Hyperoxia-induced emphysematous changes in subacute phase of endotoxin-induced lung injury in rats

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Materials and Methods

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Animal preparation. After the rats were anesthetized with diethyl ether inhalation and pentobarbital sodium (50 mg/kg ip), the right jugular vein was cannulated with a saline-filled 60-gauge polyethylene tube. The extension line from the jugular vein was routed under prolonged hyperoxia.

This study examined, in this subacute animal model, the effects of prolonged hyperoxia on the changes in alveolar structure and metabolism of the lung collagen lining the alveoli, and on the collagenase and gelatinase activities and activation of matrix metalloproteinases (MMP-2 and MMP-9), which have been implicated in the pathogenesis of acute lung injury in several recent studies (8, 11, 26, 32).

MATERIALS AND METHODS

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Experimental model and chemicals. Specific pathogen-free male Sprague-Dawley rats, 10 wk of age and weighing 320–370 g, were used. LPS from Escherichia coli (serotype O55: B5 phenol extract; Sigma, St. Louis, MO) saline solution was continuously infused intravenously by an Alzet osmotic minipump 2002 (ALZA, Palo Alto, CA) at a rate of 12 μl/day to maintain endotoxemia for 10 days. A fixed concentration of O2 was set at 75% (FiO2 = 0.75), in which surgically treated rats were able to survive for 10 days. Hyperoxia was maintained in a 150 × 60 × 45-cm forced-air environmental chamber with a 12:12-h light-dark cycle. The chamber FiO2 was maintained at 0.75 using an O2 monitoring system (OM-24AN; Taieti, Tokyo, Japan). Unrestricted food and water were available to the animals.

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At a fixed pump rate, the extension line allowed the continuous infusion of saline in saline-treated animals and of endotoxin in endotoxin-treated animals. Twenty-four hours after surgery, the saline-treated animals received a continuous infusion of sterile isotonic saline while the endotoxin-treated animals received an infusion of LPS in sterile isotonic saline (500 μg·kg body wt·day\(^{-1}\)) at an isovolumetric rate of 12 μl/day. In five rats, the amount of LPS solution remaining in the osmotic pump was measured at the end of 10 days to confirm that the expected amount of LPS solution had been delivered during that period. Furthermore, sustained endotoxemia was confirmed in five animals by measurement of LPS plasma concentrations after 10 days of continuous infusion. The mean plasma concentration was ~13.0 pg/ml.

**Experimental design.** Twenty-four rats were divided into four groups. Endotoxin-treated animals (endotoxin alone and endotoxin + hyperoxia groups) received LPS (10 mg/kg body wt) suspended in 1 ml of saline as an intravenous bolus before the start of continuous infusion of LPS, and the other groups received 1 ml of saline as an intravenous bolus. The four groups were treated as follows. 1) Control group: rats received 1 ml of saline intravenously, followed by a continuous saline infusion by a minipump at a rate of 12 μl/day in room air. 2) Endotoxin alone group: rats were treated with LPS (10 mg/kg body wt iv bolus) followed by a continuous infusion of LPS by a minipump at a rate of 500 μg·kg body wt·day\(^{-1}\) in room air. 3) Endotoxin plus hyperoxia group: rats were treated with LPS (10 mg/kg body wt iv bolus), followed by continuous infusion of LPS by a minipump at a rate of 500 μg·kg body wt·day\(^{-1}\) in a chamber with a FIO\(_2\) = 0.75. 4) Hyperoxia alone group: rats were treated with a 1-ml bolus of saline intravenously, followed by continuous saline infusion by a minipump at a rate of 12 μl/day in a chamber with a FIO\(_2\) = 0.75.

All groups were killed 10 days after the onset of continuous infusion by injection of pentobarbital sodium (50 mg/kg iv) with 1,000 units of heparin. The chest was opened, and blood samples were obtained by cardiac puncture. Both lungs and the heart were resected ligated and cut sagitally into 3-μm-thick sections and stained with hematoxylin-eosin. Light microscopic morphometric techniques were applied, and the alveolar surface area per unit of lung volume (SV\(_w\)) was measured as previously described by Weibel (33) and Kawakami et al. (19). Briefly, a standard line of the same length (LT) was drawn on the field, and intersections with this line were counted (I\(_w\)). SV\(_w\) was calculated as SV\(_w\) = I\(_w\)/LT. Ten fields per section were analyzed blindly by the same investigator. Sections of lung specimens were also stained for collagen fibers by the reticulin silver impregnation method.

**Measurement of collagen content.** The frozen right lower lobe was weighed and homogenized in 1.5 ml of phosphate-buffered saline (Polytron; Kinematica, Lucerne, Switzerland). A sample of 0.4 ml was put in a test tube (Pyrex; Iwaki Glass, Chiba, Japan) and hydrolyzed with 36% HCl in an oven (Drying Sterilizer SG-62; Yamato Scientific) at 120°C for 8 h. The sample was neutralized with 6 M KOH, and particles were removed by filtering (Millipore 13-HV; 0.22 μm). The collagen content was measured by the methods of Yamato Scientific (Tokyo, Japan) at 95°C and ~270 cmH\(_2\)O for 48 h to remove any gravimetrically detectable water. Pulmonary water accumulation was the mean W/D ratio calculated from two lung pieces harvested from each lung.

**Histological and morphometric examination.** The left lobes were inflated by injection of 4% paraformaldehyde via the left main bronchus at a pressure of 25 cmH\(_2\)O, and the main bronchus was ligated and fixed. The lung was embedded in paraffin and cut sagitally into 3-μm-thick sections and stained with hematoxylin-eosin. Light microscopic morphometric techniques were applied, and the alveolar surface area per unit of lung volume (SV\(_w\)) was measured as previously described by Weibel (33) and Kawakami et al. (19). Briefly, a standard line of the same length (LT) was drawn on the field, and intersections with this line were counted (I\(_w\)). SV\(_w\) was calculated as SV\(_w\) = I\(_w\)/LT. Ten fields per section were analyzed blindly by the same investigator. Sections of lung specimens were also stained for collagen fibers by the reticulin silver impregnation method.

**Assessment of albumin leakage and pulmonary edema.** Transvascular albumin leakage (permeability index) was assessed from the ratio of albumin concentration in BAL fluid to that in plasma (albumin B/P ratio). The severity of pulmonary edema was estimated by the lung lobe W/D weight ratio. After we measured their wet weight, the lungs were completely dried in a vacuum oven (DP22; Yamato Scientific, Tokyo, Japan) at 95°C and ~270 cmH\(_2\)O for 48 h to remove any gravimetrically detectable water. Pulmonary water accumulation was the mean W/D ratio calculated from two lung pieces harvested from each lung.
of enzyme required to liberate 1 mol L-leucine equivalent from collagen.

Collagenase and gelatinase activities. Collagenase and gelatinase activities in BAL fluid were measured using fluorescein-labeled substrates, DQ gelatin from pig skin (Molecular Probes, Eugene, OR), or DQ collagen type I from bovine skin (Molecular Probes), respectively. Briefly, 100 μl of each substrate (final 2.5 μg/well) in reaction buffer (0.05 M Tris-HCl, 0.15 M NaCl, 5 mM CaCl₂, 0.2 mM sodium azide, pH 7.6) were added to microplate wells. Next, 100 μl of BAL fluid, appropriately diluted with the reaction buffer or Clostridium collagenase solution (Molecular Probes) for construction of the standard curve, were added and incubated at room temperature. Fluorescence intensity was measured at 30, 60, and 120 min using a fluorescence microplate reader (Sectrafluor Plus; TECAN, Salzburg, Austria) set for excitation at 485 nm and emission detection at 535 nm. Gelatinase and collagenase activities were both calculated from the increase in fluorescence intensity as Clostridium collagenase equivalence. One unit is defined as the amount of enzyme required to liberate 1 mol L-leucine equivalent from collagen in 5 h at 37°C, pH 7.5.

Gelatine zymography. SDS-8% polyacrylamide gels containing 1 mg/ml of gelatin were used to identify proteases with gelatinolytic activity in BAL fluid. After electrophoresis under nonreducing conditions, the gel was washed with a solution of 2.5% Triton X-100 and incubated at 37°C for 42 h in 50 mM Tris-HCl (pH 7.5) buffer containing 150 mM NaCl, 10 mM CaCl₂, and 0.02% Triton X-100. The gels were stained with 0.25% Coomassie blue R-250 in 50% methanol and 10% acetic acid and destained in 30% methanol and 5% acetic acid. A prestained molecular weight marker (Bio-Rad, Hercules, CA) and a mixture of human MMP-2/pro-MMP-2, -9 (Gelatin Zymo MMP Marker; Yagai, Yamagata, Japan) was used as reference standards.

Densitometric analysis of the gels was performed using NIH imaging software and a high-resolution scanner. The ratio of activated MMP-2 to total MMP-2 (activated MMP-2 + pro-MMP-2) and that of activated MMP-9 to total MMP-9 (activated MMP-9 + pro-MMP-9) were estimated from their gelatinolytic activities (21).

Statistical analysis. All data are presented as means ± SE. The variables were compared by one-way analysis of variance followed by Dunnett’s post hoc test to detect statistically significant differences vs. the control group. A P value <0.05 was considered statistically significant.

Fig. 3. Histopathological findings in left lung of rats from control (A), endotoxin (B), endotoxin + hyperoxia (C), and hyperoxia (D) groups after 10 days of treatment with continuous endotoxin infusion and prolonged hyperoxia. Remarkable emphysematous changes are visible in the lung of the rat in C, although mild similar changes are observed in the animal in D. In the rats in B–D, inflammatory cells are infiltrating the lungs, and the alveolar walls are mildly thickened with collagen deposition in the endotoxin-exposed rat (B, inset). Hematoxylin-eosin and reticulin silver impregnation are shown (insets). Bars = 100 μm (outside of inset) or 50 μm (inset).

Fig. 4. Alveolar surface area per unit of lung volume after 10 days of treatment with continuous endotoxin infusion and prolonged hyperoxia. Values are means ± SE. *P < 0.05 vs. control.
RESULTS

Pulmonary edema and albumin leakage. The albumin B/P ratio was significantly higher in the endotoxin, endotoxin plus hyperoxia, and hyperoxia groups than in controls (Fig. 1A). In the endotoxin plus hyperoxia group, the B/P ratio was approximately four times greater than in controls. Pulmonary edema, estimated by the lung W/D ratio (Fig. 1B), was most prominent in the endotoxin plus hyperoxia group, although not significantly different than in controls.

Cell counts in BAL fluid. The differential cell counts in BAL fluid after 10 days of endotoxin infusion and/or hyperoxia are shown in Fig. 2. The neutrophil counts in BAL fluid were significantly increased in both the endotoxin and the endotoxin plus hyperoxia groups, and most prominently in the latter. The macrophage count was also significantly increased in the endotoxin plus hyperoxia group compared with the control group.

Histological examination. The histopathology of the left lung stained with hematoxylin and eosin in the four experimental groups is illustrated in Fig. 3. Emphysematous changes were apparent in the hyperoxia group, and slight septal thickening was present in the endotoxin group. Destruction of alveoli was most prominent in the endotoxin plus hyperoxia group, as it had the most marked air space enlargement. Inflammatory cell accumulation was also observed in all three groups. In lungs stained with reticulin silver impregnation, increased collagen deposition in alveolar walls was distinct in the endotoxin group but faint in the hyperoxia and endotoxin plus hyperoxia groups.

Alveolar surface area. SV_w was estimated by morphometric examination (Fig. 4). After 10 days of endotoxin infusion and exposure to hyperoxia, there was a significant decrease (P < 0.05) in the alveolar surface area in the endotoxin plus hyperoxia group (0.035 ± 0.002 m²/cm³) compared with the control group (0.042 ± 0.001 m²/cm³).

Collagen content. Hydroxyproline was significantly increased in the endotoxin group (2,083 ± 131 ng/mg of tissue) and significantly decreased in the hyperoxia group (1,290 ± 78 ng/mg of tissue; both P < 0.05) compared with the control group (1,644 ± 96 ng/mg of tissue; Fig. 5). Hydroxyproline content in the endotoxin plus hyperoxia group was 1,516 ± 27 ng/mg of lung tissue, which was intermediate between that in the endotoxin and the hyperoxia groups.

Collagenase and gelatinase activities. In the hyperoxia-exposed animals, collagenase activity in BAL fluid was comparable in the hyperoxia alone group and endotoxin plus hyperoxia group (0.023 U/ml), and approximately five times greater than in controls with significant difference (P < 0.05, Fig. 6A).

Gelatinase activity in BAL fluid showed the same pattern as collagenase activity and was significantly increased in the hyperoxia-treated groups (P < 0.05 vs. controls, Fig. 6B).

Protease zymography. On the gelatin zymography of the supernatant of BAL fluid, two major bands were observed, which were consistent with the latent form of MMP-9 (pro-MMP-9, 92 kDa; Fig. 7A) and the latent form of MMP-2 (pro-MMP-2, 72 kDa; Fig. 7B) based on their molecular weights. These two bands were more prominent in the three lung-injured groups than in controls. The band of pro-MMP-9 was most prominent in the endotoxin and the endotoxin plus hyperoxia groups. Furthermore, another weak band of active MMP-9 was observed in the endotoxin-treated groups under the pro-MMP-9 band. On the other hand, a band of active MMP-2 was detected in the hyperoxia and endotoxin plus hyperoxia groups.

The activation ratio, estimated by quantitative gelatin zymography of MMP-9, was significantly increased in the endotoxin-treated groups (P < 0.05 vs. controls) but not in the hyperoxia group (Fig. 8A). Conversely, the activation ratio of MMP-2 was significantly increased in the prolonged hyper-
P < 0.05 vs. controls) but not in the endotoxin group (Fig. 8B).

**DISCUSSION**

The first goal of our study was to develop a model of subacute lung injury by continuously infusing endotoxin during hyperoxia for 10 days. Other attempts have been made to create chronic endotoxemia models (5, 23). However, we are not aware of a previous model in which the continuous intravenous infusion of LPS and a high-O2 concentration were simultaneously administered over several days.

The capillary permeability was persistently higher in all three groups exposed to endotoxin and/or hyperoxia than in controls, although the lung W/D ratio was not significantly increased in any group. In addition to endotoxin, the exposure to high concentrations of O2, which by itself caused lung injury, resulted in an additional increase in capillary permeability and accumulation of both macrophages and neutrophils in the endotoxin plus hyperoxia group. Hyperoxia caused greater damage to the alveolar capillary barrier in the subacute phase of endotoxin-induced lung injury. Had we selected a more sensitive method instead of the simple W/D ratio to estimate lung edema, such as subtracting the intravascular blood weight in the calculation, we might have detected significant differences in the lung W/D ratio among the groups.

In our model, the abundant emphysematous destruction of alveolar walls occurred after simultaneous exposure to endotoxin and hyperoxia. The loss of SVw, which reflects the destruction of alveolar structures, was most pronounced in the endotoxin plus hyperoxia group, mainly from emphysematous changes. Whereas hyperoxia is known to cause or enhance emphysematous changes under certain conditions (6, 20), this study demonstrated the noxious effects of hyperoxia on lung structure during the subacute phase of endotoxin-induced lung injury.

Endotoxin and hyperoxia had opposite effects on the collagen content, i.e., it was increased by endotoxin and decreased by hyperoxia. With endotoxin plus hyperoxia, the collagen content was intermediate between that observed in the endotoxin and hyperoxia groups. These results were associated with pathological changes consisting of mild alveolar wall thickening with endotoxin and emphysematous changes with hyperoxia. Hyperoxia and endotoxin similarly reduce the alveolar surface area (Fig. 4). However, in the endotoxin group, this effect seemed to be due to fibrotic rather than emphysematous changes, considering the increase in collagen content and deposition on the alveolar walls. In contrast, the most prominent histological finding in the endotoxin plus hyperoxia group was the presence of emphysematous changes, which were also present in the hyperoxia group, along with a decrease in collagen content. Therefore, we believe that the emphysematous changes in the endotoxin plus hyperoxia group are more attributable to hyperoxia.

There are a few reports describing the involvement of LPS in pulmonary fibrosis. Corbel et al. (9) recently reported that repeated exposure of mice to LPS by aerosol may lead to interstitial fibrosis with collagen deposition in the alveolar walls and that MMP appears to be associated with this process. Briggs et al. (4) reported that multiple intraperitoneal injections of endotoxin enhanced fibrosis in bleomycin-induced pulmonary fibrosis. These reports support our finding of collagen deposition in the alveolar wall induced by continuous intravenous infusion of LPS for 10 days.

Some authors have reported experimental emphysematous changes with degradation of collagen in the lung by exposure to hyperoxia for 4–60 h (20, 28). Cantor et al. (6) demonstrated that exposure to 60% O2 enhanced lung injury in elastase-induced emphysema, and Riley et al. (28) reported that lung collagen was degraded and emphysematous lesions were...
produced by relatively short exposure to high-O₂ concentrations. In our endotoxin plus hyperoxia treatment model, degradation of collagen induced by hyperoxia and deposition in the alveolar wall induced by endotoxin may occur simultaneously.

Other authors have reported that pretreatment with a single small dose of endotoxin is protective against severe acute hyperoxia at a FiO₂ between 95 and 100% (3, 13). Frank et al. (13) showed that this was related to a stimulated increase in the activity of the pulmonary antioxidant enzyme defense system. However, in our subacute model where hyperoxia at a FiO₂ of 75% and a larger amount of entotoxin were simultaneously and continuously administered over 10 days, the emphysematous changes were probably caused by activated MMPs rather than by oxidants. Therefore, antioxidants may not be effective in protecting the lung.

One group of authors (10) has previously reported that collagenase expression in the lungs of transgenic mice causes pulmonary emphysema and provided evidence that extracellular matrix proteases other than elastase could play a role in pulmonary emphysema. In our lung injury model, alveolar macrophages were the most abundant inflammatory cells found in BAL fluid, which has been recently suggested to be a major source of metalloproteinases and which may play a prominent role in the pathogenesis of lung injury in clinical or experimental emphysema (14, 27, 30). The importance of neutrophils is unequivocal. Neutrophil infiltration was observed in our model, and MMP-9 is a component of the primary granules of neutrophils as well as a major macrophage product. We have also reported that neutrophils, which infiltrate tissues in various inflammatory conditions, may play an important role in regulating tissue destruction through MMP activity in vivo. These cells could have contributed to the emphysematous changes in our model.

Elevation of collagenase activity in BAL fluid in the hyperoxia-treated groups was probably due to an increase in the activities of several collagenase subfamilies, including neutrophil collagenase (MMP-8) (15). It has recently been shown that MMP-14 (MT1-MMP) is released from some cell types and that it can also degrade type I collagen (17, 24). Furthermore, MMP-2 enhances the degradation of type I collagen by MMP-14 (2, 24). Thus these MMPs may also contribute to the elevation of collagenase activity. Gelatinase activity in BAL fluid was increased by hyperoxia, perhaps because of MMP-2 and/or MMP-9 activation. Hyperoxia markedly increased the expression of pro-MMP-2, pro-MMP-9, and the active/total MMP-2 but not the active/total MMP-9 ratio. Endotoxin treatment caused more neutrophil infiltration, an increase in pro-MMP-9, and activation of MMP-9, as well as an increase in pro-MMP-2, although less MMP-2 activation and less collagenase and gelatinase activities in BAL fluid were detected. Thus the latent forms of MMP-2 and MMP-9 may be activated differently by endotoxin and hyperoxia, despite the presence of similar types of inflammatory cells in both groups, suggesting that some microenvironmental differences may also contribute to the apparent preferential MMP activation. Gelatinase activity, measured as a degrading activity of a fluorescent-labeled substrate, did not correlate directly with zymographic level of the activated forms of MMP-9 and/or MMP-2, perhaps due to different regulations by endogenous inhibitors of enzyme activities, such as TIMPs. Consequently, hyperoxia and MMP-2 activation appeared to be more closely associated with the emphysematous changes than endotoxin and MMP-9. We hypothesize that MMP-2, together with other collagenases and gelatinases, including MMP-8, MMP-9, and/or MMP-14, may play key roles in the destruction of air space structures.

The substrate specificity of MMP-2 and MMP-9 involves the degradation of type IV collagen, the major structural component of basement membranes, denatured collagen (gelatin), and insoluble elastin. We hypothesize that endotoxin upregulates MMPs, causes lung injury (11), and plays a role in the remodeling associated with pulmonary fibrosis (8) and that hyperoxia activates MMPs (26) and causes emphysema. In our model, endotoxin and hyperoxia together may disrupt the basement membrane and cause high capillary permeability by activation of MMP-2 and MMP-9. Although endotoxin by itself can activate MMP-9, active MMP-9 alone might not be sufficient to cause the emphysematous changes in the endotoxin alone group.

Our results suggest that prolonged hyperoxia causes pulmonary emphysematous changes with persistent injury to the alveolar capillary barrier in the subacute phase of endotoxin-induced lung injury. Collagenases and MMP-2 activated by hyperoxia together with MMP-9 may play a prominent role in the disruption of the alveolar basement membranes and degradation of collagen lining the alveolar walls.

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