Induction of DNA Strand Breaks in Cultured Rat Embryo Cells by Crocidolite Asbestos as Assessed by Nick Translation

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ABSTRACT

Asbestos, a proven carcinogen, is reported to have no genotoxic effects. We hypothesized, however, in light of its clastogenic effects that one mechanism by which asbestos induces cell transformation and tumorgenesis involves the induction of DNA strand scission. Cultured rat embryo cells were exposed to low concentrations of International Union Against Cancer crocidolite and examined at intervals ranging from 2 to 48 h. The induction of DNA strand breaks was examined using the technique of nick translation followed by autoradiography or scintillation counting. Our results indicate that cells exposed to crocidolite have a higher incidence of DNA breaks and that this effect becomes apparent within 2-6 hours of exposure. Ball-milled crocidolite as well as riebeckite have a significantly lower effect while glass fibers induce a more pronounced DNA strand damage. These observations support the role fiber length plays in carcinogenesis and suggest that the classification of asbestos as a nongenotoxic carcinogen be reconsidered.

INTRODUCTION

Exposure to asbestos has been implicated in the development of bronchogenic carcinoma and malignant mesothelioma in humans and experimental animals (1-4). Asbestos induces cell transformation and chromosome aberrations in cultured cells and fiber dimension is believed to play an important role in carcinogenesis (5, 6). Cultured cells phagocytize asbestos fibers which interact with the mitotic apparatus (7, 8) and chromosomes (9) and presumably lead to chromosome nondisjunction or breakage by physical shearing. A number of studies document the induction of chromosome aneuploidies, breaks, and rearrangements (9-12) as well as sister chromatid exchanges (13-15) in cultured cells exposed to asbestos.

Recent experiments suggest an alternative mechanism which could result in damage to chromatin or microtubules after asbestos exposure. Mossman and Landesman (16) proposed that reactive oxygen metabolites, generated after asbestos phagocytosis, may induce DNA damage. Superoxide radicals have indeed been shown to induce DNA and chromosome damage (17, 18) and to be generated following exposure to asbestos (19, 20). However, asbestos fibers reportedly failed to induce unscheduled DNA synthesis or mutations in several test systems (21-23) or to generate DNA breaks as assayed by alkaline elution (24, 25).

We hypothesized that one mechanism by which asbestos fibers induce cell transformation and tumorgenesis involves the generation of DNA strand breaks. Failure to detect such damage could be related to the inadequate sensitivity of the techniques used in earlier studies. Accordingly, we undertook the present study to determine whether DNA damage is induced after exposure of rat embryo cells to a low concentration of UICC© crocidolite fibers using the technique of nick translation (26). Nick translation was initially developed to study the replication of DNA in vitro (27) and has recently been used to investigate the availability for transcription of the DNA in chromosmes (28-30) as well as in cultured cells and tissue sections (31, 32). Exogenous DNA polymerase I binds at DNA sites containing free 3'-hydroxy termini and, due to its polymerase and exonuclease activities, synthesizes a new DNA strand while digesting the nicked DNA chain. Thus, the amount of synthesis that occurs reflects the presence of strand breaks and the degree of damage sustained by DNA. This technique appears to be sufficiently sensitive to detect chemical and UV-induced DNA damage (33) and to differentiate between active and inactive chromatin (28, 34). It has been used to document heat-induced DNA damage at earlier times then was possible with alkaline elution (35).

MATERIALS AND METHODS

Fibers and Particulates. UICC standard crocidolite was washed in acetonitrile ether (1:1) for 15 min followed by acetone and graded ethanol washes and with 4-5 rinses with deionized water. The fibers were dried, baked overnight at 100°C, and sonicated for 5 min in HBSS and triturated using a 20-gauge needle. Alternatively, in some experiments crocidolite fibers that have been autoclaved and suspended in culture medium without prior washing with organic solvents were used. Crocidolite fibers were ball-milled for 40 min and sonicated for 10 min. Riebeckite, a nonfibrous particulate mineral having the same chemical composition as crocidolite, and code 100 glass fibers (Johns Manville, Denver, CO) were kindly provided by Dr. B. T. Mossman. These were suspended in HBSS, autoclaved, and triturated using a 20-gauge needle.

Rat Embryo Cells. A pregnant Fisher 344 rat (Charles River, Kingston, NY), at 16-17 days of gestation, was sacrificed by pentobarbital euthanasia. The embryos were removed, decapitated, eviscerated, and washed in HBSS containing 20 µg/ml nystatin and 50 µg/ml gentamicin. After mincing, the tissue was dissociated using 0.25% trypsin in calcium- and magnesium-free HBSS. The first aliquot was discarded and fresh trypsin was added at 10-min intervals for a total of 40-50 min. Cells were collected and cultured in a medium consisting of equal volumes of Dulbecco's minimal essential medium and F-12 (GIBCO, Grand Island, NY) supplemented with 2.5 µg/ml transferrin (Collaborative Research, Cambridge, MA), 0.1 µg/ml hydrocortisone (Sigma Chemical Co., St. Louis, MO), 2.5 ng/ml selenium (Collaborative Research), 2.5 µg/ml insulin (Sigma), 10% fetal calf serum (GIBCO), 50 µg/ml gentamicin and 20 µg/ml nystatin. Cultured cells were dissociated and aliquots were stored in culture medium containing 5% dimethyl sulfoxide over liquid nitrogen. Cells used in the different experiments were at passages 2 to 8.

Nick Translation. For autoradiography, cells were plated in flasks (Miles Scientific, Naperville, IL) and exposed to crocidolite fibers at concentrations ranging from 0.05 µg/cm² of culture area (equivalent to 0.15 µg/ml) to 2.0 µg/cm² (equivalent to 6 µg/ml). At various times between 2 and 48 h after exposure to fibers, cells were fixed in 100% ethanol in situ and air dried. The slides were incubated at 37°C for 1 h in nick translation medium containing 5 mM MgCl₂, 10 mM β-mercaptoethanol; 4 µM concentrations each of dATP, dCPT, and dGTP; 0.3 µM [³H]dTTP (42 Ci/mmol); and 10 units/ml DNA polym-
cell permeabilization. For this reason net incorporation in treated cells was used for each treatment group, and triplicate samples were measured.

Rat embryo cells were plated at 3 × 10⁶ cells/100-mm tissue culture plates and exposed to crocidolite at concentrations ranging from 0.05 to 2 µg/cm² (equivalent to 0.28 to 11.36 µg/ml, respectively). Three or four replicate dishes were used in each treatment group. After exposure to the fibers, cells were harvested by Versene-trypsin and suspended in 0.25 M sucrose buffer, containing 0.1 M Tris-HCl at pH 7.9, 10 mM MgCl₂, and 0.5 mM diithiothreitol. Cell viability was determined by trypan blue exclusion. Cells were then permeabilized by exposure to 0.02% Triton X-100 for 10 min at 0°C and resuspended at 4 × 10⁵ cells/ml in the nick translation buffer which consisted of 50 mM Tris-HCl (pH 7.9); 5 mM MgCl₂; 10 mM β-mercaptoethanol; 50 µg/ml bovine serum albumin; 0.05 mM concentrations each of dATP, dGTP, and dCTP (Pharmacia, Piscataway, NJ); and 2 µCi/ml [3H]dThd (42 Ci/mmol; Amersham). Escherichia coli DNA polymerase I (BRL) was added at 6 units/ml. The cells were incubated for 1 h at 37°C and the reaction was terminated by the addition of 5 ml of cold 5% trichloroacetic acid and 1% PP. The precipitate was collected on GF/C filters (Whatman, MA) prewetted with 2% PP, and washed three times with 5 ml trichloroacetic acid-PP, followed by 95% ethanol. After drying for 15 min under a heat lamp the filters were placed in Omnifluor and counted in a scintillation counter.

S-Phase Cells. To determine the frequency of S-phase cells, cells were cultured in flasks and incubated in Dulbecco’s minimal essential medium plus 10% fetal calf serum plus 1 µCi/ml [3H]thymidine (42 Ci/mmol; Amersham) for 1 h. The cells were fixed in situ in 100% ethanol, washed, dipped in NTB-3 emulsion, and developed after 7 days. The frequency of labeled cells was determined by scoring 1000 cells.

Statistical Analysis. In each experiment, three or four replicate plates were used for each treatment group, and triplicate samples were measured from each plate. Additionally, three samples from each plate were incubated with the nick translation mixture lacking the DNA polymerase I to determine the endogenous or nonspecific uptake of [3H]thymidine. This background level ranged from 44.3 ± 10.9 (SD) to 172.0 ± 52.4 cpm in the different experiments conducted. Net synthesis was calculated by subtracting the background levels from the polymerase-driven incorporation, which were 10 to 200 times as high. Experiments were repeated several times and the levels of incorporation varied from one experiment to another, influenced primarily by the extent of cell permeabilization. For this reason net incorporation in treated cultures was compared to the corresponding control group within each experiment which was processed simultaneously. The mean ± SD of treatment groups was compared to that of the control using Student’s t test.

RESULTS

Rat embryo cells cultured on glass slides, fixed in situ, and processed for nick translation and autoradiography, exhibited an increase in labeling after exposure to asbestos (Fig. 1). Control cells showed a moderate number of grains at 2 h and 6 h (Fig. 1, a and d), and cells exposed to 0.05 µg/cm² (Fig. 1, b and e) or 0.5 µg/cm² (Fig. 1, c and f) crocidolite exhibited a progressively higher grain density. A similar pattern was observed following 24 h (Fig. 1, g–j) and 48 h (Fig. 1, j–l) exposures. Furthermore, cells within a treatment group exhibited a similar labeling intensity (Fig. 1). Cells in control and crocidolite-treated cultures had similar morphology as did cells which did or did not phagocytize asbestos fibers. While it is difficult to exclude the presence of submicroscopic fibers in the cytoplasm, cells containing or lacking visible fibers had a similar grain density following nick translation (Fig. 2). Cultures exposed to 2 µg/cm² crocidolite were also heavily labeled.

In order to more accurately quantify DNA damage and to eliminate the problems of selection bias and grain counting, we conducted the nick translation reaction on permeabilized cells in suspension and monitored [3H]thymidine incorporation by scintillation counting. Rat embryo cells cultured in 100-mm dishes and exposed to 0.05 µg/cm² crocidolite for 24 or 48 h showed normal population doubling, averaging between 91 and 115% of the cells in control cultures. The cell count was reduced in cultures exposed to 2.0 µg/cm² crocidolite to 79 to 85% of control. Cell viability, as assayed by trypan blue exclusion, in cultures exposed to 0.5 µg/cm² crocidolite was similar to control, ranging from 91 to 98% in both groups, compared to 89 to 93% in cultures exposed to 2.0 µg/cm² crocidolite.

Exogenous polymerase-associated DNA synthesis in cells exposed to various concentrations of crocidolite exhibited a dose-related increase in the amount of DNA synthesis following nick translation (r = 0.92; Fig. 3). In the various experiments performed, cells exposed to 0.05 µg/cm² crocidolite averaged 108% of control cells, those exposed to 0.5 µg/cm² crocidolite averaged 275%, and cells exposed to 2.0 µg/cm² crocidolite averaged 393% of control values (Fig. 3; see also Tables 1–3). While we initially performed nick translation experiments at 2, 6, 24, and 48 h of exposure, the DNA-damaging effect of crocidolite was detectable as early as 6 h following exposure using scintillation counting (Table 1). In subsequent experiments, cultures were exposed to either 0.5 or 2.0 µg/cm² crocidolite and examined after 24 or 48 h. Table 1 presents the nick translation results from a representative experiment. Cells exposed to 0.5 µg/cm² crocidolite incorporated significantly higher values, ranging between 187 and 259% of control cells. Cells exposed to 2.0 µg/cm² crocidolite incorporated even higher levels, averaging between 194 and 326% of control cultures (Table 1).

Blomycin was used to confirm that the observed increases in [3H]thymidine incorporation was due to the induction of DNA breaks (Table 2). Cells cultured with 0.5 or 2.0 µg/cm² crocidolite for 24 h incorporated 451 and 653% as much as control cultures (P < 0.005) while those treated with 10 µg/ml blomycin for 1 h incorporated 3259% of control levels (P < 0.005; Table 2).

S-phase cells have a higher frequency of 3'-hydroxy free ends than G₁ or G₂ cells due to the presence of Okazaki fragments during replication, and the observed increase in the nick translation values could possibly be the result of stimulation by asbestos of cell entry into the S phase. To ascertain whether this was the case, cultures were labeled with [3H]thymidine for 1 h before fixation and processing for autoradiography. No significant differences were observed between control and asbestos-treated cultures in the frequency of labeled cells. For example, cultures exposed to 0.5 µg/cm² crocidolite had 36.4% labeled cells at 4.5 h after exposure; 33.5% at 8 h, and 24.9% at 24 h. The corresponding control cells had 36.1, 37.1, and 32.2% labeled cells at these times, respectively. Similar results were observed for cultures exposed to 2.0 µg/cm² asbestos. These results, coupled with cell counts that showed a slight decline in the cell population exposed to asbestos, suggest that the observed increase in nick translation values is unlikely to be due to cell cycle stimulation by asbestos.

We examined the effect of fiber size and composition using ball-milled crocidolite and riebeckite, a nonfibrous analogue with chemical composition similar to crocidolite. Our sample of UICC crocidolite fibers were sized by electron microscopy and averaged 14.8 ± 13.7 µm in length and 0.90 ± 0.43 µm in width. When ball-milled, the fibers average 2.8 ± 2.1 µm along their longest diameter. In our nick translation assay, cells...
DNA STRAND BREAK INDUCTION BY CROCIDOLITE ASBESTOS

Control  Crocidolite

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<th>0.05μg/cm²</th>
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<tr>
<td></td>
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<tr>
<td>2h</td>
<td>a, b</td>
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<td>24h</td>
<td>g, h, i</td>
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<tr>
<td>48h</td>
<td>j, k, l</td>
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Fig. 1. Nick translation autoradiographs of rat embryo cells. Cells were cultured on glass slides, exposed to crocidolite, fixed, incubated in nick translation mix and processed for autoradiography (see text). Grain density reflects the distribution of DNA strand breaks. Control cells (a, d, g, j) and those exposed to 0.05 μg/cm² (b, e, h, k) or 0.5 μg/cm² (c, f, i, l) crocidolite were fixed at 2 h (a–c), 8 h (d–f), 24 h (g–i), and 48 h (j–l).

DISCUSSION

The clastogenic effects of asbestos fibers on a variety of mammalian cells are well documented. Exposure to asbestos fibers was shown to result in anaphase abnormalities (7, 8), aneuploidy and chromosome aberrations (9–12), and sister chromatid exchanges (13–15). However, the mechanism by which asbestos fibers induce chromosome damage remains undefined. While it seemed reasonable to expect that such effects be mediated through the generation of DNA strand scission, previous attempts to detect DNA breaks (24, 25) or point mutations (23, 36) that are induced by asbestos have been unsuccessful. For this reason, the demonstration by nick translation of the occurrence of a substantial degree of cellular DNA damage following exposure to crocidolite fibers (Figs. 1–3; Tables 1 and 2) is significant. This effect of asbestos is evident within hours of exposure of the cells to low concentrations of fiber (Fig. 1; Tables 1 and 2). These observations suggest that crocidolite, and by extension other fibers, should be considered genotoxic.

Although some studies investigating the in vitro effects of
Concentrations of UICC crocidolite. The mean ± SD [3H]thymidine incorporation exposure to asbestos could be due to any of several possibilities.

Asbestos used high concentrations, up to 100 μg/ml (8, 14), most used the 10-μg/ml (10, 11) or the 0.5–2.0-μg/cm² concentration range (5, 9, 23, 37–39). The fiber concentrations used in the present study ranged from 0.05 to 2.0 μg/cm² and appeared to introduce minimal perturbations in cell morphology and growth. The observed effects are thus unlikely to be the result of cytotoxic effects of asbestos on cells. Although the concentrations of crocidolite used did not exceed 2.0 μg/cm² the dose-effect relationship observed (Fig. 3) suggests that more extensive DNA damage would result at higher fiber concentrations. This would be in agreement with other studies which considered the effect of crocidolite concentration on cell survival, transformation, and cloning efficiency (5, 40).

The observed increase in isotope incorporation following exposure to asbestos could be due to any of several possibilities.

DNA STRAND BREAK INDUCTION BY CROCIDOLITE ASBESTOS

Asbestos exposure might result in an increase in the proportion of S-phase cells, either by stimulating entry into or prolonging transit time through the S phase of the cell cycle (41–44). This possibility was not supported by our data. Both control and crocidolite-treated cultures had a similar labeling index and the total cell number was slightly reduced in asbestos-treated cultures. We also discount the contribution of nonviable cells to the observed increase in isotope incorporation since control and treated cultures had a small percentage of dead cells, as determined by trypan blue uptake, while the majority of cells in fiber-exposed cultures excluded the dye, were morphologically normal, and exhibited increased labeling intensity as ascertained by autoradiography (Figs. 1 and 2).

The technique of nick translation appears to be sensitive, detecting low levels of single strand breaks in treated as well as in normal cells (Fig. 1; Tables 1 and 2), and accurate, as indicated by its good repeatability and small standard error (Fig. 3). Furthermore, the treatment groups exhibited statistically significant differences. However, it should be noted that the levels recorded for control cells vary substantially in different experiments (Tables 1–3). We attribute such variation to differences in the degree of permeabilization of the cells by the detergent. We favor this possibility since a similar variation is noted in control and treated cells of the same experiment. The magnitude of such variation is reduced when the data are presented relative to control values (Fig. 3). Even though several replicate experiments were performed, the values for treated cells were compared to controls within each experiment (Tables 1–3). Additionally, 48-h cultures commonly had lower values than 24-h cultures (Table 3). This could be due to the increase in the cell population, which resulted in the consequent dilution of fibers relative to cell number, and to repair of the induced lesions.

Recently, Turvor and Brown (43) reported an increase in S₁ nuclease-digested DNA from cells exposed for 24 h to 200 μg/cm² crocidolite. Besides using excessively high concentrations

Table 1 [3H]Thymidine incorporation in cultured rat embryo cells exposed to crocidolite fibers

Background incorporation, measured by incubating the cells with the nick translation mix lacking DNA polymerase I, ranged between 96.3 ± 3.9 and 141.6 ± 12.7 cpm. These values were subtracted from the DNA polymerase I-driven [3H]thymidine incorporation. The mean ± SD (n = 3) of the net incorporation is shown for the different treatment groups. Values in parentheses (percentage) are relative to control. Cultures receiving crocidolite were compared to control using the t test.

<table>
<thead>
<tr>
<th>Exposure time (h)</th>
<th>Crocidolite (μg/cm²)</th>
<th>Bleomycin (10 μg/ml)</th>
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<tr>
<td>6</td>
<td>696.4 ± 38.8</td>
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<td>24</td>
<td>750.9 ± 45.8</td>
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<td>744.5 ± 6.3</td>
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<td>(259)</td>
<td>1944.7 ± 246.2</td>
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<td>(201)</td>
<td>1196.3 ± 190.5</td>
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<td>2376.1 ± 530.7</td>
<td>4341.4 ± 1143.7</td>
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* P < 0.005.
* P < 0.01.
* P < 0.05.

Table 2 [3H]Thymidine incorporation by nick translation in cultured rat cells treated with either crocidolite or bleomycin

Mean ± SD (n = 4) of the net incorporated [3H]thymidine (cpm) in the different treatment groups following nick translation. Bleomycin was added to the cultures 1 h before harvest. Values in parentheses (percentage) are relative to control. Statistical analysis of the data by t test.

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* P < 0.005.
of crocidolite, no difference in the effect of milled and unmilled crocidolite was observed (41). In our nick translation assay, milled crocidolite induced a much lower degree of [³H]thymidine incorporation than unmilled crocidolite. Similarly, riebeckite-induced DNA damage was lower than that caused by UICC crocidolite (Table 3). This indicates that fiber morphology plays a critical role in asbestos-induced injury and carcinogenesis. This is in agreement with other studies that emphasize the importance of fiber size, mainly its length:width ratio, in the induction of cell formation and chromosome damage (5, 6, 37). Glass fibers, on the other hand, which have pronounced cytotoxic and chromosome-damaging effects (38) induced a much higher level of synthesis in our nick translation assay (Table 3). We observed no difference in the induction of DNA breaks between crocidolite fibers which have been repeatedly washed in acetone and ether and those suspended directly in culture medium.

Asbestos fibers are phagocytized by cells (7, 9) and could possibly induce DNA breaks by physical shearing. We observed a rather uniform degree of labeling in exposed cultures (Fig. 1) and a similarity of labeling in cells with and without visible fibers in their cytoplasm (Fig. 2). While one cannot discount the possibility of submicroscopic fibers inducing such damage, these observations raise the possibility that physical contact of the fibers with the nucleus may not be necessary. Asbestos-induced DNA damage could be mediated by oxygen free radicals which are generated following exposure of the cells to asbestos (16–20, 44–46). This would account for the induction of DNA strand breaks as documented in this report and might explain the clastogenic and tumorigenic effects of asbestos fibers. Furthermore, the induction of DNA strand breaks by asbestos indicates that its classification as a nongenotoxic carcinogen (47) should be revised.

REFERENCES

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