

Asbestos-associated chromosomal changes in human mesothelial cells

(carcinogenesis/aneuploidy/cytotoxicity)

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ABSTRACT Replicative cultures of human pleural mesothelial cells were established from noncancerous adult donors. The cells exhibited normal mesothelial cell characteristics including keratin, hyaluronic acid mucin, and long branched microvilli, and they retained the normal human karyotype until senescence. The mesothelial cells were 10 and 100 times more sensitive to the cytotoxic effects of asbestos fibers than normal human bronchial epithelial or fibroblastic cells, respectively. In addition, cultures of mesothelial cells that survived two cytotoxic exposures of amosite fibers were aneuploid with consistent specific chromosomal losses indicative of clonal origin. These aneuploid cells exhibit both altered growth control properties and a population doubling potential of >50 divisions beyond the culture life span (30 doublings) of the control cells.

Epidemiological studies have established that exposure to asbestos fibers is the primary cause of mesothelioma in the industrialized world (1-3). The latency period for this disease ranges from 15 to >40 years (4) and because of the high use of asbestos during and since World War II, the mortality rate from mesothelioma has doubled since 1967. Projections indicate that the incidence will continue to increase until the year 2007 (5). Carcinogenesis studies with animals have shown that mesothelioma can be caused by either intrapleural or intraperitoneal injections of asbestos (6, 7). In addition, phagocytosis of chrysotile asbestos by rat mesothelial cells in culture has been investigated (8). However, studies with human mesothelial cells have not been reported previously. In addition, the mechanisms by which asbestos causes mesothelioma remain obscure. Thus, we elected to investigate both short- and long-term effects of asbestos fibers on replicative cultures of normal human mesothelial cells.

MATERIALS AND METHODS

Cell Culture and Growth Medium. Cultures of mesothelial cells were initiated from pleural effusions obtained from noncancerous donors who had medical indications of thoracentesis (e.g., congestive heart failure). The fluid was initially centrifuged at $125 \times g$ for 5 min. The pelleted cells were suspended in growth medium, washed by centrifugation, resuspended in growth medium, and inoculated into surface-coated 10-cm culture dishes at a ratio of 1 dish per 50 ml of pleural fluid. Semiconfluent cultures were dissociated by using trypsin (9) and either expanded by inoculating 200,000 cells per 10-cm culture dish, cryopreserved (9), or used according to experimental protocols.

Growth medium was prepared by supplementing medium M199 with hydrocortisone (0.4 μ M), zinc-free insulin (0.87 μ M), epidermal growth factor (3.3 nM), Hepes (20 mM), trace elements (9), fetal bovine serum (5%), and gentamicin (50 μ g/ml). Hydrocortisone was purchased from Steraloids (Wilton, NH); insulin and epidermal growth factor were obtained from Collaborative Research (Waltham, MA). Lux culture dish surfaces were coated (10) with a mixture of human fibronectin (10 μ g/ml), collagen (Vitrogen, 30 μ g/ml), and crystallized bovine serum albumin (10 μ g/ml) and were dissolved in M199 medium. The mixture was added to culture dishes at a ratio of 2 ml per 10 cm² of surface area. The plates were incubated at 36.5°C for at least 2 hr before the mixture was vacuum-aspirated. Fibronectin was obtained from Collaborative Research; bovine serum albumin was obtained from Miles.

The cells were identified as mesothelial cells by several criteria, including immunofluorescent staining with anti-keratin antibodies (11), a variable cell morphology depending on the presence (fusiform) or absence (cobblestone) of epidermal growth factor in the growth medium (12), histochemical staining for hyaluronic acid mucin (13), long branched microvilli as observed by scanning electron microscopy (14), and the presence of cross-linked envelopes in senescent cultures (15).

Growth Assays. Cultures were dissociated with trypsin and washed by centrifugation with Hepes-buffered saline (9) before reinoculating the cells at a clonal density (100 cells per cm²). Two replicate dishes per variable were used. After 8-10 days growth, the clones were fixed in 10% formalin and stained with 0.25% crystal violet. Colony-forming efficiency was defined as the percentage of colonies formed per number of cells inoculated. The clonal growth rate (population doublings per day) was defined as the mean \log_2 of the number of cells per 18 randomly selected colonies divided by the number of days of incubation. A modified clonal growth assay (9) was used to measure culture longevity. Initially, 10 sister clonal plates were inoculated. After incubation, 2 were stained, and both the colony-forming efficiency and average number of population doublings were determined. A subsequent series of clonal plates were then established by using the pooled cells dissociated from the remaining unstained sister plates. This procedure was continued until cells no longer formed colonies.

Cytotoxicity. Six-centimeter dishes were inoculated with 2000 pleural mesothelial cells. Twenty-four hours later, the medium was replaced with media containing increasing concentrations of fibers. Each dose was assayed in duplicate. After 3 days of exposure, the fiber-treated and control cultures were rinsed twice with growth medium, then reincubated in fiber-free medium. Ten days after inoculation, the

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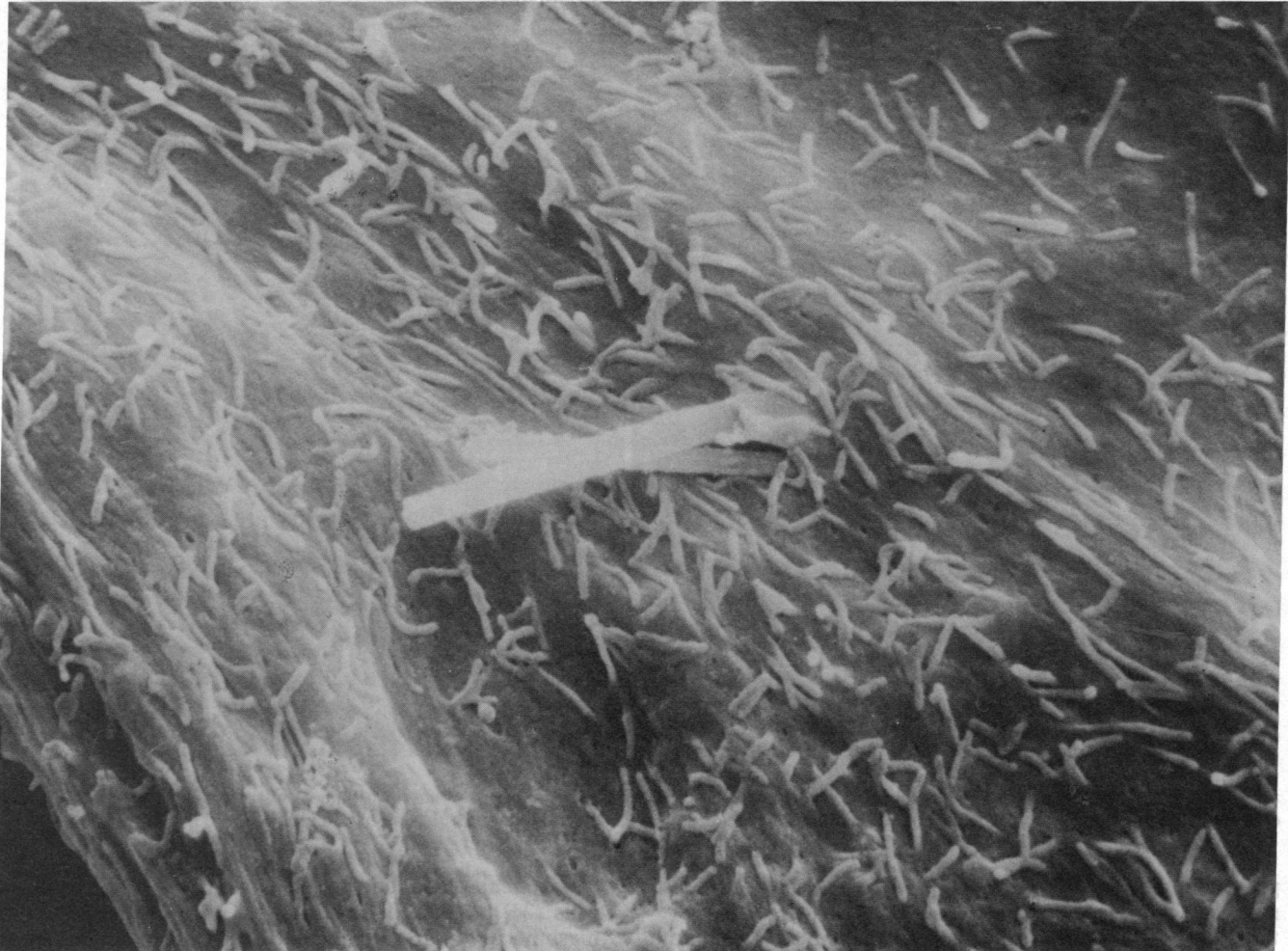


FIG. 1. Scanning electron micrograph of normal human mesothelial cells 2 hr after exposure to amosite ($0.3 \mu\text{g}$ per cm^2 of culture dish surface area). The fibers have been engulfed end first and sleeves of membranes are beginning to extend up the shaft of the fibers. Note also the numerous long and branched microvilli. ($\times 5000$.)

colonies were fixed in 10% formalin and stained with 0.25% crystal violet. The number of colonies per dish was then determined. Student's *t* test was used to evaluate the significance of differences between experimental groups. Asbestos fibers were UICC standard reference samples provided by V. Timbell (Medical Research Council, England). Dounce homogenized GF/D Whatman filters washed in 1 M HCl and water were used as glass fibers. Aliquots of fibers were prepared in H_2O and sterilized by autoclaving immediately prior to use.

Karyology. For chromosome studies, cells were exposed to Colcemid (50 ng/ml) for 2–5 hr, treated in 0.075 M KCl for 20 min, and fixed in methanol/acetic acid (3:1). The cells were then air-dried onto glass slides. Scoring of 25–50 chromosome spreads permitted determination of the modal chromosome number. For karyotypic analysis, 30–50 metaphases were stained by using a modification of Seabright's trypsin–Giemsa banding technique (16, 17).

Electron Microscopy. Cultures were fixed and processed *in situ* for scanning electron microscopy by published procedures (18).

RESULTS

Phagocytosis of asbestos fibers by human mesothelial cells proved to be rapid; fibers were observed penetrating the cells within 2 hr after exposure (Fig. 1). The fibers were engulfed end first, and a sleeve of membrane surrounded the stalk of

longer fibers ($>20 \mu\text{m}$) and then migrated up the fibers until it was surrounded. Fiber cytotoxicity, expressed as μg of fibers per cm^2 of culture dish surface area that decreased the colony-forming efficiency by 50%, was measured using clonal growth dose–response assays. Chrysotile was the most cytotoxic fiber tested and even glass fibers were markedly toxic (chrysotile, 0.06; amosite, 0.10; crocidolite, 0.40; glass fibers, 1.02). Thus, the mesothelial cells were significantly more sensitive to asbestos and glass fibers than were previously tested normal human lung cells—i.e., the amosite 50% cytotoxic doses for bronchial epithelial and bronchial fibroblastic cells were 1.02 and $10.40 \mu\text{g}$ of fibers per cm^2 of culture dish surface area, respectively (18).

For carcinogenesis studies, 10 dishes of cells (third subculture; 200,000 cells per 10-cm dish) were exposed to amosite asbestos by adding the fibers ($0.30 \mu\text{g}$ per cm^2 of culture dish surface area) to growth medium. After 4 days of incubation and at 4-day intervals thereafter, the medium was replaced with growth medium without fibers. Two weeks later, the cultures became confluent ($1\text{--}1.5 \times 10^6$ cells per dish). The cells were then dissociated with trypsin, pooled, and again inoculated at 200,000 cells per 10-cm culture dish. The following day, the protocol was repeated and the cultures were reexposed to amosite. Unexposed control cultures were studied in parallel. Two subcultures after the second exposure, numerous colonies of phenotypically altered cells were present in all of the cultures developed from treated cells (Fig. 2A); these abnormal-appearing cells were not

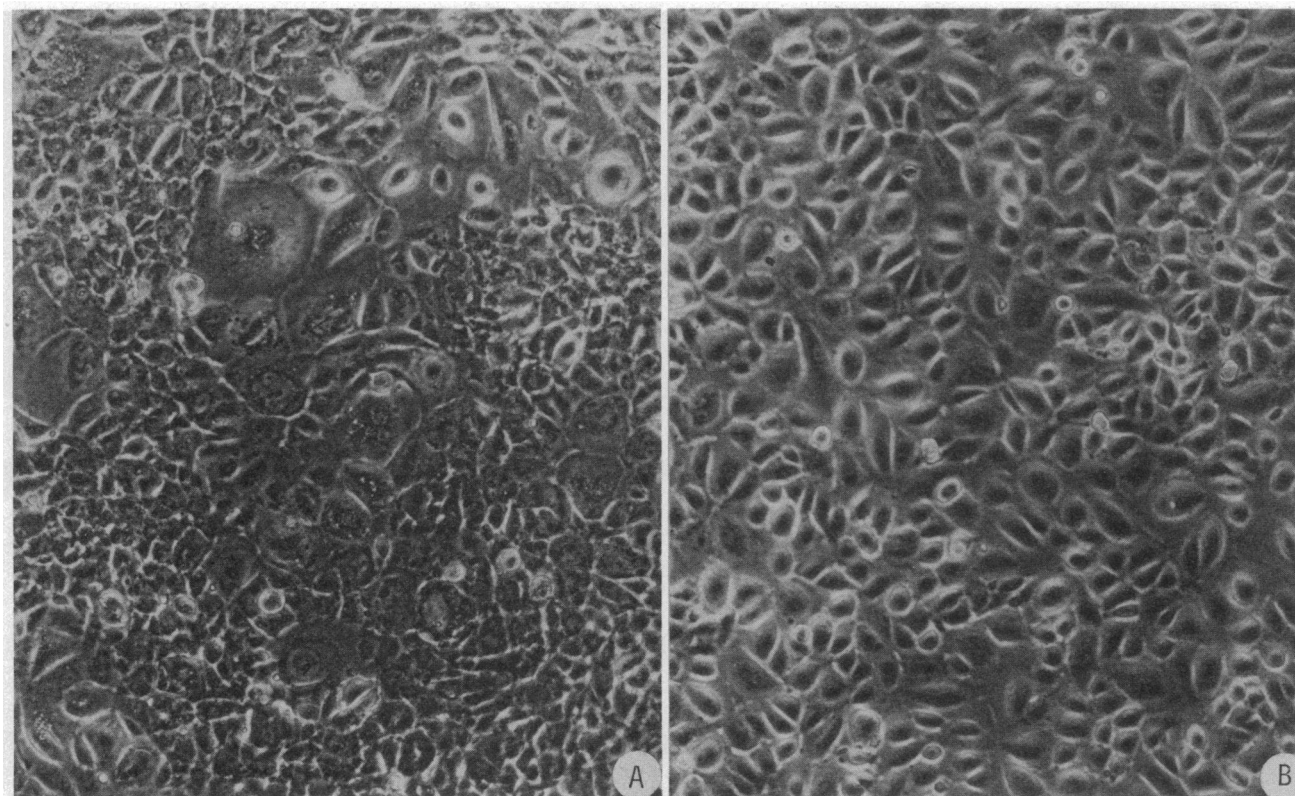


FIG. 2. Phase contrast photomicrographs of control (A) and exposed (B) cultures of human mesothelial cells three subculturing after two exposures to amosite. Note the phenotypically altered appearance of the cells depicted in the exposed culture. ($\times 200$.)

present in the control cultures (Fig. 2B). The control cultures reached senescence during the fourth subculture. However, the amosite-exposed cultures have continued to multiply for >19 subsequent subculturing (>50 population doublings) to date. Tumorigenicity of the abnormal-appearing cells was tested by injecting 11th passage post-second amosite exposure cells s.c. into adult athymic nude mice (5×10^6 cells per mouse; 9–20 mice per experiment); no tumors arose within 18 months after inoculation.

Human mesothelioma cells are aneuploid and have chromosome rearrangements (19, 20). Therefore, we examined the mesothelial cells that survived amosite exposure for chromosomal aberrations. Karyotypic analysis by Giemsa banding showed that the unexposed cultures retained the normal diploid human karyotype until senescence. In contrast, all the cells exposed to amosite by the fifth passage were pseudodiploid or aneuploid. For example, of 45 metaphases analyzed at passage 5, the majority were hypodiploid with a mode of 42 chromosomes. All of the 18 metaphases karyotyped with a hypodiploid count were missing chromosome 11 or chromosome 21, with most metaphases losing one of each chromosome (Fig. 3.) This strongly suggests a clonal origin for these cells. The remaining passage 5 metaphases analyzed had a hyperdiploid count ranging from 62 to 90. Various chromosomal abnormalities were found including dicentric chromosomes in $\approx 50\%$ of the hypodiploid and 100% of the hyperdiploid cells, respectively. Double minute chromosomes and extremely long chromosomes with similar abnormal repetitive banding patterns, possibly representing amplified DNA segments (21), were also seen in many of the metaphases (Fig. 3).

The chromosomally abnormal cells have retained histological, morphological, and ultrastructural features that are characteristic of mesothelial cells—e.g., they contain hyaluronic acid mucin and keratin and exhibit long branched microvilli. However, their generation time of 50 hr is signifi-

cantly greater than that for early-passage normal mesothelial cells (28 hr). Repeat experiments were carried out using mesothelial cultures developed from four other noncancerous donors. All have behaved similarly—i.e., they became morphologically transformed and have exhibited chromosomal rearrangements including dicentric formation within five subculturing after the second exposure to amosite.

DISCUSSION

Although asbestos fibers have been identified epidemiologically as a cocarcinogen for human malignancies other than mesothelioma, these fibers are considered to be complete carcinogens for mesothelial cells (1–3). In fact, although exposure to chemicals and radiation has produced mesothelioma in experimental animals (1), no etiologic agent other than fibrous structures—i.e., zeolites, ceramics, and occasionally glass—has been identified as a causative agent for human pleural and peritoneal mesothelioma (1–3). Mesothelial cells actively ingest asbestos in a manner analogous to human bronchial epithelial cells (18), but the resultant effects are markedly more cytotoxic. This observation suggests that the mesothelial cell has unusual properties that increase its sensitivity to fibrous agents. One unique characteristic of mesothelial cells is their remarkably plastic cytoskeletal composition—i.e., the content of keratin or vimentin in the cytoskeleton reflects the growth conditions (12, 15).

Normal human cells are characterized by chromosomal stability (22) and no increase in chromosome damage or polyploidy was noted in the metaphases of human fibroblastic cells after exposure to asbestos fibers at concentrations that induced high levels of chromosome aberrations in Chinese hamster cells (23, 24). In contrast, human mesothelial cells rapidly acquired extensive chromosomal rearrangements, particularly dicentrics, after exposure to low concentrations of amosite. Puck (25), Tsutsui *et al.* (26), and Heston and

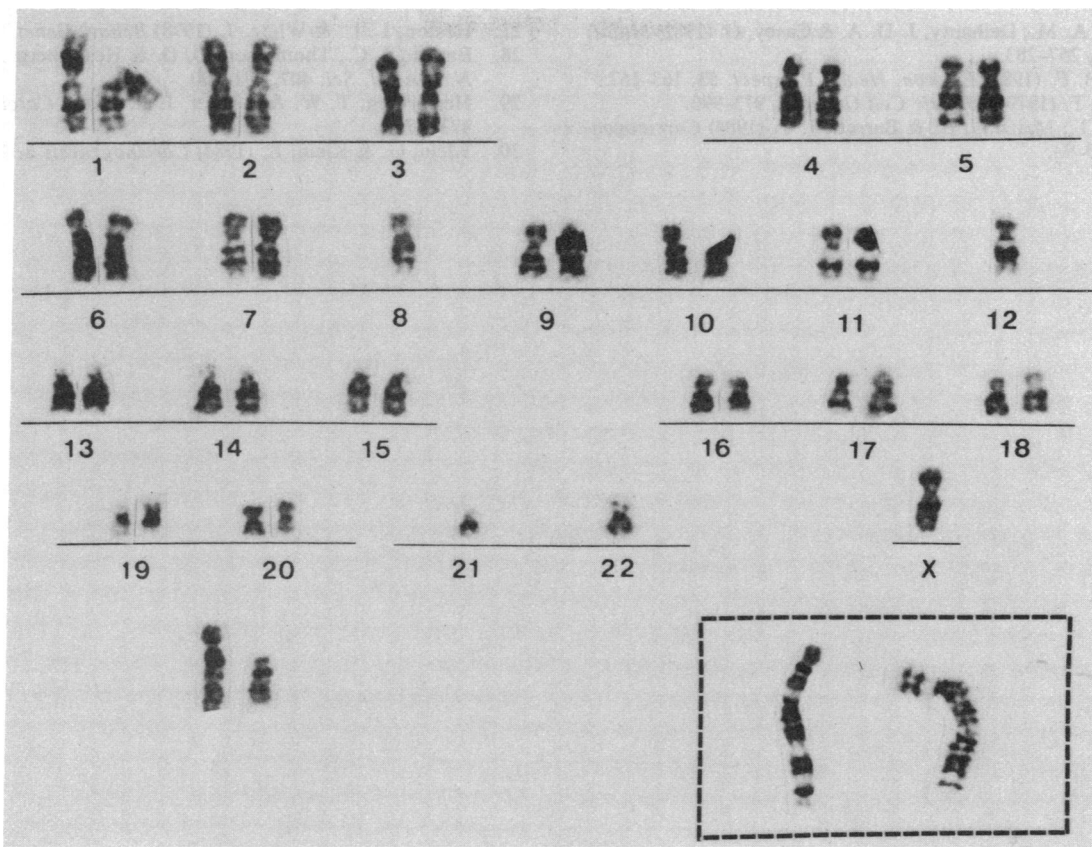


FIG. 3. Karyotype of a metaphase from a human mesothelial cell five subculturings after two exposures to amosite. This metaphase was hypodiploid, containing 43 chromosomes including two abnormal chromosomes shown at the bottom left. Among the missing chromosomes was a chromosome 21, which was lost in most of the hypodiploid metaphases examined. Two no. 11 chromosomes were present in this metaphase, although the majority of metaphases also had lost a chromosome 11. (Inset) Two long aberrant chromosomes from two additional metaphases are shown. These chromosomes had a repetitious banding pattern, which could represent amplified DNA segments.

White (27) have concluded from experimental and epidemiological data that interference with cytoskeletal functions can cause karyotypic instability. In addition, Barrett *et al.* (28) and Hesterberg and Barrett (29) have observed bizarre mitoses in Chinese hamster cells exposed to asbestos. Our cytotoxicity results suggest that the uniquely fluid mesothelial cell cytoskeleton may be very easily perturbed by penetrating asbestos fibers. This in turn would cause chromosomal instability, which could result in oncogene activation and transformation (30).

In conclusion, an *in vitro* model system has been used to explore the pathophysiological response of human mesothelial cells to asbestos. Asbestos induced clonally derived aneuploid cells with chromosomes possessing a repetitious banding pattern. These cells exhibited abnormal growth control properties, including a slower multiplication rate and a greatly extended culture population doubling potential. However, these alterations were insufficient to cause the cells to be tumorigenic.

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