips to determine 5α/5 intensities and characterization of 5α peak as a Q/RBE nanodosimeter</td>
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<td>123</td>
<td>E. Aprilie Columbia Univ., Astrophysics</td>
<td></td>
<td>Physics</td>
<td>Calibration of a liquid Xenon detector for weakly interacting massive particle (WIMPs)</td>
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<tr>
<td>133</td>
<td>S. Ghandhi J. Ahn, S. Amundson</td>
<td>CRR</td>
<td>Biology</td>
<td>Bystander effects in primary cells</td>
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<td>136</td>
<td>S. Paul A. Mezentsev S. Amundson</td>
<td>CRR</td>
<td>Biology</td>
<td>Bystander effects in 3D tissues</td>
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<td>138</td>
<td>E. Azzam J. Santos O. Kovalenko NJSMD</td>
<td>Biology</td>
<td>Investigation of the effect of mtDNA damage on apoptosis in hTERT cells</td>
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<td>140</td>
<td>L. Han (T. K. Hei) Shanghai Medical University</td>
<td>Biology</td>
<td>Study of the mechanism of radiation-induced inactivation of the FHIT gene by single cell microbeam irradiation</td>
<td>10.6</td>
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**Note:** Names in parentheses are members of the CRR who collaborated with outside experimenters.
transfer from irradiated cells, while elevated levels of chromosomal changes at later times as recorded by mFISH indicate genomic instability. Emphasis this year has been on intra-chromosomal changes in chromosome 11 in bystander cells and cells irradiated only in the cytoplasm.

Another study investigating the bystander effect was continued by Brian Ponnaiya and Charles Geard of the CRR (Exp. 106). The Track Segment Facility was used for broad-beam charged particle irradiations to examine genomic instability in irradiated and bystander hert immorlized human bronchial epithelial cells (HBEC-3kt, obtained from J. Shay). These cells were cultured on standard single-sided Mylar dishes and irradiated with half the dish covered by a thin metal shield. Cells on the non-covered portion of the dishes were irradiated with 0.5 and 1 Gy of $^4$He ions, while cells on the covered portions of the dishes were bystander cells. Irradiated and bystander populations from each dish were separated and set up in culture. At various times post irradiation (7-28 days) G2-PCCs were prepared from each culture using Calyculin A. The chromosomes were analyzed by both Giemsa staining (for gross chromosomal aberrations) and mFISH for more subtle alterations (e.g. Translocations). Giemsa staining as well as mFISH revealed that both irradiated and bystander populations had elevated yields of chromosomal changes at 7 and 14 days post irradiation.

Hongning Zhou, Yu-Chin Lien, Mei Hong and Tom Hei of the CRR continued to use the Track Segment Facility and the single-particle Microbeam Facility to try to identify the cell-to-cell signaling transduction pathways involved in radiation-induced bystander mutagenesis (Exp. 110). Using the charged particle microbeam, they found that mitochondrial DNA-depleted human skin fibroblasts ($\rho^0$) showed a higher bystander mutagenic response in confluent monolayers when a fraction of the same population was irradiated with a lethal dose of alpha particles in the nucleus compared to their parental, mitochondria-functional cells ($\rho^+$). Using mixed cultures of $\rho^0$ and $\rho^+$ cells and targeting only one population of cells with a lethal dose, decreased bystander mutagenesis was uniformly found in non-irradiated bystander cells of both cell types, indicating that signals from one cell type can modulate expression of the bystander response in another cell type. In addition, they found that Bay 11-7082, a pharmacological inhibitor of nuclear factor-kB (NF-kB) activation, and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO), a scavenger of nitric oxide (NO), significantly decreased the mutagenic frequency in both bystander $\rho^0$ and $\rho^+$ cells. Furthermore, they found that NF-kB activity and its dependent proteins, cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS), were lower in bystander $\rho^0$ cells when compared with their $\rho^+$ counterparts. These results indicate that mitochondria play an important role in the regulation of radiation-induced bystander effects and that mitochondria-dependent NF-kB/iNOS/NO and NF-kB/COX-2/prostaglandin-E2 (PGE2) signaling pathways are important to the process. Additional experiments were performed using the Track Segment Facility to irradiate several cell lines plated on “strip” dishes (described above) with $^4$He ions to identify the possible mechanism(s) of the radiation-induced bystander effect. Human-hamster hybrid (A) cells were examined for PKC expression (direct evidence) or PKC inhibitors were used (indirect evidence) to determine the involvement of the PKC pathway. Normal human lung fibroblasts/skin fibroblasts, with ($\rho^0$) or without ($\rho^+$) mitochondrial function, were examined to identify the signaling pathways. Prostate cancer cells were examined to determine if radiation can induce the bystander effect and to identify the possible mechanism.

Yigal Horowitz of Ben Gurion University of the Negev and Atara Horowitz of the Nuclear Research Center, Beer-sheva, Israel have resumed their investigation of the use of thermoluminescent dosimeters (TLDs) as nanodosimeters (Exp. 112). Recent work at Ben Gurion University has demonstrated that the major thermoluminescent dosimeter glow peak (peak 5) in the LiF:Mg,Ti (TLD-100) system is composed of at least three sub-entities of different sensitivities to ionization density. Glow peak 5a (at a temperature ~10 K less that of the main peak) is more intense following heavy charged particle irradiation and is believed to arise from localized (geminate) recombination in a molecular complex of ~20Å dimensions. The relative intensity of peak 5a to peak 5 is therefore a measure of ionization density, which has been proposed as a solid-state nanodosimeter that can, to a certain extent, mimic the ionization density dependence of radiation damage in DNA. TLD chips, both “normally cooled” and “slow cooled”, were irradiated with $10^9$ particles/cm$^2$ of low- and high-energy protons, deuterons and helium ions, as well as 0.2 and 14 MeV neutrons and 60 and 250 kV X rays. Results will be compared with full track Monte Carlo calculations using an adaptation of the program FLUKA to derive radial dose deposition profiles in nanometer-sized volumes for low energy protons and alpha particles. Initial analysis (peak-height and glow peak width) has revealed a rich degree of intriguing and previously unobserved characteristics. These will be analyzed using computerized glow curve deconvolution.

A group led by Elena Aprile of the Columbia Astrophysics Laboratory of Columbia University resumed their calibration of a liquid xenon proportional counter (Exp. 123) to be used to detect weakly interacting massive particles (WIMPs). These are heavy neutral particles that only interact weakly with matter and may be the “dark matter” that will make up the “missing” mass in the universe. Electron ion pairs formed by ionization recombine (in a proportion that is a function of the electric field applied) and eventually produce scintillation photons as well. As most of the energy lost by a recoiling xenon nucleus will be converted into atomic motion instead of transferred to electrons through ionization, the xenon scintillation signal will be smaller than that of an electron recoil of the same energy. The scintillation efficiency of the nuclear recoils can be measured by first calibrating the detector with a monoenergetic gamma source and then producing the same energy xenon recoils. Low-energy neutrons scattered at a fixed angle by the xenon nuclei were detected as a function of time after pulses produced by the neutrons in the xenon detector. Since the initial neutron energy is known, the energy imparted to the xenon nucleus can...
be calculated. Several different neutron energies and angles were used to try to calibrate the detector for the smallest pulses. Their measurements at RARAF have allowed them to make the most sensitive measurements for the search for WIMPs (limit on particle mass and cross section) in the world and their experiment was featured in an article in Nature in 2007. This group ran their experiments around the clock, sometimes for an entire weekend.

A group led by Sally Amundson of the CRR continued two types of experiments concerning radiation-induced gene expression profiles in primary human fibroblast and epithelial cell lines using cDNA microarray hybridization and other methods. One experiment, performed by Shanaz Ghandhi and Jaeyong Ahn, involved use of the track segment irradiation for comparison of gene expression responses to direct and bystander irradiation (Exp. 133). Human fibroblast cells (IMR90) and epithelial cells: (HBEC-3KT and SAEC) were plated on “strip” dishes (see above) for direct-contact bystander irradiations. The cells were irradiated with 0.5Gy of 125 keV/μm 4He ions and assayed for micro-nucleus formation. The timing of micronucleus and gene expression assays is critically important and the time windows in which to perform these assays have been optimized. Using the fibroblast model, they now have identified potential genes and pathways involved in the bystander effect that are being validated by real-time PCR. They currently are working on identifying potential genes of interest in epithelial cells from the microarray studies which, in combination with fibroblast studies, will further our knowledge of bystander responses induced by radiation.

The second experiment (Exp. 136), performed in collaboration with Alexandre Mezentsev and Sunirmal Paul of the CRR, involved irradiation of artificial human tissue samples using the Track Segment Facility. Tissue model, Epi200 (Mat-Tek, Ashland, MA) precisely imitates the structure of the epidermis. It is composed of ~20 layers of cells, each layer representing keratinocytes at a certain step of their terminal differentiation program. The tissues were irradiated with protons having an initial LET of ~10 keV/μm, either over the entire tissue surface or in a narrow line (~25 μm) across the diameter using the slit masks described above. After 48h, the tissues were removed from the culture insert and cut into narrow slices (200-400 mm) parallel to the irradiation line. Interest is focused on the lowest cell layer attached to the supporting membrane, since this is the only layer where cells divide. Irradiation of these cells allows observation of their performance and that of their descendants: undifferentiated keratinocytes (epidermal stem cells) that remain attached to the membrane, via analysis of their proliferation and mutagenesis, and differentiating cells, via study of their survival rate and metabolism. The experiments are based on three approaches: micronucleus assay, immunohistochemistry and microarray analysis. The first approach assesses the mutagenic potential of irradiation, answering the question whether irradiation interferes with cell division, and measures changes in cell proliferation rate. The second—approach detects and quantifies apoptosis in bystander and directly irradiated cells. The third approach identifies genes involved in the initiation, transition and termination of bystander effects. These analyses require multiple samples from the same tissue; often their size does not exceed a few mm². This required development of two new protocols: one for the purification of total RNA, another for the isolation of keratinocytes from 250 μm-wide tissue strips (one strip is ~3% of the tissue area). While the data analysis is still in progress, preliminary results suggest the existence of bystander effects after proton irradiation (0.10 and 2.5Gy). These effects appeared as higher frequencies of micronuclei, increased apoptotic rates and differential gene expression in bystander cells compared to non-irradiated controls.

Edouard Azzam, Janine Santos and Olga Kovalenko of the New Jersey School of Medicine and Dentistry continued to investigate whether mitochondrial DNA (mtDNA) damage by itself can trigger apoptosis in hTERT cells (Exp. 138). Parental cells carrying wild type or a nuclear-only hTERT mutant are irradiated either in the nucleus or the cytoplasm using the Microbeam Facility, allowed to recover for approximately 24h, and stained with YOPRO-1 in order to score the percentage of apoptotic cells. Using the same analysis procedure, they are also investigating radiation-induced bystander effects under conditions wherein a small fraction of cells in the exposed population is targeted through the nucleus or cytoplasm by one or more 4He ions.

Ling Han of the Second Medical Military University, Shanghai, China, in collaboration with Dr. Tom Hei of the CRR, began an experiment to determine the expression, injury and signal transduction of the FHIT (Fragile Histidine Triad) gene (Exp. 140). The project is a combination of cell irradiation techniques, radiation biology and molecular biology. The Microbeam Facility is used to irradiate single cells in the nucleus, the cytoplasm or the culture medium. In other irradiations, only a fraction of the cells are irradiated and the co-cultured unirradiated (bystander) cells are examined. FHIT gene function is studied at different stages over 50 generations after irradiation and the role FHIT plays in cell transformation is examined in any transformed cells detected. They will explore effective ways to protect FHIT against radiation-induced injury as well as methods to select anti-irradiation drugs. They intend to produce databases for further study on mechanisms of microbeam low-dose biological effect and tumor radiotherapy. Newly established methods of drug selection will help to explore the mechanisms of anti-irradiation drugs as well as find new radiation-protective drugs.

Development of Facilities

This year our development effort continued on a number of extensions of our facilities:

- Development of focused accelerator microbeams
- Non-scattering particle detector
- Advanced imaging systems
- Targeting of cells
- Focused X-ray microbeam
- New laboratory space

Development of focused accelerator microbeams

The first quadrupole triplet lens (Fig. 2), installed in
2003, has continued to operate very reliably. It has proven to be quite robust, surviving vacuum excursions caused by the occasional breakage of the ion beam exit window. An electrostatic phase space “sweeper” installed in 2006 just above the 90° bending magnet enabled us to focus a 6 MeV 4He beam down to a diameter of 2µm. By varying the voltages on the 4 electrodes of the “sweeper”, the beam is continually steered in a non-repetitive way and the object appears to the focusing system to be an isotropic source.

Two additional quadrupole triplets have been constructed and assembled into a single alignment tube as a compound lens in our machine shop by Gary Johnson. This compound lens has been installed in the beam line in place of the single lens. The lens alignment and focusing voltages are in the process of being adjusted to obtain a sub-micron beam spot diameter. At present the beam spot diameter is 1.3µm.

In order to test the alignment of the two triplet lenses, steering coils have been added in the space between the two triplets. This not only allows the beam to be steered from one triplet to the other but, by using two sets of coils with opposite fields, the beam can be displaced without changing the angle at which it enters the second lens. The current in the coils can be used to calculate in which direction and how far the bottom of the upper lens needs to be moved to align it with the lower lens.

After using this sub-micron beam for biological irradiations for a suitable period, the testing process will be repeated with a second compound lens so that we will eventually have two complete compound lenses, one of which will be used as a spare.

A second microbeam using a compound quadrupole triplet lens made from commercially available precision permanent magnets (Fig. 3) was reassembled after the construction of the 3rd floor. Because the magnet strengths are essentially fixed, only a single energy (5.3 MeV) proton or 4He ion can be focused. The pair of quadrupole triplets is similar to the one designed for the sub-micron microbeam, the major difference being that it uses magnetic rather than electrostatic lenses. This system was originally designed to focus alpha particles from a 210Po source for use during the dismantling of the Van de Graaff and the installation of the Singletron. Using a charged particle beam from the accelerator provides us with a much greater flux and a smaller beam spot size because of reduced energy spread. The endstation for our original collimated microbeam was moved from the 2nd floor to the new microbeam lab on the 3rd floor because additional room for the lens structure is required between the final bending magnet and the focal point. After realignment of the system, and without adjusting the magnets, a beam spot size of 20µm was measured, demonstrating the robustness of this design.

A magnetic steering coil “sweeper” is used to produce a beam in which the particle location and direction are not coupled, as is done for the electrostatically focused microbeam, allowing the beam to be focused to a smaller diameter and increasing the flux at the endstation. The “sweeper” used here is based on the same split-coil used for the point and shoot system described below (Targeting Systems).

The lenses have been optically aligned and the quadrupole magnet strengths used to focus the beam are being adjusted using micrometric screws to retract and extend the individual magnets of each quadrupole. After installation of the phase space “sweeper” and a smaller object aperture (0.5mm), a beam of 5.3 MeV 4He has been focused into a spot 8µm in diameter (a demagnification of x60, compared to the theoretically attainable x100). A miniature Hall probe has been purchased and will be used to map the magnetic fields of the lenses to look for aberrations and determine the octupole moment of the lenses, both of which would interfere with focusing.

This system will be used primarily for irradiations when the electrostatic system is unavailable because of development or repair and is presently being used to test the point and shoot system.

**Non-scattering particle detector**

Currently the RARAF microbeam irradiator delivers a precise number of particles to thin samples by counting the particles traversing them, using an ionization chamber placed immediately above the cells. To irradiate thick samples, such as model tissue systems or oocytes, to use particles with very short ranges, such as the heavy ions from the laser ion source, and to allow irradiation of cell monolayers without removing the culture medium, a completely non-scattering particle detector is necessary upstream of the samples. A novel particle detector has been designed on the basis of a long series of capacitive pick-up cells coupled together into a delay line. The Lumped Delay Line Detector (LD³) consists of 250 silver cylinders, each 3 mm long with
a 2.2 mm inside diameter, connected by inductors and capacitively coupled to ground. The cylinders are glued to a semi-cylindrical tube of dielectric material 1 m long for mechanical support. The dielectric has a semi-cylindrical metal tube around it that can be rotated about one edge to adjust the capacitance. If the individual capacitance is set such that the propagation velocity of the pulse equals the projectile velocity, the pulses capacitively induced in all segments by the passage of a single charged particle will add coherently, resulting in a fast electron pulse at each end of the delay line that is 125 times larger than the charge induced on a single cylinder. This easily detectable charge of at least 125 electrons will be amplified to provide the detection pulse for the particle counter. After two prototype LD^2 detectors (1/6 length) were tested, the full-length detector has been constructed. Electrical measurements indicate that the propagation velocity in the delay line corresponds well to the calculated value. The detector will be placed in a horizontal beam line for testing with charged particle beams. After testing, the detector will be mounted between the two electrostatic lenses in the microbeam and become the standard detector for all microbeam irradiations.

Advanced imaging systems

Development continued on new imaging techniques to view cells without using stain and to obtain three-dimensional images of unstained cells.

The immersion-based Mirau interferometric (IMI) objective has been designed to function as an immersion lens with standard interferometric techniques by acquiring images at four positions with sub-wavelength separations using the vertical motion of the microbeam stage. Preliminary results imaging 10 μm D polystyroid beads in air were sufficiently encouraging to warrant the effort to design the new objective. In 2006, a commercial Mirau objective was modified by filling part of the light path with water so that it could be used as a water immersion lens. Tests confirmed that the lens can provide interference fringes with sufficient contrast to perform the biological experiments. A custom Mirau objective has been constructed in our shop and several beamsplitters of different reflectivity (5-85%) have been obtained. These have been combined with spot mirrors into separate plitters, resulting in a fast electron pulse at each end of the delay line corresponding well to the calculated value. The detector will be placed in a horizontal beam line for testing with charged particle beams. After testing, the detector will be mounted between the two electrostatic lenses in the microbeam and become the standard detector for all microbeam irradiations.

A multi-photon microscope has been developed for the single-cell single-particle Microbeam Facility to detect and observe the short-term molecular kinetics of radiation response in living cells and to permit imaging in thick targets, such as tissue samples. Two photons delivered closely together in space and time can act as a photon with half the wavelength (twice the energy). The longer wavelength of the actual light beam allows better penetration into the sample while still being able to excite the fluorophor. The multi-photon capability has been integrated into the Nikon Eclipse E600-FN research fluorescence microscope of the microbeam irradiation system and will provide three-dimensional imaging. A Chameleon (Coherent Inc.) tunable titanium sapphire laser (140 fs pulses at a 90 MHz repetition rate) is the source for the multi-photon excitation. The scan head incorporates commercial scanners and a scan lens then focuses the laser beam to a point at an image plane of the microscope (a CCD camera is also placed at such an image plane for fluorescent microscopy). The incident laser beam enters the microscope through the side of the trinocular tube of the microscope (Fig. 4). A switch mirror allows us to choose between multi-photon microscopy and standard fluorescence microscopy. The scanned laser beam establishes an optical section within the specimen, where multi-photon absorption preferentially occurs. Wavelengths available from the laser can penetrate to depths of about 100 microns in a biological sample by varying the Z-position of the specimen stage. Light emitted from the specimen is selectively deflected by a series of dichroic mirrors to an array of photomultiplier tubes (PMTs). To control the multi-photon microscope, we are adopting the design and software of Karl Svoboda, Cold Spring Harbor.

Initial two-color images of stained cells are excellent. Presently only one PMT can be mounted on the system. A housing that will enable us to use two PMTs and therefore obtain simultaneous images from two fluorophors, is being constructed.

Two PMTs will allow the investigation of fluorescence resonance energy transfer (FRET). Molecules labeled with two fluorochromes normally widely separated can change their conformation by phosphorylation, positioning the two fluorochromes near each other. In this close proximity, the emission from one fluor can excite the other, changing the ratio of emissions from the two fluorochromes. A measure of the amount of phosphorylation in the sample can be determined.

Targeting of Cells

During irradiation, cells to be exposed are moved to the beam position using a combination of a high-resolution three-axis piezo-electric inner stage (Mad City Labs, Madison, WI) with a limited range and a motor-driven outer stage with a larger range but poorer accuracy. When a collimated microbeam was being used, this is a necessary but relatively time-consuming method to position cells for irradiation. Unlike a collimated microbeam, a focused microbeam is not
restricted to a single location on the exit window and therefore can be deflected to any position in the field of view of the microscope used to observe the cells during irradiation. Moving the beam to the cell position magnetically or electrostatically can be performed much faster than moving the stage.

We are developing a “point and shoots” targeting system for microbeam irradiation based on wide-field magnetic split-coil deflector system from Technisches Büro Fischer (Ober Ramstadt, Germany). A dual deflection amplifier, optimally matched to these coils, has been purchased from the same company to drive the coil. This system has been used for the microbeam facility at Gesellschaft für Schwerionenforschung (GSI), Darmstadt, Germany. One coil has been operated with this amplifier as a beam “sweeper” for the PMM, but the amplifier has been replaced by one designed and built in-house, freeing it for its original purpose. A short section of beam line has been constructed around which the coil has been placed. The coil assembly is mounted just below the upper quadrupole triplet on the PMM, where it is being tested and the deflection calibrated against the coil current.

**Focused X-ray microbeam**

There are considerable benefits in using soft X-ray microbeams for both mechanistic and risk estimation endpoints. The higher spatial resolution achievable with modern state-of-the-art X-ray optics elements combined with the localized damage produced by the absorption of low-energy photons (~1-5 keV) represents a unique tool to investigate the radio-sensitivity of sub-cellular and eventually sub-nuclear targets. Moreover, as these X rays do not suffer from scattering, by using higher energy X rays (~5 keV) it is possible to irradiate with sub-micron precision individual cells and/or part of them up to a few hundred microns deep inside a tissue sample in order to investigate the relevance of effects such as the bystander effect in 3D structured cell systems.

We have investigated expanding the microbeam to include characteristic Kα X rays generated by proton-induced emission (PIXE) from Ti (4.5 keV). The use of higher energies is not feasible due to Compton scattering effects; we are limited to X-ray energies where the predominant mode of interaction is photoelectron absorption. Charged particle beams can generate nearly monochromatic X rays because, unlike electrons, they have a very low bremsstrahlung yield.

At the suggestion of one of the members of our Advisory Committee, we have changed from a transmission design, in which the X rays used are emitted in the direction of the proton beam, to a reflection design, in which the X rays used are emitted at 90° to the proton beam direction. This eliminates several problems inherent in the previous design. The system will be mounted on its own horizontal beam line on the 1st floor of the Facility and the X-ray beam will be oriented vertically, so that the geometry of the microscope and stage will be the same as for our other microbeam systems.

The new target structure consists of a round titanium plug with an angled surface embedded in a copper cooling block. A small X-ray source (~20 µm D) will be produced by bombarding the Ti target with high-energy protons using the quadrupole quadruplet lens used for our first focused microbeam, reducing the requirements on the subsequent X-ray focusing system.

A zone plate will be used to focus the X-ray source to a beam spot 1-2 µm in diameter. The proposed zone plate will have a radius of only 120 µm, an outmost zone width of 50 nm and a demagnification factor of ~11. The final expected dose rates to the sample, based on ANSYS simulations, are 1 to 6 mGy/s.

The main elements of the system have been manufactured in our machine shop and the zone plates are scheduled to be purchased this year.

**New laboratory space**

Because of a large research and development grant received by David Brenner and Gerhard Randers-Pehrson from the National Institute of Allergy and Infectious Diseases (NIAID), the Trustees of Columbia University in 2006 contributed the funds required to build over 2000 square feet of new laboratory and office space on the third floor of the Facility. Construction was completed in December, 2006. The main lab, which comprises over half the area, has been equipped with a class II biological flow hood, incubator, refrigerators, a freezer, centrifuges and other equipment and is in regular use. The office area has had as many as 5 people occupying the desks.

The other three laboratories are also in use. The PMM facility has been reconstructed in the new microbeam lab. The microscope lab has 3 fluorescent microscopes, including a new system for doing mFISH analysis, and a NIAID pilot project is being conducted in the physics lab.

**Singleton Utilization and Operation**

Table 2 summarizes accelerator usage for the past year. The Singleton is started at 7:30 AM on most days from September through June and by 9 am the rest of the time. It is often run into the evening, and frequently on weekends for experiments, development and repair. This has resulted in a total use that exceeds the nominal accelerator availability of one 8-hour shift per weekday (~250 shifts per year).

Use of the accelerator for radiobiology and associated dosimetry was only about 2/3 that of last year (which had the highest level of use since RARAF has been at Nevis Labs), but was only slightly below the average for the last 5 years. This was due, in part, to an 80% increase in on-line developments.

**Table 2. Accelerator Use, January–December 2007**

<table>
<thead>
<tr>
<th>Usage of Normally Scheduled Days</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Radiobiology and associated dosimetry</td>
<td>30%</td>
</tr>
<tr>
<td>Radiological physics and chemistry</td>
<td>19%</td>
</tr>
<tr>
<td>On-line facility development and testing</td>
<td>46%</td>
</tr>
<tr>
<td>Safety system</td>
<td>2%</td>
</tr>
<tr>
<td>Accelerator-related repairs/maintenance</td>
<td>7%</td>
</tr>
<tr>
<td>Other repairs and maintenance</td>
<td>3%</td>
</tr>
<tr>
<td>Off-line facility development</td>
<td>25%</td>
</tr>
</tbody>
</table>
opment. About 42% of the use for all experiments was for microbeam irradiations and 24% for track segment irradiations. The Microbeam Facility continues to be in great demand because it enables selective irradiation of individual cell nuclei or cytoplasm. In addition, because of the relatively low number of cells that can be irradiated in a day, microbeam experiments usually require significantly more beam time than broad beam (track segment) irradiations to obtain sufficient biological material, especially for low probability events such as mutation and bystander effects.

The Track Segment Facility is being used very efficiently, reducing the amount of accelerator time required to satisfy user demand. Because the irradiation times for samples are often 10 seconds or less, multiple users, as many as 5, are run on a single shift, sometimes using different LETs and even different types of ions in the same day.

Radiological physics utilization of the accelerator increased by about 60% this past year. Three physics experiments made use of the accelerator (Exp. 82, 112 and 123), more than usual. The experiment for Astrophysics (Exp. 123) alone used about 24% of the experiment time mainly at night and on weekends.

Approximately 41% of the experiment time was used for experiments proposed by outside users, about twice what was used last year and 1/3 more than the average for the last five years. Again, much of this increase is due to the Aprile group (Exp. 123).

Use of the accelerator for online development increased by about 80% over last year and was slightly higher than the average use for the past 5 years. In addition to beam tests and development of the electrostatically focused microbeam, considerable effort was expended on minimizing the beam spot diameter for the permanent magnet microbeam (PMM).

Singletron maintenance and repair time was only 2/3 that of last year, the lowest it has been in about 10 years. The new Singletron accelerator has operated relatively reliably for 26 months. Until October, there were only two accelerator openings in 2007, totaling less than 6 days: one at the end of May to replace the ion source bottle, which had become dirty after 7 months of service, and one in the beginning of February. The replacement GVM also was unstable (~30 kV), even with no terminal voltage. It was discovered that the charging supply is so stable that we are able to run without terminal voltage regulation by setting the charging current to obtain the desired terminal voltage, which is how the accelerator has been running for almost 4 months.

The drift in the terminal voltage as the accelerator warms up during the day, which was discovered in 2006, has continued to be an annoyance. Because the beam energy acceptance is so narrow for the electrostatic microbeam and the PMM, beam intensity decreases rapidly as the terminal potential changes by as little as a kilovolt. A remote computer terminal was installed in the Microbeam II lab to allow the accelerator terminal voltage to be controlled from both the console and the lab until a system to maintain the temperature of the GVM can be designed and installed. Another remote computer terminal will have to be installed for the PMM.

Training

The Small Group Apprenticeship Program continued for the fourth year. Five students from Stuyvesant High School in Manhattan spent at least two half-days each week for six weeks during the summer working on projects in biology (1) or physics (4). Stuyvesant is a high school specializing in science that is open to students throughout New York City by competitive admission. The students gave professional PowerPoint presentations to our group at the end of the program. Below is a list of the titles of the work presented followed by the name of the student and the name of his or her mentor:

1. Adaptive Responses in Irradiated Cells - Farhan Nuruzzaman (Brian Ponnaiya)
2. Under-dish Detector for the Microbeam at Columbia University - Chaitanya Medicherla (Guy Garty)
3. Oxygen/Nitrogen Resonance Explosives Detector - Li Ang Zhang (Yanping Xu)
4. Design of the RARAF X-Ray Microbeam - Dawood Din (Andrew Harken)
5. Multiphoton Microscope Development - Benjamin Lerner (Alan Bigelow)

Several of the previous students have been co-authors of journal articles, including one in the prestigious Proceedings of the National Academy of Science (PNAS).

Andrei Popescu, a student at Ossining High School in Westchester County, has worked with Brian Ponnaiya for almost a year. He is studying DNA breakage and micronucleus formation in mouse cells after X-ray irradiation.

Personnel

The Director of RARAF is Dr. David Brenner, now also the Director of the Center for Radiological Research. The accelerator facility is operated by Mr. Stephen Marino, the manager, and Dr. Gerhard Randers-Pehrson, the Associate Director of RARAF.

Dr. Charles Geard, the former Associate Director of the CRR and the Senior Biologist for the P41 grant that is the major support for RARAF, continues to spend most of each day at RARAF. In addition to his own research, he collaborates with some of the outside users on experiments using

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the single-particle Microbeam Facility.

Dr. Alan Bigelow, an Associate Research Scientist, is developing a multiphoton microscopy system using a fast laser.

Dr. Guy Garty, an Associate Research Scientist, is developing an inductive detector (LD²) for single ions and the permanent magnet microbeam (PMM). He spends about half his time working on the National Institute of Allergy and Infectious Diseases (NIAID) project, for which he is the project manager.

Sasha Lyulko, a graduate student in the Physics Department at Columbia, is involved in developing methods to image cells without stain and spends about half her time working on the NIAID project.

Dr. Andrew Harken, a Postdoctoral Fellow, is developing an X-ray microbeam and working with Guy Garty on the PMM.

Dr. Yanping Xu, a Postdoctoral Fellow, has been working on the NIAID project, developing a method for determination of the number of lymphocytes in blood samples using light absorption.

Several biologists from the Center for Radiological Research are stationed at the facility in order to perform experiments:

- Dr. Brian Ponnaiya is an Associate Research Scientist performing experiments using the Track Segment and Microbeam irradiation facilities.
- Ms. Gloria Jenkins, a biology technician, performed experiments on the Microbeam Facility for Dr. Geard. Gloria retired in May after 34 years with Columbia University and 17 with the Center for Radiological Research.
- Dr. Alexandre Mezentsev, an Associate Research Scientist, is working with cultured tissue systems and spends some of his time at RARAF.
- Dr. Helen Turner, an Associate Research Scientist, is working on the NIAID project and spends at least one day per week at RARAF.

At the end of March Yigal Horowitz from Ben Gurion University of the Negev and Atara Horowitz from the Nuclear Research Center, Beersheva in Israel began a one-year sabbatical at RARAF. Their project involved the characterization of ‘slow-cooled’ LiF:Mg,Ti (TLD-100) using neutrons and charged particles. They returned to Israel in November for personal reasons.

Kenichi Tanaka, a Staff Associate, arrived in August from Hiroshima University, Japan for a one-year visit and has been working with Gerhard Randers-Pehrson on the detection of explosives. He is terminating his visit in March in order to accept a position at the University of Hokkaido.

Recent Publications of Work Performed at RARAF

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**RSO staff at various occasions**

*Front row (l-r):* Shira Abraham, Maria Taveras, Jillian Porteus and Rivka Rose; *back row:* Moshe Friedman, David Park, Ahmad Hatami, Salmen Loksen, David Rubinstein and Tom Cummings.

*Standing (l-r):* The Radiation Safety Officer and Assistant Radiation Safety Officers, Salmen Loksen, Thomas Juchnowicz and Ahmad Hatami; *sitting (l-r):* RSO Technicians, Shira Abraham and Jaclyn Marcel.

*An early morning radioactive waste shipment for GTS Du- ratek, a division of Energy Solutions, Posing with the shipper are RSO staff (l-r):* David Park, Tom Cummings, Ahmad Hatami and Roman Tarasyuk.
RSO Staff (l-r) front row: Pantea Kadkhodazadeh, Jillian Porteus, Allison Powers, Shira Abraham and Rivka Rose; back row: Roman Tarasyuk, David Park, Tom Cummings, Moshe Friedman, Salmen Loksen, Srinivasa Valluripalli, Ahmad Hatami, David Rubinstein and Charles Geraghty; not shown: Tom Juchnewicz, Bruce Emmer, Dae In Kim, Jaclyn Marcel Maria Taveras and Jennifer Curiel.

PROFESSIONAL STAFF
Salmen Loksen, CHP, DABR; Director, Radiation Safety Officer
Ahmad Hatami, DABR, DABMP; Assistant Director, Assistant Radiation Safety Officer
Thomas Juchnewicz, DABR; Assistant Radiation Safety Officer
Jacob Kamen, PhD, NRRPT, CHP; Assistant Radiation Safety Officer (through June 2007)
Bruce Emmer, DABMP, DABR; Physicist
Dae In Kim, MS, Health Physicist
Shinkyu (David) Park, MS, Radiation Protection Supervisor
Charles Geraghty, BS, Assistant Physicist
Allison Powers, BS, Assistant Physicist (through January 2008)

TECHNICAL STAFF
David Rubinstein, BS, Senior Technician
Thomas Cummings, BS, Technician B
Pantea Kadkhodazadeh, BS, Technician B
Jaclyn Marcel, MS, Technician B
Roman Tarasyuk, Technician B

ADMINISTRATIVE & OFFICE SUPPORT STAFF
Moshe Friedman, BRE, Office Administrator
Jillian Porteus, BA, Administrative Aide
Maria Taveras, BA, Senior Clerk
Jennifer Curiel, AAS, Clerk B

STUDENT/CASUAL STAFF
Shira Abraham, BS, Technician
Srinivasa Valluripalli, BS, Developer/Programmer
Rivka Rose, BA, Clerk/Secretary

Salmen Loksen, CHP, DABR, Director and Radiation Safety Officer, and Ahmad Hatami, DABR, DABMP, Assistant Director and Assistant Radiation Safety Officer, at the entrance to the Allan Rosenfield Building, 722 W. 168th Street, which houses the Joseph L. Mailman School of Public Health, as well as the Radiation Safety Office and other departments.
INTRODUCTION

On May 19, 1957 Grayson L. Kirk, President of Columbia University, distributed a memo entitled “Directive to All University Departments Having a Source of Ionizing Radiation,” advising all parties of the expanded function of the Radiation Safety Committee.

Later, a notice entitled “Radiation Safety Guide for Columbia University,” dated February 10, 1959, named Philip M. Lorio as Health Physics Officer for University Departments and Laboratories other than the College of Physicians & Surgeons, where Dr. Edgar Watts was named Health Physics Officer. The Chairman of the Radiation Safety Committee was Dr. Gioacchino Failla, who initiated the Radiological Research Laboratory in Columbia University’s Department of Radiology.

By agreement between Columbia University and New York Presbyterian Hospital in 1962, the Radiation Safety Office was established as an autonomous unit for the purpose of maintaining radiation safety. The Joint Radiation Safety Committee (JRSC), appointed by the Medical Board of the New York Presbyterian Hospital and the Vice President for Columbia University’s Health Sciences Division, was charged with the responsibility of defining and ensuring enforcement of proper safeguards in the use of sources of ionizing radiation.

Dr. Harald H. Rossi, Director of the Radiological Research Laboratory, was appointed Chairman of the Joint Radiation Safety Committee. Under his direction this committee developed a “Radiation Safety Code & Guide,” the administration of which is assigned to the Radiation Safety Officer. Dr. Eric J. Hall, the next Director of the Radiological Research Laboratory, subsequently renamed the Center for Radiological Research, followed as JRSC Chairman until his retirement from that role in 2007. Dr. David Brenner, Director of the Radiological Research Accelerator Facility, is the current JRSC Chair.

The present Joint Radiation Safety Committee of Columbia University Medical Center, New York Presbyterian Hospital and New York State Psychiatric Institute came into existence through an agreement made on February 12, 1991 between the three institutions. The agreement combined several overlapping clinical and educational programs, including all programs for ensuring radiation safety. The current Director of the Radiation Safety Office and Radiation Safety Officer, Salmen Loksen, CHP, DABR, was appointed on December 16, 1996.

The Radiation Safety Office reports to and advises the Joint Radiation Safety Committee, which meets on a quarterly basis. At the present time the Radiation Safety Officer reports on professional and technical matters to Dr. David Brenner, Chair of the JRSC, and on budgetary matters to Dr. Robert Lewy, Sr. Assoc. Dean for Health Affairs, who represents Dr. Lee Goldman, Dean of Columbia University Medical Center. In addition, the Radiation Safety Office participates in the review of research protocols for the Radioactive Drug Research Committee (RDRC) under the jurisdiction of the U.S. Food and Drug Administration.

Radiation Safety Office staff are Columbia University employees. Columbia University, New York Presbyterian Hospital and New York State Psychiatric Institute fund the Radiation Safety Office budget via a cost sharing payback arrangement.

OVERVIEW OF RADIATION SAFETY OFFICE RESPONSIBILITIES

Collectively, Columbia University Medical Center, New York Presbyterian Hospital and New York State Psychiatric Institute form a large health sciences complex with extensive teaching, research, and clinical facilities. The basic goal of the Radiation Safety Office is to ensure the implementation of all protective measures necessary to guarantee that doses from ionizing radiation to patients, visitors, students, faculty and staff on campus, as well as to the community at large, remain “as low as reasonably achievable” (ALARA). Major entities supported by Radiation Safety Office services include:

- Columbia University Medical Center
- New York Presbyterian Hospital
- New York State Psychiatric Institute
- Columbia Presbyterian Eastside
- New York Presbyterian Hospital/Allen Pavilion
- CUMC Cyclotron Facility
- Dental Facilities throughout CUMC and elsewhere as described later in this report.

The projected completion of several additional buildings, as well as the Columbia University Medical Center Integrated Imaging Center will add to the responsibilities of the Radiation Safety Office in the near future. For the purposes of this report, this collection of entities will hereafter be referred to as CUMC/NYPH/NYSPI.

Reporting to the Joint Radiation Safety Committee of CUMC/NYPH/NYSPI, the Radiation Safety Officer and the staff of the Radiation Safety Office are responsible for obtaining and maintaining licenses authorizing the possession and use of radioactive materials and obtaining and maintaining registrations and permits for the operation of radiation producing equipment. In addition, the Radiation Safety Office is responsible for obtaining and maintaining permits necessary for the safe disposal or controlled release of research and medical wastes containing radioactivity.

The Radiation Safety Office ensures that authorized users of radioactive materials and radiation producing equipment comply with all governmental regulatory requirements and guidelines by means of training, education, consultation, and by a program of internal audits and inspections of facilities. Regulatory agencies charged with overseeing the pos-
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session, use, or disposal of radioactive materials or radiation producing machines include:
- United States Environmental Protection Agency (EPA)
- United States Food and Drug Administration (FDA)
- United States Nuclear Regulatory Commission (NRC)
- New York State Department of Environmental Conservation (NYSDEC)
- New York State Department of Health (NYSDOH)
- New York City Department of Health & Mental Hygiene (NYCDOHMH), Office of Radiological Health
- New York City Department of Health & Mental Hygiene, New York State Department of Environmental Conservation, and United States Food and Drug Administration conduct periodic inspections and audits of CUMC/NYPH/NYSPI facilities operating under their licenses or permits. The Radiation Safety Office works continuously to prevent regulatory violations and swiftly implements any regulatory recommendations.

The Radiation Safety Office also ensures compliance with institutional policies and procedures published in the “Radiation Code & Guide of Columbia University Medical Center, New York Presbyterian Hospital & New York State Psychiatric Institute.”

**SUMMARY OF RADIATION SAFETY OFFICE OPERATIONS FOR 2007**

A summary of activities performed and services provided by the Radiation Safety Office is presented below. While inclusive of most major activities and services, the summary is by no means exhaustive, but is intended to provide a representative overview of departmental operations. An unabridged compilation of Radiation Safety Office activities and services may be found in the Minutes of the Quarterly Meetings of the Joint Radiation Safety Committee of CUMC/NYPH/NYSPI.

Statistical data presented are from the calendar year, January 1, 2007 through December 31, 2007. Activities are covered up to February 2008.

**New York City Department of Health & Mental Hygiene, Office of Radiological Health – Maintenance of Licenses, Registrations, Permits, and Audits and Inspections**

A primary activity of the Radiation Safety Office is the continued maintenance of City of New York Radioactive Materials Licenses, Certified Linac Registrations and X-Ray Permits. Currently this includes:
- Radioactive Materials License No. 75-2878-01 (Broad Scope Human Use)
- Radioactive Materials License No. 74-2878-03 (Non-Human Use)
- Radioactive License No. 52-2878-04 (Cyclotron Facility)
- Radioactive Materials License No. 93-2878-05 (Gamma Knife)
- City of New York Therapeutic Radiation LINAC Unit Certified Registration No. 77-0000019.
- Columbia-Presbyterian Hospital Radiation Installation Permit H96 0076353 86
- Columbia-Presbyterian-Allen Pavilion Radiation Installation Permit H96 0076383 86
- Columbia University Gymnasium, Morningside Campus or Baker Field Radiation Installation Permit H98 1005495
- Columbia University Physicians Metabolic Diseases Unit, Bone Density Permit H90 1162695

Activities performed in 2007 to maintain the City of New York Licenses, Registrations and Permits included:

**Human Use license – Philips camera:**

As noted in last year’s report, on December 13, 2006, at the regular quarterly meeting of the Joint Radiation Safety Committee of Columbia University Medical Center, New York Presbyterian Hospital and New York State Psychiatric Institute, Eric J. Hall, D Phil., D Sc., FACR, FRCR, announced his intention to resign as Chairman of the Committee, pending approval of a new Chairman by the Joint Radiation Safety Committee and the approval of a License Amendment by the New York City Department of Health and Mental Hygiene. A quorum of the Joint Radiation Safety Committee, unanimously, with the abstention of the proposed candidate, voted to approve David J. Brenner, Ph.D., as the new Chairman of the Committee. On December 20, 2006 an Amendment request for approval of this action was submitted to the NYC DOHMH.

On March 12, 2007, the Radiation Safety Office received Amendment No. 24 to Broad Scope Human-Use Radioactive Materials License No. 75-2878-01, authorizing activities throughout CUMC/NYPH/NYSPI that employ radioactive materials in the clinical diagnosis and treatment of patients and human subject research, naming David J. Brenner, Ph.D., as Chairman, Joint Radiation Safety Committee.

**Human Use license – TheraSphere:**

Humanitarian Device Exemption Use Protocols require IRB review and approval (21CFR814.124(a)). The CUMC IRB delegates to the JRSC review, on the basis of safety, those protocols involving the administration of radionuclides or radiation. CUMC IRB-AAAB8374(Y1M00), A Humanitarian Device Exemption Use Protocol of TheraSphere® for Treatment of Unresectable Hepatocellular Carcinoma, involves the use of TheraSphere®, a brachytherapy sealed source. Under the Conditions of NYC DOHMH Broad Scope Human Use Radioactive Materials License No. 75-2878-01, sealed sources are listed separately on the License. The possession limit, location of use and conditions of use for brachytherapy sealed sources and
programs (example: Iridium-192 HDR) are explicitly listed on the License. Therefore, an Amendment to NYC DOHMH Radioactive Materials License No. 75-2878-01 authorizing the possession and use of TheraSphere® brachytherapy sealed sources must also be approved by a vote of a quorum of the Joint Radiation Safety Committee. Specifically, JRSC approval is required with regard to the possession of 120 Gigabecquerals (3.24 Curies) of Yttrium-90, as glass microspheres, Model: TheraSphere®, sealed source and conditions of use as described in: USNRC Registry Of Radioactive Sealed Sources And Devices Safety Evaluation Of Device, No. NR-0220-D-113-S, dated January 24, 2005; location(s) of use to be provided by the Authorized User. The Radiation Safety Office is in the process of preparing an amendment request for submission to the NYC DOHMH to authorize the possession and use of Yttrium-90 TheraSphere®.

Human Use license – inspection:

From July 24, 2007 through August 15, 2007 New York City Department of Health & Mental Hygiene conducted an audit of records and an inspection of facilities and operations authorized by Broad Scope Human Use Radioactive Materials License No. 75-2878-01. Over the above period the NYC DOHMH inspector reviewed records of radioactive material use, audited policy and procedure manuals, inspected facilities, observed activities and interviewed staff members in Departments including: Nuclear Medicine, Nuclear Cardiology, Kreitchman PET Center, Transfusion Services, and Radiation Oncology brachytherapy service. At the exit interview the NYC DOHMH inspector commented that “there were no deficiencies” and the CUMC/NYPH/ NYSPI program appeared to be “very good” and on November 9, 2007 the Radiation Safety Office received a written report from NYC DOHMH stating: “At the time of the inspection, it was found that your facility was in compliance with the requirements of Article 175 of the New York City Health Code and the conditions of your license.”

Nonhuman Use license renewal:

Radioactive Materials License No. 74-2878-03, the Broad Scope License for Non-Human Use, had an expiration date of January 31, 2007. The Radiation Safety Office submitted an application for renewal and on January 17, 2007 the Radiation Safety Office received a letter acknowledging the timely filing of our application, and on May 31, 2007 the Radiation Safety Office received the renewal of Broad Non-Human Use Radioactive Materials License No. 74-2878-03. The renewed License is valid for a five year period and expires on January 31, 2012.

Gamma Knife license renewal:

Radioactive Materials License No. 93-2878-05 (Gamma Knife) had an expiration date of October 31, 2007. The Radiation Safety Office submitted a full renewal application \textit{ab initio}, and on October 24, 2007 received from the NYC DOHMH a Letter of Timely Renewal authorizing continued operation of the Gamma Knife while the Renewal Application is in the process of being reviewed and acted upon by the NYC DOHMH. On February 5, 2008 the Radiation Safety Office received a five-year renewal of the above License. The expiration date of the renewed License is October 31, 2012.

Gamma Knife license inspection:

On August 23, 2007 the NYC DOHMH conducted an audit of records and an inspection of facilities and operations authorized by the Gamma Knife license, which authorizes clinical patient treatment activities in the Department of Radiosurgery at CUMC/NYPH. The NYC DOHMH inspector reviewed records of radioactive material use, audited policy and procedure manuals, inspected facilities, observed activities and interviewed staff members. At the exit interview the NYC DOHMH inspector commented that the CUMC/NYPH program appeared to be “without deficiencies.” At the present time the Radiation Safety Office is still waiting to receive the inspector’s written report.

Cyclotron license renewal:

NYC DOHMH Radioactive Materials License No. 52-2878-04, which authorizes the operations of the Columbia University Medical Center Cyclotron Radiopharmacy and Radioligand Laboratory, had an expiration date of October 31, 2007. The Radiation Safety Office submitted a completed Application for the renewal and received from the NYC DOHMH a Letter of Timely Renewal authorizing continued operation of the Cyclotron Radiopharmacy and Radioligand Laboratory while the Renewal Application was in the process of being reviewed and acted upon by the NYC DOHMH. The above License Renewal Application requested that additional individuals be authorized to operate the cyclotron. On February 5, 2008 the Radiation Safety Office received a five-year renewal of the above License. The expiration date of the renewed License is October 31, 2012.

X-RAY Permit – Allen Pavilion & Dodge Fitness Center:

On April 11, 2007 the Radiation Safety Office received renewals for two Radiation Installation Permits: Permit No. H96 0076383 86 for operation of diagnostic medical X-ray equipment by the New York Presbyterian Hospital at the Allen Pavilion, 5141 Broadway, New York, NY 10034, with a new expiration date of December 31, 2008; and Permit No. H981005495 72 for operation of diagnostic medical X-ray equipment by the Columbia University Dodge Fitness Center, 3030 Broadway, New York, NY 10027 at its Morningside Heights and Baker Field Locations, with a new expiration date of March 31, 2009.

Integrated Imaging Center:

In 2007 the Radiation Safety Office continued preparations for NYC DOHMH licensing action with regard to the proposed CUMC Integrated Imaging Center (NYSTAR). The CUMC Integrated Imaging Center when completed will host on site: two PET radionuclide production cyclotrons; a PET radiopharmacy and radioligand labeling laboratory; and clinical and research PET and PET/CT imaging suites. Licensing actions will include: a) Preparation and submission of an application to the NYC DOHMH for a new City of New York Radioactive Materials License to authorize the operation of the two (2) new Siemens RDS-111 Eclipse Cyclotrons at 722 West 168th Street, New York, NY, and relocation of the
CUMC Radiopharmacy and CUMC Radioligand Laboratory from Milstein Hospital Building, B-1 Level to 722 West 168th Street, New York, NY.

b) Amendment of City of New York Radioactive Materials License No. 75-2878-01 (Broad Scope Human Use) to authorize the relocation of the PET Imaging Center from Milstein Hospital Building, 3rd Floor, 177 Fort Washington Avenue to 722 West 168th Street, 1st Floor; relocation of the PET/CT from the PET/CT Trailer located in the parking lot of 722 W. 168th St. to 722 W. 168th St., 1st Fl.; and to authorize the operation of new imaging equipment.

c) Amendment of City of New York Radioactive Materials License No. 74-2878-03 (Non-Human Use) to authorize the relocation of the Micro-PET Animal Imaging Center from Milstein Hospital Building, B-1 Level, 177 Fort Washington Avenue to 722 West 168th Street, 1st Floor; and to authorize the operation of new imaging equipment.

d) Application to the New York State Department of Environmental Conservation for a new Radiation Control Permit to authorized discharges of radio-nuclides to the atmosphere originating from operations of the proposed CUMC Integrated Imaging Center.

In addition to ensuring the above regulatory requirements are met, the Radiation Safety Office reports potential total costs associated with removal, disposal and decommissioning to the Columbia University Controllers Office to comply with Columbia University Financial Department regulations as to the locations and estimated total costs of disposal of radioactive material used and stored at Columbia University Medical Center.

Linac Amendment – Trilogy:

At the Joint Radiation Safety Committee meeting of June 12, 2007, a quorum of the JRSC voted to approve an Amendment of City of New York Therapeutic Radiation Linac Unit Certified Registration No. 77-0000019 to remove the Varian Associates linac 2100C/D listed on the Registration and authorize replacement by a Varian Associates Trilogy 2300TX. The Trilogy 2300TX was installed in Morgan Stanley-CHONY Tunnel Level Room 12. Radiation safety surveys and customer acceptance testing were completed by Radiation Safety Office Medical Health Physics staff and Department of Radiation Oncology Medical Physics staff. On December 19, 2007 the Radiation Safety Office submitted to the New York City Department of Health and Mental Hygiene the completed Amendment Application for Therapeutic Radiation Linac Unit Certified Registration No. 77-0000019. On March 3, 2008, the Radiation Safety Office received Amendment No. 6 to the Registration. Registration No. 77-0000019 expires August 31, 2008. The Trilogy 2300TX has since been placed into patient service.

Linac inspection:

A routine audit of records and inspection of facilities and operations authorized by Linac Unit Certified Registration No. 77-0000019 was conducted from November 14, 2007 through December 3, 2007 by the New York City Department of Health & Mental Hygiene. The audit and inspection consisted of selective examinations of procedures and representative records, interviews with personnel, and observations and measurements by the inspector. The findings of the audit and inspection were discussed at an exit interview, and on December 7, 2007 the Radiation Safety Officer received a written report from NYC DOHMH stating: “At the time of the inspection, it was found that your facility was in compliance with the requirements of Article 175 of the New York City Health Code and the conditions of your license.”

Increased Controls inspection:

As discussed in last year’s report, at the end of 2005 the Radiation Safety Office received an Order of the Commissioner from Thomas R. Frieden, M.D., M.P.H., Commissioner, NYC DOHMH, implementing a requirement for increased security controls “To Licensees Possessing Certain Radioactive Materials in Quantities of Concern in the City of New York.” There are several sources throughout CUMC and NYPH that require these additional controls, and in addition to the previously described measures taken for compliance, in 2007 the following actions were taken:

Supplemental surveillance camera systems and alarmed radiation monitors were installed to provide additional security. The camera systems include remote-controlled digital CCTV with recording storage capacity. The equipment and installation locations were selected with the assistance of CUMC Department of Public Safety and NYPH Security Department. As of May 31, 2007 all the work for these enhanced security measures was complete and the system was operational.

On November 20, 2007 NYC Department of Health & Mental Hygiene and NYPD Counterterrorism Division conducted an inspection of CUMC and NYPH facilities for compliance with the “increased control” requirements. A walkthrough of the CUMC and NYPH facilities possessing sources of concern was conducted and the increased controls policies, procedures and records were examined and checked for compliance. The inspectors were also shown the enhanced security changes, including the above mentioned enhanced security monitoring equipment. At the exit interview the inspectors stated that they were very pleased and impressed with the response of CUMC/NYPH to the increased control regulations, and stated that CUMC/NYPH is in full compliance and that it is a model for other New York City institutions to emulate. The Radiation Safety Office received the written report of the NYC DOHMH inspector, dated December 24, 2007, which states: “At the time of inspection, it was found that your facility was in compliance with the security requirements contained in the Order of the Commissioner and Article 175 of the New York City Health Code.”

Increased Controls – REMTrack website:

In 2007 the City of New York Department of Health and Mental Hygiene developed a new Web-based REMTrack Radioactive Material Inventory System to more efficiently track large radioactive sources and radioisotope totals at licensee facilities. The Radiation Safety Office attended a number of meetings and trainings regarding this new system. Under the authority of RCNY 175.101(k)(2), Conditions of
specific licenses. Licensees receiving, possessing, using, storing or transferring more than 500 mCi (18.5 GBq) of radioactive material will be required to submit an accurate electronic inventory to the Office of Radiological Health on a quarterly basis. Licensees handling less than 500 mCi (18.5 GBq) of radioactive material will be required to report on an annual basis. In accordance with this, the Radiation Safety Office compiled inventory records for hospital and research areas, and in a timely manner completed this requested quarterly data entry in the NYC DOHMH Web-based Radioactive Material Inventory Tracking System.

Emergency equipment grant:
In another interaction with the NYC DOHMH, as reported previously, the Radiation Safety Office and Emergency Department of New York Presbyterian Hospital, Allen Pavilion, and Children’s Hospital of New York applied for three grants for the 2006 Hospital Radiation Emergency Equipment Project. Under the terms of these awards, hospitals are able to choose equipment which they are responsible for maintaining. In the event of an emergency NYCDOHMH may requisition this equipment for its own use. Three grants of close to $24,000 each were awarded to the above institutions for the purchase of radiation safety emergency response equipment, and in December 2006 the Radiation Safety Office received the shipment of radiation equipment for New York Presbyterian Hospital and Children’s Hospital of New York. The equipment for Allen Pavilion was shipped directly to Allen Pavilion.

In 2007 the Radiation Safety Office held a number of meetings regarding this equipment with representatives of NYPH ER, NYPH Facilities, NYPH ITS, and NYPH Security to discuss relevant issues including storage, testing, staff training, installation and effective use of the equipment, emergency power, placement of area monitors and network connections. The Radiation Safety Office oversaw distribution and installation of the equipment, and developed policies, procedures and a training program for Hospital end-users. It is planned that the fixed radiation monitoring locations will soon be accessible online.

On December 11, 2007 in order to assess the current status of hospital radiation preparedness and to assist participating hospitals in achieving this goal, staff from New York City Department of Health and Mental Hygiene’s Healthcare Emergency Preparedness Program and Office of Radiological Health conducted a site visit and met with staff involved in radiation preparedness, and reviewed the installation of radiation area detectors and the facility’s response plans to alarms from any of these area monitors. Among other issues discussed during the site visit was the installation of protective coverings for the area monitors to prevent tampering with the equipment by unauthorized individuals. A copy of the draft policy & procedure for responding to the area monitors was provided to the NYC DOHMH representatives.

New York State Department of Environmental Conservation – Maintenance of Permits, and Audits & Inspections

Another primary activity of the Radiation Safety Office is the continued maintenance of New York State Department of Environmental Conservation Radiation Control Permit No. 2-6201-00005/00006.

CUMC/NYPH/NYSPI conducts medical research and clinical activities that discharge limited and controlled quantities of radioisotopes to the atmosphere and to sewage systems as per the Conditions of the Radiation Control Permit and in compliance with New York State 6 NYCRR Part 380, Rules and Regulations for Prevention and Control of Environmental Pollution by Radioactive Materials.

The entities served by the Radiation Safety Office are situated within a densely populated urban area. The quantities of radioisotopes discharged and the resulting public radiation dose are closely regulated by the New York State Department of Environmental Conservation. Radiation doses to the general public resulting from atmospheric discharges of radioisotopes are required to not exceed the USNRC Constraint Limit of 10 mrem per year. This amounts to only a fraction of the annual naturally occurring background radiation level.

CUMC/NYPH/NYSPI is currently permitted a total of 15 atmospheric emission points from which radionuclides are discharged to the atmosphere. Monitoring, analyzing, reporting, and minimizing discharges from these emission points, in order to ensure compliance with the Conditions of the Radiation Control Permit, is one of the major continuing activities of the Radiation Safety Office.

Activities performed in 2007 to maintain the NYSDEC Radiation Control Permit included:

Permit renewal:
Based on the application submitted at the end of 2006, on February 9, 2007, the Radiation Safety Office received renewal of the New York State Department of Environmental Conservation Permit No. 2-6201-00005/00006, dated February 2, 2007. The Radiation Control Permit authorizes atmospheric releases of radionuclides from diagnostic, therapeutic and research use of radioactive material at Columbia University Medical Center, New York Presbyterian Hospital and New York State Psychiatric Institute. The renewed Permit expires February 2, 2012. While the renewal letter requested the Permit be renewed as is, the attached New York State Department of Environmental Conservation NOTICE OF PERMIT ISSUANCE letter stated: “Please note that a new Special Condition No. 5 has been added, limiting total dose exposure to the public to 10 millirems/year. This condition has been added to all Part 380 permits upon renewal or modification.”

DEC inspections:
On January 17, 2007 and January 18, 2007 the New York State Department of Environmental Conservation performed an audit of records, an inspection of facilities, and met with staff at Columbia University Medical Center, New York Presbyterian Hospital and New York State Psychiatric Institute departments that are authorized to discharge radionuclides into the atmosphere under New York State Department of Environmental Conservation Permit No. 2-6201-00005/00006. Items for discussion included substantial management changes at CUMC that
involved individuals with oversight and financial responsibility for radiation safety and for operations authorized under the Radiation Control Permit. The necessity for maintaining “institutional memory” of commitments made by CUMC in correspondence with the NYS DEC was reviewed. On February 15, 2007, the Radiation Safety Office received a letter from the New York State Department of Environmental Conservation, reporting that the institution was in compliance.

2006 annual report on radioactive discharges:

On March 6, 2007 the Radiation Safety Office, in compliance with the requirements of 6 NYCCR Part 380-9.1 and the conditions of New York State Department of Environmental Conservation Permit No. 2-6201-00005/00006, submitted to the New York State Department of Environmental Conservation an Annual Report on radioactive discharges and disposal made in the previous Calendar Year 2006. The Annual Calibration of the Stack Monitoring Systems was submitted to the NYS DEC as an attachment to the Year 2006 Annual Report. On June 4, 2007 the Radiation Safety Office received a letter from the New York State Department of Environmental Conservation, Radiation Section stating that the Radiation Section had received and reviewed the Year 2006 radionuclide discharge report and determined that discharges at CUMC, NYPH and NYSPI were within the effluent limits set in the Permit and the requirements of 6 NYCCR 380-5.1(a).

Atmospheric discharges of C-11:

Regarding atmospheric discharges in 2007, the amounts of all isotopes were well below the annual limits, with the exception that discharges of C-11 from the Radioligand Laboratory became a concern. By May 31, 2007 they were at 6.828 Curies or 56.9% of the 12 Curie Permit Limit, and it was recognized that if discharges of C-11 from the Radioligand Laboratory would continue at that rate they would exceed the Permit Limit by mid-August 2007. Researchers were directed to minimize the use of C-11 until it could be determined whether the NYS DEC would increase the Permit Limit for C-11.

Upon review and approval by the Radiation Safety Officer, the Chairman, Joint Radiation Safety Committee and the JRSC management representatives of CUMC, NYPH and NYSPI, on September 5, 2007 the Radiation Safety Office submitted to the New York State Department of Environmental Conservation a Permit Modification Request, on the basis of reallocating discharges and public radiation dose from emission points well below their Permit Limit.

Since the submission of the Permit Modification Request, the Radiation Safety Office communicated several times with the NYS DEC requesting expedition in processing the Permit Modification Request. On November 5, 2007 Markus Spivak, NYS DEC Radiation Section, confirmed that patient research at CUMC is authorized to continue uninterrupted when the current C-11 Permit Limit is reached. Mr. Spivak further stated that the NYS DEC Radiation Section will exercise enforcement discretion in this regard. By December 31, 2007 the discharges of C-11 had reached 11.4 Curies or 94.73% of the original 12 Curie Permit Limit. The Radiation Safety Office is awaiting the issuance of the revised Radiation Control Permit.

Integrated Imaging Center:

With regard to the planned new cyclotron, the CUMC Integrated Imaging Center Facility (NYSTAR), on January 8, 2008 representatives of both the NYS DEC and the New York City Department of Health and Mental Hygiene arrived for a site-visit, and to inspect the cyclotron radiopharmacy and radioligand laboratories under construction and met with project participants to discuss requirements for the NYS DEC Radiation Control Permit and a NYC DOHMH Radioactive Materials License that the Radiation Safety Office is preparing to submit for this new facility.

DEC inspection with USNRC observers:

On November 14, 2007 the New York State Department of Environmental Conservation conducted a routine annual audit of records and inspection of facilities and activities authorized by Radiation Control Permit No. 2-6201-00005/00006. The Radiation Control Permit sets annual limits and other conditions for the release of radionuclides to the environment by Columbia University Medical Center, New York Presbyterian Hospital and New York State Psychiatric Institute.

The NYS DEC inspection was accompanied and observed by a representative of the US Nuclear Regulatory Commission (NRC), Division of Nuclear Materials Safety. This observational role had been requested by the NRC because the Energy Policy Act of 2005 provided for expanded regulatory authority over the NRC over radium sources, accelerator produced radioactive materials and certain naturally occurring radioactive materials, and in May 2007 the NRC published a Final Rule regarding the expansion of their regulatory authority over these radioactive materials.

At the exit interview the NYS DEC inspector stated that his inspection had found CUMC to be in compliance with NYS Part 380 regulations and the Conditions of the Radiation Control Permit, and commented on the excellence of the CUMC cyclotron program, noting the efforts of the Radiopharmacy, the Radioligand Lab, the PET Suite and the Radiation Safety Office in this regard. On December 26, 2007 the Radiation Safety Office received a letter from the NYSDEC reporting the results of the inspection. The letter, dated December 19, 2007 states: “Within the scope of the inspection, operations at CUMC were found to be in compliance with Part 380 and the conditions of its permit.” The letter from the NYS DEC dated December 19, 2007 contained a recommendation that CUMC ensure adequate professional staffing in the Radiation Safety Office.

RSO staff – training and named authorized user:

In accordance with recommendations made by the New York State Department of Environmental Conservation, the Radiation Safety Office has continued to send professional and technical staff for training in cyclotron systems and operations. Training has included manufacturer’s training by CTI in Knoxville, Tennessee and advanced training at the U.S. Particle Accelerator Summer School at various university locations. Radiation Safety Office staff have
received certificates as cyclotron operators. A Radiation Safety Office Health Physicist, Dae In Kim, has been named as an Authorized User on the Cyclotron Radioactive Materials License.

**Radioactive Material Administration: Receipt, Distribution and Radioactive Waste Disposal**

A major program of the Radiation Safety Office is the centralized administration of all authorized radioactive materials used at CUMC/NYPH/NYSPI. The use of radioisotopes by individual investigators is authorized by the Joint Radiation Safety Committee and controlled by the Radiation Safety Office. Human Use of radioactive materials is carried out by Authorized User Physicians. Authorized User status is granted following a review of credentials and a majority vote by a quorum of the Joint Radiation Safety Committee. Non-Human Use of radioactive materials by Responsible Investigators is granted after a review of applications and written permission of the Chairman of the Joint Radiation Safety Commission and the Radiation Safety Officer. In 2007 dozens of new Responsible Investigators were reviewed and approved for non-human use of radioactive materials, and scores of current Responsible Investigators received renewals and amendments of their authorizations.

Activities in 2007 to administer, receive, distribute, and dispose of radioactive materials included:

- Thousands of purchase orders for materials that contain radioisotopes were approved and 1650 packages containing radioactive material were received in addition to direct shipments to the Departments of Nuclear Medicine and Radiation Oncology. Prior approval was given for all received shipments. Package surveys and wipe tests were conducted to ensure that none of the packages were contaminated. The Radiation Safety Office maintains inventory control of all radioactive materials received and distributed through the use of a computerized database. The orders resulted in the purchase of a total of approximately 1.6 Curies of activity. $^{35}$S, $^3$H, and $^{32}$P were the isotopes purchased with the highest activities.

- Low-level aqueous radioactive waste was disposed of through the sewer during 2007. The total activity of sewer-disposal aqueous radioactive waste was 200756 mCi, of which 17.708 mCi was tritium ($^3$H), 126.029 mCi was $^{35}$S, and 14.882 mCi $^{32}$P, 1.961 mCi was $^{152}$I, and 11.226 was other isotopes. As required by 6 NYCRR Part 380 and the conditions of our NYSDEC Radiation Control Permit, the controlled sewer disposal of aqueous radionuclides was reviewed. The discharge for all isotopes was well below the yearly concentration limits of 6NYCRR Part 380-11.7 Table II.

- The Radiation Safety Office processed a total of 1210 waste shipments from CUMC, NYPH, and NYSPI, and collected 165 black bags from NYPH for Decay-in-Storage. Black bags are collected from incontinent patients who have undergone nuclear medicine procedures.

- Short-lived radioactive liquid mixed wastes are held in storage in the laboratories for decay. When the radioactivity has decayed to background levels, the wastes are transferred to the Environmental Health and Safety Office for disposal as non-radioactive hazardous waste.

On April 18, 2007 and on November 13, 2007 the Radiation Safety Office coordinated low level radioactive waste shipments, totaling 38 drums of Liquid Scintillation Vials (LSV) and other long half-life low level liquid mixed waste for disposal. The total volume of the shipments was 152.38 cubic feet, the total weight was 5520 lbs., and the total activity shipped was 8.74 mCi, of which 6.26 mCi was tritium ($^3$H), 2.05 mCi was $^{14}$C, and 0.43 mCi was other isotopes.

On February 20, 2008 the RSO shipped a total of ninety-seven (97) drums of Dry Active Waste (two 55-gallon metal drums, fifty 30-gallon metal drums and forty-one 30 gallon plastic drums) for disposal by Envirocure of Utah via GTS Duratek Super-Compaction. The same day, the Radiation Safety Office shipped a total of four (4) fiber drums of solid animal carcasses for incineration at Envirocure of Utah. The total volume of animal carcasses shipped was 16.07 cubic feet, weighing 160 pounds and containing 74.1 mCi of Tritium ($^3$H). The total volume of the dry shipment was 269.2 cubic feet, weighing 3,812 pounds. The total activity shipped was 205.50 mCi, of which 201.7 mCi was Tritium ($^3$H), 3.86 mCi was Carbon-14. The waste originated from several locations, including the New York State Psychiatric Institute Building #5 and the P&S Building.


**Personnel Dosimetry, Bioassay and Area Monitoring**

In accordance with regulatory requirements, the Radiation Safety Office operates an ALARA Program to ensure that the radiation doses resulting from operations at CUMC/NYPH/NYSPI are both within the legal limits and kept “As Low As Reasonably Achievable.” The principal methods of monitoring radiation dose include the assignment of personnel radiation dosimeters to individuals, posting of area and environmental dosimeters, and monitoring of all discharges of radioactive materials.

Immediate action is taken, as appropriate, in response to unusual or high dosimeter readings. Quarterly ALARA reports are prepared and submitted to the Joint Radiation Safety Committee. These reports present the following: a) the doses of individual workers that exceeded ALARA I limits; b) summaries of investigations of doses to individual workers that exceeded ALARA II limits; and c) discussions of trends within departments that have historically experienced high individual doses. The Quarterly Environmental ALARA report presents the quantities of radionuclides discharged to the atmosphere and the sewer system and the resulting dose to the general public.

In the past year all doses to individual workers were less than the legal annual reportable limits as specified in RCNY Article 175, Radiation Control. All doses to the general public resulting from atmospheric discharges of radionuclides were less than the USNRC constraint limit of 10 mrem per year.

Activities performed in the past year to maintain the
ALARA Program included:

The Radiation Safety Office distributed approximately 10,000 personnel radiation dosimeters each quarter, including both monthly and quarterly badges. A total of approximately 40,000 dosimeters were distributed and collected in 2007. To maintain dosimetry records, the Radiation Safety Office uses dedicated computers with internet and direct modem access to the database of the dosimeter supplier, Landauer Inc.

The Radiation Safety Office received Annual Occupational Exposure Reports (NRC Form 5) from Landauer Inc. for the year 2006 and reviewed and forwarded these reports to radiation workers as required by the New York City Department of Health regulations.

The Radiation Safety Office notified 89 employees with ALARA Level I readings and investigated 25 cases of ALARA Level II readings as reported by Landauer Inc. Particular attention was paid to occupational groups that typically exceed the ALARA limits, i.e., workers and researchers at the Cyclotron Facility, Angiography, the Cardiac Cath Lab, and physicians in the PET Suite.

The Radiation Safety Office performed 45 bioassays on radiation workers who use radioactive iodine or handle greater than 10 mCi of $^3$H or $^{32}$P.

The Radiation Safety Office provided all workers who had declared pregnancy with health physics counseling concerning the risk factors of exposure to radioactivity. Also, additional monitoring of the fetus during the gestation period was provided, and personnel radiation exposure reports were closely followed. The work environments were evaluated and modified if necessary.

**Routine Radiation Safety Compliance – Internal Inspections, Audits and Surveys**

A major activity of the Radiation Safety Office is the performance of facility inspections and audits of records at approved clinical departments and research laboratories in order to ensure compliance with regulatory requirements as well as with the guidelines and policies of the Joint Radiation Safety Committee.

Routine internal compliance activities conducted in 2007 included:

**Quarterly inventories for sealed sources:**

The Radiation Safety Office conducted quarterly inventories for sealed sources located in the following departments: Nuclear Cardiology, Nuclear Medicine-Milstein, Nuclear Medicine-Allen Pavilion, PET Imaging Suite, Cyclotron Radiopharmacy, Radioligand Laboratory and Kreitchman PET Center.

**Quarterly inspections and audits of clinical facilities:**

The Radiation Safety Office conducted all quarterly inspections and audits of CUMC and NYPH clinical facilities using radioactive materials. The audits and inspections are to ensure compliance with City of New York Radioactive Materials License conditions and with RCNY Article 175, Radiation Control. The facilities audited include: New York Presbyterian Hospital Nuclear Cardiology, Nuclear Medicine, Allen Pavilion, Nuclear Cardiology, and Allen Nuclear Medicine, PET Imaging Suite, Cyclotron Radiopharmacy, and Radioligand Laboratory.

**Quarterly inventory and biannual leak testing:**

Quarterly inventory and biannual leak testing was performed for all radioactive sources located in the following facilities: Milstein Hospital Nuclear Medicine and Nuclear Cardiology, Kreitchman PET Suite, Radioligand and Cyclotron facilities, Allen Pavilion Nuclear Medicine, and CUMC laboratories. Leak Test Certificates were generated and issued. All sealed sources were found to be in compliance with RCNY Article 175 regulations.

**Unannounced laboratory inspections:**

The Radiation Safety Office conducts unannounced laboratory inspections for the purpose of reinforcing CUMC policies prohibiting food in labs and requiring the locking of rooms containing radioisotopes when unattended. Radiation Safety Office personnel walked through all research areas including Irving Cancer Research Center, Russ Berrie Building, College of Physicians & Surgeons, Black Building, Hammer Health Sciences Center, Kolbe Building, and the New York State Psychiatric Institute. Violations if observed are documented by digital photography. Each of the labs found in violation were contacted by the Radiation Safety Office to determine corrective actions. The RSO follows up to ensure that all deficiencies are corrected to comply with regulations. The concern for compliance with regulations pertaining to food in the labs and keeping labs locked when not in use, will be emphasized in the monthly training and refresher courses that the RSO gives to radiation users.

**Annual Spill Gas Clearance Time measurement – room pressure:**

On April 27, 2007, during the annual Spill Gas Clearance Time measurement in the Milstein Hospital Nuclear Medicine Department it was discovered that room 3-219 demonstrated positive room pressure. The Nuclear Medicine Department was immediately informed that any procedures requiring aerosols were not to be performed until the room air pressure was corrected and verified by the Radiation Safety Office. Signs were posted inside and outside the room. The next day, facilities adjusted the air flow to bring the room to negative pressure with respect to the surrounding rooms. This was verified by the RSO on April 27, 2007 and regular procedures were resumed. The RSO will be testing the room pressure during quarterly audits to ensure that the rooms maintain negative pressure with respect to their surroundings.

**Routine statistics:**

846 routine radiation safety inspections and audits were performed in Columbia University Medical Center and New York State Psychiatric Institute research laboratories. The results were communicated to the Responsible Investigators. A total of 39 labs were cited with deficiencies, and these followed up to ensure compliance.

155 equipment clearance and laboratory exit/entry surveys were performed.

Airflow rates were measured in 94 fume hoods in areas where volatile radioactive materials are used. In all rooms where radioactive gases or aerosols are used, ventilation rates were measured, and spill gas clearance times were calculated and posted. Adjustments were made as required to
air supply and exhaust systems to obtain negative pressure conditions. Researchers whose hoods did not meet safe flow rate standards were instructed to have their hoods repaired or replaced. Follow-up audits confirmed that corrective actions were taken.

91 live animal and carcass surveys were performed, in order to identify potential contamination in animal facilities and cages, protect animal care staff, and ensure proper disposal of animal carcasses containing radioactivity.

Calibration and maintenance services were provided for 190 radiation survey instruments used throughout CUMC/NYPH/NYSPI. The Radiation Safety Office maintains a supply of portable survey instruments available for loan to Responsible Investigators and in case of emergency.

The Radiation Safety Office conducted surveys of 43 inpatients and outpatients treated with $^{131}I$ by the Nuclear Medicine Department and 6 surveys of patients treated with $^{137}Cs$ or $^{192}Ir$ implants by the Radiation Oncology department in 2007.

In 2007 the Radiation Safety Office interviewed 35 outpatients who were being considered for treatment with Iodine-131 for cancer of the thyroid by the Department of Nuclear Medicine. Records of interviews are on file in the Radiation Safety Office.

**Radiation Safety Training**

Pursuant to Article 175 of the New York City Health Code, the Radiation Safety Office provides initial radiation safety training to all new employees of CUMC/NYPH/NYSPI prior to their beginning work with radiation equipment or radioactive materials. The Radiation Safety Office then provides annual refresher training as well. The Radiation Safety Office also provides training in the general area of Emergency Response Preparedness as prescribed by the Joint Radiation Safety Committee. The following radiation safety courses and training sessions were presented during 2007:

- 12 initial training sessions for individual researchers
- 12 annual refresher sessions for researchers
- Annual refresher sessions for Nuclear Cardiology, Nuclear Medicine, PET Suite, Cyclotron, Radiation Oncology.
- 12 sessions for the Nursing Staff of NYPH
- Training sessions for Dental School residents and assistants
- Training sessions for all clinical departments, such as Radiology, Anesthesiology, Cardiology, Urology, Pathology, PET Suite and Oncology.
- Training sessions for the Facilities Department.
- Training for Public Safety and Security Departments

The Radiation Safety Office conducted four special training sessions for the new animal irradiator located in Irving Cancer Research Center. Researchers were trained in operation of the irradiator, safety precautions, and animal handling protocol.

For employees who could not immediately attend the regularly scheduled classes, the RSO administers a self-study program including the use of videotapes available at the Health Sciences Library. A passing grade on the online quiz (www.rascal.columbia.edu) after viewing the video qualifies an employee working in Non-Human Use applications to be issued a radiation monitoring badge. If the individual’s employment involves human use of radioactive material, a passing grade on the quiz results in obtaining a temporary badge until the next regularly scheduled new employee training session. Individuals who complete their training online must provide a copy of their training certificate to the Radiation Safety Office prior to being issued a personal radiation dosimeter. The RSO sends email reminders to all Responsible Investigators and radiation workers that they must attend a refresher course.

**Health and Safety Awareness Fair:**

On November 7, 2007 the Radiation Safety Office participated in the Health and Safety Awareness Fair sponsored by the NYP/Columbia Center and Morgan Stanley Children’s Hospital of New York (MS CHONY) Emergency Preparedness Committee. Approximately 400 people attended the fair, many of whom visited the Radiation Safety Office table where information was available regarding public safety in the event of a dispersal of radioactive materials and the use of radiation detection equipment was demonstrated.

**Medical Health Physics Professional Support Services**

The Radiation Safety Office provides professional radiation safety and health physics consultation to clinical departments, research laboratories, Authorized Users, and Responsible Investigators throughout CUMC/NYPH/NYSPI in order to ensure compliance with regulatory requirements and the ALARA program.

Specific examples of professional support provided by the Radiation Safety Office in 2007 include:

- The Radiation Safety Office investigates spills, misadministrations, and other incidents involving radioactive materials and other sources of radiation. The Radiation Safety Office ensures that, when required, timely notice of reportable incidents is made to the New York City Department of Health, Office of Radiological Health. The Radiation Safety Office responded to a number of incidents in 2007 which are on file in the Radiation Safety Office and may also be found in the Radiation Safety Office’s quarterly reports to the JRSC.

- The Radiation Safety Office provides continuing radiation safety support for the Columbia University Cyclotron Facility and the Columbia University Radioligand Laboratory for production and synthesis of PET imaging radiopharmaceuticals. This support includes maintenance of licenses and permits, basic radiation safety services, personnel dosimetry, area radiation monitoring and quantitative measurement and ALARA analysis of radiisotope releases to the atmosphere, review of Authorized User credentials, and review of system modifications.

- Other examples of professional support provided by the Radiation Safety Office include:

  **GE Lightspeed RT CT simulator:**

  The Radiation Safety Office continued to provided Medical Health Physics and Radiation Safety Support to the Department of Radiation Oncology with regard to radiation
shielding recommendations for the conversion of the former Cobalt-60 Room to a CT Simulator Room. On December 4, 2006, RSO professional staff, in conjunction with Cheng-Shie Wuu, Ph.D. of the Department of Radiation Oncology and Zheng Lu, Ph.D., of Radiology Medical Physics, assisted in performing the NYC DOHMH required radiation safety survey and medical physics acceptance testing for the newly installed GE Lightspeed RT CT Simulator. The survey was performed using a pressurized ionization survey instrument. There is a New York City Department of Health & Mental Hygiene regulatory requirement that such surveys be performed by a “qualified expert” certified in the appropriate field (RCNY 175.54(b)) and a New York State Department of Education requirement that limits the performance of appropriate radiation measurements for diagnostic and therapeutic facilities to a New York State licensed professional medical physicist. (Education Law, Article 166, Section 8701(2)(b))

On December 21, 2006, the Radiation Safety Office issued the completed Radiation Safety Survey For: GE Light Speed RT CT/Simulator Tunnel Level Room 15 CHONY. The workload corrected radiation survey revealed that the facility met the requirements of Regulations of the City of New York, Article 175, Radiation Control, Standards for protection against radiation. The regulations require that each licensee conduct operations so that the maximum radiation dose to an individual member of the public does not exceed 100 mrem in a year and 2 mrem in any one hour (175.03(d)(1)) and the occupational dose to any individual adult does not exceed a total effective dose equivalent of 5 rem in one year (175.03(c)(1)). A recommendation was made that staff should not occupy the entrance maze to the room during the operation of the Simulator.

**Integrated Imaging Center:**

During the month of February 2007, the Radiation Safety Office committed a significant amount of professional staff time to the performance of radiation shielding calculations and the drafting of radiation shielding recommendations for the CUMC Integrated Imaging Center project. There is a New York City Department of Health & Mental Hygiene regulatory requirement that radiation shielding design for diagnostic installations be performed by a “qualified expert” certified in the appropriate field (RCNY 175.54(a)) and a New York State Department of Education requirement that limits radiation shielding design for diagnostic and therapeutic facilities to a New York State licensed professional medical physicist. (Education Law, Article 166, Section 8702(1))

Radiation Safety Office professional staff, with the invaluable assistance of Peter D. Esser, Ph.D., D.A.B.R., Professor of Clinical Radiology & Chief Physicist, Kreitchman PET Center, provided the CUMC Facilities Department and the project architect with radiation shielding recommendations for: PET and PET/CT Scanning Rooms; the hot lab; the pneumatic system for transporting radiopharmaceuticals; patient administration rooms and toilets; reception areas. Specific recommendations were made with regard for CT X-ray facilities. The recommendations were made for facility workloads provided to the Radiation Safety Office.

On April 13, 2007 a substantial redesign of the First Floor Imaging Area layout was submitted to the Radiation Safety Office for estimation of the new radiation shielding requirements. Professional review required the calculation of the radiation dose contribution from eleven (11) source terms at forty-five (45) locations and the effect(s) of the installation of standard thicknesses of concrete and lead shielding between the source terms and the locations of interest. The radiation dose contribution from a redesigned pneumatic-tube dose distribution system was calculated. Recommendations were made regarding x-ray equipment requirements. On May 14, 2007 the completed Draft 4 Shielding Estimate, CUMC Integrated Imaging Facility was submitted to CUMC Facilities management, the project Architect, and Peter Esser, Ph.D., for review, and it has since been adopted for construction.

A Radiation Safety Office representative continues to attend weekly construction meetings held to review the ongoing construction of CUMC Integrated Imaging Facility (NYSTAR) in order to provide professional medical health physics support for the project. The meeting is attended by representatives of: CUMC Facilities Management, CUMC Kreitchman PET Center, J.A. Jennings and project contractors. Issues recently reviewed by the Radiation Safety Office have included: the height of the radioactive exhaust system stack, the specifications provided in the contract for lead shielding in doors and walls, and the proposed use of certain radiochemistry laboratories.

The Radiation Safety Office provided on-going extensive investigation of options and the presentation information regarding the purchase of a new radionuclide exhaust stack monitoring system versus the relocation of the existing system.

The Radiation Safety Office reviewed submittals of lead shielding installations for the new PET imaging center provided by Radiation Shielding Systems, Inc., for compliance with specifications for shielding previously provided in a Shielding Report, dated May 17, 2007 and prepared by Thomas W. Juchnewicz, M.S., D.A.B.R. and Peter Esser, Ph.D., D.A.B.R. The Radiation Shielding submittal was returned for correction.

The Radiation Safety Office has provided specifications for a radiation detector control system to activate the planned Matrix Mechanical bubble damper valve to be installed in the new cyclotron vault exhaust system. The specification references “off-the-rack” radiation instrumentation manufactured by Ludlum Measurements, Inc., to interface with the Section 15150 Automatic Control Systems Air Handling Unit.

**SPECT/CT unit in Nuclear Cardiology:**

On March 5, 2007, a radiation safety survey was performed in the rooms surrounding the new SPECT/CT unit in Nuclear Cardiology (MHB-2-020). RSO staff measured exposure rates. Lead shielding was added underneath one door to the SPECT/CT room as requested by the RSO. All calculated accumulated annual doses surrounding the SPECT/CT room were below 500 mrem/yr.

**Human Use application reviews:**

The Radiation Safety Office received scores of
JRSC/RDRC Applications for Use of Radioactive Agents in Research Involving Human Subjects and for Use of X-ray Equipment in Research Involving Human Subjects. In addition to reviewing research protocols for accuracy and completeness of human radiation dosimetry and subject consent statements for appropriateness and accuracy with regard to radiation risk, and reporting to the JRSC/RDRC. Radiation Safety Office professional staff have been consulted by researchers with regard to references and materials providing information on dosimetry, including: medical internal radiation dosimetry for radiopharmaceuticals; entrance skin exposures, organ doses, equivalent doses or CTDIs for typical diagnostic studies; and guidance on the correct uses of committed dose, effective dose and organ weighting factors. The Radiation Safety Office reviews are forwarded to the Chairman, JRSC/RDRC. The reviews are performed by NYS Licensed and Board Certified professional staff. Full reviews of the Applications for Use of Radioactive Agents in Research Involving Human Subjects and for Use of X-ray Equipment in Research Involving Human Subjects were subsequently performed on Applications by Salmen Loksen, Director, Radiation Safety Office, as a member of the Executive Committee of the JRSC. Mr. Loksen’s approval or disapproval of the protocol is reported to the Executive Committee.

Standard approaches to a written description of radiation risk:

In a note to Dr. Brenner dated June 21, 2007 Dr. Philip Alderson expressed concern with regard to the radiation risk consent form statements. Dr. Alderson recommended that investigators should receive a memo listing preferred standard approaches to a written description of radiation risk. Such standardization would add consistency to CUMC/NYPH/NYSPI consent forms.

Yorktown Heights facility:

In April 2007, the Radiation Safety Office was requested to provide assistance to the CUMC Division of Cardiology, Center for Interventional Vascular Therapy, in preparing a Radioactive Materials License Application to the New York State Department of Health in Albany, New York for authorization to operate a diagnostic nuclear cardiology facility in Yorktown Heights, New York.

Radiation Safety Office professional staff reviewed the draft application prepared by the Center for Interventional Vascular Therapy with assistance from their equipment vendor, DIS New York LLC (a subsidiary of DIGIRAD, St. Simon’s Island, Georgia) and prepared and sent a number of email memoranda with critiques of the application and a model draft application based on New York State Department of Health Licensing Guide 10.1.

On May 17, 2007, Radiation Safety Office professional staff met with Richard Gemmings, COO, Center for Interventional Vascular Therapy, Paul Early, V.P., Radiation Safety Officer, DIGIRAD, and other DIGIRAD staff to review progress with the application for the Center for Interventional Vascular Therapy. DIGIRAD’s progress in obtaining NYS DOH authorization for its nuclear medicine contract operations in New York State were also discussed. The Radiation Safety Office informed DIGIRAD of the New York State requirement that medical health physics and nuclear medicine physics functions may only be performed by a physicist licensed to practice in the State of New York. The RSO assisted with preparation of a final draft of the Center for Interventional Vascular Therapy’s New York State Radioactive Materials License Application.

On August 10, 2007 the Center for Interventional Vascular Therapy in Yorktown Heights, NY, received a letter from New York State Department of Health stating that it had completed the review of the application for a NYS DOH Radioactive Materials License No. 07-376 which allows possession of radioactive materials. The letter stated that the License “will not be issued until radiation safety equipment (survey meter, dose calibrator, etc.) is on site and you are ready to begin performing nuclear cardiology procedures.” The facility was instructed to notify NYS DOH in writing seven days before they are ready to begin operations. Upon receipt of this notification the Department will issue the Radioactive Materials License.

Orthovoltage X-ray unit survey:

A final Radiation Safety Survey was performed in the vicinity of the animal Orthovoltage x-ray unit in VC 11 Room 208. Construction of the new lab adjacent to the x-ray unit’s room is complete. All survey readings corresponded to radiation doses below 100 mrem per year.

Medical Physics work permits:

In accordance with the requirements of the NYS Education Law the Radiation Safety Office has applied for Medical Physics Work Permits for five RSO staff members to authorize them to perform necessary Medical Physics support work under the supervision of Licensed Professional Medical Physicists on staff.

Outside consulting medical physicists:

At the Quarterly Meeting of the Joint Radiation Safety Committee held June 12, 2007, there was extended discussion with regard to work at the medical center performed by outside consulting medical physicists and with regard to ongoing equipment QA programs set up by these physicists. The issue was raised by Peter Esser, Ph.D., with regard to ensuring the optimum radiation safety of patients. Variability in the quality of services provided by outside consulting medical physicists was noted. David J. Brenner, Ph.D., Chairman, JRSC stated that the credentials of, and the work performed by, outside consulting physicists must be reviewed and approved and that a memo should be sent to all appropriate department heads reminding them that if they bring in outside consulting medical physicists, they need to do so in collaboration with the Radiation Safety Office.

Radiation Safety Office professional staff has assisted Dr. Esser with the preparation of a draft policy and procedure to govern the employment of outside consulting medical physicists and the ongoing QA programs they may establish. Radiation Safety Office professional staff have specifically advised Dr. Esser regarding: NYC DOHMH regulatory requirements for the board certification of physicists performing radiation shielding design, radiation safety surveys and performing or reviewing certain QA tests; New York State Education Law requirements for the professional licensing of medical physicists performing work at diagnos-
tic or therapeutic facilities; and guidelines for the ethical practice of medical physics issued by the American Association of Physicists in Medicine (AAPM), the professional organization of medical physicists. At the September 25, 2007 meeting Dr. Brenner requested Dr. Esser to have a final draft version of the consulting medical physicist policy available for the review and approval of the Committee by the next quarterly meeting of the JRSC. The policy and procedure for hiring consulting medical physicists was voted on and approved by vote of the Committee.

Dae-In Kim, Authorized User working in Cyclotron:

On September 17, 2007, Mike Agnello, Managing Radiopharmacist, Cyclotron Radiopharmacy, informed Salmen Loksen, Director, Radiation Safety Office and Ronald Van Heertum, M.D. Director, Kreitchman PET Center, that a Cyclotron Radiopharmacy Operator/Engineer had given notice and requested that Dae-In Kim of the Radiation Safety Office be loaned to the Cyclotron Radiopharmacy until other staff could be qualified as operators and added as Authorized Users on the License. Agreement has been reached to allow Dae-In Kim to work temporarily, part-time, in the Cyclotron Radiopharmacy.

Varian Trilogy TX 2300 clinical linear accelerator:

On September 19, 2007, at the request of the Department of Radiation Oncology, professional staff from the Radiation Safety Office supervised and conducted a preliminary radiation safety survey in the vicinity of Tunnel Level Room 11, in which Varian service engineers are in the process of installing a Varian Trilogy TX 2300 clinical linear accelerator. The preliminary safety survey is limited to determining the temporary safe installation environment. RCNY 175.64(e) requires a formal protection survey by an appropriately Board-certified physicist in accordance with the requirements of RCNY 175.64(g), prior to the facility being placed into operation to treat patients. The treatment room formerly housed a Varian 2100CD clinical linear accelerator and the existing treatment room shielding had previously been evaluated by Radiation Safety Office professional staff as to its suitability for installation of the Trilogy 2300.

On September 19, 2007 measurements of X-ray and neutron radiation were taken for several machine orientations at a number of controlled and uncontrolled locations. The occupational and general public radiation dose calculated for all locations, at the installation workload provided, were well below the occupational and general public dose limits found in RCNY 175.03(c) & (d). The report of the Radiation Safety Survey was submitted to the NYC DOHMH as part of an amendment of City of New York Therapeutic Radiation Linac Unit Certified Registration No. 77-0000019 to remove the Varian Associates Clinac 2100C/D listed on the Registration and authorize replacement by a Varian Associates Trilogy 2300TX. On December 19, 2007 the Radiation Safety Office submitted to the New York City Department of Health and Mental Hygiene the completed Amendment Application for Therapeutic Radiation Linac Unit Certified Registration No. 77-0000019. On March 3, 2008, the Radiation Safety Office received Amendment No. 6 to the Registration. Registration No. 77-0000019 expires August 31, 2008. The Trilogy 2300TX has since been placed into patient service.

Patient support:

The Radiation Safety Office conducted surveys of patients treated with I-131 by the Nuclear Medicine Department, and interviewed out-patients who were being considered for treatment with Iodine-131 for cancer of the thyroid by the Department of Nuclear Medicine. All interviewees were treated as outpatients. Records of these surveys and interviews are on file in the Radiation Safety Office.

The Radiation Safety Office conducted surveys of patients treated with Cs-137 implants and surveys for Ir-192 implants by the Radiation Oncology Department.

The Radiation Safety Office has begun to provide surveys of patients involved in the procedure for the protocol titled “Phase II open-labeled, multiple dose study of intracavitary administered 131I-TM-601 in adult patients with recurrent high grade glioma.” On September 14, 2007 the first survey of this type was performed. This protocol consists of an injection of 1 mCi of 111Indium-DTPA into a VAD reservoir which leads to a surgically created resection cavity. This injection is followed by a 10 minute SPECT acquisition of the brain. After the scan there is an administration of 40 mCi of I-131-TM-601 into the same cavity. This procedure is performed at 1 week intervals for either three or six weeks.

Radiation Safety and Technical Support Services

Cyclotron decommission:

As reported at the December 13, 2006 Quarterly Meeting of the Joint Radiation Safety Committee, at the Quarterly Meeting of the Joint Radiation Safety Committee held on June 21, 2006, a quorum of the Committee voted that the Radiation Safety Office estimated the quantity of radioactivity present and the cost to decommission the existing CUMC RDS-112 Cyclotron Facility. At the March 7, 2007 Quarterly Meeting of the PET Sub-Committee Radiation Safety presented for the review of the Sub-Committee a final draft of an environmental radiological sampling protocol, a list of six (6) potential vendors, and a proposal cover letter. The PET Sub-Committee directed the Radiation Safety Office to submit the final draft documents to the review and approval of Patrick J. Burke III, Director, CUMC Facilities Management, prior to distributing the proposal to vendors to solicit bids.

The issue of decommissioning was raised in inquiries made by Alice Gleason, Deputy Controller, as to the cost of disposal of radioactive materials and the decommissioning and remediation of facilities at CUMC that utilized or housed radioactive materials. The issue of decommissioning was independently raised with regard to the CUMC–PETNET separation agreement.

Nordion Gamma Cell 40 relocation (IC):

The Center for Radiological Research internally relocated its Nordion Gamma Cell 40, a small-animal and cell irradiator, as part of the laboratory renovation. On January 5, 2007, under the supervision and with the assistance of a Service Engineer provided by the manufacturer of the Gamma Cell 40, MDS Nordion, the irradiator was moved from its location in VC room 11-204 to VC room 11-202,
which was created by partitioning off part of Room 11-204. Following the relocation the Nordion Service Engineer serviced the Gamma Cell 40, verified normal function and took Fricke dosimeter measurements to generate an updated dose map that was provided to the Center for Radiological Research.

While the relocation was in progress Radiation Safety Office staff monitored ambient radiation levels in the vicinity of the Gamma Cell 40. Radiation Safety Office staff performed radiation surveys and assayed removable contamination wipes of the irradiator prior to and after the relocation. All radiation surveys and removable contamination wipes met the RCNY 175.04, Appendix D limits for radioactive surface contamination for release of material or facilities and the RCNY 175.03(e) limits for sealed sources.

Following the relocation the RSO performed a radiation safety survey of the relocated Gamma Cell 40 in VC Room 202 using a pressurized ionization survey instrument. This survey included adjacent rooms on the 11th Floor of the VC Building and Rooms above and below on the 12th and 11th Floors of the VC Building. The workload corrected radiation safety survey revealed that the facility met the requirements of Regulations of the City of New York, Article 175, Radiation Control, Standards for protection against radiation. The regulations require that each licensee conduct operations so that the maximum radiation dose to an individual member of the public does not exceed 100 mrem in a year and 2 mrem in any one hour (175.03(d)(1)) and the occupational dose to any individual adult does not exceed a total effective dose equivalent of 5 rem in one year (175.03(c)(1)).

The CUMC Nordion Gamma Cell 40 contained at the time of its relocation approximately 2000 Curies of Cesium 137. This activity is well in excess of the Table 1 value of 27 Curies Cesium 137 for the implementation of ‘Increased Controls’ under the Order of the Commissioner, dated November 30, 2005. The Radiation Safety Office communicated the regulatory requirements for increased controls to the CUMC Public Safety Department. The CUMC Public Safety Department provided security personnel to escort non-CUMC vendors, construction contractors and riggers that needed access to the VC Building 11th floor irradiator facility during demolition and construction and during the relocation of the irradiator.

Professional Radiation Safety and Medical Physics Support for Non-Radiology X-ray Activities

Dental QA program:
The dental quality assurance program is designed to optimize the radiological safety and clinical quality of dental radiography. This program is based on recommendations for quality assurance that have been promulgated by a number of professional organizations, including the National Council on Radiation Protection & Measurements (NCRP), the Bureau of Radiological Health of the Food & Drug Administration, the American College of Radiology (ACR), and the American Academy of Dental Radiology Quality Assurance Committee. In this program, the Radiation Safety Office has primary responsibility for preliminary radiation safety shielding evaluation, acceptance testing, diagnostic quality assurance, and radiation safety surveys on all dental x-ray units installed at the following locations:

- Morningside Dental Associates (2 locations): 9 intraoral units, and 1 panoramic/cephalographic unit
- Ambulatory Care Networked Corporation (ACNC): 4 intraoral units and 1 panoramic/cephalographic unit
- Babies Hospital OR: 1 portable intraoral unit
- Vanderbilt Clinic Teaching & Research Areas: 1 panoramic unit, 1 panoramic/cephalographic unit, 23 intraoral units, and 1 intraoral–cephalographic unit
- Dentcare Clinic (Intermediate School 183): 1 intraoral unit
- Columbia Eastside: 6 intraoral units and 1 panoramic/cephalographic unit
- Columbia North: 5 intraoral units and 1 panoramic unit
- Mobile Dental Facility: 2 intraoral units
- Mannie L. Wilson Health Care Center: 5 intraoral units and 1 panoramic unit
- Odyssey House Dental Clinic: 3 intraoral units
- Harlem Children’s Health Initiative Dental Facility: 1 intraoral unit.

X-ray permits audit program:
In agreement with the New York Presbyterian Hospital, the Joint Radiation Safety Committee has assigned the Radiation Safety Office responsibility for Radiation Safety and Medical Physics support for those clinical facilities outside the Department of Radiology that use x-ray equipment. The Radiation Safety Office and the Department of Radiology Medical Physics staff jointly run the audit program for these facilities. This program is conducted in accordance with the conditions of the CUMC/NYPH/NYSPI New York City X-ray Permits, as specified in Article 175 of the New York City Health Code. In this audit program, the Radiation Safety Office is primarily responsible for ensuring that each site follows the proper QA procedures, safety practices and keeps proper records, while the Department of Radiology Medical Physics is responsible for performing all technical tests. The following locations are audited under this program:

- Urology Department, Atchley 11th Floor: 1 fluoroscopy unit
- Endoscopy Department, Atchley 13th Floor: 3 C-arm fluoroscopy units
- Spine Center, Neurological Institute, 5th Fl: 1 C-arm unit
- Sports Medicine, Dodge Fitness Center/Bakers Field: 1 mini C-arm unit
- Cystoscopy Suite, Milstein 4th Floor: 3 radiographic/fluoroscopic units
- Cardiac Care, Milstein 5th Floor: 1 C-arm unit
- Pain Management, Presbyterian Hospital 5th Floor: 1 C-arm unit
- Harkness Pavilion 9th Floor: 4 bone densitometry units, 1 Xtreme CT.

In 2006, the Radiation Safety Office provided the following support for the above programs:
Radiation safety surveys and machine performance evaluations, in addition to the standard annual QA tests described above, were performed at the following locations:

- Facility of the Columbia University College of Dental
Medicine at VC 7-214C
- Harlem Children’s Health Initiative Dental Facility
- Odyssey House Dental Clinic.

NYC DOHMH biannual inspection: On January 15, 2008 the New York City Department of Health and Mental Hygiene began its biannual inspection of CUMC/NYPH/NYSPI X-Ray Permit No. H96 0076353 86. The inspection was concluded on January 29, 2008 with an exit interview in Dr. Alderson’s Office. On March 17, 2008 the Radiation Safety Office received the written report of the NYC DOHMH inspector dated February 14, 2008. The report stated that: “Radiological equipment inspection performed on 1/29/2008 disclosed No violations of Article 175 of NYC Health Code.” No recommendations were made.

Radiation Safety Office Personnel, Meetings, Communications, Professional Training, and Facilities

The Radiation Safety Office staff continues to attend regularly scheduled meetings with the departments of Environmental Health & Safety, Public Safety and Security, and Emergency Room staff to discuss and train for emergency response to any potential emergencies.

Monthly technical staff training meetings:

Monthly technical staff training meetings continue at the RSO. During these meetings, officers and technicians gather to discuss current issues in radiation safety.

EH&S cross-training sessions:

The Radiation Safety Office and the Office of Environmental Health & Safety conduct joint training sessions in order to review fume hood certification, increased controls for radioactive materials in quantities of concern, record keeping requirements and the CUMC mixed waste program.

IACUC animal care protocol review committee:

The Radiation Safety Office participates as a member of the IACUC Animal Care Protocol Review Committee by reviewing all procedures that utilize radionuclides in animal research and reviewing other animal protocols.

Meetings at Morningside Campus:

As recommended by Dr. David Brenner, Chairman, JRSC, the CUMC Radiation Safety Office is now attending Radiation Safety Committee meetings at the Morningside Campus, and vice versa. This participation is expected to continue.

NYPH safety committee meetings:

The Radiation Safety Office participates in monthly Safety Committee meetings of New York Presbyterian Hospital/Columbia and New York State Psychiatric Institute. In addition, the Radiation Safety Office participates in frequent Emergency Preparedness Sub-Committee meetings for NYPH and NYSPI.

NYPH-NYPrepares executive emergency preparedness coordinating council meetings:

The Radiation Safety Office participates in monthly meetings of the NYPrepares Executive Emergency Preparedness Coordinating Council of New York-Presbyterian. This is a combined committee of NYP/Columbia, NYP/Weill Cornell Medical Center, NYP/Allen and NYP/CHONY. In addition, the Radiation Safety Office participates in frequent Emergency Preparedness Sub-

Committee meetings.

Integrated Imaging Center meetings:

The Radiation Safety Office, starting in May, 2007, is attending weekly construction meetings regarding the CUMC Integrated Imaging Center.

HPS mid-year topical meeting:

From January 22, 2007 through January 26, 2007, a member of the Radiation Safety Office professional staff traveled to and attended the Health Physics Society Mid-Year Topical Meeting. The focus of the Mid-Year Meeting, held in Knoxville, Tennessee was the decommissioning and decontamination of radioactive materials facilities. In addition to attending the sessions and symposia on decommissioning, the RSO representative spoke with and obtained information from vendors marketing decommissioning services and radiation safety equipment and instrumentation.

FDNY unit drill:

On April 28, 2007, the RSO participated in a Unit Drill at Health Sciences by the FDNY [Ladder 34 and Engines 67 and 84 and the Hazardous Materials (HazMat) Division], in conjunction with the EH&S and CUMC Public Safety and NYPH Security Departments. Radiation Safety Office and EH&S gave presentations, followed by leading tours of the buildings. There was ample opportunity for questions and discussion. Lessons were learned with regard to biological, chemical, radioactive, and other hazardous materials, as well as with regard to laboratory safety, fire safety, and public safety. The FDNY appreciated the opportunity to familiarize themselves with CUMC facilities.

RCRA and DOT annual update and refresher training:

On July 25, 2007 four RSO staff members attended RCRA and DOT Annual Update and Refresher training presented by the Environmental Resource Center.

RDRC symposium:

On December 11, 2007 professional staff from the Radiation Safety Office attended a one day symposium regarding Radioactive Drug Research Committee regulation and guidance hosted at Memorial Sloan Kettering Cancer Center. The presentations were made by U.S. Food and Drug Administration senior management and the symposium was attended by representatives of approximately eleven North-Eastern RDRCs, including David Brenner, Ph.D., Chairman of RDRC No. 001, which is located at Columbia University Medical Center. Topics covered included: regulatory requirements for research protocols, record keeping and reporting; requirements for radiation and pharmaceutical dosimetry and on-line dosimetry resources available; and guidance regarding areas of RDRC and IND overlap such as “Exploratory INDs.”

Electronic web format:

In June, 2007 Dr. David Brenner, Chairman, Joint Radiation Safety Committee, requested that the Radiation Safety Office produce a report on putting all radiation safety business in electronic web format with an emphasis on JRSC online support and documentation. It is the intention of the RSO to work toward this goal, continuing from and building upon previous efforts in this direction.

In the 1998-1999 the Radiation Safety Office created a strategy document for implementing web-based training for
the radiation user community and others. Since that time, some, but not all, of this functionality has been implemented university-wide. With the support of CUMC management the RSO intends to develop its web-based service offerings and to incorporate online JRSC documentation and support. Additional functionality may include, but is not limited to:

- **Forms**: latest versions will be made downloadable, with online submission capability.
- As a companion to each form, detailed instructions to enable users to properly and completely fill out and submit the forms.
- **Reference material**, including city, state and federal regulations and regulatory guides.
- Relevant documents will be placed online, including dosimetry information.
- Links to relevant sites such as NRC, etc.
- History and current membership of the JRSC/RDRC.
- Internal JRSC and RDRC operational rules, regulations, and procedures; e.g., procedures for appointing new members, conflict of interest.
- List of sub-committees and current sub-committee members of the JRSC and RDRC.
- Online availability of Agendas and Minutes of past JRSC and RDRC meetings.
- Access to RASCAL protocol document submissions for internal use.

Some access and services will be available to any member of the CUMC/NYPH/NYSPI community; some access and services will be restricted to members of JRSC and/or RDRC.  

**RASCAL system access meetings:**

The Radiation Safety Office has participated in several meetings with regard to providing JRSC and RDRC members with access to the RASCAL system. This will improve and streamline the review and approval process for the increasing number of human use protocols that are being submitted.

**Website development:**

On September 17, 2007 the Radiation Safety Office hired Srinivasa Valluripalli, a graduate student at Columbia University’s Department of Computer Science, to create websites for the JRSC and RDRC, as well as to update and redesign the RSO website. This is in accordance with the RSO’s goal of placing all radiation safety business into electronic web format, with an emphasis on JRSC online support and documentation. A new and shorter URL address for the already significantly redesigned Radiation Safety Office website, [http://rso.cumc.columbia.edu](http://rso.cumc.columbia.edu), has been assigned, and new websites for the JRSC and RDRC are operational and under development at the URL [http://jrsc.cumc.columbia.edu](http://jrsc.cumc.columbia.edu) for the JRSC and [http://rdrc.cumc.columbia.edu](http://rdrc.cumc.columbia.edu) for the RDRC. These websites are running on CUMC’s PHP SKLAD server, where it is possible to provide various advanced database functions necessary for detailed online form submission, and in order to be able to program selective logon modules for JRSC and RDRC members to access confidential information pertinent to their responsibilities in their respective committees.

**Personnel:**

Personnel changes in the Radiation Safety Office in 2007 include that Jacob Kamen, Ph.D., NRRPT, CHP, who was an Assistant Radiation Safety Officer in the Radiation Safety Office since May 1, 2000, resigned his position at the end of June 2007.

In 2008 the Radiation Safety Office is looking forward to filling all vacant positions in order to provide the best possible safety and support services to the Columbia University Medical Center, New York Presbyterian Hospital and New York State Psychiatric Institute.

![Tom Juchnewicz while conducting a walkthrough of the new CUMC Integrated Imaging Center, which will house two Siemens RDS-111 Eclipse Cyclotrons, under construction in the lower floors of 722 West 168th Street, New York, NY.](image)

*(L–r)*: Pantea Kadkhodazadeh, Ahmad Hatami and Charles Geraghty at the Health and Safety Awareness Fair sponsored by the NYP/Columbia Center and Morgan Stanley Children’s Hospital of New York Emergency Preparedness Committee.
Professional Affiliations & Activities

AMUNDSON, SALLY A., Sc.D.
Member
National Council on Radiation Protection and Measurements (NCRP)
National Academies of Science Space Radiation Shielding Study
Radiation Research Society, Finance Committee, 2008 Annual Meeting Program Committee
2nd International Systems Radiation Biology Workshop, Program Committee
Reviewer
NIH Study Section: RFA-AI-07-013 (Medical Countermeasures to Restore Gastrointestinal Function after Radiation Exposure)
Bioinformatics
Cancer Research
International Journal of Radiation Biology
International Journal of Radiation Oncology, Biology and Physics
Mutation Research
Radiation Research, Associate Editor

BALAJEE, ADAYABALAM S., Ph.D.
Member
American Association for the Advancement of Science
Radiation Research Society
Indian Association of Radiation Biology
Reviewer
Advances in Space Radiation Research
Cancer Research
Clinical Cancer Research
Cancer Research Campaign, UK
Current Medicinal Chemistry
Eurekah Bioscience
Molecular Cancer Therapeutics
Medical Science Monitor
Nucleic Acids Research
Radiation Research
Cancer Epidemiology, Biomarkers and Prevention

BIGELOW, ALAN, Ph.D.
Member
American Physical Society
Radiation Research Society
Reviewer
Nuclear Instruments and Methods in Physics Research B
Student Mentoring
New York City Stuyvesant High School summer student apprenticeship

BRENNER, DAVID J., Ph.D., D.Sc.
Member
Columbia University Radiation Safety Committee, Chairperson
National Council on Radiation Protection and Measurements (NCRP)
International Congress on Radiation Research, Program Committee
TV and radio appearances on the topic of CT examinations
Joint Radiation Safety Committee, Chairperson; Radioactive Drug Research Committee, Chairperson
Editorial Work
Radiation and Environmental Biophysics, Assoc. Editor

CALAF, GLORIA M., Ph.D.
Adjunct Faculty
University of Tarapaca; Institute for Advanced Research, Arica, Chile, Full Professor
Member
Biology Society of Chile
Mastology Society of Chile
Chilean Society of Citology
Chilean Society of Cancer
New York Academy of Sciences
Tissue Culture Association
International Association of Breast Cancer Research
American Association of Cancer Research
Society of Experimental Biology and Medicine
Radiation Research Society
Student Mentoring
Politecnical University of Madrid, Spain, Ph.D. Advisor
Grant Reviewer
FONDECYT Grants, Chile
Manuscript Reviewer
British Journal of Cancer
Mutation Research
International Journal of Radiation Oncology Biology Physics
International Journal of Radiation Biology
Environmental Health and Perspectives
Gynecologic Oncology
Cancer Detection and Prevention
Radiation Research
Endocrinology
Molecular Medicine
Endocrine Related Cancer

GEARD, CHARLES R., Ph.D.
Member
Radiation Research Society
American Society of Therapeutic Radiology and Oncology (ASTRO)
Environmental Mutagen Society
Advisory Committee on Radiobiology, Brookhaven National Laboratory
Scientific Review Panels, NASA
Editorial Work
International Journal of Radiation Biology, Editorial Board
Reviewer
Mutation Research
Radiation Research
Mutagenesis

HALL, ERIC J., D.Phil., D.Sc., FACR, FRCR, FASTRO, FSRP
Member
Royal College of Radiology
British Institute of Radiology
American Board of Radiology, Radiotherapeutic Written-Test Committee
American Society of Therapeutic Radiology and Oncology (ASTRO)
Radiation Research Society, Past President
American Radium Society, Past President
National Council on Radiation Protection and Measurements, Committee 1, Emeritus Member

Editorial Work
Intl Journal of Radiation Oncology Biology Physics, Editorial Board
International Journal of Brachytherapy
International Journal of Radiation Biology
Radiation Research
Radiology

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Student Mentoring
Doctoral Student of Environmental Heath Sciences, Columbia University, School of Public Health
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Reviewer
Cancer Research
Chemical Research in Toxicology
Environmental Health Perspective

Environmental & Molecular Mutagenesis
Free Radical Biology and Medicine
International Journal of Radiation Biology, Physics & Medicine
Mutation Research
Proceedings of the National Academy of Sciences
Radiation Research
Toxicology

Editorial Work
Advances in Space Sciences, Section Editor
Journal of Radiation Research

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Cancer Research
Carcinogenesis
Oncogene
Molecular Carcinogenesis
Journal of Biological Chemistry
BBRC
Free Radicals Biology and Medicine
International Journal of Cancer
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American Association for the Advancement of Science, Elected fellow
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Genetics Society of America
Radiation Research Society, History Committee
Sigma Xi
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Journal of Cellular Physiology, Associate Editor

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International Journal of Radiation Biology
Radiation Research
Oncogene
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International Journal of Radiation Biology

SMILENOV, LUBOMIR, Ph.D.
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Cancer Research
European Journal of Gastroenterology & Hepatology
Molecular & Cellular Biochemistry
Molecular & Cellular Biology
Oncogene
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Cancer Letters
International Journal of Radiation Biology
Mutation Research
Radiation Research

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Publications


11. Calaf GM, Parra E and Garrido F. Cell proliferation and tumor formation induced by eserine, an acetylcholi-


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52. Yin Y. Nuclear PTEN at the center of the stage. *Oncogene (invited review) 2008.*