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Exposure of Macrophages to Asbestos or Man-Made Mineral Fibers Causes Oxidative Stress through a Change in the Levels of S-nitrosothiol and Cellular Glutathione

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Abstract

Alveolar macrophages (AMs) phagocytose asbestos and/or man-made mineral fibers (MMMFs) inhaled into the alveolar space, and are stimulated to express inducible nitric oxide (NO) synthase and continuously generate a large amount of NO and other reactive nitrogen species (RNS). Moreover, AMs stimulated by pathogenic particles generate a large amount of reactive oxygen species (ROS) and free radicals. It is widely accepted that ROS and RNS exert oxidative stress on cells and tissues, and cause inflammation and other pathological changes. NO can non-enzymatically react with the free sulfhydryl groups (-SH) of reduced glutathione (GSH) and various proteins to form S-nitrosoglutathione (GS-NO) and S-nitrosothiols (RS-NO), respectively. RS-NO, including GS-NO potentially affects the function of enzymes and other proteins and causes cellular and tissue damage. However, the role of nitrosation of SH groups in GSH, enzymes and other proteins in the pathogenesis of asbestos-induced diseases remains to be further studied. In the present study, we focused on the extent of thiol-nitrosation with NO.

RAW264.7 cells and J774 cells of murine macrophage cell lines were cultured with chrysotile B (CH) asbestos, crocidolite (CR) asbestos or MMMFs comprised of glass wool (GW), rock wool (RW) or ceramic (RF1). All of these fibers significantly increased NO production and RS-NO formation in the culture with macrophages. CH, CR and GW significantly decreased the level of GSH in RAW264.7 cells. A large portion of the increased RS-NO may be in the form of GS-NO, because GSH is the most abundant thiol substance in the cell. These results indicate that macrophages exposed to asbestos or MMMFs are subject to oxidative stress not only through the generation of ROS and RNS, but also through decreases in the level of the cellular antioxidant, GSH, by GS-NO formation. The increase of RS-NO in macrophages exposed to asbestos or MMMFs may deserve more attention as the indicator of continuous oxidative stress on cells and tissues, which causes inflammation and involves the development of asbestos-induced diseases.

Introduction

It is well known that asbestos fibers cause inflammation, fibrosis and malignant tumors in the lung and pleura[1]. Therefore, occupational and non-occupational exposure to asbestos has become a serious social concern[1, 2]. However, the molecular mechanisms for the development of such asbestos-induced diseases have not been established. In addition, man-made mineral fibers (MMMFs) have come into heavy use as substitutes for asbestos, although the toxicity of MMMFs also remains obscure and requires further study[3].

Alveolar macrophages (AMs) play an important role as the first line of defense against inhaled particulates. AMs phagocytose asbestos and/or MMMFs inhaled into the alveolar space, and are stimulated to cause a respiratory burst, which results in the generation of a large amount of reactive oxygen species (ROS) and free radicals. Moreover, AMs stimulated by the pathogenic particles express inducible nitric oxide (NO) synthase and continuously generate a large amount of NO as a reactive nitrogen species (RNS) [4, 5].

It is widely accepted that ROS and RNS exert oxidative stress on cells and tissues, and cause inflammation and other pathological changes. NO can non-enzymatically react with the free sulfhydryl groups (-SH) of reduced glutathione (GSH) and various proteins to form *S*-nitrosoglutathione (GS-NO) and *S*-nitrosothiols (RS-NO), respectively[6]. GS-NO and RS-NO represent a storage pool of NO[7, 8], resulting in a decrease in intracellular GSH levels, potentially affecting the function of enzymes and other proteins and leading to cellular and tissue damage. However, the role of nitrosation of SH groups in GSH, enzymes and other proteins in the pathogenesis of asbestos-induced diseases remains to be further studied. In the present study, we focused on the extent of thiol-nitrosation with NO.

Materials and Methods

The chrysotile-B (CH, length: 2.6 ± 2.3 μm, width: 0.15 ± 1.8 μm, these represent the geometrical mean ± SD) and crocidolite (CR, length: 2.5 ± 2.0 μm, width: 0.33 ± 2.1 μm) asbestos used in the present experiment were Union Internationale Contre le Cancer standard reference materials[9]. The glass-wool (GW, length: 20.0 ± 2.58 μm, width: 0.88 ± 3.10 μm), rock-wool (RW, length: 16.5 ± 2.51 μm, width: 1.80 ± 2.32 μm) and ceramic fiber used (refractory fiber 1, RF1, length: 12.0 ± 2.36 μm, width: 0.77 ± 2.53 μm) were international standard reference samples of MMMFs provided by the Japan Fibrous Material Research Association[10]. Diaminofluorescein (DAF)-2, which is a fluorescent dye having the advantages of high-sensitivity, non-cytotoxicity and high-specificity to NO, was used to detect NO released from RS-NO (Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan). Other reagents were of analytical grade and were purchased from Sigma Chemical Co. or Wako Pure Chemical Industries, Ltd., Osaka, Japan.

RAW264.7 and J774 cells of the murine macrophage cell lines were obtained from the American Type Culture Collection (Rockville, MD). Cells were seeded at 1×10^6 cells per well of a 12-well plate (Asahi Techno Glass Co., Tokyo, Japan), and cultured in 1 ml of phenol red-free Dulbecco's modified Eagle's minimum essential medium (DMEM, Sigma Chemical Co., St. Louis, MO) containing 4% heat-inactivated (56°C, 30 min) fetal bovine serum (FBS, ICN Biomedicals Inc., Aurora, OH), 100 U/ml penicillin G and 100 μg/ml streptomycin (4% FBS-DMEM) with each type of fiber (100 μg) in a humidified atmosphere with 5% CO_2. The culture medium was collected at 24 h after the addition of fibers.

All animal experiments were performed with male Wistar strain rats weighing 300-380 g according to protocols approved by the Animal Studies Committee of the Hyogo College of Medicine. The rats were kept at 24°C and allowed free access to standard rat chow and tap water. The rats were instilled with 0.4 ml of physiological saline without/with 1 or 4 mg/rat of CH via the trachea under anesthesia with sodium pentobarbital. After 24 or 48 h of instillation, AMs were collected by

bronchoalveolar lavage (BAL) of the rats[11]. The BAL was repeated 4 times with 5 ml each of phenol red-, calcium- and magnesium-free Hanks' balanced salt solution (HBSS, Sigma Chemical Co., St. Louis, MO). The BAL fluids (BALFs) of each rat were kept on ice and spun down at 800 × g for 10 min. The supernatant obtained from the first BAL was collected as the first BALF. The resulting cell pellets were re-suspended in fresh HBSS and combined. Erythrocytes in the suspension were lysed by hypotonic shock and removed. The remaining cells were washed twice and cultured at 1×10^6 cells per well of a 12-well plate in 1 ml of 4% FBS-DMEM for 24 h.

The accumulation of nitrite (NO_2^-) was taken as a measurement of NO production by macrophages. An aliquot (200 μl) of the culture supernatant or the first BALF was mixed with 400 μl of a Griess reagent[12]. The reaction mixture was allowed to stand for 10 min at room temperature in the dark, and then absorbance of the reaction mixture was measured at 540 nm. A standard curve was constructed using $NaNO_2$.

The fluorometry assay method using DAF-2 for the RS-NO determination[13] was partly modified. The thermolysis was adopted as the way to release NO from RS-NO[14]. Briefly, the culture supernatant was first diluted 5-fold with phosphate buffered saline to avoid the influence of other substances on fluorescence. The diluted supernatant or the first BALF, in which DAF-2 concentration was adjusted to 5 μM, was heated at 80°C for 12 min, and then cooled on ice. Fluorescence intensity was then measured with a spectrophotometer (F-4500, Hitachi, Tokyo, Japan). Emission fluorescence at 515 nm was monitored upon excitation at 495 nm. The fluorescence intensity of a blank solution was subtracted from that of the sample solutions. A calibration curve was constructed using *S*-nitrosoglutathione (Dojindo Laboratories, Kumamoto, Japan).

According to the method of Anderson[15], GSH and GSSG were determined. RAW264.7 cells were cultured as described above and harvested. The cells were lysed by three freeze-thaw cycles after the addition of 10 mM hydrochloric acid (100 μl/well). After centrifugation of the lysate at 10000 × g for 5 min, 100 μl of the supernatant was mixed with 50 μl of 10% 5-sulfosalicylic acid. Precipitated protein was removed by centrifugation. The protein-free supernatant was assayed for total cellular glutathione by the GSH reductase recycling procedure. GSSG was assayed after treating GSH with 2-vinylpyridine. The difference between the amount of total glutathione and GSSG represents the amount of GSH.

All data obtained were statistically analyzed by ANOVA and a multiple comparison test. $p < 0.05$ was considered to be statistically significant.

Results and Discussion

As shown in Table 1, when RAW264.7 cells were cultured with 100 μg of CH, CR, GW, RW or RF1 for 24 h, the amount of NO_2^- in the conditioned media significantly increased. It was surely confirmed that macrophages exposed to MMMFs as well as asbestos generated NO. This observation suggests the possibility that macrophages may be damaged by NO radicals and may undergo oxidative stress when exposed to these fibers. It is conceivable that excess production of NO by MMMFs as well as asbestos is toxic.

RS-NO also significantly increased in RAW264.7 cells cultured with 100 μg of fibers for 24 h

regardless of the fiber used (Table 1). The increased RS-NO may largely consist of GS-NO, because GSH can supply a large number of free SH groups utilizable for GS-NO formation. This raises the possibility that GSH converted to GS-NO rather than GSSG in the cells generating a large amount of NO. This possibility is supported by a previous report showing that the addition of NO to human neutrophils depleted glutathione by forming GS-NO[16]. Similarly, SH groups in proteins might also convert to RS-NO. Its level would be lower than that of GS-NO because free SH groups derived from proteins in the cell are, on the whole, fewer than those from GSH. In J774 cells, the amount of NO_2^- significantly increased in the cultures with CH, GW or RF1, but not in the cultures with CR or RW, while RS-NO significantly increased in the cultures with every fiber used (data not shown).

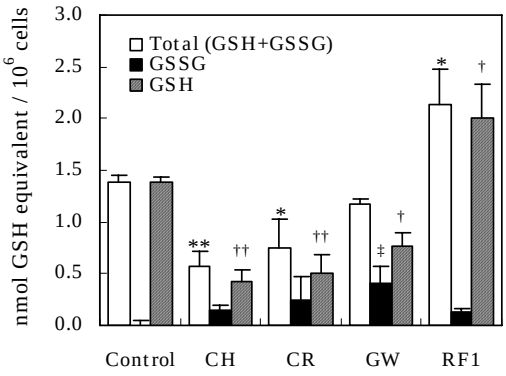
Table 1 Accumulation of NO_2^- and RS-NO in the media of RAW264.7 cells cultured with 100 μg of fibers for 24 h

Fiber	NO_2^- (μM)	RS-NO (μM) (GS-NO equivalent)
None (control)	6.2 ± 0.57	11.01 ± 3.11
CH	10.6 ± 0.95**	14.46 ± 1.37*
CR	11.5 ± 1.14**	17.65 ± 1.78**
GW	10.4 ± 0.42**	17.21 ± 1.18**
RW	8.7 ± 0.75**	15.05 ± 1.86**
RF1	9.4 ± 0.53**	15.46 ± 0.94**

CH, CR, GW, RW and RF1 denote chrysotile, crocidolite, glass wool, rock wool and ceramic fibers, respectively. Each value represents mean ± SD; n = 4; ** and * denote a significant difference from the control value at $p < 0.01$ and 0.05, respectively.

As shown in Fig. 1, when cells were cultured with CH or CR asbestos, total glutathione significantly decreased. It did not vary significantly in the cells exposed to GW, but increased significantly in the cells exposed to RF1. The level of GSH significantly decreased in the cells exposed to CH, CR or GW; contrarily, it increased significantly in the cells exposed to RF1. GSSG concentration was too low to determine precisely in the cultures without fibers. The level of GSSG significantly increased in the cells exposed to GW. In those exposed to CH, CR or RF1, GSSG concentration was higher than that in the control, though the difference from the control value was not significant. GSH, the most abundant nonprotein SH group-containing compound in eukaryotic cells, is a reducing agent and involved in the protection of cells against oxidative damage. The total amount of glutathione (GSH plus GSSG) in the cells significantly decreased with exposure to fibers, while the amount of GSSG tended to increase. It was indicated that the decrease in intracellular total glutathione, following asbestos exposure, was not due to the extracellular release of GSH and GSSG, because no rise in GSH or GSSG in the medium was observed (data not shown). This decrease suggests two possibilities. One is that GSH might be converted to a compound other than GSSG; the other is that synthesis of GSH might be decreased. In any case, decrease of the cellular GSH means enhancement of the oxidative stress in the cells exposed to asbestos or some MMMFs. Contrary to the findings described above, GSH increased in the cells exposed to RF1. This increase implies that synthesis of GSH would be up-regulated in the present experiment, but the details of its mechanism remains unclear.

Fig. 1 Glutathione levels in RAW264.7 cells exposed to 100 μg asbestos or MMMFs for 24 h



and *, † or ‡ denote a significant difference from the control value at $p < 0.01$ and 0.05, respectively.

A decrease in *de novo* GSH synthesis or an increase in decomposition of GSH may be another

reasonable interpretation for the observation of decreased GSH in the present study. Gamma-glutamylcysteine synthase, the rate-limiting enzyme of GSH synthesis, which contains an active thiol site[17], might be inactivated by NO-mediated *S*-nitrosation. Gamma-glutamyl transpeptidase and certain glutathione *S*-transferases are also induced in oxidatively stressed macrophages[18, 19]. This may be a reason for the decrease in GSH in the cells exposed to asbestos or MMMFs.

Generation of superoxide anion (O_2^-) significantly increased in the cultures with CH or RW for 3h (data not shown). This indicates that the cells undergo oxidative stress soon after the exposure to some fibers. Furthermore, it is possible that peroxynitrite anion ($ONOO^-$) is also formed, when a large amount of NO as well as O_2^- generated. It is known that $ONOO^-$ immediately decomposes and produces the reactive hydroxyl radical and nitrogen dioxide and consequently causes lipid oxidation and DNA degradation. In addition to these general effects, $ONOO^-$ may contribute to the significant increase of RS-NO, since the reaction between $ONOO^-$ and the SH groups of proteins and GSH readily forms RS-NO[20].

Findings *in vitro* were confirmed by *in vivo* experiments. As shown in Table 2, RS-NO was not detected in the BALFs obtained from group 1 rats, but was significantly increased in group 2 rats. When AMs from rats instilled with both doses of CH were cultured for 24 h, the amount of RS-NO in the conditioned media was significantly increased. The amount of NO_2^- did not increase in group 1 rats but was increased significantly in the conditioned medium cultured of AMs from group 2 rats. These findings show that AMs of CH-instilled rats should be subject to oxidative stress similar to RAW- 264.7 cells cultured with CH.

Table 2 Changes in the amount of RS-NO and NO_2^- with instillation of chrysotile into the rat trachea

Group	CH treatment	RS-NO (μM) (GS-NO equivalent)		NO_2^- in conditioned medium (μM)
		BALF	Conditioned medium	
Control	None	N.D.	3.75 ± 1.45	2.24 ± 0.60
Group 1	1 mg/rat (24h)	N.D.	7.66 ± 4.65**	3.34 ± 0.81
Group 2	4 mg/rat (48h)	0.18 ± 0.11**	19.1 ± 1.99**	5.19 ± 3.02**

Each value represents mean ± SD; $n = 4$; ** denotes a significant difference from the control value at $p < 0.01$. CH, chrysotile asbestos; BALF, bronchoalveolar lavage fluid; control rats were instilled with physiological saline; NO_2^- was not detected in the BALFs (data not shown). N.D., not detected.

In conclusion, it was demonstrated that RS-NO formation, one of the oxidative stress products, occurred by exposure to asbestos or MMMFs. Elevations in RS-NO formation and a decrease in cellular glutathione level were caused after exposure of macrophages to asbestos or MMMFs. The decrease in total glutathione results at least in part from an increase in consumption of GSH. The possibility that GSH synthesis is inhibited cannot, however, be ruled out. These phenomena suggest that not only asbestos but also MMMFs cause many kinds of oxidative stress through generation of RNS and ROS. RS-NO formation as well as RNS and ROS may be implicated in the development of asbestos-induced diseases. It remains to be further clarified whether nitrosation of thiol sites in enzymes and proteins affects their function. Moreover, future studies will be necessary to examine whether asbestos or MMMFs cause similar oxidative stress in cells other than AMs, such as alveolar epithelial cells, in the development of asbestos-induced diseases.

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