Generations of students in radiation biology have been taught that heritable biological effects require direct damage to DNA. In fact, evidence has been available for many years that this simple statement is not strictly true. As early as the 1940s there were reports that the inactivation of biological entities may be brought about equally by ionizations produced within the entity or by the ionization of the surrounding medium. By 1947, Kotval and Gray had shown that alpha particles which pass close to the chromatid thread, as well as those which pass through it, have a significant probability of producing chromatid and isochromatid breaks or chromatid exchanges.

The term used today to describe such phenomena is "The Bystander Effect", a name borrowed from the gene therapy field, where it usually refers to the killing of several types of tumor cells by targeting only one type of cell within a mixed population (Cheng et al. 1999, for example). In the radiation field, it has come to be loosely defined as the induction of biological effects in cells that are not directly traversed by a charged particle, but are in close proximity to cells that are. Interest in this effect was sparked by the report of Nagasawa and Little (1992) that, following a low dose of alpha-particles, a larger proportion of cells showed biological damage than were estimated to have been hit by an alpha particle; specifically 30% of the cells showed an increase in sister chromatid exchanges even though less than 1% were calculated to have undergone a nuclear traversal. The number of cells hit was arrived at by a calculation, based on the fluence of alpha particles and the cross-sectional area of the cell nucleus. The conclusion was thus of a statistical nature since it was not possible to know on an individual basis, which cells were hit and which were not.

The plethora of data now available concerning the bystander effect fall into two quite separate categories, and it is not certain that the two groups of experiments are addressing the same phenomenon. First, there are experiments involving the transfer of medium from irradiated cells, which results in a biological effect in unirradiated cells. Second, there is the use of sophisticated single particle microbeams, which allow specific cells to be irradiated and biological effects studied in their neighbors. With experiments in the latter category, it is possible to distinguish between a large bystander effect propagated from one cell to another when they are in gap junction communication and a smaller bystander effect when cells were far apart and signals of necessity were transmitted via the medium.

The vast majority of published data in which individual cells were irradiated with a microbeam involve high LET α-particles, and to a lesser extent protons, since the technology to focus low LET radiations onto a single cell is difficult, to say the least. Colleagues from this laboratory, using the Columbia University microbeam have demonstrated that nuclear irradiation of A_L cells by α-particles elicited a bystander mutagenic response in neighboring cells not directly traversed by a particle. Furthermore, this mutagenic response can be demonstrated at doses as low as a single α-particle and that the response can be essentially eliminated by the use of drugs that block gap junction intracellular or by using cells in which the connexion gene is inactivated.

The purpose of the project in this proposal was to determine if a bystander response could be elicited by low LET radiation. Since microbeams were not available for low LET radiations, a different technique was used. CHO cells were labeled with a dose of tritiated thymidine, so that the DNA of these cells was irradiated with short range electrons. The range is so short that adjacent cells are not irradiated. The labeled CHO cells were then mixed with human-hamster hybrid (A_L) which allow the assessment of mutations in the single human chromosome 11 that they contain. The two cell types were centrifuged to form a "cluster" with the cells in close contact and maintained in this way for 24 hours, during which time the CHO cells accumulated a significant dose of radiation. Subsequently, the clustered were dispersed and the two cell types separated and sorted by magnetic cell separation (MACS). The CHO cells were assayed for cell survival, while the A_L cells were assayed for both cell survival and the incidence of mutations.

As experiments progressed, it was found necessary to modify the protocol in one important respect. The sorting of cells by MACS proved to be about 99% efficient, which is adequate for cell survival studies down to about two decades, however, it is not adequate for the mutation assay, where the yield is typically one in 10^4 cells. Consequently all putative mutant colonies were checked by the use of a centromeric probe towards human chromosome 11, present only on the hybrid A_L cells. This process is very labor-intensive and made the project much more difficult than initially envisaged.

In the initial project, a range of activities of tritiated thymide were used, namely 10, 30, 50 and 100 μCi. At the highest activity, the fraction of self-irradiated CHO cells surviving was 0.2, while the fraction of unirradiated A_L cells surviving was 0.55, showing a substantial bystander effect. In addition the incidence of mutations on the A_L cells was 250 mutants/10^5 survivors, compared with a background level of $20/10^5$ – again a substantial bystander effect. Multiplex PCR analysis revealed the types of mutants on the bystander cells to be significantly different to those of spontaneous origin; with substantially more deletions.

To determine whether reactive oxygen species contribute to bystander mutagenesis, the free radical scavenger DMSO was incorporated into the clusters and maintained throughout the incubation period. This resulted in a 50% reduction in the mutation frequency in bystander cells, indicating that free radicals participate in the pathway to mutagenesis. By contrast, the use of the gap junction inhibitor Lindane, or the use of A_L cells that are dominant negative for connexion 43 and lack gap junction formation essentially eliminated the bystander mutagenic response.

Success with the first series of experiments prompted us to try to repeat the experiments with substantially lower doses of tritiated thymidine, namely 0.5, 1 and 5 μCi. There is no easy way to estimate the actual absorbed doses involved since it is difficult to determine the amount of tritiated thymidine involved, and the uptake is likely to be non-uniform. Judging by the fraction of cells surviving the three levels of incorporated tritiated thymidine, and by comparison with previously published survival data for X-rays, the effective X-ray doses for 0.5, 1.0 and 5 μCi of tritiated thymidine are 0.2, 0.75

and 1 Gy respectively. Again, at these lower doses, a substantial bystander effect was apparent in the A_L cells, both in terms of cell killing and mutations in surviving cells. An added touch to these experiments was the demonstration that functional gap junctions were formed between the cells that were in intimate contact, as evidenced by the migration of the fluorescent dye Calcein M from the irradiated to the non-irradiated cells.

These data provide compelling evidence that, at doses relevant to radiation protection, low LET radiation, at least in the form of short range electrons, can induce bystander mutagenesis, and that reactive oxygen species and intercellular communication have a modulating role. The principal goals of the proposal were achieved.

Publications

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