MEETING REPORT---NASA Radiation Biomarker Workshop

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ABSTRACT

A summary is provided of presentations and discussions from the NASA Radiation Biomarker Workshop held September 27-28, 2007, at NASA Ames Research Center in Mountain View, California. Invited speakers were distinguished scientists representing key sectors of the radiation research community. Speakers addressed recent developments in the biomarker and biotechnology fields that may provide new opportunities for health-related assessment of radiation-exposed individuals, including for long-duration space travel. Topics discussed include the space radiation environment, biomarkers of radiation sensitivity and individual susceptibility, molecular signatures of low-dose responses, multivariate analysis of gene expression, biomarkers in biodefense, biomarkers in radiation oncology, biomarkers and triage following large-scale radiological incidents, integrated and multiple biomarker approaches, advances in whole-genome tiling arrays, advances in mass-spectrometry proteomics, radiation biodosimetry for estimation of cancer risk in a rat skin model, and confounding factors. Summary conclusions are provided at the end of the report.

INTRODUCTION

On September 27-28, 2007, the NASA Ames Research Center hosted a workshop on Radiation Biomarkers with support and participation from the Space Radiation Project Element (SRPE) of the Human Research Program, NASA Johnson Space Center. The overall goal of the workshop was to provide an update of the radiation biomarker research across key sectors of the radiation research community—academia, clinical medicine, DOE labs, DOD labs, and NASA, with an eye toward potential future applications in space. This was a first in a series of workshops to provide periodic updates and help define research needs for future applications on long-duration human space missions.

With the possible exception of cataracts (*1*), there are presently no direct human data available from space-type radiation for any of the radiation-induced risks considered of highest priority by NASA for long duration human space travel, i.e., carcinogenesis, acute and late CNS risks, chronic and degenerative tissue risks, and acute radiation risks (*2*). Although information exists from Earth-based studies sufficient to recommend crew exposure limits and spacecraft design requirements for missions in low Earth orbit, there is insufficient knowledge of the health effects of space radiation to provide recommendations on crew exposure limits and design requirements for extended lunar and Mars missions (*3*). A major focus of the NASA space radiation effort is basic and fundamental research to expand the knowledge base and reduce the uncertainty inherent in

current exposure limits and design requirements. At present, this includes a large number of radiobiological research projects mostly performed by peer reviewed principal investigators in academia using accelerator-based simulated space radiation at the NASA Space Radiation Laboratory, Brookhaven, NY. Biomarkers (biodosimetry) is embedded within the radiobiology research. It is anticipated that information on the mechanisms and processes involved in space radiation damage and repair will reveal specific indicators of space radiation exposure. The NASA Strategic Program Plan for Space Radiation Health Research (3) indicates that biomarkers/biodosimetry will be specifically pursued during Phase 3 (2010 - 2023).

Biomarkers to assess radiation response (and dose) in astronauts have been used since the early 1960's. The first such assessments were made by Bender et al. (*4,5*) who measured dicentric chromosome aberrations in peripheral blood lymphocytes of crewmembers of Gemini-3 and Gemini-11. These flights lasted only 5 h and 3 d, respectively, and no significant increase in chromosome damage was observed when comparing pre-flight and post-flight samples. More recently, a substantial database has emerged using various cytogenetic methods, including fluorescence *in-situ* hybridization (FISH) techniques, to evaluate pre- and post-mission blood lymphocyte samples from crewmembers on Mir and ISS (*6-9*). Results indicate that radiation dose accumulated over a period of a few months or more can induce a measurable increase in the yield of chromosome damage. However, shorter missions of a few weeks or less appear

to be below the detection limit for cytogenetic effects.

Advances in genomics, proteomics, and experimental low-dose radiobiology, are providing new opportunities for radiation biomarker development. Given the lead-time required for biomarker development and NASA's plan to return humans to the Moon by 2019 and onward to Mars by 2030, it is important to discuss the potential utility of biomarkers in space and the extent to which uncertainties in space radiation risk assessment could perhaps be reduced by biomarker-based research studies. Further discussions should include how the large number of animal radiobiology studies supported by several federal agencies could partner with human biomarker studies to facilitate cross-species extrapolation, and ultimately extrapolation to humans.

THE SPACE RADIATION ENVIRONMENT

The radiation environment in space is complex. Radiation includes charged particles such as hydrogen and iron, and a myriad of secondary radiation including neutrons produced by charged particle interactions with materials (e.g., spacecraft, astronauts, the surface of the Moon, etc.). During periods of low-solar activity, the major contributor to dose in deep space or on the lunar surface is galactic cosmic radiation (GCR), which is composed primarily of high-energy protons (in the GeV range). GCR also includes heavier charged particles ranging from helium to iron nuclei. During periods of high-solar activity

(approximately 11 year cycle) the probability for a significant solar particle event (SPE) is elevated. A large SPE can release a very high flux of charged particle radiation---about 98% consists of protons, which are typically less than ~150 MeV.

High-energy GCR radiation is very penetrating and therefore difficult to shield, i.e., a large fraction of the charged particles are in the GeV/nucleon range (*10*). For example, a 1 GeV proton has a range of 324 cm in water and a 1 GeV/nucleon ^{56}Fe nucleus has a range of 27 cm water. In contrast, solar protons typically penetrate less than about 10 cm in water, and the vast majority penetrate less than 1 cm in water, barely enough to penetrate a lunar extravehicular activity (EVA) spacesuit (*11*). In addition to their differences in penetration power, the high-energy particles from GCR produce more secondary radiation via spallation reactions in materials. In some cases, these secondary radiations have larger relative biological effectiveness (RBE) than the primary radiations. Hence, shielding of GCR radiation poses a challenge for long-term space travel as well as for human habitation of a base on the Moon.

During non-SPE solar minimum conditions, the dose-equivalent rate in interplanetary space is estimated to be in the 0.5 to 1.4 Sv/y range (*12*). Due to shielding by the Moon, the dose-equivalent rate on the lunar surface is estimated to be less than in interplanetary space. However, lunar surface dose modeling is complicated due to secondary radiation (e.g., neutron) production in the lunar

regolith. It is estimated that a 6-mo stay on the Moon would result in exposure to about 0.15 Sv (*13*). A 2.5-y roundtrip to Mars may result in an integrated dose-equivalent on the order of 1 Sv (*12*). These dose estimates (which assume no significant SPEs during the mission) are not expected to result in significant short-term risk, but they may increase the long-term stochastic health risks that are associated with radiation exposure. Because these doses are primarily from GCR, they are unlikely to be substantially reduced by shielding.

In contrast, SPEs pose a different challenge. Although protons from SPEs are of much lower energy than those from GCR (and therefore can be more readily shielded), they pose an acute health risk for astronauts who are exposed during extravehicular activity (EVA). For example, if an astronaut were participating in an EVA on the Moon during the August 1972 SPE and received the full radiation from that event, the doses are calculated to be 40 Sv to the skin, 5 Sv to the yellow marrow, and 1.7 Sv to the red marrow (*11*). These doses would have greatly exceeded the current 30-day dose limits established for LEO of 1.5 Sv for skin and 0.25 Sv for marrow. The dose rates during a large SPE range from ambient to ~0.5 Sv/h for marrow and to more than 10 Sv/h for skin. Although these dose rates are considered to be high for most radiation protection conditions, they are not considered radiobiologically acute (defined as 1 Gy/min), and are expected to have an effectiveness higher than chronic, but lower than acute. Thus, the SPE radiation would have the additional complexity of intermediate and varying dose rate and response.

INDIVIDUAL SUSCEPTIBILITY

Dr. William Morgan (University of Maryland) addressed challenges associated with biomarkers of radiation sensitivity and individual susceptibility. He noted that when attempting to identify individuals at risk for developing radiation-induced cancer, one must consider the genetic complexities involved in carcinogenesis. Thus, it may be important to identify the genes responsible for initiation, promotion and progression of cancer. However, the actual variants contributing to such complex diseases are unknown. The most common type of variation in the genome is the single nucleotide polymorphism (SNP). SNPs occur once in every 300-500 nucleotides (*14*). SNPs give rise to individual gene variants that alter susceptibility to common diseases. Consequently, mapping complex traits requires determining which of the myriad of SNP's influence disease risk. Rapidly developing technologies will facilitate identification of risk-related SNPs. However, any genetic variation may be complicated by gene-gene and/or gene-environment interactions. Each SNP is a rare event. Therefore, studies of a very large human population are required to identify SNPs that may be useful markers of disease susceptibility.

Nevertheless, there is little question of the role of DNA repair in ameliorating the effects of radiation-induced DNA damage and minimizing the incidence of cancer. Many of the cancer genes identified in family studies have a role in DNA

replication and/or repair. The loss of function for many DNA repair or repair-related genes is incompatible with normal development and often results in embryonic lethality. A more challenging question is the extent to which any alteration in the ability to repair damaged DNA contributes to the sporadic incidence of cancer. Given that most individuals show extensive sequence variation in their DNA repair genes, it is likely that susceptibility will vary among individuals, depending on the particular combination of inherited alleles.

Evidence for the importance of a moderate reduction in DNA repair is accumulating from animal models. BALB/c mice are sensitive to radiation-induced mammary tumors. Genetic linkage analysis indicates that this sensitivity is associated with allelic variation in the catalytic subunit of DNA-PKcs, a gene involved in the non-homologous enjoining pathway (*15*). In addition, a number of transgenic knock-out mouse models have provided direct evidence for a significant role of DNA repair related gene function in carcinogenesis. For example, mice that are heterozygous for a mutation in ATM, the gene involved in the disease ataxia-telangiectasia (AT), have heightened susceptibility to cancer. Such mice are more sensitive to high -dose ionizing radiation than are their wild-type counterparts (*16*). It is estimated that ~1% of the human population is heterozygous for ATM. Data in mice suggest that ATM heterozygotes are susceptible to radiation-related cancer.

However, a number of *in vitro* experiments using cultured cells from AT heterozygotes have failed to demonstrate enhanced sensitivity to ionizing radiation, particularly at low doses (*17*). Most techniques are able to detect *a shift in the average* response of AT heterozygote cells, compared with normal cells, but with considerable overlap between the two groups. Although somewhat controversial, one assay that appears to provide excellent discrimination involves x-irradiating cells in G_2 and quantifying radiation-induced cytogenetic damage (*18*). Furthermore, haplo-insufficiency is only one factor that may induce susceptibility to radiation exposure. Allelic imbalance in gene expression levels can be caused by other factors such as cis acting regulatory polymorphisms in coding, intronic or regulatory sequences, as well as by differential DNA methylation or histone acetylation. In addition to genetic factors, non-genetic factors might possibly add to the complexity of radiation susceptibility. Such factors include lifestyle, diet, smoking and reproductive history.

While it is likely that one day it will be possible to identify radiation sensitive / resistant individuals, such radiation responses may be normally distributed within the population. In adequately identifying such a phenotype one must then consider how radiation responses are modulated by given genetic and epigenetic considerations as well as environmental impacts, but also a host of social, ethical, and legal considerations.

MOLECULAR SIGNATURES OF LOW-DOSE RESPONSES

As summarized by Dr. Andrew J. Wyrobek (Lawrence Berkeley National Laboratory), systems biology approaches have been employed to evaluate cellular responses to low-dose radiation using genomic gene-expression technologies and bioinformatics tools. The objective of radiation system biology approaches is to improve the knowledge of early cellular responses to low-dose radiation and to reduce the uncertainties of assessing genetic health risk at low-dose levels (*19,20*). The early transcriptome profiling studies underscore the complexity of gene-expression phenotypes and response pathways that are modulated in cells and tissues after low doses. Complex gene networks and pathways have been identified for low-dose exposures (*21*) and for cellular protection mechanisms against radiation-induced cytogenetic damage (*22*). Bioinformatics analyses have identified similar gene networks after low-dose exposures in both mouse and human models, suggesting that there are robust low-dose radiation responses across tissues and species. Radiation gene expression profiles have also been characterized for the proteome (*23*).

Low dose effects on cells have been evaluated experimentally using a variety of exposure regimens including acute, low dose rate or chronic, and adaptive response (AR) exposure regimens. AR regimens are important because they show that under certain circumstances and with some variability not yet understood, low dose exposure can confer radioprotection against subsequent exposures. Typical AR regimens use a low dose (priming dose) followed some

hours later by a high dose (challenge dose) to determine whether the priming dose conferred protection against damaging effects of the challenge dose. Radioadaptive protection has been described for cell killing, DNA damage, chromosomal aberrations, cancer latency and other cellular phenomena in a variety of biological models, most notably as summarized below.

Transcriptome analyses have been performed in human cell lines irradiated in vitro. Analyses of a detailed dose-response curve (1-400 cGy) in human lymphoblastoid cells from two unrelated individuals identified a set of ~300 low-dose responsive genes (1-10cGy), several of which did not have a significant dose slope, consistent with plateau-like responses in the low-dose range. Bioinformatics analyses suggest that low-dose-responsive gene products are associated with cellular homeostasis mechanisms, special signal transduction pathways, and various subcellular locations.

Transcriptome analyses of the cytogenetic AR have also been performed in human cells. More than 100 genes were identified whose expression was associated with the AR (*22*). This study provided molecular insights into the mechanisms of cellular protection against radiation-induced chromosomal damage. It was hypothesized that the pathways associated with these genes are the basis of an AR molecular switch that controls the degree of protection against radiation-induced chromosome damage in irradiated cells. Research is in

progress to test this molecular switch in mice after whole-body radiation exposures.

Baseline gene expression has been evaluated in the tissues of unirradiated mice. Baseline transcriptional profiles were characterized for preselected genes associated with DNA damage recognition and repair processes among several tissues of healthy adult mice (testis, brain, liver, spleen and heart). Significant tissue variation was found in the baseline expressions of stress response, damage control and DNA repair-associated genes (*21*). Overall, stress response genes exhibited the greatest variation among tissues with the highest expression in liver and heart while DNA repair genes exhibited the least variation among tissues. A multitude of factors, including metabolic activity, immunological and inflammation status and oxidative damage may affect the expression of stress response genes. Damage control genes associated with cell cycle regulation and DNA repair genes generally had the highest expression in testis. Variations in basal expression of DNA damage recognition and repair-associated genes among healthy tissues provided the foundation for investigating their differential response to genotoxic agents and susceptibility to genetic disease.

Transcriptome analyses have been performed to study the effects of whole body radiation on mouse brain tissue. Cellular functions associated with altered transcript profiles were characterized for mouse brain exposed to low-dose *in-vivo* gamma irradiation. Whole-body exposure of male mice to low-LET radiation

altered the transcript expression in their central nervous system, with distinct time- and dose-dependent clusters and identified low-dose unique gene sets (*24*). Advanced bioinformatics was applied to identify the major gene networks and biochemical pathways that were uniquely associated with low-dose versus high-dose exposures as well as pathways shared across doses. Brain irradiation modulated the expression patterns of over 1000 genes, of which >800 showed more than 1.5-fold variation. About 30% of genes showed dose-dependent variations, including genes exclusively affected by 0.1 Gy. About 60% of genes showed time-dependent variation with more genes affected at 30 min than at 4 h. Early changes involved signal transduction, ion regulation and synaptic signaling. Later changes involved metabolic functions including myelin and protein synthesis. Low-dose radiation also modulated the expression of genes involved in stress response, cell-cycle control and DNA synthesis/repair. This study demonstrated that doses of 0.1 Gy induced changes in gene expression that were qualitatively different from those at 2 Gy. The findings suggest that low-dose irradiation of the brain induces the expression of genes involved in protective and reparative functions, while down-modulating genes involved in neural signaling activity. *In situ* analyses in tissue sections provide important validation approaches to assess variations in radiation responses among different neuronal cell types (e.g., *25*). Our results support the model that brain tissue exposed to low-dose radiation employs unique molecular response pathways not observed after high doses, which underscore the problems that will be encountered when using high–dose data to infer low-dose mechanisms and to

assess low-dose CNS radiation risks.

Dr. Wyrobek concluded that systems radiation biology approaches with advanced bioinformatics are showing substantial promise for improving the molecular understanding of the early cellular responses to low-dose radiation and to help to reduce the uncertainty of assessing risk at low-dose levels. The finding of low-dose unique genes and pathways in both human cells and mouse brain tissues sets the foundation for identifying risk predictors for genomic instability and disease susceptibility in tissues irradiated *in vivo*.

MULTIVARIATE ANALYSIS OF GENE EXPRESSION

Dr. Nicholas Dainiak (Yale University) presented his work on multivariate analysis of cytokine gene expression after low dose radiation exposure. He concluded that while DNA microarray analysis may provide insight into gene function within and across biological networks, meaningful data can be generated only when studies are appropriately controlled and when the state of a living system is well defined. Parameters that are critical for the study of radiation effects include radiation quality, dose, dose rate, cell type and tissue type. Owing to the enormity of the data set, appropriate methods of data analysis must be applied, including those that determine inherent grouping (hierarchical cluster analysis or HCA and principal component analysis or PCA) and those that define known class membership (*26*).

HCA uses the entire data set to extract natural clusters without reducing dimensionality of the data. Consequently, HCA becomes computationally unfeasible with very large data sets having an indirect relationship with a covariate. Methods that reduce dimensionality and eliminate non-significant information include PCA and projection pursuit (PP). A disadvantage of PCA is that it is unable to determine maximum probability for heteroscedastic (i.e. non-uniform) uncertainties that may be correlated with each other. It is difficult for PCA to distinguish noise (i.e., spot variation) from systemic variance (i.e., bias in the microarray). Studies with large within-group variance may be better analyzed by projection pursuit, a technique that has been used to analyze chemical data sets (*27,28*). Accordingly, clustering may be revealed when within-group variance is large.

Whereas for PCA, latent variables are transformed into space according to the singular value decomposition algorithm, PP employs principle component scores to obtain sequential maximized chi-square indices. The resulting PP factors are used to generate two candidate planes that are evaluated by the method of Posse, and the PP index with the least variance is defined as the new starting plane. The algorithm iterates until the most informative structure is obtained. Since PP seeks departure from normality, it is not sensitive to outliers.

Using an in-house PP algorithm and MATLAB and PP functions (Computational Statistics Toolbox), scores plots were compared from PCA and PP in microarrays prepared from mRNA of human subjects exposed to 0.18-49.00 mSv as a result of the Chernobyl Nuclear Power Plant catastrophe. Improved clustering was obtained when PP was used, both for the groups of identified genes and for intra-cluster variance (*29*). **Table 1** shows that PP detects the expression of genes in seven distinct groupings at 11-13 years after low-dose exposure.

The biological relevance of clusters identified by PP is striking. For example, IL-8, MCP-1, TNF-α and IL-10 negatively regulate hematopoietic stem/progenitor cell proliferation. It is possible that such cytokines mediate cytopenias occurring after irradiation. In addition, TNFRSF (Fas) and its cognate ligand are overexpressed on the cell surface after irradiation (*30,31*), an interaction that is required for radiation–induced apoptosis in lymphocytes (*32*). Furthermore, identification of overexpressed ligands (i.e., IL-8 and TNFα) and their respective receptors in one or more clusters, suggests that the pathways in which these molecules participate, are involved in the biological response to ionizing radiation. It is unknown whether a gene in a cluster activates another gene of the cluster (i.e., overexpression of the two genes may be independent of each other). Regardless, PP has the potential to identify expression profiles that may possibly explain biological effects of radiation exposure.

Table 1. Comparison of clusters from PCA and PP

In conclusion, microarray technology has revolutionized genome-scale data collection by increasing the throughput of information in a short period of time. The plethora of information provided by microarrays must be assessed with tools that not only account for inherent noise components but also provide sufficiently robust analysis. The application of new tools such as PP and methods that are based on known class membership, may address both of these issues and provide structures (i.e., gene clusters) that are more biologically relevant than those provided by traditional methods such as HCA and PCA. Since there is no consensus regarding the best method to analyze a multivariate data set, it is recommended that microarray data be submitted to public databases where information can be reevaluated and interpreted, as new methods for data analysis are applied (*33*).

BIOMARKERS IN BIODEFENSE

Dr. Ken Turteltaub (Lawrence Livermore National Laboratory) discussed their extensive work on biodefense biomarkers. Over the last decade, interest in developing biomarkers for use in measuring hazardous exposures, risk from such exposures, and for use in early disease diagnosis has grown exponentially. New technologies allow rapid high capacity analysis of genes, proteins and most recently small molecules. While significant progress is being made, a number of studies have raised issues on intra- and inter-laboratory reproducibility, and the

influence of confounding factors on the ability to use biomarkers as a diagnostic test in the field.

Approximately 6 years ago, LLNL initiated a project to test the feasibility of detecting and diagnosing an infectious disease using changes in the levels of biomolecules in peripheral blood. LLNL proposed that the underlying biochemistry of an organism changes under the influence of a stressor such as an infectious agent. It was reasoned that these biochemical changes would begin as soon as the host cells began interacting with the pathogen and that a change in physiology would trigger changes in the levels of molecules residing inside cells and body fluids. Detection of these changes might be used to both detect a developing disease early, possibly in the presyndromic or prodromal period, and allow more rapid intervention with the result being reduced morbidity and mortality. Thus, a series of studies were conducted in rodents and in human samples to assess quantification capabilities of several high throughput technologies such as arrays, mass spectrometry, gel electrophoresis, solution-based multiplexed antibody assays and RT-PCR. A controlled experiment in rodents was conducted to discover which gene transcripts and proteins in plasma change in expression when challenged with a virus and when these changes would be first detectable following exposure. This was then explored in an apparently healthy human population to determine how variable a set of blood gene transcripts would be and how stable they were expressed over time. Finally, gene transcript changes and protein levels were measured in several

human cohorts, including individuals with HIV, rheumatoid arthritis, dialysis patients, bacteremia, and in a group of apparently healthy individuals during a marathon run. These groups simulated a series of potentially confounding factors such as individual variability, pre-existing conditions, and the effects of general physical stress on the levels of protein and nucleic acid transcript levels in blood. All these situations are relevant to potential factors that could influence an individual's response to radiation in space including co-exposure to infectious agents, physical stress from living in an extreme environment and individual differences in response.

The results in inbred laboratory animals suggest that under laboratory controlled conditions it is possible to detect pathological states with biomarkers a few days prior to development of overt symptoms. It is also possible to detect overtly ill humans using single biomarkers. Detection of prodromal disease states were much more difficult to impossible to detect or diagnose based on a single biomarker. Use of multiple markers combined into a panel can produce a signature that discriminates early pathological states from healthy people in some situations.

A variety of factors made discrimination of early pathological states difficult in the human studies including sample collection, storage and shipping methods, sites of sample collection from the individual, and the method used for quantification. Significant changes in blood protein levels were found in the marathon runners

both during and after the run. Significant changes were found in mice in gene expression depending on whether the blood was collected from the tail vein or by retro-orbital bleeding. Large differences were seen among rheumatoid arthritis patients in gene expression patterns. Thus sample collection, physical stress, pre-existing conditions and the method of analysis can impact the levels of potential biomarkers found in blood. It was concluded that use of molecular signatures to detect and diagnose disease in the presyndromic and prodromal phases of a disease's pathology is possible but significant attention needs to be paid to understanding factors that affect levels of potential markers as well as the factors that affect their analysis. Attention should be placed on finding both sets of molecules that are sensitive to the disease state which will likely be sensitive to other biological factors as well as ones that are refractory to confounding factors (which may be less sensitive to the disease state). Combination of these two groups of markers into panels may provide a signature that would be useful in (i) assessing exposure to hazardous environments, (ii) determining risk from that exposure, and (iii) diagnosing a developing disease early.

BIOMARKERS IN RADIATION ONCOLOGY

Dr. Srinivasan Vijayakumar, Dr. Andrew Vaughan, and colleagues from the UC Davis Medical Center presented the radiation oncology perspective on biomarkers. Advances in the field of radiation treatment of tumors both clinically and as regards general radiation effects on living systems require an integrated

approach that combines studies of basic biological mechanisms, the physics of dose deposition and measurement with the assessment of relevant endpoints of acute and chronic biological change. Biological responses have the potential to be applied to individual patient treatment.

From the perspective of basic biology the utility of biological markers of radiation effects may be viewed in different ways. The most direct utility of a biological marker is one that records the presence of a radiation exposure at some time in the past. Of these, levels of specific gene transcripts or proteins have been studied by a number of groups and radiation-responsive genes identified in a number of tissues – most commonly circulating cells of the blood. However such studies are complicated by the assumption that the dose to an individual is uniform. Nonuniform dose dramatically increases the complexity of dose analysis and thus the ability to predict biological response. In an effort to gain some information on regionally defined irradiation, the response of buccal cavity cells was examined in patients undergoing irradiation for head and neck tumors. Such cells comprise the inner lining of the mouth and are easily sampled by gentle brushing of the mouth cavity and immediate extraction of RNA. Samples were taken from four quadrants (upper/lower, back/front) both before and after the first radiation dose of an approximately 30-fraction course. Such dose to the tumor target might peak at around 2 Gy with doses to the sampling positions within the buccal mucosa ranging from this dose downward. The dose to each quadrant was determined by the planning computers used to configure the

treatment and confirmed by MOSFET detectors placed within a mouth-guard at the sampling sites. Samples produced RNA of suitable quality for analysis by qPCR. Using 50 cGy dose as a threshold, 12 genes known to respond to radiation exposure were assessed. Transcripts for HSPC132A, MDM2, PCNA, CDKN1A and CCNG1 were significantly elevated after exposure. This study indicates the potential for buccal cavity transcript monitoring as a guide to radiation exposure. However in terms of analysis, issues still remain in both the transient nature of transcript elevation and the biological significance of a positive result.

To better address the complexity of the biological response to irradiation, an alternative approach was considered. Many tumors, including those of the head and neck as well as breast and elsewhere, exhibit loss of heterozygosity (LOH) at chromosome11q23. This location may be the site of one or more tumor suppressor genes. LOH events are a known consequence of DNA double strand breaks and therefore, may be induced by irradiation. Using a mucoepidermoid cell line (H292) as a model, cells were irradiated with either 4 or 8 Gy, and the surviving cells were passed through two rounds of cloning. LOH at 11q23 was detected, using polymorphic markers for either the maternal or paternally derived chromosome that generated specific PCR products. This experiment showed that 11q23 was highly susceptible to LOH after irradiation, with 10-20% of all irradiated clones showing LOH at 11q23, but not elsewhere on chromosome 11. This analysis has distinct advantages over conventional transcript or protein

profiling. First, the aberration is a marker linked to the transformation process. Second, the marker is open to rapid screening of affected cells, using PCR based techniques. Third, the change is a permanent alteration in the genome (i.e., screening may be carried out on historically exposed individuals). Finally, unaffected cells within the sampled region provide an appropriate internal control. Therefore, this approach measures an individual biological response.

RADIATION BIOMARKERS AND TRIAGE---GENE EXPRESSION

Biomarkers and applications from the DHS perspective were discussed by Dr. Sally Amundson (Columbia University). In the event of a large-scale radiological incident, there would be a critical need for rapid, high-throughput radiation biodosimetry, both because of the need for medical triage, and as an active reassurance measure to decrease panic among those not actually exposed. Currently available biodosimetry approaches are not adequate for these needs, so some have suggested the development of gene expression profiles as a biodosimetry approach amenable to development of high-throughput and fieldable assays (*34-36*).

Microarray analysis was previously used to identify 55 genes responding in human peripheral blood 24 hours after *ex vivo* exposure to γ-rays, and demonstrated linear induction of *CDKN1A, DDB2* and *XPC* at doses from 0.2 to 2 Gy at 24 and 48 hours after exposure (34). Dose dependent increases in the

expression of these genes were also detected at 4 and 72 hours post exposure, but exhibited less linearity. In a later study, 85 genes responded *in vivo* in humans after the first or second 1.5 Gy fraction of total body irradiation (TBI), and showed dose-dependent increases of *CDKN1A, DDB2, FCGR1A*, and *CXCL10* through successive fractions (*36*).

More recent studies have used the Agilent whole-genome microarray platform (37). Global gene expression profiles of *ex vivo* irradiated human peripheral blood from unrelated healthy donors were measured at several times after irradiation. This study spanned a range of γ -ray doses relevant to medical decisions in a radiological triage situation, and identified hundreds of genes responding to radiation. Quantitative real-time PCR of *CDKN1A* and other responding genes indicated a biphasic dose-response, quite similar to that seen previously in the ML-1 human myeloid cell line (*38*), with linear kinetics up to 2 Gy and further increases with a decreased slope through 8 Gy. There was also good agreement between gene induction using different irradiation and culture protocols, different donor pools, and different gene expression measurement techniques.

In order to make such gene expression signatures useful for triage, a collaborative effort is underway to develop microfluidic cartridges (*3*9) to take a blood sample and automatically perform a chemo-luminescence based gene-expression assay. The cartridges contain all necessary reagents, pumps, valves

and control electronics, do not rely on molecular amplification methods such as PCR, and deliver highly consistent results (CV <10%). A hand-held, microprocessor-controlled prototype has been developed for sample preparation, and a commercial chemo-luminescence reader is being modified for the microfluidic cartridges. This biodosimetry concept was tested at the Coyote Crisis Campaign 2006, a disaster preparedness exercise in Scottsdale, Arizona.

The standalone version of the microfluidic gene expression-profiling cartridge could potentially be adapted to provide rapid turnaround biodosimetry to support extended space exploration missions. If, for instance, a solar particle event (SPE) occurred during a sortie on the lunar surface, rapidly available biodosimetric information could be used to help determine if an individual should be restricted to shielded areas for the remainder of the mission, or in extreme cases, if a mission should be cut short. Targeted biodosimetry studies, such as characterizing the gene expression response to SPE spectrum protons, and validation at lower doses, would also be needed to establish the usefulness of such an approach.

Additional studies are still needed to fully develop gene expression for any radiation biodosimetry application. The *in vivo* responses must be more thoroughly characterized, including understanding to what extent the cancer patients undergoing TBI can be used as a model for healthy individuals. Animal studies, such as some already performed in mice (*40*) or planned studies in non-

human primates, will also be critical. Another very important area is determining the radiation specificity of the defined biodosimetry signatures. Since a large proportion of the *in vivo* response to ionizing radiation comprises p53 regulated genes, and cytokines and genes involved in immune response (*36*), we need to be sure that infection, burns, general injury responses, or exposure to other toxins will not produce false positive radiation exposure signals. The prototyped gene expression cassettes must also be thoroughly tested and their sensitivity and specificity determined. Despite the remaining questions, current findings strongly support the usefulness of gene expression signatures and a biochip approach for radiation biodosimetry.

RADIATION BIOMARKERS AND TRIAGE---MULTIPARAMETER APPROACHES

William F. Blakely (USU/AFRRI) in his talk entitled: “Space Exploration Biodosimetry – Use of Integrated and Multiple Biomarkers” and co-authored by his Armed Forces Radiobiology Research Institute (AFRRI) colleagues, illustrated the potential dual-use of AFRRI’s integrated biodosimetry for space flight biodosimetry. Using a scenario of a radiation exposure during a Mars mission, Dr. Blakely showed how dynamic, space-flight deployable (*41*), and integrated multiparameter biodosimetry can provide key contributions in the medical management of acute-radiation sickness (ARS) injuries. He recommended deployment of these or alternative software applications and

consideration for use of blood cell counters and radiation-responsive protein bioassays for use on long-duration and other space flight missions where radiation over-exposures risks are possible.

Effective medical management of suspected radiation exposure incidents requires the measurement of dynamic medical data and physical dosimetry in order to provide diagnostic information to the treating physician and dose assessment for personnel radiation protection records. The accepted generic multiparameter and early-response approach includes observing prodromal signs and symptoms; obtaining complete blood counts with white blood cell differential; measuring radioactivity and monitoring the exposed individual; bioassay sampling, if appropriate, to determine radioactivity contamination; sampling blood for the chromosome-aberration cytogenetic bioassay using the "gold standard" dicentric assay for dose assessment; and using other available dosimetry approaches. AFRRI's Biodosimetry Assessment Tool (BAT) is a comprehensive software application developed for recording diagnostic information in suspected radiological exposures (*42*). AFRRI is also developing a First-responder Radiological Assessment Triage (FRAT), for use on hand-held personal digital assistant devices, that provides data collection templates and ability to integrate results for analysis of clinical signs and symptoms, lymphocyte counts, physical dosimetry, radioactivity, and location-based dose estimates (*43*).

Identification and validation of early-phase radiation biomarkers are needed to provide enhancement in biological dosimetry capability to assess individuals suspected of exposure to ionizing radiation (*44*). This need is of great importance to provide quantitative indications for early initiation (20-h after radiation exposure) of cytokine therapy in individuals exposed to life-threatening radiation doses as well as to provide effective triage tools for first-responders in mass-casualty radiological incidents (*45*). Monitoring of radiation exposure by biological dosimetry systems is complimentary to physical dosimetry, since they can weigh radiation quality and dose rate according to biological efficacy. Molecular biomarkers are used as diagnostic endpoints in environmental health and cancer. Hofmann and colleagues (*46*) reported radiation-induced increases of serum amylase in 41 patients following either whole-body irradiation or irradiation of the head and neck region. Mal'tsev and colleagues (*47*) measured serum C-reactive protein (CRP) in Chernobyl radiation victims within 1-9 days after exposure and correlated its levels to ARS severity. Dr. Blakely's research group's working hypothesis is that hematological changes, gene expression, and encoded protein biomarkers detected in biological samples (peripheral blood) can: a) distinguish the concerned public from individuals exposed to radiation and b) triage exposed individuals by assessing radiation dose and injury. Their research strategy involves use of both *ex vivo* (human) and *in vivo* (murine, non-human primate) radiation model systems. They have employed quantitative methodology to measure changes in blood cell (e.g., lymphocytes, neutrophils, etc.) counts, multiple gene-expression and encoded-protein targets as well as

blood serum enzyme activities. Blood cell counts were measured using a clinical hematology analyzer. Gene expression targets (Gadd45a, Ddb2, Bax, Bcl-2, CDKN1A [p21WafCip]) were quantified by real-time reverse-transcriptase polymerase-chain-reaction (RT-PCR) bioassay (*48,49*). Encoded proteins (ras p21, raf, Gadd45a, Bax, Bcl-2, p21WafCip, IL-6, CRP) were detected by the enzyme-linked immunosorbent and microsphere (Luminex^{TM})-based assays (41,50). Blood serum levels of amylase activity were measured using conventional commercial reagents used in blood chemistry analyzers (*42*). Radiation-induced changes in peripheral blood cell counts as well as up-regulation of gene expression and protein targets from varied pathways (DNA damage and repair, cell-cycle checkpoints, apoptosis/anti-apoptosis, cytokine, etc.) and radiosensitive tissue (salivary gland) from selected radiation model systems will be presented (*41,48,49-51*). These results support the proof-of-concept that use of multiple early-response biomarkers can provide useful diagnostic indices for medical management of radiation casualties.

ADVANCES IN WHOLE GENOME TILING ARRAYS

Dr. Viktor Stolc (NASA Ames Research Center) discussed the cutting-edge genomics capabilities of the Ames Genome Research Facility (http://phenomorph.arc.nasa.gov/) and their recent work on high-density oligonucleotide tiling arrays. Identification of the transcribed regions in model organisms as well as the human genome is one of the major challenges of

postgenomic biology for understanding human physiology. Empirical transcriptome mapping, using whole-genome tiling microarrays has been shown to be the most comprehensive and unbiased approach. This novel method uses high-density oligonucleotide microarrays with probes chosen uniformly from both strands of the entire genomes including all gene-coding and intergenic regions. By hybridizing the microarrays with tissue specific or pooled total RNA samples, a genome-wide picture of transcription can be derived. The comprehensive transcriptome analysis enables identification of the genetic basis of biological phenotypes and revealed transcribed sequences not detected by other methods. Advancement of microarray design to enable probing of polygenetic samples will significantly improve medical diagnostics and efficacy of treatment in human diseases.

Comprehensive genome-wide analysis of transcription can be performed rapidly with high-density oligonucleotide microarrays designed to encode unique sequences that hybridize only to their intended complement RNA sequences. Significant computational resources are required to compute all possible sequence variants for the microarray design. The NASA Ames Genome Research Facility, in collaboration with several academic laboratories produced optimized specificity and sensitivity of the oligonucleotide probes for detection and discrimination of very low abundance transcripts (52-55). For example, in the sea urchin embryo, they were able to detect and discriminate the differential expression of very low abundance RNA transcripts even for genes known to be

expressed at low levels in only a few cells (52). Dr. Stolc stated that a recently completed high-resolution map of the mouse transcriptome produced using the same technique that they applied to map the human genome (53), and the sea urchin embryo (52), revealed significant regions of novel RNA expression that are syntenous between the mouse and human genomes. This comparative analysis also resulted in identification of short transcribed regions in the human genome, previously undetected solely from the human data due to lower statistical significance. Thousands of previously uncharacterized transcripts in the mouse genome enabled identification of several hundred previously un-described human transcripts (55).

ADVANCES IN MASS SPECTROMETRY PROTEOMICS

Dr. Julie Leary (University of California, Davis) provided an overview of the state-of-the-art in mass spectrometry (MS) proteomics analysis. Mass spectrometric data from proteomics analysis were presented of both large molecular clusters and small peptides from proteins containing post-translational modifications (*56,57*). New developments involving organic derivatizing agents can be used to unambiguously determine the sites of post-translational sulfation on proteins and peptides (*56*). In a somewhat related fashion, metal affinity columns are extremely successful enriching proteins and peptides containing low level phosphorylation (*57*). Since phosphorylation and sulfation are isobaric, this combination of solution sample preparation and mass spectrometry can be paramount in distinguishing these important changes.

Mass Spectrometry technology can be very effective for the analysis of various biomarkers, particularly peptides and proteins. Modern advances in this field have now produced instrumentation that is capable of analyzing large molecular weight (megadalton) multi-subunit architecture. This can be very powerful for investigating how proteins interact in both normal and diseased tissue as well as measuring stoichiometric changes to these proteins during radiation exposure. One can easily envision isolating protein biomarkers from serum samples of exposed individuals and tracking both the various post-translational modifications that may differ, as well as comparison and contrast of proteins and peptides that may differ from exposed and non-exposed individuals.

RADIATION BIODOSIMETRY FOR ESTIMATION OF CANCER RISK IN A RAT SKIN MODEL

Dr. Fredric Burns (New York University) presented his work on radiation biodosimetry of cancer induction in rat skin. DNA double strand breaks (DSBs) have potential relevance to carcinogenesis because of the tendency that such breaks join, most frequently by non-homologous end joining, to create chromosome rearrangements and elevated genomic instability (*58-61*). Although DSBs occur spontaneously, their highly efficient induction by ionizing radiation is probably unique among carcinogenic agents (*62*). DSBs are also strongly linked to the cytotoxic action of ionizing radiation which competes with carcinogenesis, particularly at higher radiation doses (*63,64*).

Radiation ionizations occur either within dense tracks (if the incident ion is heavy) or scattered randomly (if the incident particle is light). As LET increases in dense tracks, DSB joinings become more numerous because of the lower average distance between DSBs. For randomly distributed DSBs joinings increase with radiation dose to the power of 2, because each DSB is produced in proportion to radiation dose. The above considerations lead to an expression describing the expected yield of DSB joinings for any type of radiation as follows:

$$\text{Yield}(D,L) = CLD + BD^2 \qquad \text{(Equation A)},$$

where L = LET, D = radiation dose and B and C are to be evaluated empirically. Equation A is the well-known linear-quadratic function, except for the L in the linear term.

While it is plausible that Equation A might correctly describe the yield of DSB joinings, a leap is required to imagine that this same functional form might also be applicable to cancer induction. Surely the extensive genomic alterations associated with cancer progression would obliterate all traces of any initial DNA damage with causative relevance to the cancers. But what if Equation A fits both cancer and DSB joining yields across a broad range of LETs and doses? Wouldn't that be a contradiction of initial lesion obliteration and raise the possibility that DSB joinings are the long-sought, causative DNA alterations of radiation carcinogenesis? At present, the latter is an unproven postulate in any other organ or species, but in rat skin it appears to be well-founded based on

carcinoma yields and estimates of DSBs joinings in surrogate keratinocytes. One explanation how Equation A could explain both cancer and DSB induction would be that the cancers originate in a small fraction of irradiated cells most probably stem cells with just the right joining that permits long-term survival with elevated levels of genomic instability. It is the latter that eventually produces the additional genomic alterations required for malignancy.

As surrogates for rat keratinocytes *in vivo*, rat skin keratinocytes were irradiated with either ^{56}Fe ions (L = 125 keV/µ), x-rays (L = ~0.4 keV/µ) or protons (L = 25 keV/µ) and DSBs were quantified by means of the *in situ* γ-H2AX antibody technique. The results for ^{56}Fe ions showed that many DSBs (H2AX-positive foci) were aligned along straight, parallel tracks oriented in the beam direction for at least 4 h after exposure. Interestingly, straight tracks persisted in parallel formation as nuclei rotated slowly relative to their orientation at time zero. The γ-H2AX antibody technique provides a way to estimate the yield of DSB joinings so that quantitative comparisons with cancer yields for the same doses and radiation types become possible. The linear term in the DSB version of Equation A was estimated based on total DSBs per track for the 3 radiation types studied as follows: 2 doses (1.5 and 3.0 Gy) of ^{56}Fe ions, roughly equicarcinogenic doses (4.5 and 9.0 Gy) of x-rays and Bragg peak protons (0.3 and 1.1 Gy). Of course at higher doses every epithelial cell is expected to exhibit multiple DSBs, while the cancer probability is only on the order of 1 per 10^6 cells exposed. It is an advantage for biodosimetric purposes that DSBs are very frequent in comparison

to the cancers, but proportionality between the 2 endpoints must be verified empirically throughout dose and LET ranges typical of space radiation.

When DSB joinings in surrogate keratinocytes and skin cancer yields for 3 diverse LET values were plotted as a function of radiation dose an almost exact superimposition was apparent when proportionality was fixed at 100 DSBs per keratinocyte = 1.0 carcinoma per rat at 1 year. A plot of the cancer and DSB yields on the same coordinates showed cancer yields falling within the error bars of the DSB estimates at all 6 available dose points. If both DSBs and cancers show dose and LET dependencies as described by Equation A, a useful tool for predicting carcinogenic outcomes of various, possibly even complex, radiation exposure scenarios might become available on the basis of a comparatively simple short-term *in vitro* assay.

CONFOUNDING FACTORS

Dr. Terry C. Pellmar (Armed Forces Radiobiology Research Institute) discussed factors that may confound biomarker analyses following radiation exposure. Many factors influence biodosimetric assessments. Dose- and time-dependence of a biomarker are clearly characteristics that must be addressed for accurate biodosimetry. While some biomarkers might increase throughout the radiation dose range of interest, others might level off or even begin to decrease as dose goes up. Time of sampling can significantly impact measurements, as

well (*41*). Some markers increase slowly and are sustained; others increase quickly but only transiently. Quality of radiation also impacts the dose response curve for biomarkers. For example, the calibration curves for cytogenetic biodosimetry using dicentrics show that fission neutrons are more effective than gamma rays for an equivalent absorbed dose (*65*). Accurate interpretation of the assay requires information on radiation quality. In addition, the possibility of partial body exposure needs to be considered. High radiation doses to small areas may not be revealed by a biomarker that reflects whole body changes. Differentiation of partial-body from total-body exposures will be important for treatment decisions.

The specificity of a biomarker and the variability within the population must also be considered in development of biodosimetric assays. For example, the prodromal symptoms, nausea and vomiting, are excellent indicators of radiation exposure but are symptoms of other common conditions. In addition, the inter-individual variability in the emetic response to radiation is large (*66*). Some people may vomit early to a relatively low dose while others may not vomit at all, despite a serious exposure. Biomarkers that respond robustly to radiation may also respond to other conditions. Baseline levels of the biomarkers may be widely disparate in the population, making small changes difficult to discern (*43*). Changes in biomarkers may be altered by the health status of an individual and by any drugs that have been taken (*36*). Furthermore any injuries that occur in addition to the radiation exposure could have an impact on biomarker levels.

Biomarkers are very useful in defining a radiation exposure but many confounding factors exist that must be considered in their interpretation.

DISCUSSION AND CONCLUSIONS

The workshop presentations stimulated the following discussion and conclusions.

Cytogenetic analyses in peripheral blood lymphocytes have been used to reconstruct radiation dose to astronauts in space (e.g., see *4-9*). Although physical radiation monitoring is employed on all human missions in space---and more sophisticated technologies will be available for return to the Moon—the advantage of biomarkers is measurement of the biological response in the individual, which includes contribution from dose, dose rate, radiation quality, and biologically-based modifiers of response such as DNA repair. Hence, biomarkers provide a measurement that would be expected to correlate better with health outcome than a physical dosimeter alone. An accurate biomarker (biodosimeter) response could be critical for treatment management if an astronaut has received a large acute exposure from a SPE.

It would be particularly helpful for long-duration human space exploration to have biomarkers that measure individual susceptibility to the major health risks associated with radiation exposure in space--- carcinogenesis, acute and late

CNS risks, chronic and degenerative tissue risks, and acute radiation risks (*2*). Such markers may be used, for example, to select astronauts for long-duration missions who may have low susceptibility to the major radiation-induced health risks. The risk that can be estimated presently from biomarkers (biodosimetry) is related to average population risk, not individual risk, and can therefore not be used to select resistant individuals. However, available biomarkers can be used to identify individuals with unusually high radiosensitivity, such as those with certain known DNA repair deficiencies.

Astronaut biodosimetry and biomarker evaluations would be aided by relatively few individuals on a space mission and the ability to obtain pre and post samples (and perhaps during mission samples on very long missions). However, these advantages are tempered by possible confounding factors. For long-duration missions, the temporal stability of the biomarker would be particularly important. Temporal instability can result from both biological and physical factors. In the case of a lunar mission, during a large SPE, when an accurate biodose may be most urgently needed, the SPE proton dose will be highly skewed toward the first couple of cm of tissue due to the low proton energies. This inhomogeneous dose distribution will tend to result in (a) reduced initial biomarker frequency (as compared with uniform whole-body dose) due to dilution with unirradiated biomarkers in more shielded body compartments (this phenomenon is clearly observed in partial-body exposures), and (b) variable time course profiles of changes in transitory radiation-responsive biomarker concentration over time due

to differential stem cell doses. Importantly, this would be the case even for biomarkers that are stable following uniform whole body dose (*67*).

Given that most individuals show extensive sequence variation in their DNA repair genes, it is likely that susceptibility will vary between individuals depending on the particular combination of alleles inherited. For example, mice heterozygous for a mutation in ATM have heightened susceptibility to cancer and are more sensitive to ionizing radiation. However, other factors may cause increased or decreased sensitivity to radiation (e.g., allelic imbalance in gene expression).

Systems biology approaches are showing substantial promise for improving the molecular understanding of the early cellular responses to low-dose radiation and to help to reduce the uncertainty of assessing risk at low-dose levels. The finding of low-dose unique genes and pathways in both human cells and mouse brain tissues sets the foundation for identifying risk predictors for genomic instability and disease susceptibility in tissues irradiated *in vivo*.

While DNA microarray analysis may provide insight into gene function within and across biological networks, meaningful data can be generated only when studies are appropriately controlled and when the state of a living system is well defined. Since there is no consensus regarding the best method to analyze a multivariate data set, it is important that microarray data be submitted to public databases

where information can be reevaluated and interpreted, as new methods for data analysis are applied.

Additional studies are needed to fully develop gene expression for any radiation biodosimetry application. The *in vivo* responses must be more thoroughly characterized, including understanding to what extent the cancer patients can be used as a model for healthy individuals. Animal studies will also be critical. Also, since a large proportion of the *in vivo* response to ionizing radiation comprises p53 regulated genes, and cytokines and genes involved in immune response (*29*), we need to be sure that infection, burns, general injury responses, or exposure to other toxins will not produce false positive radiation exposure signals.

Studies in support of biodefense programs clearly illustrate the normal variability in both gene expression and proteins, and demonstrate the need for multiple simultaneous markers. Multiple early-response biomarkers can also provide useful diagnostic indices for medical management of radiation casualties. Results from studies in rat skin carcinogenesis indicate that enumeration of DSBs in surrogate cells *in vitro* may, when properly calibrated, become a biodosimetric tool for estimating cancer risks associated with space radiation.

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