Susceptibility of p53-deficient Mice to Induction of Mesothelioma by Crocidolite Asbestos Fibers

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Exposure of mesothelial cells to asbestos fibers in vitro has been shown to induce DNA damage mediated by oxidants. An early cellular response to DNA damage is increased expression of the p53 protein. This protein induces transcription of genes that activate cell cycle checkpoints or induce apoptosis. A murine mesothelial cell line that spontaneously acquired a point mutation in the p53 gene shows increased sensitivity to DNA damage induced by crocidolite asbestos fibers. It is hypothesized that p53-deficient mice will show increased sensitivity to the genotoxic effects of asbestos and accelerated development of malignant mesotheliomas. — Environ Health Perspect 105(Suppl 5):1069–1072 (1997)

Key words: p53 tumor suppressor gene, asbestos, mesothelioma

Introduction

Diffuse malignant mesothelioma is a lethal neoplasm arising from the pleura, peritoneum, or pericardium. Most cases of mesothelioma are associated with a history of occupational exposure to asbestos; cases occurring after household or neighborhood exposure have also been reported (1). Asbestos fibers are classified in two categories: serpentine or chrysotile asbestos and amphiboles. Chrysotile consists of curly, white fibers and accounts for 90 to 95% of asbestos used commercially. Amphiboles are long, straight fibers and include crocidolite, amosite, anthophyllite, and tremolite. Although tremolite is not used commercially, this amphibole is a common contaminant of some deposits of sand, talc, and chrysotile asbestos. Malignant mesothelioma has also been associated with environmental exposure to tremolite asbestos or to erionite (2).

The mechanisms leading to the development of malignant mesothelioma are unknown. It is hypothesized that fiber geometry and dimensions, chemical composition, surface reactivity, and biopersistence in the lungs are important parameters related to toxicity (3). Asbestos fibers generate reactive oxygen species in a variety of cell-free assays [reviewed by Moyer et al. (4)]. Macrophages exposed to asbestos fibers in vitro release reactive oxygen (5) and nitrogen species (6). The availability of iron at the surface of fibers is a critical parameter in catalyzing the generation of these highly reactive oxygen and nitrogen radicals (7). Ferric and ferrous cations are major components of amphibole asbestos fibers; iron may also be present as surface impurities on serpentine asbestos or some man-made fibers. Asbestos and other mineral fibers such as erionite may release or acquire iron from the surrounding medium, depending on the presence of chelators or reducing agents (8).

Several investigators have obtained evidence that reactive oxygen and nitrogen species contribute to DNA damage induced by asbestos fibers using a variety of target cell populations in vitro. Exogenous catalase or superoxide dismutase prevented DNA damage induced by asbestos in rat pleural mesothelial cells (9) or a human–hamster hybrid cell line (10). Crocidolite asbestos fibers induced formation of micronuclei in murine peritoneal mesothelial cells (11); this damage was also prevented by exogenous scavenging enzymes. Both reactive nitrogen intermediates and iron contribute to DNA damage induced by asbestos fibers in a human lung carcinoma cell line (12).

DNA breaks induced by oxidants, ionizing radiation, and chemotherapeutic drugs trigger a sequence of responses that arrest cells in the G1 phase of the cell cycle or induce apoptosis (13). A key event leading to these responses is increased expression of the p53 protein, followed by expression of additional genes that mediate cell cycle arrest or apoptosis. Cells lacking functional p53 protein as a result of gene deletion, formation of complexes with viral proteins, or point mutation are defective in cell cycle arrest or apoptosis triggered by DNA-damaging agents (14). Cistulli et al. (11) have shown that a murine mesothelial cell line that spontaneously acquired a point mutation in the p53 gene has increased susceptibility to induction of micronuclei by exposure to ionizing radiation or crocidolite asbestos fibers in vitro.

Several strains of transgenic mice have been developed that carry one or two copies of a disrupted p53 allele (15). These mice show increased susceptibility to induction of micronuclei and tumors by ionizing radiation (16). Preliminary studies indicate that p53-deficient mice show increased micronuclei in proliferating mesothelial cells after ip injection of crocidolite asbestos fibers. Therefore, we hypothesize that p53-deficient mice will show increased susceptibility to induction of mesotheliomas by asbestos fibers.

Methods

In Vivo Studies

Homozygous (−/−), heterozygous (+/−), and wild-type (+/+), mice derived from the 129/Sv strain (17) were purchased from Jackson Laboratories (Bar Harbor, ME). Groups of 10 to 30 mice 6 to 8 weeks of age were injected weekly with 200 μg (5.8 × 10⁶ fibers) of Union Internationale Contre le Cancer (UICC) crocidolite asbestos suspended in 1.0 ml saline for 35 weeks. Mice were sacrificed as they developed ascites or weight loss. All experiments were conducted according to the guidelines established by the National Institutes of Health Guide for the Care and Use of

This paper is based on a presentation at the Sixth International Meeting on the Toxicology of Natural and Man-Made Fibrous and Non-Fibrous Particles held 15–18 September 1996 in Lake Placid, New York. Manuscript received at EHP 27 March 1997; accepted 27 May 1997.

This research was supported by grants R01 ES05712 and R01 ES0721 from the National Institute of Environmental Health Sciences.

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Abbreviations used: TdT, terminal deoxynucleotidyl transferase; UICC, Union Internationale Contre le Cancer.
Laboratory Animals. Histologic sections of all abdominal and thoracic organs were examined for the presence of tumors using the criteria described by Davis et al. (18). Malignant mesotheliomas were confirmed by expression of cytokeratins as evaluated by immunohistochemistry (19).

**In Vitro Studies**

Primary murine mesothelial cells were isolated from peritoneal lavage fluid or following trypsinization of the peritoneal cavity as described previously (11). Wild-type (+/+) and homozygous p53-deficient (-/-) mesothelial cells were maintained for 4 to 16 passages in vitro. Growth curves were generated as described previously (11). Preparation of metaphase spreads was carried out as described previously (20).

Apoptosis was induced in wild-type and p53-deficient murine mesothelial cells by exposure to crocidolite asbestos (7.5 µg/cm²) (21) or camptothecin (10 µg/ml) for 24 hr. Two assays for induction of apoptosis were used. DNA breaks with exposed 3’-OH ends were labeled by incorporation of biotinylated dUTP catalyzed by TdT; dUTP incorporation was detected by avidin-peroxidase immunohistochemistry using a kit purchased from Oncor, Inc. (Gaithersburg, MD). This assay was confirmed by agarose gel electrophoresis of low molecular weight DNA isolated from control and treated mesothelial cells. After ethidium bromide staining, a characteristic DNA ladder at 180-bp intervals was seen in apoptotic cells (22).

**Results and Discussion**

**Induction of Mesotheliomas in p53-deficient Mice**

After 22 weekly ip injections, 12.5% of the homozygous p53-deficient mice developed mesotheliomas. The remainder of this cohort died with thymic lymphomas or hemangiosarcomas that develop spontaneously in p53-/- mice (23). After a mean latency of 44 weeks, 76% of the heterozygous p53-deficient mice developed mesotheliomas compared to 32% of wild-type mice (Table 1). No spontaneous development of mesotheliomas has been observed in these genetically engineered mouse strains (23).

Accelerated development of malignant mesothelioma induced by asbestos fibers in p53-deficient mice could be explained by three mechanisms. First, p53-deficient mesothelial cells could have a selective growth advantage over wild-type cells (24). Second, p53-deficient mesothelial cells could have increased genetic instability secondary to loss of G1 arrest and incomplete repair of DNA damage (17). Genetic instability would predispose p53-deficient cell populations to acquisition of additional mutations or deletions in cell cycle regulatory genes or tumor suppressor genes. Finally, p53-deficient mesothelial cells may be resistant to apoptosis (25). These mechanisms were explored in vitro.

**Table 1. Incidence of malignant mesotheliomas in p53-deficient mice**

| p53 Genotype | -/- | +/- |
|--------------|-----|-----|
| Fraction of mice | 1/8 | 13/17 | 9/28 |
| with tumors* | (12.5%) | (76%) | (32%) |
| Latency, weeks | 10 | 44 ± 24 | 67 ± 1 |

*The incidences of tumors and the genotypes are not independent; p = 0.003 using Fisher’s exact test.

**Growth and Apoptosis of p53-deficient and Wild-type Mesothelial Cells in Vitro**

Growth of early passage p53 +/+ and -/- murine peritoneal mesothelial cells was compared in vitro. The doubling time of p53 +/+ cells was approximately 24 hr, compared to only 12 hr for p53 -/- cells (Figure 1). The p53 +/+ cells were nearly diploid with a mean chromosome number of 41 ± 7, while p53 -/- cells were nearly tetraploid with a mean chromosome number of 72 ± 6 (Figure 2). This observation is consistent with loss of a spindle checkpoint in p53-deficient cells (26).

Induction of apoptosis by ionizing radiation and chemotherapeutic drugs is p53 dependent (14). Early passage p53 +/+ mesothelial cells treated with crocidolite asbestos or camptothecin for 24 hr showed evidence of apoptosis as indicated by dUTP incorporation by TdT at 3’-OH ends of DNA (Table 2) and appearance of a DNA ladder on agarose gels (data not shown). In contrast, p53 -/- mesothelial cells were resistant to induction of apoptosis by exposure to crocidolite asbestos or camptothecin in vitro. Experiments are in progress to

**Table 2. Induction of apoptosis by camptothecin in wild-type and p53-deficient murine mesothelial cells in vitro**

| p53 genotype | -/- | +/- |
|--------------|-----|-----|
| Control | 0 ± 0 | 0.45 ± 0.23 |
| Camptothecin | 0.13 ± 0.13 | 12.2 ± 2.2 |

*Percent of cells positive for apoptosis using 3’-OH end labeling; mean ± SEM from three experiments.

*The difference between homozygous +/- and wild-type +/+ mesothelial cells is statistically significant; p = 0.002 using Fisher’s PLSD test.
assess whether p53-deficient mesothelial cells show comparable resistance to apoptosis in vivo.

Relevance of This Murine Model to Human Malignant Mesothelioma

Point mutations and deletions in the p53 tumor suppressor gene are relatively rare in human (27) and murine malignant mesothelioma cell lines (28). Patients with the Li-Fraumeni syndrome carry one mutated p53 allele and have an increased risk of developing sarcomas and brain, bone, breast, and adrenal cancers. A recent case-control study revealed a slightly increased risk of mesothelioma in people who were exposed to asbestos and had first degree relatives with the Li-Fraumeni syndrome (29). Despite infrequent point mutations in the p53 gene, human malignant mesotheliomas show overexpression of the p53 protein (30–32). A possible explanation for this paradoxical finding is a recent report that SV40-like DNA sequences and T-antigens have been identified in some human malignant mesotheliomas (33). Viral proteins such as SV40 T-antigen bind to the p53 protein, prolong its half-life, and inactivate its normal functions (14). Human malignant mesotheliomas are frequently aneuploid and show multiple cytogenetic abnormalities (34). This observation is consistent with increased genetic instability due to inactivation of p53 and abrogation of cell cycle checkpoints.

Figure 3 proposes the following hypothesis: The initial response of the mesothelial lining to asbestos fibers is injury that is repaired by proliferation (35). Production of oxidants by macrophages attempting to phagocytize asbestos fibers trapped at the mesothelial surface has been demonstrated in situ (36). It is hypothesized that macrophages release reactive oxygen and nitrogen species that induce DNA damage and apoptosis in mesothelial cells. Abrogation of p53 function by targeted deletion (p53-deficient mice) or inactivation by viral proteins (humans) results in loss of cell cycle checkpoints and resistance to apoptosis in proliferating mesothelial cells. Functional inactivation of p53 or other components of the G1 cell cycle checkpoint (32) would accelerate later stages in the development and progression of malignant mesotheliomas induced by asbestos fibers.

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