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Antimacrophage chemokine treatment prevents neutrophil and macrophage influx in hyperoxia-exposed newborn rat lung

Michael A. Vozzelli, S. Nicholas Mason, Mary H. Whorton, and Richard L. Auten, Jr. Antimacrophage chemokine treatment prevents neutrophil and macrophage influx in hyperoxia-exposed newborn rat lung. Am J Physiol Lung Cell Mol Physiol 286: L488–L493, 2004. First published February 14, 2003; 10.1152/ajplung.00414.2002.—Macrophage-derived cytokines may provoke the inflammatory response in lung injury. Because macrophage influx is a prominent feature of the cellular inflammatory response accompanying the development of bronchopulmonary dysplasia, we hypothesized that blocking macrophage influx would reduce overall cellular influx and oxidative damage. Newborn rats were exposed at birth to 95% O2 or air for 1 wk, and hyperoxia-exposed pups were injected with anti-monocyte chemoattractant protein-1 (MCP-1) or IgG control on days 3–5. MCP-1 was increased in bronchoalveolar lavage fluid and in histological sections from the 95% O2-exposed. IgG-injected pups compared with air-exposed controls. At 1 wk, anti-MCP-1-treated pups had reduced leukocyte numbers, both macrophages and neutrophils, in bronchoalveolar lavage fluid compared with IgG-treated controls. Cytokine-induced neutrophil chemoattractant-1, the rat analog of IL-8, was not significantly decreased in lavage fluid but was reduced in lung cells in anti-MCP-1-treated pups. Tissue carbonyls, a measure of protein oxidation, were decreased in anti-MCP-1-treated pups. Anti-MCP-1 treatment prevented neutrophil influx and reduced protein oxidation in hyperoxia-exposed newborn rats.

 bronchopulmonary dysplasia; oxidative stress; white blood cell

EARLY INFLAMMATION MAY CONTRIBUTE to disrupted lung development in premature newborns who later develop bronchopulmonary dysplasia (BPD). Elevations of leukocyte chemokines have been observed in tracheal aspirates of newborns who later develop BPD (21, 22). Elevations of monocyte chemoattractant protein-1 (MCP-1), for example, have been associated with the development of BPD (2).

Macrophages are important sources of leukocyte chemokines, such as chemokine cytokine-induced neutrophil chemoattractant-1 (CINC-1) and macrophage inflammatory protein-2 (MIP-2) in rats (13) and interleukin-8 (IL-8) and growth-related oncogene-α in humans (23). They are among the first extrapulmonary cells to infiltrate following injury and during acute infant respiratory distress syndrome (19). Hyperoxia exposure is used to model the oxidative stress experienced by newborns who develop BPD, since it produces neutrophil and macrophage influx, followed by disrupted alveolar development (1, 9, 29). Hyperoxia induces MCP-1 expression in several species (10, 11, 15). Blocking macrophage influx by neutralizing MCP-1 may therefore reduce neutrophil influx and reduce oxidative damage.

We hypothesized that early treatment with neutralizing antibody against MCP-1 would prevent both neutrophil and macrophage accumulation in the airways of hyperoxia-exposed newborn rats. We found that hyperoxia induced elevated MCP-1 expression in newborn rats, particularly in bronchiolar and alveolar epithelium. Anti-MCP-1 treatment reduced both macrophage and neutrophil accumulation in hyperoxia-exposed newborn rat lungs. CINC-1 was diminished in airway epithelium following anti-MCP-1 treatment, as was protein oxidation.

METHODS

Animals. All procedures were approved by the Institutional Animal Care and Use Committee. Timed-pregnant Sprague-Dawley rats were exposed on the day of delivery to air or 95% oxygen along with recombined litters as previously described (13). Nursing dams were exchanged daily between air- and hyperoxia-exposed litters. Litter size was adjusted to maintain numerical balance among litters.

MCP-1 expression. Air- and hyperoxia-exposed pups were killed on days 2, 4, 6, and 7 with an overdose of pentobarbital sodium given intraperitoneally, and we perfused the pulmonary artery with 0.9% NaCl and 1 mM EDTA, pH 7.5, after clipping the left atrium. A tracheal cannula was ligated in place, and four 0.5-ml bronchoalveolar lavages (BAL) were slowly performed, and the samples were pooled. After centrifugation at 2,000 g for 10 min, supernatants were snap-frozen in liquid nitrogen and later analyzed for rat MCP-1 by ELISA with a commercial kit (Opt-EIA; Pharmingen, San Diego, CA). To evaluate the effects of hyperoxia on histochemical expression of MCP-1, we killed air- and oxygen-exposed animals on day 7, and lungs were inflation fixed at 30 cmH2O pressure for 30 min and then immersed overnight in 10% phosphate-buffered formalin, paraffin embedded, and sectioned at 4-μm thickness. Random sections were incubated with rabbit anti-MCP-1 (1:1,000; Torrey Pines Biolabs, Houston, TX) or nonimmune rabbit serum as a negative control and then detected with goat-anti-rabbit antibody (1:1,000) followed by substrate visualization with the ABC Elite system and VIP peroxidase color substrate (Vector, Burlingame, CA). Sections were counterstained with methyl green. Three sections from three animals group were evaluated.

Anti-MCP-1 treatment. On days 3, 4, and 5, hyperoxia-exposed pups, n = 10/group, were injected daily with 0.1 ml of PBS, pH 7.4,
containing control (vehicle only), 5 or 25 μg of rabbit anti-rat MCP-1, a neutralizing antibody (Torrey Pines Biolabs), or 5 μg of nonimmune rabbit IgG given intraperitoneally. Exposures continued until animals were killed on day 7.

**BAL fluid analysis.** BAL fluid (BALF) was obtained as above, and cells were counted with a hemacytometer. At least 200 cells/animal were evaluated following cytospin and modified Wright’s staining. Pooled BALF supernatants from each pup were snap-frozen in liquid nitrogen for cytokine analysis.

**CINC-1 expression.** CINC-1 concentrations were measured in BALF from 10 animals in each treatment condition by an ELISA we previously described in detail (13). We chose three random sections from three animals in each treatment group: air-, hyperoxia-exposed, and hyperoxia + 25 μg anti-MCP-1 treatment.

**Protein carbonyl measurement.** To determine the effect of anti-MCP-1 treatment on oxidant stress during hyperoxia exposure, we homogenized lungs in an anti-protease cocktail (Complete, Roche) with an antioxidant, butyl-hydroxytoluene. Homogenates were derivitized with dinitrophenylhydrazine (DNPH) to detect carbonyl residues formed by protein oxidation. Homogenates were reacted with 10 mM DNPH in 6 M guanidine hydrochloride, 0.5 M potassium phosphate buffer, pH 2.5, and the DNPH-derivitized proteins were detected by ELISA using the method described by Buss et al. (5).

**Statistical analysis.** Results are expressed as means ± SE. Treatment group differences were compared with ANOVA, and significant differences were identified by post hoc analysis using Tukey-Kramer. Significance was accepted at \( P < 0.05 \), assuming an \( \alpha \)-error = 0.05 and \( \beta \)-error = 0.10.

**RESULTS**

Hyperoxia-exposed rats were smaller than air-exposed rats (14.5 ± 0.2 vs. 16.5 ± 0.3 g, \( P < 0.001, n = 20 \)/group). There were no significant differences among the oxygen-exposed control groups, and there was no effect of anti-MCP-1 at either dose on body weight. There was mortality only in the oxygen-exposed groups (air: \( n = 188, 100\% \) survival; hyperoxia: \( n = 200, 93\% \) survival; \( P = 0.0001 \)) and no differences in mortality among each of the oxygen-exposed treatment groups.

**MCP-1 expression.** 95% \( \text{O}_2 \) exposure significantly induced MCP-1 accumulation in BALF at all time points measured, with a doubling at day 2, and between fifteen- and fifty-fold increases at days 4, 6, and 7 (\( n = 9 \)/group, Fig. 1).

MCP-1 immunostaining was plainly apparent in bronchiolar and alveolar epithelium, and staining in both of these cell types was increased by 95% \( \text{O}_2 \) exposure at 1 wk (Fig. 2). Both ciliated and nonciliated bronchiolar epithelium were labeled, and higher magnification views showed septal-tip staining, particularly in the hyperoxia-exposed animals. We did not quantify staining differences among cell types.

**Anti-MCP-1 treatment: BALF leukocytes.** Hyperoxia (control, sham, and IgG-injected) induced significant increases in BALF leukocytes, both neutrophils and macrophages. Anti-MCP-1 treatment at both doses significantly reduced the BALF neutrophil counts, \( n = 6 \)/group. Only pups injected with 25 μg of anti-MCP-1 showed reductions in total and macrophage cell counts, as well as neutrophil counts (Fig. 3).

**Anti-MCP-1 effect on CINC-1 expression.** Hyperoxia induced significant increases in CINC-1 in BALF. At the highest dose, anti-MCP-1 reduced CINC-1 concentrations in BALF, although this did not reach statistical significance (Fig. 4). CINC-1 expression was increased in both alveolar and bronchiolar epithelium, as well as in macrophages in hyperoxia-exposed rats compared with air-exposed rats. Anti-MCP-1 treatment decreased CINC-1 expression in all cell types (Fig. 5), although this was not strictly quantified.

**Anti-MCP-1 treatment: protein carbonyls.** Hyperoxia induced significant increases in lung homogenate protein carbonyls detected by DNPH derivitization. Anti-MCP-1 at 5 μg had no effect but at 25 μg completely prevented the hyperoxia-induced increase in carbonyls (Fig. 6).
DISCUSSION

We have shown that blockade of hyperoxia-induced MCP-1 with neutralizing antibodies can prevent both macrophage and neutrophil accumulation in the lungs of hyperoxia-exposed newborn rats. This resulted, at the highest anti-MCP-1 dose, in reduced protein oxidation in lung tissue at the highest dose. There were also effects on BALF leukocytes and neutrophils at the lower anti-MCP-1 dose that were not apparent in saline- or nonimmune IgG-treated control groups. We did not administer nonimmune IgG at the higher 25-μg dose, so we cannot completely exclude nonspecific effects of IgG that we believe are nevertheless unlikely. Indeed, intermediate or higher anti-MCP-1 doses or pretreatment with anti-MCP-1 may be as effective or more effective. Resident alveolar macrophages and recruited macrophages take part in host defense following lung injury. Clinical and experimental studies have demonstrated prompt influx of macrophages in newborn lung injury, particularly respiratory distress syndrome (7, 19). Recruitment of macrophages is under the control of macrophage chemokines such as MCPs 1–4 and MIP-1α, some of which chemoattract macrophages through receptors CC-R1 and CC-R2 (23). The relative contribution of each of the potential macrophage chemoattractant pathways may differ depending on the inflammatory stimulus.

Hyperoxia has previously been shown to stimulate MCP-1 mRNA and protein accumulation in the lung in a number of species, in both adults and newborns (10–12, 15, 20). Likewise, lung injury induced by mechanical ventilation also increased MCP-1 (6). Clinical studies have implicated its participation in neonatal lung injury associated with BPD (2).

Blockade of MCP-1 or its receptors has had mixed results, depending on the injury model. Anti-MCP-1 did not affect macrophage or neutrophil recruitment in a model of immune complex-induced lung injury in adult rats (3). Lethality was increased along with decreased IL-10 and increased TNF-α and IL-12 in adult mice given anti-MCP-1 before endotoxin challenge. The reverse trends were observed in mice given exogenous MCP-1 (30). Because macrophages are necessary...
for clearance of apoptotic neutrophils (17), it may be that premature blockade of macrophage influx permitted neutrophils to proceed to necrosis, leading to elaboration of damaging proteases.

We thought that decreasing macrophage influx might decrease neutrophil influx by decreasing a significant cellular source of neutrophil chemokines (13). Although there was a trend toward reduced CINC-1 in BALF in anti-MCP-1-treated rats that did not achieve significance, we did note decreased alveolar and bronchiolar epithelial CINC-1 immunohistochemical labeling in high-dose anti-MCP-1 95% O₂-exposed rats. We did not test for the presence of other chemokines that are secreted by macrophages, such as MIP-2, which could be influencing neutrophil recruitment.

Decreasing macrophage and monocyte activation by treatment with anti-MCP-1 could have reduced neutrophil influx in other ways. Maus and colleagues (24) have shown that MCP-1-stimulated monocyte influx without accompanying injury does not provoke neutrophil emigration into the lung. However, selective depletion of already resident alveolar macrophages did reduce airway neutrophil recruitment and neutrophil chemokine in an endotoxin injury model (26). It may be that sequential analysis of neutrophil chemokine abundance and localization of expression would clarify mechanisms of neutrophil-macrophage cross talk in this model, since neutrophils themselves secrete neutrophil (16) and macrophage chemokines (14).

In contrast, we observed no anti-MCP-1-associated differences in mortality among the hyperoxia-exposed newborn rats or significant blockade of both macrophages and neutrophils at the highest anti-MCP-1 dose in our model of newborn lung injury. Because the hyperoxia effects are less intense than those provoked by endotoxin (chemokine elevations accumulate in days, not hours), the contribution of macrophages to counterregulate or resolve acute inflammation may be relatively less important.
The nature of the injury is likely to determine the effect of macrophage blockade. If the macrophages themselves are principal sources of neutrophil chemokines, then it may explain why anti-MCP-1 had beneficial results in our model. Maus and colleagues (25) found that knockout mice lacking CC-R2, a principal MCP-1 receptor, did not mobilize neutrophils in response to intratracheal lipopolysaccharide (LPS). They also found that the physiological benefits of MCP-1 blockade were neutrophil dependent (26). This is consistent with our finding that neutrophils and CINC-1 were reduced in animals that neutrophil dependent (26). This is consistent with our finding that neutrophils and CINC-1 were reduced in animals that

Timing of maneuvers designed to block macrophage influx may also be crucial. For example, resolution of pulmonary inflammation, particularly clearance of neutrophils, depends on neutrophil signaling of alveolar macrophages (27). Late administration of antimacrophage chemokine antibodies might delay resolution of inflammation, possibly allowing damaging contents of neutrophils to persist in the lung. Anti-MCP-1 might accelerate clearance of already resident macrophages, since macrophage survival following activation may depend on continued MCP-1 transduction (18).

Chronic lung inflammation may lead to pathological repair that is accompanied by failed alveolization and fibrosis (8). Repair depends on macrophage-expressed mitogens such as platelet-derived growth factor and others (4), but the timing of these processes is not well understood (27). It is conceivable that attenuation of late macrophage influx could interrupt maladaptive repair, preventing the development of pulmonary fibrosis, as has been done in bleomycin-challenged mice treated with antimacrophage chemokines (28).

In conclusion, we report that hyperoxia-induced pulmonary leukocyte accumulation in newborn mice can be partly prevented by treatment with anti-MCP-1. At the highest dose, anti-MCP-1 also prevented protein oxidation in lungs from hyperoxia-exposed mice. We speculate that properly timed administration of macrophage chemokine blockade can attenuate inflammation and its consequent biomolecular damage, which impairs lung development in BPD.

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GRANTS

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