Involvement of Membrane Signaling in the Bystander Effect in Irradiated Cells

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ABSTRACT

We have shown previously that when confluent cultures of mammalian cells are exposed to very low fluences of α-particles, fluences whereby only 1–3% of the cell nuclei are traversed by a particle, genetic effects, including specific gene mutations and sister chromatid exchanges, are induced in neighboring, nonirradiated (“bystander”) cells (H. Nagasawa and J. B. Little, Cancer Res., 52: 6394–6396, 1992; H. Nagasawa and J. B. Little, Radiat. Res., 152: 552–557, 1999). The present experiments were designed to determine whether signaling pathways arising in the cell membrane may mediate this effect. Cells were irradiated in the presence of Filipin, an agent that disrupts lipid rafts, effectively inhibiting membrane signaling, and the induction of sister chromatid exchange and HPRT mutations by very low fluences of α-particles (mean doses 0.17–0.5 cGy) was measured. Filipin completely suppressed the induction of both genetic effects in bystander cells. After exposure to 10 cGy, when most mutations occurred in directly irradiated cells, no suppressive effect of Filipin was observed. These results suggest that membrane signaling may play an important role in the bystander effect of radiation. On the other hand, the effects in directly irradiated cells do not appear to be mediated via the cell membrane.

INTRODUCTION

We have shown earlier that when cultured CHO cells are exposed to very low fluences of α-particles, fluences by which only ≤1% of the cell nuclei was traversed by a single α-particle, as many as 20–40% of the cells in the population showed increased frequencies of SCEs (1). The occurrence of such genetic changes in neighboring, nonirradiated cells has been termed the “bystander effect.” This finding was confirmed subsequently (2), and evidence has since been presented for the occurrence of multiple effects in bystander cells, including mutations (3, 4), malignant transformation (5), and changes in gene expression (6). It has also been reported in medium transfer experiments that extracellular factors released by irradiated cells can produce genetic changes in nonirradiated cells (7, 8).

Despite the observations that multiple genetic changes may occur in bystander cells, very little is known about the nature of the signal or its target(s) in these nonirradiated cells. Evidence has been presented for the involvement of gap junctions in the transmission of the signal between irradiated and nonirradiated cells in a monolayer population (4, 6, 9), although this would obviously not apply in the medium transfer experiments. Evidence has also been presented for the involvement of oxidative stress in the phenomenon (10), and we have found that up-regulation of proteins in the MAPK pathways occurs in bystander cells.4 Activation of MAPK takes place in the cell membrane; this could occur as a result of oxidative stress in the bystander cells or as a direct consequence of the interaction of bystander signals with the cell membrane.

Several lines of evidence have suggested that signaling via some cell surface receptors involves receptor aggregation and clustering in membrane structures known as GEMs or rafts. GEMs are composed of glycosphingolipids, sphingomyelin, cholesterol, and specific membrane proteins (11, 12), and raft assembly has been implicated in membrane sorting in polarized cells (13, 14), endocytosis (15, 16), cholesterol trafficking, and signal transduction from cell surface receptors (17–23). It has been suggested that these specialized lipid microdomains provide a milieu for spatial segregation of specific sets of proteins and enhance the efficacy and specificity of interactions between enzymes involved in signal transduction. Disruption of rafts with cholesterol-depleting agents, such as Methyl-β-cyclodextrin, Filipin and Nystatin, has been shown to abrogate signaling through this compartment in a variety of distinct cell types (24–27).

The present experiments were designed to examine whether cell membrane signaling via GEM is involved in the induction of SCE and gene mutations in bystander cells. We used Filipin, a macrolide polycyclic antibiotic (28, 29), to disrupt the cholesterol-rich rafts. Induction of SCE and HPRT mutations was examined in monolayer cultures of CHO cells irradiated with very low fluence of α-particles in the presence or absence of Filipin. Filipin completely inhibited the induction of both genetic effects in bystander cells, suggesting a role for the cell membrane in the signaling of the bystander effect.

MATERIALS AND METHODS

Cells and Cultural Conditions. CHO cells were grown in MEM supplemented with 10% FCS, penicillin (50 units/ml), and streptomycin (50 mg/ml) in a humidified 95% air 5% CO2 atmosphere at 37°C. For experiments, −105 cells from stock cultures were seeded on 1.5-mm-thick Mylar-based dishes coated with fibronectin to facilitate attachment. When the cell density reached −30% confluence, the culture medium was removed and replaced with isoleucine-deficient MEM containing 5% of the dialyzed serum to facilitate synchronization of the cells in the G1 phase of the cell cycle (30). These cultures were then incubated at 37°C until they reached either 80% confluence or full confluence, when the cells were irradiated.

Irradiation and Filipin Treatment. For α particle irradiation, the Mylar dishes were placed over a Mylar window in the exposure well of a specially constructed irradiator providing a uniform source of well-characterized 3.7 MeV α particles as described previously (3, 31). Filipin was obtained from Sigma Chemical Co., suspended in HBS, and maintained in solution at −20°C. One h before irradiation, the growth medium was removed and replaced with either HBS or fresh growth medium with 10% serum containing 0.5% or 1 mg/ml Filipin. Cells were maintained in this medium until 15 min after irradiation, when the cells were subcultured in complete MEM; this medium contained 10−3 M bromodeoxyuridine for measurement of SCE.

Measurement of SCE. The subcultured cells in 10−3 M bromodeoxyuridine were cultured at 37°C for two rounds of cell replication. Colcemid (0.2 mg/ml) was added to the culture for 4 h before fixation. After drying in air, the differential staining of SCE was carried out by the fluorescence plus Giemsa technique (32). The number of SCEs was scored in 50 cells for each data point in each of four experiments. The data are presented as the mean number of SCE per chromosome, based on a modal chromosome number of 21.

Measurement of HPRT Mutations. For each treatment group, the cells in 25–50 separate Mylar dishes were suspended by trypsination 15 min after α-rays.
particle irradiation and transferred to a similar number of P-100 plastic Petri dishes containing normal, nonselective medium. The cells were cultured for 8–10 days to allow for phenotypic expression as previously described (3). The cells from each dish were then seeded in 10 new P-100 plastic Petri dishes as a density of $2 \times 10^6$ cells and cultured in complete medium containing 6-thioguanine (5 μg/ml) for 10–12 days. HPRT mutant colonies were thus scored in a total of 250–500 dishes for each of the nine treatment/dose groups. Mutation frequencies were calculated based on the number of mutant colonies scored and the cloning efficiency at the time of seeding in selective medium.

RESULTS

Four separate experiments were carried out for the measurement of SCE. In each experiment, the cells were irradiated with three mean doses of α particles (0, 0.17, or 0.5 cGy) and incubated with three concentrations of Filipin (0, 0.5, or 1 μg/ml). Two experiments were carried out in confluent cultures and two in 80% confluent cultures, and the cells were incubated with Filipin either in HBS or in complete medium with 10% bovine serum. As the results in each experiment were similar, the data are combined in Table 1, where the results are presented as the mean ± SD of the results of the four experiments. As can be seen, a significant increase in SCE occurred in the irradiated cultures with no Filipin, when only 1–3% of the cell nuclei were traversed by a single α particle. On the other hand, no increase occurred in the cultures incubated with either concentration of Filipin.

These results are presented graphically in Fig. 1, where the data points represent the mean induced frequency of SCE ± 1 SE of the results of the four experiments. The dashed line represents the dose response relationship we reported in 1992 (1) for the induction of SCE by multiple small doses of α particles. As can be seen, the two data points from the present experiment lie exactly on this line, indicating the consistency between the results of the previous study and the present one. It is evident from both Fig. 1 and Table 1 that Filipin by itself appeared to induce a low frequency of SCE; however, no further increase was observed in the irradiated cultures.

To confirm that the increase in SCE measured in irradiated cultures actually occurred in bystander cells, we determined the distribution of SCE frequencies among individual cells for each data point in Table 1. The open bars on the left side of the control panel in Fig. 2 represent the distribution of spontaneously arising SCE in nonirradiated cells. The open bars in the irradiated groups in this panel represent the background frequencies of SCE based on the distribution in nonirradiated cultures, whereas the hatched bars represent SCE induced by the irradiation. It is evident from these results that the increased frequency of SCE occurred in a high percentage of the cells; thus, the increased frequency seen in Table 1 and Fig. 1 are the result of SCE occurring in bystander cells rather than in the 1–3% of the cell’s nuclei actually traversed by an α particle. The results in Fig. 2, however, clearly show no difference in the distribution of SCE among the irradiated as compared with the nonirradiated cells after treatment with Filipin.

Results of an experiment measuring the induction of HPRT mutations by α radiation are shown in Table 2. The mutation frequencies induced by mean doses of 0.5 or 10 cGy are identical to those reported previously (3). As was the case for SCE (Table 1), Filipin completely inhibited the induction of mutations by 0.5 cGy, when most of the induced mutations occurred in bystander cells (33). After irradiation with 10 cGy when most mutations occurred in directly irradiated cells (33), there was no suppressive effect of Filipin. These results suggest that the cell membrane may play an important role in the pathway by which signals from irradiated cells lead to genetic effects in bystander cells. On the other hand, they imply that the effect in directly irradiated cells is not mediated by the membrane. The observation that most mutations arising in cells irradiated with 10 cGy involved deletions, rather than point mutations as occurs in bystander cells (33), is consistent with the conclusion that these mutations result from the direct interaction of the α particles with the cell nucleus.

DISCUSSION

We have found previously5 that the MAPK signaling pathway, including c-Jun-NH2-terminal kinase, ELK1/2, and p38 MAPK, are activated in bystander cells. As these pathways are activated by signals arising in the cell membrane, we hypothesized that membrane signaling might be important in the induction of genetic changes in bystander cells. We examined two genetic end points, SCE and HPRT mutations, which we have shown previously are induced in bystander cells (1, 3). Differences in the spectrum of mutations arising spontaneously as compared with those induced by α particle irradiation of either 20% of the nuclei or of the cytoplasm have been reported by Zhou et al. (4) with a different mutation assay. Our studies of mutational spectra indicate that >90% of the mutations induced in bystander cells are point mutations, whereas those induced in directly irradiated cells are primarily a result of total and partial gene deletions.

Fig. 2. Distribution of mean frequencies of SCE per chromosome among individual cells: 1 = <0.1; 2 = 0.1–0.45; 3 = 0.61–0.8; 4 = >0.8. Hatched bars in histograms represent SCE induced by mean doses of 0.17 or 0.5 cGy of α radiation, whereas open bars represent background frequencies of SCE based on distribution in nonirradiated (0 cGy) cells. No significant change in background frequencies occurred in irradiated cells incubated with 0.5 or 1 μg/ml Filipin.

indicate that mutations in bystander cells arise by a different mechanism.

SCE also appeared to result primarily from point mutations (34). Thus, they are inefficiently induced by ionizing radiation as compared, e.g., with cross-linking agents or UV light, which induces primarily single base changes (pyrimidine dimers and 6:4 photoproducts; Ref. 34). In reality, the frequency of SCE induced by α radiation peaked at the dose of ~2–4 cGy and then falls off (35). This would be consistent with the hypothesis that SCE induced by α particle irradiation occurs largely in bystander cells. The induction of SCE in bystander cells was inhibited completely by incubation with Filipin (Figs. 1 and 2), suggesting the involvement of signals arising in the cell membrane in the effect. This conclusion is supported by the results shown in Table 2; the induction of mutations in bystander cells was also suppressed completely by incubation with Filipin.

Membrane signaling has been implicated in the apoptotic response to ionizing radiation in several but not all cell types (36–38). This effect is mediated by the Sphingomyelin pathway and involves sphingomyelinase activation and generation of the second messenger ceramide (27, 36, 37). Recent studies have shown that multimerization of Fas in caps is mandatory to initiate apoptotic signaling and that capping depends specifically on acid sphingomyelinase-derived ceramide generation (27). Disruption of rafts by the cholesterol-depleting agent Filipin prevented both Fas capping and apoptosis. The present studies are consistent with a GEM model, although evidence for involvement of the sphingomyelin pathway is not provided.

To our knowledge, such a membrane-based signaling mechanism has not yet been involved in the induction of genetic changes, such as SCE and gene mutations. The results in Table 2 suggest, however, that membrane signaling is restricted to the induction of mutations occurring in bystander cells. When cells were irradiated with a high fluence of α particles such that most of the mutations occurred in directly irradiated cells, membrane signaling did not appear to be involved as evidenced by the lack of a suppressive effect of Filipin. The involvement of membrane signaling in bystander cells is of interest in light of the observation that the ATM-dependent up-regulation of the p53 damage response pathway in bystander cells appears to involve signals transmitted through gap junctions (9). It has been reported that lipid raft structures in the membrane may play a role in gap junction-mediated intercellular communication.

The mechanisms by which signals that arise from irradiated cells may be transmitted to nonirradiated cells leading to genetic changes are likely varied and complex. For some end points, such as apoptosis, signals may apparently be transmitted through the culture medium (7, 8). The present results, however, provide evidence for a role of the cell membrane in the signaling pathways leading to genetic changes in bystander cells.

REFERENCES


Table 2 Effect of Filipin on the frequency of HPRT mutations induced by a radiation exposure

<table>
<thead>
<tr>
<th>Dose (cGy)</th>
<th>Control</th>
<th>Filipin (1 μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.27 ± 0.04 (39)</td>
<td>0.27 ± 0.04 (40)</td>
</tr>
<tr>
<td>0.5</td>
<td>0.71 ± 0.10 (50)</td>
<td>0.23 ± 0.06 (30)</td>
</tr>
<tr>
<td>10.0</td>
<td>1.48 ± 0.30 (25)</td>
<td>1.68 ± 0.37 (25)</td>
</tr>
</tbody>
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* Mean ± 1 SE × 10^-1 of data from multiple mylar dishes (number of dishes for each group shown in parentheses).


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