Mitotic Disturbances and Micronucleus Induction in Syrian Hamster Embryo Fibroblast Cells Caused by Asbestos Fibers

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Asbestos and other mineral fibers have long been known to induce lung cancer and mesothelioma. However, the primary mechanisms of fiber-induced carcinogenesis still remain unclear. We investigated the occurrence of mitotic disturbances induced by asbestos (amosite, crocidolite, chrysotile) in an in vitro approach using Syrian hamster embryo (SHE) fibroblast cells. The following endpoints were investigated: micronucleus formation as a result of mitotic disturbances and characterization of the induced micronuclear population by kinetochore staining and visualization of the spindle apparatus. Supravital UV-microscopy was used to analyze changes in interphase chromatin structure, impaired chromatid separation, and blocked cytokinesis. All three asbestos fibers induced a high frequency of micronucleus formation in SHE cells (>200/2000 cells) in a dose-dependent manner (0.1–5.0 μg/cm²), with a maximum between 48 hr and 66 hr exposure time. At higher concentrations (more than 5.0 μg/cm²) the micronucleus formation decreased again as a result of increased toxicity. Kinetochore staining of micronuclei revealed that 48 ± 2% of asbestos-induced micronuclei reacted positively with CREST (antikinetochore) serum. Additionally, spindle apparatus deformations occurred in cells with disturbed metaphases and anaphases, while the spindle fiber morphology appeared unchanged. Our results show that asbestos fibers may cause both loss and breakage of chromosomes in the absence of direct interaction with spindle fibers. Key words: asbestos fibers, kinetochore, micronuclei, mitotic disturbances, SHE cells, supravital UV-microscopy. Environ Health Perspect 103:268–271 (1995)

Based on sufficient evidence from epidemiological studies and animal experiments, asbestos is now regarded as an established carcinogen (1,2); however, despite numerous investigations, the mechanism of its carcinogenic action remains unclear (3). Asbestos fibers do not cause gene mutations, but they may act as tumor promoters (4–6).

Alternatively, because asbestos treatment alone induces tumors and fiber dimensions appear to be important in this process (2,7), asbestos may affect cells by more direct mechanisms. In this respect, aneuploidy is a common characteristic of asbestos-induced tumors, and it has been hypothesized that such a shift in chromosome complement plays a major role in the early stages of neoplastic development (6,8). This condition can also be caused by chemicals whose targets include components of the cytoskeleton and chromosome condensation or the spindle apparatus. Once inside an interphase cell after phagocytosis, asbestos is accumulated preferentially in the perinuclear region (3,9). As a result, asbestos fibers are frequently identified within the mitotic apparatus. These observations have led to the hypothesis that asbestos causes aneuploidy primarily by interfering with the normal course of mitosis (3,10).

Due to these disturbances during mitosis, micronucleus formation is observed (11). Micronuclei originate either from acentric chromosome fragments or from whole chromosomes or chromatids that are not incorporated into daughter nuclei when cell division is completed (12). The results of our previous investigations show that the Syrian hamster embryo (SHE) micronucleus assay is a short-term test of high predictive value (13). In combination with the immunofluorescent staining of kinetochores in micronuclei using antikinetochore (CREST) serum, the SHE assay allows the detection of clastogenic events, as well as those that affect the regular distribution of chromosomes in mitosis. Furthermore, supravital-UV microscopy (11) allows direct observation of movement of chromosomes and chromatids during mitosis.

In our experiments, we used three different types of asbestos fibers: amosite, crocidolite, and Rhodesian chrysotile (UICC standard). Average dimensions for chrysotile, crocidolite, and amosite, respectively, were 0.10 μm, 0.25 μm, and 0.24 μm in diameter; and 2.24 μm, 1.71 μm, and 2.50 μm in length. The percentage of fibers with length ≥5 μm was approximately 5% for all three types of fibers. Number of fibers, expressed as millions per microgram, were 11.2 (chrysotile), 1.4 (crocidolite), and 2.0 (amosite). These data basically agree with those reported by Coffin et al. (14).

SHE cell cultures were established as described by Pienta et al. (15). For detection of micronuclei (16) SHE cells were grown on coverslips in a humidified atmosphere (12% CO₂ in air at 37°C), fixed in cold methanol (−20°C), and stained with bisbenzimidase (Hoechst 33258, 1 μg/ml).

Only structures smaller than one-third of the nucleus by area were counted to avoid confusion with nuclei of dividing cells. In addition, only micronuclei clearly separated from the cell nucleus were taken into consideration (Fig. 1). For each dose of asbestos fibers, the number of micronuclei (MN) was determined per 2000 cells in at least 3 experiments. Ten to 15% of treated cells showed more than one MN/cell. More than three MN/cell occurred in 2–8% of cases.

The results of the micronucleus assay showed that chrysotile, crocidolite, and amosite induce micronuclei in SHE cells in a dose–dependent manner. The MN frequency depends on exposure time, reaching a rather late maximum between 48 and 66 hr (frequency up to 15%). The time course is similar for the different types of fibers. This is shown in Tables 1 and 2, and in histogram form for better visibility in Figure 2. In contrast, the concentration dependence appears different (Tables 1 and 2; Fig. 2). Amosite is the most potent fiber, with the highest MN frequency at the lowest level of fiber concentration, compared with crocidolite and chrysotile (Fig. 2). This result correlates with the known carcinogenic potency of amosite (17). Chrysotile, crocidolite, and amosite, respectively, reached a maximum in MN formation at 5.0 μg/cm² (fiber concentration) and 66 hr (exposure time), 5.0 μg/cm² and 66 hr, 0.25 μg/cm² and 66 hr (Tables 1 and 2; Fig. 2). At higher concentrations and longer exposure times, the occurrence of MN decreased again as a result of an increased cytotoxicity. We tested fiber concentrations up to 184 μg/cm² (chrysotile), 200 μg/cm² (crocidolite), and 120 μg/cm² (amosite). At these concentrations we observed a very high toxicity and only 24.5 ± 3.7 MN/2000 cells. Lower amosite concentrations than 1.0 μg/cm² were tested because the maximum of MN formation was found at 0.25 μg/cm². As a negative control, we treated SHE cells with calcium sulfate (CaSO₄; Table 3).

For further analysis of the induced micronuclei, kinetochores were stained. This was carried out by incubating the fixed cell preparations with CREST serum (60 min) in a humidified chamber at 37°C. After rinsing with phosphate-buffered saline, the cells were incubated with fluorescein isothiocyanate (FITC) conjugated goat anti–human antibodies before applying

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Mitotic disturbances caused by asbestos fibers

Asbestos dose (µg/cm²) | Treatment duration (hr) | 18 | 24 | 48 | 66 | 72
--- | --- | --- | --- | --- | --- | ---
1.0 | 86.0 ± 0.5 | 99.3 ± 11.7 | 145.3 ± 5.0 | 119.7 ± 50.9 | 106.0 ± 46.6 |
5.0 | 117.5 ± 0.7 | 153.0 ± 9.9 | 164.0 ± 25.5 | 218.5 ± 80.3 | 128.0 ± 52.3 |
10.0 | 119.0 ± 15.5 | 126.0 ± 25.5 | 145.3 ± 18.9 | 192.0 ± 28.3 | 107.5 ± 39.2 |
Control | 31.3 ± 4.0 | 32.0 ± 5.6 | 30.8 ± 7.6 | 32.3 ± 4.2 | 29.3 ± 6.4 |

Table 1. Induction of micronucleus formation in Syrian hamster embryo cells by crocidolite asbestos

Data represent the mean of 3 counts of 2000 cells (± SD) of different experiments. The difference in micronucleus formation between asbestos-treated and untreated cells is in all cases significant (p<0.001).

Figure 1. Typical micronucleus formation (arrow) and chromatin budding in fixed Syrian hamster embryo cells after treatment with asbestos fibers.

Figure 2. Micronucleus formation in Syrian hamster embryo cells after treatment with various concentrations of amosite asbestos (0.0–15.0 µg/cm²) and different treatment durations (18–72 hr). The experiments were repeated several times with consistent results (SD ±12%).

Using bisbenzimide. The numbers of kinetochore-positive (K⁺) and kinetochore-negative (K⁻) micronuclei were nearly equal (Table 4) in cells treated whith asbestos fibers. In this case the different fiber concentrations were used only for induction of a high number of micronuclei.

In addition, we stained the spindle apparatus to investigate a possible interaction between asbestos fibers and tubulin structure. This was achieved by incubating the fixed cells with tubulin antibodies (human anti-α-tubulin and FITC-anti-mouse antibodies, 60 min at 37°C) before applying bisbenzimide.

Disturbances during mitosis caused by asbestos fibers were directly monitored by supravital-UV microscopy. This method allows the observation of the spatial arrangement and movement of chromatin elements throughout mitosis in living cells. The intensity of the UV light is attenuated by neutral density filters. The faint cellular DNA signal is picked up by a silicon intensifier target camera and further improved by digital computer enhancement (11).

For fluorescence microscopy analysis, SHE cells were plated in special chambers (11) and treated with amosite (0.25 µg/cm²), crocidolite (1.0–5.0 µg/cm²), or chrysotile (1.0–5.0 µg/cm²). Exposure time was 24 hr–48 hr. Live cells were stained with 0.1 µg/ml bisbenzimide (here Hoechst 33342 was used) and the course of mitosis was observed. Figure 3 shows micronucleus formation following a disturbed mitosis.

We also observed typical changes in interphase chromatin structure (chromatin budding (Fig. 1), compaction, and nuclear holes) and impaired chromatid separation in mitosis. This frequently resulted in the formation of chromatin bridges and chromosome displacement in metaphase and anaphase. In numerous cases, cytokinesis was blocked. We observed an increase in mitotic disturbances during and after the second cell cycle (delayed, blocked, or uncompleted mitoses and stickylagging chromosomes). Abnormal events of this type were not observed in untreated cells (more than 50 mitoses were analyzed).

Visualization of the spindle apparatus of amosite-treated SHE cells revealed that about 10% showed a slightly deformed spindle apparatus, although the spindle fibers appeared to have normal morphology. However, a deformed spindle apparatus occurs only in connection with disturbed metaphases and anaphases. This effect may be due to impaired connection or disconnection at the spindle fibers.

In conclusion, we found that all three types of asbestos fibers are taken up by phagocytosis by the cells within 24 hr after administration. This is in agreement with Hesterberg et al. (19). Intracellular fibers accumulate at the perinuclear region of the cells 24–48 hr after exposure (18). When cells undergo mitosis, the physical presence of the fibers results in interference with chromosome segregation. This explains the late maximum of MN formation of 66 hr. The frequency of micronucleated cells reflects chromosomal damage (19). In the present study, chrysotile and crocidolite increased the level of MN sevenfold after
Table 2. Number of micronuclei (per 2000 cells) induced by various concentrations of chrysotile fibers\textsuperscript{a}  

| Asbestos dose (µg/cm\textsuperscript{2}) | 18  | 24  | 48  | 66  | 72  |
|----------------------------------------|-----|-----|-----|-----|-----|
| Chrysotile                             | 73.0 ± 11.8 | 87.5 ± 12.5 | 93.4 ± 7.8 | 121.0 ± 37.6 | 98.5 ± 6.4 |
| Amosite                                | 106.0 ± 2.8  | 114.0 ± 18.4 | 137.0 ± 26.6 | 210.0 ± 56.5 | 151.5 ± 13.2 |
| Crocidolite                            | 94.0 ± 5.3   | 101.3 ± 17.9 | 126.7 ± 17.5 | 125.0 ± 1.7  | 107.0 ± 7.2  |
| Control                                | 28.0 ± 1.4   | 29.0 ± 9.5   | 33.0 ± 8.0   | 28.0 ± 4.1   | 30.8 ± 7.2   |

\textsuperscript{a}Data represent the mean of 3 counts of 2000 cells (± SD) of different experiments. The difference in micronucleus formation between asbestos-treated and untreated cells is in all cases significant (p<0.001).

Table 3. Occurrence of micronuclei in Syrian hamster embryo cells after treatment with calcium sulfate (CaSO\textsubscript{4}, negative control)\textsuperscript{a}  

| CaSO\textsubscript{4} dose (µg/cm\textsuperscript{2}) | 18  | 24  | 48  | 66  |
|-------------------------------------------------------|-----|-----|-----|-----|
| 0.5                                                   | 47.0 ± 4.2 | 45.5 ± 1.7 | 59.1 ± 5.0 | 48.0 ± 2.0 |
| 1.0                                                   | 56.6 ± 9.2  | 46.0 ± 9.9 | 58.2 ± 8.5 | 51.0 ± 15.5 |
| 5.0                                                   | 46.5 ± 5.6  | 40.3 ± 5.5 | 60.5 ± 18.9 | 57.5 ± 10.6 |
| Control                                               | 41.3 ± 4.0  | 49.0 ± 5.6 | 50.8 ± 7.6 | 45.3 ± 6.4 |

\textsuperscript{a}Data represent the mean of 3 counts of 2000 cells (± SD) of different experiments. In all cases, the differences in micronucleus formation between CaSO\textsubscript{4}-treated and untreated cells are not significant.

Table 4. Presence of kinetochores in micronuclei in asbestos treated Syrian hamster embryo cells\textsuperscript{a}  

| Asbestos fiber | Fiber concentration (µg/cm\textsuperscript{2}) | No. of cells | No. of micronuclei | %CRMN\textsuperscript{a} |
|----------------|-----------------------------------------------|--------------|-------------------|--------------------------|
| Chrysotile     | 10                                            | 3520         | 220               | 48.2 ± 3.7               |
| Amosite        | 5                                             | 1662         | 133               | 47.4 ± 5.9               |
| Crocidolite    | 1                                             | 1869         | 100               | 49.0 ± 1.0               |
| Control        | 0                                             | 36000        | 300               | 27.0 ± 4.0\textsuperscript{b} |

\textsuperscript{a}No. of cells, total number of cells scored for micronuclei, no. of micronuclei scored for presence of kinetochores; %CRMN\textsuperscript{a}: percentage of the detected micronuclei that react positively to anti-kinetochore serum (± SD).

\textsuperscript{b}Data taken from Schiffmann and De Boni (17) for comparison.

Figure 3. Supravital UV–microscopy analysis showing micronucleus formation during disturbed mitosis (anaphase) after treatment (24 hr) of Syrian hamster embryo live cells with chrysotile (1 µg/cm\textsuperscript{2}).
studies should be carried out to analyze the direct interaction between asbestos fibers and chromatin or chromosomes.

REFERENCES

1. WHO. Environmental health criteria 53. Asbestos and other natural mineral fibers. Geneva: World Health Organization, 1986.
2. Stanton MF, Layard M, Tegeris A, Miller E, May M, Morgan E, Smith A. Relation of particle dimension to carcinogenicity in amphibole asbestos and other fibrous minerals. J Natl Cancer Inst 67:965 (1981).
3. Barrett JC, Lamb PW, Wiseman RW. Multiple mechanisms for the carcinogenic effects of asbestos and other mineral fibers. Environ Health Perspect 81:81–89 (1989).
4. Sincock AM, Seabright M. Induction of chromosome changes in Chinese hamster cells by exposure to asbestos fibers. Nature 257:56–58 (1975).
5. Jaurand MC, Renier A, Guadichet A, Kheuang L, Magne L, Bignon J. Short-term tests for the evaluation of potential cancer risk of modified asbestos fibers. Ann NY Acad Sci 534:741 (1988).
6. Lechner JF, Tokiwa T, LaVeck M, Benedict WF, Banks-Schlegel S, Yeager H Jr, Banerjee A, Harris CC. Asbestos-associated chromosomal changes in human mesothelial cells. Proc Natl Acad Sci USA 82:3884–3888 (1985).
7. Hesterberg TW, Barrett JC. Dependence of asbestos–mineral dust–induced transformation of mammalian cells in culture on fiber dimension. Cancer Res 44:2170 (1984).
8. Barrett JC, Thomasen DG, Hesterberg TW. Role of gene and chromosomal mutations in cell transformation. Ann NY Acad Sci 407:291 (1980).
9. Hesterberg TW, Butterick CJ, Oshimura M, Brody AR, Barrett CJ. Role of phagocytosis in Syrian hamster cell transformation and cytogenetic effects induced by asbestos and short and long glass fibers. Cancer Res 46:5795 (1986).
10. Oshimura M, Hesterberg TW, Tsutsumi T, Barrett JC. Correlation of asbestos-induced cytogenetic effects with cell transformation of Syrian hamster embryo cells in culture. Cancer Res 44:5017 (1984).
11. Schüffmann D, De Boni U. Dislocation of chromatin elements in prophase induced by diethylstilbestrol: a novel mechanism by which micronuclei can arise. Mutat Res 246:113–122 (1991).
12. Degrassi F, Tanzarella C. Immunofluorescent staining of kinetochores in micronuclei: a new assay for the detection of aneuploidy. Mutat Res 203:339–345 (1988).
13. Fritzenschauf H, Kohlhop M, Rusche B, Schüffmann D. Testing of known carcinogens in the Syrian Hamster embryo (SHE) micronucleus test in vitro; correlations with in vivo micronucleus formation and cell transformation. Mutat Res 319:47–53 (1993).
14. Coffin DL, Cook PM, Creason JP. Relative mesothelioma induction in rats by mineral fibers: Comparison with residual pulmonary mineral fiber number epidemiology. Inhal Toxicol 4:723–300 (1992).
15. Pienta RJ, Polley JA, Leberz WB. Morphological transformation of early passage golden Syrian hamster embryo cells derived from cryopreserved primary cultures as a reliable in vitro bioassay for identifying diverse carcinogens. Int J Cancer 19:642–655 (1977).
16. Schmuck G, Lieb G, Wild D, Schüffmann D, Henschler D. Characterization of an in vitro micronucleus assay with Syrian hamster embryo fibroblasts. Mutat Res 203:397–404 (1988).
17. Lechner JF, Gerwin BI, Redd RR, Gabrielson EW, Van der Meer EN, Limeinmaa K, Somers ANA, Harris CC. Studies on human mesothelial cells: effects of growth factors and asbestos fibers. In: Cellular and molecular aspects of fiber carcinogenesis (Harris CC, Lechner JF, Brinkley BR, eds). Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1991:119–129.
18. Jensen CG, Jensen LCW, Ault JG, Osorio G, Cole R, Rieder CL. Time–lapse video light microscopic and electron microscopic observations of vertebrate epithelial cells exposed to crocidolite asbestos. In: Cellular and molecular effects of mineral and synthetic dusts and fibres (Davis IM, Jaurand MC, eds). NATO ASI series, vol 85. Heidelberg: North Atlantic Treaty Organization, 1994:63–78.
19. Heddle JA, Hite M, Krikhart B, Mavournin K, MacGregor JT, Newell GW, Salamone MF. The induction of micronuclei as a measure of genotoxicity. Mutat Res 125:61–118 (1983).
20. Barrett JC. Role of chromosomal mutations in asbestos–induced cell transformation. In: Cellular and molecular aspects of fiber carcinogenesis. (Harris CC, Lechner JF, Brinkley BR, eds). Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1991:27–39.
21. Jaurand MC, Gaudichet A, Halpern S, Bignon J. In vitro biodegradation of chrysotile fibers by alveolar macrophages and mesothelial cells in culture: comparison with a pH effect. Br J Ind Med 41:389–395 (1984).
22. Lund LG, Aust AE. Iron mobilization from crocidolite asbestos greatly enhances crocidolite-dependent formation of DNA single-strand breaks in X174 RFI DNA. Carcinogenesis 13:637–642 (1992).
23. Kamp DW, Graceffa P, Pryor WA, Weitzman SA. The role of free radicals in asbestos–induced diseases. Free Rad Biol Med 12:293–315 (1992).
24. Dizdaroglu M. Chemical determination of free radical–induced damage to DNA. Free Rad Biol Med 10:225–242 (1991).
25. Takeuchi T, Morimoto K. Crocidolite asbestos increased 8-hydroxydeoxyguanosine levels in cellular DNA of a human promyelocytic leukemia cell line, HL60. Carcinogenesis 15:635–639 (1994).
26. Hesterberg TW, Barrett JC. Induction by asbestos fibers of anaphase abnormalities: mechanism for aneuploidy induction and possibly carcinogenesis. Carcinogenesis 6:473–475 (1985).