The Inhibitory Effect of Phytochemicals on the Oxidative DNA Damage in Lymphocytes by Chrysotile

A-Reum Ryu · Jum-Ji Kim · Mi-Young Lee

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Abstract We investigated the cytotoxicity and oxidative DNA damage by chrysotile, one of the asbestos, in this investigation. Chrysotile enhanced malondialdehyde (MDA) levels and intracellular reactive oxygen species generation in human airway epithelial cells. Furthermore, asbestos-induced oxidative DNA damage in lymphocytes was evaluated by single cell gel electrophoresis and quantified as DNA tail moment. Notably, phytochemicals such as curcumin, berberine, and sulforaphane presented inhibitory effect on the asbestos-induced oxidative DNA damage in lymphocytes.

Keywords asbestos · lipid peroxidation · phytochemicals · reactive oxygen species

Introduction
Asbestos, including chrysotile, amosite, crocidolite, tremolite, anthophyllite and actinolite, is a family of naturally occurring silicate fibers used commercially in such applications as electrical insulation and in building insulation (Frank, 1993). Asbestos exposure in humans is associated with inflammatory and malignant disease in the lung, including malignant lung cancer, mesothelioma and asbestosis (Park et al., 2008; Loomis et al., 2010; Seo et al., 2012). Cytokines and growth factors, such as tumor necrosis factor (TNF-α), interleukin (IL)-1α, IL-8, and platelet-derived growth factor, are strongly implicated as mediators of asbestos-related pathophysiologic responses (Bonner et al., 1993; Driscoll et al., 1993; Li et al., 1993; Perkins et al., 1993). The exact molecular mechanisms responsible for these processes are not clear (Rom et al., 1991), but largely involved in the reactive oxygen species (ROS) generation via the interaction of iron, present as a silicate-iron complex on the fiber surface (Simeonova and Luster, 1995; 1996). Generally, high level of ROS induces cytotoxicity and plays a direct role in tissue damage, whereas moderate oxidative stress can activate events in the cell signal transduction pathways leading to the production of inflammatory mediators (Simeonova et al., 1997). Previous studies have yielded conflicting data on the cytotoxic effects of asbestos on mesothelial cells. For example, increase in intracellular oxidation have not been found in studies of asbestos-exposed mesothelial cells (Gabrielson et al., 1986; Ollikainen et al., 1999), although upregulation of antioxidant enzymes and oxidative changes in DNA suggest a role for oxidative damage to the cell and to cellular DNA (Janssen et al., 1994). In addition, DNA strand breakage, an expected toxic effect of oxidative damage to DNA, has not been found in previous studies of asbestos-exposed mesothelial cells (Gabrielson et al., 1986; Kannala et al., 1994), although other DNA lesions an indirect measures of DNA damage have been reported (Renier et al., 1990; Liu et al., 2000).

A variety of antioxidants and medicinal herbs have been known to reduce the generation of reactive oxygen species. Curcumin is the principal curcuminoid of the popular Indian spice tumeric (Choi, 2009). Its antioxidant, anti-inflammatory, antiviral, and antifungal actions, as well as an anticancer effect were reported (Biswas and Rahman, 2008; Seo and Lee, 2011). Berberine, the major constituent of Coptis Chinensis, is an isoquinolin alkaloid which has wide range of pharmacological and biochemical effects. Berberine has demonstrated the significant antimicrobial activity and antitumor activity (Letasiová et al., 2005). Sulforaphane, isolated from broccoli, is not a direct-acting antioxidant or prooxidant, since it is unlikely that the isothiocyanate group might participate in oxidation or reduction reactions under physiological conditions (Barton and Ollis, 1979). There is, however, substantial and growing evidence that sulforaphane administration acts indirectly to increase the antioxidant capacity of animal cells, and their abilities to cope with oxidative stress (Fahey and Talalay, 1999). In this study, chrysotile-induced cytotoxicity in NCI-H292 cells and oxidative DNA damage in lymphocytes were measured. The suppressive effect of phytochemicals such as curcumin, berberine, and sulforaphane on the DNA damage by chrysotile was also investigated.
Materials and Methods

Cell culture and chrysotile treatment. The human airway epithelial cell line NCI-H292 was purchased from ATCC (American Type Culture Collection, USA) and maintained in Dulbecco’s modified essential medium (DMEM) supplemented with 10% heat inactivated FBS and 1% penicillin-streptomycin at 37°C in atmosphere of 5% CO₂. Powdered chrysotile was suspended in DMEM medium, and sonicated and triturated through a 22-gauge needle to obtain a homogeneous suspension, and added directly to culture dish for 24 h treatment. Curcumin was dissolved in DMSO, and berberine and sulforaphane were each dissolved in phosphate buffered saline (PBS).

Trypan blue exclusion assay. To determine the cytotoxicity of chrysotile on the NCI-H292 cells, a trypan blue exclusion assay was performed. NCI-H292 cells were seeded and treated with 0, 0.1, 0.5, 1, 2.5 and 5 µg/cm² of asbestos for 24 h. We evaluated cell viability using trypan blue exclusion assay.

Malondialdehyde (MDA) determination. MDA level in NCI-H292 cell was measured by the thiobarbituric acid method (Heath and Packer, 1968).

Confocal analysis of intracellular ROS. ROS detection is additional tools in the assessment of cytotoxicity caused by asbestos fibers (Park et al., 2007). NCI-H292 cells seeded into poly-L-lysine coated cover slip and treated with 2.5 and 5 µg/cm² asbestos for 24 h. Asbestos treated NCI-H292 cells were washed twice with PBS and then incubated with 5 µM 2',7'-dichlorofluorescein diacetate (DCFH-DA) for 30 min at 37°C in 5% CO₂ incubator, and were then washed with PBS. DCFH-DA, aliquoted in DMSO at a stock solution of 5 M, was stored in the dark at −20°C, and then diluted in PBS immediately before the experiment. The intracellular ROS was detected by Confocal microscope (460–495 nm/510 nm excitation/emission).

Determination of DNA damage by comet assay. The alkaline comet assay was performed according to Singh et al. (1988) with slight modifications, as reported previously (Park et al., 2005). The Comet assay has been considered a sensitive method in the assessment of DNA single-strand breaks in the oxidant-exposed cells (Seo et al., 2010). The isolated lymphocytes from rat were mixed with 75 µL of 0.7% low-melting-point agarose and added to slides precoated with 1.0% normal melting-point agarose. After the agarose solidified, the slides were covered with 100 µL of 0.7% low-melting-point agarose and immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% sodium lauryl sarcosine, 1% Triton X-100, and 10% DMSO) for 1 h at 4°C. The slides were placed in an electrophoresis tank containing 300 mM NaOH and 10 mM Na₂EDTA (pH 13.0) for 20 min to allow the DNA to unwind. Electrophoresis was performed at 25 V/300 mA for 20 min at 4°C. The slides were washed with neutralizing buffer (0.4 M Tris-HCl pH 7.5) three times for 5 min at 4°C, and then treated with ethanol for 5 min.

Image analysis and statistical analysis. The slides were stained with ethidium bromide (20 µg/mL) and cover slipped. Measurements were made by image analysis using Komet 5.5 software (Kinetic Imaging, UK) and fluorescence microscope (Leica, Germany). To quantify DNA damage in the comet assay, the olive tail moment was calculated as: (Tail.mean-Head.mean)×Tail% DNA/100 (Sul et al., 2008). A total of 150 randomly captured comets were examined from each slide.

The comet assay data are the means of three determinations and were analyzed using the SPSS package for Windows version 13 (SPSS Inc., USA). The mean values of DNA damage (olive tail moment) for each treatment were compared using one-way analysis of variance (ANOVA) followed by Duncan’s multiple range test. Erythrocyte damage was measured using Duncan’ multiple ranges test. p<0.05 was considered significant.

Results

Cytotoxicity of chrysotile in human airway epithelial cells. The cytotoxicity of chrysotile in human airway epithelial cells (NCI-H292) was determined using trypan blue dye exclusion assay. The assay is based on the principle that live cells possess intact cell membranes that exclude trypan blue dye, following exposure to...
various concentrations of chrysotiles for 24 h. Chrysotile exposure reduced cell viability in a concentration-dependent manner as shown in Fig. 1. The exposure of NCI-H292 cell to 1, 2.5 and 5 mg/cm$^2$ asbestos resulted in an approximately 15.5, 28.1 and 50.2% decrease in cell viability, respectively.

**MDA level and ROS generation in NCI-H292 cells exposed to chrysotile.** In order to determine intracellular MDA levels of NCI-H292 cells following chrysotile exposure, we performed modified thiobarbituric acid method (Heath and Packer, 1968). As shown in Fig. 2, chrysotile enhanced MDA levels of NCI-H292 cells, showing lipid peroxidation increase by chrysotile in dose-dependent manners. To analyze whether the generation of reactive oxygen species (ROS) may be related with asbestos-induced lipid peroxidation in NCI-H292 cells, the cells were incubated with an
9-fold increase in DNA damage at 20µg/mL chrysotile induced severe DNA damage. The olive tail moment at 1990; Kamp et al., 1992). Another potential mechanism leading to asbestos-related cell and DNA damage occurred via nitric oxide (NO). NO itself has been shown to cause oxidant-related DNA damage, inhibition of repair processes, and/or increase of various genotoxic species in cells (Wink et al., 1998). However, the role of NO-mediated mechanisms in the development of DNA damage due to asbestos fibers has remained unclear (Puhakka et al., 2002).

Previous studies have suggested the importance of hydroxyl radicals and irons in the fenton reaction in the pathogenesis of asbestos-related cell and DNA damage (Goodglick and Kane, 1990; Kamp et al., 1992). Another potential mechanism leading to asbestos-related cell and DNA damage occurred via nitric oxide (NO). NO itself has been shown to cause oxidant-related DNA damage, inhibition of repair processes, and/or increase of various genotoxic species in cells (Wink et al., 1998). However, the role of NO-mediated mechanisms in the development of DNA damage due to asbestos fibers has remained unclear (Puhakka et al., 2002).

The measurement of oxidant generation is difficult, since most previous methods are non-specific. They detect mainly the net release of various oxidants, such as H₂O₂, form the cells or measure the products of oxidant-mediated reaction, such as lipid peroxidation. Most recent studies have used an oxidation-sensitive fluorescent probe, 2',7-dichlorodihydrofluorescin (DCDHF)-diacetate (DA), which diffuses into cells and is oxidized to a fluorescent compound by ROS and reactive nitrogen species (RNS) (Liu et al., 2000).

The present study confirms the recent findings that asbestos fibers cause an increase in the mean tail extent moment in the Comet assay, a similar result shown in DNA single-strand breaks in cultured human mesothelioma cells (Ollikainen et al., 1999). Chrysotile-induced cytotoxicity and intracellular ROS and MDA

oxidative-sensitive fluorescent probe DCFH-DA using Confocal microscope. Figure 3 shows a marked and dose dependent increase in the ROS levels in NCI-H292 cells treated with 2.5 and 5µg/cm² chrysotile, suggesting that enhanced lipid peroxidation might be attributable to the generation of intracellular ROS. Chrysotile-treated cells were strongly positive for DCFDA staining compared to the medium control.

**Chrysotile-induced oxidative DNA damage in lymphocytes.** Chrysotile-induced oxidative DNA damage of lymphocytes was evaluated by Comet assay. Lymphocytes treated with 5, 10, and 20µg/mL chrysotile exhibited DNA damage, as determined by the olive tail moment (Fig. 4A). Chrysotile at low concentrations did not cause DNA damage, whereas high concentrations of chrysotile induced severe DNA damage. The olive tail moment at 20µg/mL chrysotile was about 28.04±2.03, compared with 3.03±1.23 in the PBS-treated control, indicating an approximately 9-fold increase in DNA damage at 20µg/mL chrysotile. The in vitro oxidative DNA damage caused by chrysotile increased in a dose-dependent manner. When catalase was treated (Fig. 4B), however, the tail lengths of lymphocyte DNA was reduced to the control. These results served as positive control for the current experiment to assess the antioxidative activities of phytochemicals.

**Suppressive effects of phytochemicals on the chrysotile-induced DNA damage.** Curcumin, berberine, and sulforaphane were used for their suppressive effects against chrysotile-induced oxidative DNA damage in lymphocytes (Fig. 5). The characteristics of the phytochemicals used in this investigation were described in Table 1. The addition of phytochemicals significantly inhibited the oxidative DNA damage caused by 20µg/cm² chrysotile, as demonstrated by a reduction of the olive tail moment in comet assay. Sulforaphane effectively suppressed the oxidative DNA damage in concentration-dependent manners. 3 and 5µg/mL curcumin could suppress the DNA damage, on the contrary, 8µg/mL curcumin could not inhibit the damage in this experimental condition. Berberine was the most effective in reducing the olive tail moment in this system. Vitamin C, a general antioxidant, also inhibited the DNA damage. This result raises the possibility that curcumin, berberine, and sulforaphane could be developed as therapeutic chemicals to suppress the chrysotile-induced oxidative DNA damage and asbestos-related diseases.

**Discussion**

Asbestos fiber has been heavily used in industry since World war II because of their durability, heat-resistance, and low cost. However, in 1987, the International Agency for Research on Cancer (IARC) designated asbestos fibers as a Group 1 (definite) carcinogen for human, and asbestos fibers were banned in many western countries in the 1990s (Roggli et al., 2004; Dadson and Hammmar, 2006).

Curcumin, berberine, and sulforaphane could be developed as therapeutic chemicals to suppress the chrysotile-induced oxidative DNA damage and asbestos-related diseases.

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<tr>
<th>Phytochemicals</th>
<th>Structure</th>
<th>Characteristics</th>
<th>Reference</th>
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<tr>
<td>Sulfuraphane</td>
<td><img src="image1" alt="Sulfuraphane" /></td>
<td>• It is anticancer, antidiabetic, and antimicrobial compound. • Identified in broccoli sprouts. • In numerous clinical trials including a phase II trial for prostate cancer.</td>
<td>Zhang et al., (1992); Fahey et al., (2002); Hayes et al., (2008)</td>
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<tr>
<td>Curcumin</td>
<td><img src="image2" alt="Curcumin" /></td>
<td>• It has antioxidative and anti-inflammatory activity. • Effects on the biotransformation enzymes involved in carcinogen metabolism. • Inhibition of tumor invasion and angiogenesis.</td>
<td>Choi et al., (2006); Shukla et al., (2008); Senft et al., (2010); Ströfer et al., (2011)</td>
</tr>
<tr>
<td>Berberine</td>
<td><img src="image3" alt="Berberine" /></td>
<td>• Isolated from several herbs like as barberry, goldenseal, and so on. • Beneficial effects on the cardiovascular system and significant anti-inflammatory activities. • Suppress the growth of a wide variety of tumor cells.</td>
<td>Marin-Neto et al., (1988); Zeng et al., (2003); Tang et al., (2009); Kuo et al., (2004); Zhang et al., (2010)</td>
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generation of cells were enhanced in a dose dependent manner. Next, we performed a comet assay to investigate the suppressive effects of phytochemicals on the chrysotile-induced oxidative DNA damage. Notably, however, various phytochemicals inhibited the chrysotile-induced oxidative DNA damage. Similar inhibitory effects were reported previously with red-ginseng and Acanthopanax in oxidative DNA damage induced by herbicide (Ollikainen et al., 1999).

There have been considerable evidences that asbestos may initiate ROS production by Fenton-like reaction in exposed cells, stimulating phagocytic cells to release extracellular ROS through membrane-associated-NADPH oxidase, and by mitochondrial oxidative phosphorylation (Huang et al., 2012). In this study, treatment with chrysotile caused DNA damage, indicated by increased tail lengths, however, catalase repaired the injury, evidenced by decreased tail lengths to the control level. The repair of the H$_2$O$_2$-induced DNA damage by catalase was reported in showing antioxidative effect of mush room (Park et al., 2009). Our results indicate that phytochemicals have antioxidant properties in oxidative stress by radical scavenging. Therefore, those data presented in this study may provide the possible therapeutic values of the curcumin, berberine, and sulforaphane for the prevention of oxidative DNA damage progression by chrysotile.

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