Cigarette smoke exposure potentiates bleomycin-induced lung fibrosis in guinea pigs

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The role of tobacco smoking in the development and outcome of pulmonary fibrosis is uncertain. To approach the effects of cigarette smoke on bleomycin-induced lung fibrosis, we studied five groups of guinea pigs: 1) controls, 2) instilled with bleomycin (B), 3) exposed to tobacco smoke for 6 wk (TS), 4) bleomycin instillation plus tobacco smoke exposure for 6 wk (B+TS), and 5) tobacco smoke exposure for 6 wk and bleomycin after smoking (TS/B). Guinea pigs receiving bleomycin and tobacco smoke exposure exhibited higher fibrotic lesions including a significant increase in the number of positive α-smooth muscle actin cells compared with bleomycin alone (B+TS, 3.4 ± 1.2%; TS/B, 3.7 ± 1.5%; B, 2.3 ± 1.5%; P < 0.01). However, only the TS/B group reached a significant increase in lung collagen compared with the bleomycin group (TS/B, 3.5 ± 0.7; B+TS, 2.9 ± 0.4; B, 2.4 ± 0.2 mg hydroxyproline/lung; P < 0.01). Bronchoalveolar lavage (BAL) from TS/B showed an increased number of eosinophils and higher levels of IL-4 and tissue inhibitor of metalloproteinase-2 (P < 0.01 for all comparisons) and induced a significant increase in fibroblast proliferation (P < 0.05). Importantly, smoke exposure alone induced an increase in BAL neutrophils, matrix metalloproteinase-9, and fibroblast proliferation compared with controls, suggesting that tobacco smoke creates a proinflammatory milieu that may contribute to the increased bleomycin-induced fibrosis.

PULMONARY FIBROSIS is the final common pathway of a variety of disorders affecting the lung parenchyma and grouped as interstitial lung diseases (ILD) (39). Cigarette smoking, a leading cause of chronic obstructive pulmonary disease (COPD), also exerts an effect on parenchymal lung inflammation and fibrosis. Thus it may promote or inhibit these pathological processes, and, in turn, it may influence the incidence, severity, or natural history of a wide array of ILD (25). For example, it is strongly associated with some diseases that seem to be likely caused by smoking, such as respiratory bronchiolitis-associated ILD, desquamative interstitial pneumonia, and pulmonary histiocytosis X (25, 26, 47, 49). Likewise, current and former smokers show an increased risk for developing idiopathic pulmonary fibrosis (IPF), although smokers appear to have a better survival rate than nonsmokers (3, 21). By contrast, cigarette smoking seems to have a protective effect on the development of hypersensitivity pneumonitis, a lymphocytic alveolitis provoked by the exposure to organic particles, and sarcoidosis, an inflammatory disorder of unknown etiology (10, 11, 25, 41). In addition, a number of clinical and experimental studies suggest that cigarette smoking reduces the frequency of radiation-induced pneumonitis (18, 28).

The mechanisms by which cigarette smoking affects in one or another way the inflammatory and fibrotic responses of the lung remain to be elucidated. Cigarette smoke seems on one hand to suppress some macrophage activities but, on the other, to promote systemic priming of neutrophils (2, 6).

Tobacco smoke may participate in lung remodeling by increasing the expression of a number of cytokines and by inducing the release of profibrotic growth factors bound to extracellular matrix through the increase of the expression of matrix metalloproteinases (MMP) (24).

It has been shown that epithelial cells from small airways of smokers and COPD patients highly express transforming growth factor (TGF)-β1 mRNA and protein (46). This finding may contribute to the fibrotic reaction in targeted areas of the lungs. Thus, in a study comparing smokers and nonsmoking males of various ages who died suddenly, it was found that bronchiolar inflammatory changes were evident in smokers of all ages, and bronchiolar fibrosis was clearly associated with chronic cigarette use, both lesions occurring independently of emphysema (1).

An important consideration in the putative interactions between cigarette smoking and incidence or course of an ILD is related with the timing relationship between tobacco smoke exposure and the fibrogenic injury. In this context, the aim of this study was to examine the effect of previous and simultaneous side-stream cigarette smoking on bleomycin-induced lung fibrosis in guinea pigs.

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CIGARETTE SMOKE AND FIBROSIS

MATERIALS AND METHODS

Tobacco smoke and bleomycin exposure. Guinea pigs (~400 g) were exposed to the smoke of 20 filtered commercial cigarettes (Marlboro 100, Cigatam SA de CV, Mexico; Philip Morris Products) per day, 5 days/week for 6 wk as previously described (43). Bleomycin (3 units; Bristol-Myers, Syracuse, NY) was administered by simple intratracheal instillation.

Experimental design. Five groups of guinea pigs were studied: (1) treated with bleomycin and killed 6 wk after (B); (2) exposed to tobacco smoke for 6 wk and killed after treatment (TS); (3) instilled with bleomycin and then exposed to tobacco smoke during 6 wk (B+TS); (4) exposed first to tobacco smoke for 6 wk and then instilled with bleomycin and killed 6 wk after (TS/B); and (5) control age-matched guinea pigs exposed to room air and instilled with saline solution (C). The Committee on Use and Care of Animals from the National Institute of Respiratory Diseases approved the protocol.

Eight animals from each group were anesthetized with 50 mg/kg ip pentobarbital sodium, and lungs were obtained for histology and hydroxyproline quantification.

Histology. Lungs were lavaged with saline solution through the main pulmonary artery, and the right lung was removed and inflated with 4% paraformaldehyde at continuous pressure of 25 cmH2O. Tissues were embedded in paraffin and processed for conventional light microscopy and immunohistochemistry. The whole left lung was removed and homogenized in 10 ml of PBS, and aliquots (1 ml) of lung homogenates were kept at −80°C until used.

Hydroxyproline measurement. Left lungs from eight guinea pigs of each group were analyzed for hydroxyproline content as an estimate of collagen content. Aliquots of 1 ml of homogenate were hydrolyzed in 1 ml of 12 N HCl for 24 h at 110°C, and hydroxyproline colorimetric analysis was performed as described by Woessner (48). All assays were done in triplicate, and data are expressed as micrograms of hydroxyproline per lung.

Bronchoalveolar lavage. In a parallel experiment, left lungs from six animals of each group were lavaged through a tracheal cannula with three aliquots of 5 ml of sterile saline solution at 37°C. The bronchoalveolar lavage (BAL) fluid was centrifuged at 400 g for 10 min at 4°C, and the supernatant was kept at −80°C until use. The pellet was resuspended in PBS and used for total cell count. Differential cell counting was performed after fixation with 50% ethyl alcohol and 2% Carbowax (50% polyethylene glycol) in slides stained with hematoxylin and eosin. Recovered fluid was used to evaluate fibroblast proliferation, IL-4, tissue inhibitor of metalloproteinase (TIMP)-2, and cell profile. Right lungs were used for zymography.

Lung tissue zymography. Lung samples (25 mg/ml) were homogenized in 10 mM 3-[3-cholamidopropyl(dimethylammonio)]-1-propanesulfonate, 20 mM HEPES (pH 7.5), and 150 mM NaCl and then centrifuged at 9,500 g for 20 min at 4°C. Lung supernatants containing 15 μg of protein were analyzed by SDS-PAGE gels (8.5%) containing gelatin (1 mg/ml) and a final concentration of 0.3 mg/ml heparin (51). Serum-free conditioned medium from human lung fibroblasts was used as an MMP-2 marker, and conditioned medium from FMA-stimulated human osteogenic sarcoma bone (U2-OS cells from American Type Culture Collection (ATCC), Rockville, MD) was utilized as an MMP-9 marker.

Quantification of IL-4 and TIMP-2 in BAL fluid. BAL fluid samples from six animals of the several groups were 4× concentrated, and IL-4 concentration was determined by enzyme-linked immunosorbent assay (ELISA) with the kit Quantikine HS (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. Levels of TIMP-2 were determined in the same samples by a human ELISA system (Amersham Pharmacia Biotech, Piscataway, NJ) that can detect TIMP-2 from guinea pigs. The assays were performed in quadruplicate.

Lung fibroblast growth rate. Guinea pig lung fibroblast cell line JH4 was purchased from ATCC. Fibroblasts (passages 5–8) were cultured in T-25 cm² Falcon flasks with Ham’s F-12 medium (GIBCO-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, GIBCO-BRL), 100 U/ml penicillin, and 100 mg/ml streptomycin at 37°C in 5% CO2-95% air. Fibroblasts were plated in 96-well culture plates at a cell density of 7 × 10³ cells/well under the same culture conditions and, after 12 h, replaced with new medium plus 1% FBS alone or with 1% FBS and 20% of 4× concentrated and sterilized (PVDF 0.22-μm Millex-GV; Millipore, Molsheim, France) BAL fluid from control or experimental guinea pigs. After 96 h, cell growth was determined with the cell proliferation reagent WST-1 (Boehringer Mannheim) as previously described (37). Absorbance was measured with an ELISA plate reader at a wavelength of 450 nm using a reference wavelength of 620 nm. Absorbance changes were taken as percentage of growth rate increase related to basal values (day 0). All assays were performed in sixuplicate. To analyze inhibition of cell proliferation, we ran a parallel experiment using anti-IL-4 neutralizing antibody (R&D Systems).

Immunohistochemistry. Sections were incubated with monoclonal mouse anti-human α-smooth muscle actin (SMA; DAKO, Carpinteria, CA), anti-human TIMP-2 (Oncogene, San Diego, CA), and anti-human IL-4 (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight. A secondary biotinylated anti-immunoglobulin followed by horseradish peroxidase-conjugated streptavidin (BioGenex, San Ramon, CA) was used according to the manufacturer. 3-Amino-9-ethylcarbazole (BioGenex) was used as substrate (44). The primary antibody was replaced by nonimmune serum for negative control slides. In addition, we determined the number of α-SMA-positive cells in lung sections of B, B+TS, and TS/B animals by evaluating 20 fields of fibrotic and inflammatory sections.

Evaluation of α-SMA. The ratio of α-SMA-positive cells in lung sections of B, B+TS, and TS/B animals was determined in 20 fields of fibrotic and inflammatory sections from six animals in each group. Photographs were taken with a ×40 objective on a Nikon Eclipse E600 interfaced to a Sony SSC-DC54A digital camera system. Each image was processed into the image analysis program MetaMorph 4.5 (Universal Imaging). A threshold was set automatically, and total cell number in each image was determined by nuclei counts, then a new threshold was set on the positive-staining cells, and smooth muscle cells of the bronchioles, blood vessels, and alveolar rings were manually removed from the data set. Approximately the same number of cells was counted for all slides (3,304–4,074).

Statistical analysis. All values are expressed as means ± SD. Differences between means values were assayed by a one-way analysis of variance, using Tukey’s method.

RESULTS

Lung histopathology and α-SMA-positive cells. Lung tissue sections were prepared for immunohistochemistry using α-SMA antibody and counterstained with hematoxylin to appreciate tissue architecture. As shown in Fig. 1, control animals exhibited normal parenchymal histology, and α-SMA-positive cells were
apparent in alveolar rings. Lungs from guinea pigs exposed to tobacco smoke showed a moderate peribronchial, alveolar, and interstitial inflammation consisting mostly of mononuclear cells and some areas with mild to moderate emphysematous lesions. As in controls, in these animals α-SMA-positive cells were mainly located in smooth muscle cells from vessels and in alveolar rings. At 6 wk after bleomycin instillation, the lungs displayed focal areas of inflammation and interstitial fibrosis, with the presence of α-SMA-positive cells in areas of thickened alveolar septa. Guinea pigs in the B+TS group showed a more evident loss of lung architecture with large zones of inflammation and interstitial fibrosis where an increased number of α-SMA-positive cells was apparent. In guinea pigs that were exposed to cigarette smoking first and then to bleomycin (TS/B), there was a more severe inflammatory and fibrotic lesion and numerous α-SMA-positive cells in the areas of fibrosis.

We determined the ratio of α-SMA-positive cells per 100 cells in lung sections of B, B+TS, and TS/B animals by digital imaging, counting 20 fields of fibrotic and inflammatory sections from six animals in each group. Lungs from both groups that were exposed to tobacco smoke and bleomycin exhibited a significant increase in the number of these cells compared with animals treated only with bleomycin (TS/B, 3.7 ± 1.5%; B+TS, 3.4 ± 1.2% vs. B, 2.3 ± 1.5%; P < 0.01).

**Lung hydroxyproline content.** Lung collagen was quantified by measuring hydroxyproline content (Fig. 2). Lungs from TS guinea pigs did not show differences with controls (1.4 ± 0.2 vs. 1.2 ± 0.2 mg hydroxyproline/lung). At 6 wk after bleomycin instillation, a significant increase in lung hydroxyproline was noticed in all three groups receiving bleomycin when compared with controls (B, 2.4 ± 0.2; B+TS, 2.9 ± 0.4; TS/B, 3.5 ± 0.7 mg/lung vs. 1.2 ± 0.2 mg/lung from C; P < 0.01). When these three groups were compared, a significant increase in lung hydroxyproline was found in those guinea pigs that received tobacco smoke first and bleomycin after, compared with the group that received bleomycin alone (3.5 ± 0.7 vs. 2.4 ± 0.2 mg/lung; P < 0.01).

**BAL cell profile.** Results of cell profile analysis from BAL are shown in Fig. 3. A significant increase in the
percentage of neutrophils was observed in TS and B+TS compared with C (9.5 ± 4.9, 8 ± 4.8, and 0.8 ± 0.8%, respectively; P < 0.05). The group receiving first tobacco smoke and then bleomycin revealed a significant increase in the percentage of eosinophils (TS/B, 29.7 ± 10%; B+TS, 15.4 ± 12%; B, 6.1 ± 6.0%; TS, 6.2 ± 5.9%; and C, 4.8 ± 4.4%; P < 0.05).

Fibroblasts growth rate. To determine whether BAL fluid derived from the different experimental groups had an effect on lung guinea pig fibroblast growth rate, we determined cell number 96 h after BAL fluid exposure, using the cell proliferation reagent WST-1.

As shown in Fig. 4, fibroblasts incubated with BAL from control animals exhibited a 6.3 ± 1.2% increase in growth rate over fibroblasts in presence of 1% FBS. When cells were incubated with BAL derived from the different experimental groups (TS, B, B+TS, and TS/B), all of them displayed significant increases in growth rate compared with control (TS, 53.8 ± 1.8%; B, 50.6 ± 4.5%; B+TS, 66.6 ± 2.6%; TS/B, 83.4 ± 4.6%; P < 0.05). In addition, BAL from TS/B displayed a significant increase compared with tobacco smoke or bleomycin alone (P < 0.01). Anti-IL-4 antibody significantly inhibited cell proliferation in the TS/B group (62.7 ± 3.4%, P < 0.01).

IL-4 and TIMP-2 in BAL fluid. ELISA was performed to determine whether protein levels of chemokines/cytokines were present in BAL fluids that might be associated with fibroblast proliferation. From the several molecules analyzed, only IL-4 and TIMP-2 revealed some differences among the groups tested. As shown in Fig. 5A, there was a significant increase in IL-4 levels in TS/B when all the groups are compared [18.1 ± 5.5 (TS/B) vs. 12.4 ± 1.9 (C); 11.2 ± 2.8 (TS); 8.3 ± 2.7 (B) and 11.3 ± 2.7 (B+TS) pg/mg protein; P < 0.05].

Figure 5B shows the results of TIMP-2. A significant increase in BAL fluid TIMP-2 levels was observed in the TS/B group (213.8 ± 53.5 ng/mg protein) compared with the other groups, in which TIMP-2 exhibited very similar values (72.3 ± 5.1 in C, 68.3 ± 13.4 in TS, 80.7 ± 9.2 in B, and 107.1 ± 14.6 in B+TS; P < 0.01).

Immunolocalization of IL-4 and TIMP-2. To examine the cell source of IL-4 and TIMP-2, we evaluated the expression of the immunoreactive proteins in control guinea pigs and in those exposed to bleomycin alone and to tobacco smoke and bleomycin. As illustrated in Fig. 6, IL-4 and TIMP-2 are mainly expressed by interstitial and alveolar macrophages in bleomycin-instilled animals (Fig. 6, C and D) as well as in TS/B lungs (Fig. 6, E and F). Normal lungs were usually negative, as exemplified in Fig. 6, A and B, for IL-4 and TIMP-2, respectively. Samples incubated with nonimmune serum were negative as shown in Fig. 6G.
Lung tissue zymography. To analyze gelatinases activity, we examined lung tissues obtained from controls and experimental groups by gelatin zymography. A representative sample per group and a densitometric quantification of the surface and intensity of the lysis bands of zymograms derived from four different animals in each group are illustrated in Fig. 7. Total MMP-9, representing pro-MMP-9 and its lower-molecular-weight active form, was increased about threefold in lung samples from bleomycin group compared with control and about sixfold in the three experimental groups that were exposed to tobacco smoke. An active MMP-9 band represented 23–41% of total MMP-9 and was more highly present in the groups receiving tobacco smoke at the time of death (TS and TS+B). Likewise, total MMP-2 was increased two- to threefold in lung samples from all experimental groups compared with control. The active lower-molecular-weight form represented 2–7% of total MMP-2 lysis bands.

DISCUSSION

The mechanisms by which exposure to cigarette smoking affects in one way or another the incidence or severity of various interstitial lung diseases are not entirely clear (25). Likewise, although smoking causes a number of cellular alterations in the airways, its role in parenchymal lung fibrogenesis has not been pre-
It reduces the risk to confer some protection for survival, putatively because smoking seems to be a risk factor for IPF (3), it appears to be more precisely determined. Thus, for example, although smoking may develop first pulmonary emphysema and then pulmonary fibrosis of unknown etiology (14).

In this work, we observed that tobacco smoke exposure increased the number of myofibroblasts in the alveolar septa and exacerbated the lung fibrotic response to bleomycin. However, this effect was more severe in guinea pigs where smoking preceded the exposure to the fibrogenic injuring agent. It is important to emphasize that this experimental group is different from the other that also received tobacco smoke and bleomycin in the fact that the lungs were already injured when they received the bleomycin.

An earlier report by Osanai et al. (32) found that tobacco smoke reduced the fibrotic response to bleomycin. However, this study was performed on hamsters and is difficult to compare directly with ours, which is on guinea pigs. Both our model and the hamster model exhibited statistically significant increases in the neutrophil counts when animals were treated with tobacco smoke and bleomycin. In addition to the species difference, in the hamster model, considerably lower levels of tobacco exposure were used, and animals were studied only 10 days after bleomycin instillation in contrast to the 6 wk used in our study. Perhaps, cigarette smoke in the early phases of bleomycin injury reduces the fibrosis but after prolonged injury leads to increasing damage and increasing fibrosis.

Several mechanisms may account for the smoke-related increase of fibrosis, some occurring by smoking itself whereas others appeared when both aggressions were combined. Regarding the first situation, three events that may enhance a fibrotic response took place when animals were exposed to cigarette smoke alone: 1) a noteworthy increase in neutrophils, 2) an increase in MMP-9 lung activity, and 3) the presence of fibroproliferative factors in BAL.

A higher fibrotic response has been associated with marked accumulation of neutrophils, both in experimental models and human diseases (8, 19), and increased tissue neutrophils loaded with MMP-8 and MMP-9 have been implicated in the fibroptic reaction of patients with chronic hypersensitivity pneumonitis (33).

Upregulation of MMP-9 has been previously documented in human lungs from smokers with COPD as well as in guinea pigs exposed to cigarette smoke (40, 42). There might be a linkage between elevation in MMP-9 activity and increased neutrophils since these inflammatory cells are a major source of MMP-9. Therefore, in pathological processes where neutrophils are abundant, as we found in the BAL of tobacco smoke-exposed guinea pigs, increased MMP-9 is also found. Additionally, MMP-9 is synthesized by a number of different lung cells after injury, including alveolar epithelial cells and macrophages (42, 44). A growing body of evidence strongly suggests that increased activity may contribute to fibrogenesis. MMP-9-deficient mice injured with bleomycin develop considerably fewer lung fibrotic lesions compared with MMP-9+/- littermates (4). Likewise, mice deficient in γ-glutamyl transpeptidase, a key enzyme in glutathione and cysteine metabolism, developed a markedly lesser fibrotic response to bleomycin, which was at least partially associated with decreased neutrophils and lower MMP-9 activity in lung tissues (34).

Considering its substrate affinity for type IV collagen, increased MMP-9 activity may lead to the loss of integrity of the basement membranes, which in turn, after a second injury such as bleomycin exposure, could enhance its fibrogenic effect. Another important putative profibrotic effect is related to the ability of cell surface-localized MMP-9 to cleave and activate latent TGF-β, a potent profibrotic growth factor (50).

A third possible mechanism that may be involved in the cigarette smoke enhancement of lung fibrosis is the increased fibroblastic growth rate activity observed in...
the BAL fluid. This was a surprising finding, since, at least in vitro, cigarette smoke extracts reduce viability and inhibit fibroblast proliferation and migration (16, 27, 29).

The characterization of the experimental group with the highest fibrotic response shows that this group displayed an important increase in BAL eosinophils as well as in IL-4 and TIMP-2. IL-4 and TIMP-2 were also found to be increased in the injured lung tissues primarily expressed by interstitial and alveolar macrophages. Both molecules and eosinophils have been documented to play an important role in fibrotic response. IL-4 is a pivotal molecule in the T lymphocyte helper 2 (Th2) response that is able to provoke a profibrotic milieu. IL-4 is a potent activator of the synthesis of a number of extracellular matrix proteins, including collagen and tenasin (13, 23, 35, 45), and also increases fibroblast chemotaxis (36). Furthermore, IL-4 stimulates fibroblast proliferation, and it could have contributed to the further increase of fibroblast proliferation stimulating activity found in the BAL fluid of the TS/B group. Supporting this notion, the use of anti-IL-4 antibody partially decreased the proliferating activity obtained in the BAL fluid of these animals. Therefore, IL-4 could have played a role in the exaggerated lung fibrotic repair caused by smoking and bleomycin. Studies in vivo also support a profibrogenic role for IL-4. Thus the use of neutralizing anti-IL-4 antibodies reduces murine Schistosoma mansoni-induced hepatic fibrosis and sclerodermia (9, 31). However, a recent report stresses this concept since the severity of bleomycin-induced hepatic fibrosis and sclerodermia (9, 31). Furthermore, TIMP-2 appears to be specifically synthesized by fibroblasts/myofibroblasts in the characteristic fibroblastic foci of IPF lungs (12, 44). Interestingly, an important link between IL-4 and TIMP-2 has been recently reported (15). From a variety of cytokines and growth factors assayed, only IL-4 was demonstrated to induce a dose- and time-dependent induction of TIMP-2 mRNA and protein by dermal fibroblasts.

Another important finding in guinea pigs from the TS/B group is the noteworthy increase in BAL eosinophils, a process that may be at least partially associated with the increase in IL-4 and the Th2-like cytokine network. Eosinophils have been associated with a number of tissue fibrotic conditions (30) and have been shown to act as direct modulatory cells in fibroblast proliferation, collagen synthesis, and lattice contraction, in part, through TGF-β (22). Furthermore, TGF-β1-producing eosinophils were predominantly localized within areas of active fibrosis in bleomycin-induced lung injury (52). It has been suggested that fibroblasts and alveolar epithelial cells may modulate eosinophil recruitment into the lung in this experimental model (38). The presence of eosinophils in the lungs of patients with pulmonary fibrosis has been correlated with poor prognosis or resistance to therapy (5).

In summary, our results demonstrate that exposure to cigarette smoke enhances lung fibrosis induced by bleomycin. This effect seems to be at least partially related with an increase in BAL neutrophils, MMP-9, and fibroproliferative activities. Guinea pigs that were exposed to smoke first and then were injured with bleomycin exhibited the highest fibrotic reaction, which was characterized by a Th2-like milieu.

DISCLOSURES

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