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Enhanced Macrophage–Fibroblast Interactions in the Pulmonary Interstitium Increases Fibrosis After Silica Injection to Monocyte-Depleted Mice

I. Y. R. Adamson, H. L. Letourneau, and D. H. Bowden

From the Department of Pathology, University of Manitoba, Winnipeg, Manitoba, Canada

The role of interstitial* vs. *alveolar macrophages in the generation of pulmonary fibrosis after silica was examined. Using whole body irradiation to delay the inflammatory response and so retard particulate clearance, many more instilled silica particles reached the interstitial macrophages in the first 2 weeks than after silica alone. This was followed by greatly increased fibroblast proliferation and deposition of collagen in the irradiation plus silica group, which developed large interstitial granulomas at the sites of silica retention. Although alveolar macrophages containing silica were seen in both silica groups, more interstitial particles were observed after combined irradiation and silica, significantly more silica was recovered in a residue from the lungs at 16 weeks, and pulmonary fibrosis at 8–16 weeks was greater than in all other groups. The results indicate that increased fibroblast growth and collagen synthesis** in vivo ***are associated with phagocytosis of silica by interstitial macrophages rather than by free alveolar macrophages. It is suggested that transfer of a macrophage-derived growth factor to fibroblasts is more efficient when it occurs within the pulmonary interstitium.
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Deposition of silica in the lung is associated with the development of pulmonary fibrosis. In this and other examples of environmental lung disease, it is believed that the interaction of particulates with the alveolar macrophage (AM) is responsible for the generation of growth factors for fibroblasts.[1,2] The demonstration of macrophage-derived factors has been made after *in vitro* exposure of normal AM to particles[3,4] and using supernatants of AM lavaged from fibrotic lungs.[5,6]

Pulmonary fibrosis is, however, an interstitial lung disease and it is not clear whether factors secreted by macrophages in the alveoli can reach and act on fibroblasts in the interstitium, or whether secretory activity of the interstitial macrophage plays the critical role in fibroblast stimulation. We have previously observed that, in mice, short particles of crocidolite asbestos are ingested by AM with no subsequent fibrosis[7] whereas the same dose of long fibers, many of which reach the interstitial macrophages, induce pulmonary fibrosis.[8] These results lead to the hypothesis that the interaction between particles and macrophages within the interstitium may be more important than that occurring in the alveolar space in stimulating the fibrotic process. The close contact between macrophages and fibroblasts within the interstitium may permit more efficient transfer of macrophage-derived growth factors for fibroblasts than would secretion by macrophages into the alveolar space.

To evaluate the relative roles of alveolar versus interstitial locations of the same particle in stimulating fibrosis, we chose to administer a single dose of silica to mice. In this group of animals, most particles were phagocytized by the AM. In a second group of mice, we created a situation in which reduced phagocytosis and clearance of silica from the alveoli allowed a greater number of particles to reach the interstitium. This was done using whole-body irradiation to reduce the leukocyte number, thereby limiting the inflammatory response and the clearance of subsequently instilled silica. This allowed increased transport of silica to the pulmonary interstitium for phagocytosis by interstitial macrophages. The cellular responses seen in the alveoli, the cytokinetics of various pulmonary cell types, and the development of fibrosis were correlated

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Address reprint requests to Dr. I. Adamson, Pathology Department, University of Manitoba, 770 Bannatyne Avenue, Winnipeg, Manitoba, Canada R3E 0W3.

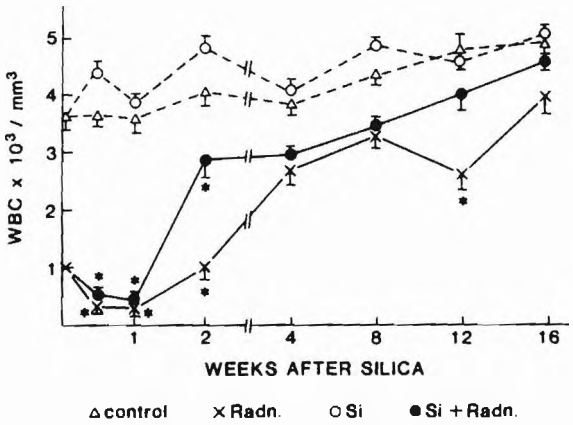

Figure 1. *White blood cell (WBC) counts in the various groups of mice. Irradiation was given to 2 groups 3 days before time zero. *P < 0.01 compared with controls at the same time. ×, irradiation only; ○, silica only; ●, silica after irradiation; △, no treatment*

with the location and retention of silica in the lung, and compared in the various groups.

Materials and Methods

A group of 50 Swiss-Webster mice (25 g males) were placed in separate sections of a plexiglas box and received 650 rads of whole-body irradiation.[9] Three days later the mice received an intratracheal instillation of 1 mg silica (crystalline quartz, Dowson and Dobson, South Africa) in 0.1 ml sterile water while under mild nembutal anesthesia.[10] Other groups of mice received silica only, irradiation only, or no treatment. The animals, in groups of 4, were killed at days 0 and 3 then at weeks 1, 2, 4, 8, 12, and 16 with time counted from the point of silica injection. Each mouse received 2 μCi/g tritiated thymidine 1 hour before death.

The heart was punctured and 0.3 ml blood withdrawn and the total white blood cell count (WBC) was determined using a Coulter counter. A tracheotomy was then performed, and the lungs were washed four times with 1.0 ml saline. The lavage fluid was pooled for each animal, and the total number of cells was counted on a hemocytometer. The cell suspension was centrifuged, and a smear was made and stained; differential counts of polymorphonuclear leukocytes (PMN) and AM were made on 500 cells per slide. The total number of cells of each type was calculated for each time studied, and the mean ± SE of the total cells per lung in the four mice per group was plotted against time. The supernatant of the lavage fluid from each mouse was used to determine total protein.[11]

After lavage, the bronchus leading to the right lung was clamped; this lung was removed, weighed, and frozen for biochemical analysis. The left lung was inflated with 0.5 ml 2% buffered glutaraldehyde and removed; most of the tissue was processed for embedding in glycol methacrylate. Sections (0.75 μ thick) from three random blocks per animal were prepared for autoradiography with the use of Kodak NTB2 emulsion. We determined the percentages of ^{3}H-thymidine-labeled cells at each time point by counting 3000 lung cells per animal. The means and standard error were calculated for each group. These sections were thin enough to allow identification of pulmonary cell types, and differential counts of labeled cells were performed on 300 labeled cells per animal. The product of the differential percentage and the total labeling percentage gave the labeling index for each cell type. The index for epithelial, interstitial, and endothelial cells was calculated at each time studied.

The right lung of each mouse was homogenized in water, and biochemical assays were performed on duplicate samples. Determinations of DNA and total protein were performed by conventional methods. As an index of collagen content, hydroxyproline levels were determined after hydrolysis with hydrochloric acid.[12]

At 16 weeks, 4 extra mice from each group were killed and the lungs removed immediately. These were chopped up and incubated in 40% KOH overnight in a 80 C waterbath. When the tissue was completely digested, the solution was centrifuged at 1500 rpm for 15 minutes and a residue was obtained. This was washed twice in distilled water, resuspended in water, and 1 drop placed on a copper grid for examination by electron microscopy. The remainder was transferred to a weighed tube, dried down, and the weight of the residue was determined.

Results

Blood

The numbers of WBC recovered from the various groups are shown in Figure 1. The values for controls and silica-injected animals were equal and rose slightly over the 16-week period. The other two groups were irradiated 3 days before the time zero on the graph. Irradiated mice received silica when the WBC count was at 1×10^3. The WBC fell to low values and did not rise until after a further week. The counts were in the normal range at 4 weeks.

Bronchoalveolar Lavage

In the lavage from groups of normal and from irradiated mice, very few if any PMN were recovered at any time

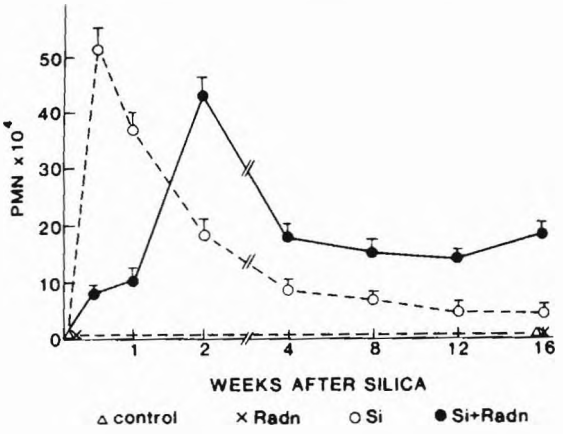

Figure 2. *The numbers of PMN recovered in BAL fluid up to 16 weeks. The values from control and irradiated groups were at or near zero so that all values shown are significantly greater than control.*

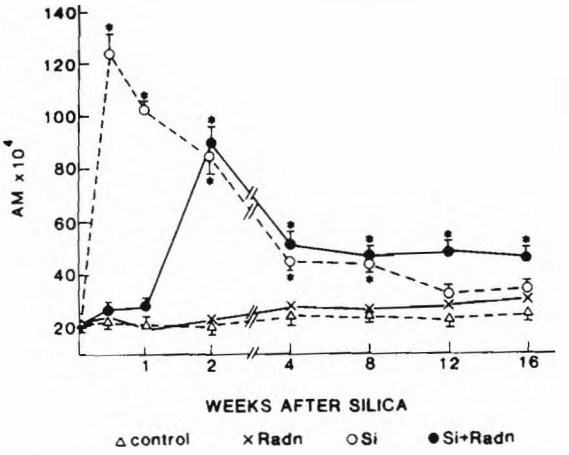

Figure 3. *The number of AM recovered in BAL fluid up to 16 weeks.* * $P < 0.01$ *compared with controls.*

(Figure 2). In contrast, mice that received silica alone showed a rapid eflux of PMN, which peaked at 5×10^5 in 3 days. The number of these cells dropped rapidly but never reached zero up to 16 weeks later. When irradiated mice received silica, there was a small increase in PMN in the BAL fluid over the first week then a sharp increase to a peak at 2 weeks (Figure 2). Although the number of PMN fell subsequently, it was always higher than that found in all other groups to 16 weeks.

The AM response to silica in irradiated mice was also delayed. The AM numbers in normal and irradiated animals were constant at about 2×10^5 (Figure 3). After silica alone, there was a rapid large increase in AM that declined over the next few weeks; however, the number recovered remained above normal up to 12 weeks after silica. When irradiated mice were instilled with particles, little effect on AM numbers was seen initially, then a fourfold rise occurred (Figure 3). Although the values fell somewhat, the number of AM recovered remained above normal over the remainder of the 16 week experiment.

The protein level in the bronchoalveolar lavage (BAL) supernatants was measured as an indicator of lung injury. The 650 rads of irradiation alone did not result in a significant increase in protein over that lavaged from control mice (Figure 4). Silica administration alone resulted in a fourfold increase in BAL protein at 3 days and the level rapidly returned to control values. The combined irradiation plus silica group showed a much higher peak in BAL protein and the increase was maintained for several weeks. The protein level was still significantly higher than in controls at 16 weeks, indicating ongoing lung injury (Figure 4).

Morphology

Animals that received irradiation only showed morphologic changes similar to these described earlier.[9] There was some evidence of endothelial injury and interstitial edema in the first 2 weeks whereas the epithelium was normal. From 8 weeks on there were focal areas of linear fibrosis resulting in a thickened air–blood barrier, especially at perivascular locations.

After silica alone, there was an initial inflammatory response with many AM and PMN seen in alveolar spaces. After 1 week the silica appeared confined to these cells with small amounts only in the pulmonary interstitium. Focal areas of type 2 epithelial cell proliferation were also seen and the interstitium showed a mixed population of macrophages and fibroblasts. From 4 weeks on, small focal interstitial granulomas were observed (Figure 5). These became less cellular and more fibrotic with time and resembled those described earlier in the pulmonary response to silica.[13] Free alveolar macrophages containing silica were seen in the cytospin preparations of lavaged cells and in lung sections up to 16 weeks. In the peripheral alveolar regions of the lung, there was no evi-

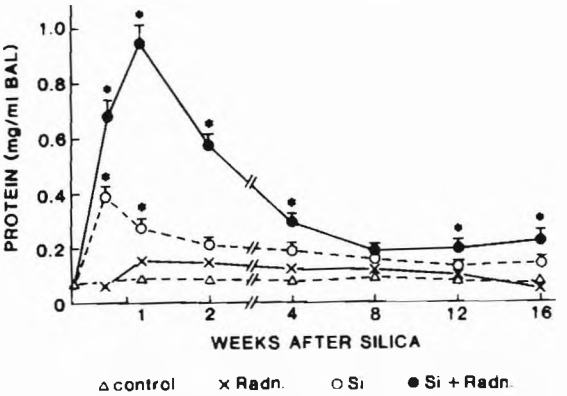

Figure 4. *Protein levels in BAL fluid over 16 weeks in the various groups.* * $P < 0.01$ *compared with controls.*

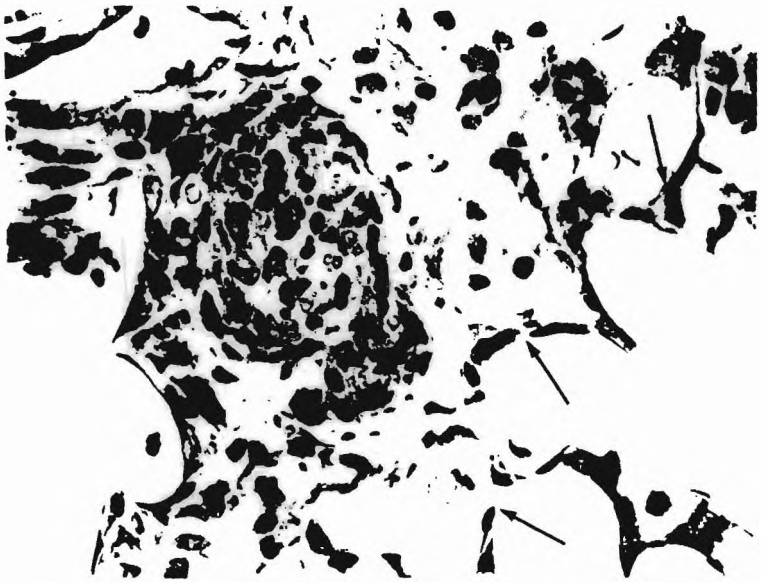

Figure 5. *Section of lung 8 weeks after silica alone. A small interstitial granuloma composed of macrophages and fibroblasts is seen. Alveolar walls appear thin as usual (arrows, ×850).*

dence of fibrosis in the alveolar walls, and the air–blood barrier was thin as in controls, even at areas where these appeared to be trapped AM containing silica.

When irradiated mice received silica, the normal inflammatory response was impaired and fewer AM and PMN were seen. Free silica particles were found up to 2 weeks in the alveoli and many particles reached the interstitium, where they were phagocytized by macrophages. Many large interstitial granulomas composed of macrophages and fibroblasts formed as early as 2–4 weeks after silica injection (Figure 6). In these areas many of the macrophages contained silica crystals that could be identified using polarized light. These large granulomas became more fibrotic with time although they still retained a high silica content. In these lungs, fibrosis seemed limited to the interstitial granulomas and little or no change in thickness was seen in alveolar walls even in regions of high AM content.

Autoradiography

The percentage of thymidine-labeled cells in normal mouse lung was approximately 0.3% (Figure 7). After irradiation alone, labeling increased in a biphasic manner over a 4-week period. From the radiographic indices, it

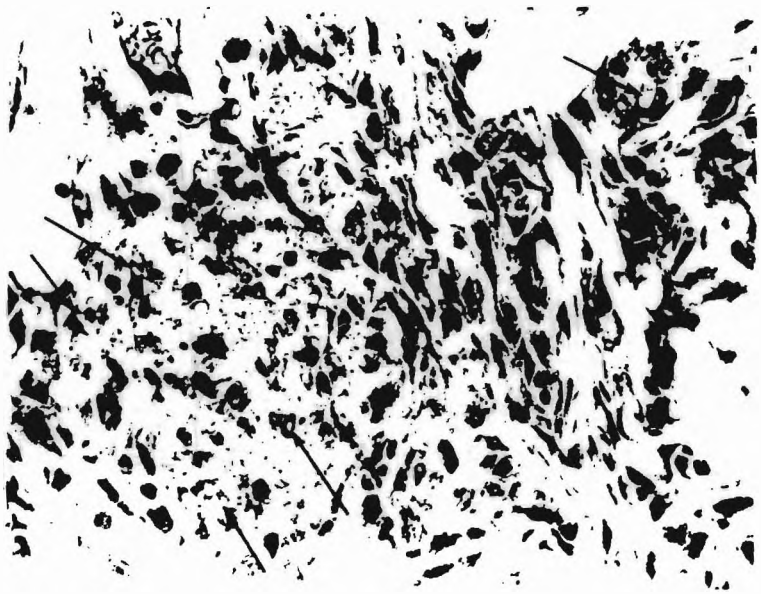

Figure 6. *Lung 4 weeks after silica with prior irradiation. Interstitial granuloma is very large and cellular. Many macrophages containing silica (arrows) are observed (×850).*

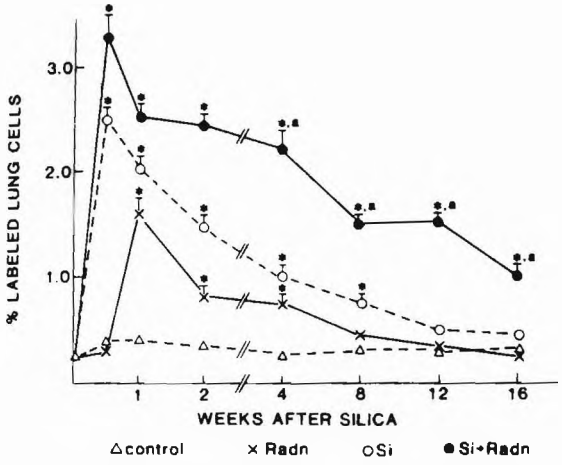

Figure 7. *Percentages of ^3H thymidine labeled nuclei in lung sections.* *P < 0.01 *compared with controls; a,* P < 0.01 *compared with all other groups.*

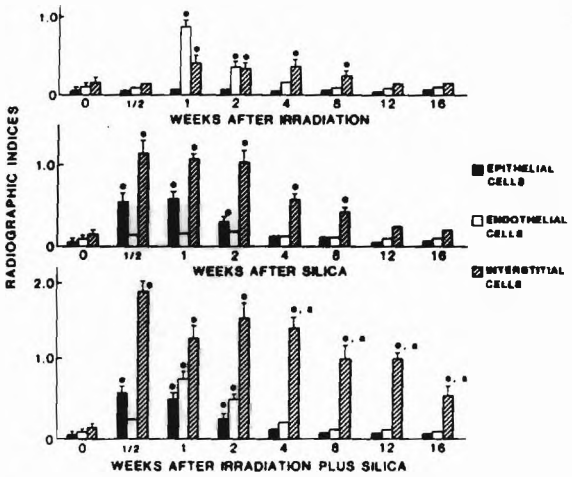

Figure 8. *Radiographic indices for epithelial, endothelial and interstitial cells in the various treatment groups.* *P < 0.01 *compared with controls; a,* P < 0.01 *compared with all other groups at the same time.*

was found that the initial peak was due to an increase in endothelial cell labeling subsequent to the endothelial damage, whereas the later phase reflected a period of increased fibroblast growth (Figure 8). After silica alone, there was an immediate increase in labeling to 2.5% of lung cells. This value dropped steadily to normal after 8 weeks (Figure 7). Differential counts showed that there was an early phase of type 2 epithelial cell proliferation that probably followed type 1 cell necrosis.[10] Interstitial cell labeling was much more prominent, however (Figure 8). Up to 2 weeks after irradiation most of these labeled cells resembled mononuclear phagocytes, whereas after 2 weeks the majority of labeled interstitial cells resembled fibroblasts.

The greatest increase in cell proliferation in the lung was seen in the combined irradiation-silica group. The initial peak was over 3% and a large increase over normal was seen throughout; from 4 weeks DNA synthesis was significantly higher than in all other groups (Figure 7). These mice showed periods of endothelial and epithelial regeneration equivalent to that seen after irradiation or silica alone, but the large increase in labeling was due to thymidine uptake by interstitial cells (Figure 8). Initially the labeled nuclei were a mixture of mononuclear cells resembling macrophages and fibroblasts (Figure 9). After 2 weeks, however, it appeared that most of the interstitial labeling was due to fibroblast proliferation (Figure 10).

Biochemistry

To quantitate pulmonary fibrosis, total hydroxyproline (HYP) content of the right lung was measured (Figure 11). Irradiation or silica exposure alone resulted in increased HYP beginning at 4 weeks and giving a greater than 50% increase at 16 weeks. The combination of irradiation followed by silica injection produced an increase in HYP by 2 weeks and the level continued to rise and remained 100% greater than that found in controls. After 8 weeks the level was significantly higher than all other groups. A similar increase in fibrosis was seen when the results were expressed as HYP/protein (Figure 12).

Figure 9. *Autoradiograph of lung 1 week after silica (10 days after irradiation). Thymidine uptake occurred in interstitial mononuclear cells, many resembling macrophages (arrows, × 1000).*

Figure 10. *Autoradiograph of lung 4 weeks after silica and irradiation. Labeled cells are mostly fibroblasts (arrows; × 1000).*

Tissue Residue

The weights of residues recovered after tissue digestion are shown in Table 1. A small increase in residue was found at 16 weeks in control and irradiated mice, probably due to insoluble elastin. The residue was amorphous and always weighed less than 0.2 mg for controls and for irradiated mice that showed some fibrosis. The residue from silica-treated lungs was white in appearance and was significantly heavier. After silica plus irradiation, a white powder was obtained after lung digestion. This residue was almost all particulate, as seen in the electron microscope, and was twice the weight of the residue from the silica only group and was four times greater than the weight of the control material.

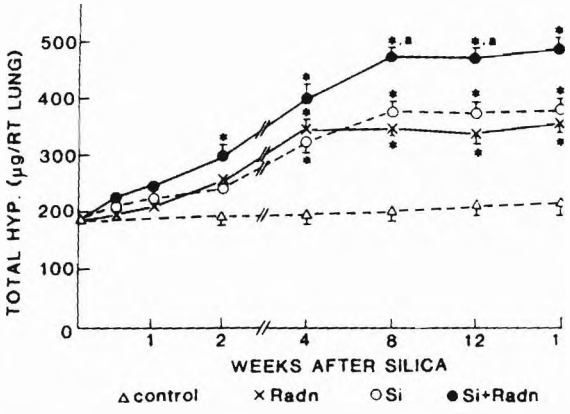

Figure 11. *Hydroxyproline (HYP) content of the right lung to 16 weeks* *P < 0.01 *greater than controls, a,* P < 0.01 *greater than all other groups.*

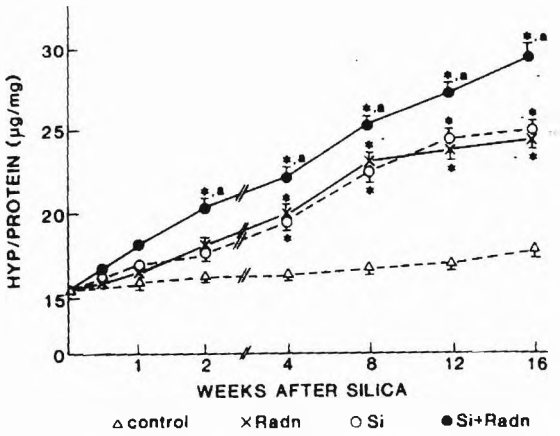

Figure 12. *The ratio of HYP to protein in right lungs of the various groups.* *P < 0.01 *greater than controls, a,* P < 0.01 *greater than all other groups.*

Discussion

In general, small amounts of inhaled particulates can be phagocytized and cleared from the lung by alveolar macrophages with no pathologic sequelae. Larger loads require an adaptive increase in the number of AM involving migration of blood monocytes to the alveoli supplemented by division and migration of mononuclear phagocytes from the interstitium.[14] These cells, plus the initial influx of PMN, constitute the normal inflammatory response and usually clear the airways. Some particles, however, especially at higher dose levels, escape phagocytosis and cross the type 1 epithelial cell to reach the interstitial macrophage.[10,13] Although macrophagic uptake of particles such as silica is associated with the production and secretion of various fibroblast growth factors (FGF), the relative importance of macrophage-particle in-

Table 1. *Weight of Residue After Total Lung Digestion*

Group (N = 4)	Dry weight (mg ± SE)
Control, week 0	0.05 ± 0.02
Control, week 16	0.12 ± 0.04
Irradiation	0.17 ± 0.05
Silica	0.40 ± 0.07*
Silica and irradiation	0.78 ± 0.09*†

* Expenmental greater than control, $P < 0.01$
† Value greater than all other groups, $P < 0.01$

teractions in the alveolus and in the interstitium to fibroblast stimulation *in vivo* is not known.

Macrophage-derived growth factors have been demonstrated in pure *in vitro* systems[15] and in studies using supernatants of AM lavaged from silicotic lungs.[2,5] This may be a general property of macrophages because FGF are associated with various types of pulmonary fibrosis[6] and may be common to a variety of chronic inflammatory states.[16] In the lung, most studies have involved the readily accessible AM and its secretion under serum-free test conditions. However, whether a secretion by free alveolar macrophages *in vivo* can reach and affect interstitial fibroblasts is not known and the possibility of direct transfer of FGF from interstitial macrophages to neighbouring fibroblasts has not been investigated. The model used in the present experiments allows comparison of the fibrotic response to silica *in vivo* when particles are either largely confined to AM or are located predominantly within interstitial macrophages. This experiment is based on our earlier finding that reduction of the alveolar inflammatory response to intratracheal carbon significantly increases the transfer of free particles to the interstitium.[17]

After the instillation of silica to the lung, we observed a delayed inflammatory response in irradiated mice, with few cells in the alveoli to 2 weeks and a large increase in the translocation of silica to the interstitium. Many particles were found in interstitial macrophages over the 16-week period, in association with fibroblasts within collagen forming granulomas. Even in these lungs, regions where silica was confined to free AM within the air sacs did not show thickening of alveolar walls. When the lung tissue at 16 weeks was digested, twice as much residue, mainly silica, was recovered from the irradiation plus silica group. The prolonged retention of silica mostly in interstitial macrophages over 16 weeks was associated with a significant increase in fibroblastic proliferation and collagen deposition. Thus, the enhanced translocation of silica to interstitial macrophages results in greater pulmonary fibrosis than when the same dose of silica is predominantly handled within the air sacs by the AM.

Although the possibility that the enhanced fibrotic response results from synergistic action of silica and irradiation on lung cell injury cannot be ruled out, there are several arguments against this possibility. The injury induced by irradiation alone is small, as shown by unchanged alveolar protein levels, and is confined to the endothelium, which is rapidly repaired (Figure 8). After irradiation followed by silica, the same degree and timing of endothelial repair is seen, indicating an equivalent amount of endothelial injury to the irradiation alone group. In addition, epithelial damage associated with this level of silica administration is also repaired within 2 weeks in the silica plus radiation group, so that the subsequent progressive fibrosis is not likely the result of continuing injury to the cells of the air–blood barrier. The type of granulomatous fibrosis seen after combined irradiation plus silica is clearly an exaggeration of the pulmonary response to silica, and more in keeping with the observed increase in interstitial silica. The predominant dividing cell in the lung after 2 weeks is the fibroblast and the continued high level of fibroblast proliferation and collagen deposition in the lung in the silica plus irradiation group is more likely to reflect ongoing secretion of a FGF by particle laden interstitial macrophages trapped within the granulomas.

These results demonstrate that limiting the initial inflammatory response is a useful method of increasing transepithelial passage of particles and their subsequent long-term retention in the lung. This provides a useful model for exaggerating the fibrotic response to particles such as silica, and suggests that, *in vivo*, the interstitial macrophage may play a more important role than the alveolar macrophage in fibrogenesis. In examining the properties of pulmonary macrophages, almost all studies have been carried out *in vitro*, usually on the alveolar macrophage; only recently has the interstitial macrophage received much attention. It is more difficult to isolate a pure population of these cells and it appears that the proliferative capacity and some functional characteristics are different from those of the AM.[18,19] Further *in vitro* experiments will be required on pure populations to determine whether interstitial macrophages are more potent secretors of FGF than alveolar macrophages. It is also possible that secretion by these two cell types is equal, but that *in vivo*, growth factors secreted into the alveolar space are more likely to be inactivated by serum factors for example, whereas close contact between activated macrophages and fibroblasts in the pulmonary interstitium may allow more efficient transfer of FGF. In either case, the results of the present *in vivo* study suggest that the key events in the generation of severe pulmonary fibrosis are the translocation of particulates across the epithelium and the stimulation of the interstitial macrophage.

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