

FINAL REPORT
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Project Title: Low Dose Studies with Focused X-rays in Cell and Tissue Models: Mechanisms of Bystander and Genomic Instability Responses

Principal Investigator: Barry D. Michael

Institution: Gray Cancer Institute

Co-Principal Investigator: Kathryn D. Held

Institution: Massachusetts General Hospital

Co-Investigators: Kevin M. Prise and Melvyn Folkard

Institution: Gray Cancer Institute

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Grant Project Officers: Arthur Katz and Justine Alchowiak

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2. Table of Contents

	<u>Page</u>
1. Cover Sheet	1
2. Table of Contents	2
3. Executive Summary	3
4. Research Objectives	8
5. Methods and Results	9
6. Relevance, Impact and Technology Transfer	18
7. Project Productivity	20
8. Personnel Supported	21
9. Publications	22
10. Interactions	24
11. Transitions	28
12. Patents	28
13. Literature Cited	28
14. Feedback	28

3. Executive Summary

Overview

The management of the risks of exposure of people to ionizing radiation is important in relation to its uses in industry and medicine, also to natural and man-made radiation in the environment. The vast majority of exposures are at a very low level of radiation dose. The risks are of inducing cancer in the exposed individuals and a smaller risk of inducing genetic damage that can be transmitted to children conceived after exposure. Studies of these risks in exposed populations indicate that they are low. As a result, the risks are impossible to detect in population studies with any accuracy above the normal levels of cancer and genetic defects unless the dose levels are high. In practice, this means that our knowledge depends very largely on the information gained from the follow-up of the survivors of the atomic bombs dropped on Japanese cities. The risks calculated from these high-dose short-duration exposures then have to be projected down to the low-dose long-term exposures that apply generally. Recent research using cells in culture has revealed that the relationship between high- and low-dose biological damage may be much more complex than had previously been thought. The aims of this and other projects in the DOE's Low-Dose Program are to gain an understanding of the biological actions of low-dose radiation, ultimately to provide information that will lead to more accurate quantification of low-dose risk.

Our project is based on the concept that the processes by which radiation induces cancer start where the individual tracks of radiation impact on cells and tissues. At the dose levels of most low-dose exposures, these events are rare and any individual cells only "sees" radiation tracks at intervals averaging from weeks to years apart. This contrasts with the atomic bomb exposures where, on average, each cell was hit by hundreds of tracks instantaneously. We have therefore developed microbeam techniques that enable us to target cells in culture with any number of tracks, from one upwards. This approach enables us to study the biological basis of the relationship between high- and low-dose exposures. The targeting approach also allows us to study very clearly a newly recognized effect of radiation, the "bystander effect", which appears

to dominate some low-dose responses and therefore may have a significant role in low-dose risk mechanisms.

Our project also addresses the concept that the background of naturally occurring oxidative damage that takes place continually in cells due to byproducts of metabolism may play a role in low-dose radiation risk. This project therefore also examines how cells are damaged by treatments that modify the levels of oxidative damage, either alone or in combination with low-dose irradiation.

In this project, we have used human and rodent cell lines and each set of experiments has been carried out on a single cell type. However, low-dose research has to extend into tissues because signaling between cells of different types is likely to influence the responses. Our studies have therefore also included microbeam experiments using a model tissue system that consists of an explant of a small piece of pig ureter grown in culture. The structure of this tissue is similar to that of epithelium and therefore it relates to the tissues in which carcinoma arises. Our studies have been able to measure bystander-induced changes in the cells growing out from the tissue fragment after it has been targeted with a few radiation tracks to mimic a low-dose exposure.

Accomplishments

A phenomenon that may influence low-dose risk is “low-dose hypersensitivity”. Cells exhibiting this effect show a more sensitive response to low doses of radiation than one would predict from their high dose response. The variation in sensitivity is believed to be due to an adaptive response to radiation whereby cellular defenses (e.g., repair) are only fully activated once the cell has sustained a certain level of damage. Using our focused soft x-ray microbeam with human cells, we have found that there is much less low-dose hypersensitivity than is seen after conventional x-ray exposures and this finding applies whether we target the focused x-rays just to a very small region of the cell nucleus, or defocus it across the nucleus. In experiments using conventional x-rays in which we have altered the levels of defense against oxidative damage, either by adding a scavenger or by depleting the cell’s own pool of protective molecules, we find

that the hypersensitive response is reduced by partial removal of the cell's natural defense against oxidative attack. In this respect, an increased level of oxidative damage appears to act like a small priming dose of radiation that activates the cell's defenses against radiation.

Another concept is that the cell's defenses against the low levels of oxidative damage that are continuously induced may allow it to tolerate low-dose radiation damage and therefore exhibit a threshold-type of radiation response (i.e., low-dose *hyposensitivity*). We have carried out a systematic study comparing oxidative and radiation damage in a number of cell lines to see whether the kinetics and pathways are similar. The results show that different pathways are involved, indicating that the cell's patterns of processing and expression of radiation and oxidative damages are different and therefore unlikely to lead to a threshold-type of response for ionizing radiation.

The bystander effect is potentially important for low-dose risk as a mechanism that can amplify the number of cells that respond to the passage of individual radiation tracks through tissue. Much of the research on bystander effects has used α -particles (densely ionizing radiation, i.e., high LET) but our unique focused soft x-ray microbeam has enabled us to study the effect using a radiation that approximates much more closely to the sparsely ionizing radiation (low LET) that comprise most of the low-dose exposures that are of public concern. We have found that, when viewed in terms of dose to the cell nucleus, low-LET radiation is of similar effectiveness to high LET in inducing a bystander response. However, the dose deposited by a single track of low-LET radiation is very low and the data obtained in the course of this project indicate that these tracks may be substantially less effective than single tracks of high-LET radiation as triggers of the bystander response. Further work will be needed in cell and model tissue systems to evaluate exactly how effective single tracks of low LET are in relation to the bystander effect. We have also found that it is the *dose per hit cell* that is the main determinant of a bystander response and the *number of cells hit* has less influence. Another determinant is the age of the cell. We have found that cells in the latter part of their life cycle, where they have replicated their DNA, are more likely to respond to bystander signals emitted by hit cells.

We also report data demonstrating the successful development of a model tissue system (pig ureter explant) as an *in-vivo*-like model of low dose response. This is an important step towards linking the processes that we and others have observed using cell culture systems to the effects that low-dose exposures may induce in intact organisms, including man. So far, we have been able to validate the ureter explant system using microbeam targeted high LET and the data show that the bystander mechanism operates and, as with cells in culture, it amplifies the number of cells showing damage. Interestingly, we have found an additional and major component of bystander response in this system that appears to be protective. This effect is an increase in the level of maturation (differentiation) of the progeny cells and *in vivo* this would represent a decrease in the number of cells that could become malignant, indicating a decrease in overall risk. Our focused soft x-ray microbeam has recently been upgraded to provide a range of x-ray energies, some sufficient to penetrate 3D models (ref. DE-FG02-01ER63236) to enable the studies on model tissue systems to be extended down to the low-LET radiation range of greatest relevance to the aims of the Low Dose Program.

Relevance, Impact and Technology Transfer

As outlined above, the work executed in the project addresses key issues in the problem of quantifying low-dose risk. One of these is to understand the relationship between the actions of the infrequent single low-LET radiation tracks per cell that are typical of low-dose exposures and the high-dose, many-tracks-per-cell exposures from the atomic bombs that are the source of most of the human risk data. This relationship is central to the correct extrapolation of the known high-dose risks down into the low-dose range. The project also addresses several processes that may influence the relationship and these include the interplay between radiation damage and the damage that arises continually from byproducts of metabolism, the adaptive response, which may increase low-dose sensitivity, and the bystander effect, which may amplify the number of cells that are damaged, or, as we show for the ureter system, may reduce the number of cells in which malignant change can be induced. Thus at this point, as we report, there are processes that may increase low-dose risk above the levels currently predicted by LNT (linear-no-threshold)

extrapolation of the known high-dose risks, but there are also processes that may work in the opposite direction and decrease the risk. Further research is needed to determine the overall balance and particularly to carry the studies across into model tissue systems.

The data and methodologies that we report are directed at an improved understanding of low-dose risk mechanisms. Along with the results of other studies in the program, including the development of improved mechanistic models, they will ultimately aid the regulatory agencies in their deliberations of permissible and recommended exposure limits to be applied in the management of radiation risk.

Collaborations

The project itself was a collaboration between Gray Cancer Institute (GCI) and Massachusetts General Hospital (MGH). For the microbeam dosimetry, we collaborated with Dr WE Wilson (WSU) (grant ref. DE-FG03-99ER62860). The work has led to an integrated modeling and experimental study with Dr A Chatterjee (LBNL) (PI), Dr LA Braby (Texas A&M) and Dr KD Held (MGH) (grant ref. DE-FG02-02ER63305). The studies with the porcine ureter model were in collaboration with Dr CE Mothersill, DIT, Dublin, Eire. GCI's low-dose research is also linked with projects at a number of centers in the European Union through its Nuclear Fission Safety program.

The research activities of the Low Dose Program have led the International Commission on Radiation Units and Measurements to commission a report "Approaches to Dosimetry at Low-Dose Exposure to Ionizing Radiation". The production of this report is to receive funding from the Low Dose Program. The committee that will write the report will include a number of researchers engaged on projects funded by the Program, including from this project and the collaborations outlined above.

4. Research Objectives

Accurate evaluation of the risks associated with exposure to ionizing radiation remains one of the major challenges facing environmental science. While exposure of people to radiation or radioactivity can be measured with better precision than exposures to many other toxins, the environmental risks to individuals and populations remain poorly understood. This is partly because radiation is a weak carcinogen as far as the population as a whole is concerned, and, therefore, the increase in cancer incidence in exposed populations over cancers arising due to other causes is difficult to detect with accuracy. Current knowledge of the carcinogenic and genetic risks associated with radiation exposures is based mainly on data from the atomic bomb survivors. These risk data have had to be extrapolated down to the very much lower dose levels and dose rates that apply in most environmental and occupational exposures. They also have to be transferred to populations with different natural incidences of cancer. Because of lack of mechanistic information, the risk models that have been applied in these extrapolations have had to be based on the most simple assumptions. This may have led to the adoption of conservative dose limits, notably, a public limit for man-made non-medical exposures that is less than the average natural background and substantially less than the geographical variation in background levels.

The object of this research has been to provide mechanistic information at extreme low doses that will contribute to the development of more refined models to extrapolate high dose epidemiological risk data into the mGy range. The research has had two interlinked hypotheses: that cells exposed to dose levels equivalent to their being traversed by a few electron tracks (i.e., in the mGy range) do not respond in simple proportion to the number of tracks (i.e., not proportional to dose); and that any deviations from low dose linearity detected in testing the first hypothesis are related to levels of damage induced by ROS. Using similar methods, we have determined how damage signals induced by ROS and/or low doses of radiation are transmitted within cells and between them (bystander effects). To achieve these ends, the work has made use of unique microbeam irradiation techniques to determine how cells respond to and are damaged by isolated low LET tracks of radiation, mimicking the type of exposure that cells at risk receive in environmental and occupational dose levels. It has combined these radiation studies with studies in which low levels of oxidative stress are used to determine whether the resulting damage is similar to that from low levels of ionizing radiation.

The project included seven specific goals: (1) Determine the response of individual cells to low doses of ionizing radiation from a focused soft X-ray beam with a 250 nm diameter beam spot. (2) Determine the response of cells to ROS generated by chemical agents in a fashion that mimics the endogenous cellular generation of ROS. (3) Study the interaction between cellular oxidative processes and ionizing radiation. (4) Determine the importance of the subcellular distribution of ROS from focused soft X-rays on cellular response. (5) Determine whether damage deposited in individual cells by focused soft X-rays or by chemically-generated ROS can elicit a response in other, surrounding, untreated cells, a “bystander” effect. (6) Quantify the low dose response and the targets involved in the genomic instability phenotype in cells exposed to low LET radiation and the relationship with the bystander response. (7) Develop tissue explant systems for the measurement of low dose effects in multicellular systems.

5. Methods and Results

Low dose hypersensitivity

It has been shown by others that hypersensitivity to low doses of radiation exists in a range of animal and human tumor cell lines. However, little is known about the response of primary human cells. In this work, primary human skin fibroblasts (AGO1522B) were exposed to low doses of conventional and focused soft X-ray irradiation. The results show that at doses of 0.2 Gy and below of conventional X-rays, hypersensitivity with respect to cell clonogenicity was observed (Fig. 1). Furthermore, a similar hypersensitive response to the same doses of conventional X-rays was found when the production of micronuclei was measured (Fig. 2). When individual cells were irradiated through the nucleus with a focused carbon-K soft X-ray microprobe, cells were more radiosensitive compared to conventional X-rays as measured by both the clonogenic survival and micronucleus formation assays at doses greater than 0.2 Gy. However, no hypersensitivity to low doses of focused soft X-rays was observed (Fig. 1). To test whether induction of intracellular reactive oxygen species and oxidant-antioxidant balance are

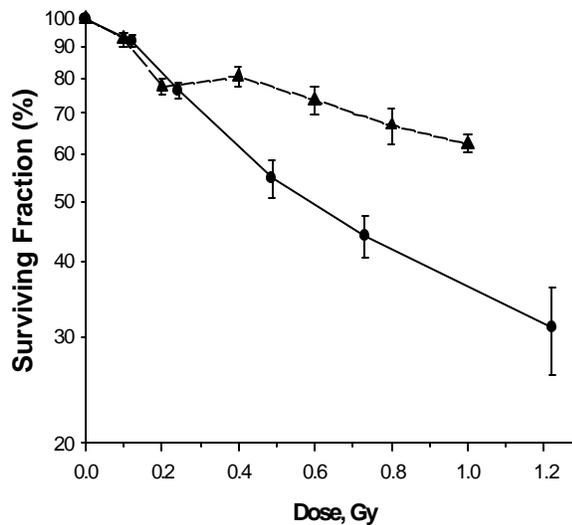


FIG. 1. Cells irradiated through the nucleus with focused soft X-rays show greater radiosensitivity to low doses than cells irradiated with conventional radiation. AGO1522 cells were seeded as single cells and after 4-5 h irradiated individually through the nucleus with focused carbon-K soft X-rays microprobe (solid line). The next day cells were transferred to a new dish and cultured for 12 days. Colonies were stained, counted, and their numbers were corrected for plating efficiency. Data are mean \pm S.E.M. from 3-8 experiments. Dotted line is for conventional X-rays.

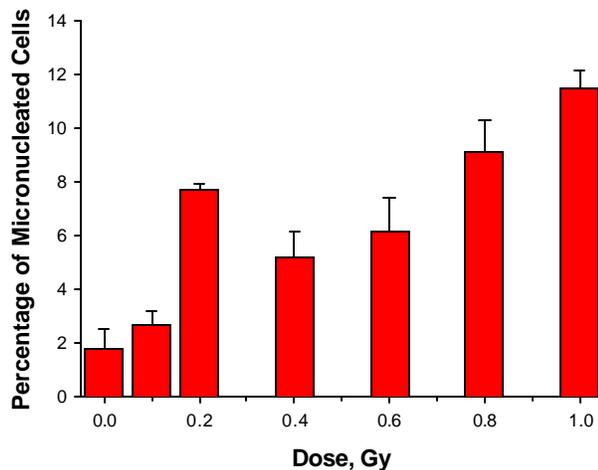


FIG. 2. Primary human fibroblasts exposed to 0.2 Gy show hypersensitivity with respect to chromosomal damage. Cells were irradiated on Petri dishes, and 3 days later were fixed and stained with acridine orange. Percentage of micronucleated cells was determined using fluorescence microscopy as detailed in Methods. Data are mean \pm S.E.M. from 3 separate experiments.

involved in the mechanism of hypersensitivity to conventional X-rays dimethyl sulfoxide, a hydroxyl radical scavenger, and buthionine sulfoximine, a suppressor of intracellular glutathione production were used. Dimethyl sulfoxide had no protective effect on the hypersensitive response of cells to conventional X-ray irradiation (Fig. 3). However, pretreatment of cells with buthionine sulfoximine before irradiation had a radiosensitizing effect with respect to cell survival at all doses, and hypersensitivity below 0.2 Gy was not observed (Fig. 4). Collectively, these results show that a primary human cell line is hypersensitive to conventional X-rays at doses below 0.2 Gy. These cells were much more sensitive to soft X-ray irradiation through the nucleus at doses higher than 0.2 Gy, while survival rates were similar at lower doses. Moreover, our data suggest that while the deviation from linearity in a dose-effect response to conventional X-rays at doses less than 0.2 Gy is not due to DMSO-scavengeable oxidants, glutathione levels do appear to influence the response in these cells.

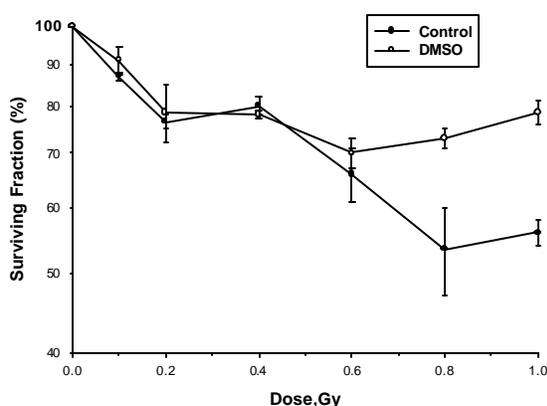


FIG. 3. DMSO treatment reduces radiosensitivity of cells irradiated with 0.8 and 1 Gy conventional X-rays. AGO1522 cells were incubated with 8 % DMSO for 10 min prior to being exposed to the indicated doses of ionizing radiation. DMSO was removed 40 min after irradiation by replacement of media. The samples were cultured for 12 d prior to fixation and staining. Data are mean \pm S.E.M. from 3 experiments.

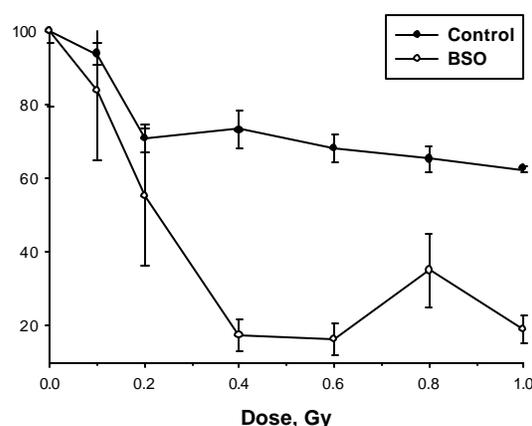


FIG. 4. The glutathione inhibitor BSO radiosensitizes AGO1522 cells at low doses of conventional X-rays. AGO1522 cells were incubated with 10 mM BSO for 16 h and exposed to conventional IR. BSO was removed 4 h after irradiation by replacement of media. The samples were cultured for 12 d prior to fixation, stained and counted. Data are mean \pm S.E.M. of 3 separate experiments.

Comparison of ROS-induced cellular damage with that by IR

Because both endogenous oxidative processes and ionizing radiation produce predominantly the same three ROS, namely $O_2^{\bullet-}$, H_2O_2 and $\bullet OH$, it has been suggested that cells may have some threshold level of tolerance to low levels of radiation because the cells are adapted to low levels of ROS. We have been investigating this using different means to produce ROS and comparing the effects with those produced by ionizing radiation after low and high doses. The ROS include bolus addition of H_2O_2 , treatment with dithiothreitol (DTT) which

produces ROS, including H₂O₂ and •OH (Biaglow et al., 1997; Kachur et al. 1997), and treatment with the photosensitizer rose bengal and light to produce singlet oxygen (work of our collaborator in another project, Dr. Irene Kochevar). As endpoints, we have investigated several aspects of apoptosis induction, including pathways and timing for the appearance of damages. Our results with the human leukemia cell line HL-60 are summarized in Table 1. The results clearly indicate that low and high doses of ionizing radiation differ in the pathways to apoptosis they initiate, and both differ from H₂O₂ induced apoptosis.

Table 1. Effects of low and high dose ionizing radiation and various modes of generating H₂O₂ on apoptosis induction in HL-60 cells.

	Direct ROS generation	Time to maximal late stage apoptosis	Mitochondrial involvement	Caspase 3 activation
H ₂ O ₂	<5 – 30 min	1 – 4 h	0.5 – 1 h	0.5 – 2 h
DTT	5 – 60 min	5 h	None	2 – 4 h
Low dose IR	Immediate	4 – 5 h	2 – 4 h	None
High dose IR	Immediate	72 – 96 h	48 – 72 h	48 – 72 h
Rose bengal*	Immediate	1 – 4 h	2 – 3 h	1 – 4 h

* (data from Kochevar et al. 2000 and Zhuang et al 2000)

We have also been developing methods for chronic production of low levels of ROS. One method uses glucose/glucose oxidase to product H₂O₂ continuously outside cells, and the other method uses tyramine as a substrate for monoamine oxidase (MAO) to produce H₂O₂ inside cells at the mitochondrial membrane. Preliminary studies on induction of apoptosis in human lymphoblastoid WTK1 cells show glucose oxidase to cause apoptosis in a time frame similar to bolus H₂O₂, i.e., maximal apoptosis at about 24 h, but intracellular ROS production by MAO stimulation with tyramine or by use of a mitochondrial-localizing photosensitizer causes apoptosis rapidly, maximal effect by 4 h. In contrast, in these cells ionizing radiation is less effective at causing apoptosis, and the maximum appearance of apoptosis does not occur until 72 h after irradiation. Again, the data indicate that ROS do not cause the same damages as ionizing radiation.

We have also obtained clonogenic survival information in several cell lines with bolus H₂O₂ and the “slow release” H₂O₂ methods. – Loss of clonogenicity in AGO 1522 fibroblasts, keratinocytes and retinal pigment epithelial (ARPE-19) cells treated with H₂O₂ bolus is shown in Fig. 5. The AGO cells and the keratinocytes are relatively similar in sensitivity to each other and to several other cell lines we have tested, while ARPE-19 cells are somewhat more resistant at low H₂O₂ doses. As can be seen by comparison with the data in Fig. 1 where 1 Gy results in a surviving fraction of about 60% in AGO cells, approximately 25 μM H₂O₂ is equivalent to 1 Gy of X-rays in terms of loss of clonogenicity. It is interesting to note, however, that whereas 1 Gy of X-rays causes about 6-fold increase in micronuclei (Fig. 2), H₂O₂ up to 100 μM does not produce any micronuclei in AGO cells in our studies. Again, there appears to be a disconnect between damage induction by ionizing radiation and chemically generated ROS. We have also begun to test the sensitivity of these cells to “slow release” H₂O₂ using glucose oxidase and tyramine as a substrate for monoamine oxidase. Preliminary data in Table 2 show the same three

lines vary somewhat in the sensitivity to loss of clonogenicity by these slow release agents, and the variations are not in the same fashion as to bolus H₂O₂.

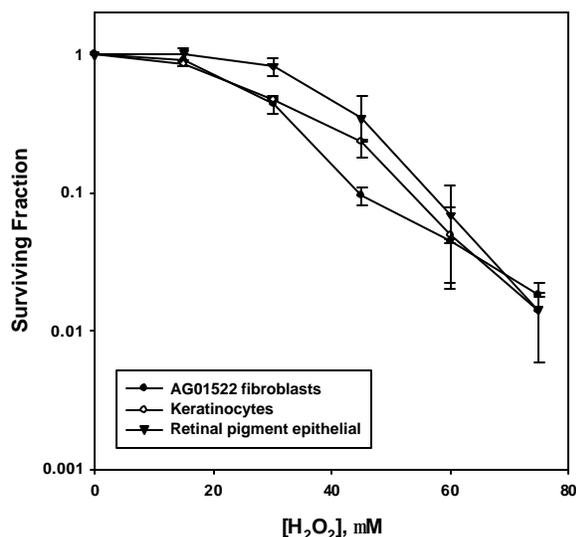


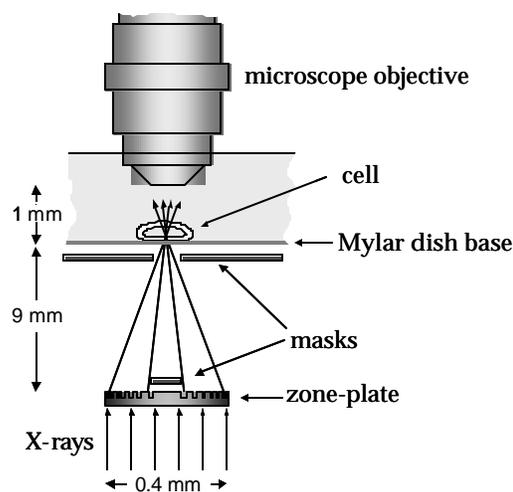
Figure 5. Loss of clonogenic cell survival in cells treated for 2 h with varying concentrations of H₂O₂. Data are means ± SD from 1-4 experiments.

Table 2. Surviving fraction (clonogenic assay) for three cell lines treated with H₂O₂ “slow release” agents.

Treatment	AGO1522	Keratinocytes	ARPE-19
1 mU/ml GO, 2 h	0.62 ± 0.07	0.24 ± 0.03	0.97 ± 0.08
4 mU/ml GO, 2 h	0.0048 ± 0.0020	0.0090	0.48
2.0 mM tyramine, 2 h	0.18	0.64	0.41

Radiation-induced bystander responses to targeted soft X-rays

The role of bystander responses, where cells which have not been exposed to radiation respond to their neighbors being targeted, is of considerable interest to low dose studies of radiation. Our own studies during this project have utilized a unique focused soft X-ray source developed at the Gray Cancer Institute. The essential components of the system are shown in figure 6. This uses zone-plate technology, commonly found in soft X-ray microscopes that focus a beam of carbon-K characteristic X-rays to a 250nm spot size. This can be targeted to cells at specific locations using a computerized imaging and revisiting system. Also, soft X-rays provide a unique model for quantifying the effectiveness of the terminal track electrons of conventional low LET radiations. Our cellular studies have monitored the effectiveness of the soft X-rays at inducing cell killing in V79 cells exposed under conditions where either every cell



Apparatus for targeting cells individually with focused soft X-rays.

or only one cell within a population was targeted. 278 eV carbon-K soft X-rays are highly attenuated within cells so dosimetry has to be performed carefully. To calculate the nuclear dose delivered by these X-rays, optical sections of cells are generated using a two-photon microscopy system to allow the nuclear thickness and, more importantly, the cytoplasmic thickness between the Mylar substrate and the cell nucleus to be measured. Taking into account the attenuation and knowing the flux of photons being produced at the cell position from the zone-plate, it then possible to calculate the dose delivered. Figure 7 shows the survival curves under these condition normalized to the dose delivered to the nucleus of the exposed cells. For the situation where every cell is targeted, survival decreases rapidly with increasing dose, essentially following a linear quadratic relationship, with little evidence for low dose radiation hypersensitivity. The overall sensitivity is higher

than that observed for conventional X-rays (data not shown) comparable to what we observed with human fibroblasts (see figure 1). Importantly however, when only a single cell is targeted, a significant level of cell killing is observed due to a bystander effect. Typically when only a single cell within a population or around 150 starting cells is targeted at the centre of the dish, approximately 10% cell killing is observed equating to an additional 15 non-viable cells. An increasing level of bystander mediated cell

killing was observed at 50 and 100 mGy which reached a plateau at 200 mGy and above. At these low doses, there was little difference in the response between the situation where either one or every cell was targeted, suggesting that at low doses, bystander responses predominate. These experiments were performed with cells seeded at low density over an area of 25 mm². Analysis of the distribution of non-viable colonies over the area of the dish showed that there was an equal probability of a non-viable colony being produced anywhere on the dish. This suggests that the factors released are highly stable and active. Further analysis of the distribution of damaged colonies has shown that some clustering of damaged cells is observed, within an increased probability of clusters of damaged cell being found within a 500µm radius of each other above that expected on the basis of purely random distribution.

These observations imply that certain cells have an increased probability of reacting to the bystander signal than others and potentially releasing further bystander signals leading to clustering of effect. The studies presented here have been performed with asynchronous cells. Due to the use of Hoechst DNA binding dyes for the imaging of cell nuclei, it is possible to classify cells which are found by the microprobe cell finding system according to the position in the cell cycle. This was done after cells sorted by flow cytometry were imaged after attachment

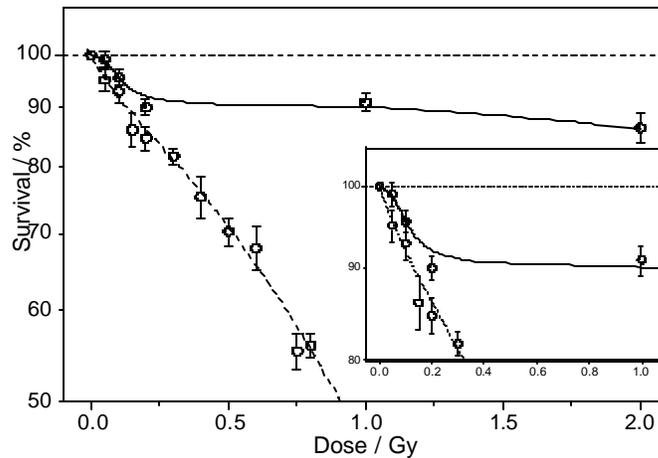


Figure 7. Cell survival of V79 cells targeted with focussed C-K soft X-rays through the cell nucleus. Curves are shown for the situation where every cell was targeted (open symbols) or only one cell within the population was targeted (closed symbols).

on the microbeam and fluorescent signals recorded to calibrate the system. In a series of experiments individual G1 or G2 cells were selectively irradiation within an asynchronous population and then cell cycle phase of the cells which did not survive was assessed. Typically the degree of bystander mediated cell killing was independent of the cell cycle phase of the targeted cell. Taken together with the information that targeting a single cell within a population induces an effect this shows that every cell within a population can release a bystander signal when irradiated. However, of the cells which were killed due to the bystander effect there was an increased probability of G2 cells responding (see figure 8).

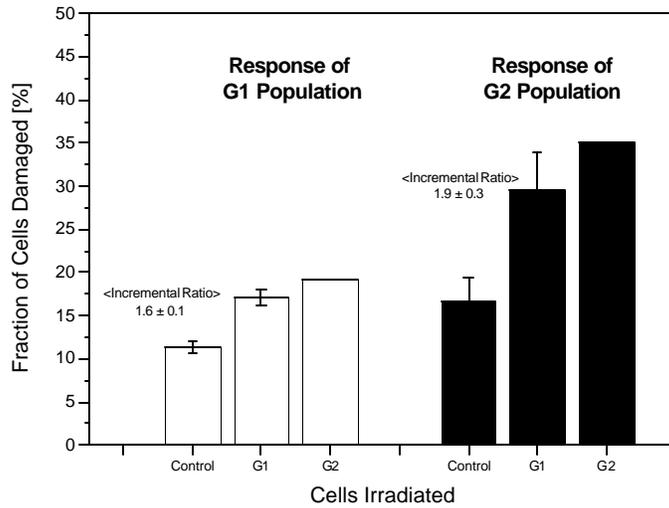


Figure 8. Fraction of G1 or G2 damaged cells produced when either a G1 or G2 cell is initially targeted

Further studies were performed where the number of cells irradiated within the population was varied. Figure 9 (left panel) shows the degree of bystander response measured as a loss of clonogenic survival which was obtained when 1, 2, 5 or

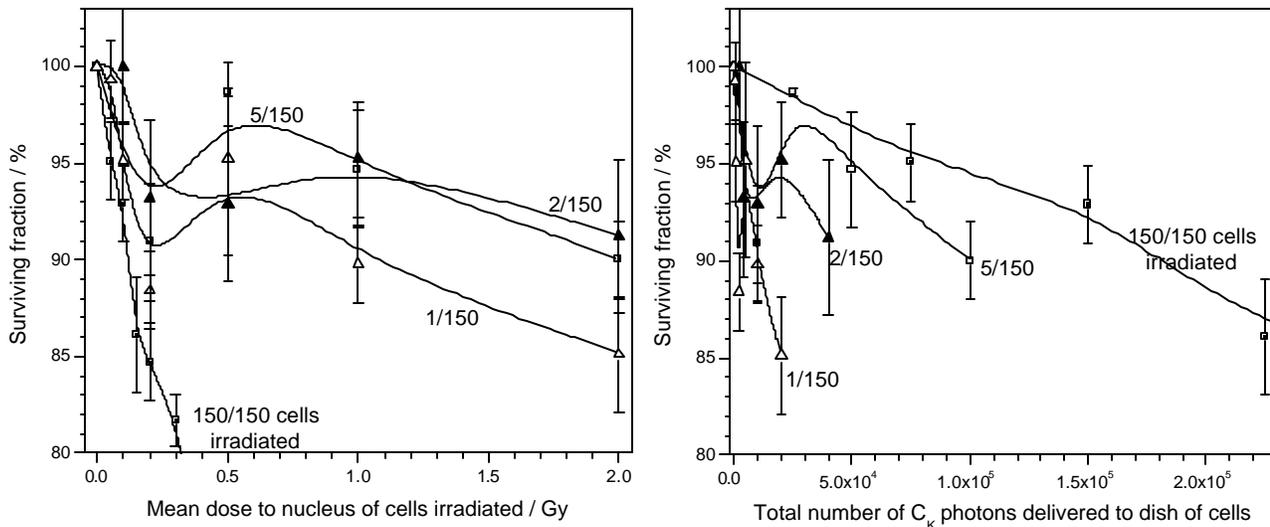


Figure 9. Comparison of the effect of increasing numbers of cells targeted and the degree of bystander-induced cell killing observed based on mean dose to the cell nucleus (left panel) or total number of photons delivered to a dish of ~ 150 cells where either 1, 2, 5 or all had been irradiated (right panel).

all the cells within the population were targeted with doses per targeted cell nucleus varying between 50 and 200 mGy. Importantly, the level of bystander response does not vary when the

number of cells targeted is increased from 1 to 5. An alternative way to consider the data shown in figure 9 is to plot the loss of cell survival versus the total number of photons delivered to the complete population of cells within the dish. As depicted in figure 9 (right panel), when plotted this way the maximum effect is observed when only a single cell is targeted. An important conclusion from this is that is the dose to an individual cell nucleus which determines the degree of bystander response rather than the number of cells targeted. Thus, in this cell system, it is the concentration of energy deposited per irradiated cell that governs the bystander response rather than the total energy received by the cell population as a whole.

We have also compared the bystander response measured with C-K soft X-rays with that observed with charged particles delivered by the Gray Cancer Institute Charged particle microbeam. For two proton energies, 1.0 and 3.2 MeV we also measure a significant level of bystander induced cell killing when only a single cell is targeted within a population. To

compare this with the soft X-ray data we have normalized to the dose delivered to the cell nucleus under these conditions. Figure 10 shows the comparison between the three radiation qualities for both every cell and only one cell targeted. For these situations, it is clear that little difference is observed between the radiation types at low dose when dose is expressed per nucleus. For bystander responses, some difference in the saturation level of cell killing is observed, however, an overall conclusion from this is that it is energy delivered to the nucleus which determines the level of bystander response rather than radiation quality.

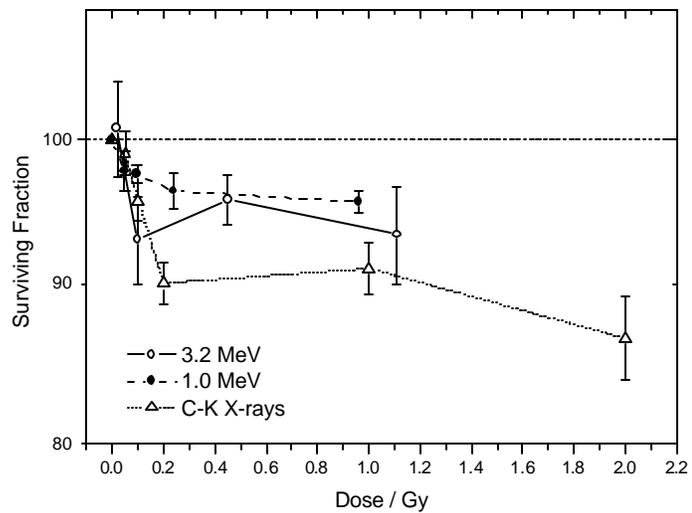


Figure 10. Comparison of bystander-induced cell killing observed when a single cell is targeted with either 3.2 or 1MeV protons or C-K X-rays.

Dose distribution studies with focused and defocused soft X-rays

With the soft X-ray microprobe used here the radiation dose is focused into a small spot of ~250 nm in size, so the local dose in that region is very high. We have compared the effect of changing the focus spot size of the soft X-rays within the cell nucleus, but delivering the same number of photons overall, Figure 11, shows the effect of this for cell killing. No significant difference is observed when the same number of soft X-ray photons are delivered to a localized region or spread throughout the cell nucleus. This implies that dose distribution of electron tracks of this energy does not influence direct effect when this is distributed over increased numbers of chromatin domains within a cell and that it is the localized energy deposition, in this case in 7 nm volumes which is more important.

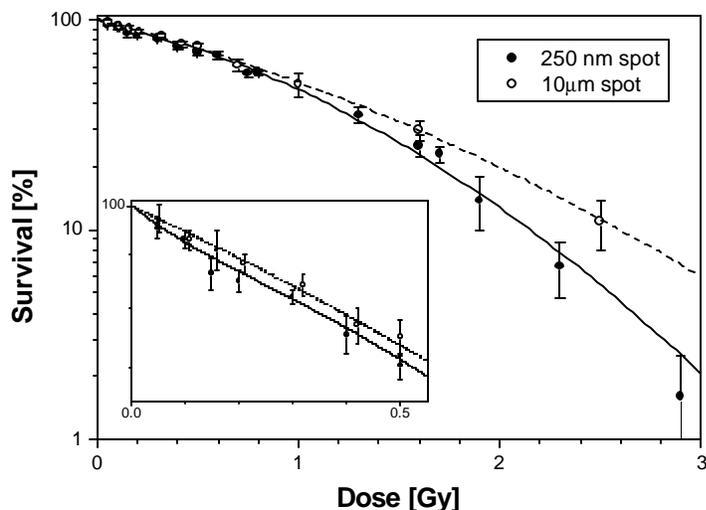


Figure 11. Comparison of the effects of two different beam spot sizes of C-K X-rays on cell survival.

Targeted tissue studies of bystander responses

The latter part of this project aimed to develop tissue models for measuring radiation effects at low doses. Pilot studies have been performed with charged particles, although it was not possible to extend these to soft X-ray studies. For this we used a porcine ureter model (in collaboration with Dr C Mothersill, DIT, Dublin, Eire). The ureter consists of highly organized layers of urothelium cells consisting of stem cells, pluripotent and fully differentiated cells. Sections of ureter were irradiated and then cultured under conditions where urothelial cells formed an explant outgrowth over a period of 7 days. The tissue was irradiated locally at a single location with individual helium ions such that between 4 and 8 urothelial cells were irradiated. In the explant outgrowth several thousand micronucleated and apoptotic cells were scored 7 days later.

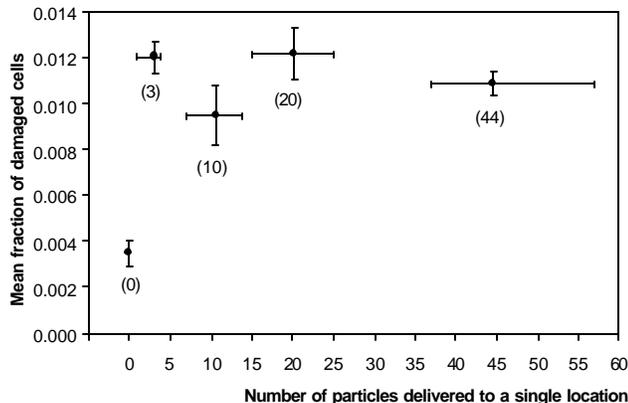


Figure 12. Fraction of damaged cells scored in a urothelial explant outgrowth 7 days after a single 2 μm location was targeted with 10 helium ions.

Although this is a large increase in the absolute numbers of cells responding due to a bystander effect, it represents a small proportion of the total cells present in the explant outgrowth, typically less than 1 %. An example of the dose response curve obtained when the number of particles targeted to a single location of the ureter explant is varied is shown in figure 12. The level of bystander induced cell damage is independent of the number of particles delivered, similar to what is observed in isolated cell systems.

In this model a major fraction of the cells within the explant outgrowth are undergoing differentiation to form terminally differentiated urothelial cells. Using a specific marker, uroplakin III for the differentiated cells the yields and distributions of these have been quantified in the explant outgrowths after the starting tissue was irradiated at only a single location. The level of differentiated cells in control sample explants varies between 50 and 60% in different explant sample. After irradiation of the tissue section this increase to between 70 and 90%. This is a massive increase in the absolute numbers of differentiated cells present in the explant outgrowth, despite the fact that only a few urothelial cells were exposed in the tissue fragment originally. This is an important observation which suggests, that at least in this model, that a highly protective removal of dividing cells which could be potentially damaged is occurring via a premature differentiation process. These studies have been predominantly performed with helium ions but will be extended to X-rays with the development of our soft X-ray source to produce focused beams of higher energy 1.5 keV aluminum and 4.5 keV titanium energies.

6. Relevance, Impact and Technology Transfer

a & b. A major challenge faced by DOE environmental science is evaluating the risks from exposure to ionizing radiation, as might occur for the general public, as well as radiation workers, including individuals involved in clean up. The data derived as part of these studies show, in particular, that low doses of low LET radiation cause low dose hypersensitivity to cell killing and micronuclei formation in normal human fibroblasts and significant bystander damage in the form of micronuclei in unirradiated neighboring cells of irradiated cells. Both these effects suggest that a simple, linear back extrapolation from cancer risk data derived at relatively high radiation doses may underestimate the effects at low doses. Furthermore, our data showing differences between damage induced by low LET IR and chemically-generated ROS suggest that endogenous ROS may not mimic IR and thus may not be involved in producing a threshold or adaptive response to IR. It must be pointed out that these conclusions, although provocative, are still preliminary and additional research is greatly needed. Furthermore, the development of the unique microbeam radiation sources as part of this work is important since it is the only such facility in the world and has special capabilities in allowing us to determine cellular damage and response to isolated low LET tracks.

c. The research in this project is hypothesis-driven, fundamental, basic studies applicable to aiding in understanding of mechanisms of radiation-induced carcinogenesis. Until additional mechanistic knowledge is gained it remains premature to apply this to technology development or cleanup approaches.

d. Some results obtained in these studies have already been presented at meetings and the remainder will be published in the literature. Hence, they will be available immediately for assessment and follow-on studies by other individuals, labs, and institutions, as well as serving as a basis for additional studies by ourselves. It is anticipated that additional studies by ourselves and others will result in further understanding of the mechanisms of radiation-induced cancer that will eventually lead to improved risk assessment at low radiation doses.

e. Additional studies to follow-on with the observations made here are sorely needed, and it is important that such studies include more in-depth investigation of molecular mechanisms, to increase understanding of radiation-induced carcinogenesis. The development of the unique microbeam radiation sources as part of this work is important since it is the only such facility in the world and has special capabilities in allowing us to determine cellular damage and response to isolated low LET tracks.

f. This project has led to collaborations with scientists at other institutions, as described in section 10, below.

g. The data derived as part of these studies show, in particular, that low doses of low LET radiation cause low dose hypersensitivity to cell killing and micronuclei formation in normal human fibroblasts and significant bystander damage in the form of micronuclei in unirradiated neighboring cells of irradiated cells. Both these effects suggest that a simple, linear back extrapolation from cancer risk data derived at relatively high radiation doses may underestimate

the effects at low doses. Furthermore, our data showing differences between damage induced by low LET IR and chemically-generated ROS suggest that endogenous ROS may not mimic IR and thus may not be involved in producing a threshold or adaptive response to IR.

h. It must be pointed out that the conclusions reached in these studies, although provocative, are still preliminary and additional research is greatly needed before decisions can be made about low dose risk assessment.

i. No.

7. Project Productivity

Substantial productivity has been made on this project in three years, as indicated by the number of presentations given and papers submitted or in preparation. In some instances, experimental set-up and initial studies required longer times than originally expected, hence, the work plan was revised to eliminate some planned experiments in the course of the project. Goal 1, to determine responses of individual cells to focused soft X-rays, was largely accomplished, as described above. Progress was made on goal 2, to determine the response of cells to ROS generated by chemical agents, as described above, although some of these studies are still in progress, now partially supported by other funding mechanisms. In short, accomplishment of this second goal was slow because it took greater effort to do the drug dose range finding studies than expected. Goal 3, to determine the interactions between ROS and IR, was not begun, because of the incomplete nature of the studies in goal 2, to date. Goal 4, to determine the importance of the subcellular distribution of ROS, turned out to be particularly problematic. This work planned to use apoptosis as an endpoint to study damage after focused soft X-rays to mitochondrial regions of cells. Hence, the work required attached cell lines, but the AGO fibroblasts show little apoptosis and the RKO cells detach quickly after irradiation, making both cell lines of no use for the proposed studies. Secondly, a critical aspect of this goal was determining the initial site of ROS production and following its movement in cells. This has turned out to not be possible given current technologies. We had anticipated using DCFH-DA, which gets into cells then fluoresces upon reaction with ROS, to demonstrate sites of production of ROS and their movement. However, studies have shown that after ROS-producing treatments, DCF fluorescence appears throughout the cells within seconds. This suggests either the ROS being produced in localized spots or the fluorescent DCF product diffuse rapidly throughout the cell. Significant progress was made on goal 5, to determine the bystander effect from focused soft X-rays, as described above. Furthermore, important information on the nature of the bystander effect has been derived from comparison of the results with the lower LET focused soft X-rays with those from high LET charged particle irradiation, e.g., alpha particles. The sixth goal, to quantify genomic instability, was deleted in our revised plan because of insufficient time. Lastly, significant progress was made with regards to goal 7, development of tissue explant systems. As described above, explant systems have been developed and tested with the particle microbeam; studies with these systems using the focused soft X-ray microprobe are planned.

8. Personnel Supported

Gray Cancer Institute

Barry D. Michael, Ph.D.
Kevin M. Prise, Ph.D.
Melvyn Folkard, Ph.D.
Giuseppe Schettino, Ph.D.
Laurence Tartier, Ph.D.
Sejal Patel

Principal Investigator
Co-Investigator
Co-Investigator
Post-doc
Post-doc
Research Assistant

Massachusetts General Hospital

Kathryn D. Held, Ph.D.
Elena Rusyn, M.D., Ph.D.
Aruna Karkala, Ph.D.
Yvonne McCarey

Co-Investigator
Post-doc
Post-doc
Technician

9. Publications

a. Peer-reviewed

- Prise KM, Folkard M & Michael BD. The use of microbeams in radiation biology: An overview. In *Radiation Research*, Moriarty, M., Mothersill, C., Seymour, C., Edington, M., J.F., W. & Fry, R.J.M. (eds), Vol. 2. pp. 174-177. Allen Press: Lawrence, KS. (2000).
- Tartier L, McCarey YL, Biaglow JE, Kochevar IE, Held KD. Apoptosis induced by dithiothreitol in HL-60 cells shows early activation of caspase 3 and is independent of mitochondria. *Cell Death and Differentiation* **7**: 1002-1010 (2000).
- Folkard M, Schettino G, Vojnovic B, Gilchrist S, Michette AG, Pfauntsch SJ, Prise KM & Michael BD. A focused ultrasoft x-ray microbeam for targeting cells individually with submicrometer accuracy. *Radiation Research* **156**: 796-804 (2001).
- Folkard, M., Vojnovic, B., Prise, K.M., Gilchrist, S., Schettino, G., Belyakov, O.V., Ozols, A., and Michael, B.D., The impact of microbeams in radiation biology. *Nuclear Instruments and Methods in Physics Research B* **181**: 426-430 (2001).
- Prise KM, Belyakov OV, Newman HC, Patel S, Schettino G, Folkard M & Michael BD. Non-targeted effects of radiation: bystander responses in cell and tissue models. *Radiation Protection Dosimetry* **99**: 223-226 (2002).
- Prise, K.M, Belaykov, O.V., Folkard, M., Ozols, A., Schettino, G., Vojnovic, B., and Michael, B.D., Investigating the cellular effects of isolated radiation tracks using microbeam techniques. *Advances Space Research* **30**: 871-876 (2002).
- Schettino G, Folkard M, Prise KM, Vojnovic B & Michael BD. Upgrading of the Gray Laboratory soft X ray microprobe and V79 survival measurements following irradiation of one or all cells with a CK X ray beam of different size. *Radiation Protection Dosimetry* **99**: 287-288 (2002).

b. Unreviewed

- Folkard, M., Schettino, G., Vojnovic., Michette, A.G., David, C., Pfauntsch, S.J., Prise, K.M. and Michael, B.D., Development and application of a focussed ultrasoft X-ray probe for radiobiological applications. *Proceedings of SPIE*, **4499**, 10-18. (2001),
- Schettino, G., Folkard, M., Prise, K.M., Vojnovic, B and Michael, B.D., Upgrading of the Gray Laboratory soft X-ray microprobe with aluminium K-shell X rays and measurement of the effect of carbon K-shell X-ray beam of different size focused into V79 cell nuclei. *Radiation Research*, **158**, 374-375. (2002),
- Michael, B.D., Schettino, G., Folkard, M., Prise, K.M., Held, K.D and Vojnovic, B., Charged-particle and focused soft X-ray microbeams for investigating individual and collective radiation responses of cells. *Radiation Research*, **156**, 439-440. (2001).
- Schettino, G., Folkard, M., Michette, A.G., Prise, K.M., Vojnovic, B., and Michael, B.D., A focussed soft X-ray microbeam for investigating the radiation responses of individual cells. In *Workshop on X-rays from electron beams*, (H. Prade, ed.), FZR, Dresden, FZR -287 pp 229 – 246. ISSN 1437-322x (2000),

c. Submitted for publication

- Rusyn EV, Schettino G, Folkard M, Prise KM, Michael BD, Held KD. Low dose hypersensitivity in human fibroblasts: A comparison of conventional and focused soft X-rays. *Radiation Research* In revision.
- Prise, K.M., Belyakov, O.V., Folkard, M., Ozols, A., And Michael, B.D., Non-targeted effects of radiation: some considerations of the influence of radiation quality and dose-effect relationships. *Advances in Space Research*.
- Schettino, G., Folkard, M., Prise, K.M., Vojnovic, B., Held, K.D., And Michael, B.D., Low dose studies of bystander cell killing with targeted soft X-rays. (*Radiation Res.*)
- Prise, K.M., Folkard, M and Michael, B.D., A review of the bystander effect and its implications for low dose exposure. *Radiation Protection Dosimetry*.
- Prise, K.M., Folkard, M., and Michael, B.D., Targeting radiation at the subcellular, cellular and tissue levels : Future Strategies In *Radiation Research: Science for the Future*.

d. In preparation

- McCarey YL, Karkala A, Tartier L, Held KD. Comparison of pathways to apoptosis in HL-60 cells exposed to high and low dose ionizing radiation. To be submitted to *Radiation Research*.

10. Interactions

a. Participation/presentations at meetings, etc.

Michael BD, Held KD, Folkard M, Prise KM. Low dose studies with focused X-rays in cell and tissue models: Mechanisms of bystander and genomic instability responses. Poster presented at US DOE Low Dose Radiation Research Program Workshop I, November 1999.

Michael, B.D., Schettino, G., Folkard, M., Michette, A.G., Prise, K.M., and Vojnovic, B., focused soft X-ray microbeam for investigating the radiation responses of individual cells, invited lecture, Workshop on X-rays from Electron Beams, Forschungszentrum Rossendorf, Germany, February 2000

Michael BD, Held KD, Folkard M, Prise KM. Low dose studies with focused X-rays in cell and tissue models: Mechanisms of bystander and genomic instability responses. Poster presented at US DOE EMSP National Workshop, April 2000.

Prise, K.M. Folkard, M. Belyakov, O.V. Malcolmson, A.M. Newman, H.C. Ozols, A., Schettino G. and Michael, B.D., The use of microbeams in the study of radiation-induced bystander effects. Invited Talk, Radiation Research 2000, Bristol, UK, April, 2000.

Michael, B.D., Prise, K.M., Belyakov, O.V., Folkard, M., Ozols, A., Schettino, G., and Vojnovic, B., Investigating the cellular effects of isolated radiation tracks using microbeam techniques, invited lecture, Committee on Space Research 33rd COSPAR Scientific Assembly, Warsaw, Poland, July 2000

Michael, B.D., Prise, K.M., Belyakov, O.V., Folkard, M., and Ozols, A., Non-targeted effects of radiation: some considerations of the influence of radiation quality and dose-effect relationships invited lecture, Committee on Space Research 33rd COSPAR Scientific Assembly, Warsaw, Poland, July 2000

Held KD. Invited participant in US DOE Low Dose Radiation Research Program Computer Modeling Workshop, September 2000.

Michael, B.D., Schettino, G., Folkard, M., Michette, A.G., Prise, K.M., and Vojnovic, B., Microbeam probes of cellular radiation response, invited lecture, IUPAP Conference on Biological Physics and Synchrotron Radiation: Medical Applications, Grenoble, France, October 2000

Michael, BD, Introductory remarks on effects of dose and radiation quality in relation to non-targeted effects, LH Gray Workshop on Radiation-induced Bystander Effects, Dublin, December 2000

Michael, B.D., Held, K.D., Schettino, G., Folkard, M., Prise, K.M. and Vojnovic, B., invited lecture, Microbeam irradiation with light ions and focused soft x-rays, NCI Workshop: Probing Individual Cells: Applications to Signaling, Structure and Function, Bethesda, March 2001

Held, KD. Biological consequences of low dose radiation exposure: Current controversies. Invited seminar, MIT Department of Nuclear Engineering, April 2001.

McCarey YL, Held KD. Rapid vs. delayed radiation-induced apoptosis in HL60 cells. Poster presented at the 48th Annual Meeting of the Radiation Research Society, April 2001.

Prise, K.M., Tracks to DNA: Watch out for the Neighbours, Michael Fry Award Lecture, 48th Annual Meeting of the Radiation Research Society, San Juan, Puerto Rico, USA. April, 2001.

Rusyn EV, Schettino G, Folkard M, Prise KM, Michael BD, Held KD. Low dose hypersensitivity in human fibroblasts: A comparison of conventional and focused soft X-rays. Poster presented at the 48th Annual Meeting of the Radiation Research Society, April 2001.

Michael, B.D., Folkard, M., Prise, K.M., Schettino, G., and Vojnovic, B, Charged-Particle and Focused Soft X-Ray Microbeams for Investigating the Radiation Responses of Cells, invited lecture, 13th Symposium on Microdosimetry, Stresa, Italy, May 2001

Prise, K.M. Belyakov, O.V. Newman, H.C. Patel, S., Schettino G., Folkard, M. and Michael, B.D., Non-targeted effects of radiation, Invited Talk, 13th Symposium on Microdosimetry - An Interdisciplinary Meeting on Radiation Quality, Molecular Mechanisms, Cellular Effects and Health Consequences of Low Level Ionising Radiation, Stresa, Italy, May, 2001.

Held KD, McCarey YL, Tartier L, Rusyn EV, Schettino G, Folkard M, Prise KM, Michael BD. Comparison of IR and ROS for induction of damage to cells. Poster presented at the DOE/NASA Low Dose Radiation Investigators Workshop, June 2001.

Prise, KM, Newman, H., Pinto, M., and Michael, B.D., DNA damage by high let radiation: role of clustering, invited lecture, 7th International Workshop on Radiation Damage to DNA, Orléans, France, September, 2001

Michael, B.D., Belyakov, O.V., Folkard, M., Ozols, A., Prise, K.M, Schettino, G., and Vojnovic, B., Targets for radiation-induced genomic instability, the bystander effect and other non-targeted responses, invited lecture, Workshop on Radiation-Induced Genomic Instability, Nagasaki, Japan, February, 2002

Folkard M, Vojnovic B, Schettino G, Prise KM, Michael BD. A variable-energy soft X-ray microprobe to investigate mechanisms of the radiation-induced bystander effect. Poster presented at DOE Low Dose Radiation Research Program Workshop III, March 2002.

Rusyn EV, Schettino G, Folkard M, Prise KM, Michael BD, Held KD. Low dose hypersensitivity and bystander responses in human and mouse fibroblasts: A comparison of conventional and focused soft X-rays. Poster presented at DOE Low Dose Radiation Research Program Workshop III, March 2002.

Prise, K.M, Folkard, M, and Michael, B.D., Targeted versus non-targeted cellular responses at low doses, Invited Talk, 49th Annual Meeting of the Radiation Research Society, Reno, NV, USA, April, 2002.

Schettino G, Folkard M, Prise KM, Vojnovic B, Held KD, Michael BD. Upgrading of the Gray Laboratory microprobe with higher energy X-rays and investigation of the bystander effect through the cell cycle. Poster presented at the 49th Annual Meeting of the Radiation Research Society, April 2002.

Schettino G, Newman HC, Prise KM, Folkard M, Held KD, Michael BD. Microbeam studies of relationships between bystander, direct and adaptive responses in V79 cells. Poster presented at the 49th Annual Meeting of the Radiation Research Society, April 2002.

Michael, B.D., Folkard, M, and Prise, K.M, Schettino, G., and Vojnovic, B., Techniques for micro-irradiation of cells and model tissue systems and their applications in radiation biology, invited lecture, Conference on Biological Effects of Ion Beam Irradiation, Xinjiang, China, July, 2002

Michael, B.D., Folkard, M, and Prise, K.M, Schettino, G., and Vojnovic, B., The new generation of probes of radiation actions on cells and tissues, teaching lecture, European Society for Therapeutic Radiology and Oncology, Prague, Czech Republic, September, 2002

Prise, K.M, Folkard, M, and Michael, B.D., Experimental studies of bystander responses: Challenging fundamental mechanisms, Invited Lecture, 4th International Conference on Health Effects of Low-Level Radiation, Oxford, UK, September, 2002.

b. Consultative and advisory functions

Held KD. Service on Scientific Advisory Committee for Radiobiology, Brookhaven National Laboratory, June 2002 – present.

Held KD. Member, NIH Radiation Study Section, 1998-2002.

Held KD. Member, USA MRMC Breast Cancer Research Program, RON Peer Review Panel, 2001.

Held KD, Member, Scientific Advisory Committees, Seventh and Eighth International Workshops on Radiation Damage to DNA, 1999-2001 and 2002-2004, respectively.

Michael, BD: Working Group for Department of Trade and Industry NMS Ionizing Radiation Program, 1992 – present.

Michael, BD: Member of International Commission on Radiation Units and Measurements, 1997 – present.

Michael, BD: National Institutes of Health Study Section site visiting RARAF, Columbia University, New York, NY, 1999

Michael, BD: Co-ordinator of EC Consortium “Induction, Repair and Biological Consequences of DNA Damages Caused by Radiations of Various Qualities” 2000-2003

Michael, BD: Co-ordinator of EC Consortium “European MSc Course in Radiation Biology”
2000-2003

Michael, BD: Member of Working Group on Impact and Applications of Nuclear Science: Life Sciences, Nuclear Physics European Collaboration Committee, 2001 – present.

Michael, BD: Member of Advisory Board for Therapy Project, GSI, Darmstadt, Germany, 2001 – present.

Michael, BD: Member of EC Cost Action “Radiation Damage in Biomolecular Systems”, 2003 –

Prise, K.M: Member, British Institute of Radiology Radiation Protection Committee, 1998 - 2001

Prise, K.M: International Scientific Committee, European Society for Radiation Biology, Dresden, 2000 – 2001

Prise, K.M: Scientific Committee, 4th International Conference on Health Effects of Low-Level Radiation, Oxford, UK, 2001 – 2002

c. Collaborations

Related to this project, the Gray Cancer Institute has also received a DOE grant in 2000 entitled “A variable-energy soft X-ray microprobe to investigate mechanisms of the radiation-induced bystander effect, PI: M. Folkard.

Also, as a result of this project, a collaboration developed that resulted in the funding of DOE grant in 2001 entitled “Mechanistic Modeling of Bystander Effects: An integrated theoretical and experimental approach”, PI: A. Chatterjee (Lawrence Berkeley National Laboratory); Co-PI’s (at individual institutions): BD Michael, KD Held, L Braby (Texas A & M University); Co-I’s: KM Prise, J Ford (Texas A & M University).

During this project, we also collaborated with Dr W.E. Wilson on aspects of low-LET microbeam dosimetry related to the focused soft X-rays. W.E. Wilson, J.H. Miller, D.J. Lynch, K. Wei and A. Kurtulus, Washington State University-TriCities, Richland, WA 99352, USA DE-FG03-99ER62860

The studies with the porcine ureter model were in collaboration with Dr CE Mothersill, DIT, Dublin, Eire. Ureters were kindly supplied by Dr M Rezvani, Churchill Hospital, Oxford, UK

The International Commission on Radiation Units and Measurements (ICRU) has agreed to commission a report “Approaches to Dosimetry at Low-Dose Exposure to Ionizing Radiation”. Funding to support this activity has been requested from and approved by the Low Dose Program . The PI of the present project, BD Michael, is a member of ICRU and is a co-sponsor of the report. Membership of the report committee will include several investigators in the Low Dose Program, including from among the above collaborations.

11. Transitions – Not applicable

12. Patents - None

13. Literature Cited

Biaglow JE, Manevich Y, Uckun F, Held KD. Quantitation of hydroxyl radicals produced by radiation and copper-linked oxidation of ascorbate by 2-deoxy-D-ribose method. *Free Radic Biol Med* **22**:1129-1138 (1997).

Kachur AV, Held KD, Koch CJ, Biaglow JE. Mechanism of production of hydroxyl radicals in the copper-catalyzed oxidation of dithiothreitol. *Radiat Res* **147**:409-415 (1997).

Kochevar IE, Lynch MC, Zhuang S, Lambert CR. Singlet oxygen, but not oxidizing radicals, induces apoptosis in HL-60 cells. *Photochem Photobiol* **72**: 548-553 (2000).

Zhuang S, Demirs J, Kochevar IE. p38 mitogen-activated protein kinase mediates bid cleavage, mitochondrial dysfunction, and caspase-3 activation during apoptosis induced by singlet oxygen but not by hydrogen peroxide. *J Biol Chem* **275**: 25939-25948 (2000).

14. Feedback

Feedback to DOE was provided through the provision of annual written reports and presentations given at 4 DOE workshops listed above in item 10.