Chrysotile Causes Human Bronchial Epithelial Cell Apoptosis in Response to the Fas-Mediated Apoptosis Pathway

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Keywords

Mineral fibers · Cytotoxicity · Apoptosis

Abstract

Aims: Asbestos is harmful to human health. However, the pathogenicity of chrysotile is a controversial matter. This study aimed to investigate the apoptosis of a human bronchial epithelial cell line (BEAS-2B) exposed to chrysotile that may function in part through the Fas death receptor pathway. **Methods:** Cultured human BEAS-2B cells were treated with chrysotile and cell viability was evaluated by CCK-8 assay. The induction of cell apoptosis was evaluated by FACS analysis. mRNA expression levels of Fas, caspase-3, and caspase-8 were evaluated quantitatively by real-time PCR. The expression of Fas, caspase-3, and caspase-8 proteins were evaluated by Western blot. Meanwhile, cells were preincubated with various concentrations of anti-Fas antibody (CH11) and antagonistic anti-Fas antibody (ZB4). **Results:** Chrysotile inhibits proliferation, induces apoptosis, and up-regulates the expression of Fas, caspase-3, and caspase-8. The role of Fas as a regulator of chrysotile-induced apoptosis in BEAS-2B cells was tested by the prominent increase in and partial blockade of the apoptotic rate with CH11 and ZB4. When CH11 was pretreated, a synergistic effect was appar-

ent on chrysotile-induced apoptosis and the mRNA and protein expression levels of Fas and cleaved caspase-3. **Conclusion:** Chrysotile causes the apoptosis of BEAS-2B cells via the Fas death receptor pathway. The Fas-mediated apoptosis pathway plays an important role in chrysotile-induced apoptosis of BEAS-2B cells in vitro.

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Introduction

Asbestos causes a high incidence of lung cancer through a slow and progressive pathology. The most commonly occurring form of asbestos is the serpentine mineral chrysotile, which is the main fiber detected in asbestos machining plants and outdoor air [1]. Previous epidemiological studies have shown that exposure to chrysotile and other general mixed amphibole fibers in workers is associated with carcinogenic effects [2]. Some experimental studies argue that chrysotile asbestos is less biopersistent in the lung than the amphibole forms of asbestos (e.g., crocidolite, amosite, and tremolite). There

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is a belief that chrysotile is less potent for the induction of lung cancer than amphiboles, which is strongly opposed to the argument that the lower biopersistence of chrysotile in the lung does not necessarily imply that it would be less potent than amphiboles for lung cancer, considering the relatively low percentage of amphibole fibers in commercial asbestos [3–5]. Moreover, the difference in lung cancer risk between chrysotile and amphibole cohorts is only 2-fold among asbestos textile workers [4, 5].

In animal experiments, bronchial carcinomas [6] and pleural mesotheliomas [7] have been observed in rats after exposure to chrysotile fibers. Furthermore, Davis [8] found the highest tumor incidences (21–38%) in chrysotile-exposed Wistar rats due to the relatively longer chrysotile fibers used in the experiment. Programmed cell death plays critical roles in a wide variety of physiological processes during fetal development and in adult tissues. Two of the major pathways involved in apoptosis in mammalian cells are the Fas/FasL pathway and mitochondrial pathway [9]. Asbestos can induce cell apoptosis in *in vitro* experiments and too much apoptosis contributes to the potential carcinogenicity and physiological cell death [10]. To date, apoptosis has been demonstrated to play an important role in the toxicity of asbestos, but the potential mechanism of chrysotile is rarely reported. In addition, alveolar epithelial [11] and mesothelial cells [12] are known to undergo apoptosis via the mitochondrial pathway when cells are exposed to asbestos. Moreover, it has been demonstrated that the exposure of chrysotile at a lower level induced the activation of caspase-8, the death receptor-activated caspase, and activation of caspase-9, a mitochondrial-activated caspase, in asbestos-exposed alveolar epithelial cells [11]. However, chrysotile may be mediated not only by the Fas-related apoptotic pathway, but also a mitochondrial pathway in human peripheral blood mononuclear cells [13]. Results indicate that apoptosis induced by chrysotile may have an association with the Fas/FasL pathway. However, the mechanism underlying chrysotile-induced apoptosis in bronchial epithelial cells is unclear.

In the present study, we suppose that chrysotile is cytotoxic as the amphibole for human bronchial epithelial cells (BEAS-2B) and explore the pathway involved in the apoptosis of chrysotile. The main goal of this study was to determine whether the mechanism of chrysotile-induced bronchial epithelial cell death could be mediated by Fas activation in order to provide some useful clues for the further study of chrysotile-induced cytotoxicity.

Methods

Cell Culture and Fiber Preparation

A commercially available human bronchial epithelial cell line (BEAS-2B) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). BEAS-2B cells were revived in bronchial epithelial growth medium (Cambrex Bio Science, Walkersville, MD, USA) and grown on fibronectin-coated plates (0.5 mg/mL; Sigma, Gillingham, UK) at 37°C in 5% CO₂ and 100% humidity according to the ATCC guidelines. After serial passage culture of the BEAS-2B cells, they were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (Life Technologies), L-glutamine (2 mM), and nonessential amino acid (2 mM) at 37°C in 5% CO₂ and 100% humidity. For tests, cellular suspension of 5×10^5 , 1×10^5 , and 5×10^4 BEAS-2B cells/mL were respectively plated onto 6-, 24-, and 96-well plates and cultured for 24 h before the fiber exposure. Chrysotile provided by Japan Fibrous Materials Research Association was suspended in distilled water by ultrasonic agitation, and processed by autoclave sterilization.

Cell Viability Detection

Cell viability detection using the CCK-8 assay was performed as previously described [14]. Cells were exposed to different concentrations of chrysotile at various time points. Three independent experiments were performed. Unexposed cells were maintained in the same conditions. To avoid the fiber interference on absorbance tested by spectrophotometry, BEAS-2B cells were seeded in 24-well culture plates for the experiments. A total of 40 µL of CCK-8 solution was added to each well for a further culture at 37°C for 4 h. Next, 100 µL of supernatant were added to 96-well plates and the colorimetric changes of CCK-8 were assayed by optical density measurement at 450 nm.

Detection of Cell Apoptosis by Flow Cytometry

After exposure to 10 µg/cm² of chrysotile at 6, 24, 48, and 72 h, the cell density of 2×10^6 cells/mL were collected, centrifuged, and washed with cold PBS before staining with YO-PRO-1 (100 µM) and PI (1 mg/mL; Molecular Probes, Eugene, OR, USA) for 20 min on ice in the dark. Soon after, the cells were analyzed by flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA, USA) using 488 nm excitation with green fluorescence emission for YO-PRO-1 (530/30 bandpass) and red fluorescence emission for PI (610/20 bandpass), with gating on cells to exclude debris.

Immunofluorescence Assay

The cells were plated on Lab-Tek Chamber Slides (Thermo Fisher Scientific, Waltham, MA, USA). They were fixed with 4% paraformaldehyde for 15 min at room temperature, perforated with 0.1% Triton X-100 for 15 min, washed with PBS and blocked in 10% serum for 30 min. Finally, they were incubated with the Fas antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. The cells were then incubated with a matched secondary antibody (Invitrogen, Grand Island, NY, USA) and Hoechst 33342 solution (2 µg/mL in PBS) for 30 min in the dark and visualized and recorded using fluorescence microscopy (DMI4000B, Leica, Wetzlar, Germany) at a magnification of $\times 40$.

Quantitative Real-Time PCR

Total RNA was extracted using a high-purity RNA Isolation kit (Qiagen, Hilden, Germany) and then total RNA was converted

into cDNA (Invitrogen Life Technologies). Quantitative PCR was performed in the StepOne Plus machine using the SYBR Green PCR mix (Qiagen, Hilden, Germany). The primers used were as follows: Fas, forward 5'-TTCACCTTCGAGGATTGCTC-3', reverse 5'-GGCTTATGGCAGAATTGGCC-3'; caspase-3, forward 5'-TGGTTCATCCAGTCGCTTTGT-3', reverse 5'-CAAATTCTGTTGCCACCTTTTCG-3'; caspase-8, forward 5'-TGTTGATTTGGGCACAGACT-3', reverse 5'-ATGGGGAAGGTGAAGGTGCG-3', and GAPDH, forward 5'-GGAGTCAACGGATTTGGT-3', reverse 5'-GTGATGGGATTTCCATTGAT-3'. The relative copy number was carried out according to the manufacturer's protocol. The values of target genes were normalized to the values of a housekeeping gene, GAPDH, and the relative expression values were calculated from Ct values using the $\Delta\Delta C_T$ method with untreated cells as a control. Each sample was set up in triplicate, and the experiment was repeated at least twice. The statistical significance of differences in the target gene expression levels between different treatments was assessed by *t* test, at $\alpha = 0.01$. Cells were preincubated with various concentrations of anti-Fas antibody (CH11; Upstate, Lakeland, NY, USA) and antagonistic anti-Fas antibody (ZB4; Upstate) for 2 h.

Western Blotting Analysis

In total, 40 μ g of proteins were loaded on 13% polyacrylamide gels and electrophoresed. After transfer to nitrocellulose membranes, the membranes were blocked with 5% nonfat milk in TBS (20 mM Tris-HCl and Tween 0.1%) and incubated overnight at 4°C with anti-Fas (1:1,000, Santa Cruz), anti-caspase-3 (1:1,000), anti-caspase-8 (1:500, Cell Signaling Technology, Beverly, MA, USA), or anti-GAPDH antibodies, respectively. GAPDH was probed as an internal loading control. Goat anti-mouse IgG or goat anti-rabbit IgG were used as second antibodies. Membranes were washed with TBS and incubated in enhanced chemiluminescence detection reagents to visualize the proteins of interest on a Light Gel Imaging System (FluorChem HD2, Alpha Innotech, San Leandro, CA, USA). The cells were preincubated with various concentrations of CH11 and ZB4 for 2 h.

Statistical Analysis

The statistical significance of differences between the individual treatments was evaluated by 1-way ANOVA Tukey test (SPSS 17.0, SPSS Inc., Chicago, IL, USA). Data are the means $\pm$ SD of 3 independent experiments. Statistical comparisons were performed at a significance level of $p < 0.05$.

Results

Cell Viability of Chrysotile on BEAS-2B

We tested the degree of chrysotile on bronchial cell viability by CCK-8 analysis using 5, 10, 20, 40, and 100 μ g/cm² of chrysotile at 48 h. We noticed no significant inhibition of viability in BEAS-2B with 5, 10, and 20 μ g/cm² of chrysotile. However, there was a significant decrease in cell viability with 40 and 100 μ g/cm² of chrysotile in a dose-dependent manner (Fig. 1a). No significant inhibition of cell viability was demonstrated with 10 μ g/cm² of

Fig. 1. The inhibition of cell viability related to chrysotile in a human bronchial epithelial cell line (BEAS-2B). **a** Adherent cells were incubated with different concentrations of chrysotile at 48 h and cell viability was determined by CCK-8 assay. **b** Cultured BEAS-2B cells were treated with 10 μ g/cm² of chrysotile for the indicated times. Results are compared with the untreated control group. Values are the mean $\pm$ SD of 6 determinations. * $p < 0.05$ compared with the control.

chrysotile-treated cells at 6, 24, 48, and 72 h (Fig. 1b). Similar results were further confirmed using MTT assay. Therefore, in further study we used 10 μ g/cm² of chrysotile as the nontoxic dose.

Apoptosis of Chrysotile on BEAS-2B

We quantified the degree of chrysotile on bronchial cell apoptosis by FACS analysis using 10 μ g/cm² of chrysotile at 6, 24, 48, and 72 h. We noticed no significant increase of apoptosis in BEAS-2B for the 6-h treatment compared with untreated cells. A significant increase of apoptosis was seen at later time points. Chrysotile significantly increased BEAS-2B apoptosis in a time-dependent manner (Fig. 2). Respectively, there were 6, 11, and 15% increases in BEAS-2B apoptosis of chrysotile-treated cells at 24, 48, and 72 h.

Fig. 2. The induction of apoptosis in BEAS-2B cells exposed to chrysotile. Cultured BEAS-2B cells were treated with $10 \mu\text{g}/\text{cm}^2$ of chrysotile for the indicated times. Apoptotic cells were examined by flow cytometry. Results are compared with the untreated control group. Each value is the mean $\pm$ SD of 3 determinations. * $p < 0.05$ compared with the control.

Fig. 3. Chrysotile-induced Fas expression in cultured BEAS-2B cells. Cells were treated with $10 \mu\text{g}/\text{cm}^2$ of chrysotile for 48 h. Fas mRNA and proteins were detected by real-time PCR (**a**) and Western blotting (**b**). Band densities were measured by imaging software. * $p < 0.05$ compared with the control.

Fig. 4. Fas proteins were detected by immunofluorescence assay. Chrysotile significantly increased Fas expression in BEAS-2B cells (**a**) compared with the control group (**b**). Original magnification, $\times 400$.

Expression of Fas mRNA and Protein in Chrysotile-Treated BEAS-2B Cells

In order to confirm the Fas-mediated apoptosis pathway involved in chrysotile-induced apoptosis, we used quantitative real-time PCR and Western blot analysis to measure the expression of Fas expression in mRNA and protein, respectively. The level of Fas mRNA in BEAS-2B cells was upregulated with $10 \mu\text{g}/\text{cm}^2$ of chrysotile at 48 h compared with untreated cells (Fig. 3a). The results also showed the same tendency for the expression level of Fas protein (Fig. 3b). In a previous study, the expression of β -actin mRNA was not stable in chrysotile-treated BEAS-

2B cells [15]. GAPDH was therefore chosen as the internal control in the quantitative PCR experiments and Western blot analysis. We also measured Fas protein expression in chrysotile-treated and untreated cells by immunofluorescence analysis (Fig. 4). In chrysotile-treated BEAS-2B cells, a remarkable increase in immunoreactive staining of Fas protein was seen compared with untreated BEAS-2B cells. Together with the Western blot results, this confirmed that chrysotile increased the expression of Fas significantly in BEAS-2B cells.

Fig. 5. CH11 (Fas antibody) significantly increased chrysotile-induced apoptosis in BEAS-2B cells. CH11-treated BAES-2B cells displayed a higher apoptosis than untreated cells in a dose- (a) and time- (b) dependent manner. * $p < 0.05$ compared with the control group; + $p < 0.05$ compared with the group only treated with chrysotile.

The Fas Death Receptor Pathway May Be a Possible Pathway of Chrysotile-Mediated Apoptosis

To investigate the role of the Fas death receptor signaling pathway in chrysotile-induced apoptosis, we used various concentrations of anti-Fas antibody (CH11) and antagonistic anti-Fas antibody (ZB4). When cells were pretreated with different concentrations of the CH11 antibody, the apoptotic effect of chrysotile ($10 \mu\text{g}/\text{cm}^2$) was effectively increased compared with control group at 48 h (Fig. 5a). After incubation with CH11 (500 ng/mL) for 6, 24, 48, 72 h, the apoptotic rates of the chrysotile-treated groups were significantly higher than the nonincubated control group (Fig. 5b). The apoptotic effect of chrysotile was synergistically increased by CH11 only compared with chrysotile-treated cells. Therefore, CH11 increased chrysotile-induced apoptosis in a dose- and time-dependent manner. As shown in Figure 6a, when different concentrations (100–300 ng/mL) of ZB4 were incubated with $10 \mu\text{g}/\text{cm}^2$ of chrysotile for 48 h, apoptosis was slightly

Fig. 6. a ZB4 did not attenuate chrysotile-induced apoptosis significantly in BEAS-2B cells. **b** The antagonistic anti-Fas antibody ZB4 showed a slight inhibition of chrysotile-mediated apoptosis in BEAS-2B cells. * $p < 0.05$ compared with control group.

decreased (Fig. 6a). When BAES-2B cells were pretreated with 300 ng/mL of ZB4 and incubated with chrysotile for 6, 24, 48, and 72 h, no significance apoptosis difference was observed compared with the group only treated with chrysotile (Fig. 6b). It was therefore shown that ZB4-inhibited chrysotile did not induce apoptosis in a significant concentration- and time-dependent manner.

CH11 Increased Fas, Caspase-3, and Caspase-8 Expression in Chrysotile-Treated Cells

When chrysotile was incubated with 500 ng/mL of CH11 for 48 h, increased expression of Fas, caspase-3, and caspase-8 in mRNA and protein were shown. When pretreated with the CH11 antibody, the mRNA expression of Fas, caspase-3, and caspase-8 was effectively increased compared with the group only treated with chrysotile (Fig. 7). Chrysotile and CH11 antibody-treated BAES-2B cells had a higher protein expression of Fas than the control cells. Chrysotile and CH11 decreased the pro-

Fig. 7. Expression of Fas, caspase-3, and caspase-8 mRNA in BEAS-2B cells pretreated with 500 ng/mL of CH11. Relative mRNA expression of Fas, caspase-3, and caspase-8 in BEAS-2B cells treated with either chrysotile or CH11 demonstrated increased levels compared with untreated controls. The Fas, caspase-3, and caspase-8 mRNA expression levels are relative to GAPDH expression. * $p < 0.05$ compared with control group; + $p < 0.05$ compared with the group only treated with chrysotile.

Fig. 8. Western blots for Fas, caspase-3, and caspase-8 activation: lane 1, BEAS-2B cells incubated in control medium; lane 2, BEAS-2B cells treated with CH11 (500 ng/mL); lane 3, BEAS-2B cells stimulated with chrysotile for 48 h; lane 4, 10 $\mu\text{g}/\text{cm}^2$ of chrysotile and 500 ng/mL of CH11-treated BEAS-2B cells for 48 h. GAPDH was used to standardize the amount of protein.

tein levels of procaspase-3 and procaspase-8, and increased those of cleaved caspase-3 (Fig. 8). CH11 significantly regulated chrysotile-mediated Fas, caspase-3, and caspase-8 expression in BEAS-2B cells, suggesting that Fas-dependent apoptosis is critically involved in chrysotile-induced bronchial epithelial cell death.

Discussion

Chrysotile is the only asbestos fiber type being mined today and the chrysotile-related lung cancer burden should be analyzed in the future. In this study, we provided evidence that chrysotile can inhibit cell proliferation in a cultured BEAS-2B cell line, and induce cell apoptosis in a time- and dose-dependent increase. Furthermore, we showed that the expression of Fas, caspase-3, and caspase-8 were increased in BEAS-2B cells cultured with chrysotile. These results suggest that chrysotile-induced apoptosis is mostly associated with the Fas death pathway.

It is known that apoptosis is an intrinsic cell suicide program through a distinct form of cell death control to remove cells from the body without inducing inflammation [15, 16]. It seems to be a physiological mechanism for apoptosis in mitochondria-dependent and mitochondria-independent pathways. In general, asbestos induces apoptosis mostly linked to the mitochondrial-regulated death pathway in various kinds of cells, such as alveolar

epithelial cells [17]. Although abundant information on cells during the mitochondria-regulated apoptosis is available, little is known about the action of Fas-mediated signaling related with asbestos, especially chrysotile fibers.

Fas/APO-1 (CD95) belongs to the tumor necrosis factor/nerve growth factor receptor family, which is present in the control of the Fas death receptor signaling pathway after cells are bound by a specific antibody or natural Fas ligand [18, 19]. Human bronchial epithelial cells are the most important site of fiber stimulation, and thus thought to be the main cells in malignant transformation [15]. Our data demonstrated that chrysotile induces BEAS-2B cell apoptosis via caspase-8 and caspase-3 activation and has a synergistic effect with the CH-11 agonistic anti-Fas antibody. However, in this study we have also observed that ZB4, an antagonistic anti-Fas antibody, is not effectively decreased in chrysotile-induced apoptosis. The different results are due to a variety of causes. It is possible that the partial inhibition of apoptosis in BEAS-2B cells was due to the existence of other pathways of apoptosis induced by chrysotile [20], the dose of ZB4 was low or the irreversible fiber phagocytosis induced apoptosis [21]. These results suggest that the Fas apoptotic pathway is pivotal in the apoptosis of BEAS-2B in vitro, which may have some importance in the clearing of overproliferative BEAS-2B cells and cytoprotection. Furthermore, we observed that CH11 regulated the expression of Fas, caspase-3, and caspase-8 in chrysotile-treated cells. There-

fore, the Fas death receptor signaling pathway may contribute to the apoptosis induced by chrysotile as a result of an imbalance between cell proliferation and cell death. Besides the mitochondrial apoptotic pathway and Fas death receptor apoptotic pathway, autophagy has also been shown to be a type of programmed cell death involved in chrysotile-treated cells [22]. The autophagic pathway also plays a different role in cell death, suggesting that an early activation of apoptosis on human bronchial epithelial cells induced by chrysotile may be dependent on several pathways in order to induce death.

The mitochondrial apoptotic pathway usually relates to intracellular reactive oxygen species, the level of cytosolic cytochrome *c*, and cleavage of caspase-9 and caspase-3 [22]. The Fas death receptor apoptotic pathway may partly contribute to certain physical characteristics (length, diameter, and iron and ferritin concentrations) [23, 24], the level of Fas, and cleavage of caspase-8 and caspase-3 [25]. Moreover, chrysotile not only induces cell apoptosis via the Fas-mediated pathway, but also stimulates the pleural mesothelial cells to secrete IL-1 β and IL-6 [26], causing cell proliferation and migration. Costello et al. [27] showed Fas-induced apoptosis could improve tumor immunogenicity.

Acencio et al. [26] found that crocidolite and chrysotile both induce the acute inflammatory response in cultured mesothelial cells. They speculate that cytokines may contribute to the asbestos-induced inflammatory environment and damaged cells may somehow survive asbestos injury, leading to pleural inflammation, fibrosis, and eventually to a tumor. It is important in the balance between cell proliferation and apoptosis because the failure of apoptosis can trigger an inflammatory reaction or ma-

lignant transformation. However, exuberant apoptosis may also develop epithelial barrier dysfunction and lung inflammation [28].

The findings of our pivotal study are significant because they show the toxic effect of chrysotile on human bronchial epithelial cells in terms of apoptosis and the regulation of the Fas death receptor pathway. Fas or FADD siRNA may be used in further studies to confirm the role of the Fas receptor on chrysotile stimulation due to the low effect of the antagonistic anti-Fas antibody.

In conclusion, chrysotile is able to cause BEAS-2B apoptosis through the Fas-mediated pathway. Therefore, it seems reasonable to state that chrysotile triggers apoptosis pathways like other types of asbestos. We believe the bronchial epithelium may play an important role in the defense against chrysotile stimulation. The results of this study, which strongly suggest that Fas-mediated apoptosis is involved in the stimulation of chrysotile to BEAS-2B cells, will further our understanding of the pathogenesis of pulmonary diseases associated with fiber stimulation. In addition, this finding implies the importance of anti-Fas treatment for a therapeutic approach in human airway epithelial cells.

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