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Accelerated Progression of Asbestos-Induced Mesotheliomas in Heterozygous $p53^{+/-}$ Mice

Charles A. Vaslet, Norma J. Messier, and Agnes B. Kane[1]

Department of Pathology and Laboratory Medicine, Brown University, Providence, Rhode Island 02912

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Asbestos fibers produce diffuse malignant mesotheliomas in chronic rodent inhalation assays or after direct intrapleural or intraperitoneal injection. *In vitro* models have provided evidence that asbestos fibers are genotoxic carcinogens that can directly or indirectly generate reactive oxygen- and nitrogen-derived species that cause DNA damage. Heterozygous $p53^{+/-}$ mice show an increased incidence and reduced latency of malignant mesotheliomas induced by weekly intraperitoneal injections of crocidolite asbestos fibers. In this study, we investigated whether loss of heterozygosity (LOH) at the *p53* tumor-suppressor gene locus contributes to accelerated tumor progression. LOH was found in 50% of the tumors produced in heterozygous $p53^{+/-}$ mice. In contrast to tumors that arise in $p53^{+/+}$ mice or those that retained one *p53* allele, LOH was associated with large tumor masses with central areas of necrosis, local invasion, and penetration of lymphatics. Increased tumor size was not associated with increased levels of cell proliferation as determined by BrdU incorporation, but it was correlated with a reduction in apoptosis as determined morphologically and by the TUNEL assay. Wild-type p53 protein is essential for cell cycle arrest in response to DNA damage and in maintenance of genomic stability. Cell lines established from tumors that showed LOH at the *p53* tumor-suppressor gene locus were nearly tetraploid. These results suggest that *p53* haplo-insufficiency sensitizes mice to the clastogenic or aneuploidogenic effects of crocidolite asbestos fibers, resulting in a shorter latent period. As solid tumors develop, spontaneous loss of the wild-type allele accompanied by decreased apoptosis and genetic instability is associated with accelerated tumor growth, invasion, and lymphatic dissemination.

Key Words: asbestos fibers; mesothelioma; *p53*-deficient mice; apoptosis; tumor progression; crocidolite asbestos; genetic instability.

Genetically engineered mice carrying activated oncogenes or inactivated tumor- suppressor genes have been used to assess the role of specific genetic alterations involved in the initiation, promotion, and progression of cancer (Macleod and Jacks, 1999). Transgenic mice carrying one copy of a disrupted allele at the *p53* tumor suppressor gene locus ($p53^{+/-}$ heterozyotes) have been proposed as a replacement for the two-year rodent carcinogenesis assay (Tennant *et al.*, 1995). Heterozygous $p53^{+/-}$ mice develop spontaneous lymphomas, sarcomas, and adenocarcinomas, depending on the genetic background, after a latency of 10 months (Harvey *et al.*, 1993a). In contrast, after exposure to genotoxic agents such as ionizing radiation, these heterozygous mice rapidly develop tumors after exposures as short as 26 weeks (Harvey *et al.*, 1993b; Lee *et al.*, 1994). The tissue specificity of genotoxic carcinogens is similar in both wild-type and $p53^{+/-}$ mice (French *et al.*, 2001). These heterozygous *p53*-deficient mice are useful for human risk assessment, taking into account interindividual variations in genetic sensitivity to carcinogens (Preston, 1996).

Asbestos fibers induce malignant mesotheliomas in chronic rodent-inhalation assays, as well as after direct intrapleural or intraperitoneal injection (reviewed in Kane, 1996). *In vitro* models have provided evidence that asbestos fibers have properties of genotoxic as well as nongenotoxic carcinogens. The geometry of long, thin fibers has been associated with genotoxicity in target cells *in vitro*. The surface reactivity of asbestos fibers and their ability to catalyze iron-dependent generation of oxygen- and nitrogen-derived species has been linked to production of oxidized bases, DNA breaks, mutations, and deletions (reviewed in Kane, 1996). Asbestos fibers have also been shown to be mitogenic, both by binding to specific surface receptors and by activating intracellular signaling pathways (reviewed by Robledo and Mossman, 1999). Asbestos fibers also cause apoptosis that may trigger compensatory hyperplasia (Broaddus, 1997). Activation of signal transduction pathways and induction of apoptosis by asbestos fibers in pleural mesothelial cells are dependent on the generation of reactive oxygen species (Robledo and Mossman, 1999).

The p53 protein plays a major role in coordinating cell-cycle arrest and DNA repair or apoptosis induced by oxidants or ionizing radiation. Cells that lack a functional p53 protein as a result of gene deletion, point mutation, or complex formation with viral proteins are defective in cell-cycle arrest and resistant to induction of apoptosis by DNA-damaging agents (Ko and Prives, 1996). We have shown that a murine mesothelial cell line that spontaneously acquired a point mutation in the

[1] To whom correspondence should be addressed. Fax: (401) 863-9008. E-mail: agnes_kane@brown.edu.

p53 gene during passaging *in vitro* is defective in the G1 cell cycle checkpoint induced by ionizing radiation. This cell line also shows increased sensitivity to induction of micronuclei by direct exposure to crocidolite asbestos fibers *in vitro* (Cistulli *et al.*, 1996). Therefore, we hypothesized that *p53*-deficient mice would show increased sensitivity to the genotoxic and carcinogenic effects of crocidolite asbestos fibers *in vivo*. We have reported previously that heterozygous $p53^{+/-}$ mice show an increased incidence and reduced latency of malignant mesotheliomas induced by crocidolite asbestos fibers (Marsella *et al.*, 1997). In this study, we report that 50% of the tumors induced in $p53^{+/-}$ mice show loss of heterozygosity (LOH) at the *p53* tumor suppressor gene locus, increased tumor size, and extensive invasion. The accelerated progression of malignant mesothelioma in heterozygous $p53^{+/-}$ mice could be explained by three mechanisms. First, *p53*-deficient mesothelioma cells could have a selective growth advantage over wild-type or heterozygous $p53^{+/-}$ mesothelioma cells. Second, *p53*-deficient mesothelial cells may acquire increased genetic instability secondary to loss of cell-cycle checkpoints and accumulation of oxidant-induced DNA damage. Third, *p53*-deficient mesothelial cells may be resistant to apoptosis. Evidence for or against each of these three mechanisms was evaluated *in vitro* and *in vivo*.

MATERIALS AND METHODS

Animals. Homozygous $p53^{-/-}$, heterozygous $p53^{+/-}$, and wild-type $p53^{+/+}$ male mice derived from the 129/Sv strain on a 75% C57B1/6 background were purchased from Jackson Laboratories (Bar Harbor, ME). These transgenic mice carry a targeted neo insert replacing exons 2–6 of the *p53* tumor suppressor gene (Livingston *et al.*, 1992). Mesotheliomas were induced by weekly intraperitoneal injections of 200 μg of UICC crocidolite asbestos fibers as described previously (Goodglick *et al.*, 1997). Fiber suspensions prepared in phosphate-buffered saline (PBS) were handled under a laminar flow hood equipped with Hepa filters with external exhaust. Mice were housed and treated according to guidelines established by the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Histopathology. Complete necropsies were performed on all mice and histopathologic diagnosis of malignant mesotheliomas was carried out as described previously (Goodglick *et al.*, 1997). For analysis of cell proliferation, mice were injected with BrdC (1 mg/1ml PBS, ip), which is more soluble than BrdU, one h before sacrifice. BrdC is converted to BrdU *in vivo*. Paraffin-embedded sections of tumors were processed for immunohistochemistry as described previously (Macdonald and Kane, 1997). The thickness of tumors growing on the serosal surfaces was measured at a magnification of 100× using an eyepiece equipped with a micrometer. An *in situ* assay was developed to visualize micronuclei in mesothelial cells using whole mounts of the diaphragm. In order to distinguish between micronuclei associated with mesothelial cells and debris from inflammatory cells, proliferating mesothelial cells were labeled with BrdU as described above. After 14 h (the cell cycle time of mesothelial cells is 18–24 h), mice were sacrificed and whole mounts of the diaphragm were prepared for BrdU immunohistochemistry. Micronuclei were counted at 40× magnification; at least 1000 proliferating mesothelial cells were counted per diaphragm. Micronuclei were counted if they were in the same focal plane as the nucleus and less than 25% of the diameter of the parent nucleus.

Isolation of mesothelioma cell lines. Cell lines were isolated from heterozygous $p53^{+/-}$ or wild-type mice after development of ascites or weight loss following weekly intraperitoneal injections of crocidolite asbestos fibers, as described previously (Goodglick *et al.*, 1997). Cell lines were established from ascites or peritoneal lavage fluid and analyzed after 2–6 passages *in vitro*.

Tissue microdissection and DNA extraction. Fixed, paraffin-embedded tumors were cut into 15 μm sections, mounted on glass microscope slides, and dissected with a #11 surgical blade under an inverted phase-contrast microscope at 10× magnification. Tissue samples were deparaffinized in xylene, followed by 100% ethanol, air-dried, and digested in 100 mM NaCl, 10 mM Tris, pH 8.0, 25 mM EDTA, 0.5% SDS, and proteinase K at 100 μg/ml for 4 h at 37°C. DNA was extracted in phenol/chloroform/isoamyl alcohol (25:24:1). The aqueous phase was collected and the salt concentration adjusted to 250 mM NaCl. DNA was ethanol-precipitated overnight at −20°C, washed, resuspended in water, and stored at −80°C.

PCR and RT-PCR analyses. Genomic DNA was isolated from microdissected tumors, tail biopsies, or cultured mesothelioma cell lines, as described above. For PCR amplification, 200 ng of genomic DNA was amplified using the following primers: X7: 5′-tatactcagagccggcct-3′ and X6.5: 5′-acagcgtggtggtaccttat-3′ to amplify the wild-type *p53* gene and X7 and neo 19: 5′-ctatcaggdcatagcgttgg-3′ to amplify the disrupted *p53* allele. For microdissected tumors, only the wild-type *p53* allele was amplified, due to limited amounts of DNA. Sufficient tumor samples free of stromal cells were available from only 3 mice. PCR amplification was performed at 95°C for 5 min.; 95°C, 1 min; 60°C, 2 min; and 72°C, 3 min for 30–35 cycles, followed by 72°C for 10 min. PCR products were separated on a 3% agarose (TAE) gel. Total RNA was isolated from subconfluent cultures of mesothelioma cell lines using RNazol™, and used for RT-PCR analysis as described previously (Goodglick *et al.*, 1997). The following primers were used for *p53* RT-PCR: 5′-acaggaccctgtcaccgagacc-3′ and 5′-gaccteegtcatgtgctgtgac-3′. Oligonucleotide primers for murine β-actin were purchased from Clontech Laboratories (Palo Alto, CA).

p53 *Genomic DNA sequencing.* Genomic DNA, obtained from early passages of mesothelial cell lines isolated from one wild-type and 4 $p53^{+/-}$ mice that retained the wild-type allele, was PCR-amplified for exons 5–8 of the *p53* gene, using Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA) and primers derived from intron 4 (forward) and exon 11 (reverse). These products were cloned into a pCRII-Topo vector (Invitrogen) and cycle sequenced using *p53* intron-specific forward and reverse primers.

Metaphase chromosome spreads. Mesothelioma cell lines were maintained for 2–6 passages *in vitro* and exposed to colcemid (0.2 μg/ml for 1.5 h.). Metaphase spreads were prepared as described previously (Marsella *et al.*, 1997); at least 20 metaphase spreads were counted to determine chromosome numbers in each cell line.

Apoptosis assays. Tumor samples from heterozygous $p53^{+/-}$ mice were fixed in Omnifix, embedded in plastic, sectioned at 1 μm, and stained with toluidine blue for histologic analysis of necrosis or apoptosis. Paraffin-embedded tumor tissues were sectioned and analyzed for incorporation of biotinylated dUTP (TUNEL assay kit, Oncor, Inc., Gaithersburg, MD). Apoptotic cells were identified using avidin-peroxidase immunohistochemistry as described previously (Marsella *et al.*, 1997).

RESULTS

Increased Sensitivity of p53*-Deficient Mice to Induction of Micronuclei by Crocidolite Asbestos*

An *in situ* assay was developed to visualize micronuclei in mesothelial cells using whole mounts of the diaphragm, as illustrated in Figure 1. Induction of micronuclei was assayed three days after a single intraperitoneal injection of 200 μg of crocidolite asbestos fibers; this is the peak of mesothelial cell proliferation induced in this murine model (Moalli *et al.*, 1987). As summarized in Table 1, an increased number of

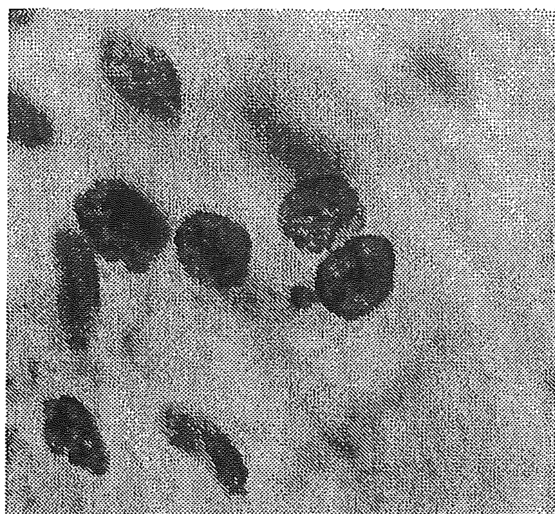

FIG. 1. Induction of a micronucleus in a proliferating mesothelial cell identified by BrdU immunohistochemistry on a whole mount of the diaphragm, as described in Materials and Methods; magnification ×400.

micronuclei was induced in $p53^{-/-}$ mice three days after injection of asbestos fibers, although no increase in micronuclei was observed in saline-injected controls. Wollastonite is a soluble calcium silicate that is not tumorigenic. Injection of 2 mg of wollastonite fibers elicited a comparable inflammatory response as asbestos (Macdonald and Kane, 1997); however, the number of micronuclei induced was not statistically elevated over saline-injected controls. The magnitude of the inflammatory response elicited by intraperitoneal injection of asbestos was similar in wild-type and *p53*-deficient mice (data not shown). Although *p53*-deficient mice are susceptible to the development of thymomas and sarcomas, they do not develop spontaneous malignant mesotheliomas (Donehower *et al.*, 1992). However, because *p53*-deficient mice showed increased sensitivity to asbestos-induced genotoxicity, we hypothesized that they would be more sensitive to induction of mesotheliomas by weekly intraperitoneal injections of 200 μg of crocidolite asbestos fibers.

TABLE 1
Induction of Micronuclei in Proliferating Mesothelial Cells *in Vivo*

Treatment	% Mesothelial cells with micronuclei: Wild type	% Mesothelial cells with micronuclei: *p53*-Deficient
Saline	0.49 ± 0.49	0.28 ± 0.95
Asbestos	1.35 ± 0.05*	3.48 ± 0.52**
Wollastonite	0.75 ± 0.09	Not tested

Note. Wild-type ($p53^{+/+}$) or deficient ($p53^{-/-}$) mice were injected ip with saline (1 ml), crocidolite asbestos (200 μg/1 ml PBS), or wollastonite (2 mg/1 ml PBS) fibers and were sacrificed after 3 days. Micronucleus induction in proliferating mesothelial cells was determined on whole mounts of the diaphragm dissected from 3 mice for each treatment (as described in Materials and Methods). Values are expressed as mean ± SD.

*Significantly different in asbestos-injected mice from saline-injected controls ($p < 0.05$).

**Significantly different in *p53*-deficient mice from wild-type mice ($p < 0.05$).

Malignant Mesotheliomas Induced by Crocidolite Asbestos in Heterozygous p53-*Deficient Mice*

The time course of induction of malignant mesotheliomas by weekly injections of crocidolite asbestos fibers is shown in Figure 2. After a mean latent period of 44 weeks, 76% of the heterozygous *p53*-deficient mice developed mesotheliomas. In contrast, only 32% of the wild-type mice developed mesotheliomas after a mean latent period of 67 weeks. The incidence of tumors in $p53^{+/-}$ vs. $p53^{+/+}$ mice was dependent on the genotype ($p = 0.003$ using Fisher's exact test). The difference between the mean latent periods was also statistically significant ($p = 0.002$ using analysis of variance and Fisher's PLSD test).

Distinct morphologic stages in tumor progression have been described in this murine model (Craighead and Kane, 1994). Focal, well-localized tumors limited to the serosal surfaces were the earliest mesotheliomas observed in $p53^{+/+}$ or $^{+/-}$ mice. These tumors spread diffusely over the peritoneal lining or grew as papillary projections within the abdominal cavity. Invasion was limited to the injection site or mesenteries. In 50% of the $p53^{+/-}$ mice, malignant mesotheliomas with extensive invasion were found (Fig. 3). Tumors in these mice were

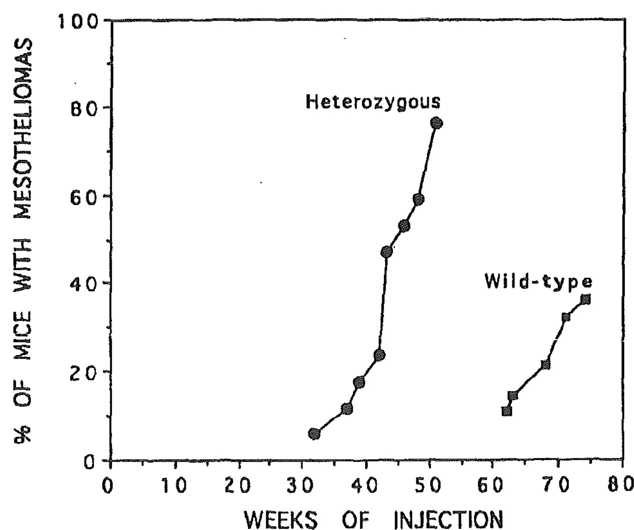

FIG. 2. Time of development of malignant mesotheliomas in heterozygous $p53^{-/-}$ or wild-type $p53^{+/-}$ mice induced by weekly ip injections of crocidolite asbestos fibers (200 μg/1 ml PBS). Mice were sacrificed when they developed massive ascites or weight loss; all abdominal organs were examined histopathologically for the presence of diffuse malignant mesothelioma. These data were originally presented in tabular form in Marsella *et al.* (1997).

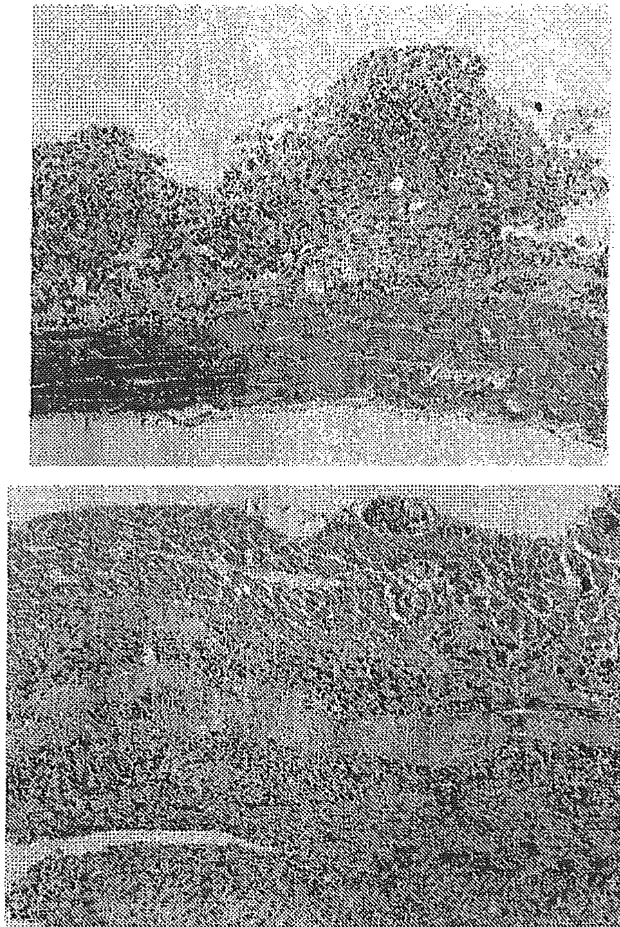

FIG. 3. Histologic appearance of diffuse malignant mesotheliomas in heterozygous $p53^{+/-}$ mice that lost the wild-type allele. Upper panel, solid tumors on the abdominal wall showing central areas of necrosis. Lower panel, infiltration of the diaphragm and penetration into lymphatics; magnification ×100. Hematoxylin and eosin stain.

larger than those in $p53^{+/+}$ mice: the tumor thickness on the serosal surfaces ranged between 0.40–0.76 mm (n = 4 mice) compared to 0.20–0.36 mm (n = 4 mice) in the other $p53^{+/-}$ mice and 0.05–0.25 mm (n = 5 mice) in the $p53^{+/+}$ mice. Lymphatic invasion and penetration through the diaphragm to involve the pleura was also found (Fig. 3, lower panel), in addition to metastasis to abdominal lymph nodes. Invasion of the diaphragm and lymphatic metastases have rarely been observed in rodent mesotheliomas induced by intraperitoneal injection of asbestos fibers (Craighead and Kane, 1994).

Malignant Mesotheliomas Induced by Asbestos in p53$^{+/-}$ *Mice Show Loss of the Wild-Type Allele*

The accelerated rate of growth and invasion in 50% of mesotheliomas arising in $p53^{+/-}$ mice raises the possibility that the wild-type allele was lost in these tumors. This was investigated using mesothelial cell lines established from these mice and microdissected samples of malignant mesotheliomas identified histologically on serial paraffin-embedded sections (Fig. 4, upper and center panels). The genotypes of the donor mouse and the tumor were compared using PCR analysis of DNA extracted from the tail or from early passages of the corresponding cell line. As shown in Figure 4 (upper panel), primer sets designed to amplify exons 6–7 of the p53 gene generated the expected product at 400 bp in $p53^{+/+}$ or $^{+/-}$ mice, but not in $p53^{-/-}$ mice. In addition, a second product at 600 bp was detected in $p53^{+/-}$ and $^{-/-}$ mice using primers that amplified the neo-insert in the disrupted *p53* allele. Fifty percent of the tumor-cell lines established from the donor $p53^{+/-}$ mice had lost the wild-type *p53* allele. This PCR analysis of DNA isolated from cell lines established from *p53*-deficient mice with asbestos-induced mesotheliomas was confirmed by RT-PCR analysis for *p53* expression (Fig. 4 lower panel). RNA was isolated from early passages of these cell lines, treated with DNase to eliminate priming from the p53 pseudogene, reversed-transcribed, and amplified with primers to detect expression of *p53* or actin as a control for integrity of the isolated RNA. All RNA samples showed amplification of actin and 80% showed expression of WT1, which is characteristic of murine malignant mesotheliomas (Goodglick *et al.*, 1977).

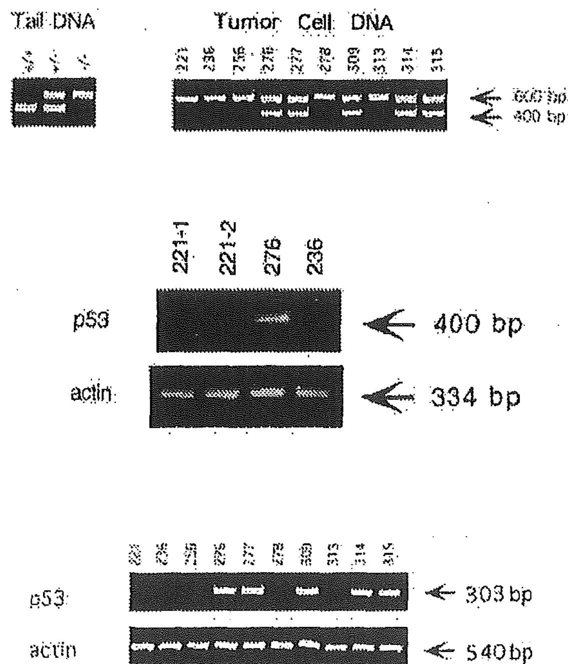

FIG. 4. Inactivation of *p53* in mesothelioma cell lines isolated from heterozygous $p53^{+/-}$ mice after weekly ip injections of crocidolite asbestos fibers, as described in Materials and Methods. Upper panel, genotypes of mice and tumor cell lines using PCR analysis of DNA; center panel, genotypes of tumors microdissected from wild-type (276) and $p53^{+/-}$ mice (221, tumors 1 and 2 and 236); lower panel, mRNA expression of p53 and actin using RT-PCR analysis of RNA.

Expression of *p53* corresponded with PCR analysis of DNA extracted from these cell lines in all cases.

PCR analysis of mesothelioma cell lines was confirmed by direct genotyping of the primary tumors (Fig. 4, center panel). Sections of mesotheliomas free of contaminating stroma were microdissected, deparaffinized, and digested overnight. Phenol-extracted DNA was used to determine the *p53* genotype using PCR analysis. A tumor sample microdissected from a $p53^{+/+}$ mouse showed retention of the wild-type allele. In contrast, 3 tumor samples isolated from 2 $p53^{+/-}$ mice showed loss of the wild-type allele. Mesothelioma cell lines isolated from these mice corresponded to those that showed loss of the wild-type allele using PCR or RT-PCR analysis as described above.

Four of the 5 cell lines derived from $p53^{+/-}$ mice that retained the wild-type allele were analyzed for mutations in exons 5–8 of the *p53* gene. No missense mutations or deletions were detected.

Growth of p53*-Deficient and Wild-Type Mesothelioma Cells* in Vivo

If *p53*-mutant- or -deficient cells have a selective growth advantage *in vivo*, we would predict increased mesothelial-cell proliferation in tumors induced by asbestos in *p53*-deficient mice. Mesothelial-cell proliferation was assessed by BrdU labeling using immunohistochemistry. Sections of mesotheliomas were studied from $p53^{+/+}$ and $^{+/-}$ mice, including tumors that had lost the wild-type allele. The BrdU labeling index ranged from 6–9%, with no statistically significant difference between $p53^{+/+}$, $^{+/-}$, or $^{-/-}$ tumors.

Genetic Instability of Mesothelioma Cell Lines Derived from p53*-Deficient Mice*

Tumors that develop in $p53^{-/-}$ mice are characterized by aneuploid and subtetraploid populations and abnormal karyotypes (Donehower *et al.*, 1995). Therefore, we hypothesized that malignant mesotheliomas induced by asbestos in *p53*-deficient mice would show genetic instability. Cell lines were established from ascites or peritoneal lavage fluid collected from mice as described in Cistulli *et al.* (1996). Metaphase spreads were prepared from cells at passages 2–6. Normal $p53^{+/+}$ mesothelial cells are nearly diploid ($2n = 40$). Cell lines derived from all of the $p53^{+/-}$ mice that had lost the wild-type allele were tetraploid (mean chromosome number = 81 ± 3.6). This observation is consistent with a *p53*-mediated spindle checkpoint (Cross *et al.*, 1995). In contrast, cell lines derived from $p53^{+/+}$ and $p53^{+/-}$ mice that retained the wild-type allele were aneuploid (mean chromosome number = 55 ± 11). The difference between $p53^{+/+}$ and $p53^{-/-}$ mesothelioma cell lines is statistically significant ($p = 0.01$). The $p53^{-/-}$ mesotheliomas showed nuclear pleomorphism with binucleate and multinucleated tumor cells and abnormal mitotic figures (Fig. 5).

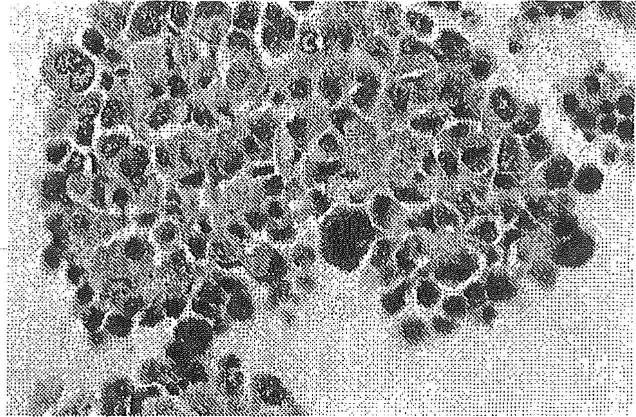

FIG. 5. Histologic section of a diffuse malignant mesothelioma induced in a heterozygous $p53^{+/-}$ mouse that had lost the wild-type allele. Anaplastic tumor cells with multiple nuclei and abnormal metaphases are visible. Original magnification ×400. Hematoxylin and eosin staining.

Apoptosis in $p53^{+/+}$ *and* $p53^{-/-}$ *Mesotheliomas*

Loss of p53 function has been hypothesized to contribute to aggressive growth during later stages of tumor progression in choroid plexus tumors (Symonds *et al.*, 1994) and islet cell carcinomas (Naik *et al.*, 1996) in *p53*-deficient mice. Therefore, we hypothesized that loss of the wild-type allele in heterozygous *p53*-deficient mice would be associated with decreased apoptosis. The extent of apoptosis in malignant mesotheliomas induced by asbestos in *p53*-deficient mice was evaluated by the TUNEL assay and nuclear morphology. As illustrated in Figure 6, tumors in $p53^{+/+}$ mice showed isolated cells with crescentic margination of chromatin and numerous apoptotic bodies while tumors that arose in $p53^{+/-}$ mice that showed loss of the wild-type allele had confluent areas of central necrosis. These morphologic changes were consistent with the percentage of cells in apoptosis as detected by the TUNEL assay performed on paraffin-embedded tumors as illustrated in Fig. 7: $12 \pm 2\%$ of the tumor cells in $p53^{+/-}$ mice that retained the wild-type allele or $p53^{+/+}$ mice showed apoptosis compared with $1.7 \pm 1\%$ of the tumors in $p53^{+/-}$ mice that lost the wild-type allele. This difference is statistically significant ($p < 0.05$).

DISCUSSION

We have previously reported that the incidence of mesotheliomas is statistically increased in $p53^{+/-}$ mice in comparison to wild-type mice with a reduced latent period (Marsella *et al.*, 1997). This chronic carcinogenicity assay in heterozygous *p53*-deficient mice supports the hypothesis that crocidolite asbestos fibers act as a genotoxic carcinogen in this model system. Since crocidolite asbestos fibers produce both persistent mesothelial cell proliferation and chronic inflammation, it is impossible to distinguish between a direct clastogenic or aneuploidogenic

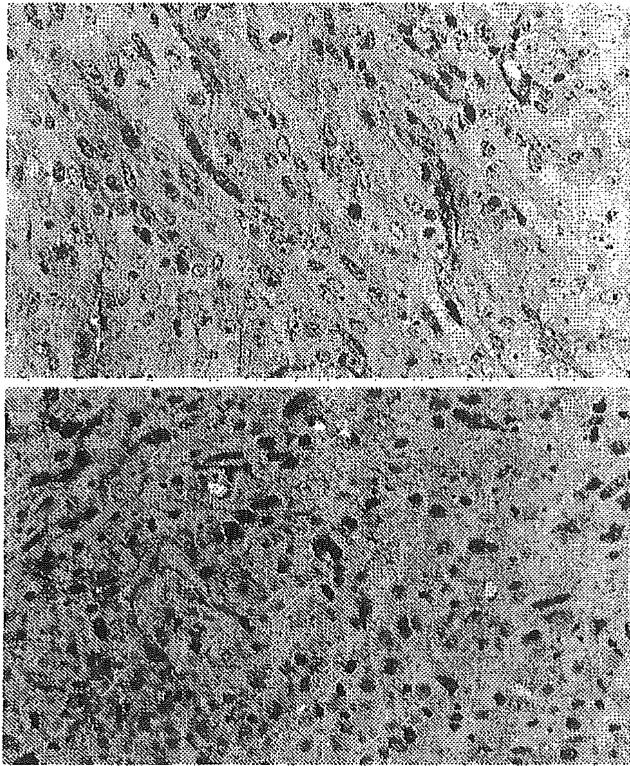

FIG. 6. Morphologic evidence of apoptosis or necrosis in diffuse malignant mesotheliomas induced by weekly ip injections of crocidolite asbestos fibers. Upper panel, tumor from a wild-type $p53^{+/+}$ mouse showing isolated condensed nuclei with fragmentation and margination of chromatin. Lower panel, tumor from a heterozygous $p53^{+/-}$ mouse that had lost the wild-type allele showing confluent areas of necrosis with loss of cell outlines, cytoplasmic swelling, and nuclear debris. Original magnification ×400. One μm plastic sections, toluidine blue stain.

effect of asbestos fibers and an indirect genotoxic effect secondary to chronic inflammation characterized by activated macrophages (Macdonald and Kane, 1997). The simplest explanation for the increased sensitivity of heterozygous *p53*-deficient mice to asbestos-induced carcinogenicity is loss of cell cycle checkpoints, resulting in accumulation of oxidant-induced DNA damage and genetic instability (Marsella *et al.*, 1997). However, the prolonged latent period before development of malignant mesotheliomas, even in heterozygous *p53*-deficient mice, suggests that additional mutational events are required for the development of this tumor (Macleod and Jacks, 1999).

The most striking pathologic finding in this chronic carcinogenicity study in heterozygous *p53*-deficient mice was accelerated tumor progression. Half of these mice had large tumor masses with central areas of necrosis, extensive local invasion, and penetration of lymphatics; these features are rarely seen in wild-type rodent tumors produced by direct intraperitoneal injection of asbestos fibers (Craighead and Kane, 1994). Cell lines were successfully established from 8 of the heterozygous $p53^{+/-}$ mice; 50% of these cell lines showed loss of *p53* expression associated with loss of the wild-type allele. LOH at the *p53* tumor suppressor gene locus occurs in approximately 50% of the tumors that arise in Li-Fraumeni families, as well as in lymphomas induced by ionizing radiation (Kemp *et al.*, 1994) or skin tumors induced by DMBA (Kemp *et al.*, 1993). However, not all tumors induced by carcinogens in heterozygous *p53*-deficient mice develop LOH or other mutations at the *p53* tumor suppressor gene locus. For example, neither mammary tumors induced by DMBA (Jones *et al.*, 1997) nor lymphomas induced by uv irradiation of the skin (Jiang *et al.*, 2001) have any apparent mutations in the remaining wild-type *p53* allele. Only 50% of the mesotheliomas induced by asbestos fibers had LOH; therefore, LOH is not essential for the development of mesotheliomas. However, LOH does appear to be associated with accelerated progression of these tumors.

Potential mechanisms responsible for accelerated tumor progression in *p53*-deficient mice have been explored in other model systems. Increased tumor size could be due to increased cell proliferation, reduced apoptosis, or both. In a mouse mammary tumor model (Wnt1-ITG mice crossed with $p53^{+/+}$, $^{+/-}$, or $^{-/-}$ mice), increased tumor size occurred in the Wnt-ITG $p53^{+/-}$ mice and was accompanied by loss of the wild-type allele in 50% of the tumors. These tumors had an increased mitotic index but no change in the level of apoptosis in comparison with the Wnt-1 TG $p53^{+/+}$ or Wnt-1 TG $p53^{-/-}$ tumors (Jones *et al.*, 1997). Although we were unable to measure tumor size directly in this intraperitoneal model because malignant mesotheliomas spread diffusely over the peritoneal surfaces (Craighead and Kane, 1994), larger tumor masses and increased tumor thickness were seen in half of the heterozygous $p53^{+/-}$ mice. In contrast to the mouse mammary tumor model, no statistically significant differences in the BrdU la-

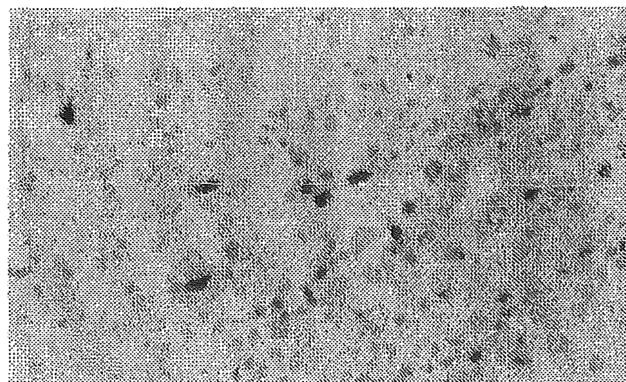

FIG. 7. Spontaneous apoptosis in a diffuse malignant mesothelioma induced in a wild-type $p53^{+/+}$ mouse by weekly ip injections of crocidolite asbestos fibers. Apoptotic cells as detected by TUNEL assay show dark staining of the nuclei. Some of the nuclei are pyknotic or show margination of the chromatin; original magnification ×400. DAB immunohistochemical reaction as described in Materials and Methods.

beling index were observed in *p53*$^{+/+}$, *p53*$^{+/-}$, or *p53*$^{+/-}$ tumors that had LOH. Instead, a statistically significant decrease in the apoptotic index was observed in *p53*$^{+/-}$ tumors that had lost the wild-type allele. This observation confirms previous studies in *p53*-deficient mice; for example, increased growth of choroid plexus tumors in *p53*$^{-/-}$ transgenic mice expressing a truncated SV40 large-T antigen has been attributed to a reduction in the apoptotic index (Symonds *et al.*, 1994).

In addition to induction of apoptosis, wild-type p53 protein is essential for cell-cycle arrest triggered by DNA damage and maintenance of genomic stability. Induction of the G1 cell cycle checkpoint depends on post-translational modification, stabilization, and translocation of the p53 protein to the nucleus where it activates transcription in conjunction with other co-activators (Prives and Hall, 1999). Activated transcription of *p21* inhibits cyclin-dependent kinases resulting in hypophosphorylated Rb and G1 arrest. Other transcriptional targets of p53 include mdm-2, which binds to p53 itself in an autoregulatory fashion, and GADD45, which interacts with PCNA and prevents entry into S phase (Ko and Prives, 1996). In addition to the G1 cell cycle checkpoint, p53 also regulates a G2 checkpoint, duplication of centrosomes, and a spindle checkpoint during mitosis (Fukasawa *et al.*, 1996). Therefore, inactivation of p53 function in response to DNA damage would result in unrepaired DNA breaks leading to aneuploidy and multiple rounds of DNA synthesis in the absence of chromosome segregation, resulting in tetraploidy (Cross *et al.*, 1995; Ko and Prives, 1996).

Histologic examination of mesotheliomas in heterozygous *p53*-deficient mice that had lost the wild-type allele showed multinucleated tumor cells and abnormal mitotic figures. Mesothelioma cell lines isolated from these mice showed a subteraploid or tetraploid number of chromosomes. Tetraploid or nearly tetraploid tumor cell populations have been reported previously in mammary tumors induced in transgenic Wnt-1 TG *p53*$^{-/-}$ mice; these tumor cell populations also had evidence of genetic instability as revealed by karyotyping and comparative genomic hybridization (Donehower et al., 1995).

It is hypothesized that *p53* haplo-insufficiency sensitizes mice to the clastogenic or aneuploidogenic effects of crocidolite asbestos fibers, resulting in a shorter latent period. In solid tumors, spontaneous loss of the wild-type allele, accompanied by decreased apoptosis and accumulation of additional mutations, accelerates tumor growth, invasion, and lymphatic dissemination, as suggested by Macleod and Jacks (1999).

This genetically engineered murine model is highly relevant to the pathogenesis of human malignant mesotheliomas. Although *p53* mutations, rearrangements, or deletions are rare in either human or rodent malignant mesotheliomas (Lechner *et al.*, 1997; Murthy and Testa, 1999), some investigators have demonstrated SV40 T-antigen and viral sequences in up to 80% of human mesothelioma tissues examined (reviewed in Carbone *et al.*, 1999). Antisense constructs directed against large T-antigen have been shown to inhibit proliferation of human mesothelioma cell lines (Waheed *et al.*, 1999). Although these findings are still controversial (Pipas and Levine, 2001), this murine model demonstrates a potential role for inactivation of p53 function in the development of asbestos-induced malignant mesotheliomas. This murine model provides the opportunity for additional mechanistic studies to investigate whether SV40 virus and asbestos fibers are cocarcinogens.

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