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In Situ Visualization of DSBs to Assess the Extranuclear/Extracellular Effects Induced by Low-Dose α-Particle Irradiation

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Extranuclear/extracellular effects may have a significant effect on low-dose radiation risk assessment as well as on the shape of the dose–response relationship. Numerous studies using different end points such as sister chromatid exchanges, micronuclei and mutation have shown that this phenomenon exists in many cell types. However, these end points mostly reflect the late events after radiation damage, and little is known about the early response in this phenomenon. DNA double-strand breaks (DSBs) induced by ionizing radiation or carcinogenic chemicals can be visualized *in situ* using γ-H2AX immunofluorescence staining, and there is evidence that the number of γ-H2AX foci can be closely correlated with DSBs induced. Here we used γ-H2AX as a biomarker to assess the extranuclear/extracellular effects induced by low-dose α particles *in situ.* The results show that a greater fraction of positive cells with DSBs (48.6%) was observed than the number of cells whose nuclei were actually traversed by the 1-cGy dose of α particles (9.2%). The fraction of DSB-positive cells was greatly reduced after treatment with either lindane or DMSO. These results suggest that *in situ* visualization of DSBs can be used to assess radiation-induced extranuclear/extracellular effects soon after irradiation. Moreover, the *in situ* DSB assay may provide a means to evaluate the spatial effect on unirradiated cells that are located in the neighboring region of cells irradiated by α particles.

INTRODUCTION

DNA double-strand breaks (DSBs) induced by ionizing radiation and other carcinogenic chemicals are considered to be the most relevant lesion for mutations and carcinogenesis. Unrepaired and misrepaired DSBs are considered to be a serious threat to genomic integrity (*1*, *2*). The existence of DSBs could result in chromosomal aberrations, which can affect many genes simultaneously, and lead to cellular malfunction and death (*3*). In normal living organisms, a few DSBs may also be induced continuously as a consequence of oxidative metabolism (*4–6*).

One of the earliest steps in the cellular response to DSBs is the phosphorylation of serine 139 of H2AX, a subclass of eukaryotic histone proteins that are part of the nucleoprotein structure called chromatin (*7*). Using a fluorescent antibody specific for the phosphorylated form of H2AX (γ-H2AX), discrete nuclear foci can be visualized at the sites of DSBs, either induced by exogenous agents such as ionizing radiation (*8*, *9*) or generated endogenously during programmed DNA rearrangements (*10–12*). Initial studies have shown a close correlation between the number of γ-H2AX foci and the number of DSBs expected after irradiation with 0.6 Gy of γ rays (*8*). Recently, a direct correlation was observed between the number of foci and the number DSBs produced by decay of ^{125}I incorporated into cellular DNA (*13*), suggesting that each focus may represent an individual break and that each DSB may form a focus. Furthermore, Rothkamm *et al.* have shown that DSBs can be detected after X irradiation with doses as low as 1 mGy (*14*). Thus the visualization of DSBs, which employs γ-H2AX focus formation as a biomarker, may be a new approach to assess the risk of low doses of radiation.

The extranuclear/extracellular effect was first reported in 1992 by Nagasawa and Little (*15*). They observed an increase in the frequency of sister chromatid exchanges (SCEs) in about 30% of the cells, even though less than 1% of cell nuclei were calculated to have been traversed by one α particle. This phenomenon is now known as the bystander effect, in which cells that received no radiation show biological effects resulting from irradiation of their neighboring cells. Bystander effects induced by low-dose or low-dose-rate radiation are a great challenge for traditional radioprotection modeling since it has long been thought that the genotoxic effects of ionizing radiation are due mainly to DNA damage caused by the direct traversal of cell nuclei by the radiation. In the past decades, numer-

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ous studies using different end points such as SCEs, micronuclei and mutation have shown that this phenomenon exists in many cell types and can be induced by many different radiation qualities (*15–19*). However, these end points mostly reflect the late events of the radiation damage, and little is known about the early response in this phenomenon.

DSBs induced by radiation have been detected by *in situ* visualization of γ-H2AX foci from 0.05 to 24 h postirradiation (*14*). It is therefore of interest to determine whether an *in situ* DSB assay can be used to assess the extranuclear/extracellular effects induced by low-dose radiation in a rapid and quantitative manner. In the present studies, a DSB assay based on immunofluorescence staining of γ-H2AX foci was used to assess the extranuclear/extracellular effects induced by low-dose α-particle radiation.

MATERIALS AND METHODS

Cell Culture and Alpha-Particle Irradiation

AG1522 normal human diploid skin fibroblasts, received as a kind gift from Dr. Barry Michael (Gray Laboratory, UK), were maintained in α-Eagle's minimum essential medium (Gibco) supplemented with 2.0 m*M* L-glutamine and 20% FBS (Hyclone) plus 100 μg/ml streptomycin and 100 U/ml penicillin (Gibco) at 37°C in a humidified 95% air/5% CO_2 incubator. For irradiation, approximately 1×10^4 exponentially growing AG1522 cells in passage 11–14 were seeded into each specially designed rectangular dish (10×6 mm^2) consisting of a 3.5-μm-thick Mylar film bottom to which the cells attached. The culture medium was replaced every 2 days and the cells were irradiated with doses of 0.5, 1 and 10 cGy, respectively, at day 4 under confluent conditions. The average energy of α particles from the ^{241}Am radiation source was 3.5 MeV measured at the cell layer, and the particles were delivered at a dose rate of 1.0 cGy s^{-1}. After irradiation, cells were kept in the incubator for another 30 min. Cultures were then fixed for immunochemical staining. Sham-irradiated AG1522 cells were treated similarly to serve as a control.

Immunochemical Staining of Cells (γ-H2AX) and DSB Measurement

Immunochemical staining of cells was performed as described (*20*). Briefly, 30 min after irradiation, cells in the dish were removed from the incubator, washed with PBS three times, fixed in a 2% paraformaldehyde solution in PBS for 15 min at room temperature, and then rinsed three times with PBS again. Prior to immunochemical staining, cells were incubated for 30 min in TNBS solution (PBS supplemented with 0.1% Triton X-100 and 1% FBS) to improve their permeability and then incubated with anti-γ-H2AX antibody (Upstate Biotechnology) in PBS$^+$ (PBS supplemented with 1% FBS) for 90 min, washed in TNBS for 3×5 min, and incubated in PBS$^+$ containing the FITC-conjugated goat anti-mouse secondary antibody (Sigma) for 60 min. After another wash with TNBS for 3×5 min, cells were counterstained with Hoechst 33342 at a concentration of 5 μg/ml for 20 min at room temperature. After a final wash with TNBS, the stained cells on the Mylar film were mounted by 50% of glycerol-carbonate buffer (pH 9.5) for microscopy.

The stained rectangular dishes were loaded on a 35-mm-diameter glass-bottom dish (glass thickness: 0.17 mm) that was used as a bracket. Immunofluorescence images were captured with a confocal laser scanning microscope (Leica, TCS SP2). For quantitative analysis, the cells with γ-H2AX foci were regarded as the positive cells and the fraction of positive cells was calculated (cells with DSBs/total cells) (*21, 22*). Five images were recorded for each sample, and at least 300 cells in each image were counted.

Treatment with Lindane or Dimethyl Sulfoxide

To examine whether reactive oxygen species (ROS) or gap junctional intercellular communication (GJIC) also play a role in DSB induction, cells were treated with 40 μ*M* lindane (Sigma) 2 h before, during and 30 min after irradiation or with 1% DMSO 15 min before, during and 30 min after irradiation with 1 cGy α particles. After treatment, cells were fixed to visualize the DSBs as described above. The dose of the two chemicals used is effective and has previously been shown to be non-toxic and non-genotoxic to the cells under the conditions used in the present studies (*18, 23, 24*).

Statistics

Data are presented as means and standard errors of the mean. Significance levels were assessed using Student's *t* test. A *P* value of 0.05 or less between groups was considered to be significant.

RESULTS

DSB Induction by Radiation

Figure 1 shows a representative fluorescent image of the positive cells with DSBs (red γ-H2AX foci in the cyan nucleus region). It was clear that the number of positive cells with DSBs increased with increasing dose 30 min after α-particle irradiation. In general, the majority of cells irradiated with doses of 0.5 or 1 cGy contained a single γ-H2AX focus. Two or more DSB foci could be visualized in some cells after a higher dose of radiation (for example 10 cGy, Fig 1D). The fraction of positive cells with DSBs as a function of dose is shown in Fig. 2. The frequency of γ-H2AX focus induction increased in a dose-dependent manner, and the induction appeared to level off at the highest dose examined (10 cGy).

The fractions of γ-H2AX-positive cells with DSBs induced by different doses of α-particle radiation are shown in Table 1. The yield was 80.4% for 10 cGy α particles and 48.6 and 42.8% for 1 and 0.5 cGy, respectively. Table 1 also shows the fractions of cells whose nuclei would be traversed by an average of one α particle after a dose of 0.5, 1 or 10 cGy based on dosimetry calculations. Although there was a 20-fold increase in the fraction of cells whose nuclei were traversed by an average of one α particle for 10 cGy compared with 0.5 cGy, only a 1.9-fold increase in the fraction of DSB-positive cells was detected in the cell population. This observation suggested that at the lower dose, some cells other than those whose nuclei were actually traversed by a single α particle contributed to the observed increase in DSB-positive cells. It also suggested that the effects resulting from an average of one α-particle traversal were not restricted to direct nuclear damage. The large number of DSB-positive cells could be the result of either cytoplasmic damage or a bystander effect induced by α-particle radiation. However, at doses of 0.5 and 1 cGy, the contribution of the cytoplasmic component to the induction of γ-H2AX was minimal (Table 1). At a dose of 0.5 cGy, 34.2% of the cells were estimated to have had their cytoplasm traversed by a single particle, while at a dose of 1 cGy, this proportion increased to 68.5%. This

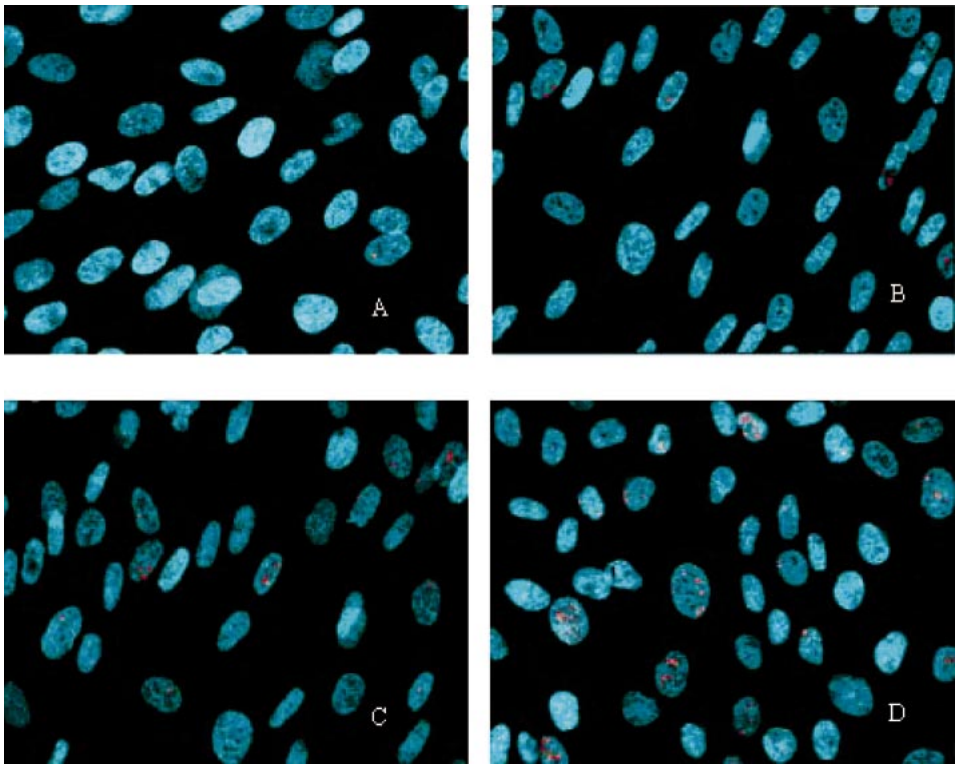

FIG. 1. Image of DSB-positive cells (red γ-H2AX foci in the cyan nucleus region) after irradiation with 0 cGy (panel A), 0.5 cGy (panel B), 1 cGy (panel C) and 10 cGy (panel D) α particles.

twofold increase in cytoplasmic traversal, however, did not results in a significant increase in incidence of γ-H2AX foci (Table 1).

Attenuation of DSB Formation by Lindane or DMSO Treatment

Pretreatment of cells with either the gap junction communication inhibitor lindane (40 μ*M*) or with the free radical scavenger dimethyl dioxide (1% v/v) reduced the fraction of DSB-positive cells (Table 2). In cells irradiated with 1 cGy of α particles, addition of lindane and DMSO reduced the number of cells containing γ-H2AX foci by 60%, from 48.6% to 19.8% and 18.7%, respectively ($P < 0.05$). At a dose of 10 cGy, the percentage of cells with DSBs increased to 80.4%. However, addition of lindane and DMSO reduced the number of cells containing γ-H2AX foci by only 20%, from 80.4% to 66.8% and 64.1%, respectively ($P < 0.05$). The decrease in DSB-positive cells after treatment with chemical agents suggested that GJIC or ROS might play important roles in induction of the extranuclear/extracellular effects.

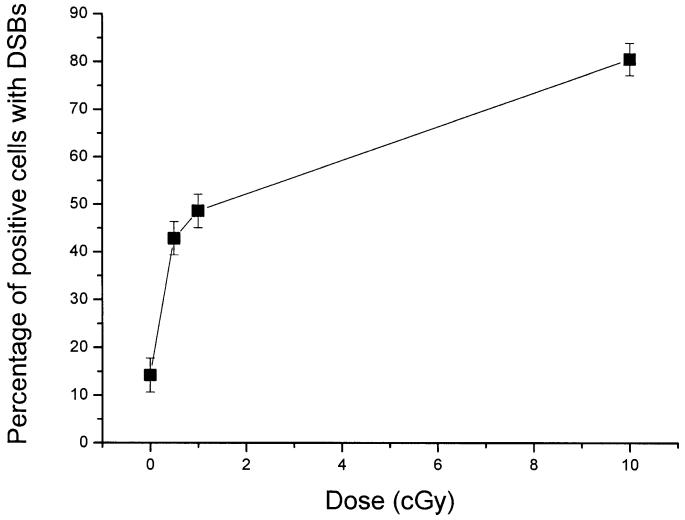

FIG. 2. Induction of DSBs (γ-H2AX foci-positive cells) as a function of dose of α particles. Data from 10 individual experiments were pooled. Bars represent ±SEM, $P < 0.05$.

DISCUSSION

The recent work of Sedelnikova *et al.* (*13*) demonstrated a close correlation between the number of γ-H2AX foci and the expected number of DSBs after irradiation, that each focus may represent an individual break, and that each DSB may form a focus. In our experiment, using the immunochemical staining of γ-H2AX as a biomarker of DSBs, one or more DSB foci could be visualized clearly in each AG1522 cell 30 min after high-dose α-particle irradiation (>20 cGy, data not shown). However, a greater fraction of DSB-positive cells was also observed after irradiation at the low doses of 0.5 or 1.0 cGy, while the fraction of cells whose nuclei were actually traversed by an

TABLE 1
Dosimetry for α-Particle Radiation and Average Increase in DSB-Positive AG1522 Cells

Dose (cGy)	Percentage of cells in which the nuclei are traversed by an average of one α particle[a]	Percentage of intact cells traversed by an average of one α particle[a]	Percentage of cells in which only the cytoplasm is traversed by an average of one α particle[a]	Percentage of positive cells with DSBs
0	0	0	0	14.2 ± 3.60
0.5	4.6	38.8	34.2	42.8 ± 3.52*
1	9.2	77.7	68.5	48.6 ± 3.54*
10	92.0	100.0	8.0	80.4 ± 3.41*

Notes. Data were pooled from 10 individual experiments. Values (means ± standard errors of the mean) with * are significantly different ($P < 0.05$) from unirradiated control based on the Student's *t* test.

[a] CR-39 track-etch plastic was used for measurements of particle fluence and field uniformity. The dose rate was 0.034 α-particle track/μm^2 min^{-1}, and the average size of the nucleus or the whole cell growing in confluence after fixing, staining and measurement by confocal microscopy was 163 ± 5 μm^2 and 1371 ± 4 μm^2, respectively, yielding 0.092 α-particle track/nucleus min^{-1} and 0.7756 α-particle track/cell min^{-1}.

average of one α particle was 4.6% and 9.2%, respectively. These results suggested that cells whose nuclei were not traversed also formed DSBs, indicating that the extranuclear/extracellular effects could be assessed with the DSB assay *in situ* and probably reflected an early-stage damage induced by radiation in potentially non-hit cells. Kashino *et al.* have also demonstrated indirectly a bystander DSB induction in cells of the cell line xrs5, which is DNA double-strand break repair-deficient, with targeted soft X rays irradiating a single cell within a population (*25*).

Many studies have shown that GJIC and ROS play important roles in mediating radiation-induced bystander effects (*26–28*). In our studies, the treatment with lindane, an inhibitor of GJIC, reduced the fraction of DSB-positive cells induced by 1 cGy radiation, and the fraction of DSBs positive cells in the cell population was similarly decreased after treatment with 1% DMSO, a scavenger of ROS (shown in Table 2). These results suggest that both ROS and GJIC play important roles in mediating the production of DSBs in cells. The decrease in the fraction of DSB-positive cells by two drugs proves indirectly that using the visualization of DSBs was feasible to assess the extranuclear/extracellular effects induced by low-dose radiation.

The conventional dogma in radiation biology has been that the nucleus is the quintessential target for the radiobiological effects of ionizing radiation. However, using a precision microbeam, our previous studies and one carried out recently at the Gray Cancer Institute show, respectively, that targeted cytoplasmic irradiation induces mutations in the nucleus of hit cells (*29*) and that cytoplasmic irradiation can induce a bystander micronucleus response in glioblastoma cells (*30*). These data suggest that cytoplasmic damage may also contribute to the bystander effect. Thus the next question for our studies concerns the origin of these excess DSBs and what the likely contribution is from cytoplasmic damage. As shown in Table 1, the fraction of intact cells traversed by an average of one α particle is much higher than that of cells whose nuclei are traversed by an average of one α particle based strictly on microdosimetric estimation of a Poisson distribution of particle traversals to specific cellular sites. The observation that a twofold increase in cytoplasmic traversals resulted in no increase in the fraction of γ-H2AX foci (Table 1) suggests that the cytoplasmic or extranuclear contribution to the increase in foci is minimal. Moreover, the ability of lindane, which blocks GJIC, to further decrease the fraction of γ-H2AX foci suggested that an extracellular event, i.e. a bystander effect, contributes to the increase in foci in our study. However, direct evidence whether cytoplasmic irradiation could induce DSBs in the cell nucleus or in neighboring cells may need to be validated using microbeam irradiation since particles can be delivered to specific sites in the cells (i.e. nucleus or cytoplasm).

TABLE 2
Fraction of DSB-Positive Cells after α-Particle Irradiation and Treatment with either Lindane or DMSO

Dose (cGy)	No drug treatment (%)	Treatment with 40 μM lindane (%)	Treatment with 1% DMSO (%)
0	14.2 ± 3.6	12.7 ± 2.3	10.6 ± 1.0
1	48.6 ± 3.5	19.8 ± 1.0*	18.7 ± 0.6*
10	80.4 ± 3.4	66.8 ± 1.9*	64.1 ± 1.7*

Notes. Data were pooled from 10 individual experiments. Values are means ± standard errors of mean. The symbol * indicates statistically significant differences ($P < 0.05$) between controls and DMSO- or lindane-treated cells.

The percentage of positive cells with DSBs at the dose of 10 cGy was only about 80.4%, which was less than the percentage of cells whose nuclei were estimated to be traversed by an average of one α particle based on dosimetry calculations (92%). This result could be due to the random nature of the Poisson distribution of particle tracks. Following a Poisson distribution, if a population of cells in culture is exposed to an average of one ^{241}Am α-particle traversal per cell, only 37% of the cells will be likely to receive a single traversal, 26% of the cells will receive two or more particle traversals, and 37% will receive no particle. As a result, these calculations suggest that at a dose of 10 cGy, only 57.9%, not 92%, of the nuclei were actually traversed by an α particle. When cells were treated with lindane or DMSO and 10 cGy α particles, the formation of DSBs in

the cells also decreased to the level predicted by Poisson distribution (Table 2). Moreover, the fraction of DSB-positive cells, which was found to be about 14.2% in the sham-irradiated cell population, might be induced by ROS, which were produced during the process of normal oxidative metabolism, including mitochondrial respiration (*6*). On the other hand, during the process of DNA replication and transcription, DNA breaks are an unavoidable transient phenomenon (*31, 32*).

To be relevant, the existence of DSBs in a cell should have a close relationship with the formation of micronuclei, chromosomal translocations, mutation and apoptosis. Studies have shown that when DSBs are induced by radiation or carcinogenic chemicals, and if the unrepaired DSB remains, the chromosome fragments lacking centromeres (acentric fragments) can be observed in the binucleated stage of the cell cycle and form micronuclei (*33*). Also, there is experimental evidence for a causal link between the generation of DSBs and the induction of mutation and chromosomal translocations (*34–38*) as well as the triggering of apoptosis (*39*). As a result, the assay of DSBs not only could be used to assess the extranuclear/extracellular effects as another biological end point assay but also might provide information to explain why other damage end points might be induced and what kinds of early steps were involved.

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