Final Report of GCI group DOE project number DE-FG02-02ER63305

Determine the yield of micronucleated cells in primary human fibroblasts exposed to focused soft X-rays.

This project was a small part of a larger collaborative study headed by Dr Aloke Chatterjee, (Lawrence Berkley National Laboratory) and including Drs Les Braby, John Ford (Texas A&M) and Kathy Held (MGH Boston), which was developing an integrated theoretical and experimental model of the radiation-induced bystander response. Our part of the study has been to determine the effectiveness of soft X-rays at inducing chromosomal damage under conditions of direct and bystander exposure. The aim was to compare this with the effectiveness of the low energy 60 kV electron microbeam available at Texas A&M. Previous studies have been performed with primary human fibroblasts measuring micronuclei formation to determine the relative yields of direct versus bystander mediated micronuclei formation after cells were individually irradiated utilising our novel focussed soft X-ray microprobe, which is capable of producing localised submicron beams of carbon-K (278 eV) X-rays. Only a brief overview is given here as the study has been published in several papers (see below).

Our original hypothesis was to study yields of bystander-induced micronucleated cells in both wild-type and mutant fibroblast from mouse embryo fibroblasts. Difficulties with the level of background micronuclei in the MEFs prevented systematic studies of bystander responses in the laboratories involved in the collaboration. We then performed these studies with AG1522 primary human fibroblast cells using a siRNA approach developed by John Ford at Texas A&M to knock down DNA PKcs in the first instance.

Our soft X-ray source has been in routine use for carbon-K X-rays and is now available with Aluminium-K (1.49 keV) and titanium-K (4.5 keV), although the dose-rate from titanium is still too low at present for most experiments, where large numbers of cells need to be exposed. A separately funded project developed a new soft X-ray microprobe which will give much greater flexibility for changing energies and giving high dose-rates for exposures (See DE-FG02-01ER63236). However, we performed pilot studies measuring bystander responses with titanium-K. To date we have performed studies with V79 cells measuring cell survival as an endpoint and are starting studies in our human fibroblasts to measure micronuclei yields. A significant bystander response is observed in the V79 cells under conditions where only a single cell within a population was irradiated either with carbon-K or titanium-K X-rays. Typically around 10% cell killing is observed under these conditions. These studies are now being extended to measure micronuclei yields in the AG1522 cells under direct and bystander conditions. Our work has suggested that the yield of micronuclei in fibroblasts exposed to soft X-rays may be reduced in comparison to conventional X-ray exposures (Prise et al., 2003). Although further studies are required to confirm this using a range of scoring times.

Determine the role of replication dependent damage in bystander response

We have developed techniques for determining the cell cycle phase of cells at the time of irradiation, *in situ* on the microbeam stage. This involves measuring the fluorescence signal from the Hoechst staining at the time of irradiation. To date this has only been tested in the V79 cells and need to be extended to the AG1522 cells. We are also planning to try pulsed BUdR labelling of cells to determine the response of S-phase cells present at the time of irradiation. We then aim to determine whether any phase of the cycle has increased sensitivity to bystander-induced micronuclei formation.

For mechanistic studies of the formation of micronuclei we are quantifying the micronuclei produced in terms of whether they contain dsb. Two approaches are being used. One involves staining for kinetochores (CREST) to determine whether acentric fragments are present and the other is to use γ-H2AX as a marker to detect the presence of dsbs indirectly. Analysis of conventionally irradiated cells shows little difference in the distribution of kinetochore positive and negative micronuclei relative to control populations. These studies are now being extended to soft X-ray exposures and to low doses.

A complimentary approach is to use γH2AX as a marker for the production of DNA dsb in both directly exposed and bystander cells after soft X-ray exposure. Pilot studies have characterised the induction response of γH2AX production in primary human fibroblasts directly exposed to radiation and the kinetics of its removal during repair. For cells exposed to focused Al-K soft X-rays delivered through the nucleus, a linear relationship is observed with dose, for the induction of γH2AX foci, with a yield twice as high as that induced by conventional X-rays (~70 foci per cell per Gy (AL-K) versus ~35 foci per cell per Gy (240kV X-rays). The removal of γH2AX foci follows biphasic kinetics over 24 hours for both radiation types. For conventional X-ray exposure we see evidence for the production of larger foci at delayed times after irradiation. These may be a consequence of misrepair or residual un-repaired dsb and may be a potential marker for cell lethality. The use of γH2AX foci appears to offer a sensitive detector of exposure. We have detected direct induction of γH2AX foci with doses as low as 1cGy of Al-K X-rays giving an average of around 1 foci per cell (Hamada et al., 2006).

We have also detected significant levels of γH2AX foci in bystander cells as reported by several other groups. Critically, these γH2AX foci are not produced in bystander cells as a consequence of ATM or DNA-PK dependent phosphorylation (which is the case in directly irradiated cells). Using Seckel-cell fibroblasts it was observed that ATR plays a key role and this is restricted to S-phase cells confirming that stalled replication forks in response to DNA damage are playing a key role (Burdak-Rothkamm *et al.,* 2005).

Determine a role for RNS/ROS in the bystander response

Recent studies have suggested that nitric oxide may play an important role in mediating the bystander response. Some studies have reported a protective response when cells were prevented from releasing nitric oxide. To determine the role of nitric oxide in bystander responses after microbeam irradiation , we have monitored two cell lines. One a T98G radioresistant glioma and the other, AG1522 primary human fibroblasts being used in this project. When micronuclei are induced in either cell line

under bystander conditions, the presence of a NO scavenger at the time of exposure completely removes the bystander response. For the primary human fibroblasts, ROS species are also produced as the addition of DMSO can also remove the bystander response. A significant observation is that the phenotype of the cell receiving the bystander signal was important in determining the overall level of effect. These data also input into the cytokine/ROS studies which were performed by Dr Held at MGH Boston.

Publications

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SCHETTINO, G., FOLKARD, M., MICHAEL, B.D., and PRISE, K.M., (2005), Low-dose binary behaviour of bystander cell killing of a single cell with focused C_k X-rays. *Radiation Research*, **163,** 332-336.

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BURDAK-ROTHKAMM, S., SHORT, S.C., FOLKARD, M., ROTHKAMM, K., and PRISE, K.M., ATR-dependent radiation-induced γ-H2AX foci in bystander primary human astrocytes and glioma cells. *(Oncogene*) **in press**