Silica-induced Apoptosis in Alveolar and Granulomatous Cells in Vivo

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Silica is a toxicant that can stimulate cells to produce various cellular products such as free radicals, cytokines, and growth factors. Silica and its induced substances may induce apoptosis to regulate the evolution of silica-induced inflammation and fibrosis. To examine this hypothesis, groups of Wistar male rats were intratracheally instilled with different doses of Min-U-Sil 5 silica (Silica, Berkeley Springs, WV). Ten days after the instillation, we obtained cells by bronchoalveolar lavage and placed them on slides by cytospin preparation. The slides were stained with Diff-Quik (Lab Aids, Sydney, NSW, Australia) and examined under oil immersion. A substantial number of cells with apoptotic features were identified in all silica-instilled rats and the apoptosis was confirmed by agarose gel electrophoresis. The number of apoptotic cells was clearly related to silica dosage. Engulfment of apoptotic cells by macrophages was also noted. Neutrophil influx in silica-instilled rats could be saturated with the increase of silica dosage and the number of macrophages in different dose groups changed in parallel with the proportion of apoptotic cells. Fifty-six days after instillation, morphologically apoptotic cells could be identified in granulomatous cells of lung tissue from silica-instilled rats. We conclude that intratracheal instillation of silica could induce apoptosis in both alveolar and granulomatous cells, and the apoptotic change and subsequent engulfment by macrophages might play a role in the evolution of silica-induced effects.

Methods

Animals

Specific pathogen-free male Wistar rats (200–230 g) were obtained from the University of New South Wales (Little Bay, NSW, Australia) breeding facility. The rats were held in cages (five rats per cage) where food and water were freely accessible. The lighting in the animal house was set to a 12-hr on/off cycle.

Experimental Design

Twenty rats were randomly distributed into four intratracheal instillation treatment groups (five in each group): a) control 0.5 ml saline, b) 2.5 mg silica (Min-U-Sil 5 crystalline silica, Silica, Berkeley Springs, WV) suspended in 0.5 ml saline; c) 7.5 mg silica in 0.5 ml saline; d) 22.5 mg silica in 0.5 ml saline. The Min-U-Sil 5 particle size range was 0.6 to 8.0 μm and 98% < 5 μm in diameter; purity was 99.5% α-quartz by X-ray diffraction. The dust was suspended in saline and autoclaved for sterilization before the experiment. The preparation was free of endotoxin.

Intratracheal Instillation

Rats were anesthetized with ip injection of a mixture of 100 mg/kg ketamine and 3.3 mg/kg xylazine (Sigma Chemical, Sydney, NSW, Australia) dissolved in saline. Intratracheal instillation was performed by a tracheal exposure procedure. After it was shaved, the skin in the ventral aspect of the neck was incised in the midline. Through blunt dissection, the trachea was exposed. Using a 1-ml disposable syringe with a 26-gauge needle, 0.5 ml dust suspension or saline was injected. The incision was sutured carefully with interrupted silk sutures immediately after the injection and the rat was kept in a 30°C incubator until it regained consciousness.

Introduction

Silica is known to induce inflammation and fibrosis in both human and experimental animals. It also induces lung tumors in rats and is now classified as a human carcinogen. However, the underlying mechanism for the development of silica-induced pathological changes is not fully understood.

In silica-induced effects, leukocyte infiltration into the alveolar space and granuloma formation in lung tissue are two marked reactions (1,2) that make the cell number in the lung increase significantly.

Mechanisms to remove these redundant cells are presumed to exist, as cell number and composition of bronchoalveolar lavage (BAL) cells change, and replacement of cells in lung granuloma by noncellular elements occurs, during the evolution of silica-induced effects. Clarification of these mechanisms will provide a better understanding of the development of silica-induced pathological changes.

When exposed to silica particles, various cytokines, growth factors, and free radicals are generated in the lung (3–6). These generated substances play important roles in the development of silica-induced inflammation, as well as in fibrosis and carcinogenesis, and also induce apoptosis i.e., programmed cell death.

Apoptosis is one of the pathways of cell death and is controlled by genes. Since it is an active process and is considered cell suicide, apoptosis is distinctly different from necrosis, which is accidental. Apoptosis is important in maintaining cell numbers in tissues or organs. Because apoptosis-triggering substances are generated and cell numbers in the lung are increased concurrently in silica-induced reactions, it is quite possible that the generated substances counteract the increase of cell number and regulate the evolution of silica-induced effects through the induction of apoptosis. More importantly, apoptosis seems to play a role in determining the efficiency of tumor initiation by some carcinogens (7). The purpose of the present study is to examine the role of apoptosis in the development of silica-induced effects.
**Bronchoalveolar Lavage**

Ten days after the instillation, rats were anesthetized with 75 mg/kg pentobarbital by ip injection. Laparotomy was performed and the abdominal aorta exposed. Rats were killed by transection of the aorta and BAL was performed with 2 aliquots of 5 ml phosphate-buffered saline after in situ perfusion of the lung. The recovered fluid volume was recorded.

**Histology**

Lavage fluid (100 μl) was placed on a slide by cyospin centrifugation immediately after the lavage. At least two slides were made for each rat. The slides were stained with Diff-Quick (Lab Aids, Sydney, NSW, Australia).

**Total Cell Number and Lactate Dehydrogenase Activity**

The total cell number was counted with a hemocytometer and the remaining fluid was centrifuged (3000 rpm, 10 min). The supernatant was collected to measure lactate dehydrogenase (LDH) activity by a kit method (Trace, Sydney, NSW, Australia).

**Differential Counting**

The slides were read under oil immersion (×1000). Five hundred leukocytes were counted to determine the frequency of different cell types by their morphology.

**Counting Apoptotic Cells**

A minimum of 1000 leukocytes were counted for the occurrence of cells with apoptotic features. Apoptotic features included formation of condensed chromatin bodies with sharp edges and convolution of the cell surface (8,9).

**Electrophoresis and Extraction of Genomic DNA from Bronchoalveolar Lavage Cells**

Extraction of genomic DNA of the cells in lavage fluid was conducted by standard methods (10). The alveolar cell pellet obtained was fixed by 70% alcohol immediately after lavage and centrifugation. The fixed cells were treated by lysis buffer and nuclear lysis buffer to obtain DNA. Agarose gel electrophoresis and ethidium bromide staining were used to ascertain whether there was any ladder development during the electrophoresis of the extracted DNA.

**Histology of the Lung**

A separate group of five rats (body weights approximately 500 g) was intratracheally instilled with 12.5 mg Min-U-Sil 5 silica to obtain tissues for histopathologic observation. Five randomly selected rats of similar body weight were instilled with saline as a control group. The rats were sacrificed at day 56 by ip injection of pentobarbital (75 mg/kg) and transection of the abdominal aorta. The lung was removed from the chest cavity and weighed. Lung tissue was fixed by inflation with 10% formalin and sectioned after paraffin embedding. The sections were stained with hematoxylin and eosin and examined under the microscope for pathologic changes. Under oil immersion, lung sections were examined to identify histologic signs of apoptosis in silica-induced granulomata in lung tissue.

**Statistical Analysis**

An unpaired two-sided t-test (Welch t-test if applicable) was used to compare the means of various indices measured by planned comparison. A two-sided p value of 0.05 was adopted as the criterion for statistical significance.

**Results**

**Inflammatory Reaction**

Pulmonary inflammatory reaction was measured by the total cell number and LDH activity in lavage fluid as well as by the cell types of inflammatory cells. The recovered volume of lavage fluid ranged from 9.0 to 9.7 ml and no significant difference in recovered volume could be detected among the four groups. LDH activity and total cell number in lavage fluid of the three silica-instilled groups were significantly higher than those of the control group (Table 1).

**Leukocyte Apoptosis**

There were few apoptotic leukocytes in the saline-instilled group. In silica-instilled groups, however, typical nuclear changes of apoptosis in BAL cells were identified (Figure 1). The number of apoptotic cells in BAL fluid increased with dose (Figure 2). There was a small number of apoptotic cells identified as macrophages (Figure 3) by their morphological characteristics, but the majority of apoptotic cells appeared to be neutrophils (Figure 4). Macrophage engulfment of apoptotic cells was also noted in this study (Figure 5) and all these findings could still be identified in the rats sacrificed 56 days after silica instillation.

![Figure 1. Apoptosis in bronchoalveolar lavage cells from silica-instilled rats (oil immersion, ×1000).](image-url)
Changes in Neutrophil and Macrophage Number
The number of neutrophils increased dramatically in the silica-instilled groups compared with that of the saline group. Among silica-instilled groups, the number of neutrophils increased with initial dosage and could be saturated when the dose reached 7.5 mg to 22.5 mg (Figure 6A). The number of macrophages, in contrast to the number of neutrophils, did not exceed the saline control level in the 2.5 and 7.5 mg groups but significantly increased in the 22.5 mg group compared with the control group (Figure 6B). The proportion of apoptotic cells in silica-instilled groups paralleled the number of macrophages in these groups (Figure 6C).

Electrophoresis of DNA from Bronchoalveolar Lavage Cells
A typical ladder was obtained in the electrophoresis of extracted genomic DNA from silica-instilled rats at each dose level. In saline-instilled rats, however, only one band could be seen that paralleled the main bands of silica-instilled rats, and no ladder could be detected (Figure 7).

Apoptosis in Lung Tissue
Fifty-six days after silica instillation, granulomas composed mainly of epithelial-like cells were identified in lung tissue. Apoptotic cells were identified in the granulomas (Figure 8).

Discussion
We studied an animal model of silica-induced inflammation that was evidenced by influx of neutrophils into alveolar space as well as by the elevation of LDH activity and increase of total cell numbers in BAL fluid. We demonstrated that apoptosis occurs in BAL cells of silica-instilled rats both morphologically and biochemically. The number of the apoptotic cells in BAL fluid was clearly related to silica dosage. As silica-induced increase in cell number and LDH activity in BAL fluid was also dose-related, the apoptosis might also be an aspect of silica-induced inflammation.

To our knowledge, this is the first documentation of apoptosis in silica-induced effects. Because cell death frequently occurs in silica-induced effects and is considered the outcome of silica toxicity, it is significant to note that cell death can proceed not only by necrosis but also by apoptosis. The apoptosis in our experiment could be detected 10 and even 56 days after silica administration, which indicates that apoptosis can also occur in chronic inflammation. Since silica-induced chronic inflammation resolves poorly and evolves into fibrosis, the role of apoptosis
in this process is worthy of clarification. Two cytokines that play important roles in silica-induced fibrosis, tumor necrosis factor alpha (TNF-α) and transforming growth factor beta (TGF-β) (5,6,11), can also induce apoptosis in different types of cells (12–14). TNF can also induce apoptosis in neutrophils (15). TNF and TGF may regulate the silica-induced response via induction of apoptosis.

We also noted the engulfment of apoptotic cells, which was consistent with the findings of Cox and co-workers (16). The apoptotic cells, mostly neutrophils, may be deleted by this mechanism, which maintains a balance against the influx of circulating neutrophils. In this study, we noted interesting quantitative relationships between neutrophil number, apoptotic cell proportion, and macrophage number. The neutrophil numbers, initially increasing with the increase of silica dose, can be saturated with the further increase of silica dose, whereas the macrophage number decreased initially with the silica administration but increased with dose increase, which paralleled the proportion of apoptotic cells. This finding suggests that the increase in proportion of apoptotic cells may regulate the macrophage recruitment to engulf and delete the apoptotic neutrophils. This would maintain a certain level of intra-alveolar neutrophils, the main components of alveolar cells in inflammatory conditions. The finding also indicates that apoptosis and subsequent engulfment by macrophages is consistent with a homeostatic function.

In lung tissue, granuloma formation was observed and lung weight in the silica-instilled group increased significantly (data not shown). This could be attributed at least partially to the granuloma formation—a hyperplasia reaction. In the development of silica-induced fibrosis, granulomatous cells are replaced ultimately by noncellular elements. Apoptosis might be a pathway to remove these cells during their replacement by other substances such as immunoglobulin or collagen.

In induced lung granulomata where histological signs of apoptosis were identified, the majority of granulomatous cells were considered to be differentiated macrophages. However, type II epithelial cells can proliferate markedly and line the newly formed granulomata to incorporate them into lung tissue during silica-induced pathological evolution (17). The possibility that type II epithelial cells differentiate and incorporate into the granulomata cannot be ruled out. Abnormally transformed type II epithelial cells are the progenitors of...
APOTOPSIS IN CELLS FROM SILICA-INSTILLED LUNGS

Figure 8. Apoptosis in lung granuloma of silica-instilled rats (oil immersion, ×1000).

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