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Secreted Cytokines as Possible Biomarkers of Exposure to Ionizing Radiation

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Canada

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In conducting the research described in this report, the investigators adhered to the 'Guide to the Care and Use of Experimental Animals, Vol. I, 2nd Ed.' published by the Canadian Council on Animal Care.

Abstract

The quest for reliable and unique biomarkers of radiation exposure is an ongoing challenge in the field of radiation biology. Once identified they will provide an invaluable tool for both clinicians wanting to assess therapeutic outcomes of patients as well as first responders and medical personnel needing to rapidly triage populations of potentially exposed people following an radiation/nuclear (RN) event. It has been reported that there is an acute inflammatory response following exposure to ionizing radiation. We hypothesize that this is initiated in part by cytokines secreted from various cell types within the body and that these secreted cytokines may serve as biomarkers for exposure. The various studies described in this report examine the cytokine response following radiation exposure from a single cell line model through to animal studies.

Résumé

La recherche de biomarqueurs fiables et uniques indicatifs d'exposition aux rayonnements est un défi dans le domaine de la radiobiologie. Une fois identifiés, ils fourniront un outil inestimable aux cliniciens voulant évaluer les perspectives thérapeutiques des patients ainsi qu'aux premiers intervenants et au personnel médical lors du triage d'individus potentiellement exposés lors d'un événement radiologique/nucléaire. Il a été rapporté qu'il y a une réponse inflammatoire aiguë après l'exposition aux rayonnements ionisants. Nous formulons une hypothèse selon laquelle ce processus est amorcé en partie par des cytokines sécrétées par différents types de cellules dans le corps et que les cytokines sécrétées peuvent servir de biomarqueurs indicatifs d'exposition aux rayonnements. Les études décrites dans ce rapport examinent la réponse des cytokines suivant une exposition aux rayonnements sur un modèle de lignée cellulaire ainsi que sur un modèle animal.

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Executive summary

Secreted Cytokines as Possible Biomarkers of Exposure to Ionizing Radiation

Hillary Boulay Greene; Kate Daniel; Aimee Jones; Louise Prud'homme-Lalonde; DRDC Ottawa TR 2012-175; Defence R&D Canada – Ottawa; September 2012.

Introduction or background: The quest for reliable and unique biomarkers of radiation exposure is an ongoing challenge in the field of radiation biology. Following an overexposure to ionizing radiation, occupationally or terrorist-related, it is imperative to obtain an accurate evaluation of absorbed dose or at the very minimum an indication of exposure especially during the first few days after exposure when identification of those individuals who would benefit most from medical intervention is key.

The challenge in predicting radiation health effects in humans is to understand how cellular responses occurring in a multi-cellular context are integrated to produce a response at the organism level. Experimental designs for studying radiation effects at the cellular level typically employ single cell types or isolated tissues and to this end many signaling pathways and molecules have been implicated in the radiation response. However, how these individual events translate into a systemic response fails to be completely understood. Gaining a better understanding of the early biochemical response of irradiated cells and tissues will allow for the opportunity to exploit these biochemical changes for the development of molecular signatures or "biomarkers" of exposure.

Results: In the series of studies presented here, we examined the potential of secreted cytokines to serve as diagnostic markers of radiation exposure based on previous research findings demonstrating activation of the peripheral immune response following IR exposure. Investigations began in a simple single cell line model and increased in complexity progressing to studies in a murine model allowing us to examine the robustness of the cytokine response in complex systems. Results thus far suggest that the chemokines MCP-1 and MIP-1β may prove to be indices of radiation exposure however inflammatory signalling cascades are complex and further investigation is needed to better understand and validate the robustness of this response.

Significance: Once identified, biomarkers of radiation exposure will provide an invaluable tool for both clinicians wanting to assess therapeutic outcomes of patients as well as emergency and medical personnel needing to rapidly triage populations of potentially exposed individuals following an RN event.

Future plans: Future work will focus on expanding the number of cytokines examined using multi-plex analysis and increasing the number of data sets from complex systems (animal models and clinical samples) to further refine and validate the potential for cytokines to be used as diagnostic markers of radiation exposure. Studies to date have yielded an extensive amount of data and biostatistical analysis to explore possible "signatures" and identification of a panel of 4-6 cytokines to be used as a diagnostic tool will be the aim of future endeavours.

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Secreted Cytokines as Possible Biomarkers of Exposure to Ionizing Radiation:

Hillary Boulay Greene; Kate Daniel; Aimee Jones; Louise Prud'homme-Lalonde ; DRDC Ottawa TR 2012-175 ; R & D pour la défense Canada – Ottawa; septembre 2012.

Introduction : La recherche de biomarqueurs fiables et uniques indicatifs d'exposition aux rayonnements est un défi dans le domaine de la radiobiologie. Suite à une surexposition aux rayonnements ionisants, professionnellement ou liée au terrorisme, il est impératif d'obtenir une évaluation précise de la dose absorbée ou, à tout le moins, une indication d'exposition en particulier au cours des premiers jours suivant l'exposition quand l'identification des individus qui profiteraient le plus de l'intervention médicale est primordial.

 Le défi dans la prévision des effets sur la santé d'individus exposés aux rayonnements ionisants est de comprendre comment des réponses cellulaires se produisant dans un contexte multi-cellulaire sont intégrées pour produire une réponse au niveau d'organisme. Les modèles expérimentaux typiquement utilisés pour étudier les effets des rayonnements ionisants au niveau cellulaire emploient des types cellulaires simples ou des tissus isolés, et à cette fin beaucoup de voies et de molécules de signalisation ont été impliquées dans la réponse à la radiation. Cependant, le processus par lequel ces événements individuels se traduisent par une réponse systémique n'est pas complètement compris. L'obtention d'une meilleure compréhension des réponses biochimiques initiales dans les cellules et les tissus irradiés permettra d'exploiter ces changements biochimiques dans le développement de signatures moléculaires ou "biomarqueurs" d'exposition.

Résultats : Dans la série d'études présentées ici, nous avons examiné le potentiel des cytokines sécrétées comme marqueurs d'exposition basé sur des données expérimentales démontrant l'activation de la réaction immunitaire périphérique après l'exposition aux rayonnements ionisants. Les études ont débuté sur un modèle de lignée cellulaire simple et ont progressé vers l'étude sur des rats afin d'examiner la robustesse de la réponse de cytokine dans des systèmes complexes. Les résultats suggèrent jusqu'à présent que les chimiokines MCP-1 et MIP-1ß puissent s'avérer être des indices d'exposition aux rayonnements, cependant les cascades de signaux inflammatoires sont complexes et des études supplémentaires sont nécessaires pour mieux comprendre et valider la robustesse de cette réponse.

Importance : Une fois identifiés, les biomarqueurs d'irradiation fourniront un outil inestimable aux cliniciens voulant évaluer les perspectives thérapeutiques de patients aussi bien qu'au personnel de secours et médical lors du triage d'individus potentiellement exposés lors d'un événement radiologique/nucléaire.

Perspectives : Les études futures se concentreront sur l'expansion du nombre de cytokines examinées avec l'analyse multiplexe et sur l'augmentation des ensembles de données des systèmes complexes (modèle animal et échantillons cliniques) afin d'améliorer et valider le potentiel des cytokines comme marqueurs diagnostiques d'exposition. Les études ont jusqu'à

présent rapporté une vaste quantité de données et d'analyse biostatistique qui nous permettra de concentrer nos activités futures sur l'exploration de « signatures » possibles et l'identification d'un panel de 4 à 6 cytokines comme outil diagnostique.

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Acknowledgments

Introduction

The quest for reliable and unique biomarkers of radiation exposure is an ongoing challenge in the field of radiation biology. Following an overexposure to ionizing radiation, occupationally or terrorist-related, it is imperative to obtain an accurate evaluation of absorbed dose or at the very minimum an indication of exposure especially during the first few days after exposure when dose information would identify those individuals who would benefit most from medical intervention [1]. In reality, the majority of individuals are likely to receive low and biologically insignificant doses however identifying these individuals is of equal importance in a mass casualty situation where medical resources may be limited and need prioritization.

A number of approaches for dose estimation are currently available including physical dosimetry (personal dosimeters), clinical signs and symptoms and biological dosimetry however they are not without their practical limitations. It is suggested that these capabilities for dose assessment are inadequate for immediate evaluation of radiation injury and would be insufficient in a mass-casualty situation where there is potential for thousands of individuals to be exposed. The reality is such that accurate physical dosimetry is often not available, especially in accident situations. Clinical estimation is based on the observation of symptoms (such as nausea, vomiting, fatigue, hypotension), but these are only typically displayed at high dose exposures (>2 Gy) and can take days to weeks to present themselves depending on the individual. Moreover, diagnosis is easily complicated by possible chemical/biological exposure, physical injury, psychosomatic effects and fatigue. Lymphocyte (white blood cell) depletion kinetics has been demonstrated to be useful for dose estimation soon after an exposure however due to the large variation in lymphocyte counts among the population it is recommended that serial measurements over time be taken and this is not conducive for use during a mass casualty situation [2]. Cytogenetic analysis, the gold standard for biological dosimetry, is highly radiation specific and relies on the examination of radiation-induced damage in chromosomes of peripheral lymphocytes. The assay is accurate, highly sensitive and analysis can provide dose estimates at lower dose exposures (down to 0.2 Gy) [3], however these assays are very time consuming, labour and expertise intensive and inappropriate for rapid surveillance and use in the field as dose estimations can take days-weeks to achieve. Therefore, it is clear that there is an urgent need to develop novel emerging technologies to supplement the current capabilities for rapidly identifying radiologically-exposed individuals and furthermore assessing emergency radiation doses for triage purposes.

Over the years, an extensive amount of research has focused on gaining a better understanding of the early events following ionizing radiation (IR) exposure (DNA damage induction, free radical production, and gene and protein activation) that lead to cellular and tissue injury. What has become evident is that the biochemical response of mammalian cells to radiation exposure is extremely complex [1]. The increased understanding of the early biochemical response of irradiated cells and tissues provides the opportunity to exploit these biochemical changes for the development of molecular signatures or "biomarkers" of exposure.

Several biological studies have highlighted the importance of the peripheral inflammatory response following radiation exposure. Over the past 3 decades it has been discovered that proteins can regulate communication among cells of the immune system. These proteins, called cytokines, are produced by a wide variety of cell types either constitutively or after induction and

are involved in virtually all aspects of inflammation, including development and function of the immune system, cell proliferation and differentiation, cellular recruitment and activation and regulation of cellular interactions [4]. Cytokines act as signalling molecules between cells and tissues and there is increasing evidence that they are part of the systemic inflammatory response following radiation exposure [5, 6, 7, 8].

It has been stated by the Department of Homeland Security that "within the first 72 hours tools are needed that can detect whole body does of between 1-8 Gy" [9]. Many approaches to developing such tools have included different cytogenetic end-points – premature chromosome condensation [10] and micronuclei assays [11], as well as protein biomarkers including histone phosphorylation (γ-H2AX) [12, 13], and global proteomic changes [14]. Changes in gene expression have also been examined as a promising approach for dose estimation and biomarker use [15, 16]. It is believed that selected molecular biomarkers are likely to provide a more robust approach for high-throughput, minimally invasive radiation biodosimetry when compared to existing capabilities [17].

The objective of the work presented here was to identify potential biomarkers of radiation exposure in order to greatly simplify and accelerate the diagnosis of radiation injury. We hypothesized that following exposure to ionizing radiation the induced changes in cytokine expression and secretion may serve as potential biomarkers of exposure. Once identified these markers could be useful for future assay development.

Once identified, biomarkers of radiation exposure will provide an invaluable tool for both clinicians wanting to assess therapeutic outcomes and sensitivities of patients as well as emergency and medical personnel in the field needing to rapidly triage populations of potentially exposed individuals following an RN event.

1   Study Design

1.1   Rationale

The challenge in predicting radiation health effects in humans is to understand how cellular responses occurring in a multi-cellular context are integrated to produce a response at the organism level. Experimental designs for studying radiation effects at the cellular level typically employ single cell types or isolated tissues and to this end many signaling pathways and molecules have been implicated in the radiation response. As noted previously, the response to ionizing radiation exposure is highly complex involving many cellular events and signaling cascades. However, how these individual events translate into a systemic response fails to be completely understood.

Limited by the availability of appropriate animal models and ethical implications of using non-human primates, there is a need to develop *in vitro* model systems which take into consideration the multi-cellular response of an organism following radiation exposure. As part of this study we embarked on the development of a multi-cell culture system to meet these needs.

To determine the potential for secreted cytokines to serve as radiation-responsive biomarkers, we examined the cytokine response following IR exposure in various model systems. From a simple single cell line system through to whole-body exposures in rats, we were able to examine the robustness of the signal through increasingly complex systems.

1.2   Model systems

1.2.1   Human Cell lines

In the development of the multi-cell culture system, six (6) human established cell lines were selected to simulate the peripheral circulatory/immune system. These included fibrobasts (lung, normal; HFL1), myeloblasts (HL-60), lymphoblasts (TK6, K-562), osteoblasts (SaOS-2) and endothelial cells (umbilical vein, normal; HUV-EC-C). Three of the cell lines grew as adherent cultures (HFL1, HUVEC-C, SaOS-2) and 3 were grown in suspension (TK6, K-562, HL-60) (Figure 1). All cell lines were purchased from ATCC.

Prior to examining the radiation response of the multi-cell culture system, preliminary investigations focused on the characterization of each of the individual cell lines in the model. Their independent growth kinetics and cellular radiation responses (viability and cytokine profiles) were examined following exposure to gamma radiation (Co-60).
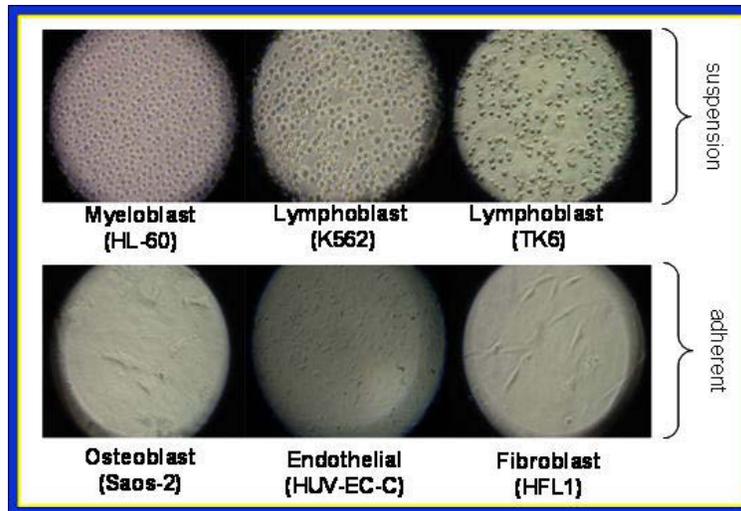
Figure 1: Human cell lines used to develop multi-cell culture system for studying the systemic response to ionizing radiation and biomarker identification

1.2.2   Multi-cell Culture System

In the body, various cell types with varying kinetic cycles, cellular distribution patterns and post-irradiation responses contribute to the overall response of the system. The multi-cell culture model allows us to examine the radiation response of an *in vitro* system that more closely approximates the human systemic response to ionizing radiation in hopes of identifying novel biological markers of radiation exposure.

As cell-to-cell communication has been described as a critical component in the early radiation response, the system was designed with this in mind. A six well plate and trans-well inserts were used to keep the cell types segregated while permitting the free exchange of media, growth factors, cytokines and other soluble peptides across the membrane (0.4 μm pore size), mimicking the cross-talk and exchange that might be observed in the body (Figure 2).
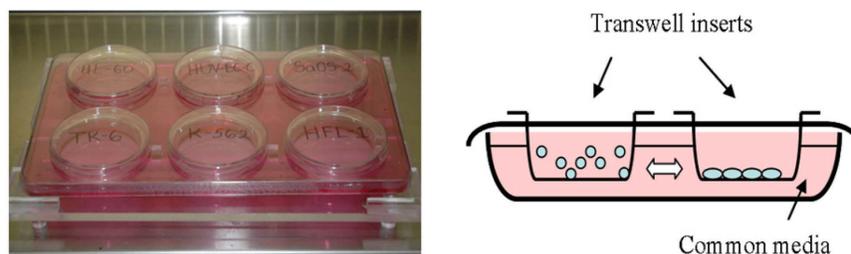
Figure 2: Multi-cell culture system design

Each of the cell lines were brought to the appropriate concentrations for culture, irradiated individually and then plated in the multi-cell culture system where they were cultured for 96 hrs. To examine the potential of secreted cytokines being used as a biomarker of exposure, the cytokine profile of the shared media was examined following IR exposure (0-2 Gy) at various time points throughout the experiment. Growth response curves for each of the cell types were also examined to see how the cells responded to the culture system.

1.2.3   Co-Culture System

As an alternative model to the multi-cell culture system, additional experiments were conducted where all physical barriers were removed and the six cell lines were allowed to grow in proximity and share a common environment (Figure 3). All cells were seeded in a 25cm^2 flask at the same ratios used in the trans-well system. The culture protocol (radiation exposure and length of culture) was the same as the multi-cell culture system and the common media was sampled at various time points for cell counts and cytokine analysis. Within this model, individual cell types could not be monitored for growth kinetics and thus there was no way of determining if one cell type or another was dominating the culture. In the future cell surface markers will be used to distinguish and identify the various cell types in the system so that it can be sufficiently characterized and monitored.
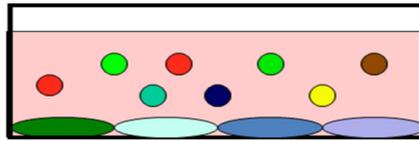
Figure 3: Schematic of co-culture system showing cell lines grown without barriers.

1.2.4   Human Engineered Skin Equivalents

The skin is the first line of defence for radiation exposure and depending on the penetration of the insult can illicit an extensive immune reaction. The cutaneous radiation response is an important and often limiting factor to radiation therapy and the dermal inflammatory response has been implicated in the pathophysiology of radiation injury [4]. It is therefore an ideal model for biomarker investigation.

In the current research we employed the commercially available *in vitro* human skin model, EpiDerm FT (full thickness) to examine the multi-cellular dermal cytokine response following radiation exposure.

The EpiDerm FT model (MatTek Corp, MA, USA) is a normal human-derived 3D skin equivalent composed of highly differentiated epidermal keratinocytes and dermal fibroblasts. The cells are cultured to form a multi-layer model of human dermis and epidermis that is ultra-

structurally parallel to human skin including the presence of a well developed basement membrane and *in vivo*-like lipid profile (Figure 4). EpiDerm FT consists of organized basal, spinous, granular and cornified epidermal layers analogous to those found *in vivo*. The model is mitotically and metabolically active and has growth characteristics that are uniform and highly reproducible making it an ideal *in vitro* system to assess dermal responses to various stressors including ionizing radiation [18, 19]. The cells are cultured on specifically prepared cell culture inserts making them easy to handle and transfer during experiments. The inserts are placed in culture dishes containing serum free media where the membrane (0.4 μm pore size) the cells are grown on is in contact with the growth media, leaving the upper cell layers exposed to the air.
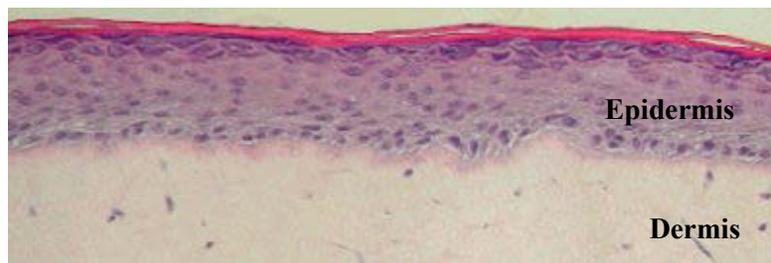
Figure 4: Representative histology of EpiDerm FT model (Photo courtesy of MatTek)

In the current study, skin inserts were exposed to significant doses of gamma radiation (0-16 Gy) and cell viability and cytokine profiles of the growth media were monitored over the course of the experiment.

1.2.5   Animal model

To evaluate the potential for using circulating cytokines as biomarkers for radiation exposure, we made use of on-going experiments at Atomic Energy of Canada Limited (AECL)'s Chalk River Laboratories. As part of a collaborative study with AECL and Health Canada, male Wistar rats received whole body exposures to ionizing radiation (0-5 Gy). Serum samples were collected at various time points, including prior to exposure as well as 24, 48, and 72 hrs following irradiation. Samples were shipped on dry ice back to DRDC Ottawa where cytokine analysis was performed.

All animal work was conducted at AECL under the approval of the animal care committee and strict adherence to protocols [20].

2   Materials and Methods

2.1   Cell Culture

All cell lines were maintained in a humidified incubator at 37°C, 5% CO_2 , 95% air in 75 cm^2 flasks. Roswell Park Memorial Institute (RPMI)-1640 media supplemented with 2 mM L-glutamine and 10% fetal bovine serum was selected as the common media for the multi-cell culture system. For characterization studies each of the cell lines were suspended in media to the appropriate concentration required (6.0×10^4 - 2.0×10^5) and exposed to gamma radiation (0, 1, 2 Gy). Immediately following irradiation, cells were seeded into 6 well culture plates in 2mL complete media and cultured over 96 hrs. At 24 hr intervals, cells were harvested for counting and viability analysis and supernatant collected for cytokine analysis. Growth curves and doubling times for each of the cell lines was assessed.

During the multi-cell culture system experiments, following irradiation (0-2 Gy) each of the 6 cell lines were seeded (Table 2) onto 24 mm cell culture inserts with a transparent PET membrane (0.4 μm) (Becton Dickinson) in 2 mL media and placed into a custom manufactured (CRC Model shop) 6-well culture insert companion tray containing 60 mL of complete RPMI media. Adherent cell were allowed to adhere for 2 hrs before the culture system plates were placed on a Stovall rocking shaker set on low in the incubator (37°C, 5% CO2, 95% air) to promote diffusion of signalling molecules across membranes into the shared media. To facilitate harvesting of the cells, 1 plate per time point (24, 48, 72, 96 hr) per dose (0, 1, 2, Gy) was used for a total of 12 plates. At each time point, aliquots of the shared media were collected and stored at -80°C for cytokine analysis. Cells were harvested from the inserts accordingly (adherent cells required trypsinization) for counting and viability analysis. Cell pellets were collected, treated with Trizol and stored at -80°C for future genomic or proteomic work.

Table 1:   Cell densities used in the multi-cell culture system.

	Cell Line	Cell concentration	# cells / insert
Suspension	HL-60	1×10^5/mL	2×10^5
Suspension	K562	5×10^4/mL	1×10^5
Suspension	TK6	1×10^4/mL	2×10^4
Adherent	HFL1	3×10^4/mL	6×10^4
Adherent	HUVEC	4×10^4/mL	8×10^4
Adherent	SaOS-2	5×10^4/mL	1×10^5

For the co-culture system, cell densities were modified to fit in a 25 cm^2 culture flask. Appropriate concentrations of the suspension cell lines and appropriate numbers of adherent cells were added to the culture flask for a total volume of 12 mL complete RPMI media and were irradiated (0, 1, 2 Gy) on Day O. One T-25 flask per dose was used. Following irradiation the flasks were returned to the incubator. At 24 hr intervals, 150 μL of the culture media was collected for cytokine analysis and stored at -80°C. Viability of all cells was assessed after 96 hrs.

2.1.1   Human Skin Equivalents

Tissues purchased from MatTek Corp were allowed to equilibrate overnight at 37°C, 5%CO$_2$, 95% air in EFT-Assay media (Modified DMEM, MatTek) prior to the beginning of the experiments. Skin inserts (1.2 cm diameter) were exposed to gamma radiation (2-16 Gy, sham-irradiated tissues serving as controls) on Day 0, transferred to 6-well culture dishes with media and placed back in the incubator. Tissue inserts were placed on culture stands in the culture dish to allow for 5mL of media to be used for feeding of tissues throughout the duration of the experiments. Inserts were maintained in culture for 96 hrs. At 24 hr intervals, a non-destructive viability assay was performed on each insert and a 500 μL sample of supernatant was collected for cytokine analysis. An equivalent volume of fresh media was added back to the well to maintain a constant volume.

2.1.2   Irradiation

Samples (cells in suspension and skin inserts) were exposed to varying doses depending on experimental design (0-16 Gy) using a Co-60 gamma beam-150C irradiator (JL Shepherd and Associates, San Fernando, CA). The dose rate was periodically checked and verified using a calibrated electrometer (Farmer model #2670A; Nuclear Enterprise Technology Ltd., Beenham, UK) and an ionization chamber (NE model #2581; Nuclear Enterprise Technology Ltd.). All irradiations were performed to ensure homogenous exposure to the samples.

2.1.3   Viability Analysis

2.1.3.1   Guava ViaCount®

To assess cell number and viability of the cells in culture, a Guava EasyCyte flow cytometer (Guava Technologies Inc, Hayward, CA) and ViaCount reagent (Millipore) were used. The assay allows for rapid, highly reproducible cell counting and assessment of apoptotic populations within the culture. The assay distinguishes viable and non-viable cells based on differential permeabilities of two DNA-binding dyes in the reagent. Briefly, 20 μL aliquots of cell suspension were incubated with ViaCount reagent for 5 min in the dark at room temperature. The suspension is then run on the flow cytometer with appropriate gate settings for the assay. Instrument software provides total cell counts (in cells/ mL) and the number of viable cells in the cell population for each sample.

2.1.3.2 AlamarBlue®

Health of the skin inserts was assessed on a daily basis using a fluorescent metabolic activity assay alamarBlue®. This non-toxic, water soluble reagent is used to measure metabolic activity of the cells and can be used in-situ with out cell lysis so that cell viability can be repeatedly monitored over time. Briefly, skin inserts were placed in a clean 6 well-plate with 2 mL fresh media and prepared alamarBlue solution was added. Tissues were incubated at 37°C for 1 hr to allow uptake and processing of the reagent. Following incubation, the inserts were removed, rinsed in PBS and returned to their original culture dishes. AlamarBlue/medium samples were pipetted into replicate wells of a 96-well black plate with clear bottom and read at 530nm excitation/590nm emission on a BioTek spectrophotometer. Cell free controls were used as background and subtracted from sample absorbance values. All values were compared to the 0 Gy exposure group.

2.2 Animal studies

Male Wistar rats were kept in a metabolic cage (Tecniplast Inc. BUGUGGIATE (VA) Italy) with food and water supplied ad libitum for 24 hours prior to exposure and for 72 hours following exposure to whole body ionizing radiation.

2.2.1 Irradiation

Animals were exposed to external radiation (at approximately 8.5 weeks of age) using the Gamma Cell 200 (Co-60) at AECL Chalk River Laboratories for either 9.1 minutes (1 Gy), 27.6 minutes (3 Gy) or 46.1 minutes (5 Gy). Control animals were placed into the gamma cell chamber for 10 minutes but were not exposed to the source. Animals were supplied with breathing air while in the gamma cell chamber.

2.2.2 Blood collection

Blood samples were collected at -24hr, +24hr, +48hr and +72hr time points. Blood was taken from the saphenous vein (lateral or medial) using an animal lancet (Goldenrod animal lancet, MEDIpoint International, INC. Mineola, NY USA) and collected into BD Microtainer® Tubes (Cat #365956, BD Diagnostics. Franklin Lakes, NJ USA) and allowed to clot for 30 minutes. After clotting serum was collected by centrifugation at 10000 RPM for 5 minutes and transferred into 0.5mL eppendorf tubes. Samples were stored at -80°C before being shipped to DRDC Ottawa for cytokine analysis.

2.3 Cytokine Analysis

Cytokine analysis was performed using the Bio-Plex 200 suspension array system (Bio-Rad, Hercules, CA) that allows for multiplex analysis on a single sample. Secreted cytokine levels in the samples were assessed using a multi-plex assay prepared according to the manufacturer's instructions (Bio-Rad). Table 1 outlines which assays were used for different experimental models.

Supernatants of irradiated cells and skin inserts, and rat serum samples were collected at different time points following exposure (0-96 hrs) and stored at -80°C until analysis could be performed. Media aliquots or serum samples were thawed on ice, vortexed and centrifuged for 10 min at 1000X g. Serum samples were diluted 1:4 prior to use. Total sample volume was 50 μL and all samples were run in duplicate. In brief, cytokine antibody-conjugated magnetic beads were added to each well of a black flat and clear-bottom 96-well plate. Samples were pipetted into the wells, incubated and washed using a Bio-Plex Pro wash station (Bio-Rad Inc). Diluted detection antibody was added and allowed to incubate for 30 min. The plate was washed again to remove excess detection antibody and streptavidin-phycoerythrin was added. Following a 10 min incubation, the plate was washed and assay buffer was added to resuspend the beads for data acquisition. Fluorochromes bound to the magnetic beads were quantified by the Luminex-200. Cytokine quantification was determined by assessment against prepared standards and plotted standard curves for each analyte. Intensity of the fluorescence was directly proportional to the concentration of the cytokine in the sample. All calculations were done internally to the Bio-Plex Manager software version 6.0 (Bio-Rad Inc).

Table 2: Multi-plex Cytokine Assays used for different model systems

Model System	Multi-plex Assay	Analytes examined
Individual Cell lines	Human 17-plex	IL-2, IL-4, IL-6, IL-8, IL-10, GM-CSF, IFN-γ, TNF-α, IL-1b, IL-5, IL-7, IL-12, IL-13, IL-17, G-CSF, MCP-1, MIP-1β
Multi-cell Culture System		
Human Skin Equivalents		
Animal studies - Rat	Rat 23-plex	IL-1a, IL-1b, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12 (p70), IL-13, IL-17A, IL-18, EPO, G-CSF, GM-CSF, GRO/KC, IFN-γ, M-CSF, MIP-1α, MIP-3α, RANTES, TNF-α, VEGF

2.4   Statistical Analysis

Mean with standard error of the mean is reported as applicable. A minimum of 3 independent experiments were conducted for all cell based experiments. For each cytokine examined, two-way analysis of variance (ANOVA) was used to determine interactions between time and radiation dose. At each time point cytokine levels of the irradiated groups were compared to non-irradiated controls (0 Gy) using Bonferroni post-tests. Significance was set at 5%. GraphPad Prism (GraphPad Software Inc, La Jolla, CA) was used for all statistical analyses.

3 Results

3.1 Characterization of Cell lines Used in Multi-Cell Culture System

3.1.1 Growth Characterization and Radiation Sensitivity

Each of the cell lines to be used in the multi-cell culture system was characterized for their radiation response and growth in RPMI media as this was the media selected to be used in the culture system. Cell lines were exposed to Co-60 gamma rays and growth performance was monitored over 96 hrs in culture. Cell viability of each of the cell lines following radiation exposure is shown in Figure 5. Doubling times were calculated from the linear portion of the curve and are shown in Table 3. Data are presented as the mean number of viable cells ± SEM and represent three independent replicates (n=3).
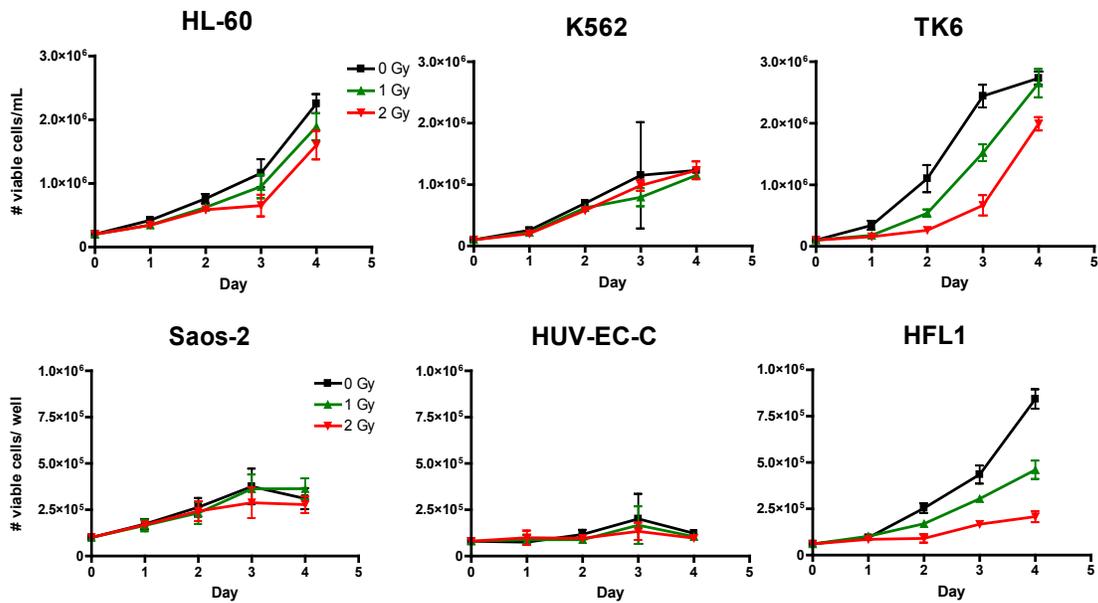

Figure 5: Growth response curves for each of the cell lines used in the multi-cell culture system with or without exposure to ionizing radiation (n=3).

Doubling Time (hr)			
	Dose (Gy)		
Cell line	0	1	2
HL-60	28.5	30.5	34.8
K562	17.5	23.4	21.1
TK6	15.5	18.0	27.9
Saos-2	42.2	43.1	54.4
HUV-EC-C	41.8	58.4	62.9
HFL1	27.8	39.4	76

It can be seen from the data that the three different adherent cell lines and the three suspension cell lines differ in their sensitivity to ionizing radiation. Radiation exposure affected the growth of the cells to varying degrees and ultimately reduced their doubling time. The least to most sensitive for the adherent cells was SaOS-2 › HUV-EC-C › HFL1 while for the suspension cells it was HL-60 › K562 › TK6.

3.1.2   Cytokine profiles of individual cell lines

To identify potential biomarkers of radiation exposure, cytokine profiles for each of the cell types in the multi-cell culture system were assessed following exposure to radiation. Not all of the cytokines in the 17-plex assay were expressed by the cell lines and only those cytokines that were expressed at detectable levels are presented (Figures 6-10). Cytokine expression of the endothelial HUV-EC cells was highly variable between replicates and is not presented here.

Expression of several cytokines was significantly effected by radiation dose over time. Data are presented as the mean cytokine concentration ± SEM and represent four independent replicates of the experiment (n=4). Significance levels are expressed as * $p<0.05$, ** $p<0.01$, *** $P<0.001$ and are relative to the 0 Gy controls.

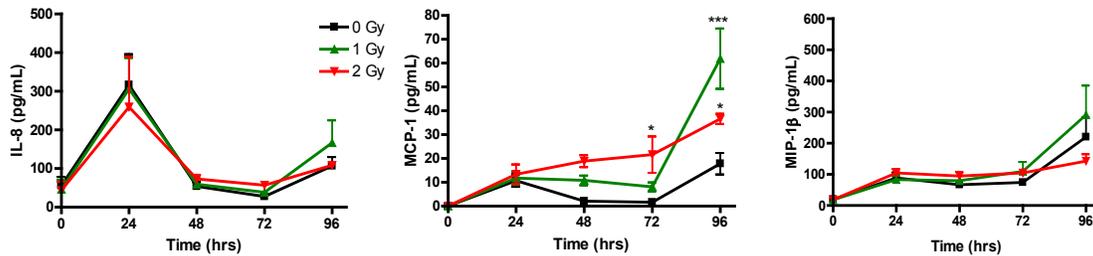*Figure 6: Cytokine profiles for HL-60 myeloblast cells exposed to ionizing radiation (0, 1, 2 Gy)*
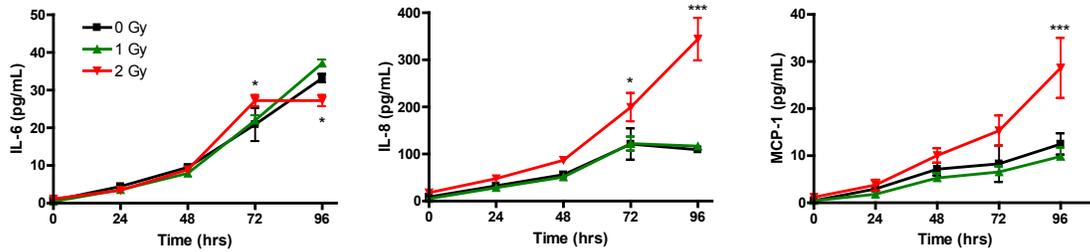
Figure 7: Cytokine profiles for K562 lymphoblast cells exposed to ionizing radiation (0, 1, 2 Gy).
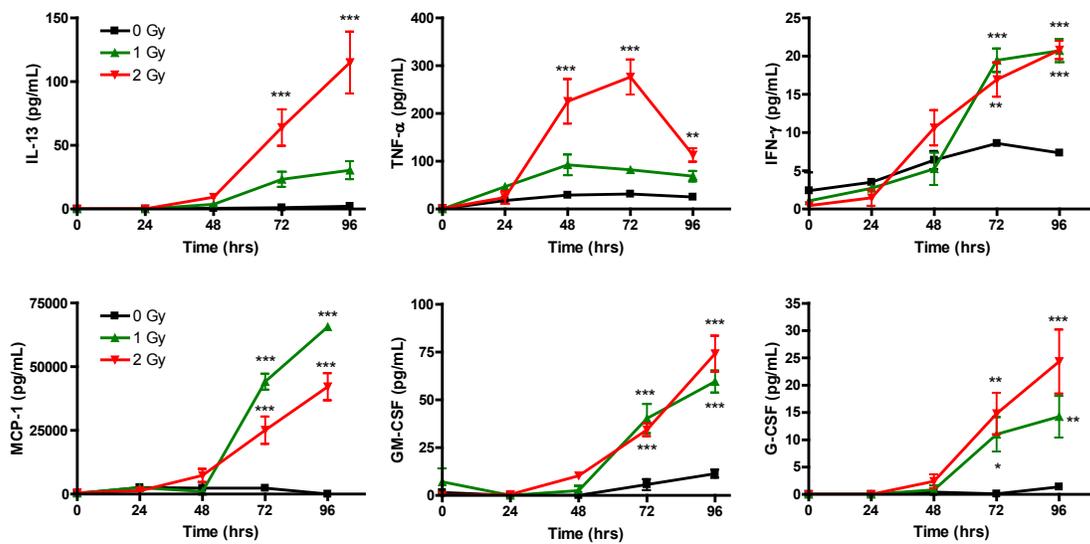
Figure 8: Cytokine profiles for TK6 lymphoblast cells exposed to ionizing radiation (0, 1, 2 Gy)
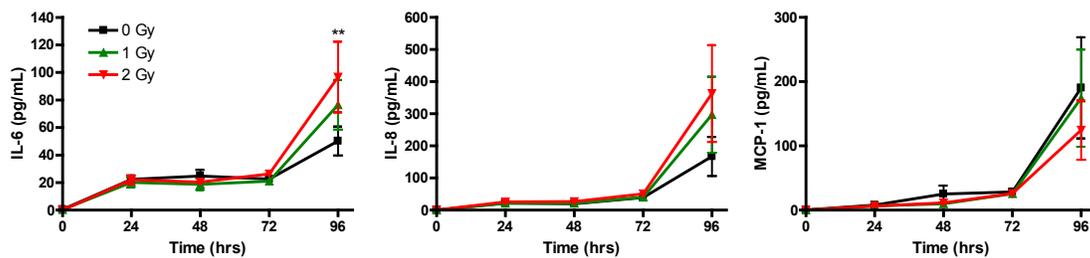
Figure 9: Cytokine profiles for SaOS-2 osteoblast cells exposed to ionizing radiation (0, 1, 2 Gy)
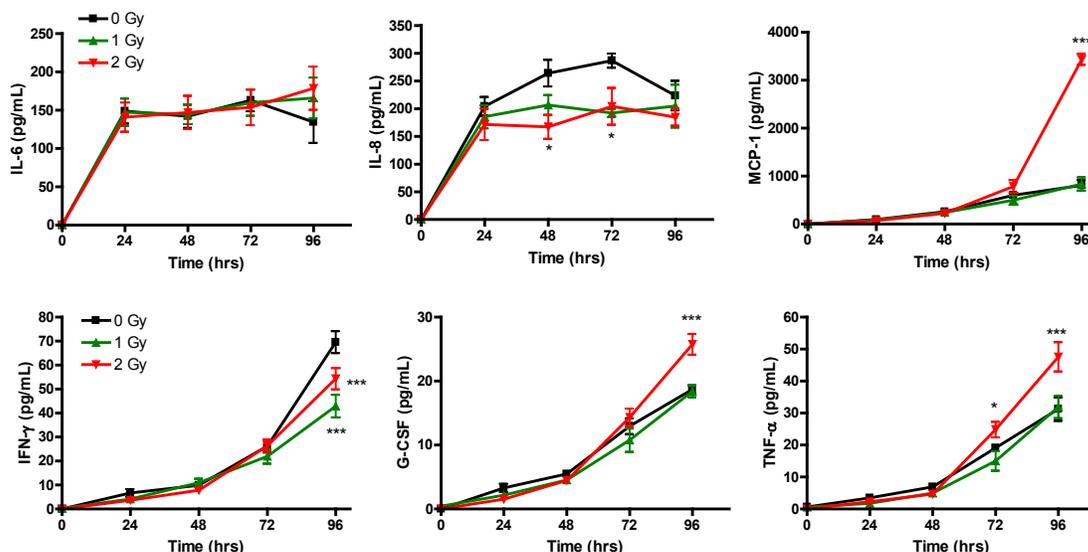
Figure 10: Cytokine profiles for HFL1 fibroblast cells exposed to ionizing radiation (0, 1, 2 Gy).

Although not all cell lines expressed all of the cytokines examined, several cytokines were significantly increased following radiation exposure including IL-6, IL-8, IL-13, MCP-1, TNF-α, IFN-γ, GM-CSF, and G-CSF. Not surprisingly, radiation-induced cytokine expression was most prominent in most radiosensitive cell lines, TK6 (lymphoblast) and HFL1 (fibroblast).

3.2   Multi-cell Culture Systems

3.2.1   Growth response curves

The growth kinetics and radiation response of the cell lines were examined when the cells were grown in shared media in the 6-well multi-cell culture system and exposed to ionizing radiation (Co-60, 0-2 Gy; Figure 11). Data are presented as mean number of viable cells ± SEM and represent six independent replicates of the experiment (n=6). Similar to the observations from the cell line characterization studies, TK6 and HFL1 were the most radiation sensitive cells and their growth was significantly decreased following IR exposure. Interestingly, the myeloblast cell line HL-60 had an increased sensitivity to radiation in the multi-cell culture system compared to the characterization studies when grown independently. Significance levels are expressed as ** $p < 0.01$, and *** $p < 0.001$ relative to 0 Gy time points.
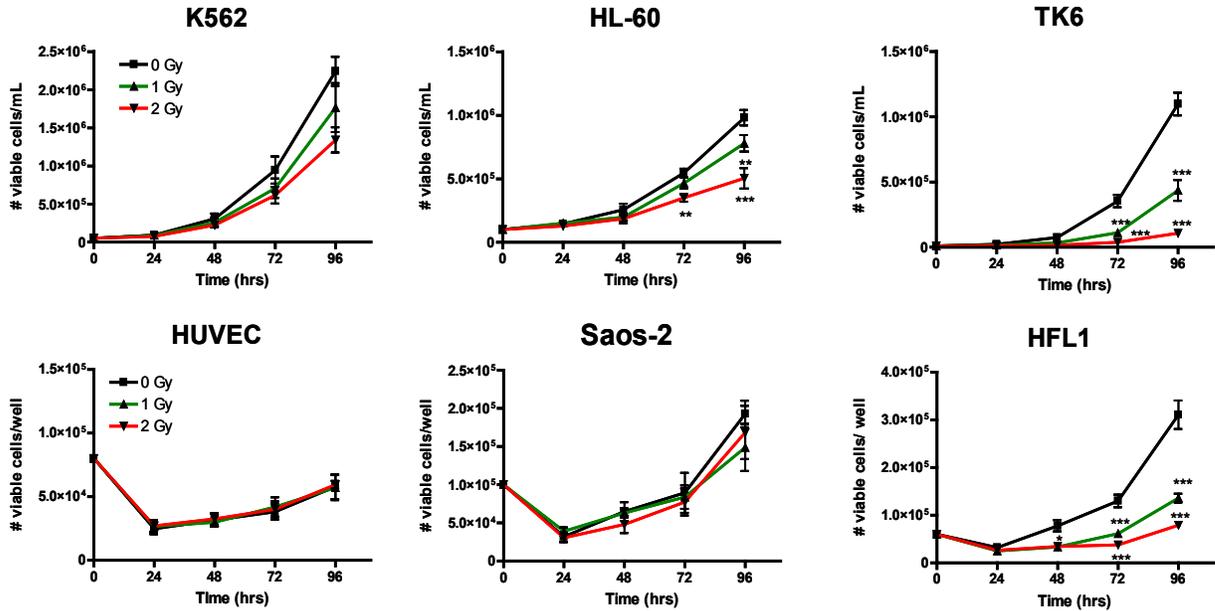
Figure 11: Effect of radiation exposure on growth of each of the cell lines used in the multi-cell culture system (MCCS)

Growth kinetics were also examined when the cells were grown under the co-culture conditions (in the same 25 mL flask; Figure 12) however it was not possible to determine which cells were contributing to the viability counts as the different cell lines could not be differentiated. The overall response of all cells in culture (the "system") is depicted in Figure 12. Data is expressed as the mean number of viable cells $\pm$ SEM and represents three independent replicates (n=3).
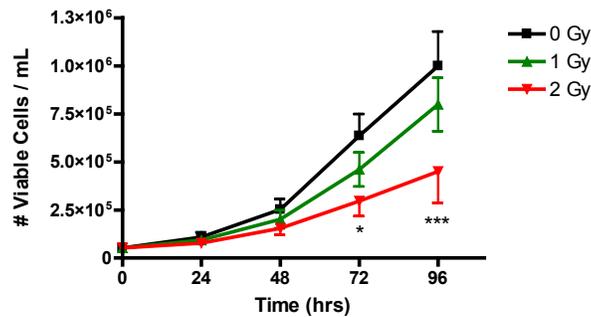
Figure 12: Effect of radiation exposure on the growth response curve of cells in the co-culture system (CCS)

3.2.2 Cytokine profiles of shared media

3.2.2.1 Multi-cell culture system

To examine the systemic response of the culture system following radiation exposure and evaluate the potential of secreted cytokines serving as biomarkers, cytokine analysis was performed on the shared media of the multi-cell culture system. Of the 17 cytokines examined, detectable levels for only 4 cytokines were observed and their expression pattern following IR is shown in Figure 13.

Interestingly several cytokines, MCP-1 and MIP-1β were significantly (* $p<0.05$, *** $p<0.001$) decreased over time following IR exposure. The data are presented as mean cytokine concentration ± SEM and represent six independent replicates (n=6). Overall, cytokine levels in the shared media were quite low, likely due to the volume of media used in the system (50 ml).
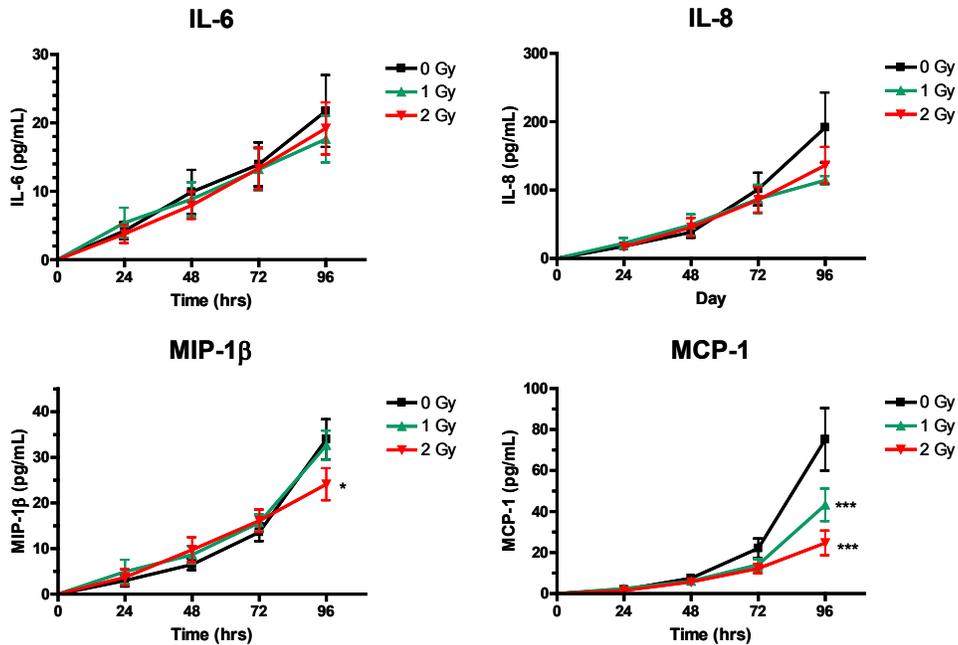
Figure 13: Effect of radiation exposure on the cytokine profile of the shared media in the multi-cell culture system (MCCS)

3.2.2.2 Co-culture system

Cytokine analysis was also performed on the shared media from the co-culture system and is shown in Figure 14. A greater number of cytokines were detectable in the media from the co-culture system and in contrast to the multi-cell culture system several cytokines demonstrated a significant increase following exposure to ionizing radiation, including MCP-1 and MIP-1β. Data are presented as mean cytokine concentration $\pm$ SEM and represent three independent replicates (n=3).
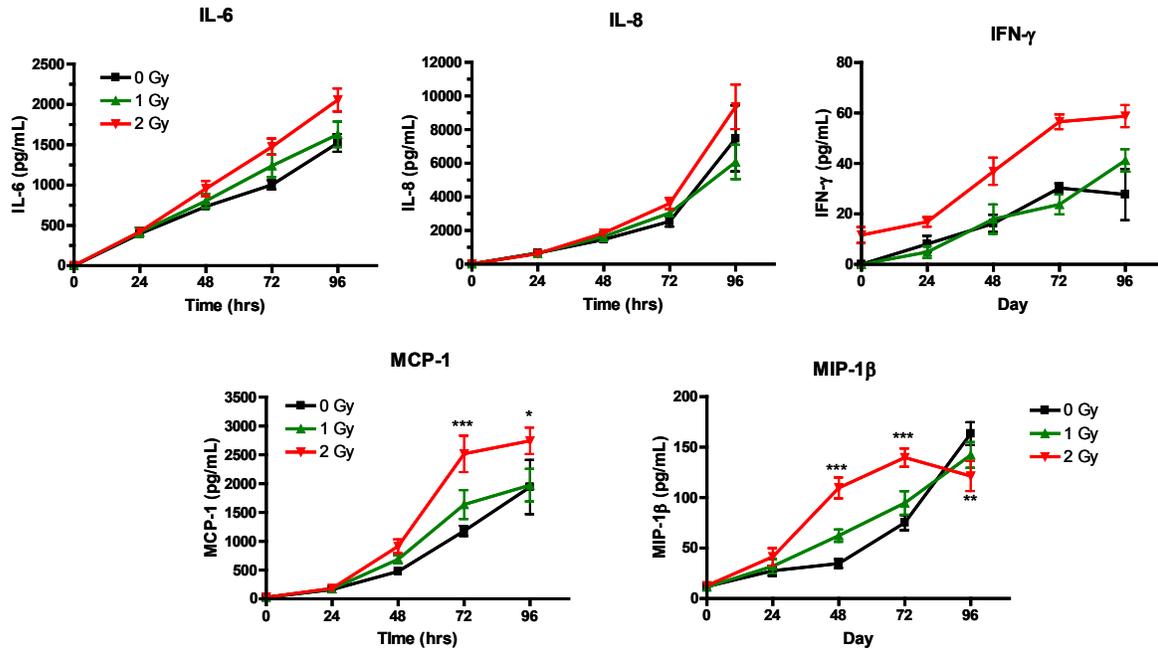
Figure 14: Effect of radiation exposure on the cytokine profile of the shared media in the co-culture system (CCS)

3.3 Summary of Cell Model Radiation-induced Cytokine Response

To investigate the potential for secreted cytokines to serve as biomarkers of radiation exposure, early studies focused on cell line-based models. Three models were investigated including the characterization of the radiation response of six individual cell lines, the multi-cell culture system (MCCS) where the cells were grown in trans-well inserts with shared common media, and the co-culture system (CCS) where the cells were grown together in the same culture flask. An abundant amount of data was generated and for those cytokines expressed at detectable levels, the results were presented in the previous sections. As a summary and for ease of comparison the results of all three "systems" are shown in Table 4. It can be seen that each cell line used in the model has a unique pattern of cytokine expression and the majority of cytokines responded with increased expression following ionizing exposure. In these studies, the pro-inflammatory chemokine MCP-1 was the most commonly up-regulated cytokine following radiation exposure.

Table 4: Cytokine expression in the three culture models following exposure to ionizing radiation

		Cytokines								
		IL-6	IL-8	IL-13	G-CSF	GM-CSF	IFN-γ	MCP-1	MIP-1β	TNF-α
Cells Cultured Individually	HL-60							↑		
	K-562	↑	↑					↑		
	TK-6			↑	↑	↑	↑	↑		↑
	HFL1		↓		↑		↓	↑		↑
	HUV-EC-C									
	Saos-2	↑								
MCCS								↓	↓	
CCS		↑	↑				↑	↑	↑	

3.4 EpiDerm FT Human Skin Equivalents

To examine the dermal radiation response and evaluate the potential of secreted cytokines to serve as biomarkers of radiation exposure, engineered human skin equivalents were exposed to varying doses of gamma radiation (Co-60, 0-16 Gy) and cultured over 96 hrs. Cytokine concentrations in the growth media were analyzed using BioPlex protein suspension array technology.

3.4.1 Viability of skin inserts following radiation exposure

To monitor the health and viability of the skin inserts through out the duration of culture, the non-toxic alamarBlue assay was used (Figure 15). Data for each exposure dose was normalized to the 0 hr time point and expressed as mean viability ± SEM. Four independent replicates of each experiment were performed (n=4).
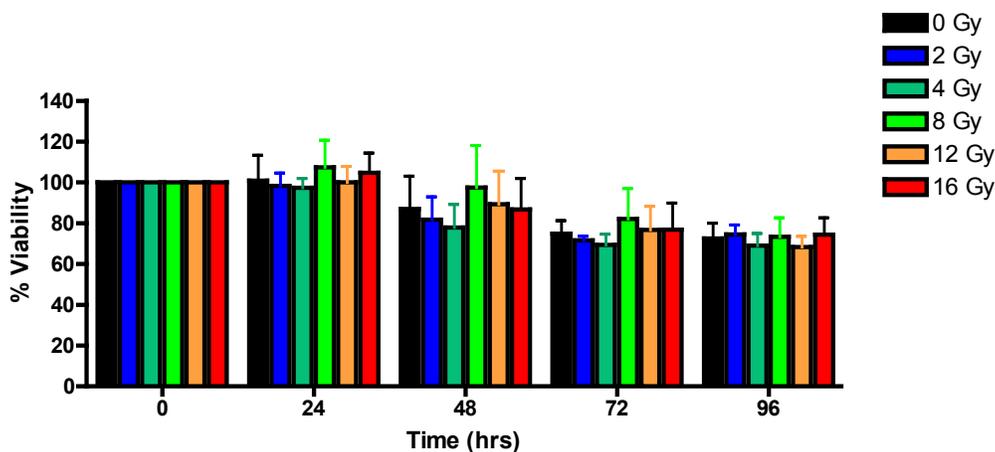
Figure 15: Viability of human skin equivalents in culture following exposure to ionizing radiation.

Viability of the skin tissues was maintained throughout the culture period and did not appear to be significantly affected by the gamma radiation exposure. This is consistent with previous findings that suggest the skin is able to withstand significant doses of radiation (> 10 Gy) without detriment in the short term [21]. Towards the end of the culture period the viability of the inserts decreased to just below 80% however this was independent of dose and was anticipated based on the manufacturer's instructions as the media was not replenished daily as suggested.

3.4.2 Cytokine response of human skin inserts

Secreted cytokines from the engineered human skin inserts were evaluated in the growth media at different time points following irradiation. Of the 17 cytokines examined 9 were expressed by the skin tissues at detectable levels. Their response following exposure to ionizing radiation is shown in Figure 16 below. Data are presented as mean cytokine concentration ± SEM and represent five independent replicates of the experiment (n=5).
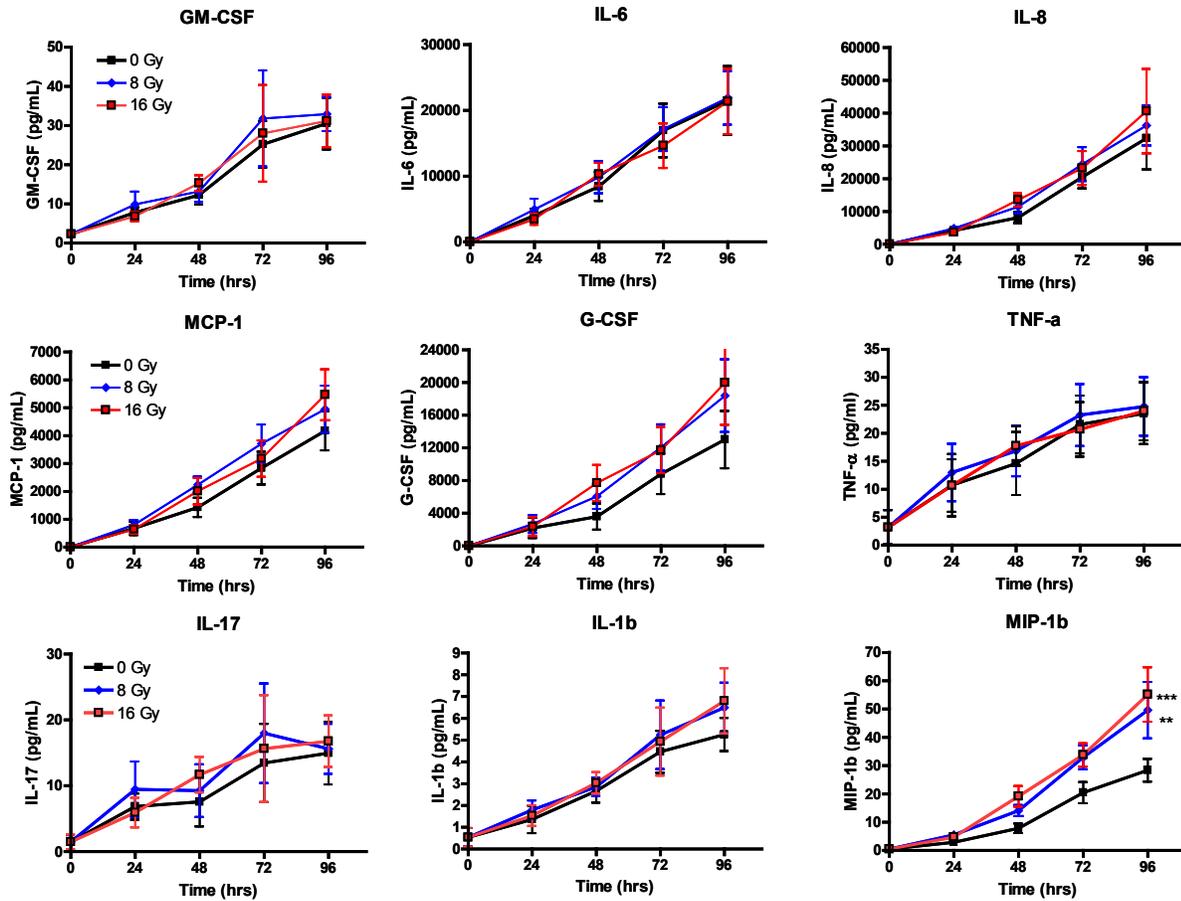
Figure 16: Effect of ionizing radiation on the secreted cytokine profiles of human skin inserts

At 96 hrs post exposure, MIP-1β was the only cytokine that was significantly increased relative to the 0 Gy control. In general cytokine levels increased over time and although not significant there was a general trend for increased expression with increasing radiation dose.

3.5 Animal Studies

To evaluate the potential for cytokines to serve as markers of radiation exposure, cytokine analysis was performed on serum samples from rats exposed to whole body gamma radiation at various time points post-irradiation. All 23 cytokines examined were expressed at detectable levels in the serum, however little to no effect was observed from radiation exposure. Cytokine levels remained constant over the time period examined. Representative data for the cytokine response is shown in Figure 17. Data for the remaining cytokines examined is not shown. Data are presented as mean cytokine concentration ± SEM and represent 5 -7 animals per dose.

Figure 17: Effect of whole body ionizing radiation exposure on serum cytokines in Wistar rats

4   Discussion and Conclusions

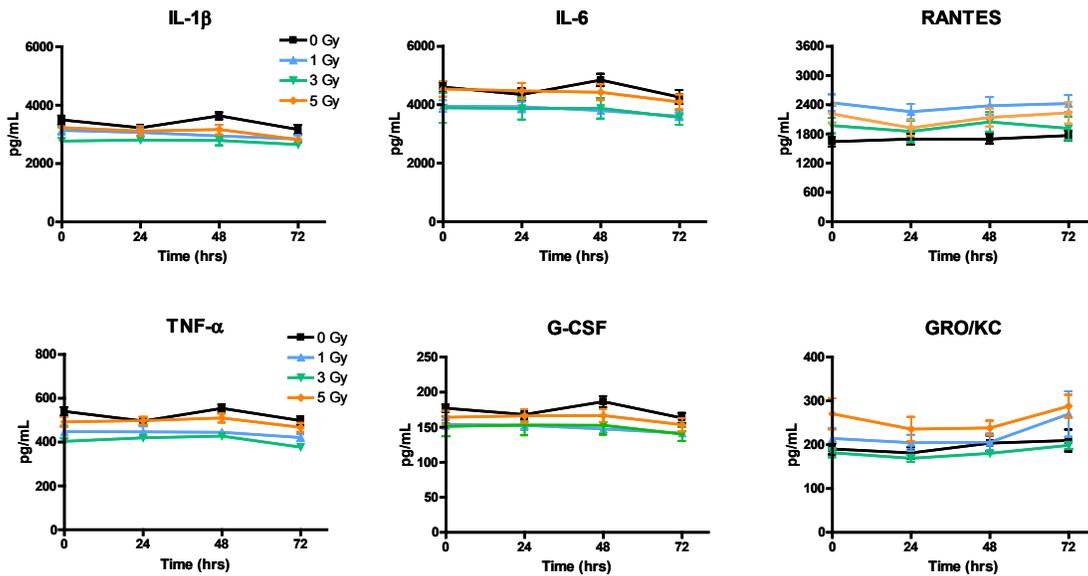Understanding the systemic response of the body following exposure to ionizing radiation will provide insight for biomarker identification. In the series of studies presented here, we examined the potential of secreted cytokines to serve as diagnostic markers of radiation exposure based on previous research findings demonstrating activation of the peripheral immune response following IR exposure.   Investigations began in a simple, single cell line model and increased in complexity progressing to studies in a murine model allowing us to examine the robustness of the cytokine response in complex systems.

As a means to study the systemic radiation response, without using an animal model, a multi-cell culture system was developed. Six human cell lines were selected to be included in the system that attempted to simulate the peripheral immune system. Following characterization studies, it was seen that the cell lines had differing growth kinetics and radiation sensitivity. This is ideal as cells within the human body differ vastly in their radiation response and susceptibility to radiation injury thus validating, in part, our system as a representative model for *in vivo* studies.

4.1   Cell line-based models

In the cell line models, several cytokines demonstrated radiation-induced expression including IL-6, IL-8, IL-13, G-CSF, GM-CSF, TNF-α, IFN-γ, MCP-1 and MIP-1β. These findings were consistent with previous studies where these cytokines had been shown to have a role in the cellular radiation response.

The multi-cell culture system was developed with the objective of examining the "systemic" cell response following radiation exposure. In these studies, very few of the cytokines examined were present in the shared media at detectable concentrations. A larger volume of media was needed to ensure coverage of the 6-well culture dish and it is possible that the molecular signals were just too diluted for an effect to be observed. Interestingly, in contrast to the individual cell line studies and the co-culture system, decreased expression of MCP-1 and MIP-1β was observed following radiation exposure. This may be explained by the effect of the radiation on the cell types responsible for their expression in combination with the dilution of the signal in a large volume of media. Investigating the use of other commercially available culture dishes for our purposes may be worthwhile and allow us to reduce the volume of media needed in the system and thus improve signal detection.   Cell communication and cytokine signalling can occur by several ways including paracrine signalling where a "messenger" molecule from one cell is secreted and interacts on a distant or near-by cell. The requirement for cell-to-cell communication may be a critical component in the radiation response and the segregation of the cells in the multi-cell culture system may have inhibited this paracrine signalling by creating too far of a distance for the signal to be recognized by neighbouring cell types.   For this reason the co-culture system may represent a more suitable model for simulating *in vivo* studies as the cells were allowed to interact in close proximity to each other and molecular signalling was barrier free. In this system, several more cytokines were expressed at detectable levels including IL-6, IL-8, interferon-γ, MCP-1 and MIP-1β. Both MCP-1 and MIP-1β were significantly increased in the growth media following radiation exposure while the others exhibited a general trend in the same direction.

It is evident from the studies that both culture systems proved to be useful models for studying the *in vitro* systemic radiation response however both could benefit from optimization in future work. Reducing the media volume in the multi-cell culture system may allow for better amplification of the systemic "signal" while being able to positively identify the individual cell types contributing to the response via appropriate cell markers in the co-culture system would also be beneficial and allow for a better characterized system from a QA/QC perspective.

4.2   Skin inserts

In general the cytokines expressed by the engineered human skin inserts, including IL-6, IL-8, GM-CSF, MCP-1, G-CSF, IFN-γ and MIP-1β were consistent with those expressed by the fibroblast cell line HFL-1 in previous studies. This was to be expected as fibroblasts represent the major radiosensitive cell type found in the skin. Although not reaching statistical significance there was a general trend towards increased cytokine expression with increasing dose particularly for IL-1β, IL-8, G-CSF and MCP-1. All of these cytokines had shown radio-responsiveness in the cell model systems examined previously and the findings are consistent with others in the literature. IL-1B signalling has been shown to be a critical component of radiation induced skin fibrosis [22] while IL-1, IL-6, IL-8, TNF-α, and GM-CSF have been shown to be the major cytokines in the response of skin cells to ionizing radiation [4]. The pro-inflammatory chemokines, monocyte chemotactic protein -1 (MCP-1) and macrophage inflammatory protein-1β (MIP-1β) have not traditionally been shown to be involved in the dermal radiation response however a study by Hasewaga and colleagues [23], demonstrated to the involvement of these cytokines in the development of pulmonary fibrosis in patients with systemic sclerosis, a connective tissue disorder characterized by fibrosis of the skin and visceral organs. Serum levels of these cytokines were significantly elevated in observed in patients with the disease relative to normal controls. Fibrosis is the major manifestation of radiation injury in both the skin and lung and thus the early response of these cytokines may represent novel biomarkers of radiation exposure.

During recent years much progress has been made in dissecting the complex cytokine network however the role of cytokine in the pathophysiology of the cutaneous radiation reaction is only beginning to be understood. The human skin inserts represent a more complex model than the cell line-based systems and with that comes variability. In our experiments, some of the cytokines examined showed variability between replicates and this could explain, in part, the results seen in our work.

In the skin experiments, it is possible that the observation period may have been too short. The cutaneous radiation reaction can take days to weeks to manifest and show clinical symptoms. The newly conceived paradigm for the induction of radiation-induced skin damage suggests an early cascade of cytokines is initiated immediately after exposure, decreases and then persists at high levels for long periods of time leading to the development of late radiation damage (fibrosis) [24]. By looking at 24 hrs and onward we may have missed the initial cytokine peak that is thought to occur.

4.3   Animal studies

The animal studies conducted as part of this work were done to evaluate the potential for using circulating cytokines as biomarkers for radiation exposure and to examine the robustness of the response in a more complex "system". Interestingly, all of the 23 cytokines examined were expressed in the rat serum samples however there was no change in their levels over time nor following radiation exposure even at the highest dose tested, 5 Gy. Unfortunately, the rat homologs for MCP-1 and MIP-1β, two cytokines which showed promise as biomarkers of exposure in the cell based systems and skin equivalents were not available in the multi-plex assay used on the serum samples. Therefore the robustness of their signal in the rat model could not be validated. Future experiments using the rat model will investigate the response of these secreted chemokines following ionizing radiation through ELISA or other appropriate assays. In addition, investigating the radiation-induced cytokine response in a mouse model may provide further validation.

As with all of the studies presented here it is possible that the time frame selected may not have been ideal for observing peak cytokine responses. Our primary interest was in identifying biomarkers that would be indicative of radiation exposure in the first few days following radiation exposure when this information is most relevant for medical personnel and critical for treatment outcomes. For this reason the experiments were designed accordingly with time points starting at 24 hrs out to 72 or 96 hrs post exposure.   Previous cell line based studies and some animal studies have shown that the cytokine response following radiation exposure occurs very quickly. In a recently published study by Singh et al, maximum levels of serum granulocyte-colony stimulating factor (G-CSF) in mice exposed to 9.2 Gy of whole-body gamma radiation were seen at 8 hrs after irradiation while IL-6 levels peaked at 12 hrs post irradiation [8]. Both cytokines returned to basal levels by 24 hrs. Interestingly, G-CSF peaked again at 48 hrs however in our study no such peaks were observed for any of the cytokines examined. The dose of radiation used in the Singh study was similar to the LD_{50} for mice, whereas in our study the upper dose (5 Gy) was significantly below the lethal limit in rats (7 Gy). Perhaps if a more challenging radiation dose had been used in the rat model, we would have seen a greater, more sustained inflammatory response.

4.4   Way Forward

The research presented here has focused on individual cytokines as potential biomarkers of radiation exposure. While results from the cell culture and dermal tissue models have identified several cytokines that appear to be radiation responsive including IL-6, IL-8, MCP-1, MIP-1β, G-CSF, TNF-α and interferon β, the robustness of this response has not been fully validated and warrants additional investigation in complex systems. Further animal work and potentially analysis of clinical samples from patients undergoing radiation therapy is needed to further refine and validate the potential for cytokines to be used as diagnostic markers of radiation exposure. In addition, employing additional analytical techniques such as genomic and proteomic analysis will help characterize and validate the robustness of the response. Investigation of the specificity of the observed radiation-induced cytokine response is also warranted to eliminate potential confounding factors such as infection or stress. To date, only the response from exposure to gamma radiation has been examined as it was thought to most likely induce a "systemic"

response. Examination of the cytokine response following exposure to other radiation qualities (alpha exposure, internal contamination) could provide further characterization and validation.

Furthermore, after years of biomarker research it has been accepted that a single robust marker of exposure is not likely to exist and that a more parametric approach (unique signature of multiple markers) is more likely to be successful. This has been shown in both gene expression [15-17] and protein profiling studies [1, 14]. Follow-on work will focus on data analysis using biostatistical tools and data mining to evaluate possible cytokine signatures following radiation exposure and to identify a representative panel of 4-6 cytokine biomarkers that can be used as a diagnostic tool. In addition, expanding the data sets to included additional cytokines (17-plex to 27-plex) and increasing the number of data sets from complex systems (animal models) will provide further insight into the radiation-induced systemic cytokine response.

4.5   Conclusion

The aim of the current studies was to identify novel markers of radiation exposure while gaining a better understanding of the systemic radiation response. We were successful in developing and employing an *in vitro* cell line-based culture system to simulate an *in vivo* model. Additional complex models were used to examine the radiation-induced cytokine response and validate the potential for their use as biomarkers of exposure. Results thus far suggest that the chemokines MCP-1 and MIP-1β may prove to be indices of radiation exposure however inflammatory signalling cascades are complex and further investigation is needed to better understand and validate the robustness of this response.

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List of symbols/abbreviations/acronyms/initialisms

ARP	Applied Research Program
Co-60	Cobalt-60
CCS	Co-culture system
CRTI	Chemical Biological Radiological and Nuclear Research and Technology Initiative
DNA	Deoxyribonucleic Acid
DND	Department of National Defence
DRDC	Defence Research & Development Canada
ELISA	Enzyme-linked immunosorbent assay
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony stimulating factor
GRO/KC	Growth regulated protein (CXC ligand-1)
Gy	Gray
γ-H2AX	Phosphorylated histone H2AX
IFN-γ	Interferon - gamma
IL-1β	Interleukin-1β
IL-2	Interleukin-2
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-13	Interleukin-13
IR	Ionizing radiation
MCSS	Multi-cell culture system
MCP-1	Monocyte chemotactic protein-1
MIP-1β	Macrophage inflammatory protein-1β
RANTES	Regulated and normal T cell expressed and secreted protein (CCL5)
RN	Radiation/nuclear
SEM	Standard error of the mean
TNF-α	Tumor necrosis factor-α

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The quest for reliable and unique biomarkers of radiation exposure is an ongoing challenge in the field of radiation biology. Once identified they will provide an invaluable tool for both clinicians wanting to assess therapeutic outcomes of patients as well as first responders and medical personnel needing to rapidly triage populations of potentially exposed people following an radiation/nuclear (RN) event. It has been reported that there is an acute inflammatory response following exposure to ionizing radiation. We hypothesize that this is initiated in part by cytokines secreted from various cell types within the body and that these secreted cytokines may serve as biomarkers for exposure. The various studies described in this report examine the cytokine response following radiation exposure from a single cell line model through to animal studies.

La recherche de biomarqueurs fiables et uniques indicatifs d'exposition aux rayonnements est un défi dans le domaine de la radiobiologie. Une fois identifiés, ils fourniront un outil inestimable aux cliniciens voulant évaluer les perspectives thérapeutiques des patients ainsi qu'aux premiers intervenants et au personnel médical lors du triage d'individus potentiellement exposés lors d'un événement radiologique/nucléaire. Il a été rapporté qu'il y a une réponse inflammatoire aiguë après l'exposition aux rayonnements ionisants. Nous formulons une hypothèse selon laquelle ce processus est amorcé en partie par des cytokines sécrétées par différents types de cellules dans le corps et que les cytokines sécrétées peuvent servir de biomarqueurs indicatifs d'exposition aux rayonnements. Les études décrites dans ce rapport examinent la réponse des cytokines suivant une exposition aux rayonnements sur un modèle de lignée cellulaire ainsi que sur un modèle animal.

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ionizing radiation, biomarkers, multi-cell culture system, cytokines

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