Alveolar Macrophage Stimulation of Lung Fibroblast Growth in Asbestos-Induced Pulmonary Fibrosis

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Asbestotic lesions are characterized by macrophagic accumulation, fibroblast proliferation, and collagen deposition. To evaluate the potential involvement of alveolar macrophages in the subsequent fibrogenic reaction, the authors studied the effects of macrophages from normal and asbestos-treated rats upon lung fibroblast proliferation *in vitro*. Culture supernatants from bronchoalveolar (BAL) cells (99% macrophages) of normal rats stimulated lung fibroblast DNA synthesis and growth in a dose-dependent manner. Fibroblast growth factor (FGF) release by alveolar macrophages (AMs) was rapid (within 1 hour of incubation) and dependent on the number of AMs in culture. Moreover, culture supernatants from BAL cells of animals exposed to asbestos (single intratracheal injection) stimulated fibroblast proliferation to a greater degree than culture supernatants from BAL cells of control animals. Enhanced FGF production occurred 1 week after asbestos instillation and persisted up to 24 weeks. This change was accompanied in the early stages (1–4 weeks) by an increase in the total number of BAL cells which returned to control values by 12 weeks. Differential analysis of BAL cell populations showed a transient infiltration of neutrophils in the bronchoalveolar compartment followed by a significant accumulation of macrophages which persisted up to 1 month. Furthermore, lungs of asbestos-treated animals showed evidence of pathologic alterations characterized by fibroblast proliferation and collagen deposition. This study demonstrates that increased production of fibroblast growth factor by alveolar macrophages *in vitro* coincides with the development of asbestos-induced fibrosis. Prolonged stimulation of FGF release may contribute to excessive fibroblast proliferation and fibrosis. (Am J Pathol 1986, 122:205–211)

ASBESTOSIS is characterized by a chronic inflammatory reaction and diffuse interstitial fibrosis. Although intensive efforts have been made to elucidate the pathogenesis of the disease, the cellular events leading to the fibrogenic reaction are largely unknown. Depending on their localization, inhaled asbestos fibers can react with many different cell populations potentially important in lung connective tissue metabolism. Thus, inhaled asbestos fibers have been shown to reach the pulmonary interstitium and were found in interstitial macrophages and fibroblasts.[1] Such interaction of fibers with interstitial constituents, especially fibroblasts, may directly mediate fibrogenic reactions. On the other hand, the macrophage is a primary target cell in asbestosis,[2] and several investigators have suggested that this cell type may provide a link between initial asbestos deposition in the lung and the subsequent fibrosis.[3,4] However, to our knowledge, very few studies have been performed to address this issue.

In support of such an assumption, previous work has demonstrated that long and intermediate chrysotile fibers may be deposited at alveolar duct bifurcations and that significant numbers of pulmonary macrophages accumulate at sites of asbestos deposition.[5] In addition, macrophages have been shown to play a crucial role in the tissue response to injury and connective tissue repair.[6] Among other activities, macrophages produce soluble mediators which influence the rate of replication of multiple cell types.[7] Several recent studies, including ours,[8–11] have shown that pulmonary macrophages obtained by lung lavage have the ability to release growth factor(s) for lung fibroblasts.

Because pulmonary fibrosis may result from an increased number of collagen-producing cells, we investigated the interactions between alveolar macrophages

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(AMs) and lung fibroblasts during the development of asbestos-induced fibrosis. In a previous study, we observed that asbestos exposure in sheep enhanced the release of a fibroblast growth factor (FGF) by bronchoalveolar (BAL) cells.[11] In the present report we confirm this observation using a rat model of asbestos-induced lung injury. We further demonstrate that increased production of FGF by AMs coincides with the presence of lung lesions characterized by fibroblast proliferation and collagen deposition. We also show that stimulation of FGF is not a transient change, but a rather persistent reaction that may contribute to the maintenance and the irreversibility of the fibrotic response.

Materials and Methods

Animals

Male Wistar rats weighing 250–300 g were purchased from Charles River Canada Inc. (St-Constant, Québec). These animals were derived from a pathogen-free colony, shipped behind filter barriers, and housed in isolated temperature controlled quarters. They were given Labchows (Ralston Purina) and water *ad libitum* and were used 1 week later. After sacrifice, no control animals or animals given injected asbestos fibers showed evidence of infection when evaluated by histologic examination of lung sections.

Lung Exposure to Asbestos

UICC standard sample of chrysotile B was obtained from the National Research Institute for Occupational Diseases, Johannesburg, South Africa. This preparation has been well characterized and contains 21% of fibers longer than 10 μ.[12] This material was autoclaved for 45 minutes and suspended in sterile phosphate-buffered saline (PBS, pH 7.4) with a Dounce glass homogenizer prior to instillation into the animals. All injections were made by the transtracheal route. Rats were lightly anesthetized with a mixture of ketamine–xylazine (85–15%, 100 mg/kg). The trachea was exposed surgically, and the asbestos suspension (5 mg) or saline was briskly injected with a 3-ml syringe fitted with a 22-gauge needle in a final volume of 0.5 ml fluid and 0.5 ml air. At least 5 animals in each group were sacrificed at various intervals (1 week to 24 weeks) after instillation and evaluated for histologic changes and bronchoalveolar analyses.

Bronchoalveolar Lavage

Animals received a lethal dose of sodium pentobarbital (30 mg/rat). The abdominal aorta was severed, and the trachea was cannulated. A total volume of 50 ml of PBS in 5-ml aliquots was infused in each animal, 90% (45 ml) of which was recovered in control rats. For asbestos-treated rats, lavage fluid recovery ranged from 75% to 93% throughout the study (Day 1, 83%; 1 week, 83%; 2 weeks, 75%; 3 weeks, 80%; 4 weeks, 91%; 12 weeks, 90%; 24 weeks, 93%). The bronchoalveolar cells were obtained by centrifugation at 200*g* at 4 C for 10 minutes, washed in PBS, and finally resuspended in Dulbecco's modified Eagle's medium (DMEM) (GIBCO). Cells were counted in a hemocytometer chamber, and viability was determined by trypan blue exclusion. For comparison, values of total cell counts in lavage fluid at each time point studied were corrected for the corresponding fluid recovery at that particular interval, and all values were expressed as cell counts per 45 ml of lavage fluid recovered. Differential counts of lavage cells were made from cytocentrifuge smears stained with Wright–Giemsa stain.

Bronchoalveolar Cell Production of Fibroblast Growth Factor

Equal numbers of bronchoalveolar cells (0.5×10^6 unless indicated otherwise) from control and asbestos-exposed rats were incubated in 1 ml of DMEM supplemented with 0.5% fetal bovine serum (FBS) for various intervals at 37 C in a humidified 95% air, 5% CO_2 atmosphere. After the incubation period, the culture supernatants were collected, centrifuged, and frozen at −80 C until assayed. Cell viability was determined by trypan blue exclusion.

The culture supernatants were tested for fibroblast growth-promoting activity as described previously.[11] Briefly, control media (0.5 ml) and culture supernatants (0.5 ml) from BAL cells of control and asbestos-exposed rats were added to 5×10^4 human embryonic lung fibroblasts WI-38[13] (Flow Laboratories) at passage level 22–24 in a final volume of 1 ml DMEM supplemented with 0.5% FBS. After 48 hours of incubation at 37 C, the fibroblast cultures were pulsed with 1 μCi/ml tritiated thymidine (^{3}H-TdR, sp. act. 2.0 Ci/mM, New England Nuclear, Boston, Mass) for 4 hours. Thymidine incorporation was measured by counting the radiolabeled TCA-precipitable material. Each assay was done in triplicate. In parallel, triplicate cultures of fibroblasts were counted in a hemocytometer chamber, and cell viability was determined by trypan blue exclusion.

Lung Morphology

Histologic examination was performed in all animals. After lavage, the lungs were removed and a median lon-

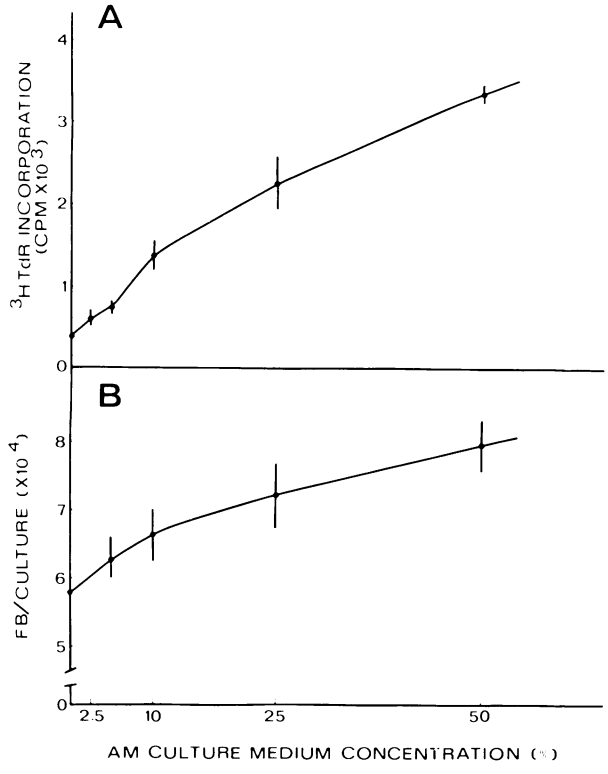

Figure 1A—Stimulation of lung fibroblast growth by culture supernatants of normal rat AMs. BAL cells (99% AMs) were incubated for 4 hours in DMEM supplemented with 0.5% FBS. Am culture supernatants were then incubated at various concentrations with 5 × 10^4 lung fibroblasts in the same medium. Proliferation was estimated at 48 hours by measuring the incorporation of ^{3}H-TdR. **B**—Fibroblast proliferation was also measured at 72 hours by direct cell counts. Values represent the mean ± SEM of three separate determinations.

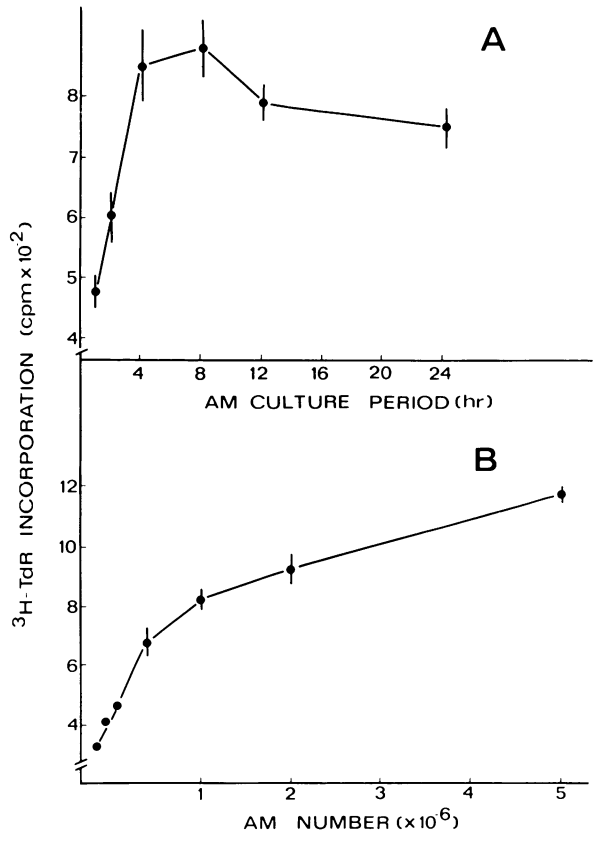

Figure 2A—Kinetics of lung fibroblast growth factor release by rat AMs. AMs (0.5 × 10^6) were incubated for 1, 2, 4, 8, 12, and 24 hours in DMEM containing 0.5% FBS. **B**—Growth factor production as a function of AM density. Varying numbers of AMs were incubated for 4 hours in DMEM with 0.5% FBS. Culture supernatants were then added to 5 × 10^4 fibroblasts at a concentration of 50% in a final volume of 1 ml. ^{3}H-TdR incorporation was measured at 48 hours. Values represent the mean ± SEM of three separate determinations.

gitudinal section of the upper left lobe (1 mm thick) was fixed by immersion in phosphate-buffered 4% formaldehyde, 1% glutaraldehyde solution. After fixation, lung tissue was embedded in paraffin, and representative sections 5 μ thick were cut and stained with hematoxylin and eosin.

Statistical Analysis

Results are expressed as mean values ± standard error of the mean (SEM). The statistical significance of differences between asbestos-treated and control groups was determined with the use of the Student *t* test.

Results

Release of Fibroblast Growth Factor by Rat AMs

Incubation of lung fibroblasts with culture supernatants of normal rat AMs resulted in a dose-related increase in fibroblast thymidine incorporation (Figure 1A). Concentrations of culture supernatants ranging from 10% to 50% caused significant stimulation of fibroblast proliferation, whereas lower concentrations had no significant effect. Although thymidine incorporation is a more sensitive assay of fibroblast replication, cell counts were also significantly increased in fibroblast cultures exposed to the same concentrations of AM culture supernatants (Figure 1B).

In order to study the kinetics of growth factor production, AM-conditioned media were collected at various intervals after the initiation of the cultures. Figure 2A shows that fibroblast growth-promoting activity was already detectable after 1–2 hours of incubation and was maximal by 4 hours. Therefore, the growth factor appears to accumulate rapidly in the culture medium, and its production remains constant for 24 hours under these experimental conditions. Viability of the AM cultures at each time point was at least 92%, which indicates that growth factor production is associated with viable macrophages.

As shown in Figure 2B, the fibroblast growth-

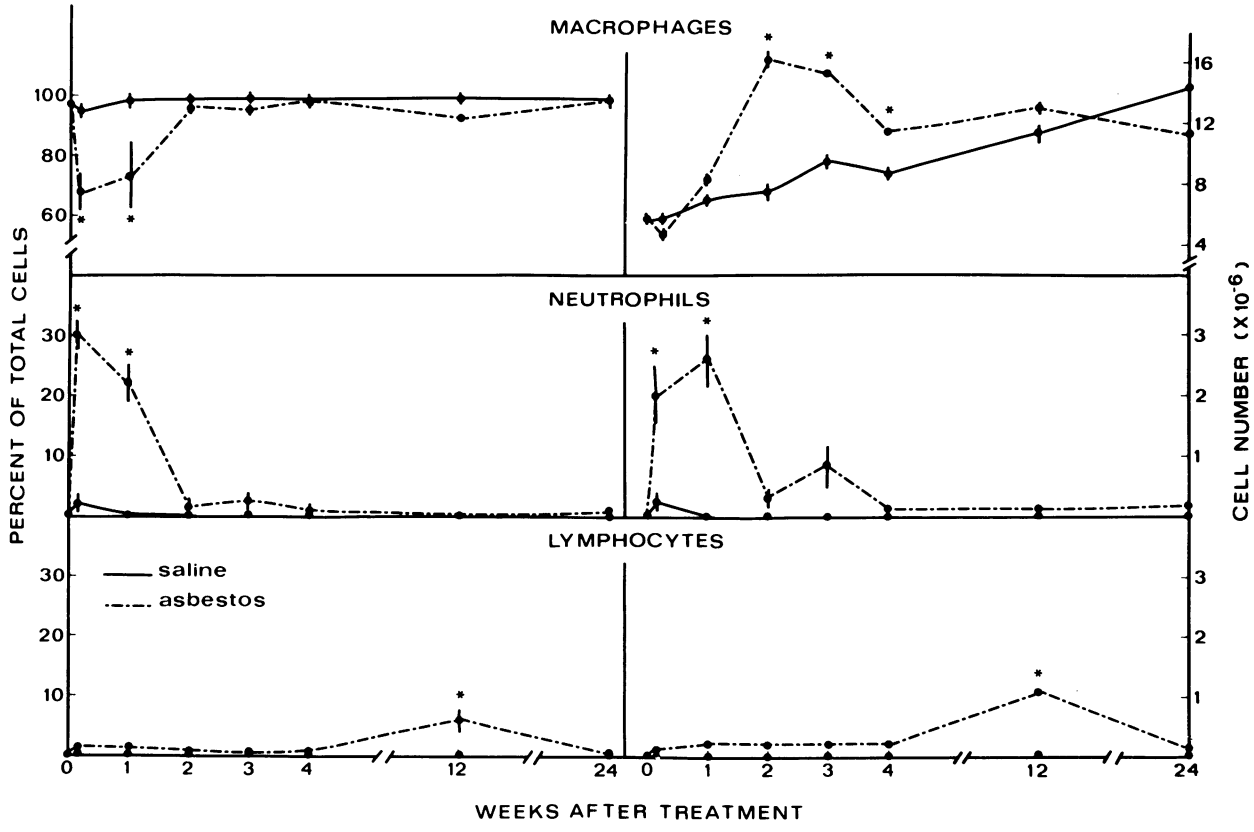

Figure 3—Analysis of BAL cell populations at various intervals following intratracheal instillation of saline or chrysotile asbestos. Cell populations were identified on cytocentrifuged smears stained with Wright–Giemsa stain. Differential cell counts were performed on 200 cells. Cell populations were enumerated as the percentage of total cells (**left panel**). Absolute numbers (**right panel**) were derived by multiplying these percentages by the total cell counts. Values represent means ± SEM of at least 5 animals per group.

promoting activity produced by AMs cultured for 4 hours was linearly proportional to the number of AMs over a range of 2–5 × 10^5 cells. Although higher densities of AMs (1–5 × 10^6) produced more growth activity, production was not linearly increased, which suggests that growth activity was not completely stable in the conditioned medium. Preliminary characterization reveals that AM-derived growth factor for fibroblasts is nondialyzable (>12,000 mol wt) and resistant to treatment at 56 C for 30 minutes. However, this activity is destroyed by heating at 80 C for 30 minutes, by acidic conditions (pH 2), and by repeated freezing and thawing.

Cellular Analysis of Bronchoalveolar Lavage Fluid Obtained at Various Intervals After Asbestos Exposure

Lavage fluids obtained from control animals yield an average of 6 × 10^6 cells. Although lavage recovery was identical at each time point studied (90% ± 1%), we observed a significant increase in the number of total cells recovered as a function of age and body weight. Rats lavaged at 24 weeks yield twice as many cells as those lavaged at the beginning of the study.

Soon after asbestos exposure (1 day), the average number of BAL cells recovered was similar to normal. However, at 1 week, 2 weeks, and 3 weeks after treatment, rats exposed to asbestos demonstrated a significant increase in the absolute number of total BAL cells, compared with saline-exposed rats. By the fourth week, BAL cell number was still significantly higher than in controls but tended to decrease to normal, where it remained thereafter. BAL cell viability in all asbestos groups was not significantly different from controls (91% ± 1%, compared with 93% ± 1%).

Although the bronchoalveolar cell population of normal rat is mainly composed of macrophages (99%), with very few neutrophils and lymphocytes, differential analysis revealed significant changes in the BAL cell populations after asbestos exposure (Figure 3). During the first week, neutrophils appeared in the bronchoalveolar compartment, where they represented 23–30% of all cells. This increase, however, was rather transient, with the proportion of neutrophils returning to normal at 2 weeks. It was followed by a significant increase in the absolute number of macrophages, which persisted up to 4 weeks after asbestos exposure. No significant change in lymphocyte population was ob-

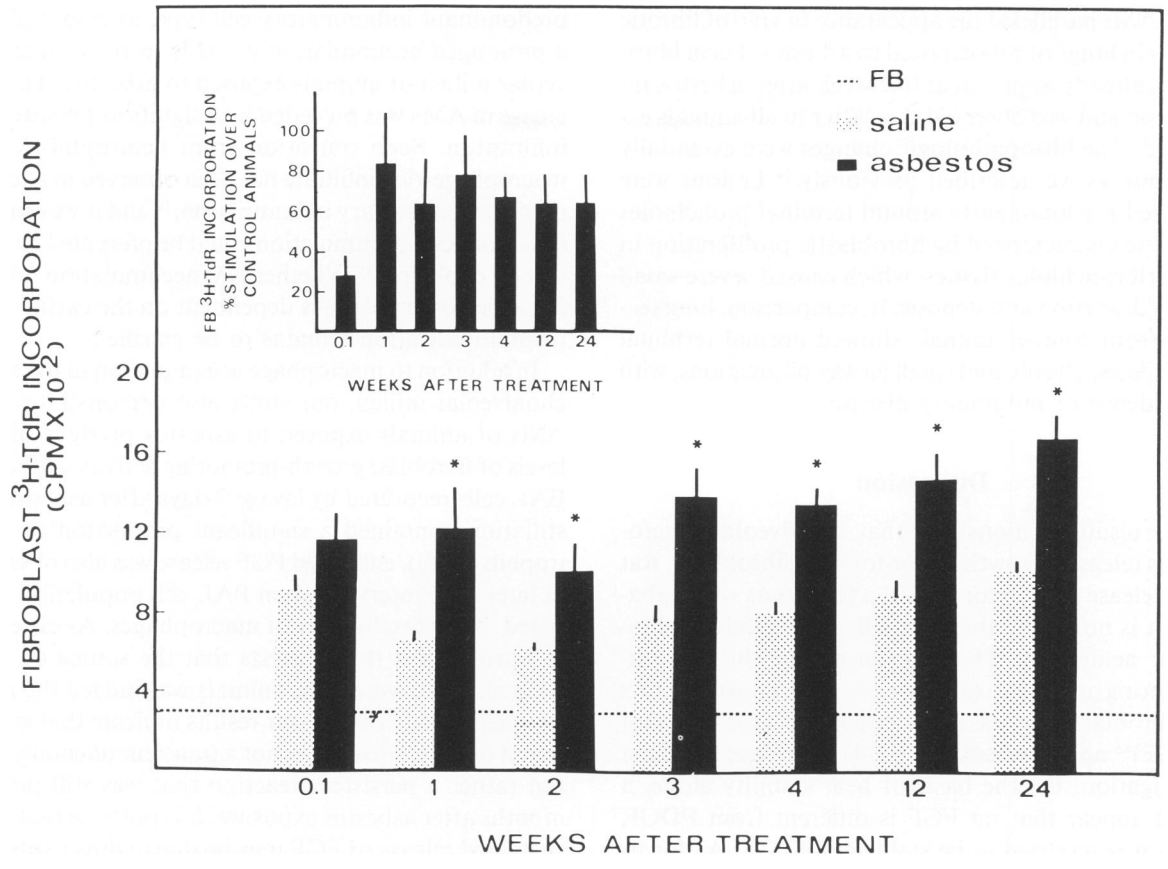

Figure 4—Kinetics of *in vitro* production of fibroblast growth factor by rat AMs after intratracheal instillation of saline or asbestos. BAL cells were obtained by lung lavage and were incubated at a density of 0.5 × 10^6/ml for 4 hours. The culture media were collected, centrifuged, and incubated (50% concentration) with 5 × 10^4 fibroblasts. ^{3}H-TdR incorporation was measured at 48 hours. Values represent means ± SEM of 5 to 10 animals per group. *Significantly different from control at $P < 0.05$.

served during the first weeks of the study, although a small but significant increase in the proportion and the absolute number of lymphocytes was apparent 12 weeks after asbestos exposure. By contrast, control animals had normal proportions of macrophages, neutrophils, and lymphocytes throughout the study. Thus, characterization of the bronchoalveolar cellular response revealed an increase in the absolute number of inflammatory cells in the lung, particularly macrophages.

FGF Production by Rat AMs During the Development of Asbestos-Induced Fibrosis

BAL cells obtained by lung lavage at various intervals after intratracheal instillation of saline or asbestos were incubated for 4 hours, and the effects of their culture supernatants on fibroblast growth were tested. The results shown in Figure 4 indicate that growth-promoting activity derived from cultures containing equal numbers of AMs increased significantly after asbestos exposure. Macrophages that were obtained from individual animals 1 week after asbestos instillation stimulated fibroblast proliferation to a greater degree than normal rat AMs. Conditioned media from normal AMs stimulated ^{3}H-thymidine incorporation twofold, whereas asbestos-treated rat AMs resulted in a threefold to fourfold stimulation. Moreover, this 80% stimulation over control animals was not a transient phenomenon; indeed significant, higher stimulation of fibroblast replication was observed up to 24 weeks after asbestos exposure. Thus, in our experimental conditions, asbestos exposure was associated with a sustained enhancement of fibroblast growth factor release by AMs. Interestingly, a small but gradual increase in FGF production by AMs from normal rats was seen over the 24-week period of the study, which suggests that AMs may develop a greater capacity to produce FGF with age.

Lung Morphology of Saline- and Asbestos-Treated Rats

After bronchoalveolar lavage, a lung section of the left lobe from each animal in both groups was examined for histopathologic changes. Our results indicate that increased production of fibroblast growth factor

by rat AMs paralleled the appearance *in vivo* of fibrotic lesions in lungs of rats exposed to asbestos. Focal fibrosis was already apparent at one week after asbestos instillation and was observed thereafter in all animals examined. The histopathologic changes were essentially the same as we described previously.[14] Lesions were localized predominantly around terminal bronchioles and were characterized by fibroblastic proliferation in the peribronchiolar tissues, which caused severe small airway distortion and stenosis. In comparison, lung sections from control animals showed normal terminal bronchioles, alveoli, and small airway bifurcations, with no evidence of pulmonary disease.

Discussion

Our results demonstrate that rat alveolar macrophages release a growth factor for lung fibroblasts. Rat AMs release this factor within a few hours of incubation. It is nondialyzable, partially heat-stable, and labile at acidic pH. The relationship of this growth-promoting activity to other well-known growth factors for fibroblasts such as platelet-derived growth factor (PDGF)[15] and interleukin-1 (IL-1)[16] is currently under investigation. On the basis of heat stability alone, it would appear that rat FGF is different from PDGF, which was reported to be stable at 100 C.[17] As for its possible analogy to IL-1, studies from our laboratory indicate that both the kinetics and the modulation of the release of FGF and IL-1 by rat AMs are different (submitted for publication). Further characterization in terms of molecular weight, retention on anion exchange column, and progression versus competence activity would be necessary to better define the growth-promoting activity elaborated by rat AMs.

Alveolar macrophages from various origins, including man,[8] sheep[11] and guinea pig,[10] have been shown to release FGF, which indicates that production of fibroblast-stimulating activity is not species-dependent and that animal models may be useful for study of the *in vivo* role of such activity in lung connective tissue regulation. In this regard, Leibovitch and Ross[6] were the first to demonstrate that the macrophage plays a major role in fibroplasia and wound healing and that it may exert this important regulation possibly via the release of soluble mediator(s) capable of stimulating fibroblast proliferation.[18] However, the role of macrophage-derived growth factor in pathogenesis characterized by inadequate tissue repair such as fibrosis is unknown.

In the present study, we investigated the *in vitro* production of fibroblast growth factor by AMs following asbestos exposure. In our rat model of asbestos-induced pulmonary fibrosis,[14] the macrophage is the predominant inflammatory cell type, as evidenced by a prolonged accumulation of AMs in the bronchoalveolar milieu of animals exposed to asbestos. This increase in AMs was preceded by a significant neutrophil infiltration. Such transition from neutrophil-rich to macrophage-rich infiltrate has been observed in another model of pulmonary inflammation,[19] and it was shown that monocyte accumulation could be prevented by neutrophil depletion.[20] Whether AM accumulation following asbestos exposure is dependent on the earlier neutrophil infiltration remains to be clarified.

In addition to macrophage accumulation in the bronchoalveolar milieu, our study also demonstrates that AMs of animals exposed to asbestos produce higher levels of fibroblast growth-promoting activity. Although BAL cells recovered by lavage 7 days after asbestos instillation contained a significant proportion of neutrophils (23%), enhanced FGF release was also observed at later time intervals when BAL cell population consisted almost exclusively of macrophages. As observed in normal rats, this suggests that the source of FGF production in asbestotic animals was indeed the macrophage. Furthermore, our results indicate that stimulation of FGF release was not a transient phenomenon, but rather a persistent reaction that was still present months after asbestos exposure. It is possible that such sustained release of FGF may be due to direct stimulation of AMs by asbestos fibers retained in the lung. Long-term retention of asbestos fibers in the lung, especially the longer ones, has been well documented,[21-22] and the presence of these residual fibers may stimulate macrophages, in a chronic fashion, to produce FGF. In separate *in vitro* experiments, we observed a modest stimulatory effect of chrysotile asbestos fibers in a dose range of 5–25 μg/ml on FGF release by rat AMs (unpublished data). However, this effect was not consistent, possibly because of technical difficulties in working with insoluble fibers of various lengths. Other possible mechanisms of FGF stimulation may involve the participation of additional inflammatory cell types such as lymphocytes[23] or soluble mediators released from the injured lung.

More importantly, our results further demonstrate that enhanced release of FGF by rat AMs coincides with fibroblast proliferation seen histologically at 7 days and thereafter in lung lesions of animals exposed to asbestos. Taken together, our observations suggest that stimulation of FGF release may contribute to the proliferation of fibroblasts seen in asbestotic lesions. While production of FGF under rate-limiting conditions such as in the normal repair process may be beneficial, chronic stimulation of FGF release would be detrimental and might lead to excessive fibroblast replication and fibrosis.

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