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The Bystander Response in C3H 10T½ Cells: The Influence of Cell-to-Cell Contact

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Although radiation-induced heritable damage in mammalian cells was thought to result from the direct interaction of radiation with DNA, it is now accepted that biological effects may occur in cells that were not themselves traversed by ionizing radiation but are close to those that were. However, little is known about the mechanism underlying such a bystander effect, although cell-to-cell communication is thought to be of importance. Previous work using the Columbia microbeam demonstrated a significant bystander effect for clonogenic survival and oncogenic transformation in C3H 10T½ cells. The present study was undertaken to assess the importance of the degree of cell-to-cell contact at the time of irradiation on the magnitude of this bystander effect by varying the cell density. When 10% of cells were exposed to a range of 2–12 α particles, a significantly greater number of cells ($P < 0.0001$) were inactivated when cells were irradiated at high density (>90% in contact with neighbors) than at low density (<10% in contact). In addition, the oncogenic transformation frequency was significantly higher in high-density cultures ($P < 0.0004$). These results suggest that when a cell is hit by radiation, the transmission of the bystander signal through cell-to-cell contact is an important mediator of the effect, implicating the involvement of intracellular communication through gap junctions.

INTRODUCTION

It has been a long-held tenet of radiation biology that cellular DNA damage required direct interaction of radiation with DNA. This ''targeted'' DNA damage occurred either by direct ionization or by production of hydroxyl radicals in water molecules adjacent to DNA. However, over the past decade, considerable evidence has emerged for the existence of a ''non-targeted'' phenomenon that has been termed the bystander effect (*1*). The bystander effect is defined as the observation of a biological response in cells which have not been directly traversed by ionizing radiation but which results from signals initiating in cells in which energy has been deposited. This may be an important phenomenon influencing the shape of the dose–response curve, particularly at low doses, where there are many nontraversed cells, and several models are available which assess the significance of such effects at low doses (*2, 3*). Although the effect of such bystander responses on the low-dose cancer risk is not fully understood (*4*), they are thought to represent a balance between protective mechanisms such as apoptosis and differentiation (*5*) and potentially harmful mechanisms, in which DNA damage and potential genomic instability are mediated through bystander signals (*3, 6*).

To date, many studies, primarily using single cell *in vitro* systems but also *in vivo*, have confirmed a bystander response for several end points including clonogenic survival, oncogenic transformation, micronucleus induction and gene expression (reviewed in ref. *7*). These studies have employed three distinct protocols: the conventional irradiation of cells with low fluences of α particles, the transfer of medium from irradiated onto unirradiated cells, and the use of charged-particle microbeams, which have made it possible to define precisely which cells are traversed by an exact number of α particles (reviewed in ref. *8*). However, the specific factor(s) produced by irradiated cells responsible for the bystander response remains unknown, although several candidate molecules have been suggested (*9–11*).

It seems likely that the bystander effect is mediated by two distinct mechanisms that depend on the experimental protocol employed and that both may contribute to the final response (*12*). The first involves the transmission of a secreted, soluble extracellular factor from irradiated to unirradiated cells, often over some considerable distance (*13, 14*). The second is direct communications between adjacent cells through gap junctions (*15, 16*).

Previous studies using the Columbia microbeam have shown a significant bystander effect for the end points of clonogenic survival and oncogenic transformation in C3H 10T½ cells (*17, 18*). The aim of the present study was to assess whether the magnitude of this effect was dependent upon cell-to-cell proximity at the time of irradiation. To achieve this, cells were plated at both high and low density,

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targeted with a range of α particles aimed at the centroid of the nucleus, and assessed for clonogenic survival and oncogenic transformation.

MATERIALS AND METHODS

Microbeam Irradiation

The Columbia microbeam system and the irradiation procedures have been described in detail previously (*19*). Briefly, cells were attached to the thin bases (3.8 μm polypropylene) of 6.3-mm-diameter miniwells to give a final cell density of either 200 or 2000 cells per well. Individual nuclei were then identified and located with a computer-controlled optical image analysis system. For each dish, a computer/microscope-based analysis system first automatically locates and records the *x,y* coordinates of the nuclei of cells on the dish. Next the dish is moved sequentially under computer control such that the first cell nucleus is positioned over a highly collimated α-particle beam. The beam shutter is opened until the required numbers of α particles are detected (with a gas-filled ion counter mounted on the microscope lens) to have passed through the nucleus. The shutter is then closed and the next cell is moved over the beam. The overall spatial precision of the beam, including positioning and beam spread, is about ±3.5 μm, compared with an average nuclear cross-sectional area of the cells of approximately 200 μm^2 and a cellular cross-sectional area of >500 μm^2 (*20*). In the present study, 5.3 MeV α particles accelerated by a Van de Graaff accelerator were used for the irradiations. The average stopping power of the α particles traversing the cells was 90 keV/μm. The search and irradiate software can be instructed to expose any given proportion of the cells, selected at random, to any desired number of α particles. To assess clonogenic survival, either 10% or 100% of the cells were exposed to a range of α particles from 2–12 through the nucleus and for oncogenic transformation 10% of the cells were exposed to 8 α particles. Dishes used to assess plating efficiency were sham-irradiated, i.e. handled in an identical fashion except that the beam shutter was not opened. Irradiation times for each dish were approximately 6–10 min.

Cell Culture

Prior to irradiation, C3H 10T½ mouse fibroblast cells between passages 9 and 11 were grown in Eagle's basal medium supplemented with 10% FBS and penicillin/streptomycin. Eighteen hours before exposure, 200 or 2000 exponentially growing cells were plated into miniwells as described above. Prior to irradiation, cells plated at the high density formed a confluent culture with a high degree of cell-to-cell contact, in contrast to the low-density cultures in which the cells were individuals, separated from their neighbors. The attached cells were stained for 0.5 h with 50 n*M* of the vital nuclear dye Hoechst 33342, enabling individual nuclei to be identified and located using the optical image analysis system. Immediately prior to irradiation, cells were washed with serum-free medium to avoid fluorescence from serum components and irradiations were carried out in the presence of a thin film of serum-free medium. After irradiation, cells were washed twice with PBS and trypsinized from the irradiation container. For assessment of clonogenic survival, approximately 100 viable cells were plated into 100-mm culture dishes and incubated for 2 weeks, and the resulting colonies were stained with 2% crystal violet to determine both the plating efficiencies and surviving fractions of the control and irradiated cells. To assess oncogenic transformation, cells were replated at a low density of about 300 viable cells per dish. The cells were incubated for 7 weeks with culture medium changed every 12 days before they were fixed and stained with Giemsa to identify morphologically transformed type II and III foci, as described elsewhere (*21*).

Data from a minimum of three independent experiments were pooled. All data for clonogenic survival were presented as means with standard errors. Surviving fractions measured at the doses tested were fitted with the linear-quadratic equation. The statistical significance of differences between groups was tested by Student's *t* test and between survival curves using multiple regression analysis.

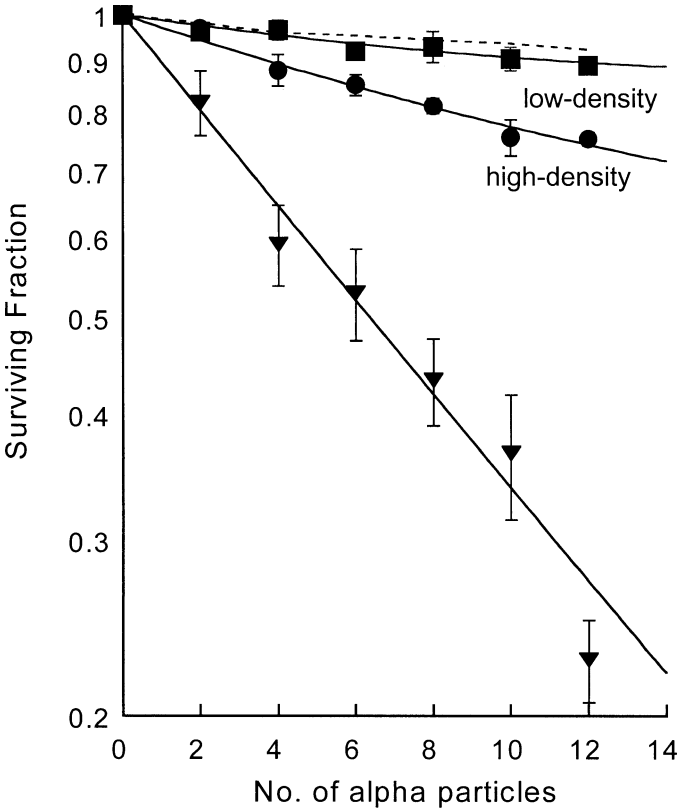

FIG. 1. Clonogenic survival of C3H 10T½ cells after nuclear traversals by 5.3 MeV α particles. Data points represent the means ± SEM from at least three repeat experiments. The triangles indicate survival when all cell nuclei on the dish are exposed to a range of α-particle traversals, from 2 to 12 per nucleus. The dotted line shows the percentage of cells that would be expected to survive when 10% of the cells are irradiated and is calculated from the survival observed when all cells are irradiated. The circles and squares show survival when 10% of the cells are irradiated at high and low density, respectively. The extent to which survival seen at the two densities falls below the dotted line indicates the magnitude of the bystander effect.

RESULTS

The results of the experiments to compare clonogenic survival for cells exposed at high and low density are shown in Fig. 1. The clonogenic survival obtained when 100% of cells are hit by various numbers of α particles is indicated by the solid triangles and is in agreement with that seen previously in C3H 10T½ cells (*18*).

The dotted line shows the percentage of the cells that would be expected to survive when 10% of the cells are irradiated in the absence of any bystander effect. It is calculated by applying the 100% survival curve to the 10% of cells that were actually irradiated. The results are compared with those obtained from the irradiation of 10% of the cells at either high or low density, which are shown by the solid circles and squares, respectively. The extent to which these lines fall below the dotted line indicates the magnitude of

TABLE 1
Clonogenic Survival Rates, Number of Dishes Exposed, Numbers of Viable Cells Exposed in Transformation Studies, Number of Transformed Clones Produced, and Transformation Frequencies for Microbeam Irradiations

No. of cells plated, no. of α particles	Clonogenic surviving fraction (plating efficiency) (±SEM)	No. of dishes exposed	No.[a] of viable cells exposed/10^4	No. of transformants produced	Transformation frequency/10^4 surviving cells
200, 0	(0.23 ± 0.02)	71	0.9	1	1.1
2000, 0	(0.17 ± 0.03)	60	2.1	1	0.5
200, 8	0.93 ± 0.02	155	1.8	6	3.3
2000, 8	0.81 ± 0.01	73	1.5	14	9.6

[a] Estimated, accounting for plating efficiency and clonogenic survival.

the bystander effect, although only the cell survival observed in high-density cultures is significantly lower ($P < 0.0001$ compared to $P = 0.11$ at low density). At both cell densities, the surviving fractions fall progressively as more α particles traverse the nucleus, but the amount of cell killing is significantly greater at the high cell density compared with low-density cultures ($P < 0.0001$).

Results from experiments conducted to evaluate the effect of cell density at the time of irradiation on oncogenic transformation are shown in Table 1. In these studies, a total of approximately 3.1×10^5 cells were individually imaged, positioned and irradiated. At high density, a transformation frequency of $9.6/10^4$ viable cells was seen, which is similar to that found previously in high-density cultures (*17*). Using previously published data (*22*), it is possible to calculate that when 10% of the cells in a population are irradiated with 8 α particles, the expected transformation frequency in the absence of a bystander effect would be $2.1/10^4$ viable cells. This is lower than that seen in the present study at both cell densities, although again the difference is only significant in the case of the high-density cultures ($P < 0.0001$ compared to $P = 0.28$ at low density). A statistically significant threefold decrease in the transformation frequency was observed in the low-density cultures relative to high-density cultures ($P < 0.0004$).

DISCUSSION

It is now widely accepted that radiation-induced heritable effects in mammalian cells are not solely the result of direct damage to DNA, and there is now evidence for a number of non-targeted effects, including the bystander response, which do not require a direct nuclear exposure (*1*).

Although reproducible bystander effects have now been demonstrated for a range of biological end points, the mechanisms by which the biological insult is transmitted from targeted to non-targeted cells have not been fully elucidated (reviewed in ref. *8*).

One causative agent may be the secretion from irradiated cells of a soluble factor(s) into the medium that then elicits a biological response in adjacent, unirradiated cells (*13*). Alternatively, when densely cultures are irradiated, the signal may be transmitted through cell-to-cell communication between adjacent cells (reviewed in ref. *7*). When cells are in close contact, they can communicate through gap junctions which are intercellular membrane channels that permit the direct exchange of small molecules (<1.2 kDa) between adjacent cells (*23*).

It now seems apparent that for a given cell line, transmission of the bystander signal by cell-to-cell contact elicits a more pronounced bystander effect compared with the transfer of an extracellular factor through the medium, suggesting that these are two separate phenomena. Evidence for this comes from mutation studies in A_L cells using two different protocols. Either 20% of densely cultured cells (≈70% of cells in contact) were irradiated with the Columbia microbeam, or cells on one surface of a double Mylar dish were irradiated while cells on the other side of the dish acted as bystanders. The results suggest that the cytotoxic factor(s) released from the cells into the culture medium using the latter protocol had a small, barely significant effect on the mutagenic response of the bystander cells (*24*), whereas the microbeam-based studies showed a threefold elevation of mutation incidence in bystander cells, which was significantly reduced in the presence of the gap junction inhibitor lindane (*15*).

A similar conclusion has been implied from experiments performed with V79 cells in two different laboratories. In one study carried out at Columbia University, cells were irradiated with various numbers of α particles and a considerable degree of cell killing was seen in non-hit cells, with survival reduced to 60% at the highest dose of 16 α particles per nucleus (*25*). This was in contrast to data from the Gray Cancer Institute, UK, where only 5 to 10% lethality was seen (B. Michael, personal communication, 2001), and it was concluded that this discrepancy was a result of the cells being plated at a lower density in the latter study. Here there was little contact between cells, and therefore the bystander effect observed was assumed to be due to the release of a soluble factor into the medium affecting nonirradiated cells.

However, there are several differences between the protocols used at Columbia and the Gray Cancer Institute for microbeam experiments that, in addition to the cell density at the time of irradiation, may have contributed to the observed discrepancy in survival.

The present study is the first to examine the effect of cell density under the same experimental conditions, allowing us to conclude that any observed differences are solely a result of cell density at the time of irradiation. When approximately 2000 cells were plated on a microbeam dish, the vast majority (>90%) of the cells were in direct contact with neighbors through membranes and intercellular gap junctions when they were irradiated 18 h later. In contrast, when 200 cells were plated using the same protocol, very little contact between cells (<10%) was seen, with the majority of cells appearing as isolated entities, separated by many tens of micrometers from their neighbors.

The results of the present study confirm those seen previously in C3H 10T½ cells when irradiation of 10% of the cells (≈80% confluent at time of irradiation) on a dish with an exact number of α particles (>4 α particles/nucleus) resulted in a surviving fraction of less than 90%, indicative of a substantial bystander effect (*17*).

In the present study, at both high and low cell density, the surviving fraction fell progressively as the number of α particles traversing 10% of the cells increased. This suggests that as more damage is inflicted on the cells, there is an increase in transmission of the bystander signal either through increased gap junction communication (high density only) or increased secretion of a cytotoxic factor(s) into the culture medium. However, the amount of cell killing, and by implication the magnitude of the bystander effect, was significantly greater in the high-density cultures, with an approximately 2.5-fold increase in the amount of cell killing at the highest dose tested. A significant increase in the transformation frequency was also observed at the high density. These data indicate that the magnitude of the bystander effect is dependent on cell density in C3H 10T½ cells, implicating the involvement of gap junction-mediated intercellular communication in transmitting the bystander effect. Several studies have now shown that inhibition of this gap junction activity in cells irradiated in close contact results in decreased levels of the bystander effects for a variety of biological end points (*15, 16, 26*). An alternative, but unlikely, explanation is that the observed effect is due to some factor released into the medium, which, because of a very short half-life, can migrate only small distances from the irradiated cell. This is unlikely because it has been estimated that for the irradiation protocol used in the present study, any bystander signal induced could travel over a large distance through the medium during irradiation (approximately 600–700 μm) (*27*).

The results obtained for low-density cultures did deviate from those expected in the absence of a bystander effect, suggesting that such an effect may still be operative. Considering oncogenic transformation, in the absence of a bystander effect, a transformation frequency of $2.1/10^4$ viable cells is expected that is less (although not significantly) than the observed frequency of 3.3 (Table 1). A similar result was seen for clonogenic survival with a non-significant increase in cell killing (Fig. 1). However, any bystander effect evident in the low-density cultures is likely to result from interaction of a secreted cytotoxic factor with unirradiated cells rather than from direct communication due to the very low frequency of cell-to-cell contact. This has been confirmed in a previous study of low-density cells in which a random distribution of damaged cells throughout the population was seen, suggestive of an extracellular factor (*14*).

In conclusion, the present study confirms that when cells are exposed to low doses of α particles, the degree of cell-to-cell contact at the time of irradiation is important in transmission of the bystander signal. When cells are in close contact, gap junctions play a major role, whereas if the degree of contact is low, the bystander effect is mediated by the release of factors into the surrounding environment.

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