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Functional Alteration of Alveolar Macrophages Exposed to Asbestos Fibers: the Production of TGF-β, Apoptosis and the Generation of Multinucleated Giant Cells

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Abstract

Alveolar macrophages (AMs), which can produce TGF-β as well as inflammatory cytokines, have been thought to play a critical role in evoking lung fibrogenesis in asbestosis. In the present study, we performed experiments involving intratracheal instillation of male Wistar rats with chrysotile B (CH) and in vitro exposure of AMs to CH, to examine the production of TGF-β1, apoptosis and generation of multinucleated giant cells (MGCs) by the AMs. Five days after instillation with 4 mg of CH, bronchoalveolar lavage fluids (BALFs) recovered from the rats showed increases in the production of TGF-β1 and the percentages of annexin V $(Anx)^{+}PI^{-}$ early apoptotic cells, $Anx^{+}PI^{+}$ late apoptotic cells and DNA-degraded cells, and showed the generation of MGC. The AMs in these BALFs produced a significantly higher amount of TGF-β1 after culture for 5 days than those from the control group of rats. The AMs from the control group produced a high amount of TGF-β1 in the culture with 10 μg/ml CH. However, apoptosis of AMs was not induced at this concentration of CH, while 50 μg/ml CH markedly induced increases in the percentages of $Anx^{+}PI^{-}$, $Anx^{+}PI^{+}$ and DNA-degraded apoptotic cells. CH and the apoptotic cells did not directly induce the generation of MGCs by AMs in vitro, in contrast to the results obtained after intratracheal instillation with CH. These results indicate that AMs can autonomously augment the production of TGF-β1 regardless of the interaction with lung epithelial cells or fibroblasts. In addition, it was found that the exposure of AMs to CH can induce the two different responses of apoptosis and TGF-β1 production, dependent on the CH dose, and that these responses are independent of each other. Suppression of AMs with such fibrogenic ability may be crucial in preventing the progress of lung fibrogenesis.

Introduction

Chrysotile B can induce a number of different responses related to inflammation and fibrogenesis in the lung, in which alveolar macrophages (AMs) are thought to play a critical role (1). In particular, the production of TGF-β1 by AMs is very important for the progress of pneumoconiosis because of its ability not only to promote the production of extracellular matrix but also to suppress immune functions. In the present study, we examined the responses of apoptosis, TGF-β1 production and multinucleated giant cell (MGC) generation in rat AMs exposed to chrysotile *in vivo* and *in vitro*. Then, we discussed the effects of the interaction between AMs and other types of cells in the bronchoalveolar space and the concentration of chrysotile on those responses in AMs.

Material and Methods

Chrysotile instillation and bronchoalveolar lavage

The Canadian chrysotile B (length: 2.6 ± 2.3 μm, width: 0.15 ± 1.8 μm, these represent the geometrical mean ± SD) used in the present study was Union International Contre le Cancer standard reference material [2]. Male Wistar rats (Oriental Yeast Co., Ltd., Tokyo, Japan) were instilled with 4 mg chrysotile or saline via trachea [3]. Five days after instillation, the lungs and the heart were removed from the rats, and bronchoalveolar lavage fluids (BALFs) were collected by washing the lungs with calcium- and magnesium-free Hank's balanced salt solution (HBSS) [4]. All animals were handled according to protocols approved by the Animal Studies Committee of Hyogo College of Medicine.

Preparation and culture of alveolar macrophages

AMs adhering to the culture plate were obtained by pre-incubation of the cells recovered from BALFs for 1 hr and the subsequent removal of non-adherent cells. The culture medium used was Dulbecco's modified MEM supplemented with 4 % fetal calf serum (FCS). To eliminate the effect of TGF-β1 included in FCS, 4 % serum-replacement (Sigma, St. Louis, MO) was used in the culture of AMs for the assay of TGF-β1 production. The prepared AMs were cultured at 4.5 x 10^5 cells per well in 24-well culture plates with or without 10 μg/ml chrysotile. After 5 days of culture, the supernatants were assayed for the production of TGF-β1, and the cells adhering to the well bottom were stained by May-Grünwald-Giemsa to evaluate the generation of MGCs. To examine apoptosis induced by the *in vitro* exposure of chrysotile, AMs were cultured with 10 or 50 μg/ml chrysotile, and assayed for apoptosis after 5 or 24 hr of culture.

Measurement of TGF-β1

The BALFs and the culture supernatants were assayed for the production of TGF-β1 by Quantikine ELISA kit (R&D Systems, Minneapolis, MN) in accordance with the manufacturer's instructions. Finally, the absorbance at 450 and 570 nm in each well was measured by a Corona Microplate reader MTP100 (Corona Electric, Ibaragi, Japan).

Flow cytometric analysis of apoptosis

AMs were harvested by flushing at 0, 5 or 24 hr of culture and assayed for apoptosis by several methods. In method one, an Annexin-V-FLUOS Staining Kit (Roche Diagnostics, Mannheim, Germany) was used to assay annexin V-positive (Anx^+) propidium iodide-negative (PI^-) early apoptotic cells and Anx^+PI^+ late apoptotic cells by flow cytometry. In the second method, the harvested cells were fixed with cold 70 % ethanol and stained with 50 μg/ml PI. Cells with a lower DNA content than diploid cells, which lost some degraded DNA, were counted as apoptotic cells with DNA degradation by flow cytometry.

Statistical analysis

Significant differences between culture conditions were determined by ANOVA and repeated measures ANOVA. The statistical analysis was performed by StatView 5.0J (SAS Institute, Cary, NC) software referring to Statistical Principles in Experimental Design [5].

Results

TGF-β1 production and apoptotic cells in BALFs

BALFs collected from the rats 5 days after chrysotile instillation were assayed for the production of TGF-β1 and the percentages of several stages of apoptotic cells (Table 1). The intratracheal

instillation with chrysotile significantly increased the production of TGF-β1 in BALFs compared with saline instillation. In addition, chrysotile instillation significantly increased the populations of Anx^+PI^- early, Anx^+PI^+ late apoptotic cells and DNA-degraded cells.

Table 1. The production of TGF-β1 and the percentages of several stages of apoptotic cells

intratracheal instillation	TGF-β1 (pg/ml)	Annexin V^+PI^- (%)	Annexin V^+PI^+ (%)	DNA-degraded (%)
Saline	55.31 ± 10.53	2.73 ± 0.49	3.94 ± 0.27	6.93 ± 0.81
Chrysotile	234.61 ± 65.10 *	11.14 ± 1.51 **	11.81 ± 0.67 **	42.39 ± 7.17 **

The data represents means ± SE from six rats. Statistically significant differences between instilled materials are shown (**p < 0.01, *p < 0.05)

The generation of MGCs induced by chrysotile instillation

The cells in BALFs from the rats pre-treated with intratracheal instillation of chrysotile showed the generation of MGCs, but their frequency was very low and most had a low degree of multinucleation (not shown). However, the culture of AMs prepared from these BALFs, which included few MGCs, accelerated the generation and the enlargement of MGCs (Fig. 1).

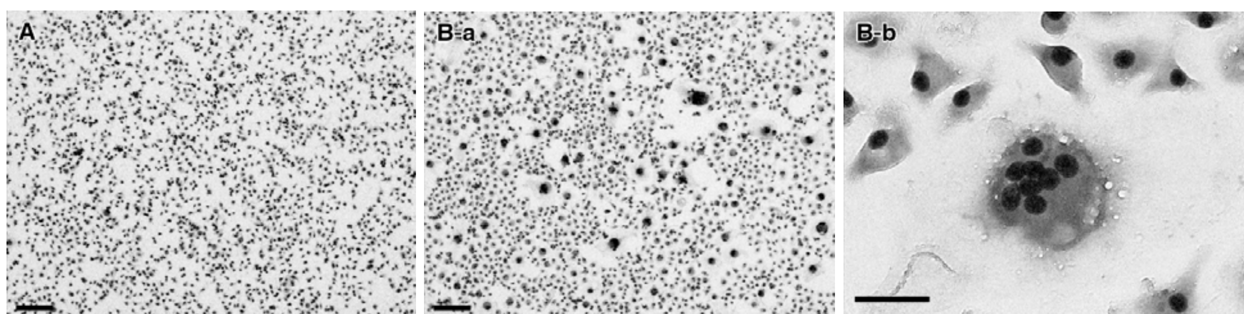

Figure 1. The generation of MGCs induced by intratracheal instillation with chrysotile These are representative micrographs of the AMs cultured for 5 days. A and B show the AMs from the rats instilled with saline and chrysotile, respectively. MGCs can be frequently observed in B. The scale bars show 150 and 30 µm in A and B-a and in B-b, respectively.

TGF-β1 production in the culture of alveolar macrophages

AMs from rats instilled with chrysotile or saline were cultured with or without 10 µg/ml chrysotile, and assayed for the production of TGF-β1 (Table 2). The AMs from the rats instilled with chrysotile produced a significantly increased amount of TGF-β1. The additional stimulation with chrysotile *in vitro* could not augment the amount of TGF-β1 produced by these AMs. However, the AMs could produce a high amount of TGF-β1 by *in vitro* exposure alone to chrysotile at the same level as the AMs exposed *in vivo*.

Table 2. TGF-β1 production in the culture of AMs

	Condition I	Condition II	Condition III	Condition IV
Intratracheal instillation with 4 mg chrysotile	-	-	+	+
In vitro exposure to 10 µg/ml chrysotile	-	+	-	+
TGF-β1 production (pg/ml)	262.46 ± 9.95	495.65 ± 16.23 **	506.91 ± 6.90 **	488.84 ± 31.29 **

AMs were cultured for 5 days and assayed for TGF-β1 production. The data is means ± SE from triplicate culture wells. This is the representative data in the four independent experiments. Statistically significant differences from Condition I are shown (**p < 0.01)

Apoptosis of alveolar macrophages exposed to chrysotile in vitro

AMs were cultured with direct exposure to chrysotile and assayed for apoptosis by flow cytometry (Table 3). AMs could not undergo apoptosis at 10 µg/ml of chrysotile, which could augment the production of TGF-β1 *in vitro* as shown in Table 2. However, 50 µg/ml of chrysotile strongly induced apoptosis in AMs showing a significant difference from the culture without chrysotile. The

percentage of Anx^+PI^- early apoptotic cells reached a peak at 5 hr, and the percentages of Anx^+PI^+ late apoptotic cells and DNA-degraded cells gradually increased from 5 to 24 hr.

The de novo generation of MGCs in the culture of AMs with chrysotile

It is known that macrophages can fuse and generate several types of MGCs in the various conditions of culture [6]. Therefore, we examined the *de novo* generation of MGCs in the culture of AMs with chrysotile at several concentrations from 10 to 100 µg/ml. After 5 days of culture, however, MGCs could not be induced specifically by chrysotile (not shown), and AMs mostly just died by apoptosis at more than 50 µg/ml of chrysotile, as described above (Table 3). These results prompted us to hypothesise that early apoptotic cells induced by chrysotile might be involved in the generation of MGCs. To confirm this hypothesis, AMs cultured with chrysotile at 50 µg/ml for 4 hr were diluted fivefold, to give a concentration of chrysotile of about 10 µg/ml, and added to the culture of AMs without any additional chrysotile. Contrary to our supposition, the apoptotic cells could not specifically induce the *de novo* generation of MGCs (not shown).

Table 3. The percentages of apoptotic cells in the AMs exposed to chrysotile *in vitro*

		The percentage of apoptotic cells		
		Annexin V^+PI^-		
Chrysotile (µg/ml)	Incubation	0 hr	5 hr	24 hr
0.0		2.44 ± 0.82	2.45 ± 0.09	4.10 ± 0.49
10.0		2.44 ± 0.82	8.54 ± 2.82	11.65 ± 2.30
50.0		2.44 ± 0.82	33.91 ± 1.38 **	19.11 ± 1.64 **
		Annexin V^+PI^+		
Chrysotile (µg/ml)	Incubation	0 hr	5 hr	24 hr
0.0		3.56 ± 0.33	6.96 ± 1.24	8.67 ± 1.29
10.0		3.56 ± 0.33	10.71 ± 1.95	17.85 ± 5.37
50.0		3.56 ± 0.33	30.48 ± 2.76 **	64.02 ± 0.64 **
		DNA-degraded		
Chrysotile (µg/ml)	Incubation	0 hr	5 hr	24 hr
0.0		5.96 ± 1.00	7.43 ± 1.75	12.89 ± 4.18
10.0		5.96 ± 1.00	9.05 ± 2.27	16.02 ± 3.90
50.0		5.96 ± 1.00	17.53 ± 3.15	55.43 ± 3.55 **

AMs were cultured with or without 10 or 50 µg/ml chrysotile for 5 or 24 hr, and assayed for apoptosis by flow cytometry. The data represent means ± SE from three experiments. Statistically significant differences from the culture without chrysotile are shown (**$p < 0.01$)

Discussion

AMs exposed to chrysotile only *in vitro* can produce a high amount of TGF-β1; at the same level as the AMs obtained from the rats pre-treated with intratracheal instillation of chrysotile. These results indicate that AMs can autonomously augment the production of TGF-β1 regardless of the interaction with lung epithelial cells or fibroblasts. In concurrence with the previous studies [7], AMs cultured with the high dose of chrysotile showed the representative progress of apoptosis, which is the translocation of phosphatidylserin to the outer layer of the plasma membrane visualized by fluorescein-conjugated Anx and the subsequent DNA-degradation. Apoptosis of AMs is thought to be an important event in the initiation of pneumoconiosis [7, 8]. However, the low dose of chrysotile 10 µg/ml could induce only negligible levels of apoptosis in AMs, although their production of TGF-β1 could be definitely augmented even at the low dose. These results indicate that the exposure of AMs to chrysotile can induce the two different responses of apoptosis and TGF-β1 production according to dose and that these responses are independent of each other. In contrast with the production of TGF-β1, the generation of MGCs could not be induced specifically by the *in vitro* exposure of AMs to chrysotile, which suggests the need of the interaction between AMs and other types of cells in the bronchoalveolar space for the generation of MGCs. Taken

together, our present study demonstrated that such functional alterations in AMs induced by direct exposure to the low, but not the high, dose of chrysotile can play a critical role in initiating and accelerating lung fibrogenesis. Further studies on the responses in AMs induced by low doses of chrysotile and other toxic fibers or particles will be able to contribute to revealing the mechanism of lung fibrogenesis.

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