Poster Session I

Carcinogenesis and Genomic Instability

6:10 p.m. – 8:00 p.m.
Bayles and Willse Rooms
DNA double-strand break repair is attenuated in three-dimensional human lung epithelial cell culture exposed to ionizing radiation

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DNA damage and consequent mutations initiate the multi-step carcinogenic process. Most mechanistic studies of the DNA damage response in vitro have been performed in monolayer (2D) cultures. Although these systems provide useful and powerful information, 2D systems do not recapitulate the three-dimensional (3D) structural organization or functional differentiation of the cells in vivo. We have developed a novel human lung 3D model and have assessed cellular responses to DNA damage induced by low- and high-linear energy transfer (LET) ionizing radiation (IR). We provide evidence that the number of double-strand breaks (DSBs) formed in the 3D lung model, as measured by the number of EGFP-53BP1 foci formed, was linear with γ-radiation dose from 10 to 100 cGy but was not linear with high-LET radiation dose. Though the number of DSBs induced after low- and high-LET IR was comparable between 2D and 3D structures, DSBs were repaired with slower kinetics in 3D structures as compared to 2D. Furthermore, unlike 2D, a large fraction of DSBs induced by high-LET radiation persisted for several days in 3D structures and these persistent DSBs are found in the differentiated cells, providing mechanistic insight into the differential DSB repair in 2D and 3D cultures. Interestingly, in contrast to cells in 2D, cells in 3D structures were less sensitive to IR and displayed limited types of gross-chromosome aberrations, suggesting that cellular response to IR is attenuated in 3D human lung epithelial cell culture. Thus, this study will have important implications for assessing cancer risk due to radiation exposure.

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Microsatellite Mutations in Mouse Tissues Induced by Low and High LET Radiation

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Mismatch repair (MMR) is important for maintaining genome integrity and loss of MMR gives rise to microsatellite instability (MSI) and a strong predisposition to cancer. Radiation exposure has been shown to increase mutations in coding and non-coding microsatellite repeats and may thus contribute to gene inactivation and carcinogenesis. To determine the effect of radiation on microsatellites in the presence and absence of MMR, we exposed a cohort of $Msh2^+/+$ and $Msh2^{-/-}$ mice to $^{56}$Fe ions or $\gamma$-rays and calculated microsatellite mutation frequencies (MF) at different time points. Spontaneous MF was significantly higher in $Msh2^{-/-}$ mice as expected and varied for both genotypes by tissue type, corresponding to differences in cellular turnover rates ($buccal>colon>spleen>blood>liver>brain$). MF in $^{56}$Fe irradiated tissues 3 days after exposure increased in $Msh2^+/+$ mice in all tissues, but increased only in colon of $Msh2^{-/-}$ mice. Previous data from our lab showed a dose dependent increase in MF in $Msh2^{-/-}$ mice after 10 weeks, thus mutation response might be time dependent. The kinetics of mutation induction revealed dramatic differences between low and high LET radiation in wildtype mice; although both types of radiation increased mutations over time, this increase was apparent 3 days following $\gamma$-rays but was not observed until 12 months following $^{56}$Fe exposure. This data suggests that although both types of radiation produce MSI, the effect is immediate and persistent following low LET radiation, but is latent subsequent to high LET exposure. In summary, these experiments reveal that radiation exposure causes a statistically significant increase in MF in MMR proficient cells that is tissue dependent. Further, the MF in irradiated $Msh2^+/+$ tissues increased faster than background MF, suggesting ongoing MSI, which could increase carcinogenesis risk.
Telomeres are the natural ends of linear chromosomes. In order to maintain genomic stability, telomeric DNA ends must be protected from degradation and loss, as well as from end-joining events. In striking contrast, broken DNA ends, damaged for example by exposure to ionizing radiation, must be correctly and rapidly rejoined. Recent years have witnessed increasing evidence that these seemingly contradictory requirements trigger a highly integrated cellular response, and in the process have been redefining traditional roles for protein players more commonly regarded as acting in a particular pathway, or at a specific type of DNA end. Such observations return us to a fundamental question first recognized by Muller and McClintok, how does a cell know which end’s which? The sometimes surprising answers, such as we report here, have important implications in the study of not only telomeres, but also of DNA repair, cancer and aging.

We have shown that mammalian telomeric end-capping function requires proteins more commonly associated with repair; i.e., the non-homologous end-joining (NHEJ) protein complex, DNA-dependent protein kinase (DNA-PK) [1]. Our continued characterization of uncapped (as opposed to shortened) dysfunctional telomeres in cells deficient for the catalytic subunit of DNA-PK (DNA-PKcs) provided the first evidence that such uncapped telomeres are inappropriately detected and processed as DSBs, thus contributing to genomic instability and carcinogenesis, specifically murine mammary carcinoma following exposure to ionizing radiation (IR) [2].

Intrigued by the dynamics of the seemingly contradictory yet integrated cellular responses to the requisites of preserving telomere integrity while also efficiently repairing damaged DNA, we investigated roles of the telomeric PARP tankyrase 1 in telomere function and the DNA damage response following exposure to ionizing radiations. Utilizing siRNA knockdown of tankyrase 1 in human cells, we found its reduction resulted in increased levels of telomeric recombination, specifically telomere sister chromatid exchange (T-SCE), exclusively in telomerase negative backgrounds. Consistent with defective DNA damage response, we also observed increased sensitivity to ionizing radiation-induced cell killing, mutagenesis and chromosome aberrations. Most unexpected however, was the finding that tankyrase 1 depletion also led to rapid reduction of DNA-PKcs protein levels, while Ku86 and ATM levels remained unchanged; DNA-PKcs mRNA levels were also unaffected. We demonstrate that depletion of tankyrase 1 results in rapid DNA-PKcs proteasome-mediated degradation, likely explaining the associated radiosensitivity phenotype. Our results also suggest that the requirement of tankyrase 1 for DNA-PKcs protein stability reflects the necessity of PARP enzymatic activity (PARsylation). While reciprocal interactions between PARP1 and DNA-PKcs have been previously reported, we provide the first evidence to our knowledge for tankyrase 1 – DNA-PKcs interaction, and in so doing reveal a novel aspect of DNA-PKcs regulation [3].

Interestingly, PARP has also been linked to aging via a strong interaction with WRN, the protein mutated in the premature aging Werner syndrome, raising the possibility of tankyrase 1 involvement in aging, in addition to its involvement in the other age-related processes of telomere maintenance, and as we demonstrate here, DNA repair via interaction with DNA-PKcs. Consistent with this view, we found elevated telomeric recombination associated not only with tankyrase 1 depletion, but also with premature aging syndromes like Werners [4]. Our results continue to provide support for the functional significance of telomere and IR-induced DNA repair/damage response dependencies - and the importance of individual genetic susceptibility considerations in ionizing radiation-induced cancer risk models.

This research was supported by NASA grants NNJ04HD83G and NNX08AB65G.

References
Human Cell Neoplastic Transformation and Clustered DNA Damage Induced by HZE Particles and SPE Protons

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Human space travelers flying to low earth orbit and beyond can accumulate significant doses of ionizing radiation from exposure to HZE particles and high energy protons. In addition, significant doses from protons, in some cases acute, can be incurred in a relatively short period of time during solar particle events (SPEs) posing imminent health risk. Both types of exposures, low dose rate, and SPE exposures, have the potential for increasing the lifetime risk of cancer morbidity and mortality to exposed individuals, which is projected to occur many years after the original mission. Thus, understanding the biological consequences of multiple particle exposures is essential for assessing radiation risks of space travel. Our research focuses on understanding the effect in human cells exposed to various types of space radiation present at low earth orbit and in deep space at levels relevant to space environment. Using a megabase pair DNA model, we have shown that the yields of clustered DNA damages calculated per particle fluence increase with increasing linear energy transfer (LET) values of charged particles of different Z and same kinetic energy. This indicates a direct relation between the Z and the levels of clustered damage induction. At a cellular level, we have used a primary human cell neoplastic transformation model, in which irradiated cells acquire growth properties of cancer cells. Among HZE particles, C, O, Si, Ti and Fe are found in abundance in GCR radiation. In addition, charged particles—both protons and light ions—are increasingly used in tumor radiotherapy. We determined both clonogenic survival and transformation frequencies produced by exposure to low doses of 290 MeV/n C, 1000 MeV O, 600 MeV/n Si, 1000 MeV/n Ti or 1000 MeV/n Fe ions corresponding to ~1 or fewer ion traversals per cell nucleus. The dose responses of each beam for the transformation frequencies as a function of dose in cGy had similar slopes. However, fluence-based analysis revealed the relative transforming effect of each ion species. The slopes of the resulting dose-response function depended strongly on LET of the ion.

We next focused on how DNA damage clusters might play a mechanistic role in the transformation frequencies observed with iron ions, an abundant component in GCR radiation. Major DNA damages induced by low radiation doses are complex, multi-lesion clusters of strand breaks, oxidized bases and abasic sites. Because they are proposed to be repair-resistant and thus persistent, they present a potential cause of radiation-induced growth changes, killing, mutation, carcinogenesis, degenerative diseases and premature aging.

In addition to investigating risk and mechanism of HZE particle exposures, we studied the effects of medium and high energy protons which represent the major component of the GCR and SPE fluence spectrum. Compared to HZE particles, protons over a wide range of energies have LET values that are much more similar. We tested the transforming efficiency of protons of descending kinetic energies, 1GeV, 500 MeV, 250 MeV, 100 MeV and 50 MeV. On a per particle basis, we discovered that 50 MeV protons have the greatest potential for human cell transformation. This energy is one of the most abundant species in an SPE spectrum and therefore we subsequently chose it to study clonogenic survival, human cell transformation (presented separately) and the induction of clustered damage. Studies were performed using an SPE related dose rate of 1.65 cGy min⁻¹, and for comparison with a high dose rate of 33.3 cGy min⁻¹. A distinct effect of dose rate on the induction of clustered DNA damage was observed.

**This work is dedicated to the memory of Dr. Betsy Sutherland, a true pioneer in the field of Low Dose Radiation Studies, whose unfailing promotion and support of the NASA Space Radiation Laboratory is a continuing inspiration to all of us who were fortunate to have known and worked with her.

We thank Drs. Adam Rusek, Michael Sivertz and I-Hung Chiang for dosimetry and development of the low dose rate proton beams. We also thank Mr. James Jardine, Drs. Deborah Keszenman, Mamta Naidu and Stefan Tafrov for expert facility support.

Research was supported by grants from Exploration Systems Mission Directorate of the NASA division of Human Research in Radiation Health (NJ07HC73I) and the Low Radiation Dose Program of the Office of Biological and Environmental Research of the U.S. Department of Energy (BO-089) to BMS.
LOSS OF p15/Ink4b ACCOMPANIES TUMORIGENESIS TRIGGERED BY PARTICLE RADIATION

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Ions of high atomic number and energy (HZE particles) pose a potential cancer risk to astronauts on long-duration space missions. These ions are also being used for targeted cancer therapy in the clinic, although not much is known about the risk of secondary cancers from such charged particles. We previously demonstrated that DNA double-strand breaks (DSBs) induced by high-LET Fe ions are repaired slowly and incompletely, while those induced by low-LET gamma-rays are repaired efficiently by mammalian cells. To determine whether complex HZE-induced DSBs are more potently tumorigenic than gamma ray-induced breaks, we irradiated “pre-initiated” murine astrocytes that were deficient in Ink4a and Arf tumor suppressors and injected the surviving cells sub-cutaneously into nude mice. Using this model system, we find that Fe ions are potently tumorigenic, generating tumors with significantly higher frequency and shorter latency compared to tumors generated by gamma rays. Tumor formation by Fe-irradiated cells is accompanied by rampant genomic instability and multiple genomic changes, the most significant of which is loss of the p15/Ink4b tumor suppressor due to deletion of a chromosomal region harboring the CDKN2A and CDKN2B loci. The additional loss of p15/Ink4b in tumors derived from cells that are already deficient in p16/Ink4a bolsters the hypothesis that p15 plays an important role in tumor suppression, especially in the absence of p16. Indeed, we find that re-expression of p15 in tumor-derived cells significantly attenuates the tumorigenic potential of these cells, indicating that p15 loss may be a critical event in particle-induced tumorigenesis.
Carcinogenic Transformation and Neuroendocrine Differentiation Induced by Space Radiation in Human Prostate Cells

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Prostate cancer is the most often diagnosed type of cancer in male astronauts [NASA Longitudinal Study of Astronauts Health, 2004], as well as the most common cancer in men in the United States and the second leading cause of cancer deaths. The relationship between exposure to ionizing radiation, including space radiation, and the development of prostate cancer is not yet known. Several lines of evidence suggest that conversion of normal prostate or prostate cancer cells into cells secreting cell growth hormones, so called neuroendocrine cells, stimulates cancer development and/or progression. Such conversion was recently reported in prostate cells subjected to low doses and long exposures of low-LET ionizing radiation [Deng et al., 2008]. We postulate that prolonged exposure to radiation during space flights could increase the risk of prostate cancer development through two interconnected pathways: the direct transformation of prostate epithelial cells into cancer cells, or the differentiation into cancer-supporting neuroendocrine cells. Since neuroendocrine cells seem to play an important role in the development of other common types of solid tumors, such as breast, lung or colon cancers, the insight gained during this study should also be applicable to these diseases. The results obtained during the proposed studies will allow NASA to better estimate the cancer health risk(s) of space exploration, as well as provide clues that will allow the development of novel preventative measures to protect astronauts.

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Response of human hematopoietic stem and progenitor cells to energetic carbon ions

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BACKGROUND: The increasing application of heavy ions in radiotherapy and the growing interest in protracted
space travels are a strong motivation to expand the fundamental research in radiation biology. With respect to long
term effects in different cell systems, in the majority of studies the occurrence of chromosomal aberrations is
assessed, but only in lymphocytes. However, the investigation using hematopoietic stem and progenitor cells
(HSPC) which are responsible for the permanent renewal of all blood cells, seem to be more appropriate. Recent
results revealed a low relative biological efficiency of carbon ions (< 2). The observed differentiation pattern of the
progeny of exposed cells suggests that the normal lineage distribution of circulating blood cells may be altered after
irradiation. In addition, in the major part of surviving cells, radiation induced chromosomal aberrations were
transmitted clonally. In the following step, we set out to investigate the relevance of these results for the situation in
vivo and assessed possible changes in the differentiation pattern after xenotransplantation of irradiated human HSPC
into NOD/SCID mice. Based on the isolation of colonies formed by single lineage restricted progenitors we
investigated the transmission of chromosomal aberrations to the following generations.

METHODS: HSPC were G-CSF mobilized into the peripheral blood of healthy donors and positively selected for
the stem cell specific CD34 antigen. Irradiation of CD34+ cells was performed with different doses of X-rays [16
mA, 250 kV] or carbon ions [29 keV/µm monoenergetic beam], mimicking radiotherapy conditions. To investigate
radiobiological features immediately after exposure in vitro, the cells were cultured in a methylcellulose based 3D
culture system, where cytokines were supplemented (CFU assay). Two weeks later, the number of colonies formed
was enumerated and the clonogenic survival was determined. In addition, the differentiation potential of the lineage
restricted progenitors of the erythroid, granulocytic and monocyte-macrophage pathways were assessed. The
occurrence of chromosomal aberrations in the progeny of irradiated HSPC was investigated by isolation of single
colonies from the CFU assay. Metaphase spreads were prepared from each colony and analyzed after 24-colour
fluorescence in situ hybridization (mFISH). In addition, HSPC were irradiated with 1 Gy of X-rays or 1 Gy of
carbon ions and xenotransplanted into NOD/SCID mice (Epo, Berlin, Germany). The engraftment of the HSPC was
measured every 4 weeks by human specific DNA amplification (centromere-specific fragments of human
chromosome 17). After 14 weeks the mice were sacrificed and cells from blood, spleen and bone marrow were
analysed. In addition, a CFU assay was performed with the engrafted human bone marrow cells, the surviving
colonies were classified and the transmission of chromosomal aberrations to subsequent generations (isolation of
colonies from CFU-Assay) was investigated.

RESULTS: We observed in the descendants of exposed cells, which were cultured in vitro, an enrichment of cells of
the erythroid lineage and a depletion of cells of the granulocyte/macrophage lineages. The yield of chromosomal damage in the progeny
of exposed cells was not different comparing X-rays and carbon ions. Both types of radiation predominantly induced
reciprocal translocations, detected in 2/3 of all surviving colonies, whereas in 1/3 of the surviving colonies no
aberrations were observed. Remarkably, in all these clones chromosomal aberrations were transmitted clonally to
the progeny of the irradiated cells. In contrast, the analysis of HSPC bulk cultures by mFISH revealed a difference
between both radiation qualities, in that the fraction of complex-type aberrations was higher following carbon ion
compared to X-ray exposure. The relevance of the described results will be discussed in the light of our first results
on differentiation pattern and transmission of chromosomal aberrations obtained in engrafted HSPC that were
xenotransplanted into mice after radiation exposure. Interestingly, the engraftment potential, the haematologic
profile of the transplanted HSPC and other physiological effects (body weight and weight of the spleen of the mice)
were different after xenotransplantation with irradiated compared to control cells.
Silicon Ions Have a Significantly Higher Biological Effectiveness than Iron Ions for the Induction of Chromosome Damage in Human Lymphocytes

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Chromosome aberrations were measured in human peripheral blood lymphocytes after in vitro exposure to ²⁸Si– ions with energies ranging from 90 to 600 MeV/u, or to ⁵⁶Fe– ions with energies ranging from 200 to 5,000 MeV/u. The LET of the various Fe beams in this study ranged from 145 to 440 keV/µm and the LET of the Si ions ranged from 48 to 158 keV/µm. Doses delivered were in the 10- to 200-cGy range. Dose-response curves for chromosome exchanges in cells at first division after exposure, measured using fluorescence in situ hybridization (FISH) with whole-chromosome probes, were fitted with linear or linear-quadratic functions. The relative biological effectiveness (RBE) was estimated from the initial slope of the dose-response curve for chromosome damage with respect to γ-rays. The estimates of RBEmax values for total chromosome exchanges ranged from 4.4±0.4 to 31.5±2.6 for Fe ions, and 11.8±1.0 to 42.2±3.3 for Si ions. The highest RBEmax value for Fe ions was obtained with the 600-Mev/u beam, and the highest RBEmax value for Si ions was obtained with the 170-MeV/u beam. For both ions the RBEmax values increased with LET, reaching a maximum at about 180 keV/µm for Fe and about 100 keV/µm for Si, and decreasing with further increase in LET.
Modulation of Esophageal Epithelial Cell Behavior by Irradiated Stromal Fibroblast:
Effects of Radiation Quality

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The stromal microenvironment, composed of fibroblasts, vascular components, immune cells and extracellular matrix molecules, forms a supportive framework providing a rich source of biologically active compounds that control the normal growth and differentiation of an epithelial tissue. Likewise, in the context of a tumor, the microenvironment has a profound impact on the pathogenesis of the disease. Cross talk between the epithelial compartment and the stromal environment can influence many aspects of malignant progression, including tumor cell proliferation, migration, invasion and recruitment of new blood vessels. We are using hTERT-immortalized human esophageal epithelial cells and genetic variants grown in co-culture with esophageal stromal fibroblasts to study the effects of ionizing radiation on the microenvironment and intercellular communication as it relates to esophageal carcinogenesis [1]. We hypothesize that one path whereby radiation is affecting esophageal cancer development is through non-targeted mechanisms involving alterations in the stromal fibroblasts that drive preneoplastic changes in esophageal epithelial cells.

To test this hypothesis, we have initiated studies that evaluated how irradiation of stromal fibroblasts affected epithelial cell migration and invasion, behaviors associated with cancer promotion and progression. These studies were performed using both modified Boyden chamber assays and scratch wound healing assays where esophageal epithelial cells were exposed to conditioned media from irradiated esophageal stromal fibroblasts. Our results showed a dose-dependent increase in migration of epithelial cells when exposed to conditioned media from low-LET gamma irradiated vs. non-irradiated fibroblasts. We also observed enhanced invasion through a basement membrane matrix in similarly treated cells. Antibody-capture arrays and ELISAs were used to identify hepatocyte growth factor (HGF) and interleukin-8 (IL-8) as major secreted proteins in the conditioned media of irradiated fibroblasts. Both of these factors are known pro-angiogenic and pro-metastatic effectors. Wound healing assays using recombinant HGF and IL-8 confirmed the positive effect of these proteins on epithelial cell migration in this system.

Currently, the impact of radiation quality on these processes is being addressed using experimental samples derived from esophageal stromal fibroblasts that were exposed to 220 MeV/n proton and 250 MeV/n silicon ion beams during a run conducted at the NASA Space Radiation Laboratory (NSRL10A). Secretion of HGF and IL-8 will be quantified in these experimental samples and gene expression studies will also be used to identify additional alterations. These results should further our understanding of the mechanisms by which radiation impacts the tissue microenvironment and its role in cancer development.


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Dose-Response and Time-Course Expression of Cancer Cell Markers in Adult Human Fibroblast and Epithelial Cells Exposed to Light and Heavy Ions.

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By using biochemical, molecular, and live cell imaging techniques we have demonstrated that proton beam and iron ion radiations delivered to a variety of normal cells induce alterations in cell cycle parameters, produce persistent DNA damage, and induce alterations in cell morphology and division properties manifested by the fact that the irradiated cells become large multinucleated (polyploid) cells that are able to undergo multipolar division. The end result of successful multipolar division events is often the production of cell progenies able to maintain proliferative capacity. The fact that normal cells exposed to space radiation undergo MC but are able to escape death and originate cell progenies has great implications for the carcinogenic risk of exposure to these type of radiations. Delving into the molecular mechanisms that permit depolyplloidization in the irradiated cells, we have found that meiosis-specific proteins involved in chromosome pairing and cross-over, cohesion, meiotic recombination, DNA cleavage and repair (all phenomena that allow genetic redistribution), such as DMC1, SPO11, SYCP3, and γ-H2AX, are expressed in the irradiated cells at times that match the live cell imaging measurements of multipolar division events. Moreover, at later times post-irradiation, presumably in the progeny of the cells that has undergone successful multipolar divisions, the expression of the promoter of cell division cyclin B1, and of the cancer stem cell marker nestin, are also detected. Cyclin B1 is the regulatory subunit of the maturation-promoting factor (MPF) and, when phosphorylated by the MPF catalytic subunit cdc2, it plays a role both during meiosis and mitosis: it is responsible for meiotic maturation of oocytes and for the G2/M phase transition during the mitotic cell cycle. Nestin is a Class VI intermediate filament protein that regulates the assembly and disassembly of intermediate filaments in the developing central nervous system (CNS) and participates in cell remodeling. Nestin is both a marker for CNS cancer stem cells and an angiogenic marker of neovascularity of endothelial cells in tumors. These findings support the hypothesis that normal cells that have undergone space radiation-induced MC escape death by activating unusual pathways of cell division that utilize meiosis-specific proteins, and retain proliferative abilities by acquiring stem cell competence. Thus, it seems possible that silenced gametogenic programs are activated in cells exposed to charged particle radiation and that this switch may play a role in radiation carcinogenesis.

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Immediate and Delayed Cellular Effects of Single or Sequential Exposures to Low Doses of X-rays in Human Skin Fibroblasts

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INTRODUCTION

In space, humans may be exposed to low protracted and/or repetitive doses of ionizing radiation of different qualities. On Earth, analogous exposures may be experienced by patients submitted to fractionated radio therapeutic regimes or medical diagnostic procedures. The magnitude and mechanisms underlying the risks of development late health effects produced by this type of radiation exposures such as carcinogenesis, degenerative diseases and premature aging is a fertile ground for research. Using different biological endpoints to study the effects of dual exposures to sparsely and/or densely ionizing radiation, previous studies have shown adaptive responses, synergistic as well as additive interactions or even no interaction depending on interval between cell exposure, characteristic of radiation (quality, dose and dose rate) and the order of the radiation species in the case of exposures to different radiations [1, 2]. To study the cellular responses to low doses of sequential exposures to ionizing radiation, we have determined the effects on the survival probability and cell proliferation after single or sequential exposures to low doses of X-rays as immediate cellular responses. To evaluate late cellular effects we have assessed the induction of cellular transformation using cell anchorage-independent growth assay.

MATERIALS AND METHODS

Primary human fibroblasts initiated in our laboratory from neonatal foreskins were used as cell model system. Irradiations with X-rays were carried out using Philips RT-100 Therapy unit 100 kVp, LET 2 keV.µm⁻¹) at a dose rate of 11cGy.min⁻¹ monitored using a Radcal MDH meter (Model 1015, MDH Industries, Inc. Monrovia, CA). Subconfluent cell cultures were irradiated once (single exposure) trypsinized and split in two aliquots. One was used for immediate survival determinations. The other one was further cultured in a T25 flask, incubated for 48 h and then irradiated with the same dose. Each irradiation within the sequential treatment constituted a fraction of the total dose received by the cells after multiple sequential irradiations. Cells of the same passage were used as controls. Cell lethality was assessed by measuring the clonogenic survival immediately after each radiation treatment. Cell proliferation was determined through vital count using erytrosin-B exclusion 24 h after a single exposure to different doses of radiation. Cellular transformation assayed by the ability to form anchorage independent colonies in soft agar [2] was determined 14 days after single exposure to different radiation doses or after the multiple irradiation fractions.

RESULTS AND DISCUSSION

Cell survival as a function of dose to single exposure of X-rays at a moderate dose rate of 11cGy.min⁻¹ assayed immediately after treatment showed an increase above the control level at low doses (5 cGy ≤ D ≤ 20 cGy). Cell proliferation determined 24 h after single radiation exposure increased at low doses and it was progressively inhibited above 20 cGy. The clonogenic survival remained without important modifications after sequential fractions of X-rays even after a total delivered dose of 160 cGy achieved after 8 fractions of 20 cGy. Cells of the corresponding passage irradiated with single dose (2, 5, 20 cGy) used as a control showed similar response. However, the number of soft-agar transformats per survivor increased with the total dose after 8 fractions. These results suggest that DNA repair mechanisms may be processing potentially lethal damages ensuring survival. However, due to the increased cellular transformation frequencies observed, either these repair mechanisms are of low fidelity leading to the accumulation of misrepaired DNA damages (aging?) and/or there is an increasing induction of potentially mutagenic DNA damages due to progressive modifications of the intracellular oxidative metabolism.

* In memory of B.M. Sutherland, an untiring Radiation Biology Scientist.

REFERENCES


ACKNOWLEDGEMENTS

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SPACE RADIATION AND EMESIS STUDIES IN THE FERRET MODEL – PRELIMINARY RESULTS

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A major solar particle event (SPE) may place astronauts at significant risk for the acute radiation syndrome (ARS). The ARS may be exacerbated when combined with other injuries, resulting in compromise of crew health or the mission. As part of the NSBRI Center of Acute Radiation Research (CARR), the ferret animal model is being used to study the effects of proton radiation at different doses and dose rates to evaluate the risk for the prodromal symptoms (retching and vomiting) for astronauts under these conditions.

Thus far, experiments have been performed at the Loma Linda University Medical Center (Loma Linda, CA) under an approved Institutional Animal Care and Use Committee Protocol with either gamma photons from a ⁶⁰Co source or 110 MeV protons with a spread out Bragg peak (SOBP) in the clinical facility. 110 MeV protons were determined to be a homogenous dose for the width of the chamber in which the ferrets were irradiated. The ferrets are video-taped one or two at a time during the irradiations and for another 3-7 hours (two at a time) afterward in a separate facility. Non-irradiated animals are observed for 7 hours in restraint boxes identical to those used for the irradiation runs. The recorded data on disk are returned to AFRRI, rendered into videos and the animals are observed for retching and vomiting events. These and other behaviors are keyed by an observer into the AFRRI Incident Response Program (IRP; linked to EXCEL) for individual and group analyses.

The key parameters used for analysis are: 1) proportion of animals in a group retching or vomiting or both, 2) the mean number of retches or vomits or both in a group, 3) the mean latency to the first retching or vomiting event, and 4) the mean duration of the prodromal period (i.e., that time interval between the first and last event).

The first immediate goal is to establish a Relative Biological Effectiveness value for the prodromal response in ferrets for 110 MeV protons, with gamma-photons used as the reference radiation. Preliminary data will be presented from the following experimental groups: 1) gamma photons – 1 Gy and 0.25 Gy, both at 0.5 Gy/min and 0.5 Gy/hr (n = 6/group), 2) 110-MeV protons – 2 Gy @ 0.5 Gy/min (n = 5), and 1 Gy and 0.25 Gy, both at 0.5 Gy/min and 0.5 Gy/hr (n = 5-12/group). Examples of data collected from the proton high dose rate group (0.5 Gy/minute) include the observation that there are statistically significant differences (determined by the Mann-Whitney test) between the results obtained for the number of vomits and retches compared to controls at the 1 Gy and 2 Gy doses, and for the numbers of vomits compared to controls at the 2 Gy dose.

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This is a new research project to explore the effects of radiation quality on the dose-response relationships for telomere aberrations and gene mutations. It will relate these two potential cancer development processes by determining whether telomere-double strand break fusions are a significant contributor to the overall mutagenic response of a cell to ionizing radiation. Finally, it will explore various aspects of radiation-induced bystander effects; novel aspects of the work will include determining whether different radiation qualities vary in their abilities to induce bystander signals, and whether telomere-associated aberrations can be induced as a bystander response.

Mutations in cancer include point mutations and large-scale events that unmask recessive alleles by producing loss of heterozygosity via deletional or recombinational processes. In addition, a third potential mechanism would be erroneous joining of a double strand break and a telomere, which could wholly or partially delete a key gene and thus unmask a recessive allele. Telomere dysfunction has become a popular subject for study and telomere-driven aberrations are sometimes discussed as making an important contribution to genomic instability. There are two important considerations that will be explored in this research. First, there has not yet been a detailed study of how different radiation qualities induce aberrations involving telomeres. Second, it is not yet established whether telomere-double strand break fusions are a significant contributor to viable chromosome alterations that actually produce the mutational events that could drive carcinogenesis.

Therefore, the goals are to (i) understand how radiation quality affects the induction of telomere aberrations and gene mutations, (ii) to determine whether a link may exist between telomere-double strand break fusions and mutagenesis, and (iii) to explore whether these radiations induce bystander effects.

Aim 1 will be to produce detailed dose-response curves for mutagenesis and telomere-aberrations including telomere-double strand breaks. The radiations to be examined include \( \gamma \)-rays, 1000 MeV protons, 250 Mev/n He, 250 Mev/n O, 400 Mev/n Si, 1000 Mev/n Ti, and Fe at 300, 600 and 1000 Mev/n. The doses will be 0, 10, 20, 30, 40, 50, 75, 100 cGy. These studies will be done in human lymphoblast cells. Mutagenesis experiments will be at the heterozygous thymidine kinase locus, and will be performed using a protocol designed to circumvent problems in quantification that could arise from the non-homogeneous damage to the irradiated cell populations. Telomere aberrations also will be examined; these experiments will be done under conditions where the telomeres are either stable or unstable, using a chemical inhibitor to reduce the levels of the protein DNA-PKcs.

Aim 2 is to extend the study of radiation quality on mutagenesis and telomere aberrations to the realm of bystander effects. Several questions will be addressed, including:

a. Is bystander mutagenesis, known to be induced by low LET radiation, also radiation-quality dependent? How do bystander responses affect the overall shape of the dose-response curves for mutagenesis, for different radiation qualities?

b. Are telomere-aberrations, including double strand break fusions, induced by bystander signals? Is there dependence on radiation-quality?

c. There is a question whether radiation-induced double strand breaks could trigger the production of bystander signals. Does a single double strand break in a fraction of cells produce a measurable bystander signal?

d. As an extension of (c), if a single double strand break can generate a bystander signal, and a dysfunctional telomere can act like a double strand break, does a dysfunctional telomere produce bystander signal?

Aim 3 is to determine if telomeres join with double strand breaks to produce viable mutants. Therefore this aim is designed to link telomere-dysfunction with the production of the types of mutations that can drive carcinogenesis. These experiments will make use of a lymphoblast derivative that has a yeast I-Sce1 restriction site engineered into the second intron of the active tk allele (this cell line, called TK6-sce, has been constructed and is ready for use). Therefore introduction of the I-Sce1 enzyme will produce a double strand break in that one site in the genome and nowhere else (fortuitously the 18 base pair I-Sce1 sites do not occur randomly in the human genome). As in aim 1, the experiments will be done under conditions where the telomeres are stable or unstable. Mutants that have lost tk function will be analyzed first by fluorescence in situ hybridization to determine the fraction that has joined tk sequence with telomere sequence. Then, DNA sequencing will determine the exact structures of mis-joining events.
Use of a Non-Targeted Radiation Leukemogenesis Model for a Mechanistic Understanding of Radiation Quality Effects for NASA Relevant Radiations

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During the last 10 -15 years, the “Linear No Threshold” hypothesis has been challenged by a large number of observations of the so-called “non-targeted effects” i.e., bystander effect, genomic instability, which consists of the induction of damage in cells not directly traversed by radiation, most likely as a response to molecular messengers released by directly irradiated cells. The role of non-targeted effects (NTE) and the possible unique patterns of cellular and tissue damage produced by protons and HZE nuclei decrease confidence in the use of a risk assessment model based on radiation quality factors and linear dose response. Non-targeted radiation effects from these types of space radiations have not been studied in an in vivo carcinogenesis model. Use of a non-targeted radiation effects leukemia model to study a variety of radiation qualities and underlying mechanisms, can play an important role in understanding qualitative and quantitative differences between space radiation and low LET radiation leading to uncertainty reduction and ultimately the use of new systems biology models of cancer risk that are further applicable for pharmacological countermeasure testing. A murine model for the development of acute myeloid leukemia (AML) has been developed by Duhrsen and Metcalf which allows an analysis of the non-targeted effects of irradiation without the complicating mutagenic effects on hematopoietic cells. In this model, cells of the IL-3-dependent line, FDC-P1, consistently transformed to myeloid leukemia cells when injected into DBA/2 mice irradiated with 1 to 3.5 Gy gamma radiation (Duhrsen and Metcalf, 1988; Miller et al., 2005). Changes in the microenvironment favor the outgrowth of preexistent abnormal cells and promote their definitive leukemic transformation. Our laboratory recently demonstrated that this model was applicable to other types of radiation and to heavy metals (Miller et al., 2005, Miller et al, 2009). DBA/2 mice exposed to internalized depleted uranium (DU), an alpha particle emitter, also developed myeloid leukemia after injection of factor-dependent FDC-P1 cells. FDC-P1 cells are bone marrow stem cells. Concomitant to the development of leukemia, changes in rasP21 oncoprotein serum levels and intra-chromosomal aberrations in murine chromosomes 2 and 11 were measured. This model is currently used in our laboratory to test pharmacological countermeasures to low-LET radiations.

Current preliminary studies in our laboratory demonstrate 1) that 60Co radiation can induce myeloid leukemia in the DBA/2/FDC-P1 mouse model; 2) that 60Co-induced leukemia is dose and dose rate- dependent 3) that the incidence of leukemia and the latency to leukemia development is radiation quality dependent based on neutron and 60Co results; 4) that DNA hypomethylation occurs during 60Co-induced leukemogenesis; and 5) that ongoing chromosomal instability occurs during 60Co-leukemogenesis.

To further evaluate this model, we have examined the role that irradiated supportive bone marrow stromal cells play in the transformation process of hematopoietic stem cells like FDC-P1 cells. Co-cultivation of gamma-irradiated stromal cells with non-irradiated FDC-P1 cells induces transformation of the FDC-P1 cells measured using a colony formation assay.
These data provide evidence for a role of non-targeted radiation effects in the FDC-P1 leukemogenesis mouse model.

The use of this non-targeted leukemogenesis mouse model will provide new information in the search to better understand the role that non-targeted radiation plays in space radiation risk assessments.


In vivo Bystander Signaling in the Nematode C. elegans

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We quantify genotoxic damage in the nematode intestine by irradiating larvae and count stable anaphase bridges 60 hrs later the binucleate cells of young adults. This endpoint is dose & LET dependent and analysis of individual intestinal (E) cells (which in C. elegans have an invariant lineage and anatomy) shows that they have unique radiosensitivities. We previously showed that germline signaling may modulate E cell radiosensitivity. Statistical analysis of responses for pairs of E cells now shows that damage to one E cell substantially enhances the probability of damage in its neighbors as a function of cell-cell distance. Precision irradiations of intestine cells with charged particles confirms that signals originating in vivo in the intestine spread longitudinally along the endothelium. A combination RNA interference and mutant screens is being used to identify genes required for the signaling and includes genes for: DNA damage response, cell junctions, extracellular matrix and signal transduction. Analysis has been completed for over 50 genes. So far, genes in the NHEJ DNA repair pathway have been implicated in repairing damage leading to anaphase bridge whereas 9-1-1 response and homologous repair genes are not. Surprisingly, the atm-1 and atl-1 genes that serve as master regulators for DNA damage detection did not seem to be required. Another surprise was the observation that inhibition of trt-1, a telomerase reverse transcriptase, reduces bridge frequency which was counter to the expectation that improper telomere maintenance would lead to chromosome fusions in conjunction with the bridge-break-bridge cycle and result in higher frequencies. Inhibition of gap junction genes expressed in the gut so far have not resulted in modification of the bridge frequency patterns. Inhibition of Notch (lin-12), MAP kinase (sek-1) and other signal transduction genes has increased overall bridge frequency but not altered the observed spatial patterns. We are currently testing genes regulating microRNA metabolism because as many as 19% of all worm genes may be under miRNA regulation and recent publications have demonstrated robust regulation of gene expression by miRNAs in mammalian systems. MiRNA processing genes include argonaut genes alg-1 and alg-2 as well as rde-1 & rde-4 which are dsRNA binding proteins that act as accessories to the dsRNA ribonuclease, dicer (dcr-1). We will summarize of the current results from the genetic screens as well as more recent precision irradiation experiments.
Radiation Quality Dependent Effects on Phospho-protein Kinetics and Surrogate Cancer Endpoints

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Identifying relationships between early and late DNA damage cell signaling profiles, surrogate cancer endpoints, and radiation qualities will aid in obtaining a more accurate risk assessment for cancer formation due to space radiation exposure. We are studying critical phosphorylation signaling at early and late time points and relating these findings to surrogate cancer endpoints of telomere length changes, and genomic stability measured by centrosome amplification, at days post exposure using flow cytometry based assays. Isogenic epithelial and fibroblast cells from the same individuals are being studied to better understand the etiology of cancer, and why certain cell types more readily become tumorigenic. Detailed comparisons of endpoints following multiple energies and ions are being performed to understand the heterogeneous nature of high LET radiation. Energy deposition from high LET ions is dense in the track cores, resulting in closely spaced and complex damages, with delta rays originating from the cores providing additional exposures more similar to X-rays. Using carefully chosen charge, energy and LET of particles to obtain differing populations of cells hit by tracks and delta rays alone we will investigate how phospho-kinetic profiles change. Studies to date reveal both radiation quality and cell type is determinant in phospho-protein kinetics. The magnitude of telomere length changes following radiation exposure also appears to be cell type specific. Additional experiments are in progress to determine how radiation quality affects the telomere length changes observed. Interestingly, centrosome defects and γH2AX levels both show an upward trend with increasing dose many days post exposure. Additional studies will define how radiation quality affects both the phospho-protein signaling and the surrogate cancer endpoints, and how cell type modifies these results.
Progress Towards Development of a New Technique to Detect Recurrent Point Mutations; Intended Use in a Murine Model of Radiation-Induced AML.
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RADIATION-INDUCED AML IN SUSCEPTIBLE MICE
In mouse strains that are prone to the development of acute myeloid leukemia (AML) after exposure to ionizing radiation, one common denominator of subsequent AML is a large deletion on one chromosome 2 homolog. The deletion always encompasses one copy of the PU.1 (Sfpi1 in mouse) gene. This gene encodes a transcription factor that in this model behaves like a tumor suppressor gene. Loss of the second allele almost always occurs through a loss of function point mutation. This mutation occurs in the region of the gene encoding targeted DNA binding. The most common recurrent point mutation in the second allele of PU.1 is R235C. The mechanism and timing of the occurrence of this mutation in the remaining allele of the PU.1 event is of interest to the Radiation Carcinogenesis NSCOR.

DEVELOPMENT OF A NEW TECHNIQUE
Peptide Nucleic Acid (PNA) probes have been shown to have superior hybridization characteristics. They have been used to distinguish single base mismatches. Labeling procedures for PNA probes are more difficult, costly, and less effective than procedures to label similar DNA probes. Using a proprietary form of fluorescently-labeled PNA oligonucleotide probes (developed by KromaTiD Inc.) we initiated FISH experiments to determine if a reliable method could be developed to identify mouse bone marrow cells that have the R235C mutation in a background of normal cells. The fluorescent probes were designed to target the PU.1 cellular mRNA, specifically to the region around the R235C recurrent point mutation. Cells isolated from a mouse (8016) with radiation-induced AML were used for comparisons with normal mouse bone marrow cells. The 8016 cell line is ideal for probe development because they are a relatively homogeneous population of cells having a large deletion of chromosome 2 encompassing one PU.1 allele and the remaining allele has the R235C mutation.

In hybridization experiments analyzed using fluorescence microscopy, probes were observed around the nuclei. Fluorescent signals were not observed when cells were treated with RNase prior to FISH, indicating that the probes are binding to RNA. Fish experiments comparing wild type and mutant probes consistently showed that probes with sequences exactly matching expected RNA sequences could be discriminated from those containing a one base mismatch. Competition experiments, analyzed using Laser Scanning Cytometry demonstrated a reproducible and expected difference in signal intensity between the exactly matched probe and the corresponding mismatched probe. Future experiments will include spiking bone marrow cells with known numbers of 8016 cells to show that mutant cells can be detected at expected frequencies. After resolving technical difficulties inherent in such a sensitive assay, we plan to detect the commonly occurring R235 point mutations at early time points after irradiation of mice in order to determine the timing of the mutation during the development of radiation-induced murine leukemogenesis.

BROADER APPLICABILITY
Common point mutations that occur during neoplastic lineage evolution and in human tumors can be detected using specialized assays, but these assays typically destroy cellular context. Cellular context include, DNA content, gene copy number, gene expression levels, immunophenotype, and additional cancer-related mutations occurring in the same cell in which the tested mutation occurs. Detection of point mutations on a per cell basis is desirable because cancer is the result of genome evolution within a common lineage of cells. Therefore it is important to determine if two or more oncogenic changes, each required for malignancy, are found in the same cell or in different cells. If the technique to detect recurrent point mutations can be successfully applied to the hemizygous murine AML model, its applicability recurrent heterozygous point mutations will be tested.

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Neoplastic Transformation In Vitro by High Energy Protons and Iron Ions

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Our current research has been focusing on high and low dose-rate studies on the 250 MeV proton beam at Loma Linda University as well as the shape of the dose-response curve for 1 GeV protons and 1 GeV/n iron ions at NSRL. In addition, we have examined the effect of sequential irradiation with protons and iron and vice versa at NSRL, so-called mixed beam effects. Our findings are as follows:

1. **Dose-rate effect for 250 MeV protons.** Dose-rates of 50 cGy/min and 20 cGy/h were examined over the dose-range 1-100 cGy. No evidence for a dose-rate effect was found over this range.

2. **Dose-response for 1 GeV protons.** The dose-response curve is similar to that found earlier for 250 MeV protons (IJRB 81: 291-297, 2005) in that it is non-linear with evidence for a dose threshold. Repeated assays of samples from a single NSRL run indicate that 1 GeV protons may be more biologically effective than the 250 MeV protons available at Loma Linda at the higher doses of 50 and 100 cGy. This needs to be confirmed.

3. **Dose-response curve for 1 GeV/n iron ions.** Earlier experiments have revealed a threshold type dose-response curve which we have attributed to an adaptive response as a consequence of the delta ray penumbra (Radiat. Res. 171: 764-770, 2009). Recent studies have extended the dose range examined to less than 1 cGy and there is evidence of a possible hormetic effect at these doses.

4. **Mixed beam effects.** These experiments involved examining the effect of 10 cGy 1 GeV protons followed either immediately (10 minutes) or after a delay (16-24 h later) by 100 cGy of 1 GeV/n iron ions as well as the reverse sequence of low dose iron followed by high dose protons. Contrary to our hypothesis, no evidence for induction of an adaptive response by low dose (10 cGy) protons against a high challenge dose (100 cGy) of iron was found. Surprisingly, there was evidence for an adaptive response for the reverse sequence, again contrary to our original hypothesis. It was also noted that this adaptive response was larger when the proton challenge dose (100 cGy) was delivered within 15 minutes of the iron adapting dose (10 cGy).

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Effects of space radiation on genetic instability, neuromotor function, carcinogenesis and longevity in Ataxia telangiectasia deficient mice

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As human space exploration becomes a priority for NASA, the need to understand the biological effects of space radiation are pertinent. Long term effects of high LET radiation are not well understood. How genetics may play a role in sensitivity to high LET radiation is also poorly understood. To study the biological effects of space radiation, we used accelerated $^{56}$Fe particles, considered to be among the most biologically relevant components of high-LET space radiation. To understand genetic susceptibility to radiation, we used mice in which three amino acids of the Atm (Ataxia-telangiectasia mutated) protein ($Atm\Delta SRI$) are deleted, inactivating the kinase domain. ATM is important in the recognition and repair of DNA double-strand breaks. ATM deficiency in humans results in Ataxia-telangiectasia (AT) characterized by loss of motor skills, a compromised immune system, and an increased and premature risk of cardiac damage and cancer. Although AT is rare, ATM heterozygosity is estimated to be present in approximately 1% of the population and evidence is accumulating that ATM heterozygous people have a higher cancer rate than the general population. Using $Atm\Delta SRI$ heterozygous, homozygous, and wildtype mice we looked at the effects of 1 Gy, 1 GeV $^{56}$Fe particles on lifespan, carcinogenesis, neuromotor performance, and cardiovascular damage. We found that $Atm$-deficient and heterozygous mice do not seem to be more sensitive to 1 Gy, 1 GeV/n $^{56}$Fe particles than wildtype mice in terms of lifespan, cancer incidence, neuromotor capacity, or DNA damage. However, we found that female $Atm$-heterozygous and wildtype mice have a decreased lifespan compared to their male counterparts after exposure to high-LET radiation. In addition, we found that although cancer incidence is not affected by exposure to high energy iron ions, cancer latency is differentially affected depending on genotype. Cancer latency in wildtype mice is not affected by exposure to high-LET radiation while cancer latency is shortened in $Atm$ heterozygous mice and prolonged in $Atm$-deficient mice. Our results present an extensive investigation into the long-term effects of high-LET radiation exposure in a sex- and genotype-dependent manner and will contribute to the understanding of long-term risks astronauts may face.
**Bardoxolone Methyl (BARD) is Both a Countermeasure and Mitigator of Radiation-induced Neoplastic Transformation of Normal Human Epithelial Cells**

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Exposure to radiation induces oxidative stress and chronic inflammation, which are critical initiators and promoters of carcinogenesis. Many studies have demonstrated non-steroidal anti-inflammatory drugs and anti-oxidants can reduce the risk of radiation-induced cancer development. In this study we found that a synthetic triterpenoid, Bardoxolone methyl (BARD, also termed as RTA402 and CDDO-Me), was able to protect immortalized human epithelial cells (HCEC CT and HBEC KT) against radiation-induced damage and transformation. HCEC cells can be experimentally transformed with exposure to 2 Gy of Proton (1 GeV/n) followed 24 hours later by 50 cGy of $^{56}$Fe (1 GeV/n). Transformed cells showed an increase in proliferation rate and in both anchorage-dependent and anchorage-independent colony formation ability. Remarkably pre-treatment of cells with 50 nM BARD which has been shown to activate Nrf2 and induce anti-oxidative enzymes prevented radiation-induced neoplastic transformation. We also found that BARD mitigates radiation-induced apoptosis by treatment within 30 minutes after gamma-irradiation.

Taken together these results demonstrate that an oral available non toxic compound, BARD, can be used to protect against radiation-induced neoplastic transformation. This study also demonstrates that experimentally immortalized human epithelial cells are valuable premalignant cellular reagents that can be used to study radiation-induced carcinogenesis.

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Discerning the Cellular and Molecular Events Associated with Lung Carcinogenesis after Exposure to HZE Particles Using the Human Bronchial Epithelial Cell Line HBEC3-KT and its Isogenic Variants.

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The goals of this research program are to discern the molecular and cellular events associated with or that participate in the multi-step process of lung cancer, particularly after exposure to the unique radiations associated with long-term manned space exploration. The data generated here, as well as other data from the UTSW NSCOR, will be used to assist in the development of more accurate models of lung cancer risk from HZE radiation exposures.

In the first phase of this study we are examining the early molecular events that drive the response of lung epithelial cells to low and high LET radiation exposures using isogenic variants of the bronchial epithelial cell HBEC3-KT including HBEC3-KTR53, a variant that contains a mutant KRASV¹² gene and down-regulated p53. (HBEC3-KT was established by introducing hTERT and cdk4 into normal human bronchial epithelial cells from a 65 y.o. non-smoking female.) Cellular survival, gene expression and ultimately cellular transformation were examined. High LET radiations included 1GeV Fe, O and Si. Gene expression analysis was determined 1, 4, 12, and 24h after radiation at doses that rendered equivalent biological effect using Illumina Whole Genome BeadChips. The results from principal component analysis segregated samples based on the type of radiation exposure. Significantly changed genes after radiation were determined by applying a time course analysis model (maSigPro) in order to build a model (Support Vector Machine) that predicts Fe, Si and γ-ray exposures. The best model was chosen by repeatedly partitioning sample groups for building and cross-validation of the models. The cross-validation results showed 98% accuracy in predicting the three radiation types. An independent testing set was collected in the latest BNL run and the prediction results are being analyzed. We are collecting and processing data from a series of other particle and energy combinations to generate a series of LET and particle energy comparisons to determine whether LET is the dominant factor that defines the different expression profiles from different particles.

In the second phase of this study, the rate of cellular transformation (anchorage-independent growth of cells in soft agar) after HZE irradiation was determined for HBEC3-KT, HBEC3-KTR53 and the HBEC3-ET cell line (immortalized by introducing the HPV16 E6/E7 and hTERT genes). These 3 cell lines were irradiated with doses from 0 to 5 Gy with γ-rays or up to 1 Gy with Fe or Si particles (1 GeV). Surviving cells were maintained for at least 4 months post-irradiation and soft agar colony formation was examined at monthly intervals. The spontaneous rates of transformation varied between cell lines: HBEC3-KT, 2 x 10⁻⁷; HBEC3-KTR53, 10⁻³; and HBEC3-ET, 0.14. Interestingly, γ-rays had little impact on transformation frequency, although a slight increase was seen in the HBEC3-KT cell line. The response of HBEC3-KT to Fe and Si, however, was dramatic, peaking at 0.25 Gy and declining thereafter, although the frequency at 1 Gy was still 2-3 fold higher than the γ-ray value. Fractionated and low dose rate exposures are either ongoing or planned. Our preliminary data for transformation after a 1 Gy dose of Fe given over 5 days, (0.2 Gy per day) suggests that it is equivalent to the single dose transformation frequency.

We have recently isolated over 100 soft agar clones representative of the spectrum of radiation types and doses received. The clones were filtered based upon the ability to recapitulate the anchorage-independent phenotype. Cellular and molecular characterization and radioresponse of these clones is ongoing, however, we have noted several findings. 1) There are no mutations evident for the RAS gene in the HBEC3-KT derived clones. 2) Approximately one-third of the HBEC3-KTR53 derived clones showed phenotypic and molecular evidence for epithelial-mesenchymal transition (EMT), including some derived from the control samples (spontaneously induced). 3) Some of the clones display radioresistance, and although the number examined to date is limited, there does not appear to be a correlation between radioresistance and EMT status. While the cellular and molecular characterizations are ongoing, in particular comparing the spontaneous vs radiation-induced transformants, representative clones will be tested for their oncogenic potential by their ability to form tumors in immune-compromised mice. These tumors and the clones from which they were derived will be characterized and compared across those generated by the different radiations and those that arose spontaneously. This should provide a clearer understanding of the carcinogenic process following radiation exposure beginning with the initial radiation response to the development of lung tumors, provide the community with information that can be used to model risk, and provide models that can be used to test potential countermeasures to lung carcinogenesis.
Discrete Sets of Histone Acetyltransferase 1 (Hat1) Isoforms Expressed by Normal Human Fibroblasts and Keratinocytes

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INTRODUCTION

Human Hat1 was isolated as an enzyme responsible for acetylating histone H4 molecules destined for deposition on newly synthesized DNA molecules during replication [1]. Hat1 participates in several other processes, including interactions with the origin recognition complex, transcriptional silencing, and DNA repair [2]. Diverse protein isoforms seemingly support this breadth of Hat1’s functions. The “AceView” annotation database (www.ncbi.nlm.nih.gov/IEB/Research/Acembly/index.html) predicts the possible expression of four “very good” and six “good” human Hat1 protein variants. The alternative splicing databases (www.eurasnet.info/tools/asdatabases) identify at least nine possible alternative splicing mRNA isoforms originating from the human Hat1 gene, some of which utilize alternative start and stop codons, while some result from skipping internal exons. The EE-21 antibody (Sigma) specifically recognizes the C-terminal twenty amino acids of the human Hat1 protein. In protein extracts from normal human keratinocytes (NHKc), the EE-21 antibody detected two Hat1 isoforms - isoform a (64 kDa), and isoform b (52 kDa) [3]. Here, I describe the Hat1 isoforms that are present in NHKc and normal human fibroblasts (NHFb) and their distribution in unirradiated cells and cells exposed to various doses of γ-rays.

MATERIALS AND METHODS

To analyze the Hat1 protein isoforms I undertook subcellular protein fractionation followed by western blotting analyses. The protein extracts from normal human keratinocytes (NHKc) and fibroblasts (NHFbs) were prepared according to the protocol for isolating the nuclear matrix. The cells were grown to 80% confluence then exposed to 0-, 2-, 5-, or 10-Gy of γ-rays, and left to recover for 30 min in growth media. The protein fractions were obtained by permeabilizing the cells with 0.5 % triton-X-100, and the cytosolic- and soluble nuclear-proteins were collected. Digesting the cellular DNA with DNase I liberated the chromatin-associated proteins. The cellular remnants first were extracted with 0.25 M ammonium acetate, and then with 2 M NaCl. The remaining insoluble nuclear-matrix proteins were solubilized in 8 M urea, the proteins of each sample separated by 4-20 % SDS-PAGE electrophoresis, and subjected to western-blotting analysis.

RESULTS AND DISCUSSION

I utilized two anti-Hat1 antibodies to detect the Hat1 isoforms. The EK-14 antibody recognizes the N-terminal 8-21 amino acids of Hat1, and the EE-21 antibody identifies the C-terminal 20 ones, as described above. In similar protein extracts from unirradiated keratinocytes and fibroblasts, the EE-21 antibody detected three Hat1 isoforms: the 64 kDa (isoform a), the 52 kDa (isoform b), and a 38 kDa isoform. The EE-21 antibody detected the 52 kDa isoform, along with three distinct novel isoforms, viz., 50 kDa, 48 kDa, and 34 kDa. These protein isoforms show subcellular- and cell-type-specific variations in distribution that I will discuss, along with detailing the distribution of the Hat1 isoforms after exposure to γ-rays.

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Characteristics of DNA Binding Proteins Determine the Biological Sensitivity to High Linear Energy Transfer Radiation


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Non-homologous end-joining (NHEJ) and homologous recombination repair (HRR), contribute to repair ionizing radiation (IR)-induced DNA double strand breaks (DSBs). Mre11 binding to DNA is the first step for activating HRR and Ku binding to DNA is the first step for initiating NHEJ. High-linear energy transfer (LET) IR (such as high energy charged particles) killing more cells at the same dose as compared with low-LET IR (such as x or gamma rays) is due to inefficient NHEJ. However, these phenomena have not been demonstrated at the animal level and the mechanism by which high-LET IR does not impact the efficiency of HRR remains unclear. In this study, we showed that although wild type and HRR deficient mice or DT40 cells are more sensitive to high-LET IR than to low-LET IR, NHEJ deficient mice or DT40 cells are equally sensitive to high- and low-LET IR. We also showed that Mre11 and Ku respond differently to shorter DNA fragments in vitro and to the DNA from high-LET irradiated cells in vivo. These findings provide strong evidence that the different DNA DSB binding properties of Mre11 and Ku determine the different efficiencies of HRR and NHEJ to repair high-LET radiation induced DSBs.
Pathway Model of the Kinetics of the TGFβ Antagonist Smad7 and Cross-Talk with the ATM and WNT Pathways

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Abstract:

Signal transduction controls cellular and tissue responses to radiation. Transforming growth factor beta (TGFβ) is an important regulator of cell growth and differentiation and tissue homeostasis, and is often dis-regulated in tumor formation. Mathematical models of signal transduction pathways can be used to elucidate how signal transduction varies with radiation quality, and dose and dose-rate. Furthermore, modeling of tissue specific responses can be considered through mechanistic based modeling. We developed a mathematical model of the negative feedback regulation by Smad7 in TGFβ-Smad signaling and are exploring possible connections to the WNT/β-catenin, and ATM/ATF2 signaling pathways. A pathway model of TGFβ-Smad signaling that includes Smad7 kinetics based on data in the scientific literature is described. Kinetic terms included are TGFβ/Smad transcriptional regulation of Smad7 through the Smad3-Smad4 complex, Smad7-Smurf1 translocation from nucleus to cytoplasm, and Smad7 negative feedback regulation of the TGFβ receptor through direct binding to the TGFβ receptor complex. The negative feedback controls operating in this pathway suggests non-linear responses in signal transduction, which are described mathematically. We then explored possibilities for cross-talk mediated by Smad7 between DNA damage responses mediated by ATM, and with the WNT pathway and consider the design of experiments to test model driven hypothesis. Numerical comparisons of the mathematical model to experiments and representative predictions are described.
HZE-INDUCED SKIN CARCINOMA: AN IN-VIVO-LIKE MULTI-CELLULAR ETHELIAL MODEL SYSTEM FOR ANALYSIS OF RADIATION QUALITY EFFECTS AND DSB REPAIR PROCESSES

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INTRODUCTION

Carcinogenesis is a multi-step process whereby cells accumulate genetic alterations during progression towards malignancy [1]. There is considerable evidence that ionizing radiation causes non-melanoma skin cancer (NMSC), and the risk for NMSC in astronauts is elevated [2]. It is currently unknown what role high LET ions play during oncogenic initiation and progression, and the role of homologous recombinational DNA repair (HRR) has not been tested. However, it is known that X-rays induce cancer, and that HRR is essential for limiting mutagenesis during X-ray-induced carcinogenesis. HRR also is required for minimizing both mutation load and cytotoxicity after high LET iron ions, as we have shown [3].

APPROACH

Here, we are proposing to use an in-vivo-like multi-cellular epithelial system to model skin cancer risk for humans from space radiation. Our 3D model system contains collagen embedded fibroblasts co-cultured with keratinocytes, which interact such that a stratified and differentiated epithelium is formed [4]. To more accurately define skin cancer risk, we will extend our model system to include genetic susceptibility and the presence of indolent tumor cells (intraepithelial neoplasia). Individual susceptibility to skin cancer will be modeled by partially knocking-down different DNA double-strand break (DSB) repair pathways in skin keratinocytes. To address how the presence of sparsely distributed indolent tumor cells can affect skin cancer risk after space radiation, we will mix tumor cells established from different stages of skin tumor progression with normal keratinocytes in 3D co-cultures. Multiple different endpoints as surrogate markers for skin cancer progression will be studied in 2D and/or our 3D model system, including micronucleus (MN) formation, altered cell proliferation, evasion from apoptosis, abnormal differentiation and invasion. To study the longer-term effects of space radiation exposure on skin cancer processes, we will use a humanized mouse model by grafting irradiated pure or mixed cultures of keratinocytes and tumor cells onto immunodeficient mice. Late indicators of tumor progression, including invasion and proliferation in the underlying stromal compartment and presence of human cells in the mouse circulatory system, as an early marker of metastasis, will be assessed.

We hypothesize that, in basal cells, unfaithful DNA DSB repair will lead to persistent chromosomal damage, promoting altered cell growth, escape from tissue homeostasis and progression towards malignancy. Results will be presented that demonstrate significance and feasibility of this research project. We believe that the proposed approach is highly relevant for assessing the risk of skin cancer in astronauts and for modeling space radiation-induced tumor development in other epithelial tissues.

REFERENCES

Distributions of low- and high-LET radiation-induced breaks in chromosomes are associated with inter- and intrachromosome exchanges

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We exposed human epithelial cells in vitro to Cs-137 $\gamma$ rays at both low and high dose rates, secondary neutrons at a low dose rate, and 600 MeV/u Fe ions at a high dose rate to study the effect of low- and high-LET radiation on break locations within Chromosome 3. Breakpoints were identified using multicolor banding in situ hybridization (mBAND) that paints Chromosome 3 in 23 different colored bands. Breakpoint distributions were found to be similar between $\gamma$ rays of low and high dose rates and between the two high-LET radiation types. Detailed analysis of the chromosome break ends involved in inter- and intrachromosome exchanges revealed that only the break ends participating in interchromosome exchanges contributed to the hot spots found for low-LET. For break ends participating in intrachromosome exchanges, the distributions for all four radiation scenarios were similar with clusters of breaks found in three regions. Analysis of the locations of the two break ends in Chromosome 3 that joined to form an intrachromosome exchange demonstrated that two breaks with a greater genomic separation may be more likely to rejoin than two closer breaks, indicating that chromatin folding can play an important role in the rejoining of chromosome breaks. Our study demonstrated that the gene-rich regions do not necessarily contain more breaks. The breakpoint distributions are associated with whether a chromosome fragment joins with another fragment in the same chromosome or to a fragment from a different chromosome.
Cancers are not simply lumps of homogeneous cells, but are caricatures of normal tissue development. By affecting the distributions of interacting cells and communication among them through non-targeted effects, radiation can promote the outgrowth of altered cells with malignant potential. A fundamental property of cancer cells, the capacity for extensive self-renewal, is only found in small subsets of cells found in normal somatic tissues – those with “stem” or “progenitor” qualities. In more differentiated cells, this property is repressed through heritable “epigenetic” changes in function that are independent of changes in the primary DNA sequence. Early events in radiation-induced carcinogenesis must either cause the expansion of preexisting cells with extensive self-renewal potential or the acquisition of extensive self-renewal potential by cells that have repressed it. A current weakness in cancer risk assessment models is the lack of consideration of such epigenetic factors that influence the susceptibility of mixed cell populations to malignant transformation.

The intent of this project is to use a systems biology approach to directly address several of the gaps listed in NASA’s Human Research Program Integrated Research Plan. Specifically, a tissue specific risk model representing two major processes in radiation carcinogenesis will be developed using human breast cells. While it is not currently possible to simulate oncogenic progression in its entirety using human cells, aberrant differentiation and self-renewal are important surrogate endpoints that can be used directly in estimates of cancer risk and in reducing the uncertainties in radiation quality effects.

Using a three-dimensional (3D) cell culture system that supports differentiation of human epithelial cells, we are pursuing the following specific aims:

1) Determine the effects of ionization density and dose on the frequency of altered differentiation / self-renewal in primary human mammary epithelial cell (HMEC) cultures. To test the hypothesis that radiation of different qualities can stably alter the phenotypes of susceptible target populations with varying efficiencies, advanced computational methods will be extended and employed to quantify differentiation and telomerase expression by high content screening.

2) Identify biochemical pathways in primary HMEC that show persistent changes as functions of ionization density and dose. These pathways will be constructed through analyses of gene expression data corresponding to distinct subpopulations.

3) Use genetic manipulations to test the mechanistic involvement of discrete pathway components in persistent radiation quality effects. This objective will be accomplished through RNAi and cDNA overexpression, as appropriate.
An ISS Experiment: Biological Influences of Space Environments (Radiation and Microgravity)

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For human beings, environments in space are extremely different from those on earth. It is, therefore, important
to elucidate biological influences of space environmental factors for promoting a utilization of space environments.
For estimating the space-radiation effects separately from other effects, human lymphoblastoid TK6 cells were
preserved as a frozen state in the “Kibo” module of International Space Station (type 2 cells in Fig.1: a total of 134
days in space with a received dose of 72 mSv). In our post-flight assays for such cells, a radiation-sensitive
methodology, LOH analysis, was applied for detecting induced mutants. As a result, we observed a ~2.3-fold
induction in thymidine kinase (TK) deficient mutants over the ground control. In addition, the TK mutation frequency
of such cells exposed to 2 Gy X-ray was reduced to ~60% of the level of the ground-control cells. Furthermore, the
type 2 recovered cells demonstrated a more efficient repair of DNA double-strand breaks created by I-SceI
expression, ~1.9- and ~1.8-fold due to non-homologous end joining and homologous recombination, respectively.
These results suggests the possibility that frozen cells can record damage accumulated during space travel and
subsequently express the genetic and cellular responses when grown on earth. In this experiment, type 1 cells were
incubated under μG or 1 G in ‘Kibo’ for 8 days in the period of preservation. Similar post-flight analyses for such
cells demonstrated a possible effect of microgravity, ~60% reduction in both viable-cell recovery and TK mutant
population. Future experiments are required for confirmation of this possibility.
MicroRNA-21 is involved in radiation-promoted liver carcinogenesis

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Abstract

Background & aims: High-LET ionizing radiation (IR) has a higher risk than low-LET IR for liver carcinogenesis, the mechanism remains unclear. MicroRNAs (miRNAs) have a critical effect on carcinogenesis through post-transcriptional modification; however, no report links miRNA and IR-promoted carcinogenesis. We wanted to test whether miRNA changes contribute to the higher risk of high-LET IR-promoted liver carcinogenesis.

Methods: We examined miRNA expression profiles in high-LET IR-induced mouse liver tumor sample, human or mouse hepatocytes, and liver tissues by using microarray assay. We combined a set of experiments including making miR-21 construct, transfection, qRT-PCR, siRNA and xenograft model etc. to examine the relationship between low- or high-LET IR-induced miR-21 and IR-promoted liver tumorigenesis.

Results: We showed that miR-21 was the only miRNA that over-expressed in IR-promoted liver tumors. IR stimulated miR-21 expression in hepatocytes and liver tissues. The stimulation depended on IR-induced up-regulation of AP-1 (at an earlier time) and the ErbB/Stat3 pathway (at a later time). Over-expressing miR-21 in the human hepatocytes (non-irradiated, high-LET irradiated and low-LET irradiated) made these cells become tumorigenic in nude mice, however, the size of the tumors from high-LET-irradiated-cells > that of the tumors from low-LET-irradiated-cells > that of the tumors from non-irradiated-cells. These phenomena were accompanied by more significant changes of miR-21-targets.

Conclusions: IR-induced up-regulation of miR-21 plays an important role in IR (especially high-LET)-promoted liver carcinogenesis. The higher risk of high-LET IR-promoted liver carcinogenesis related to more damage left in the cells and IR (especially high-LET)-induced an environment change facilitates miR-21-induced liver tumor. (This work is supported by a NASA grant NNX09AF24G to Y.W.)
Radiation Carcinogenesis NSCOR: Tumors Arising in $^{56}$Fe Ion Irradiated Mice

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INTRODUCTION
The goal of the Radiation Carcinogenesis NSCOR is to provide the information required to develop a rational scientific basis for estimation of cancer risks in humans from exposure to radiation during space flight. It is the successor to the Leukemogenesis NSCOR and focuses on using murine model systems to study HZE radiation risks and extending the findings to risks in humans.

AML AND SOLID TUMOR INCIDENCE IN HZE IRRADIATED CBA MICE
AML sensitive male CBA/CaJ mice were irradiated with 1 GeV/n $^{56}$Fe ions at NSRL and with $^{137}$Cs $\gamma$-rays at Colorado State University and followed to 800 days of age for the development of AML. Unexpectedly, the RBE for $^{56}$Fe-induced AML was essentially 1, suggesting that cell killing effects of this HZE ion may outweigh its transformation effects in the murine radiation-induced AML model system. An additional, unexpected finding was that 1 GeV $^{56}$Fe irradiated mice tended to develop hepatocellular carcinomas (HCC) with an estimated RBE of approximately 50.

LEUKEMIA AND SOLID TUMOR INCIDENCE IN HZE IRRADIATED BALB/c MICE
In an additional, small-scale mouse HZE irradiation study, 12-week-old female BALB/cByJ mice were irradiated with 0.2 Gy of 1 GeV/n $^{56}$Fe ions or 0.5 Gy of $^{137}$Cs $\gamma$-rays, or left unirradiated. There were approximately 100 mice per group. These mice were monitored to 800 days of age. The experiment was designed to provide preliminary data on whether the spectrum of tumors that arise in $\gamma$-irradiated female BALB/c mice also arise in 1 GeV/n $^{56}$Fe ion irradiated mice of the same sex and strain, and to determine if the high risk for HCC seen in HZE irradiated male CBA mice is also found in HZE irradiated female BALB/c mice.

HCC, which is strongly induced by 1 GeV/n $^{56}$Fe ion irradiation of CBA/CaJ male mice, was absent in similarly irradiated BALB/cByJ female mice. Whether the difference in HCC incidence is due to the strain difference or the sex difference will require further experiments, but the results suggest that HZE irradiation only induces this tumor type in mice that are susceptible because of their genetic background and/or sex. Since this pilot study was limited to only one dose for $\gamma$-rays and $^{56}$Fe ions, 0.5 and 0.2 Gy respectively, RBEs cannot be estimated for the tumors that arose. However, from our limited results it seems unlikely that the RBEs for mammary and ovarian tumorigenesis will be higher than one. Indeed, 1 GeV/n $^{56}$Fe ions may be less effective than $\gamma$-rays in inducing these tumors. Further work is required to determine this.

HZE EFFECTS ON MYELOID PROGENITOR CELLS
The results from the large-scale mouse irradiation leukemogenesis study were complemented by those from our cytogenetic studies of HZE irradiated mouse bone marrow cells. In these studies we found that the RBE for persistent chromosome 2 deletions in 1 GeV $^{56}$Fe irradiated bone marrow cells was about 1. These deletions are likely the initiating events for radiation-induced AML. We also found that murine strain differences in susceptibility to radiation leukemogenesis were reflected by strain differences in the persistence of bone marrow cells with chromosome 2 deletions.

CHARACTERIZATION OF HZE- AND GAMMA-INDUCED AML
The $\gamma$-ray and HZE induced leukemias were assayed for chromosome 2 deletions, mutations in the $PU.1$ ($Sfpi1$ in the mouse) gene, microsatellite instability, gene expression and genomic loss or gain. Chromosome 2 deletions were seen in both $\gamma$- and HZE-induced leukemias, but because there are few HZE-induced leukemias to analyze we cannot determine if there is a difference in the frequency of deletions. As previously reported by others, mutations, usually C>T transitions at R235, were commonly seen in the remaining $PU.1$ allele in the $\gamma$-ray induced leukemias. Mutations in the remaining $PU.1$ allele were seen in two HZE-induced leukemias; both were C>A transversions at R235. We found that both $\gamma$- and HZE-induced AML are characterized by an increase in ongoing microsatellite instability. AML induced by these two radiation qualities cannot be distinguished by array CGH or by gene expression profiles.

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The Role of Mir-107 in the Cellular Response to Ionizing Radiation Exposure

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MicroRNAs (miRNAs) have been shown to regulate multiple developmental and physiological processes by modulating protein production. An increasing number of miRNAs have been implicated in cancer with both oncogenic and tumor suppressor roles for specific miRNAs. Mir-107 was previously found to regulate the cell cycle by targeting CDK6 in non-small cell lung cancers. Induction of Mir-107 expression in lung cancer cell lines leads to cell cycle arrest and Mir-17 can be silenced by epigenetic means. Our recent data suggested that the expression of mir-107 was involved in the early stages of the radiation response in both human normal bronchial epithelial cells (HBEC3-KT) and skin fibroblasts (HSF), and that inhibiting mir-107 activity resulted in increased radiation sensitivity.

The up-regulation of mir-107 after γ-ray radiation was identified by miRNA profiling using Illumina Human Universal-12 BeadChips for miRNAs. In this study, HBEC3-KT cells were irradiated using a ¹³⁷Cs source at Brookhaven National Lab during NSRL campaigns 08C and 09A. Total RNA was extracted at 1, 4 and 24 hours after radiation with a modified Qiagen protocol that retains short RNA fractions. The raw data were background subtracted and normalized using the MBCB algorithm as designed for Illumina arrays. The results indicated that the most robust miRNA changes occurred 1 hour after radiation. Mir-107 was found increased nearly 4 fold in both NSRL runs with a p-value less than 0.01.

We performed clonogenic assays using cell transfected with anti-Mir-107 or control RNAs. Transfection with the Mir-107 inhibitor resulted in increased radiation sensitivity compared to cells transfected with control RNAs. We inhibited Mir-107 expression in a series of experiments to investigate the role of Mir-107 in cell cycle check point control, DNA double strand break repair and chromosome integrity after radiation. Our data indicated that the abolishment of Mir-107 expression after radiation resulted in an accumulation of cells in S-phase; delayed DNA double strand break repair as depicted by persistent phosphorylated γH2AX foci; and an increase in the number of chromosomal aberrations with characteristic dicentric chromosomes and chromatid breaks.

Mir-107 has been shown to be under-expressed in non-small cell lung cancers. In connection with that, we examined mir-107 levels in HZE-transformed HBEC3-KT cell clones. These clones were collected in soft agar where they showed anchorage-independent growth 4 months after Fe-particle irradiation. Mir-107 expression was decreased in soft agar clones when compared with non-irradiated cells which were cultured for same period of time.

In an effort to study the molecular mechanisms, we are currently investigating other potential Mir-107 targets that are associated with the processes described above. It is our intention to determine the role that Mir-107 plays in the early response to radiation exposure and whether the silencing of Mir-107 is associated with radiation-induced cancer initiation and promotion.

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