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Am J Physiol Lung Cell Mol Physiol 288:L488-L496, 2005. First published 19 November 2004; doi:10.1152/ajplung.00123.2003

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Essential role of p53 in silica-induced apoptosis

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Submitted 25 April 2003; accepted in final form 5 November 2004

Wang, Liying, Linda Bowman, Yongju Lu, Yon Rojanasakul, Robert R. Mercer, Vince Castranova, and Min Ding. Essential role of p53 in silica-induced apoptosis. *Am J Physiol Lung Cell Mol Physiol* 288: L488–L496, 2005. First published November 19, 2004; doi:10.1152/ajplung.00123.2003.—Occupational exposure to mineral dusts, such as silica, has been associated with progressive pulmonary inflammation, lung cancer, and fibrosis. However, the mechanisms involved in this process are poorly understood. Because p53 is a key transcription factor regulating many important apoptosis-related genes, we hypothesized that p53 may play a key role in silica-induced apoptosis and that abnormal regulation of p53 by silica may contribute to development of lung cancer as well as silicosis. We used both in vitro and in vivo studies to test this hypothesis. Treatment of JB6 cells carrying a p53-luciferase reporter plasmid with silica caused dose-dependent p53 transactivation. Western blot indicates that silica not only stimulated p53 protein expression but also caused p53 phosphorylation at Ser392. TUNEL and DNA fragmentation analysis show that silica caused apoptosis in both JB6 cells and wild-type p53 (*p53*+/+) fibroblasts but not in p53-deficient (*p53*−/−) fibroblasts. Similar results were obtained by in vivo studies. Intratracheal instillation of mice with silica induced apoptosis in the lung of *p53*+/+ mice, whereas this induction was significantly inhibited in *p53*−/− mice. Confocal image analysis indicates that most apoptotic cells induced by silica were alveolar macrophages. These results demonstrate for the first time that silica induces p53 transactivation via induction of p53 protein expression and phosphorylation of p53 protein and that p53 plays a crucial role in the signal transduction pathways of silica-induced apoptosis. This finding may provide an important link in understanding the molecular mechanisms of silica-induced carcinogenesis and pathogenesis in the lung.

JB6 cells; particulate matter; silicosis; gene knockout mice

RESPIRABLE PARTICULATE MATTER is an occupational concern because of the potential toxicity to the lungs. Crystalline silica, a common exposure agent that causes silicosis, is generated secondarily during sandblasting, tunneling, rock cutting, and various types of mining. Epidemiological and pathologic studies have established that occupational exposure to crystalline silica is associated with the development of pulmonary silicosis (7, 34) and an increased risk for lung cancer (1, 2, 7, 29). As a known carcinogenic and fibrogenic dust, silica has been demonstrated to cause lung cancer and pulmonary inflammation. This inflammation and damage may ultimately result in progressive fibrosis, for which there is no effective clinical treatment. Silica also induces apoptosis of alveolar macrophages (21). This apoptotic potential may be an important factor in the pathogenesis of silicosis (21). However, the mechanisms involved in the silica-induced carcinogenesis and pathogenesis are unclear.

Apoptosis has been characterized as a fundamental cellular activity occurring under a wide range of physiological and pathological conditions (33, 38, 46, 48). Uncontrolled regulation of apoptosis has been implicated in a variety of human disorders including cancer, autoimmunity, and neurodegenerative disorders (14, 36). More recent studies have indicated that apoptosis is also involved in pulmonary disorders, such as acute lung injury, diffuse alveolar damage, idiopathic pulmonary fibrosis, and other lung disorders caused by bleomycin, silica, endotoxin, and the deposition of immune complexes (4, 23, 24, 28). Inhibition of apoptosis by gene deletion strategies or by caspase inhibitors abrogates the pathologic effects of these agents (4, 23, 24, 28), supporting the role of apoptosis in inflammatory lung disorders.

The p53 tumor suppressor protein is a transcription factor that regulates the transcription rate of several genes involved in the regulation of the cell cycle, DNA repair, and apoptosis (10, 24). Under normal circumstances, cells respond to external damage stimuli by activating the regulation of expression of these genes, thereby transiently delaying cell cycle progression to allow the repair of damaged DNA or leading to apoptosis if the damage cannot be repaired (10, 27, 49). The *p53* gene has come to the forefront of cancer/apoptosis research, since it is commonly mutated in human cancer and functions as a guardian of genomic integrity by inducing either cell cycle arrest or programmed cell death (6, 39, 40, 43).

Several genotoxic agents, including ionizing radiation, UV radiation, and certain chemicals, have been reported to cause p53 upregulation (14, 18). Marked decreases in apoptosis after exposure to radiation correlate with the occurrence of p53 mutations in some transgenic mice (3). It is not known, however, whether this transcription factor is also upregulated by silica. Furthermore, the possible role of p53 in silica-induced apoptosis remains to be established. Because silica has previously been shown to cause apoptosis (26) and because, in many cell systems, apoptosis is regulated by p53, we therefore hypothesize that p53 may play a key role in silica-induced apoptosis. This was investigated using various in vitro and in vivo gene knockout techniques.

MATERIALS AND METHODS

Reagents. Eagle's minimal essential medium (EMEM) and Dulbecco's modified Eagle's medium (DMEM) were obtained from Whittaker Biosciences (Walkersville, MD). Fetal bovine serum

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(FBS), gentamicin, and L-glutamine were purchased from Life Technologies, (Gaithersburg, MD). Luciferase assay substrate and the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay kit were obtained from Promega (Madison, WI). Monoclonal antibodies against p53 (Ab-1) were obtained from Calbiochem (San Diego, CA), p53 (Do-1) from Santa Cruz (Santa Cruz, CA), and antiphospho-p53 (FL-392) from Cell Signaling Technology (Beverly, MA).

Cell culture. The JB6 P^+ mouse epidermal cell line (C1 41) and its stable p53 luciferase reporter plasmid transfectant, C1 41 p53 cells, were established and reported earlier (18). The cells were cultured in monolayers at 37°C under 5% CO_2 with EMEM containing 5% FBS, 2 mM L-glutamine, and 25 μg/ml gentamicin. Wild-type embryo fibroblasts (*p53*+/+) or p53-deficient embryo fibroblasts (*p53*−/−), cells that were originally established by Dr. M. Harvey (16), were cultured in DMEM with 10% FBS, 2 mM L-glutamine, and 25 μg/ml gentamicin.

Preparation of freshly fractured silica. Crystalline silica was obtained from Pennsylvania State University, Generic Center (State College, PA). The detailed method for preparation of freshly fractured silica has been described elsewhere (8). Crystalline silica (2–10 mm in diameter) was ground for 30 min with a ball grinder equipped with agate mortar and balls. The ground silica was sieved through a 10-μm mesh filter for 20 min before use. Purity was checked by X-ray diffraction spectrometry, and diameter was determined by morphometric analyses, which indicate that fractured silica had a purity of 99.5% and a mean diameter of 3.7 μm.

Analysis of p53 activity. Confluent monolayers of JB6 C1 41 p53 cells were trypsinized, and cells were suspended in EMEM containing 5% FBS (19). The cells (5×10^4) were seeded into each well of a 48-well plate and incubated at 37°C in a humidified atmosphere of 5% CO_2. Twelve hours later, cells were starved by being cultured in the same medium containing 0.1% FBS for 12 h. The cells were exposed to different concentrations of silica (0–150 μg/cm^2) for 24 h and then extracted with 200 μl of 1× lysis buffer provided in the luciferase assay kit by the manufacturer. Luciferase activity was measured with a Monolight luminometer, model 3010. The results were expressed as relative p53 activity compared with untreated controls.

Immunoprecipitation assay. The level of p53 protein and phosphorylation of p53 were analyzed by immunoprecipitation with antibody against p53 (Ab-1) and followed by Western blot assay with specific antibodies against p53 (Do-1) or phospho-p53 (FL-392). JB6 cells, cultured in 100-mm cell culture dishes, were treated with or without silica for 30–360 min. The cells were then lysed on ice for 1 h in the lysis buffer and centrifuged at 14,000 rpm for 5 min. The lysates were immunoprecipitated using monoclonal anti-p53 (Ab-1) or antiphospho-p53 (Ser392) antibodies and protein G plus protein A agarose. The beads were washed, and the p53 protein was specifically measured by Western immunoblotting assay. The immune complexes on the membranes were detected using a chemiluminescent system (NEL-100, DuPont NEN). The expression of actin from total cell lysate was used as a control gene product.

Apoptosis determination. TUNEL analysis and DNA fragmentation assay were used to detect apoptosis. For TUNEL assay, cultured JB6 cells, fibroblast cells, or lung tissue sections were stained for apoptotic cells using the Promega TUNEL assay kit as described by the protocol of manufacturer. This system measures fragmented DNA by catalytically incorporating fluorescein-12-dUTP at the 3′-OH DNA polymeric tail, as described previously (15). The apoptotic cell nuclei were visualized as a yellow-green fluorescent signal under fluorescence microscopy. Other cell nuclei were counterstained with propidium iodide, which gives red fluorescence.

For in vivo DNA fragmentation assay, isolated lung tissues were ground in liquid nitrogen, and 50 mg of the samples were weighed out into 0.2 ml of RSB buffer (19 mM Tris, pH 8.0, 10 mM NaCl, 25 mM EDTA) with 10 μg/ml RNase at 37°C for 30 min. Then 0.05 ml of 10% SDS and 0.1 ml of 10 mg/ml proteinase K were added, and the samples were incubated overnight at 37°C. On the following day, 0.05 ml of 5 M NaCl was added and followed by chilling the sample on ice for 1 h. After a 14,000-rpm centrifugation at 4°C for 30 min, fragmented DNA in the supernatant was extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol/vol) and once with chloroform, then precipitated with ethanol and salt. The DNA pellet was washed once with 70% ethanol and resuspended in Tris-EDTA buffer, pH 8.0, with 100 μg/ml RNase at 37°C for 2 h. The DNA fragments were separated by gel electrophoresis at 20 V for 18 h, through a 2% Tris-boric acid-EDTA agarose gel containing 1 μg/ml ethidium bromide. The separated DNA fragments were visualized under a UV transilluminator and then photographed. For in vitro experiments, JB6 cells or fibroblasts cells, treated with/without silica, were harvested by centrifugation and lysed with a lysis buffer (5 mM Tris·HCl, pH 8.0; 20 mM EDTA; 0.5% Triton X-100) on ice for 45 min. The DNA fragments obtained by precipitation were separated by gel electrophoresis as described above.

Animals and administration of silica. p53 knockout (B6.129S2-Trp53^{tm1Tys}) homozygous male mice and the wild-type control strain (Trp53)-C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME) and used in this study. All animal procedures and protocols were reviewed and approved by the National Institute for Occupational Safety and Health Animal Care and Use Committee. The animals (6 wk old, ~30 g body wt) were housed separately in sterilized polycarbonate cages (four mice per cage) in an American Association for Accreditation of Laboratory Animal Care-approved facility under temperature-, humidity-, and light-controlled conditions. Food and water were available ad libitum. Freshly fractured silica (1–5 mg) suspended in 0.07 ml of sterile phosphate-buffered saline (PBS) was administrated intratracheally to the mouse according to the method described previously (11). Control mice were instilled intratracheally with 0.07 ml of PBS per mouse. On the basis of the results of a dose-response study, 1 mg/mouse was selected for the further study.

Lung tissue section. One and seven days after intratracheal instillation with silica, the mice were killed by exsanguinations under deep pentobarbital anesthesia. The left lungs were used for DNA ladder assay, and the right lungs were fixed via airway instillation with 10% buffered formalin by intratracheal instillation of formalin at 10 cmH_2O. After measurements of fixed lung volume, the lungs were embedded in paraffin and sectioned at 5 μm. The sections were mounted on glass slides, deparaffinized, rehydrated, and then stained with TUNEL to measure apoptotic cells. The percentage of apoptotic and normal cells was determined by standard morphometric methods.

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide assay. Cells were plated at a density of 1×10^4 cells/well in the 96-well plate, cultured, differentiated, and treated with or without silica. After 48-h incubation, 10 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution (Sigma, St. Louis, MO) were added in each well, and the plates were further incubated for 4 h. Then 100 μl of solubilization solution (formazan salt crystals) were added, and the plate was incubated overnight at 37°C. The optical density (OD) of the wells was measured at a wavelength of 590 nm with reference of 650 nm using an ELISA plate reader. Results were calibrated with OD measured without cell culture.

Statistical analysis. Analysis of variance and Duncan's multiple comparison tests was used to evaluate the significance between measurements. All tests were two-sided tests, and $P < 0.05$ was considered to be significant.

RESULTS

Induction of apoptosis in JB6 cells by silica. To study the mechanisms of silica-induced apoptosis and its signal transduction pathways, we used the JB6 cell line to investigate the effects of silica on the induction of apoptosis. The JB6 cell line was previously used for silica studies in our laboratory, and it

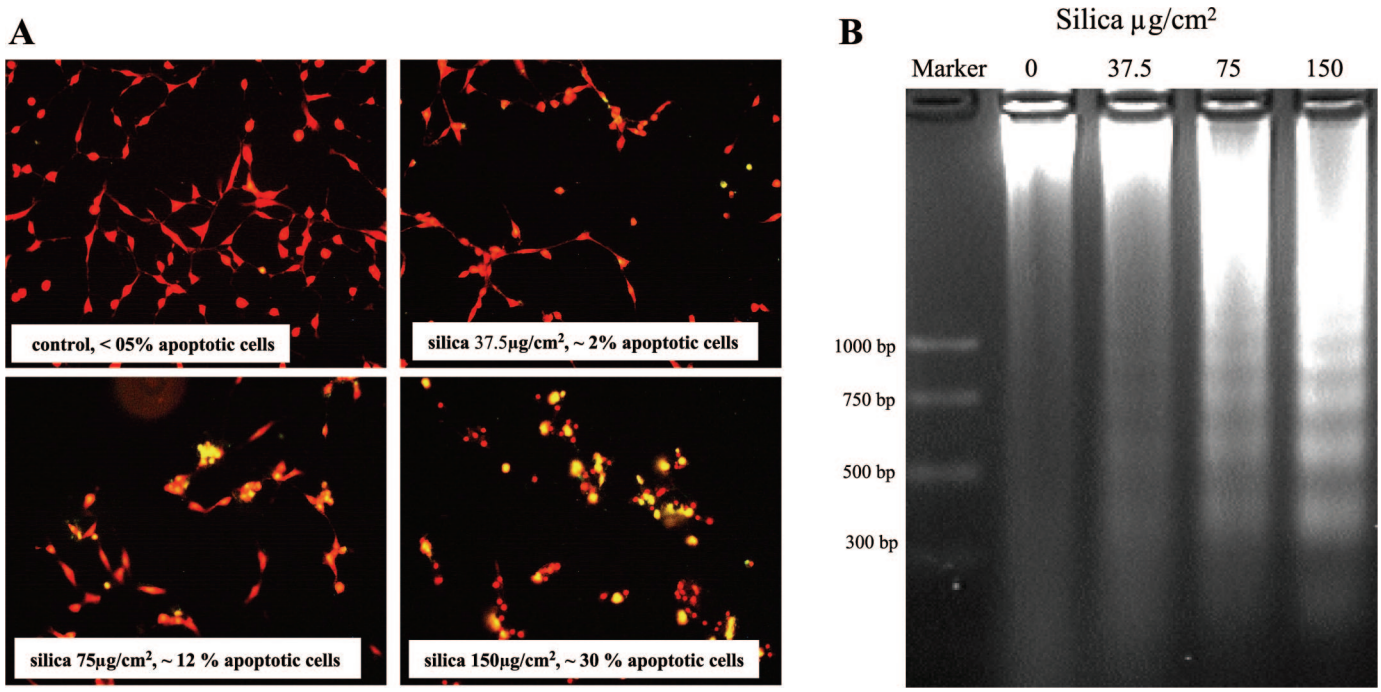

Fig. 1. Induction of apoptosis by silica. JB6 cells were treated with silica (0–150 μg/cm^2) at 37°C for 24 h. *A*: dose-dependent induction of apoptosis by silica determined by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. Apoptotic cells are indicated by a bright yellow-green fluorescence signal. The red fluorescence signal indicates propidium iodide counterstain. The percentage of apoptotic cells was determined and shown. *B*: dose-dependent induction of apoptosis by silica according to DNA fragmentation assay. The total DNA of the treated cells was also collected and subjected to electrophoresis. Note that the silica-treated samples show classic DNA ladder patterns compared with the untreated control, confirming the induction of apoptosis by silica.

has been shown to respond to a greater degree than rat lung epithelial cells (11, 12). Silica-induced apoptosis was analyzed by DNA fragmentation and TUNEL assays. Treatment of JB6 cells with silica (0–150 μg/cm^2) caused a dose-dependent increase in the level of apoptosis (Fig. 1*A*). The percentage of apoptotic cells was determined from a total of 1,000 cells stained by TUNEL. Similarly, treated cells were also tested for apoptosis by DNA fragmentation assay. Typical apoptotic DNA laddering was observed in cells treated with silica (Fig. 1*B*). This result was consistent with the TUNEL assay.

Silica induces p53 transcription activity. Previous studies indicated that p53 is one of the key regulators of apoptosis. To study the role of p53 on silica-induced apoptosis, we investigated the effect of silica on p53 activity using a JB6 cell line carrying a stably transfected p53-luciferase reporter plasmid (Cl 41 p53). The cells were exposed to various doses of silica (0–150 μg/cm^2) for 6–48 h, and then the luciferase activity was determined. The results show that silica markedly activated p53-dependent transcription activity of the cells in a dose-dependent manner (Fig. 2*A*). The maximum induction of p53 activity occurred at the concentration of silica between 50 and 100 μg/cm^2 (Fig. 2*A*). A higher concentration of silica (150 μg/cm^2) caused a decrease in p53 activity, presumably due to cytotoxic effect of silica at this high dose. Time-course studies indicate that induction of p53 was first observed after 10 h of incubation, and thereafter, the p53 activity continued to increase through 48 h (Fig. 2*B*). These results clearly demonstrate that silica could induce p53 transactivation in JB6 cells.

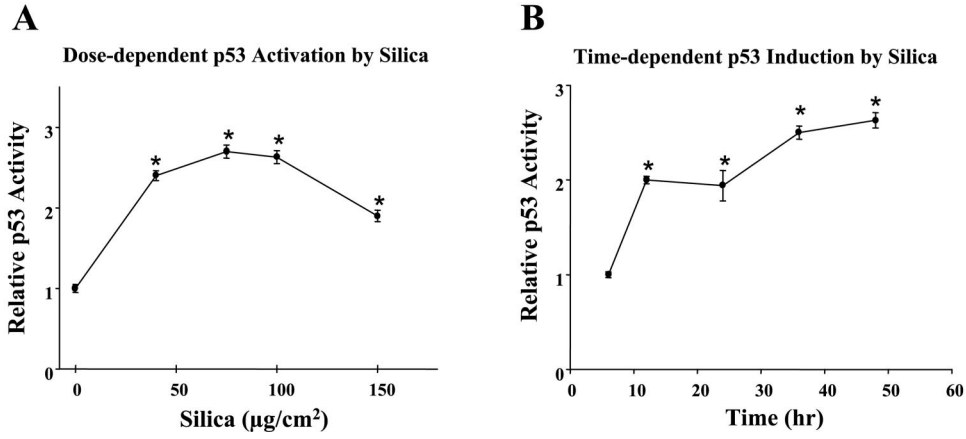

Fig. 2. Effect of silica on p53-dependent transcription activity. JB6 cells with p53 luciferase reporter stable transfectant (Cl 41 p53) were treated with silica at indicated concentrations and times. p53 activity was determined by luciferase activity assay as described in MATERIALS AND METHODS. *A*: dose-response study determined at 24 h of incubation. *B*: time-course study determined at 150 μg/cm^2 of silica. Results, presented as relative p53 activity compared with the untreated control cells, are means and SE of triplicate assays. *Significant increase from the control ($P < 0.05$).

Effect of silica on cell proliferation. To determine whether silica dust had any effect on cell proliferation, serial concentrations of silica from 18.75 to 150 μg/cm^2 were used to study the effect on cell proliferation by MTT assay by the methods described in MATERIALS AND METHODS. The results show that at the low doses of silica (18.75–35 μg/cm^2), there were no significant effect on cell proliferation, whereas higher doses inhibited cell growth (Fig. 3). The inhibition of cell proliferation may be due to the induction of apoptosis or/and cytotoxicity induced by silica.

Silica induces p53 protein expression and phosphorylation. To further explore the mechanism of silica-induced p53 activation, we investigated the effect of silica on p53 protein expression or phosphorylation. JB6 Cl 41 cells were treated with silica (75 μg/cm^2) for various times, and p53 protein expression was analyzed by immunoprecipitation and Western blot analysis using the monoclonal anti-p53 antibody. The results showed that silica was able to induce p53 protein expression in a time-dependent manner (Fig. 4*A*). The p53 protein was elevated at 30 min postexposure, whereas untreated control cells showed modest p53 expression. Maximum p53 protein expression was observed at 360 min posttreatment.

Because phosphorylation of p53 at Ser392 has been shown to stabilize the tetramer formation of p53 and increase its transcriptional activity (35), silica-induced phosphorylation of p53 at Ser392 was investigated using a specific antiphospho-p53 (Ser392) antibody. Phosphorylation of p53 protein at the position of Ser392 was observed in cells treated with silica (Fig. 4*B*), and this effect was time dependent. Phosphorylation of p53 at Ser15 and Ser20 positions was also tested. No induction of p53 phosphorylation by silica was observed at these positions (data not shown). The above results suggest that silica-induced p53 transactivation may be caused by not only the induction of p53 protein expression but also the phosphorylation of p53 at Ser392.

p53 is required for silica-induced apoptosis. To further study the role of p53 in silica-induced apoptosis, we tested the induction of apoptosis induced by silica using the *p53*+/+ and *p53*−/− fibroblast cell lines. These cell lines were derived from mouse embryos as reported previously (16). The results of TUNEL assay showed that apoptosis (~12%) was observed in *p53*+/+ cells treated with 75 μg/cm^2 silica for 24 h (Fig. 5*A*), whereas this response was significantly reduced in *p53*−/− cells (<1%). Similar results were obtained in the DNA fragmentation assay (Fig. 5*B*). These results indicate that p53 is required for silica-induced apoptosis.

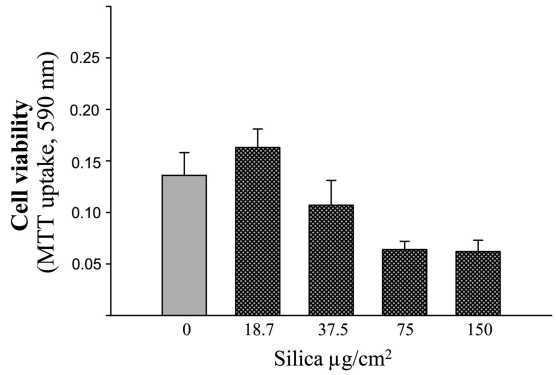

Fig. 3. Effects of silica on cell viability in JB6 cells. JB6 cells were incubated with various concentrations of silica as indicated. After 24 h of incubation, viable cells were quantified by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Data are expressed as means ± SE (n = 4).

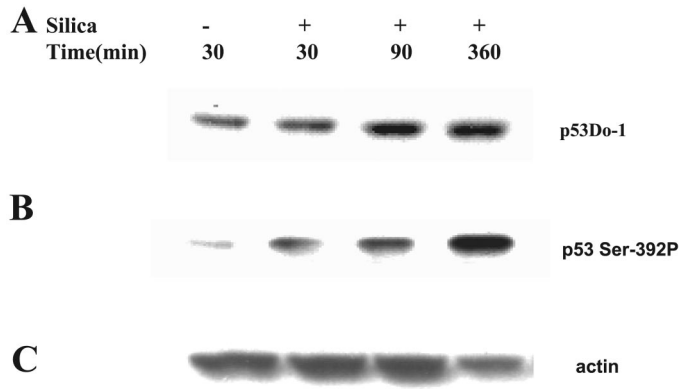

Fig. 4. Induction of p53 protein expression and phosphorylation of p53 by silica. JB6 Cl 41 cells were treated with silica (75 μg/cm^2) for indicated times. After the treatment, cells were lysed, immunoprecipitated, and subjected to Western blot analysis as described in MATERIALS AND METHODS. *A*: expression of p53 protein; *B*: phosphorylation of p53 at Ser392; *C*: expression of actin (control gene product).

Activation of p53 is essential for silica-induced apoptosis in vivo. The role of p53 in silica-induced apoptosis was also investigated in vivo in homozygous p53-knockout (p53−/−) mice. The wild-type parental strain [(Trp53)-C57BL/6J *p53*+/+ mice] was used as the control. Male p53−/− mice (6 wk old) were instilled with freshly fractured silica (1 mg/mouse), and apoptotic cells in the lung were determined by both DNA fragmentation and TUNEL assay at 1 day postexposure (Fig. 6).

DNA fragmentation assay shows that apoptosis was induced in the lung samples from *p53*+/+ mice treated with silica, whereas this response was significantly reduced in p53−/− mice (Fig. 6*A*). Similar results were obtained by TUNEL assay (Fig. 6*B*). The results of TUNEL assay indicate that the number of apoptotic cells in the lung of p53−/− mice treated with silica was 70% less than that in p53+/+ mice (Fig. 6*C*) but was still significantly elevated compared with untreated p53−/− mice. These results indicate that silica was able to induce apoptosis in the lung of animals and that p53 plays an important role in this process.

To identify the apoptotic cell type in the lung induced by silica, confocal image was performed. The result of fluorescence and confocal image of an apoptotic cell in silica-treated lung is shown in Fig. 7. This figure gives a high-magnification image of an apoptotic cell with fluorescence imaging of the TdT-positive nucleus and a corresponding differential interference-confocal image. The TdT-positive alveolar macrophage, identified by the white arrow, is in the air space of the alveolar wall and is representative of the apoptotic cells found in the silica-treated cases. Apoptotic cells with morphology typical of other air space cells, such as neutrophils, were not common. Apoptotic cells were not found in the interstitial spaces of the alveolar wall.

DISCUSSION

Occupational exposure to silica is associated with the development of silicosis and lung cancer. The molecular mecha-

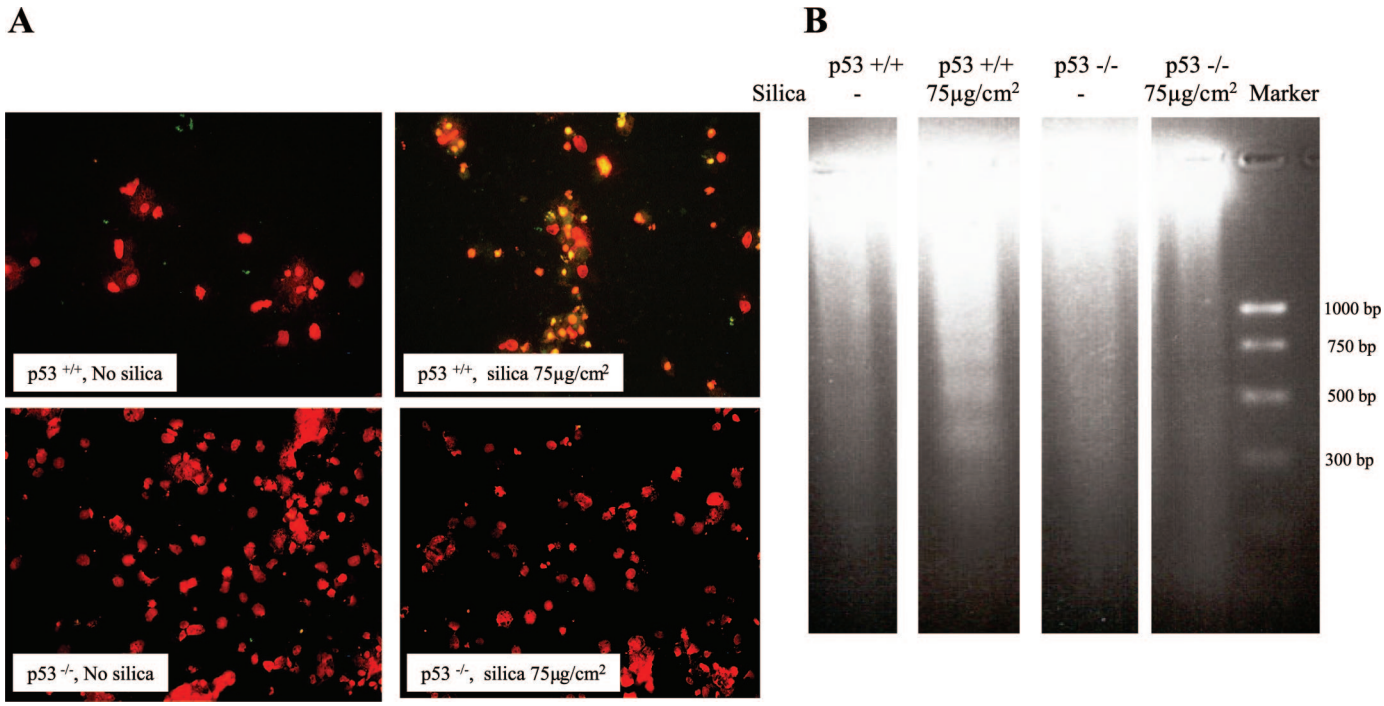

Fig. 5. Silica-induced apoptosis in wild-type (WT) p53 (*p53*+/+) vs. p53-deficient (*p53*−/−) fibroblasts. *p53*+/+ or *p53*−/− fibroblast cell lines were treated with 75 μg/cm^2 of silica for 24 h. *A*: TUNEL assay, treatment of cells with silica induced 12% apoptosis in *p53*+/+ cells, whereas the same treatment induced <1% in *p53*−/− cells. *B*: DNA fragmentation assay, cells from the same experiment were also subjected to DNA fragmentation assay.

nisms involved in silica-induced pathogenesis are poorly understood (1). Previous studies have shown that apoptosis is a fundamental cellular process occurring under a wide range of physiological and pathological conditions (38, 46) and that p53 may play a critical role in cell cycle control and the induction of apoptosis (25). Here, we investigated the effect of freshly fractured silica on cell apoptosis and the role of p53 in silica-induced apoptosis using cell cultures and p53 knockout animals. The results show that apoptosis was induced by silica concomitantly with an increased level of p53 transactivation in JB6 cells. Silica-induced p53 transactivation appears to be mediated through the increase in p53 protein expression and phosphorylation of p53 at Ser392. Silica also induced apoptosis in *p53*+/+ fibroblasts but not in *p53*−/− cells. Similarly, in vivo studies using experimental animals indicate that silica-induced apoptosis was significantly inhibited in *p53*−/− animals. These data indicate that silica could induce apoptosis in both cell culture and animal models and demonstrate for the first time that p53 is essential for silica-induced apoptosis.

Apoptosis is a crucial cellular activity in the behavior of mammalian cells in a wide range of pathophysiological conditions. It is responsible for the deletion of unwanted cells. Apoptosis of individual cells may present a protective mechanism against neoplastic development in the organism by eliminating genetically damaged cells. External signals trigger apoptosis through a signal transduction pathway, which may involve the stimulation of the receptors, activation of protein kinase/phosphates cascades, and release of secondary messengers for transcription factors that upregulate or suppress the transcription of specific genes (47). Although there are numerous external signals that are involved in regulation of apoptosis, p53 is the one of most extensively investigated pathways (31, 42, 47). Normal p53 function was shown to be crucial in the induction of apoptosis in human and marine cells following DNA damage. Hence, p53-mediated apoptosis is an important part of tumor suppressor phenotype, and subsequent p53 deficiency may permit a population of genetically damaged cells to escape the normal process of apoptotic deletion.

It has been reported that exposure of animals to silica increases p53 expression in the lung (17, 37), whereas another study by Ishihara and coworkers (20) found that a single intratracheal injection of silica to mice suppressed expression of p53mRNA. The present studies demonstrated that silica induces accumulation of p53 protein in cultured cells and also found that silica markedly activates p53-dependent transcription activity in a dose- and time-dependent manner. It is believed that p53 function is modulated by posttranslation events. The phosphorylation and dephosphorylation of p53, which are mediated by a variety of intracellular protein kinases, are the leading candidates for implementing control of p53 function (41). Phosphorylation of Ser392 stabilizes the tetramer formation of tumor suppressor protein p53 (35). This activity may result in increasing the half-life of p53 protein, elevated p53 transcription activity, and accumulation of p53 protein in the cells. However, silica failed to phosphorylate p53 at Ser15 and Ser20 positions. Early studies also indicated that DNA damage caused by UV radiation stimulated the activation of p53 Ser392 kinase. This kinase complex could phosphorylate p53 at Ser392 and promote the DNA sequence-specific binding and transactivation ability of p53 (9, 22,). Because p53 was activated in response to DNA damage and silica is a DNA-damaging agent (9, 45), the p53 transactivation may be due to DNA damage and chromosomal aberrations caused by silica. The present study also demonstrated that silica induces p53 transactivation at the same dose range that induces p53 protein expression and phosphorylation. These results suggest

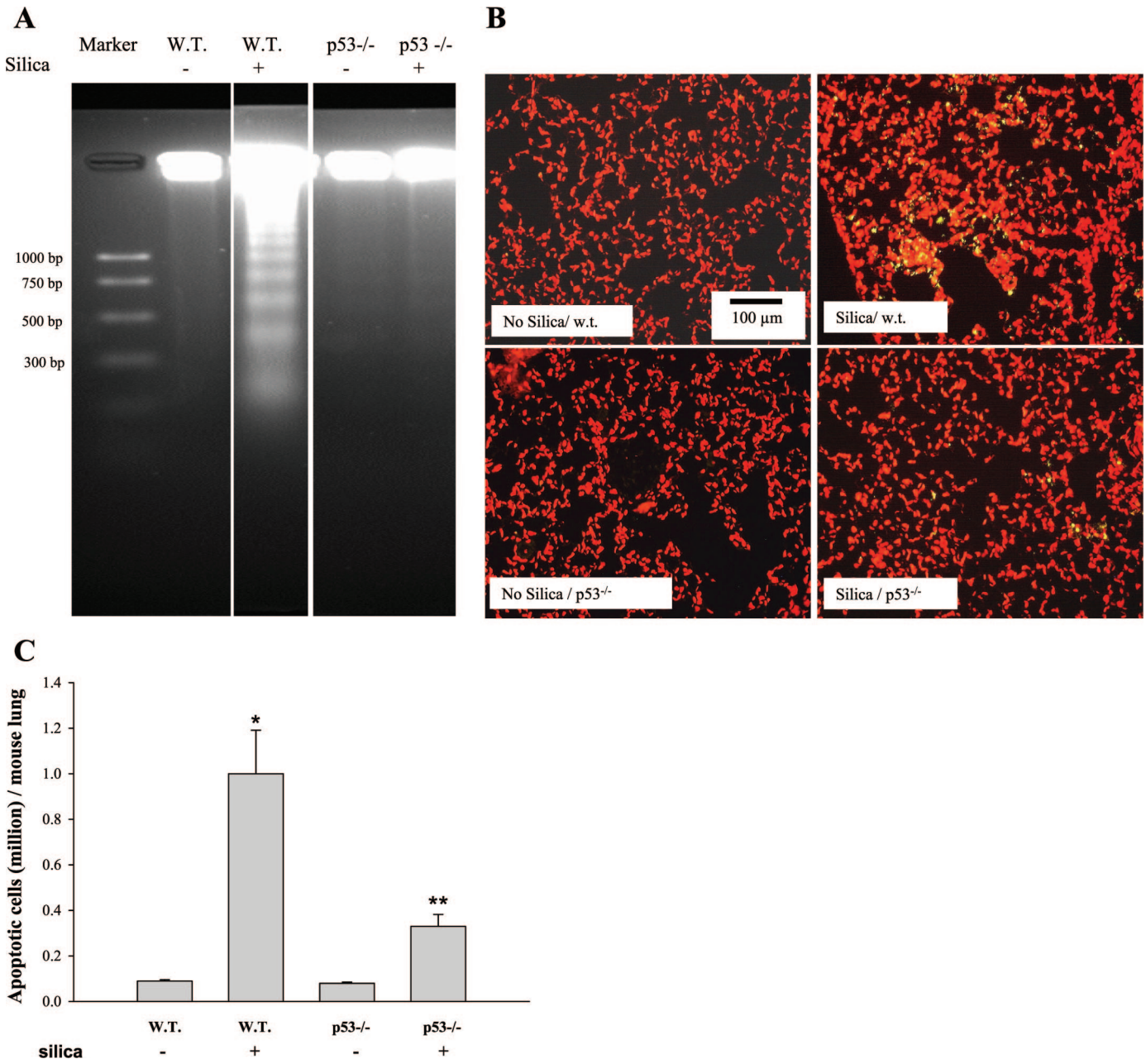

Fig. 6. Detection of silica-induced apoptosis in the lungs of p53 knockout mice. *p53*+/+ and *p53*−/− mice were treated with silica (1 mg/mouse) or PBS via intratracheal instillation. One day after the treatment, mice were killed, and the left lungs were removed for DNA fragmentation analysis (*A*), whereas the right lungs were used for TUNEL assay (*B*). Note: there was significant inhibition in apoptotic DNA fragment formation and apoptotic cell number in p53 knockout mice. The number of TUNEL-positive cells from *B* was counted by a standard morphological method. A minimum of 20 fields per lung was examined at ×100 magnification. Those values, given as mean ± SD, $n = 4$ mice/group, are shown in *C*. *$P < 0.05$ vs. saline control group; **$P < 0.05$ greater than control but less than the silica-exposed p53+/+ group. All in vivo studies were carried out on 4 mice/group and repeated separately 3 times.

that p53 transactivation induced by silica might be through an increase in the half-life and accumulation of p53 protein in the cells, as well as phosphorylation at Ser392.

The present results also suggest that the induction of wild-type p53 transcription activity is necessary to induce apoptosis by silica in normal cells. This is supported by the finding that apoptosis was induced by silica concomitantly with the increase in levels of p53 transactivation in JB6 cells. The strongest evidence for the role of p53 in silica-induced apoptosis was provided by the study using fibroblast cell lines with wild-type p53 (*p53*+/+) or p53 deficiency (*p53*−/−). Apoptosis induced by silica was observed only in *p53*+/+ cells, not in *p53*−/− cells. The essential role of p53 in silica-induced apoptosis was further demonstrated in vivo in p53 knockout mice. Homozygous p53−/− mice that were exposed to silica exhibited only mild lung cell apoptosis, whereas *p53*+/+ mice showed significantly greater apoptosis in response to silica treatment. These results strongly suggest that p53 activation plays an important role in silica-induced apoptosis. MTT assay indicates that silica inhibited cell proliferation at the concen-

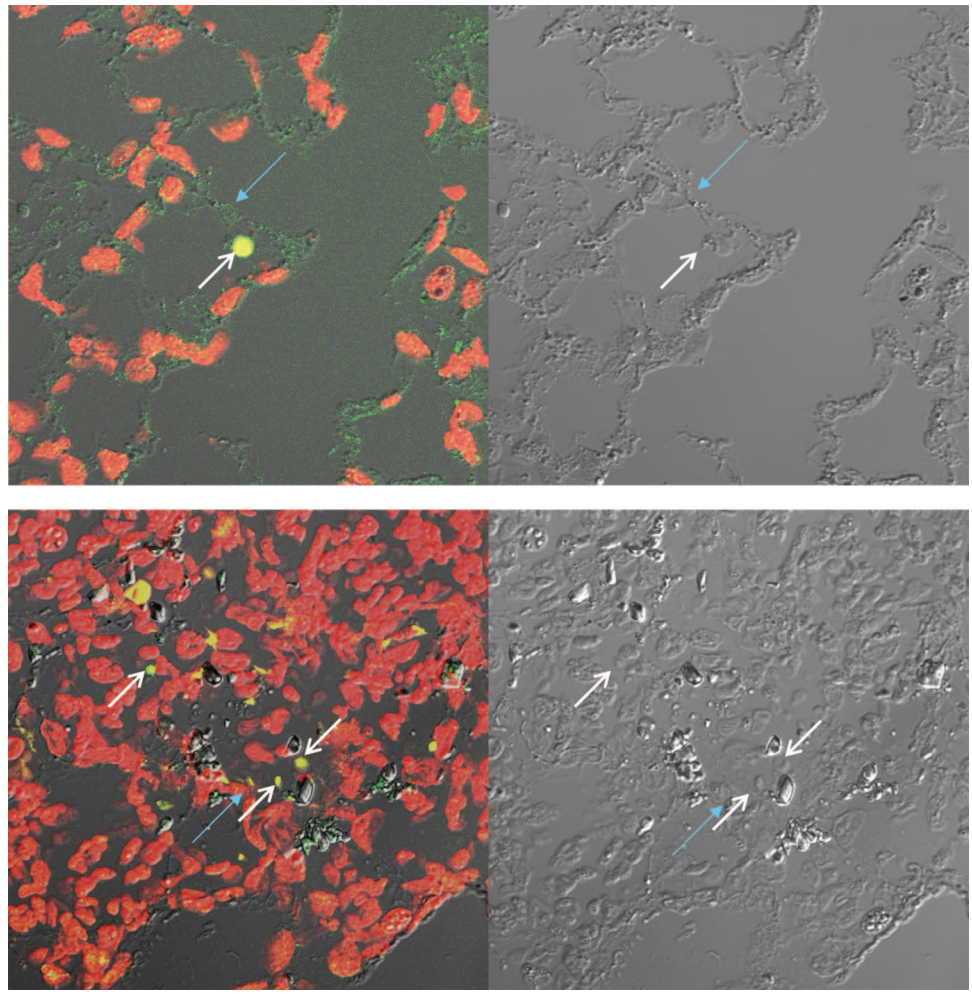

Fig. 7. TUNEL and confocal image of an apoptotic cell in silica-treated lung. The TdT-positive nucleus (*left*) and a corresponding differential interference-confocal image (*right*) are shown. Blue arrows indicate the alveolar wall, which can be identified by the confocal image. The TdT-positive alveolar macrophage, identified by white arrows, is in the air space of the alveolar wall and is representative of the apoptotic cells found in the silica-treated cases.

tration of 75 μg/cm^2 or higher. This result was consistent with the finding that silica induces dose-dependent apoptosis in cultured cells. The inhibition of cell proliferation by silica may be due not only to the induction apoptosis but also to cell cycle arrest caused by DNA damage or other cytotoxicity effects.

Although apoptosis has been implicated as a homeostatic mechanism, increasing evidence indicates that it also may play a role in human diseases. It has been suggested that apoptosis may be involved in the pathogenesis of silicosis, since apoptotic cells are frequently observed in bronchoalveolar lavage fluid in silica-induced pneumoconiosis, in both acute and chronic stages of silicosis (21). Because apoptosis-triggering substances, such as reactive oxygen species and Fas ligand, are generated and cell numbers in the lung are increased concurrently in silica-induced reactions, it is possible that the generated substances counteract the increase of cell number and regulate the evolution of silica-induced effects through the induction of apoptosis. Apoptotic DNA strand breaks have been reported in association with increased p53 expression in idiopathic and bleomycin-induced pulmonary fibrosis (5, 23). However, the direct role of p53 in these fibrotic lung disorders has not been demonstrated. The ability of silica, a known fibrogenic agent, to activate p53-dependent apoptosis suggests that this transcription factor may participate in the fibrotic process by acting as a common regulator of apoptosis. Supporting this notion is the evidence that suppression of apoptosis by caspase inhibitors also abrogates pulmonary fibrosis (5).

The mechanism by which p53 regulates silica-induced apoptosis and fibrosis remains to be elucidated. Accumulated evidence suggests that activation of the p53-dependent Fas (CD95/APO-1) death receptor may be involved in this process. In a silica- and bleomycin-induced apoptosis model, mice deficient in Fas (*lpr* mutants) or Fas ligand (*gld* mutants) were protected from lung fibrosis (32). Because Fas has been reported to be transcriptionally regulated by p53 (30) through a p53 response element located within the Fas gene (13), these studies therefore suggest a possible linkage between p53 and Fas in silica-induced apoptosis and fibrosis. However, a recent study reported that bleomycin-exposed transgenic mice that express a dominant-negative mutant form of human p53 gene display more severe lung pathology with associated collagen deposition and pronounced lung eosinophilia than simultaneously exposed nontransgenic mice (44). Long-term exposure is required to understand the role of p53 in apoptosis-related lung disorders.

Apoptotic cells by their nature are relatively devoid of intercellular organelles and other features that are present in the original cell type. This can make identification difficult, but in our experiment model, the apoptotic cells were within alveolar

air spaces and had morphology consistent with an alveolar macrophage origin, indicating that these cells were alveolar macrophages (Fig. 7). We did not identify apoptotic neutrophils in silica-treated lungs, which is significantly different from the large numbers of apoptotic neutrophils that we have found to be produced by toxic metals such as vanadium (44).

In conclusion, the present study demonstrates that silica is able to induce p53 transactivation through stimulation of p53 protein expression and phosphorylation at Ser392. Both in vitro and in vivo studies indicate that silica-induced apoptosis occurs through a p53-dependent pathway. This finding may provide an important link in understanding the molecular mechanisms of silica-induced lung pathogenesis.

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