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Biological Responses in Known Bystander Cells Relative to Known Microbeam-Irradiated Cells

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Normal human fibroblasts in plateau phase (≅95% G_1 phase) were stained with the vital nuclear dye Hoechst 33342 (blue fluorescence) or the vital cytoplasmic dye Cell Tracker Orange (orange fluorescence) and plated at a ratio of 1:1. Only the blue-fluorescing nuclei were microbeam-irradiated with a defined number of 90 keV/μm α particles. The orange-fluorescing cells were then "bystanders", i.e. not themselves hit but adjacent to cells that were. Hit cells showed a fluence-dependent induction of micronuclei as well as delays in progression from G_1 to S phase. Known bystander cells also showed enhanced frequencies of micronuclei (intermediate between those seen in irradiated and control cells) and transient cell cycle delays. However, the induction of micronuclei in bystander cells did not appear to be dependent on the fluence of the particles delivered to the neighboring hit cells. These are the first studies in which the bystander effect has been visualized directly rather than inferred. They indicate that the phenomenon has a quantitative basis and imply that the target for radiation effects cannot be considered to be the individual cell. © 2004 by Radiation Research Society

INTRODUCTION

A basic paradigm in considering the potential risk associated with exposure to ionizing radiation is that cell nuclear DNA is the target. In recent years this basic concept and consequent extrapolations of risk from high to low doses have been brought into question. An increasing amount of data from radiation studies has led to the proposal that the overall frequency at which a particular end point is observed in an irradiated population has a component from cells that are not directly hit by the initial irradiation. The nonirradiated cells that respond have been termed bystanders, a term borrowed from a similar phenomenon reported in viral transfection experiments (*1–4*).

It has been reported that exposure to very low doses of α particles initiated sister chromatid exchanges in more cells than it was calculated could have been hit by an α particle (*5, 6*). These non-hit, responding cells were "bystanders" either of directly hit cells or of energy depositions in extracellular medium. Similar types of experiments have also demonstrated the induction of specific genes in more cells than were estimated to have been hit by α particles (*7, 8*). Some subsequent studies have confirmed these results and pointed to extracellular factors as being responsible for these effects, with reactive oxygen species strongly implicated (*9–12*).

Other investigators have transferred medium from cultures of irradiated cells into cultures of unirradiated cells and have observed enhanced cell death and specific gene induction in the nonirradiated populations (*13, 14*). These results have been interpreted to indicate that the irradiated cells release factors into the medium that result in the observed changes in the unirradiated recipient cells.

More recent studies of the bystander effect have used a charged-particle microbeam. A microbeam has the ability to deliver a defined number of α particles (including a single particle) at very precise locations in a cell population. Thus it is possible to target the nuclei of individual cells with a defined number of particles. This is in contrast to whole-population "broad-beam" irradiations, where only a fraction of the particles actually traverse cell nuclei. Furthermore, the number of particles delivered to a population typically follows a Poisson distribution, and therefore any analysis of the population is by definition an average of that population. By contrast, the microbeam allows the precise analysis of each cell in a population, in which the number of α-particle traversals received by each cell is known.

In previous studies, a microbeam was used to irradiate a few cells in a population, with levels of micronuclei and of apoptosis being much higher than expected, i.e. evidence of a bystander effect (*15, 16*). Further, both mutation induction (*18*) and oncogenic transformation (*19*) have been shown to be enhanced in bystander cells after microbeam irradiation of known proportions of cells in a population.

Here we compare the cell cycle progression as well as induction of micronuclei in known human fibroblast by-

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TABLE 1
Cell Numbers and BrdU Labeling Index of Cells Stained with Hoechst 33342 and CTO Dye 2 h Postirradiation

Number of α particles	Number of cells counted (number of dishes)		Ratio of Hoechst: CTO-stained cells	Percentage cells labeled	
	Hoechst-stained	CTO-stained		Hoechst-stained	CTO-stained
0	2114 (9)	2120 (9)	0.997	2.8 ± 0.4	3.2 ± 3.1
1	2138 (9)	2366 (9)	0.904	5.5 ± 2.6	4.8 ± 1.9
2	765 (3)	668 (3)	1.145	3.4 ± 2.5	4.4 ± 2.8
5	1338 (6)	1574 (6)	0.850	3.5 ± 1.7	1.8 ± 0.8
25	1607 (7)	1884 (7)	0.853	7.0 ± 2.1	4.1 ± 1.8

stander cells to that of microbeam-irradiated cells using a modified staining technique that allows the direct identification of hit and bystander cells. This approach takes full advantage of the ability of the microbeam to target specific cells in a population, and it is the first study in which known hit and bystander cells in the same population are visualized directly and the biological responses in both sets of cells are compared.

MATERIALS AND METHODS

Cell Culture and Microbeam Irradiation

Normal human fibroblasts (Clonetics) were grown to plateau phase in T-25 flasks for 3–7 days prior to experiments. Cells were then split 1:2 and reseeded onto T-25 flasks. Once the cells had attached, one flask was stained with 100 n*M* Cell Tracker Orange (CTO, Molecular Probes, Eugene, OR) for 30 min. At this concentration the dye does not cause any measurable cell perturbations, and it gives a clear cytoplasmic signal through three to four cell divisions. The other flask was stained with 50 n*M* Hoechst 33342 for 30 min, as done routinely in microbeam studies (*18, 19*). Flasks were then rinsed and incubated with fresh medium for 30 min. Both sets of cells were then trypsinized, counted, mixed in a 1:1 ratio, and seeded in a 2-μl drop onto microbeam dishes (coated with Cell-Tak to enhance attachment of cells) at a density of 500 cells per dish (250 cells of each type). This resulted in a mean distance of 300 μm between cells. Construction of the microbeam dishes and the microbeam irradiation protocol used have been described elsewhere (*21*). Briefly, these dishes are 60-mm non-tissue-culture dishes that have a 0.25-inch hole drilled in the center to which a 3.8-μm-thick polypropylene film is attached to create a miniwell in which the cells are cultivated. Only the Hoechst-stained cells were irradiated with the indicated number of α particles, while the CTO-stained cells were the bystander cells. Cells were irradiated within 5 h of plating to minimize progression through the cell cycle and movement relative to one another.

Micronucleus Analyses

After irradiation (~7 min per dish) cells were fixed *in situ* with 100% ice-cold methanol 8, 16, 24, 32 and 48 h after irradiation. Cells were counterstained with DAPI and were scored for presence of micronuclei (blue fluorescence) in irradiated and bystander cells (orange fluorescence). Given the limited numbers of cells in a single microbeam experiment, data from nine different experiments were pooled. Differences in micronucleus frequencies between control and irradiated or bystander cells were evaluated using the χ^2 test.

Analysis of Cell Cycle Progression

After irradiation, BrdU was added (final concentration 1 μ*M* in medium) and cells were fixed *in situ* with 100% ice-cold methanol 2, 24 or 48 h postirradiation. Dividing cells, as indicated by BrdU uptake, were identified using a FITC-labeled anti-BrdU antibody (Becton Dickinson) following the manufacturer's recommendations. Cells were counterstained with DAPI and were scored for presence of BrdU uptake (green fluorescence) in irradiated and bystander cells (orange fluorescence).

RESULTS

Status of Cells at the Time of Irradiation

At each fluence, dishes were fixed *in situ* at 2 h postirradiation, and the numbers of each cell type were recorded. In addition, the cell cycle distributions of both populations were determined using BrdU uptake. These data are presented in Table 1. The number of each cell type ranged from 223 to 262 per dish, with a mean of 244 cells. As can be seen, the ratios of Hoechst-stained to CTO-stained cells ranged from 0.85 to 1.14, in line with our experimental design goal of equal numbers of irradiated and bystander cells. There were no significant differences in the BrdU labeling index between Hoechst-stained and CTO-stained cells. In both populations, the percentage of labeled cells was around 5%, indicating that most of the cells were in G_0/G_1 at the time of the irradiation. After we had determined conditions at the time of irradiation, irradiated and bystander cells were examined for the presence of micronuclei as a function of time postirradiation.

Induction of Micronuclei in Irradiated and Bystander Cells

Dishes were fixed *in situ* 8, 16, 24, 32 and 48 h postirradiation, and the incidence of micronuclei in both irradiated and bystander cells was scored. Figure 1 depicts a representative field used to score the expression of micronuclei 48 h postirradiation. As can be seen, even at 48 h, the bystander cells were clearly distinguishable from the irradiated cells, based on the fluorescence of the CTO dye. Micronuclei were also clearly visible in one of the irradiated cells as well as in the bystander cell. All cells of each type were evaluated on each dish, with data from nine microbeam experiments pooled, resulting in a total of 23,959 microbeam-irradiated cells and 27,413 bystander cells. In addition, 10,531 control cells (4,898 sham-irradiated and 5,633 bystander cells) were scored.

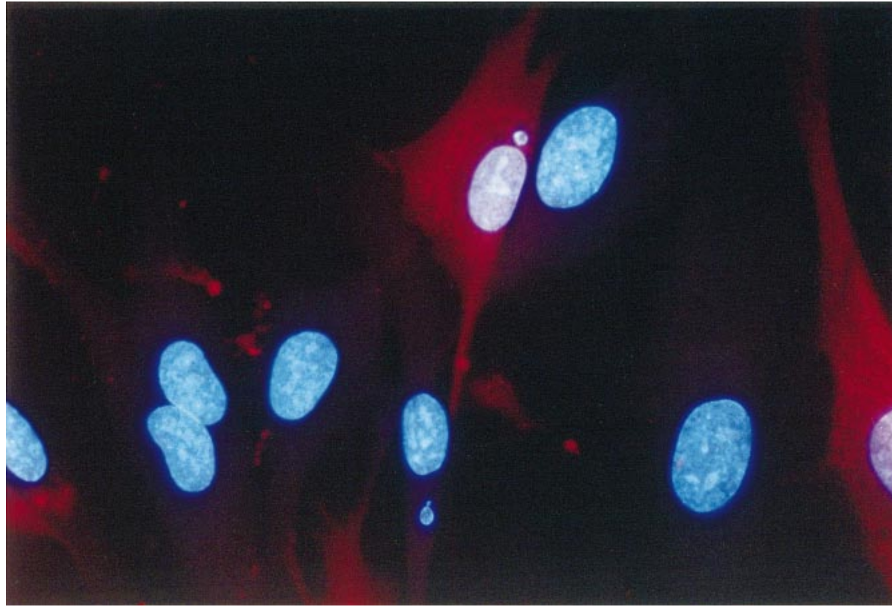

FIG. 1. Detection of micronuclei in irradiated and bystander cells at 48 h postirradiation. Bright blue nuclei, one with a micronucleus, stained with DAPI were originally stained with Hoechst 33342 dye and microbeam-irradiated. Orange fluorescent cells, one with a micronucleus, are bystander cells originally stained with CTO dye. Each cell type can be readily discriminated and categorized. As can be seen, at 48 h, the increase in cell numbers has reduced the distances between hit and bystander cells.

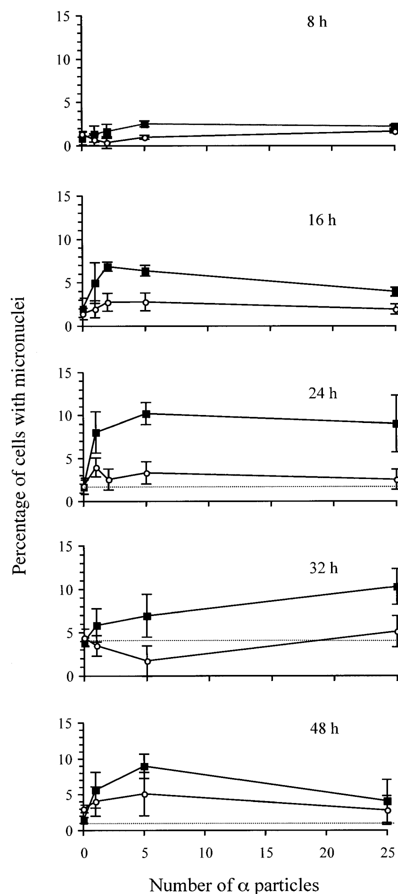

FIG. 2. Frequencies of microbeam-irradiated (■) and bystander cells (○) expressing micronuclei at various times after irradiation with α particles.

The frequencies of cells with micronuclei at the various times are shown in Fig. 2. As can be seen, there were no differences in the frequencies of micronuclei in hit, bystander and control cells at 8 h postirradiation. This is expected, given that 95% of the cells were in G_0/G_1 at the time of the irradiation, and normally growing cells have a doubling time of at least 24 h.

As expected, the frequency of irradiated cells expressing micronuclei increased with time postirradiation and was higher than in control levels for all particle fluences at all times. At 16 h postirradiation, between 4–6% of the hit cells contained micronuclei. Over the next 32 h, 4–10% of the irradiated cells demonstrated micronuclei. Importantly, the bystander cells generally expressed micronuclei at frequencies higher than that seen in controls, though lower than those in irradiated cells.

At 16 h postirradiation, the maximum frequency of cells with micronuclei was seen in the population irradiated with two α particles. This peak shifted to the population irradiated with five α particles at 24 h and to the population irradiated with 25 α particles at 32 h. At 48 h postirradiation, population irradiated with five α particles contained the most cells with micronuclei. This observation indicates that there is a particle fluence-dependent cell cycle delay in the irradiated populations. This is expected given previous reports on cell cycle delay after α-particle irradiation (*24, 25*). However, experimental limitations precluded an accurate estimation of the number of divisions that each cell expressing micronuclei had completed at the time of analysis. Therefore, the yields of micronuclei are expressed as

TABLE 2
Frequencies of Micronuclei Observed in Hit and Bystander Cells after Microbeam Irradiation

Treatment conditions	Number of cells counted	Expected number of micronuclei[a]	Number of micronuclei scored
Control			
Hoechst-stained	4898	—	81
CTO-stained	5633		113
Total	10,531		194
1 α particle			
Hoechst-stained	8221		436
CTO-stained	9205	184	256[b]
2 α particles			
Hoechst-stained	2306		114
CTO-stained	1916	38	39
5 α particles			
Hoechst-stained	6883		547
CTO-stained	8309	166	276[b]
25 α particles			
Hoechst-stained	4760		313
CTO-stained	6745	135	180[b]

[a] Expected frequencies of micronuclei for bystander cells based on corresponding control values in the absence of a bystander effect.
[b] Observed frequencies are significantly different from expected values as determined by the χ^2 test ($P < 0.001$).

the totals of those observed at 16, 24, 48 and 72 h postirradiation (Table 2).

There were no significant differences in the yields of micronuclei in control populations stained with either Hoechst 33342 or CTO dye. Further, these frequencies are similar to those reported for normal human fibroblasts (*26*), indicating that, at the concentrations used, neither Hoechst 33342 nor CTO dye induced micronuclei above background levels.

The incidence of micronuclei in the irradiated cells was somewhat higher than that determined previously in broad-beam experiments performed in this laboratory but was in agreement with frequencies observed in microbeam irradiation experiments. This may be due to the inherent heterogeneity of population irradiations compared to the more homogeneous nature of microbeam irradiations. Nonetheless, there was a fluence-dependent increase in the incidence of micronuclei. One α particle through the nucleus was sufficient to increase the incidence to more than three times that observed in control cells. The highest frequencies of micronuclei were observed in cells irradiated with five α particles (4.8-fold higher than background) that dropped to three times that of controls in cells that received 25 α particles. This decline is most likely due to the lack of cells entering mitosis, which is required for the expression of micronuclei.

The induction of micronucleus in the bystander cells was somewhat different. While all bystander populations consistently showed increased micronucleus yields compared to controls, this increase did not appear to be dependent on fluence. Micronucleus incidences in bystander cells were between 1.3- and 1.65-fold higher than controls, with no clear increase with increasing numbers of α particles delivered to the hit cells. With the exception of bystanders to cells that received two α particles, all bystander populations had significantly higher incidences of micronuclei ($P < 0.001$) than expected based on yields of control cells. Importantly, at the times of analysis, only a few random bystander cells were in contact with irradiated cells, and there was no correlation between these cells and bystander cells that expressed micronuclei.

Cell Cycle Delay in Irradiated and Bystander Cells

The data on micronucleus incidence discussed above suggested that there may be significant fluence-dependent delay in cell cycle progression in irradiated cells, and that bystander cells may exhibit similar responses. To determine whether this was indeed the case, entry of cells into S phase after irradiation was monitored using BrdU uptake as an indicator of DNA synthesis. The patterns of BrdU labeling after irradiation are shown in Fig. 3. At 24 h postirradiation (Fig. 1A), sham-irradiated and bystander control populations contained about 65% labeled cells. This is expected, given that more that 95% of the cells were in G_0/G_1 at the time of irradiation, and the labeling indices observed at 2 h postirradiation. As expected, there was a fluence-dependent decrease in BrdU-positive cells in the irradiated populations at this time.

Irradiation with one α particle was sufficient to induce a significant delay, with less than 28% of the irradiated cells showing the incorporation of BrdU. This trend in delay of

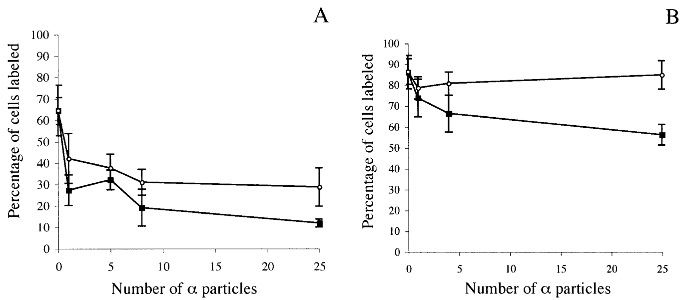

FIG. 3. Percentage of BrdU-positive cells in irradiated (■) and bystander (○) populations at 24 h (panel A) and 48 h (panel B) postirradiation.

irradiated cells continued through to irradiation with 25 α particles, where only 12% of the irradiated cells showed an incorporation of BrdU. There was also a smaller fluence-dependent decrease in the percentage of BrdU-labeled cells in the bystander populations as well. After exposure to one α particle, only 42% of the bystander cells demonstrated any BrdU uptake at 24 h postirradiation. In the absence of a bystander effect, these cells would show labeling comparable to that seen in control populations. The decrease in BrdU uptake by bystander cells was also observed after irradiation with five, eight and 25 α particles. Approximately 30% of the bystanders to cells that received 25 α particles showed uptake of BrdU.

The reductions in BrdU labeling in the populations irradiated with five and 25 α particles were apparent even at 48 h postirradiation, with only 66% and 56% of the cells demonstrating uptake, respectively (Fig. 1B). However, unlike the delay in cell cycle progression observed in the hit cells, the delay in the bystander cells seen at 24 h postirradiation did not extend to 48 h. At this time there was no difference in labeling frequencies between control populations (86%) and any of the bystander populations (79–85%).

DISCUSSION

There is now a substantial amount of data indicating that nonirradiated cells adjacent to those that are irradiated demonstrate several biological responses associated with exposure to ionizing radiation. It has been proposed that irradiated cells are responsible for the production of a signal(s) that leads to similar responses in nonirradiated bystander cells. This bystander effect has been seen to result in cell death (*13*), induction of sister chromatid exchanges (*5, 6*), mutations (*18*) and transformation (*19*) of nonirradiated cells along with other molecular/cellular end points (*7–12*). Early studies of the bystander effect relied on calculations indicating that biological responses were observed in more cells than were irradiated (*5, 6*). Since this required the use of very low fluences of particles, these types of studies were not amenable to the investigation of a dose response for the bystander effect. Further information on bystander effects has come from medium transfer experiments in which the irradiated and bystander cells are cultured separately and therefore cannot be examined together (*13, 14*). The development of a charged-particle microbeam has permitted the investigation of aspects of the bystander effect that were not possible with more traditional methods. Here we have compared microbeam-irradiated and bystander cells in the same population with emphasis on the responses observed with increasing dose/particle fluence.

Micronucleus yields after microbeam irradiation have been reported by others (*15, 16*). After irradiation of four cells with five α particles, about 2.5% of about 3,000 bystander cells showed micronuclei compared to 1% of the control cells showing micronuclei. The fraction of cells expressing micronuclei remained relatively unchanged with increased numbers of α particles (up to 15 particles) delivered to the same number of cells. From these data, induction of micronuclei by the bystander effect appears to be independent of dose starting at the lowest fluence studied, five nuclear traversals. This is consistent with our observation that induction of micronuclei in bystander cells does not appear to be dependent on the amount of damage induced in the irradiated cells.

From the data presented here, it is clear that given an equal proportion of hit and non-hit cells, even single α-particle traversals are capable of inducing cell cycle delay and micronuclei in non-hit bystander cells. This is in keeping with other studies that have demonstrated bystander effects at very low doses (*6, 9, 10, 27, 28*). In fact, these data overall are consistent with the phenomenon being more significant at very low doses. First, it is clear that the magnitude of these end points in bystander cells is much smaller than that seen in directly hit cells. In addition, it appears that the induction of the bystander effect (as seen by cell cycle delay and micronucleus incidence) is not dependent on fluence. This was certainly true for the observed increases in incidence of micronuclei in bystander cells. While there appeared to be a lesser fluence-dependent cell cycle delay in the bystander cells at 24 h postirradiation, this delay was transient and returned to control levels by 48 h. Taken together, this would indicate that, in an analysis of a population of hit and bystander cells, the effect would be observed most readily at lower doses where the response of irradiated cells is relatively small. At higher doses, the bystander effect would be masked, where the response of the hit cells would overwhelm any contribution made by the bystander cells. It follows that while at higher doses the contribution of the bystander cells may be negligible, at lower doses both hit and bystander cells contribute significantly to the frequency at which an end point is observed. This is particularly important in situations in which there is an extrapolation from higher doses to lower ones with the assumption that the response is linear with respect to dose. While it is clear that bystander responses severely alter the notion of linear dose responses at very low doses,

what impact this would have on risk assessment at low doses remains in question. The data presented here on DNA damage as well as that on enhanced mutation and transformation frequencies in bystander cells (*18, 19*) would suggest a supra-linear response. That is, at low doses, significantly more cells are damaged, mutated and transformed than would be predicted by linear extrapolation from higher doses, indicating a greater than expected risk at these doses. However, other data suggest a more protective role for the phenomenon. For example, the detection of apoptosis in unirradiated cells (*29*) would suggest that the presence of bystander responses serves to eliminate altered cells and would argue that alterations to risk estimates are unnecessary.

It is important to stress that all the experiments performed here used densely ionizing (high-LET) radiations in which a clear delineation between hit and non-hit cells can be achieved. How far these conclusions apply to sparsely ionizing (low-LET) radiation such as X or γ rays is still unclear.

There appear to be at least two different mechanisms by which bystander effects are propagated. Several investigators have suggested that in confluent cultures, gap junction communications may be responsible for responses in bystander cells (*7, 8, 14, 18*). In these situations the prevention of gap junction formation by the use of either mutant cell lines or inhibitors resulted in the suppression of the effect. While gap junction communication may be involved in the transfer of signals when irradiated and bystander cells are in direct contact, it is unlikely in the present experiments. Here cells were seeded at low numbers to allow sufficient space to progress through the cell cycle, to move and to express micronuclei after cellular division. As a result, only a small fraction of cells were in contact with each other. The bystander effects observed in this study are more likely due to the release of factors from irradiated cells that are responsible for the responses in unirradiated neighboring cells. This mechanism has previously been postulated to explain bystander effects observed in medium transfer studies in which the two sets of cells are never in contact with each other (*9, 14, 28*). It is thought that deposition of energy in the medium or in cells generates factors in the medium that can then influence nonirradiated cells that share the same environment to produce responses similar to those observed in the irradiated cells. While little is known about the precise mechanisms whereby this takes place, several candidate molecules have been proposed, including IL8, TGFB and TNFA.

Others have reported data that suggest reactive oxygen species may be the signal between hit and non-hit cells (*9–12*). Given the nature of the experiments conducted here, it is not possible to either support or discount the possibility that reactive oxygen species play a role in this observed bystander effect.

In conclusion, this study is the first to take full advantage of microbeam irradiation techniques that allow the irradiation of specific cells in a population. Using a unique staining protocol, it was possible to follow the fate of individual irradiated and bystander cells in the same population, this has allowed a direct contrast of the biological responses observed in irradiated and bystander cells.

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