Paper

THE BYSTANDER EFFECT

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Abstract—**The bystander effect refers to the induction of biological effects in cells that are not directly traversed by a charged particle. The data 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; in this case communication is by gap junction. Medium transfer experiments have shown a bystander effect for cell lethality, chromosomal aberrations and cell cycle delay. The type of cell, epithelial vs. fibroblast, appears to be important. Experiments suggest that the effect is due to a molecule secreted by irradiated cells, which is capable of transferring damage to distant cells. Use of a single microbeam has allowed the demonstration of a bystander effect for chromosomal aberrations, cell lethality, mutation, and oncogenic transformation. When cells are in close contact, allowing gap junction communication, the bystander effect is a much larger magnitude than the phenomenon demonstrated in medium transfer experiments. A bystander effect has been demonstrated for both high- and low-LET radiations but it is usually larger for densely ionizing radiation such as alpha particles. Experiments have not yet been devised to demonstrate a comparable bystander effect on a three-dimensional normal tissue. Bystander studies imply that the target for the biological effects of radiation is larger than the cell and this could make a simple linear extrapolation of radiation risks from high to low doses of questionable validity.**
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INTRODUCTION

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 1940's 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 (Dale 1940, 1942, 1943; Lea et al. 1944). By 1947, Kotval and Gray had shown that alpha particles that 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 (Randers-Pehrson et al. 2001).

Medium transfer experiments

Experiments involving the transfer of medium from irradiated to unirradiated cells have demonstrated a

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highly significant reduction in the plating efficiency of both normal and malignant epithelial cells—whether or not the cells were irradiated (Mothersill and Seymour 1997).

This bystander effect suggested that irradiated cells secreted a molecule into the culture medium that was capable of killing cells when that medium was transferred onto unirradiated cells. By contrast, medium irradiated in the absence of cells had no effect. Further experiments demonstrate that not all cells were capable of producing the toxic factor, nor were all cells capable of receiving the secreted signal (Mothersill and Seymour 1997, 2001). In later experiments using explants of human uroepithelium, Mothersill et al. (2001) show that there is considerable variation in the release of the bystander factor into the surrounding cell culture medium. The effect produced by epithelial cell cultures is dependent on the cell number at the time of irradiation, can be observed as soon as 30 min post irradiation, and is still effective if taken from the irradiated cells up to 60 h after irradiation. This bystander effect can be induced by radiation doses as low as 0.25 mGy and is not significantly increased up to doses of 10 Gy. Forty-eight hours after receiving irradiated medium there were many apoptotic bodies present, suggesting that apoptosis may be a prominent mechanism of cell death responsible for the reduced clonogenic survival. In addition to increased levels of cell death and reduced cloning efficiency, medium transfer experiments have shown an increase in neoplastic transformation as well as genomic instability in cells that have not themselves been irradiated.

Some limited progress has been made in the search for the mechanisms involved in this bystander effect. Following exposure to radiation, the first detectable effect of transferred medium on recipient cells was a rapid calcium pulse (1–2) followed 30–120 min later by changes in mitochondrial membrane permeability and the induction of reactive oxygen species (Lyng et al. 2002). Gap junction communication between cells was not required to induce killing of bystander cells, but medium from cell cultures irradiated at high densities induced the greatest amount of cell death. Furthermore, the use of apoptosis inhibitors or medium from lactate dehydrogenase or glucose-6-phosphate dehydrogenase mutant cells reduced or prevented the bystander effect. Treatment with the anti-oxidants L-lactate and l-deprenyl prevented bystander factor associated cell kill suggesting that energy/REDOX metabolism may be involved in the medium mediated bystander response.

The majority of bystander experiments involving medium transfer have utilized low-LET x or gamma rays, in contrast to microbeam experiments where alpha particles or protons have been the particles of choice.

THE BYSTANDER EFFECT DEMONSTRATED BY MICROBEAM EXPERIMENTS

Experiments described here involve the scoring of micronuclei, cell lethality, mutation, and oncogenic transformation.

Micronuclei in normal human fibroblasts

Perhaps the most direct and most dramatic demonstration of the bystander effect involves the observation of micronuclei in irradiated human fibroblasts. Cells of one population were lightly stained with cyto-orange, a cytoplasmic vital dye, while cells of another population were lightly stained blue with a nuclear vital dye. The two cell populations were mixed and allowed to attach to the culture dish, and the computer controlling the accelerator was programmed to irradiate only blue-stained cells with 10 alpha particles directed at the centroid of the nucleus. The cells were fixed and stained 48 h later, at which time micronuclei and chromosome bridges were visible in a proportion of the nonhit (i.e., orange-stained) cells (Fig. 1). This is an astonishing demonstration of the bystander effect because the development of micronuclei implies significant chromosome damage and rearrangement, which is clearly visible in nonhit cells that have been fixed in situ.

Cell lethality

Lines of hygromycin- and neomycin-resistant V79 cells were produced. Before exposure the hygromycin-resistant cells were stained with a low concentration of a

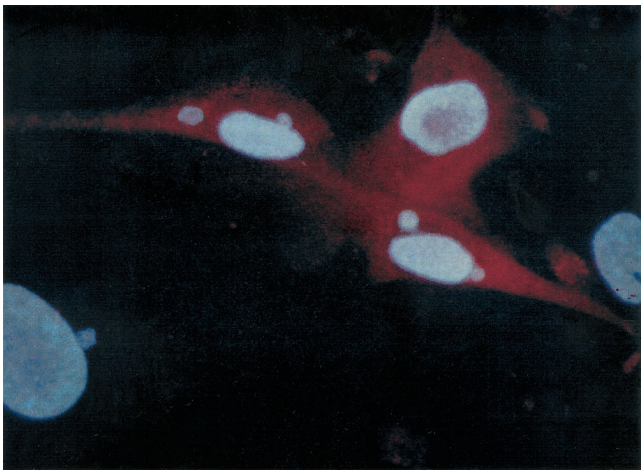

Fig. 1. The bystander effect with human fibroblasts. Cells of one population were stained with the vital nuclear dye Hoechst 3342 (blue fluorescence), and cells of another population were stained with the vital cytoplasmic dye cell tracker orange (orange fluorescence) and mixed at a ratio of 1:1. Only blue nuclei were microbeam irradiated with alpha particles; the orange cells were thus "bystanders." Cells were fixed and stained 44 h after exposure to radiation. A micronucleus is clearly visible in an orange (nonhit) cell (courtesy of Charles Geard).

vital nuclear dye. They were then plated in micro wells in the proportion nine neomycin-resistant for every one hygromycin-resistant cell. The computer was programmed to irradiate only the 10% of cells stained with a nuclear dye with various numbers of alpha particles from 1–16 aimed at the centroid of the nucleus. The cells were then removed and cultured for survival in the appropriate growth media, which made it possible to obtain survival curves for hit and nonhit cells. The data are shown in Fig. 2. There is a considerable degree of cell killing in the nonhit cells, implying a substantial bystander effect. The magnitude of the bystander effect in these studies is much greater than that reported by The Gray Institute for Cancer Research where only 5 to 10% lethality is seen in nonhit cells, using protons or soft x rays in a microbeam. The difference is probably accounted for by the cell density. In The Gray Institute studies, only about 200 cells were seeded in an area of 10 × 10 mm. The average distance between cells, therefore, was some hundreds of microns, so it is likely that communication via gap junction did not contribute to the effect observed. By contrast, in the studies reported here, 1,000 to 1,200 cells were plated in a mini-well of 6.3 mm diameter so that 50 to 60% were in contact, allowing gap junction communication that has been demonstrated to be of importance in mutation studies with the microbeam. Therefore, the current study also supports the need for gap junction communication as a mediator of bystander effects in relation to radiation-induced cell killing.

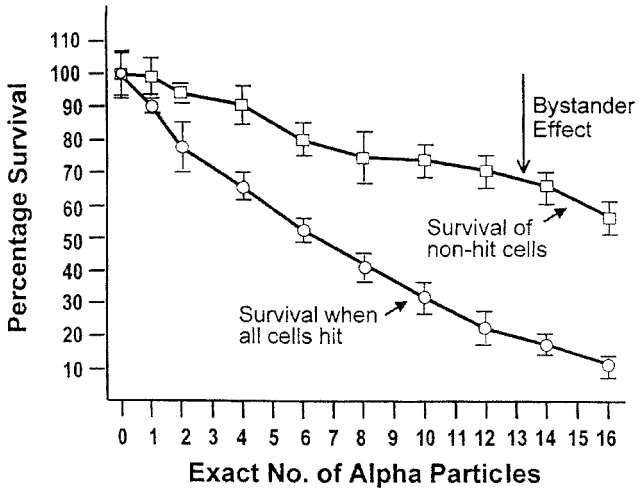

Fig. 2. The bystander effect for cell survival in V79 cells. Each data point (mean ± SE) on the line with circles refers to the survival of cells when all cell nuclei on each dish were exposed to the same exact numbers of alpha particle traversals using the microbeam system. The squares show survival for various numbers of alpha particles, from 1–16, traversing 10% of the cell population. The extent to which this falls below the 100% survival for the nonhit is an indication of the magnitude of the bystander effect. Each data point represents the mean ± SD of the clonogenic survivals from three culture plates (redrawn from Sawant et al. 2002).

Mutagenic effects in human-hamster hybrid cells

Zhou et al. (2000) reported a study in which human-hamster hybrid (A_L) cells were exposed to alpha particles by use of the Columbia microbeam. After all cells on the dish were identified and located, the computer was programmed to expose 20% of the cells, randomly selected, to 20 alpha particles directed through the centroid of the nucleus. This irradiation allows less than 1% of the cells to survive, and yet when assayed for mutations in the human chromosome 11, the mutation yield was four times that of the background (Fig. 3). These mutations must clearly arise from neighbor cells, not directly exposed, but in close proximity to irradiated cells.

A further series of experiments identified the importance of cell-cell communication via gap junctions as a mechanism of the bystander effect. When A_L cells were transfected with a dominant negative connexin 43 vector (DN6), which eliminates gap junction communication, the bystander effect essentially disappears. This is illustrated in Fig. 4.

Oncogenic transformation in mouse fibroblasts

Mouse fibroblast (C3H10T½) cells were plated in a monolayer, and the computer was programmed to irradiate either every cell, or every tenth cell, selected at random with 1–8 alpha particles directed at the centroid (Sawant et al. 2001) of the cell nucleus. The cells were subsequently removed by trypsinization, replated at low

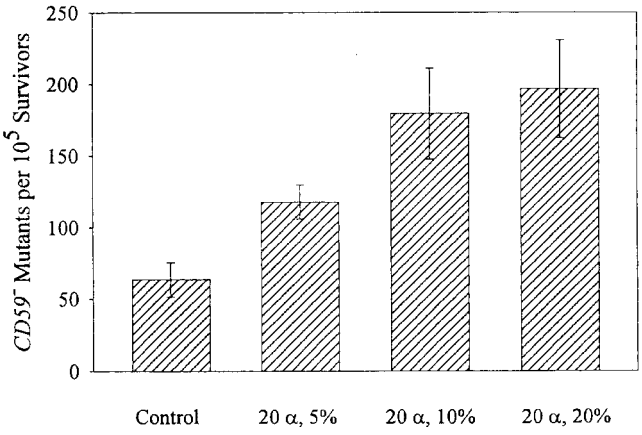

Fig. 3. The bystander effect for mutations in the human-hamster hybrid (A_L) cells when 20% of the cells receive 20 nuclear traversals by alpha particles. There is a substantial incidence of mutations over the background level, despite the fact that no irradiated cells survive (redrawn from the data of Zhou et al. 2000).

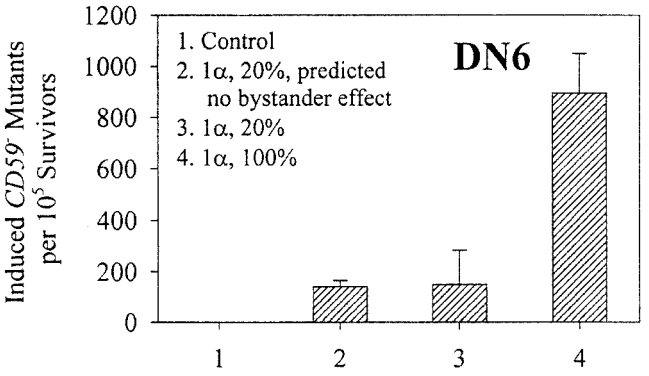

Fig. 4. Mutation fraction (M_F) from population of A_L-AH1-9 cells transfected with a dominant negative connexin 43 vector (DN6). Error bars represent ± SD. The population of AH1-9 cells used in these experiments have higher mutant induction as well as background mutant level than the parental A_L cells (redrawn from the data of Zhou et al. 2001).

density, and transformed foci were identified 6 wk later by their morphologic appearance. The results are shown in Fig. 5 and illustrate that (1) more cells can be inactivated by alpha particles than were actually traversed by an alpha particle and (2) when 10% of the cells on a dish are exposed to two or more alpha particles, the resulting frequency of induced oncogenic transformation is indistinguishable from that when all the cells on the dish are exposed to the same number of alpha particles.

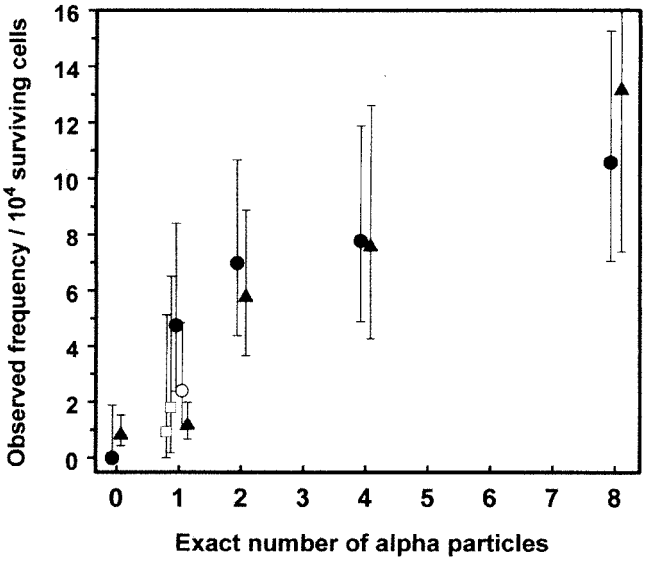

Fig. 5. Yield of oncogenically transformed cells per 10^4 surviving C3H10T½ cells produced by nuclear traversals by 5.3 MeV alpha particles. Triangles represent exposure of all cell nuclei on each dish to exact numbers of alpha particles using the microbeam system. Solid circles represent exposure of 1–10 cell nuclei on each dish to exact numbers of alpha particles. Open squares represent subsequent repeats of the experiment in which 1–10 cell nuclei were exposed to exactly one alpha particle. Open circle represents combined data for all the experiments in which 1–10 cell nuclei were exposed to one alpha particle including these repeat experiments (with caveats described in the text). Standard errors (± SD) were estimated assuming an underlying Poisson-distributed number of transformed cells (26) (redrawn from the data of Sawant et al. 2001).

Implications

It is important to note that the experimental results discussed in this paper involve laboratory model systems, since bystander experiments with in vivo systems, particularly in the human, are clearly not possible at the present time. However, if these results were applicable in vivo, they could have significant consequences in terms of extrapolation of radiation risks from high to low doses, implying that the relevant target for radiation oncogenesis is larger than an individual cell, and that the risk of carcinogenesis would increase more slowly, if at all, at intermediate doses. Thus a simple linear extrapolation of radiation risk from intermediate doses (where they can be measured) to lower doses (where they must be inferred) would be of questionable validity, at least at high-LET.

This is illustrated in Fig. 6 which combines the data of Zhou et al. (2001), in which only a proportion of cells are irradiated with a single particle (allowing the bystander effect to be manifest), together with a previous compilation of data by Zhou et al. (2000) where all cells were exposed to various numbers of particles from 1–4.

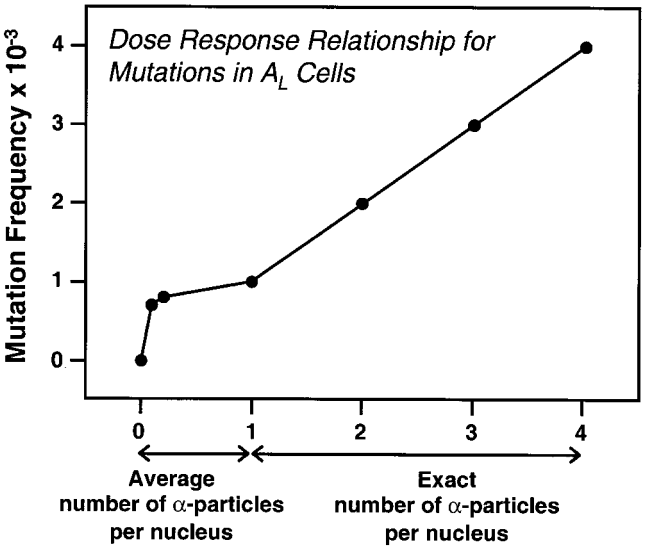

Fig. 6. Mutation frequency as a function of the number of alpha particles per nucleus (data fractions of the cell population were exposed to a single alpha particle). Due to the bystander effect which is evident when only a proportion of the population is exposed, the risk at low doses is higher than predicted by a linear extrapolation from high doses (based on the data of Zhou et al. 2000, 2001).

Under these experimental conditions, it is evident that a linear extrapolation of risks from high doses to low doses (which average less than one particle per cell) would underestimate the risks at low doses. This applies, at this stage, strictly to alpha particles, and it is not known whether it would apply in an in vivo situation to, for example, radon exposure in homes and mines.

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