

Phosphorylation of p53 Protein in A549 Human Pulmonary Epithelial Cells Exposed to Asbestos Fibers

Masato Matsuoka,¹ Hideki Igisu,¹ and Yasuo Morimoto²

Departments of ¹Environmental Toxicology and ²Occupational Pneumology, Institute of Industrial Ecological Sciences, University of Occupational and Environmental Health, Kitakyushu, Japan

We examined effects of asbestos exposure on the phosphorylation of p53 protein in human pulmonary epithelial type II cells (A549), which express wild-type p53. In cells exposed to two different types of asbestos, chrysotile (~1–6% iron content) and crocidolite (~27% iron content) fibers, at the doses of 1, 5, and 10 µg/cm² for 24 hr, the levels of p53 phosphorylated at Ser15 and p53 protein were correlated with the dose. On a per-weight basis, chrysotile was more potent in inducing Ser15 phosphorylation and accumulation of p53 protein than was crocidolite. After exposure to 10 µg/cm² chrysotile, the levels of p53 phosphorylated at Ser15 and of p53 protein increased after 18 hr. Among serines in p53 protein immunoprecipitated from A549 cells treated with chrysotile, only Ser15 was markedly phosphorylated. In contrast, no clear phosphorylation was observed at Ser6, Ser9, Ser20, Ser37, Ser46, or Ser392. Blocking of the extracellular signal-regulated protein kinase pathway with U0126 or inhibition of p38 activity with SB203580 did not suppress chrysotile-induced Ser15 phosphorylation. On the other hand, treatment with wortmannin, an inhibitor of DNA-activated protein kinase and ataxia-telangiectasia mutated, suppressed both chrysotile-induced Ser15 phosphorylation and accumulation of p53 protein. Treatment with either catalase or *N*-acetylcysteine failed to suppress chrysotile-induced Ser15 phosphorylation, suggesting that reactive oxygen species do not play a major role in the phosphorylation of p53 protein. The present results show that asbestos, particularly chrysotile, induces phosphorylation of p53 protein at Ser15 in A549 cells depending on a DNA damage-signaling pathway. **Key words:** A549 cells, chrysotile, crocidolite, p53, phosphorylation, reactive oxygen species, SB203580, Ser15, U0126, wortmannin. *Environ Health Perspect* 111:509–512 (2003). doi:10.1289/ehp.5945 available via <http://dx.doi.org/> [Online 21 November 2002]

Asbestos is a family of crystalline-hydrated silicates with a fibrous geometry, including chrysotile [Mg₃Si₄O₁₀(OH)₈], the curly serpentine-type, and crocidolite [Na₂(Fe³⁺)₂(Fe²⁺)₃Si₈O₂₂(OH)₂], the rodlike amphibole-type asbestos (Mossman et al. 1990; Mossman and Gee 1989). Clinical and epidemiologic studies have established that asbestos fibers are associated with the development of pulmonary interstitial fibrosis, lung cancer, and malignant mesothelioma (Mossman et al. 1990; Mossman and Gee 1989). Asbestos exposure induces diverse cellular events related to lung injury (Jaurand 1997; Kamp and Weitzman 1999; Manning et al. 2002). However, the molecular mechanisms of asbestos-induced fibrogenesis and carcinogenesis and of repair of lung injury are not fully understood.

The p53 tumor suppressor protein plays an important role in the control of genomic integrity or the elimination of damaged or tumorigenic cells (Bargonetti and Manfredi 2002; Levine 1997; Vousden 2000). The mutational inactivation of p53 protein is one of the most common genetic events that occur in human cancers (Hupp et al. 2000). It has been reported that the frequency of p53 protein accumulation is increased in lung carcinomas of patients with clinical or histologic asbestos exposure (Nuorva et al. 1994). Treatment with crocidolite increased the number of p53 protein-expressing cells in A549 human pulmonary epithelial cells (Johnson et al. 1997; Johnson and Jaramillo

1997), and treatment with chrysotile induced the elevation of p53 protein level in rat pleural mesothelial cells (Levresse et al. 1997). In addition, inhaled chrysotile induced the expression of p53 protein at fiber deposition sites (bronchiolar-alveolar duct bifurcations) in rat lungs (Mishra et al. 1997). These findings suggest a possible association between asbestos exposure and accumulation of p53 protein in the pulmonary tissues or cells.

The p53 protein is phosphorylated on multiple residues in both the amino- and carboxy-terminal domains by several different protein kinases (Giaccia and Kastan 1998; Lakin and Jackson 1999; Meek 1998). Among serine residues, phosphorylation at position 15 has been shown to play an important role in the stabilization and subsequent induction and transactivation function of p53 (Dumaz and Meek 1999; Shieh et al. 1997; Siliciano et al. 1997). Members of the phosphatidylinositol 3-kinase-related kinase (PIKK) family such as DNA-activated protein kinase (DNA-PK) and ataxia-telangiectasia mutated (ATM) have been implicated in the phosphorylation of p53 at Ser15 (Giaccia and Kastan 1998; Lakin and Jackson 1999; Meek 1998). Furthermore, extracellular signal-regulated protein kinase (ERK) and p38, the members of mitogen-activated protein kinase (MAPK), have been reported to induce p53 phosphorylation at Ser15 (Bulavin et al. 1999; Kwon et al. 2002; Persons et al. 2000; She et al. 2000, 2001; Shih et al. 2001; Wang and Shi 2001).

In the present study, we examined whether two different types of asbestos, chrysotile and crocidolite, induce the phosphorylation of p53 at Ser15 and other serines in A549 human pulmonary epithelial type II cells, which express wild-type p53 (Jia et al. 1997). Using inhibitors to the members of MAPK and PIKK, we also determined the protein kinases responsible for asbestos-induced p53 phosphorylation. Because reactive oxygen species have been shown to be an important mediator responsible for pulmonary toxicity of asbestos (Kamp et al. 1992; Kamp and Weitzman 1999; Quinlan et al. 1994), effects of antioxidants such as catalase and *N*-acetylcysteine on asbestos-induced p53 phosphorylation were also examined.

Materials and Methods

Preparation of asbestos fibers. Union Internationale Contre le Cancer (UICC) standard samples of Rhodesian chrysotile and crocidolite asbestos were used in the present study. The fibers were suspended in distilled water at the concentration of 1 mg/mL. Then the suspensions were passed through a 22-gauge needle eight times and sterilized by autoclaving. Before the addition to the medium, the fibers were dispersed by sonication for 10 min and vortexed.

Cell culture and treatments. A549 cells were obtained from Health Science Research Resources Bank (Japan Health Sciences Foundation, Osaka, Japan) and grown in Earle's minimum essential medium with nonessential amino acids, 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco BRL, Life Technologies, Inc., Rockville, MD, USA) in a humidified atmosphere of 5% CO₂, 95% air at 37°C. For each experiment, exponentially growing A549 cells were plated at 1.5 × 10⁵ cells/well in 12-well culture plates or 3 × 10⁶ cells/dish in 100-mm culture dishes, and cultured for 1 day before the experiments.

Address correspondence to M. Matsuoka, Dept. of Environmental Toxicology, Institute of Industrial Ecological Sciences, University of Occupational and Environmental Health, 1-1 Iseigaoka, Yahatanishi-ku, Kitakyushu 807-8555, Japan. Telephone: 81-93-691-7404. Fax: 81-93-692-4790. E-mail: masatom@med.uoeh-u.ac.jp

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A549 cells were incubated with the complete medium containing 1, 5, or 10 $\mu\text{g}/\text{cm}^2$ of fibers/cm² of culture vessel for 24 hr. As a reference, 20 μM cadmium chloride (CdCl_2 ; Sigma Chemical Co., St. Louis, MO, USA), which has been shown to induce the phosphorylation of p53 protein at Ser15 (Matsuoka and Iqisu 2001), was used. In the time course study, A549 cells were incubated with 10 $\mu\text{g}/\text{cm}^2$ of fibers/cm² for 3–24 hr. Untreated control cells were incubated with the complete medium alone and treated identically to the cells exposed to asbestos.

U0126, SB203580, and wortmannin (Calbiochem, La Jolla, CA, USA) were dissolved in dimethyl sulfoxide (DMSO). Catalase (Calbiochem) was dissolved in distilled water. *N*-Acetylcysteine (Sigma) was dissolved in phosphate-buffered saline (PBS) immediately before use, and the pH was adjusted to 7.4 with 2*N* NaOH. A549 cells were preincubated with the complete medium containing each compound for 30 min (for wortmannin), 1 hr (for U0126, SB203580, and catalase), or 12 hr (for *N*-acetylcysteine). The control cells were preincubated with the complete medium either alone or containing DMSO at the concentration used in the treated cells (0.05 or 0.08%). Then preincubated and control cells were treated with or without asbestos fibers for 24 hr.

Western immunoblotting. After the incubation with asbestos fibers or CdCl_2 , cells were washed with PBS and lysed with sodium dodecyl sulfate (SDS)–polyacrylamide gel Laemmli sample buffer. Cell lysates were collected, sonicated, and boiled for 5 min. Aliquots equivalent to 2×10^5 cells were subjected to SDS–polyacrylamide gel electrophoresis on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane (Hybond-ECL; Amersham Pharmacia Biotech, Buckinghamshire, UK). The membrane was blocked with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween 20 for 2 hr at room temperature. The antibodies

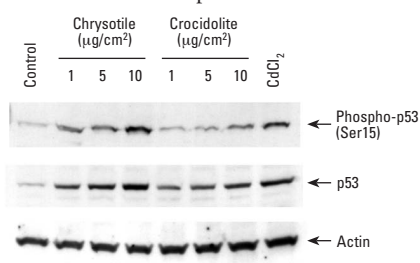


Figure 1. Effects of chrysotile or crocidolite exposure on the levels of p53 phosphorylated at Ser15 and p53 protein. A549 cells were incubated with chrysotile (1, 5, or 10 $\mu\text{g}/\text{cm}^2$), crocidolite (1, 5, or 10 $\mu\text{g}/\text{cm}^2$), or CdCl_2 (20 μM) for 24 hr. Cell lysates were subjected to Western immunoblotting using phospho-p53 (Ser15), p53, and actin antibodies. Results shown are representative of three independent experiments.

used were p53 (Pab 1801) antibody, actin (I-19) antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), and phospho-p53 (Ser6, Ser9, Ser15, Ser20, Ser37, Ser46, and Ser392) antibodies (Cell Signaling Technology, Inc., Beverly, MA, USA). The membrane was incubated overnight at 4°C with the primary antibody diluted 1:100 (for p53 antibody) or 1:1,000 (for phospho-p53 antibodies). Protein was detected with a Phototope-HRP Western blot detection kit (Cell Signaling Technology). After immunodetection, some blots were incubated with Restore Western Blot Stripping Buffer (Pierce Chemical Co., Rockford, IL, USA) for 30 min at room temperature and reprobed with actin antibody diluted 1:500 for 1 hr at room temperature.

Immunoprecipitation–Western immunoblotting. After the incubation with asbestos fibers, cells were washed with PBS and lysed in RIPA buffer (PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with 0.6 mM phenylmethylsulfonyl fluoride, 30 $\mu\text{L}/\text{mL}$ aprotinin (Sigma A6279), and 1 mM sodium orthovanadate. After sonication, the lysates were stored on ice for 1 hr and centrifuged at $10,000 \times g$ for 10 min at 4°C. Then, cell lysates equivalent to 6×10^6 cells were incubated overnight at 4°C with 10 μg of p53 (Pab 1801) antibody agarose conjugate (Santa Cruz Biotechnology). The pellet was washed four times with RIPA buffer and suspended in SDS–polyacrylamide gel Laemmli sample buffer. Phosphorylation of p53 protein at Ser6, Ser9, Ser15, Ser20, Ser37, Ser46, and Ser392 in the same samples was analyzed after SDS–polyacrylamide gel electrophoresis and immunoblotting with respective phospho-p53 antibodies. After stripping, the blots were reprobed with p53 antibody.

Results

Accumulation of p53 phosphorylated at Ser15 and p53 protein by asbestos exposure. Exposure to 20 μM CdCl_2 for 24 hr induced a clear phosphorylation of p53 at Ser15 and an

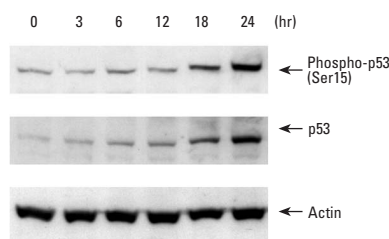


Figure 2. Time course of chrysotile-induced accumulation of p53 phosphorylated at Ser15 and p53 protein. A549 cells were incubated with 10 $\mu\text{g}/\text{cm}^2$ chrysotile for 3–24 hr. The untreated control is 0 hr. Cell lysates were subjected to Western immunoblotting using phospho-p53 (Ser15), p53, and actin antibodies. Results shown are representative of three independent experiments.

accumulation of p53 protein in A549 cells (Figure 1), as has been shown in MCF-7 human breast cancer cells (Matsuoka and Iqisu 2001). Similarly, in A549 cells exposed to 1–10 $\mu\text{g}/\text{cm}^2$ of chrysotile or crocidolite for 24 hr, accumulation of both p53 phosphorylated at Ser15 and p53 protein was found depending on the dose (Figure 1). However, chrysotile exposure induced more marked accumulation of p53 phosphorylated at Ser15 and p53 protein than crocidolite exposure at each dose of 1, 5, and 10 $\mu\text{g}/\text{cm}^2$. On the other hand, the levels of actin were not changed by exposure to chrysotile, crocidolite, or CdCl_2 (Figure 1).

After exposure to 10 $\mu\text{g}/\text{cm}^2$ chrysotile, the levels of p53 phosphorylated at Ser15 and p53 protein increased after 18 hr, whereas actin levels were not changed after 3–24 hr exposures (Figure 2). In A549 cells exposed to 10 $\mu\text{g}/\text{cm}^2$ crocidolite, clear Ser15 phosphorylation was not found at 3–18 hr (data not shown). Hereafter, we focused on p53 phosphorylation induced by exposure to 10 $\mu\text{g}/\text{cm}^2$ chrysotile for 24 hr.

Phosphorylation of serine residues in p53 protein. We examined whether serine residues other than position 15 in p53 protein can be phosphorylated in response to chrysotile exposure. In p53 protein immunoprecipitated from A549 cells exposed to chrysotile, the marked phosphorylation was found only at Ser15 (Figure 3). On the other hand, clear phosphorylation was not observed on Ser6, Ser9, Ser20, Ser37, Ser46, or Ser392 in p53 immunoprecipitated, whereas equal amounts of p53 protein were detected (Figure 3).

Effects of MAPK inhibitors on Ser15 phosphorylation in p53 protein. Treatment with U0126 (5 and 20 μM), an inhibitor of both activated and nonactivated forms of MAPK/ERK kinase (MEK1/2) (Favata et al. 1998), or with SB203580 (5 and 20 μM), a p38 inhibitor (Cuenda et al. 1995), did not

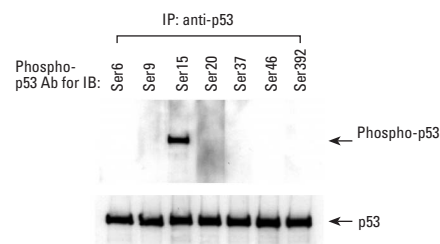


Figure 3. Effects of chrysotile exposure on the phosphorylation of serines in p53 protein. Abbreviations: IP, immunoprecipitation; Ab, antibody; IB, Western immunoblotting. A549 cells were incubated with 10 $\mu\text{g}/\text{cm}^2$ chrysotile for 24 hr, and cell lysates were incubated with p53 antibody agarose conjugate. Phosphorylation of p53 at Ser6, Ser9, Ser15, Ser20, Ser37, Ser46, and Ser392 in the same samples was analyzed after Western immunoblotting using respective phospho-p53 antibodies. After stripping, the blots were reprobed with p53 antibody. Results shown are representative of three independent experiments.

suppress chrysotile-induced Ser15 phosphorylation (Figure 4A, B). Treatment with a higher concentration of U0126 (50 μM) or SB203580 (50 μM) also failed to suppress chrysotile-induced Ser15 phosphorylation, whereas phosphorylated forms of ERK or p38 were not detected in A549 cells treated with each compound (data not shown).

Effects of wortmannin on Ser15 phosphorylation in p53 protein. Treatment with wortmannin (1 and 5 μM), an inhibitor of DNA-PK and ATM (Sarkaria et al. 1998), suppressed chrysotile-induced Ser15 phosphorylation and accumulation of p53 protein depending on the concentration (Figure 5). The levels of actin were not affected by treatment with wortmannin (Figure 5).

Effects of catalase and N-acetylcysteine on Ser15 phosphorylation in p53 protein. Treatment with catalase (1 and 5 KU/mL) or N-acetylcysteine (1 and 5 mM) did not suppress chrysotile-induced Ser15 phosphorylation (Figure 6A, B). In addition, accumulation of p53 protein in A549 cells exposed to chrysotile was not suppressed by treatment with catalase or N-acetylcysteine (data not shown).

Discussion

In A549 cells exposed to two different types of asbestos, chrysotile and crocidolite, at the doses of 1–10 $\mu\text{g}/\text{cm}^2$ for 24 hr, the levels of p53 phosphorylated at Ser15 and p53 protein were found to be elevated. Among serines in p53 protein immunoprecipitated from A549 cells treated with 10 $\mu\text{g}/\text{cm}^2$ chrysotile for 24 hr, only Ser15 was markedly phosphorylated. In contrast, no clear phosphorylation was observed at other serine residues examined (Ser6, Ser9, Ser20, Ser37, Ser46, and Ser392). Ser15 has been identified as a site on p53 protein phosphorylated in response to DNA-damaging agents such as ionizing radiation (Shieh et al. 1997; Siliciano et al. 1997), ultraviolet radiation (She et al. 2000; Shieh et al. 1997; Siliciano et al. 1997), camptothecin

(Shieh et al. 1997), cisplatin (Persons et al. 2000), chromium (Wang and Shi 2001), and cadmium (Matsuoka and Igisu 2001). The present study clearly showed for the first time that asbestos exposure induces phosphorylation of p53 protein at Ser15 in a human pulmonary epithelial cell line. Phosphorylation of p53 at Ser15 was shown to reduce the binding of murine double minute 2 (MDM2) (Shieh et al. 1997), an E3 ligase that targets both p53 and itself for ubiquitination (Vousden 2000). The level of p53 mRNA as determined using reverse transcriptase–polymerase chain reaction analysis was not elevated in A549 cells treated with chrysotile or crocidolite at 10 $\mu\text{g}/\text{cm}^2$ for 24 hr (data not shown). Therefore, asbestos-induced Ser15 phosphorylation might be responsible for the accumulation of p53 protein at least in part.

When two types of asbestos fibers were compared in their potency to induce Ser15 phosphorylation and accumulation of p53 protein, chrysotile was more marked than was crocidolite on a per-weight basis. In agreement with our results, treatment with 10 $\mu\text{g}/\text{cm}^2$ crocidolite for 24 or 48 hr induced less significant increases in p53 protein level than did treatment with chrysotile in rat pleural mesothelial cells (Levresse et al. 1997). When DNA breakage was determined using the single-cell gel (Comet) assay, chrysotile was reported to induce more abnormalities in comet parameters than did crocidolite in rat pleural mesothelial cells (Levresse et al. 2000). If this is the case in human pulmonary epithelial cells as well, a higher genotoxic potential of chrysotile asbestos might underlie the marked phosphorylation and accumulation of p53 protein observed in the present study.

The MAPKs, including ERK, p38, and c-Jun NH₂-terminal kinase (JNK), are a family of serine/threonine protein kinases that transmit extracellular signals into the nucleus (Schaeffer and Weber 1999). Exposure to chrysotile has been reported to activate ERK in rat pleural mesothelial cells *in vitro* (Zanella

et al. 1996) and mouse pulmonary epithelial cells *in vivo* (Robledo et al. 2000), although its effects on p38 and JNK are not known. On the other hand, Ser15 phosphorylation induced by various cellular stimuli such as ultraviolet radiation (Bulavin et al. 1999; She et al. 2000), cisplatin (Persons et al. 2000), L-thyroxine (Shih et al. 2001), chromium (Wang and Shi 2001), resveratrol (She et al. 2001), and 3-methylcholanthrene (Kwon et al. 2002) has been reported to be mediated by ERK and/or p38. However, in the present study, treatment with neither U0126 nor SB203580 suppressed chrysotile-induced Ser15 phosphorylation, indicating it is unlikely that ERK and p38 are responsible for p53 phosphorylation at Ser15 in A549 cells exposed to chrysotile.

In contrast to MAPK inhibitors, treatment with wortmannin suppressed both chrysotile-induced Ser15 phosphorylation and accumulation of p53 protein. These results suggest that chrysotile-induced Ser15 phosphorylation is dependent on PIKK family such as DNA-PK and ATM, and support the possible role of Ser15 phosphorylation in the accumulation of p53 protein. DNA-PK and ATM are activated after cellular exposure to agents that induce DNA double-strand breaks (DSBs) or other discontinuities in DNA (Canman et al. 1994; Gottlieb and Jackson 1993; Morozov et al. 1994). It has been reported that chrysotile exposure at doses of 8 or 16 $\mu\text{g}/\text{cm}^2$ for 24 hr induced DNA DSBs in both wild-type and DNA DSB repair–deficient mutant Chinese hamster ovary cells (Okayasu et al. 1999). Therefore, DNA damage induced by chrysotile exposure might activate the signaling pathways leading to PIKK activation, and resultant p53 activation might contribute to the protection of cells from fatal genetic injury.

The iron associated with asbestos fibers promotes the formation of hydroxyl radicals via the modified Haber-Weiss (Fenton) reaction (Kamp et al. 1992; Kamp and Weitzman 1999; Quinlan et al. 1994). Although

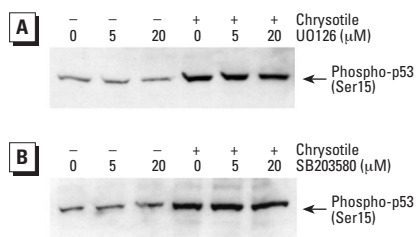


Figure 4. Effects of U0126 (A) and SB203580 (B) on chrysotile-induced phosphorylation of p53 at Ser15. A549 cells were preincubated with DMSO (0.08%), U0126 (5 or 20 μM), or SB203580 (5 or 20 μM) for 1 hr and then incubated without or with 10 $\mu\text{g}/\text{cm}^2$ chrysotile for 24 hr. Cell lysates were subjected to Western immunoblotting using phospho-p53 (Ser15) antibody. Results shown are representative of three independent experiments.

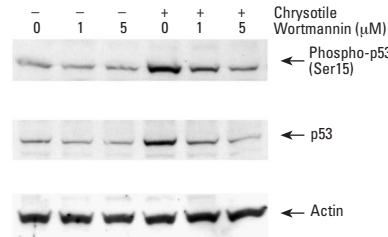


Figure 5. Effects of wortmannin on chrysotile-induced phosphorylation of p53 at Ser15. A549 cells were preincubated with DMSO (0.05%) or wortmannin (1 or 5 μM) for 30 min and then incubated without or with 10 $\mu\text{g}/\text{cm}^2$ chrysotile for 24 hr. Cell lysates were subjected to Western immunoblotting using phospho-p53 (Ser15), p53 and actin antibodies. Results shown are representative of three independent experiments.

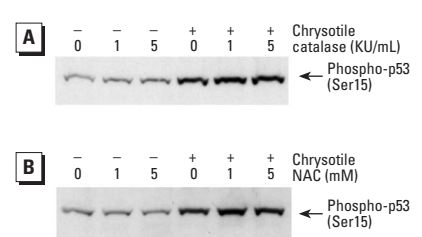


Figure 6. Effects of catalase (A) or N-acetylcysteine (NAC) (B) on chrysotile-induced phosphorylation of p53 at Ser15. A549 cells were preincubated with catalase (1 or 5 KU/mL) for 1 hr or N-acetylcysteine (1 or 5 mM) for 12 hr and then incubated without or with 10 $\mu\text{g}/\text{cm}^2$ chrysotile for 24 hr. Cell lysates were subjected to Western immunoblotting using phospho-p53 (Ser15) antibody. Results shown are representative of three independent experiments.

chrysotile contains only ~1–6% iron primarily as a surface contaminant (Kamp et al. 1992; Kamp and Weitzman 1999), this asbestos has been reported to generate hydroxyl radical-like species and cause DNA strand breaks in human pulmonary epithelial-like WI-26 cells (Kamp et al. 1995). However, treatment with catalase or *N*-acetylcysteine failed to suppress chrysotile-induced Ser15 phosphorylation in the present study. Furthermore, treatment with deferoxamine (1 and 5 mM), an iron chelator, did not suppress chrysotile-induced Ser15 phosphorylation but induced Ser15 phosphorylation significantly even in the absence of asbestos (data not shown). Consistent with these results, crocidolite asbestos, which has a high iron content (~27%) (Kamp et al. 1992; Kamp and Weitzman 1999), induced less marked Ser15 phosphorylation in A549 cells. Thus, chrysotile-induced p53 phosphorylation at Ser15 might be caused by mechanisms not based on reactive oxygen species, such as the direct physical interaction with cellular components or the generation of reactive nitrogen species (Tanaka et al. 1998).

The p53 protein plays a central role in the control of cell cycle progression or apoptotic cell death (Bargonetti and Manfredi 2002; Levine 1997; Vousden 2000). Therefore, there is a possibility that cell cycle arrest (Levrèsse et al. 1997) and apoptosis (Aikoh et al. 1998; Broaddus et al. 1996; Dopp et al. 1995; Hamilton et al. 1996; Wu et al. 2000) found in cells exposed to chrysotile might be caused by an activated form of p53 protein. On the other hand, in transgenic mice with reduced and enhanced p53 functions specifically targeted within the pulmonary epithelium, asbestos exposure induced less and more severe fibrogenic lesions in the lung, respectively (Nelson et al. 2001). These findings suggest that p53 protein in cells exposed to asbestos has the fibrosis progressive function as well as the tumor suppressive function. In other words, activation of p53 by asbestos exposure might intensify its pulmonary toxicity further by forming a positive feedback loop. To clarify the functions of asbestos-induced p53 activation in the pulmonary cells, genes, and/or pathways that are activated after phosphorylation of p53 at Ser15, the critical event for its transactivation (Dumaz and Meek 1999; Shieh et al. 1997; Siliciano et al. 1997), should be investigated.

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