Signal transduction processes in response to low dose ionizing radiation doses expected during space flight

Tracy Criswell¹, Eva Goetz², Dmitri Klokov³, Yonglong Zou⁴, Xiuquan Luo⁴, and David A. Boothman⁴,*

⁴Departments of Radiation Oncology and Pharmacology, UT Southwestern Medical Center at Dallas, 6001 Forest Park Drive, ND2.210K, Dallas, Texas 75230-8807

David.Boothman@UTSouthwestern.edu

Current Author Affiliations:

¹TC: Wake Forest Institute for Regenerative Medicine, Wake Forest, NC Richard H. Dean
Biomedical Building, 391 Technology Way Winston-Salem, NC 27101

²EG: Department of Medicine, Dana-Farber Cancer Institute, DA 1524, 44 Binney Street, Boston, MA 02115

³DK: 1Radiological Protection Research and Instrumentation Branch, Chalk River Laboratories, Atomic Energy Canada Limited, Chalk River, ON, USA;

*To Whom correspondence should be addressed

Abstract

A major issue affecting our plans to visit and one day inhabit other planets is that once outside the protective influence of our atmosphere, astronauts will experience dramatically increased exposure to ionizing radiation (IR). While shielding spacecraft can minimize effects, exposure to significant levels of both high and low linear energy transfer (LET) IR is inevitable. Understanding the effects of these doses at a mechanistic level is absolutely required to understand both dosimetry and long-term biological effects. While determining the physical dose is relatively straightforward using well worked out physic techniques, the biological effects of such exposures are far more complicated.

Evidence now supports the notion that in addition to DNA repair sensors, other cellular processes (non-DNA targets) can be activated in response to low doses of IR (0.3 to 0.6 Gy), doses approximating levels that astronauts would experience in prolonged space travel, such as in a trip to Mars (~1.2 Gy total absorbed dose at 0.3-0.6 Gy over 2 years). Furthermore, IR exposure to one cell can stimulate bystander effects in neighboring cells. For example, we will use the induction of insulin-like growth factor 1-secretory clusterin expression (IGF-1-sCLU) to discuss low dose and low dose rate effects within tissue microenvironments, including within an emerging (and most likely unknown at the time of departure to Mars). It is clear that examining cellular responses to high doses of IR (e.g., clinically-relevant doses) cannot be used to 'extrapolate down' to low doses of IR (ranging from <0.01 Gy at a low end and 3 Gy over 2 years). Signal transduction responses are affected and influenced by the microenvironment, and examining cellular responses to IR using cells in vitro, under 21% oxygen (normoxic in tissue culture, but certainly not in vivo, where 'normoxic' tension would be ~3%) is not appropriate in isolation. A major challenge of using signal transduction processes for determining biodosimetry is the short half-life of most stimulated processes. A detailed mechanistic approach is required to find micro-environmental indicators that reflect long-term biological consequences, such as fibrosis and carcinogenesis. We will discuss our identification of the IGF-1-sCLU expression...
axis as a mechanistic marker of the long-term consequences of low doses of both low and high LET IR.

**Introduction**

Signal transduction processes have evolved to coordinate cellular responses following endogenous or exogenous insults to the cell. By definition (from Webster dictionary), ‘signal transduction’ means: (i) **Signal**: n, a sign for giving notice, especially at a distance; adj, memorable, remarkable; and (ii) **Transduction**: the transforming of one form of energy into another, as by the sensory mechanisms of the body. Thus, signal transduction processes, in the context of response to IR, means that the cell detects and amplifies sensory signals to stimulate a response, such as to stimulate growth, change cellular metabolism, stop cell division, induce expression of proteins required for repair (e.g., stimulated autophagy) or cell death (e.g., stress-induced premature senescence, SIPS); compared to most other exogenous insults, IR is a poor inducer of programmed cell death (i.e., apoptosis) in human epithelial cells.

Importantly, signal transduction processes are context-dependent, and therefore, its processes cannot be investigated in their entirety in vitro using isolated cells in culture with 5-10% fetal bovine serum at 21% oxygen levels in culture. In addition, since various cell types have different sensitivities to IR, the cell type being investigated matters. The extent and type of IR used is also a major factor in the overall cell’s response. The cell cycle state is also a major determinant, and depending on what state the cell is in strongly affects cell responses to IR. Most cells in the human body, even with a growing tumor, are not dividing. Therefore, using logarithmically growing tumor cells to mimic effects of IR exposures is fraught with problems in interpreting physiological meaning. In conclusion, while initial investigations are commonly performed to determine mechanism using isolated normal or tumor cells in culture, the overall responses have to be performed in vivo using mouse models to simulate an appropriate microenvironment.

A major determinant in understanding signal transduction processes is the dose used and damage created. An examination of the literature indicates that >1000 articles on IR-induced signal transduction processes have been published since 1995. The vast majority of publications (~99%), however, have used >2 Gy as doses (Figure 1). Approximately 1% of publications, or ~10 publications have used doses of <0.01 Gy, a dose approximating exposures.
that may be experienced in space-related travel. Many studies have attempted to use high doses of IR, thinking that the responses noted can be simply extrapolated down to low doses. There are, in fact, few examples of successful extrapolation from high to low doses. In fact, there are very few examples of signal transduction processes activated by low doses of low LET IR (<0.1 Gy) in human epithelial cells \(^1, 2\), and few of these responses have been explored in detail in animal models to see if such signaling represents 'meaningful' responses \textit{in vivo}. Finally, our understanding of signal transduction processes after clustered DNA damage created by high LET IR exposure is even less understood at a mechanistic level and few reliable papers have been published. With respect to our discussion below, we know that low doses of low or high (after iron, carbon and all other ions tested) LET IR (at \textasciitilde 0.1 Gy total absorbed dose) induce the IGF-sCLU pathway by an identical manner initially stimulated by activation of ATM or TGF\(\beta\) \(^{56, 58}\).

**DNA damage and downstream responses**

While not the only target of IR in the cell, DNA damage and repair systems remain the major damage sensing and signal transduction mediators in response to IR. The wide spectrum of damage caused by IR exposure simultaneously induces several DNA sensing and repair responses. Since very low doses of IR may not induce DNA lesions above background levels (made from cell metabolism and DNA replication), it is not entirely surprising that DSB repair processes are not stimulated by low doses of IR. For this discussion we define low doses of IR as \(< 0.1\) Gy. At these low levels of DNA lesions, the major DNA sensors are \textit{Ataxia telangiectasia} mutant (ATM), the

**Figure 2.** An overview of DNA damage sensing and downstream signal transduction processes known to occur after any dose of IR. Indicated in ‘red’ are the only known signal transduction processes induced at low (<0.1 Gy) doses. ATM, \textit{Ataxia telangiectasia} mutant kinase; ATR, AT-related kinase; MMR, DNA mismatch repair (mut S homolog family members); BER, DNA glycosylase-AP endonuclease (APE) base excision repair system; NER, nucleotide excision repair; DNA-PK, DNA protein kinase complex; PARP1-Alt NEHJ, Poly(ADP-ribosyl) polymerase 1. NEHJ, nonhomologous end joining.
DNA mismatch repair (MMR) system and DNA base excision repair (BER). These DNA repair systems detect extensive formation of base damage, including elevated levels of the mutagenic DNA lesion, 8-oxoguanine (8-OG). Once activated, these DNA sensing/repair systems cause cell cycle checkpoint alterations, altered gene expression, and changes in cellular metabolism (Figure 2). Depending on the cell type and extent of damage, cells can also respond by undergoing senescence or cell death, although IR is a poor inducer of apoptosis.

DNA damage is not the only ‘signal’ created by low doses of IR that can stimulate responses in cells. Other lesions include lipid peroxidation, membrane alterations (pore composition and size changes), damage-induced conformational activation of receptors, and growth factors. These signals can ‘transduce’ (transfer) signals, including various post-translational modifications (i.e., phosphorylation, ubiquitinylation, sumoylation, S-nitrosylation), changes in protein conformation, alterations in second messengers (Ca$^{2+}$, NO, and nucleotides (e.g., AMP)) such signal are, in general, secondary to the more dramatic responses mediated by DNA damage and sensory mechanisms of damage detection (Figure 2).

Use of signal transduction processes for biodosimetry.

Many researchers have been attempting to use signal transduction processes for biodosimetry, with the formation of gamma-H2AX (γH2AX) being a major endpoint of analyses. While such analyses are possible and can be a good measure of exposure, the short half-life and problems of basal level differences in humans are major issues. Furthermore, the biological significance of these early responses is not known in terms of long-term consequences. The short half-life of the responses also limits the use of these endpoints for biodosimetry.

Signal transduction amplification: the IGF-1-sCLU secreted response

Many of the issues discussed above are highlighted by our work with the IGF-1-sCLU expression response, which occurs in a complex, yet amplified manner to alter the tumor microenvironment. However, the mechanism of regulation of sCLU induction in response to IR (including high LET IR exposures), involving IGF-1 regulation was determined in vitro using a variety of normal and tumor cells in culture.

sCLU/XIP8 induction. In the late 1980’s, using two-dimensional gel electrophoresis, a series of x-ray-inducible proteins (XIPs) were identified from growth-arrested human melanoma cells, and their mRNAs isolated using subtractive hybridization. Steady state protein analyses demonstrated that XIP8 was secretory clusterin (sCLU, aka., apolipoprotein J), a secreted protein that mediated extracellular heat shock responses in which the protein cleared unfolded proteins created by injury, including reactive oxygen species (ROS)-induced damage. Importantly, sCLU levels increased dramatically after low doses of high or low LET IR (>0.001 Gy). Second, induction was robust (>10,000-fold) and delayed, appearing in all cells after IR exposure at >48 h, with peak levels noted commonly at 96 h or later. This delayed response highlights the need, in general, to examine gene expression at both early (seconds to minutes), as well as later times (hours to days) after IR. Interestingly, peak induction times could be increased by higher doses of IR (or high LET), or lengthened by decreasing the doses of IR.
The relative steady state levels of sCLU could be used, therefore, for biodosimetry reading, if induction were defined in vivo.

**IGF-1 controls sCLU induction.** Defining the regulatory mechanisms by which a delayed inducible protein is controlled is not trivial. Working upstream and using various DNA damaging agents, we noted that while most DNA damaging agents caused induction of sCLU, those agents causing the greatest stimulation also were those that also dramatically induced ATM \(^{50, 57}\). Further research demonstrated that sCLU was induced in wild-type, but not isogenetic cells in which ATM activity was deficient (i.e., AT cells corrected or not with ATM) \(^{58}\). In contrast, loss of DNA-PK, ATR or c-abl did not influence sCLU. In addition, loss of functional p53 caused uncontrolled elevated basal levels of sCLU \(^{53, 57, 58}\), suggesting that functional p53 (a tumor suppressor) negatively regulated expression of sCLU. We ultimately showed that sCLU was, in fact, controlled by IGF-1 expression and that all of the regulation of sCLU was controlled by regulating IGF-1 expression.

**Delineating IGF-1-sCLU expression mechanism in vitro.** Use of genetic manipulation is key for investigating the mechanisms underlying the control of a gene expression axis, and without using select specific genetic alterations mechanisms are not likely to be accurately delineated. This is especially true when using inhibitors, which should only be combined with genetic alterations, to investigate given mechanisms. Finally, 'circular genetics' must be used in which genes are knocked down or deleted, and the cells are then isogenically 'corrected' using mutant versions of the cDNAs. An alternative approach is to knockdown a given gene using 3'- or 5'-untranslated regions (UTRs), where wild-type cDNAs can be re-inserted for 'correction' of isogenic cells. Using such techniques, as well as dominant-negative constructs or constitutively-active expression vectors, chromosome immunoprecipitation (ChIP) technology, DNA pull-down analyses, select point mutations of promoters and select use of specific inhibitors, we showed that IGF-1 was regulated by p53 in association with nuclear factor-Y (NF-Y) family

---

**Figure 3.** Mechanism for IGF-1-sCLU induction in response to low doses of IR.

*IGF-1 - sCLU Regulatory Pathway*

![IGF-1-sCLU Regulatory Pathway](https://three.jsc.nasa.gov/articles/Boothman.pdf)
In its resting state, IGF-1 is suppressed by p53/NF-YA binding. Following the activation of ATM by IR, elevated p21 levels stimulate the inhibition of p53 phosphorylation through cdc2 (cyclin-dependent kinase) inhibition, which is required for the phosphorylation of NF-YA and association with p53. Dephosphorylated NF-YA binds less tightly to p53, decreasing p53/NF-YA binding to the IGF-1 promoter. Increased p53 stabilization results in minor induction of Mdm2, which further degrades p53 through its ubiquitin ligase activity. Increased levels of IGF-1, stimulate the IGF-1R/Src/MAPK/Egr-1 pathway, leading to Egr-1 protein binding to the clusterin promoter, resulting in CLU promoter transactivation and finally sCLU protein induction. IGF-1 stimulation of IGF-1R receptor also stimulated the AKT1 enzyme in the cell, which positively feeds back to stimulate phosphorylation of Mdm2, leading to further p53 degradation and further stimulation of sCLU expression levels. Expression of sCLU is a prosurvival factor, and suppression of its levels can greatly enhance lethality of clinically-relevant IR doses.

**IGF-1-sCLU regulation in vivo.** Having delineated the mechanism of sCLU induction in vitro, we delineated its induction in vivo. Surprisingly, the mechanism of induction in vivo occurred unexpectedly by the activation of transforming growth factor beta1 (TGFß1), and not by the mechanism delineated in vitro as shown in Figure 3. In vivo, especially at low doses, IR activates TGFß1, which ultimately controls IGF-1-sCLU expression. TGFß1 simultaneously induces the transcriptional activation and expression of Mdm2 and LEF1. Mdm2 induces p53 degradation, while LEF1 binds ß-catenin and these together cause the stimulation of the IGF-1-sCLU expression axis. Induction was responsive in select tissue (colon, thymus, spleen, breast and bone marrow) in a robust and prolonged fashion so as to significantly alter the microenvironment of the irradiated tissue.

**Conclusions:** Overall, there are significant alterations in the microenvironment in response to low or high doses of IR, which are under intense investigation in our laboratory as well as many others. These responses are likely to become even more important after low doses of high LET IR, where there will be sparse tracks and non-uniform damaged cells. Furthermore, responses in normal tissue (devoid of tumor cells) will likely be very different from microenvironments containing micro-nodules of benign or possibly more advanced tumors that are not detectable prior to a trip to Mars. Under these conditions low dose IR could induce IGF-1-sCLU responses that would promote the growth of these benign microenvironments. In terms of our discussion in this report, the major changes that occur are: (i) activation of TGFß1, enhanced levels of IGF-1 and dramatic and long-lasting secretion of sCLU. All of these affect the irradiated cells, but by definition, also affect the microenvironment as a whole since all three are secreted, extracellular proteins. Pre-irradiation of a given tissue can cause IGF-1-sCLU expression for months in the colons of irradiated mice after as little as <0.1 Gy. Since both IGF-1 and sCLU are pro-survival proteins, such responses may affect tumor versus normal tissue responses during multiple exposures and continuous elevated (above background) doses experienced during space travel. Other implications are that these low dose inducible responses can also occur during port film exposures prior to XRT therapy, and during fractionated therapies, or during brachiotherapy.
regimens during radiotherapies. Expression of the IGF-1-sCLU expression axis can arise from stroma and/or tumor tissue directly following low or high LET IR and at various doses. Delineating these responses within a given microenvironment is essential for risk assessment, especially in individuals with given genetic variations.

**Literature Cited:**


