Letter to the Editor

Role of Fas/FasL Pathway-mediated Alveolar Macrophages Releasing Inflammatory Cytokines in Human Silicosis*

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The role of Fas/Fas ligand (FasL) in mediating alveolar macrophage (AM)-derived inflammatory cytokines in 29 human lung silicosis patients and 6 controls was studied. It showed that the serum levels of IL-1β, TNF-α, IL-8, MIP-1α, MCP-1, and TGF-β_1 were higher in silicosis patients than in controls, and increased with the progression of silicosis except TGF-β1 and IL-8. TGF-β_1, IL-8, MCP-1, MIP-1α were efficiently down-regulated by anti-FasL antibody or caspase-8 inhibitor, indicating that Fas/FasL signaling promotes the development of human silicosis by regulating the expression and release of inflammatory cytokines.

Silicosis is one of the most serious pneumoconiosis characterized by lung fibrosis. Alveolar macrophages (AM) aggregate and phagocytose silica particles in alveoli, during which AM are activated to synthesize and release a large amount of cytokines, pro-inflammatory factors, chemokines, and proteases, thus contributing to AM malfunction and disintegration, alteration of alveolar structure, damage to other cells, and culminated in pulmonary interstitial fibrosis[1]. The exact mechanism of silica inhalation underlying necrosis of AMs in humans still remains unclear.

Our previous study showed that the proportion of apoptosis is higher in silicosis patients than in controls, while the expressions of mFas and Caspase-3 increase with the progression of human silicosis[2]. AM engulfing particles such as silica cause lysosomal membrane permeabilization and release of cathepsins into the cytosol where they contribute to apoptosis signaling and inflammation. At this stage, apoptosis leads to incompletely digested particles remaining inside the lungs for an extended period of time[3]. Phagocytosis of apoptotic cells (efferocytosis) is a pivotal regulator of inflammation, because it prevents postapoptotic necrosis and suppresses release of a variety of proinflammatory mediators[4].

Cytokines are proteins or small molecular weight polypeptides, such as lymphokines, interferons, interleukins (IL), tumor necrosis factors (TNF), chemokines, and colony-stimulating factors (CSFs) and function in intercellular communication, immunoregulation, and immune responsiveness. Borges VM, et al. suggested that AM apoptosis is the pathological basis of alveolitis and fibrosis[5]. Apoptotic AM release cytokines such as IL-1, IL-8, TNF-α, and metabolic substances of arachidonic acid. These cytokines can recruit neutrophils and AM, leading to alveolitis, alveolar wall destruction and lung fibrosis. It was reported that AM that phagocytose silica particles are activated to upregulate the expression of pro-apoptotic genes[5]. FasL expressing AM and activated AM synthesize and release a great amount of pro-inflammatory and fibrotic factors, which recruit neutrophils to alveolar spaces and give rise to alveolitis. Cytotoxic and pro-inflammatory factors such as TNF-α, reactive oxygen species (ROS), and soluble Fas (sFas, sCD95) released from neutrophils and activated AM induce pulmonary injury. TGF-β released from AM upon engulfment of an apoptotic cell is involved in remodeling and progression of pulmonary fibrosis[6]. TGF-β1 directly induces epithelial cell apoptosis by activating Fas and caspase-3 and enhancing the FasL-Fas interaction. Aggregation of Fas on the surface of AM that have phagocytosed silica particles leads to IL-1β secretion, which, in turn, induces secretion of chemokines such as macrophage inflammatory protein 1 alpha and beta (MIP-1α, MIP-1β), monocyte chemotactic proteins-1 and 2 (MCP-1, MCP-2), and IL-8. These cytokines can recruit neutrophil extravasation. IL-1β-deficient mice seldom develop silicosis. IL-8 is a key cytokine involved in acute lung injury and fibrosis. FasL and TNF-α induce apoptosis of bronchial epithelium and secretion of IL-8. FasL- and TNF-α-mediated cell apoptosis is not associated with new gene expression and protein synthesis but requires IL-8 secretion.

Current evidence for the role of AM in silicosis derives mainly from *in vitro* and rodent studies, rather than from investigating inflammatory

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cytokines released from AM in human silicosis. In the present study, we hypothesized that Fas/FasL-mediated release of inflammatory cytokines by AM was involved in human silicosis. AM from whole lung lavage fluids of human silicosis patients were used as target cells to study the expression of Fas/FasL signaling proteins, the release of inflammatory cytokines, the relationship of Fas/FasL signaling protein expression with inflammatory cytokines, silica-exposure related factors, molecular mechanisms and potential preventive methods of silicosis.

In the present study, 29 silicosis patients and 13 workers exposed to silica dust (as controls) who underwent whole lung lavage in Beidaihe Sanatorium for China Coal Miners were included in this study. None of the patients exhibited clinical signs/symptoms of autoimmune diseases such as sclerotic skin, Reynaud's phenomenon, facial erythema, arthralgia, or malignancies. Background information was collected before lavage, including occupational history, silica exposure history, silicosis diagnosis, smoking history, and pulmonary complications. This study was approved by Medical Ethics Committee of China Medical University. All patients gave their written informed consent prior to lung lavage.

AM from silicosis patients and controls were isolated, purified and incubated for 24 h. The serum levels of IL-1, IL-8, TNF-α, TGF-β_1, MCP-1, and MIP-1α in silicosis patients and controls were measured in triplicate with ELISA kit according to its manufacturer's specifications.

The purified AM were divided into untreated group, Fas/FasL pathway group, anti-FasL antibody (Santa Cruz, USA) group, and caspase-8 inhibitor-V-IETD-FVM (Santa Cruz, USA) group and incubated for 24 h. The serum levels of IL-1, IL-8, TNF-α, TGF-β_1, MCP-1, and MIP-1α in AM supernatants were measured as previously described.

Data about IL-1, IL-8, TNF-α, TGF-β1, MCP-1, and MIP-1α were analyzed by Student's *t* test, rank sum test, variance analysis, SNK-*q* test, Kruskal-Wallis test or Spearman correlation analysis, using the STATA 10.0 software.

The average age of the patients enrolled in this study was 46.48±8.23 (range: 32-64) years, the average silica exposure time was 15.36±11.50 (range: 2-37) years, the average silica exposure cessation time was 4.18±4.04 (range: 0-13) years, and the smoking rate was 75.0%. No significant difference was found in age, smoking rate, silica exposure time and silica exposure cessation time between controls and silicosis patients.

In the present study, the serum levels of IL-1β, TNF-α, TGF-β_1, IL-8, MCP-1, and MIP-1α were higher in silicosis patients than in controls (P<0.05), the serum levels of TGF-β_1 and TNF-α was lower whereas that of MCP-1 was higher in silicosis patients at stage II or stage III than in those at stage I (P<0.05, Table 1). The serum levels of IL-1β, TNF-α, and TGF-β_1 were positively related to the stage of silicosis (P<0.05). The serum levels of TGF-β_1 and MIP-1α were negatively related to the silica exposure time (P<0.05) whereas those of IL-1β, TNF-α, and MCP-1 were negatively related to with the silica exposure cessation time (P<0.05).

AM promotes development of silicosis by releasing mediators, including fibrotic factors, lysosomal enzymes, ROS, cytokines, and chemokines, that interact in a complex network to affect lung injury, inflammation, and potential fibrosis[7]. Dosreis GA and Borges VM reported that the upregulation of Fas and FasL not only induces cell apoptosis, but also results in release of cytokines and chemokines, thereby recruiting more neutrophils to the alveolar space[6]. These results are consistent with those in a previous study[7]. Tumane RG, et al.[8] reported that IL-8 is one of the important neutrophil-attracting chemokines. TNF-α and IL-1 induce neutrophil activation mainly via IL-8. Kevin ED and James KM[9] demonstrated that IL-1, TNF-α, and fibronectin released from alveolar macrophages are positively related with pulmonary granuloma, inflammation, and fibrosis in F344 rats following intratracheal crystalline silica exposure. IL-1 release is associated with development of pulmonary granulomas, and TNF release is positively related with the degree of neutrophil recruitment following SiO_2 exposure. In addition, a persistent increase in fibronectin release from AM is related with development of pulmonary fibrosis. Zhai et al. showed that the serum levels of TNF-α, IL-1β, IL-8, and IL-6 are higher in silicosis patients than in controls[10]. Exposure to anti-MCP-1 and anti-MIP-1α antibodies blocks 22% and 18% of monocyte chemotactic activities, indicating that pulmonary inflammation and fibrosis are inhibited (Table 2).

Our previous study showed that mFas increases and sFas decreases with the progress of silicosis [2]. In order to study the mechanism of inflammatory cytokine secretion, AM were exposed to one of the 3 Fas/FasL pathway inhibitors (anti-FasL antibody, or caspase-8 inhibitor) to block death signal transduction. The serum levels of MCP-1 and MIP-1α decreased after Fas/FasL pathway was blocked by anti-FasL antibody (P<0.05). The serum levels of IL-1β, IL-8, MCP-1, MIP-1α, and TGF-β_1 decreased upon caspase-8 inhibition after the 3 death receptor signal pathways were blocked (P<0.05, Table 3).

Table 1. Serum Levels of Cytokines in Silicosis Patients and Controls

Groups	*n*	IL-1β (ng/L)	IL-8 (ng/L)	TNF-α (ng/L)	TGF-β1 (ng/L)	MCP-1 (ng/L)	MIP-1α (ng/L)
Controls	6	4.45±0.31	5.76±0.22	7.74±0.32	5.50±0.04	8.19±0.65	6.45±0.01
Silicosis at stage I	15	53.81±51.17*	135.66±37.39*	21.81±3.08*	70.33±27.86*	48.94±8.17*	5.01±7.95
Silicosis at stage II	8	70.66±14.67*	140.43±21.33*	15.36±2.10$^{*,\#}$	51.32±10.10$^{*,\#}$	56.84±13.01$^{*,\#}$	5.35±2.47
Silicosis at stage III	6	67.73±27.49*	146.58±43.50*	18.38±2.64$^{*,\#,\$}$	45.13±12.02$^{*,\#}$	96.99±39.05$^{*,\#,\$}$	8.14±2.52
F		3.12	19.07	10.54	11.03	19.17	0.47
P		0.0416	0.0000	0.0001	0.0001	0.0000	0.7077

Note. $^{*}P$<0.05 *vs* controls, $^{\#}P$<0.05 *vs* stage I silicosis, $^{\$}P$<0.05 *vs* stage II silicosis.

Table 2. Serum Levels of Cytokines in Relation with Silica Exposure-related Factors (r_s)

Silica Exposure-related Factors	IL-1β	IL-8	TNF-α	TGF-β1	MCP-1	MIP-1α
Age (yr)	-0.0806	0.1079	-0.2184	-0.1239	-0.1105	0.0434
Age began to silica exposure (yr)	-0.1138	0.2064	-0.2008	-0.4395*	-0.3045	-0.3529*
Duration of silica exposure (yr)	0.1612	-0.1554	0.1356	0.2898	0.1968	0.2936
Stage of silicosis (yr)	0.4804*	0.0799	0.4447*	0.4712*	0.2351	0.2331
Age at silicosis onset (yr)	0.1799	-0.1331	0.0226	0.1026	-0.0454	0.1865
Duration from silica exposure to silicosis onset (yr)	0.2173	-0.1986	0.2127	0.1099	0.2443	0.1950
Duration of silica exposure cessation (yr)	-0.4451*	0.1224	-0.3241*	0.0270	-0.3768*	0.0263
Pattern of silicosis	0.0622	-0.0088	0.2383	-0.0074	0.1967	-0.0771
Smoking	0.0821	0.0983	0.1696	-0.0673	0.1795	-0.0921

Note. $^{*}t$ test, P<0.05, n=29.

Table 3. Serum Levels of Cytokines in 3 Blocked Death Receptor Signal Pathways

Treat-ment	*n*	IL-1β (ng/mg. pro)	IL-8 (ng/mg. pro)	TNF-α (ng/mg. pro)	MCP-1 (ng/mg. pro)	MIP-1α (ng/mg. pro)	TGF-β1 (ng/mg. pro)
A	9	0.2324±0.1512	0.2237±0.0685	0.1258±0.0535	0.2776±0.0097	0.1967±0.0259	0.1745±0.0452
B	9	0.1319±0.0103*	0.1604±0.0273*	0.0993±0.0161	0.2361±0.0328*	0.1647±0.0169*	0.1454±0.0290
C	9	0.1480±0.0463	0.1814±0.0457	0.1160±0.0031	0.2424±0.0648	0.1975±0.0371$^{\blacktriangle}$	0.1650±0.0383
D	9	0.1854±0.0679$^{\blacktriangle}$	0.1313±0.0069$^{*,\blacktriangle,\blacklozenge}$	0.1128±0.0204	0.2687±0.0688	0.1820±0.0167$^{\blacktriangle}$	0.2076±0.0123$^{*,\blacktriangle,\blacklozenge}$
F		2.28	6.20	0.93	3.80	3.80	5.77
P		0.0770	0.006	0.4551	0.0103	0.0103	0.0009

Note. A: untreated; B: treated with anti-TNF-α and anti-TRAIL antibody blocking TNF/TNFR and TRAIL/TRAILR pathway; C: treated with anti-FasL antibody; D: treated with Caspase-8 inhibitor. $^{*}P<0.05$ *vs* A, $^{\blacktriangle}P<0.05$ *vs* B, $^{\blacklozenge}P<0.05$ *vs* C.

Monocytes and monocytes-derived macrophages release TNF-a and IL-8 following Fas ligation. Conditioned medium from Fas-activated monocytes and macrophages induce direct migration of neutrophils. Fas-induced monocyte cytokine responses are associated with monocyte apoptosis, nuclear translocation of NF-kappa B, cytokine gene expression, which are inhibited by caspase and IL-1 beta signaling. In contrast, Fas-induced macrophage cytokine responses occurred in the absence of apoptosis are independent of caspase, indicating that maturation-dependent differences in the Fas signaling pathways lead to proinflammatory cytokine induction[11]. Intraperitoneal injection with FasL vesicles induces apoptosis of Fas-expressing and Mac-1 resident macrophages, resulting in secretion

of IL-1β, MIP-1α, MIP-1β, and MIP-2, followed by neutrophil extravasation. Fas engagement also induces pro-inflammatory response to vascular smooth muscle cells, resulting in secretion of IL-1, IL-8, and MCP-1. Reactive oxygen intermediates (ROIs) in AM are important for FasL induction by silica. FasL protein expression and FasL mRNA transcription in T cells are highly dependent on ROIs.

In summary, the serum levels of MCP-1 and MIP-1α decrease after ROS is cleared by SOD coenzyme. Caspase-8 inhibitor blocks the 3 death signaling pathways. Fas/FasL pathway is blocked by anti-FasL antibody. The serum levels of IL-1β, IL-8, and TGF-β_1 decrease after the 3 death signaling pathways are blocked by caspase-8 inhibitor. IL-8 expression is blocked by SOD coenzyme. Fas/FasL pathway plays an important role in regulating the serum level of inflammatory cytokines, leading to lung inflammation and fibrosis.

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REFFERENCES

1. Khalil N, Churg A, Muller N, et al. Environmental, inhaled and ingested causes of pulmonary fibrosis. Toxicol Pathol, 2007; 35, 86-96.
2. Yao SQ, Rojanasakul WL, Chen ZY, et al. Fas/FasL pathway-mediated alveolar macrophage apoptosis involved in human silicosis. Apoptosis, 2011; 16, 1195-204.
3. Biswas R, Bunderson-Schelvan M, and Holian A. Potential Role of the Inflammasome-Derived Inflammatory Cytokines in Pulmonary Fibrosis. Pulm Med, 2011; 2011,105707. Epub 2011 Jun 2.
4. Vandivier RW, Richens TR, Horstmann SA, et al. Dysfunctional cystic fibrosis transmembrane conductance regulator inhibits phagocytosis of apoptotic cells with proinflammatory consequences. Am J Physiol Lung Cell Mol Physiol, 2009; 297, L677-L686.
5. Borges VM, Lopes MF, Falcão H, et al. Apoptosis underlies immunopathogenic mechanisms in acute silicosis. Am J Respir Cell Mol Biol, 2002; 27, 78-84.
6. Dosreis GA and Borges VM. Role of Fas-ligand induced apoptosis in pulmonary inflammation and injury. Inflammatory & Allergy, 2003; 2, 161-7.
7. Fubini B and Hubbard A. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) generation by silica in inflammation and fibrosis. Free Radic Biol Med, 2003; 34, 1507-16.
8. Tumane RG, Pingle SK, Jawade AA, et al. An overview of caspase: Apoptotic protein for silicosis. Indian J Occup Environ Med, 2010; 14, 31-8.
9. Kevin ED, James KM. Cytokine and Growth Factor Release by Alveolar Macrophages: Potential Biomarkers of Pulmonary Toxicity. Toxicol Pathol, 1991; 19, 398-406.
10. Zhai R, Ge X, Li H, et al. Differences in cellular and inflammatory cytokine profiles in the bronchoalveolar lavage fluid in bagassosis and silicosis. Am J Ind Med, 2004; 46, 338-44.
11. Park DR, Thomsen AR, Frevert CW, et al. Fas (CD95) induces proinflmmatory cytokine responses by human monocytes and monocyte-derived macarophages. J Immunol, 2003; 170, 6209-16.