Alpha-Particle-Induced Sister Chromatid Exchange in Normal Human Lung Fibroblasts: Evidence for an Extranuclear Target

A. Deshpande, E. H. Goodwin, S. M. Bailey, B. L. Marrone and B. E. Lehnert

Life Sciences Division, Los Alamos National Laboratory, Los Alamos, New Mexico 87545

INTRODUCTION

The increased incidence of lung cancer among uranium miners has contributed to a growing concern about the effects of α particles in the lung due to the inhalation of indoor radon and radon progeny. Recent estimates suggest that exposure to radon and radon progeny may be responsible for as many as 24,000 new cases of lung cancer yearly (1, 2). The mechanism(s) by which α particles cause lung cancer has not been elucidated, although a variety of genetic lesions, including dose-dependent chromosomal damage, have been associated with the DNA-damaging effects of α particles (3-9). Overall, α particles emitted from radon and its progeny have a high linear energy transfer (LET) and are 2.5-40 times more effective than low-LET radiation in causing biological damage. This greater effectiveness may be due to the effect that the DNA double-strand breaks caused by α particles are frequently not repaired or are mis-repaired (10). In the context of cancer, α particles can give rise to mutations and malignant transformation (11). Kadhim et al. (12, 13) have recently obtained evidence in vitro that suggests α-particle irradiation can induce the delayed occurrence of nonclonal chromatid aberrations in murine and human bone marrow cells; such genetic instability presumably could contribute to the ultimate emergence of cancerous phenotypes (14).

Many investigators have assumed that α particles cause their DNA-damaging effects upon traversal of cell nuclei. Indeed, a substantial amount of information has been obtained about the number of α-particle traversals through the nucleus that are required to kill a cell (15-18), as well as information about the effect of size, shape and/or thickness of the nucleus with respect to a cell’s susceptibility to reproductive inactivation with α-particle irradiation (19). Even so, some evidence suggests α particles may initially mediate their DNA-damaging effects by extranuclear events as well. In investigations of immortalized Chinese hamster ovary (CHO) cells that were exposed to low doses of α particles, Nagasawa and Little (5) observed increases in the frequency of sister chromatid exchanges (SCEs) in ~30% of the cells, even though less than 1% of the cells’ nuclei received a direct nuclear “hit” by an α particle. Thus it would appear that abnormal increases in SCEs, as one end point of genetic damage, may involve a target size for the α particles that is larger than only the cell’s nucleus. More recently, Hickman et al. (20) reported that α particles induced accumulations of the tumor suppressor and cell cycle-regulating protein p53 in immortalized rat lung epithelial cells in a higher percentage of the exposed population than the per-
percentage of cells that were calculated to receive a nuclear traversal by one or more α particles. In that such increases in p53 are related to DNA strand breaks, especially double-strand breaks (21), the observation by Hickman and coworkers appears to point further to the possibility that the DNA-damaging effects of α particles may not be initiated exclusively in a cell’s nuclear compartment.

In this report we describe the results of an investigation in which we used normal human diploid lung fibroblasts exposed to low doses of α particles to examine the relationship between nuclear traversals by α particles and DNA damage, as indicated by the induction of excessive SCEs (22). As will be described herein, we have obtained evidence that supports the possibility that the effects of α particles are mediated at least in part, if not exclusively, by an extranuclear mechanism.

**MATERIALS AND METHODS**

*Cell culture.* Normal human diploid lung fibroblasts (HFL1) initially obtained from a human fetus (CCL 153, American Type Culture Collection, Rockville, MD) were routinely cultured in 75 cm² tissue culture flasks in α-minimum essential medium (GIBCO-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT). All cell cultures were incubated at 37°C in humidified 5% CO₂/95% air. Cells were harvested from the flasks by trypsinization and seeded in 1.5-mm-thick Mylar-bottomed, 30-mm-diameter culture dishes (23) at an initial density of 2 X 10⁶ cells/dish 5 days prior to the exposures, with a change to fresh culture medium on the second day after plating. All of the irradiations were performed with density-arrested cells.

*Determination of cell nuclear areas.* HFL1 cells were grown in the Mylar dishes until they reached a density-inhibited plateau growth phase. The cells were then fixed on the dishes according to the protocol of Raju et al. (19) with minor modifications. Briefly, the confluent HFL1 cells were first treated with 1% glutaraldehyde for 1 h at 4°C, followed by fixation in 100% methanol for 15 min at −20°C. The cells were subsequently stained with 0.1 µg/ml Hoechst 33342 (Calbiochem-Novabiochem Corp., La Jolla, CA) for 5 min at 37°C, and the Mylar membranes were excised and mounted on glass slides using Vectashield mounting medium (Vector Labs, Inc., Burlingame, CA). Fields, viewed with a 20X Neoflor objective, containing approximately 30–50 cells were imaged digitally using a Photometrics camera (Tucson, AZ), mounted on a Zeiss Axiophot microscope.

Nuclear areas were then calculated from the images using the image analysis software provided in the public domain program “NIH Image” version 1.54, running on a Macintosh Quadra 650 computer (24).

*Exposure of cells to α particles.* Confluent HFL1 cells were exposed to doses of α particles ranging from 0.4 to 12.9 cGy at room temperature. Exposure to the α particles was performed using a 238U α-particle collimated exposure system that has been described in detail previously (19, 25, 26). With this system, the average energy of the α particles at the dishes (23) at an initial density of 2 X 10⁶ and seeded in 1.5-mm-thick Mylar-bottomed, 30-mm-diameter culture dishes was measured to be 3.5 MeV delivered at a dose rate of 3.65 cGy s⁻¹. Most doses gave less than one nuclear hit/cell, and cell survival was greater than 80% for all doses. Control HFL1 cells were sham-irradiated at room temperature.

*Sister chromatid exchange (SCE) assay.* Prior to the irradiations, most of the culture medium was aspirated from the culture dishes, and immediately after exposure it was replaced, thus preventing cell dehydration. After the exposures, the culture dishes were placed in an incubator for a period of 24 h. The HFL1 cells were then harvested by trypsinization and replated in 75 cm² flasks in 15 ml medium containing 5 µM bromodeoxyuridine (BrdU, Sigma Chemical Co., St. Louis, MO) at a density of 5 x 10⁵ cells/flask. The flasks were incubated at 37°C in 5% CO₂/95% air in the dark for 48 h. Four hours prior to the harvest of the cells, 0.1 µg/ml Colcemid (GIBCO BRL-Life Technologies, Inc., Grand Island, NY) was added to the flasks. The cells were collected by trypsinization, suspended in hypotonic potassium chloride (0.075 M) for 15 min at room temperature and then fixed in 3:1 methanol/glacial acetic acid overnight. Fixed cells were dropped onto cold wet glass slides and then stained using the fluorescence-plus-Giemsa staining method (27, 28). The numbers of SCEs per cell were scored for each α-particle dose and for sham-irradiated samples, and data from 50–60 metaphase cells/dose were obtained.

*Mathematical and statistical analyses.* The mean nuclear area of HFL1 cells was determined from the morphometric data obtained for the nuclear areas of HFL1 cells grown to confluence on Mylar (Fig. 1). This mean nuclear area value was subsequently used in conjunction with information on the α-particle fluence (19) to calculate the mean number of nuclear hits per dose (Table 1). Based on target theory (29), the average number of nuclear hits per dose was then used to calculate the percentage of HFL1 cells hit by one or more particles. This is the maximum percentage that would be expected to show excessive SCEs above the control value, assuming that SCEs are induced only in hit cells.

To test the hypothesis that SCEs are induced only in cells whose nuclei were traversed by one or more α particles, we analyzed our data using a χ² “goodness-of-fit” statistical test (30). The theoretically expected distributions of SCEs we attempted to fit to the observed data were derived assuming two random processes. First, the number of α particles/cell nucleus was assumed to be random, i.e. Poisson-distributed (8). Second, the number of SCEs induced by a single α-particle traversal was also assumed to follow a Poisson distribution. These two random processes operating simultaneously result in a Poisson-Poisson distribution of induced SCEs/cell (31). It should be pointed out that SCEs occur even in unirradiated cells. To account for this background phenomenon and thereby obtain the expected distribution of SCEs in irradiated cultures due to α particles, we mathematically imposed the distribution of induced SCEs onto the zero-dose distribution (8). For performing the “goodness-of-fit” test, we grouped some classes of cells showing SCEs, e.g. cells with 0 SCE, 1 SCE, 2 SCEs, etc. into larger sets, e.g. cells with 0–5 SCEs, so that a small number of cells at the extreme ends of an observed distribution would not influence the results of our analysis unduly.

FIG. 1. Distribution of nuclear areas of HFL1 cells that were grown to density-inhibited plateau growth phase on Mylar. The values shown in the figure were derived from nuclear measurements obtained from 100–150 cells. The mean nuclear area and SEM were 148 ± 5 m².

For statistical comparisons, Student’s t test and analysis of variance were performed where indicated using the VAX/VMS version of the statistical software package “Minitab” (30).
RESULTS

Occurrence of excessive SCEs after exposure to 1.8–12.9 cGy α particles. The mean numbers of SCEs/cell after exposure to doses of α particles ranging from 1.8–12.9 cGy are summarized graphically in Fig. 2. Compared to the sham-irradiated condition, the numbers of SCEs/cell were increased significantly (P < 0.001, t test) after exposure to the lowest dose of α particles examined in this study series. Statistical analysis (analysis of variance) of the data for SCEs/cell in Fig. 2 revealed no significant differences among the different doses of α particles. The effects of the α particles on SCEs are also illustrated in Fig. 3 in terms of the distributions of SCEs in the cell populations after the exposures to the different α-particle doses. Relative to the sham-irradiated control cells, all of the α-particle doses resulted in pronounced and significant (P < 0.001, t test) increases in the percentages of cells in the irradiated populations that showed >5 SCEs/cell. Some evidence of a dose–response effect was suggested by the observation that cells with 16–20 SCEs were evident only in cell populations exposed to a dose of α particles exceeding 2.3 cGy. However, the numbers of cells within this range were relatively low, and no cells with 16–20 SCEs were observed at 5.7 cGy. Overall, the induction of SCEs by α particles that we observed with the HFL1 cells over the range of 1.8–12.9 cGy is consistent with the data for SCE induction that Nagasawa and Little obtained previously with CHO cells.

Occurrence of SCEs after exposure to very low doses of α particles. In a subsequent study series, confluent HFL1 cells were exposed to even lower doses of α particles, i.e. 0.4–2.0 cGy, and SCE analysis was performed (Fig. 4). Even at the lowest dose (0.4 cGy), a significant increase (P < 0.05) in SCEs/cell above the control condition was observed, with the maximum level of SCE induction occurring between 1.5–2.0 cGy. This is consistent with the high-dose-range study in which the numbers of SCEs/cell were increased maximally at doses of ~2 cGy and higher. Analysis of the...
distributions of SCEs in the cell populations exposed to the lower dose range of α particles also provided further support for an α-particle dose–SCE response relationship. As illustrated in Fig. 5, the percentages of cells showing 11–15 SCEs/cell increased progressively as the dose of α particles increased over a dose range of ~0.4–1.5 cGy.

Relationship of postexposure cell sampling time to SCEs. Conceivably, the above findings could be related to the time postexposure at which we sampled cells for the SCE analyses, or, in other words, due to a time-dependent selection of irradiated subpopulations that did not represent each irradiated population of HFL1 cells as a whole. We have recently observed that α particles can cause transient delays in both the G1 and G2 phases of the cell cycle, at least when administered at higher doses than used in this study, i.e. 19 and 57 cGy (unpublished observations). In such cases, sampling of cells at a single time for analyses of SCEs could exclude those cells with transiently retarded cell growth characteristics. On the other hand, some undamaged cells may have progressed through the cell cycle earlier than the time chosen for cell sampling, which could result in an overestimation of target cell damage for a particular dose of α particles. In a second experiment designed to assess these possibilities, confluent HFL1 cells were exposed to 8.4 cGy α particles and harvested at 48, 50, 52 and 56 h. Data for SCEs were obtained for each time and then compared. Control cells in this experiment were sham-irradiated at room temperature. No significant differences were found by analysis of variance in the numbers of SCEs/cell that occurred at the various collection times compared to the 50-h time originally used in the previous study series (Fig. 6). Hence the occurrence of excessive SCEs after exposure to α particles evidently was not due to time-dependent selection of an unrepresentative subpopulation of cells.

Predicted compared to observed occurrence of excessive SCEs after exposure to α particles. In Table I the theoretically expected percentages of cells with excessive SCEs above background levels after exposure to the different doses of α particles are compared with the values observed experimentally. The actual percentages of cells that showed excessive SCEs after exposure to 1.8 and 2.3 cGy were approximately three times higher than the percentages of cells that were predicted to have experienced at least one nuclear hit by an α particle; at these two doses, the mean numbers of α particles that would be expected to traverse a
nucleus were calculated to be 0.129 and 0.173, respectively. With the single exception of the highest dose of α particles studied (12.9 cGy), all other doses also resulted in higher percentages of cells that actually showed excessive SCEs than predicted theoretically.

Table II compares the theoretically expected percentages of cells receiving one or more nuclear hits with the experimentally observed percentages of cells showing excessive SCEs for the very low dose range. The actual percentages of cells that showed excessive SCEs after exposure to 0.4 and 1.1 cGy were 8.6 and 3.6 times higher than the percentages of cells that were predicted to have experienced at least one nuclear hit by an α particle; at these two doses, the mean numbers of α particles that would be expected to traverse a nucleus were calculated to be 0.027 and 0.081, respectively. At 1.5 and 1.9 cGy, the percentages of cells showing excessive SCEs above background were approximately four times higher than the percentages of cells theoretically expected to have received one or more nuclear traversals.

<table>
<thead>
<tr>
<th>Alpha-particle dose (cGy)</th>
<th>Average number of α particles/nucleus</th>
<th>Percentage of cells receiving one or more nuclear hits</th>
<th>Percentage of cells showing excessive SCEs</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.4</td>
<td>0.027</td>
<td>2.7</td>
<td>23.3</td>
</tr>
<tr>
<td>1.1</td>
<td>0.081</td>
<td>7.7</td>
<td>28.4</td>
</tr>
<tr>
<td>1.5</td>
<td>0.110</td>
<td>10.4</td>
<td>43.3</td>
</tr>
<tr>
<td>1.9</td>
<td>0.144</td>
<td>13.4</td>
<td>45.0</td>
</tr>
</tbody>
</table>

To evaluate more formally the possibility that α-particle-induced SCEs may involve an extranuclear mechanism in addition to perhaps direct nuclear traversals, we compared the theoretically expected distribution of SCEs induced by nuclear hits for the lowest dose used in the first study (1.8 cGy) with the distribution of SCEs observed experimentally for the same dose (Fig. 7). Theoretical calculations were based on the assumption that the SCE distribution for the α-particle dose followed a Poisson-Poisson distribution, as described in the Materials and Methods. As shown in Fig. 7, the distribution obtained experimentally did not conform to the distribution predicted from the number of nuclear hits received by cells, thereby further indicating a role of an extranuclear compartment in the occurrence of excessive SCEs caused by exposure to α particles.

DISCUSSION

Using immortalized CHO cells, Nagasawa and coworkers (4, 5, 32, 33) have reported evidence consistent with the possibility that α particles, like those emitted by radon and radon progeny, can cause cytogenetic alterations in the form of SCEs by a mechanism(s) that does not solely involve direct interactions of α particles with cell nuclei. How this phenomenon may extend to normal human cells has not been assessed previously.

In our investigation, we used normal human diploid lung fibroblasts to examine further the relationship between nuclear hits by high-LET α particles like those emitted by radon and radon progeny and the subsequent occurrence of genetic damage, as indicated by sister chromatid exchanges (34). Some differences in methodology between our work and that of Nagasawa and coworkers, beyond the use of normal human cells, should be mentioned. Our cultures were synchronized through contact inhibition of growth, which is a natural cellular process, rather than isoleucine deprivation. This synchronization method eliminates any possible artifact due to amino acid starvation. Additionally, the concentration of BrdU, which itself can induce SCEs, was reduced in our study by a factor of two. Preliminary experiments (data not shown) confirmed that the relatively low concentration of BrdU used in our investigation resulted in a substantial reduction in background SCEs.
without any loss in the ability to differentiate chromatids. BrdU has also been shown to affect DNA repair (35, 36). In our experiments, we delayed subculture into medium containing BrdU for 24 h. During this time, most DNA lesions would have been repaired if reparable, thus greatly reducing any potential influence of BrdU on SCEs through an effect on DNA repair.

We have found that, as with CHO cells (5), excessive SCEs occur in higher percentages of human cells than what would be predicted from estimates of the actual percentages of cells experiencing direct nuclear traversals by α particles. In addition to this general conclusion, the induction of SCEs in HFL1 cells by α particles also appears to share some common qualitative features with what has been reported for CHO cells (5). Specifically, both cell types show a relative constancy in the percentages of cells that show excessive SCEs (Tables I and II), as well as a virtual constancy in the mean number of induced SCEs/cell (Fig. 2) that is independent of the dose of α particles administered once a dose threshold is reached. With the HFL1 cells, the distributions of excessive SCEs among the cell populations exposed to ≥1.5 Gy α particles in our study were also very similar (Figs. 3 and 5), even though the percentages of cells that received nuclear hits by the α particles increased progressively with increasing dose (Tables I and II). These collective findings suggest that the mechanism by which α particles induce SCEs in our cells became maximally operational after exposure to even a low 1.5-Gy dose of α particles. Moreover, our results provide no evidence for a role of nuclear traversals by α particles in mediating the induction of SCEs. Otherwise, one might expect that at least the percentage of cells showing excessive SCEs due to nuclear and extranuclear mechanisms would increase as more and more cells experience nuclear hits by the α particles in addition to the extranuclear mechanism, unless, of course, the percentages of HFL1 cells that can express SCEs and the magnitude of excessive SCE expression by the cells under our experimental conditions are in some manner limited. Evidence for an α-particle dose–SCE response relationship was observed in our study only after exposure to very low doses of α particles, i.e. <1.5 Gy.

Induction of SCEs depends on passage through S phase. Our work shows that α particles induce a relatively long-lived effect capable of inducing SCEs at least 36 h after exposure, which is when most HFL1 cells would be entering S phase. While an association between induction of SCEs and mutagenicity and carcinogenicity has been recognized for some time (37), the molecular mechanisms by which physical and chemical agents cause SCEs remain unclear. Existing evidence, however, indicates: (1) cleavage and reunion of DNA strand breaks or their functional equivalent are necessary for the occurrence of SCEs; (2) DNA double-strand breaks correlate with SCEs under some experimental conditions (38); (3) DNA crosslinks and monoadducts can result in SCEs (39); (4) SCEs occur during S phase (40) at or in the proximity of replication forks (41, 42); (5) chemical inhibition of the DNA strand rejoicing activity of DNA topoisomerase results in excessive SCEs (38, 42, 43); and (6) the incorporation of BrdU in DNA in itself can play a role in the expression of SCEs (44), especially in some mutant cells (45) and cells from individuals with some cancer-prone genetic disorders, e.g. Bloom’s syndrome (46, 47). Given the complexity of the numerous factors that are involved in SCE formation, we can only speculate on how α particles might initiate the process of inducing SCEs via an extranuclear compartment.

One potential mechanism may involve a stimulated production of a clastogenic cytokine. For example, tumor necrosis factor-α (TNF-α), a cytokine produced by macrophages in response to a variety of stimuli, has been shown to increase the frequency of SCEs in lymphocytes (48). However, it would seem unlikely that TNF-α accounts for the excessive SCEs found in our study inasmuch as this cytokine is produced by activated macrophages and typically not by lung fibroblasts. Even so, this does not rule out the possibility of a role for another secreted mediator akin to TNF-α in activity. Excessive SCEs resulting from α particles that do not hit cell nuclei directly may, on the other hand, be due to the generation of reactive oxygen species by radiolytic reactions outside of the nucleus, as pointed out earlier by Nagasawa and Little (5). In this regard, superoxide anions are sufficiently stable to allow diffusion within cells, and the generation of clastogenic hydroxyl radicals from these reactive species and/or hydrogen peroxide via Fenton-type reactions would not be unexpected (49). Along the same line, it is also conceivable that free radicals formed by high-LET α particles result in the initiation of lipid peroxidation in cell membranes and perhaps even in the fatty acids contained in culture medium, with the subsequent outcome being the formation of lipid peroxidation products capable of inducing SCEs when present at low concentrations, e.g. 4-hydroxynonenal (50, 51). That one or more soluble and relatively stable mediators of SCE induction may be involved in the extranuclear effects of α particles observed in our study gains some support, albeit indirect, from other observations. First, conditioned media from cells prone to increases in spontaneous chromosomal aberrations and excessive SCEs, e.g. cells from individuals with the disorders Bloom’s syndrome, Fanconi’s anemia and ataxia telangiectasia, show clastogenic activity when cultured with normal cells (52–54). Second, persistent clastogenic activity has been observed in the plasma of individuals exposed to very high doses of ionizing radiation (51, 55). Whatever the mechanism, it is clear that it is not initiated as readily by equivalent doses of low-LET ionizing radiation (5), and our results suggest that it is maximally set into place by an α-particle dose <2 Gy.

Finally, numerous microdosimetric models, including the recent Human Respiratory Tract Model for Radiological Protection (56), have been developed for assessing the α-particle radiation dose to sensitive airway cells in the lower respiratory tract or for estimating cancer risk due to the
inhalation of radon and radon progeny (57–60). A common assumption shared by these models is that it is traversals of α particles of the nuclei of the target cells, e.g. the basal and secretory cells, in the mucosa lining the airways that are of primary concern in the induction of cancer. In this regard, considerable effort has been directed toward characterizing morphometrically the distances to the cell nuclei for use in calculating the probability density in specific energy delivered to the nuclei by α particles from the decay of radon and radon progeny. The results from the present study and those of other investigators cited previously collectively suggest that the target for α-particle-induced genetic changes is larger than the nuclear compartment, perhaps considerably so. Based on data presented in the report by Nagasawa and Little (5), we have calculated that the potential target size for the SCE-inducing effects of α particles under their exposure conditions included a spatial distance equivalent to ~350 times the area of a typical CHO cell’s nucleus (calculations not shown). In that CHO cells have relatively scant cytoplasm, this estimated target size includes both the cell’s cytoplasmic compartment and an extracellular compartment as well. While it is unlikely that any physical or biological target exists with these dimensions aside from culture medium, the calculations nevertheless lend support to an extranuclear mechanism in the induction of SCEs. With our cells and the lowest dose used in this study, we have calculated the potential target size for α particles to cause excessive SCEs to be ~9 times the area of HFF1 cell nuclei.

ACKNOWLEDGMENTS

This investigation was supported by U.S. Department of Energy-funded projects entitled “Latent Expression of Genetic Damage in Human Lung Cells Caused by α particles and γ-rays,” “Mechanisms of Pulmonary Damage” and “Chromosome Damage in the One Rad Region,” and the work was conducted under the auspices of the Department of Energy. We would also like to thank Chris Coulon for his expert assistance in image acquisition and analysis. The authors also express gratitude to Drs. Michael N. Cornforth, University of Texas Medical Branch, Galveston, TX, and Larry Sklar and Carolyn Mold, University of New Mexico, Albuquerque, NM, for useful discussions over the course of this work.

Received: July 21, 1995; accepted: October 16, 1995

REFERENCES


