Parathyroid hormone-related protein reduces alveolar epithelial cell proliferation during lung injury in rats

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Hastings, Randolph H., John T. Berg, Daphne Summers-Torres, Douglas W. Burton, and Leonard J. Deftos. Parathyroid hormone-related protein reduces alveolar epithelial cell proliferation during lung injury in rats. Am J Physiol Lung Cell Mol Physiol 279: L194–L200, 2000.—Parathyroid hormone-related protein (PTHrP) is a growth inhibitor for alveolar type II cells and could be a regulatory factor for alveolar epithelial cell proliferation after lung injury. We investigated lung PTHrP expression in rats exposed to 85% oxygen. Lung levels of PTHrP were significantly decreased between 4 and 8 days of hyperoxia, concurrent with increased expression of proliferating cell nuclear antigen and increased incorporation of 5-bromo-2’-deoxyuridine (BrdU) into DNA in lung corner cells. PTHrP receptor was present in both normal and hyperoxic lung. To test whether the fall in PTHrP was related to cell proliferation, we instilled PTHrP into lungs on the fourth day of hyperoxia. Eight hours later, BrdU labeling in alveolar corner cells was 3.2 ± 0.4 cells/high-power field in hyperoxic PBS-instilled rats compared with 0.5 ± 0.3 cells/high-power field in PTHrP-instilled rats (P < 0.01). Thus PTHrP expression changes in response to lung injury due to 85% oxygen and may regulate cell proliferation.

THE PLURIPOTENTIAL CAPABILITY of alveolar type II epithelial cells plays an important role in repairing the alveolar epithelium after lung injury. Alveolar epithelial damage is a prominent feature of many inflammatory conditions (25). Type II cells are more resistant to injury than type I cells and are able to repair the defects resulting from loss of type I cells. After lung injury, type II cells can divide, spread across the denuded basement membrane, and differentiate into type I cells (1, 8). Alveolar epithelial repair is essential to prevent continued influx of proteinaceous fluid into the air spaces, to enable its clearance, and to restore normal gas exchange. Type II cells proliferate after lung injury caused by a variety of agents, including toxic chemical, fibrogenic dust, hyperoxia, and air pollutants (27). Proliferation typically begins within days follow-

ing the initial insult and persists for a variable length of time depending on the initiating agent, the severity of the insult, and whether the injury is acute or perpetuating. The mechanisms regulating type II cell proliferation after lung injury are poorly understood. Lung expression of several growth factors, such as transforming growth factor-α (TGF-α), insulin-like growth factor I (IGF-I), and hepatocyte growth factor (HGF), increase following lung injury (3, 4, 10, 15, 16, 20, 24), but a causal relationship with type II cell growth during the repair phase has not been demonstrated for any of these factors. This study investigated the role of another pneumocyte growth factor, parathyroid hormone (PTH)-related protein (PTHrP), after hyperoxic lung injury.

PTHrP is best known as the mediator of humoral hypercalcemia of malignancy. However, it exerts paracrine or autocrine growth-regulatory actions in a wide variety of normal tissues, including the lung. We have shown that type II cells produce and secrete PTHrP, express a receptor for PTHrP, and respond to changes in ambient PTHrP concentration with alterations in function and growth (12–14). The amino-terminal portion of the molecule, PTHrP-(1–34), stimulates characteristics of the differentiated type II cell phenotype, such as production of disaturated phosphatidylcholine (13). In addition, endogenous PTHrP-(1–34) is an autocrine inhibitor of type II cell growth in vivo and in vitro (14). PTHrP-(1–34) is the portion of the molecule that binds and activates the classical PTHrP-PTH receptor. Because PTHrP-(1–34) is a type II cell growth inhibitor, we conjectured that type II cell proliferation after lung injury might result from decreased lung levels of PTHrP. The purpose of this study was to investigate the response of PTHrP expression in the lung to injury and to determine whether changes in PTHrP regulate type II cell proliferation.

METHODS

Reagents. Male pathogen-free Sprague-Dawley rats (250–300 g) were ordered from Harlan Laboratories (San Diego, California 92161-5085; E-mail: rhhastings@ucsd.edu).

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CA). Evans blue, paraformaldehyde, 5-bromo-2′-deoxyuridine (BrdU), and other chemical reagents came from Sigma (St. Louis, MO). Synthetic PTHrP peptides and a rabbit polyclonal antibody to PTHrP-(1–34) (RAS6151n) were purchased from Peninsula Laboratories (Belmont, CA). Monoclonal antibody to proliferating cell nuclear antigen (PCNA), PC10, was supplied by DAKO (Carpinteria, CA). A mouse monoclonal antibody directed against PTHrP-(1–34) (8B12) was developed in our laboratory (13). A mouse monoclonal antibody to BrdU was obtained from Becton Dickinson (San Jose, CA). Rat II, a rabbit polyclonal antibody to the rat PTHrP receptor, came from Berkeley Antibody (Berkeley, CA). The antigenic peptide for the PTHrP receptor antibody, NH$_2$-ESKENKDVPTGSSRRGR-COOH (9), was purchased from Genemed Biotechnologies (South San Francisco, CA). Biotinylated goat anti-mouse and anti-rabbit IgG antibodies were purchased from Chemicon (Temecula, CA).

**Chronic hyperoxic lung injury.** All animal protocols were reviewed and approved by the animal care and use committee. Rats were housed, two to a cage, in a 50-liter Plexiglas chamber ventilated with 6 l/min of 85% oxygen in nitrogen for 1, 2, 4, 6, 8, 10, or 14 days. The oxygen concentration and carbon dioxide tension of outflow gas were measured in line daily with an Ohmeda 5250 RGM gas analyzer (Englewood, CO). The Ohmeda analyzer was checked occasionally against a Datex Capnomac Ultima gas analyzer (Helsinki, Finland). Oxygen concentrations measured by the two instruments always agreed within 1%. Oxygen concentrations were always within 85 ± 3%, and carbon dioxide tensions never exceeded 3 mmHg. As controls, additional rats were housed in the chamber ventilated with ambient air at the same flow rate for 2, 4, 8, and 14 days. Rats had free access to food and water. Cages were exchanged for clean cages every 2 days, a procedure that took less than 1 min.

In experiments where immunocytochemistry was planned, rats were injected with 25 mg/kg BrdU 2 h before death. At the end of the experimental period, rats were anesthetized with 30 mg/kg pentobarbital sodium intraperitoneally. The right main stem bronchus was ligated, and the right lung was removed for PTHrP assay and gravimetric lung water analysis. Hemoglobin concentrations were always within 85% oxygen, and 6 h later, they were injected with 25 mg/kg BrdU and 25 mg/kg pepsin to strip proteins from DNA, and a 30-min incubation in 2 N HCl to denature DNA before incubation with primary antibody. After primary antibody treatment, sections were treated with secondary antibody diluted 1:250 in PBS with 0.5% bovine serum albumin for 30 min. The staining finished with incubation with streptavidin-peroxidase enzyme conjugate, DAB, and hydrogen peroxide.

**Immunohistology.** Lung blocks stained with Evans blue were dehydrated through an ethanol-butanol series and embedded in paraffin. Sections (5 μm) were rehydrated in xylene and hydrated through a graded ethanol series. Non-specific protein binding was blocked with 10% goat serum, 0.02% rat IgG, and 0.02% sodium azide in PBS. Sections were incubated with primary antibody overnight at 4°C. PTHrP was stained with primary antibody 8B12 (10 μg IgG/ml). 8B12 is specific for PTHrP-(1–34) and does not cross-react with human calcitonin, salmon calcitonin, human calcitonin gene-related peptide (CGRP), rat CGRP, human PTH, or rat PTH. PCNA staining used a 1:400 dilution of PC10. BrdU was detected with a 1:300 dilution of anti-BrdU antibody in a solution of 0.5% Tween 20 and 0.5% bovine serum albumin, and PTHrP receptor was stained with RT II (2 μg IgG/ml). RT II is an affinity-purified rabbit polyclonal antibody specific for residues 90–107 of the rat kidney type I PTHrP receptor, a sequence in an extracellular domain of the receptor (9). The protocol for BrdU included an additional 10-min incubation in 0.0085 N HCl, 0.4% pepsin to strip proteins from DNA, and a 30-min incubation in 2 N HCl to denature DNA before incubation with primary antibody.

**Radioimmunoassay of PTHrP.** Levels of PTHrP were determined in the supernatants with a previously described radioimmunoassay (6) using a rabbit polyclonal antibody to PTHrP-(1–34). The quantity of PTHrP in the lung sample was calculated by multiplying the concentration in the lung supernatant by the total water content of the lung homogenate. PTHrP levels were expressed per gram of dry lung.

**Gravimetric lung water analysis.** The total water content of the lung homogenate and the dry lung weight were determined by gravimetric analysis. Samples of lung homogenate, lung supernatant, blood, and lysis buffer were dried to constant weight at 60°C, and water-to-dry weight ratios were calculated for each tissue or liquid. Hemoglobin concentrations were measured in lung supernatants and blood. Then the weight of extravascular lung water and the dry weight of the lung were calculated as described by Matthay et al. (18). The lung dry weight was corrected for the dry weight of the lysis buffer.

**PTHrP replacement in rats exposed to chronic hyperoxia.** To test the effects of PTHrP on cell proliferation in injured lung, rats were anesthetized with 4% isoflurane, intubated, and installed with 1 ml of PBS or 1 ml of PBS containing 7.5 μg of PTHrP-(1–34). After recovery, rats were returned to 85% oxygen, and 6 h later, they were injected with 25 mg/kg BrdU intraperitoneally. Rats were killed with 30 mg/kg pentobarbital sodium intraperitoneally 2 h later (8 h after PTHrP or PBS instillation), and the lungs were fixed for BrdU immunocytochemistry or homogenized for PTHrP determination.

**Radioimmunoassay of PTHrP.** Lungs were weighed, covered with a tissue lysis buffer (3:1 vol/wt), weighed again, and homogenized in a Waring blender. The tissue lysis buffer was 50 mM Tris·HCl, pH 7.4, with 0.25 M NaCl, 0.1% Nonidet P-40, 1% Triton X, 5 mM EDTA, 5 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 0.2 U/ml aprotinin, 1 μM leupeptin, 1 μM pepstatin, and 1 μg/ml DNase I. Samples of homogenate were removed for gravimetric analysis, and the remainder was centrifuged at 16,000 g for 30 min. PTHrP levels were determined in the supernatants with a previously described radioimmunoassay (6) using a rabbit polyclonal antibody to PTHrP-(1–34). The quantity of PTHrP in the lung sample was calculated by multiplying the concentration in the lung supernatant by the total water content of the lung homogenate. PTHrP levels were expressed per gram of dry lung.
significant differences between day 0 means and experimental group means (29). The percentage of PTHrP-immunoreactive cells was compared among rats exposed to 85% oxygen for different lengths of time by Kruskal-Wallis nonparametric ANOVA. The number of BrdU-immunoreactive nuclei per high-power field was compared between PTHrP-instilled and PBS-instilled hyperoxic rats by t-test. Differences were considered to be statistically significant if \( P \leq 0.05 \). Data are reported as means ± SE.

RESULTS

**PTHRP and PTHrP receptor expression during lung injury.** In uninjured control lung, PTHrP levels averaged 129 ± 8 ng/g dry lung (\( n = 6 \)). Levels fell after 4 days of exposure to 85% oxygen and reached a nadir of 19 ± 3 ng PTHrP/g dry lung (\( n = 6 \)) by day 8 of hyperoxia. Figure 1 shows the time course for changes in lung PTHrP in rats exposed to hyperoxia compared with room air controls. Levels of PTHrP increased at days 10 and 14 in hyperoxic rats and were not significantly different from control values. Sections of lungs from control animals and animals exposed to 85% oxygen for 1 and 2 days contained PTHrP immunoreactivity in cells in the corners of alveoli. The PTHrP-positive cells were the size, shape, and location expected for type II epithelial cells (control, Fig. 2A). More than 25% of corner cells were immunoreactive. Preadsorption of the antibody with the antigenic peptide abolished staining (data not shown). Consistent with the immunoassay results, immunoreactivity was decreased in lungs exposed to 4 or more days of hyperoxia (day 6, Fig. 2B). Fewer than 2% of corner cells contained PTHrP immunoreactivity at these time points (\( P < 0.05 \) vs. hyperoxic exposure ≤2 days). PTHrP immunoreactivity did not change with time in control animals exposed to room air (data not shown).

PTHRP receptor immunoreactivity was present in corner alveolar cells and in type I epithelial cells of control lungs and at all time points during exposure to 85% oxygen (Fig. 3). Rat kidney, the positive control, demonstrated immunoreactivity in proximal and distal tubular cells. Preadsorption of antibody with the receptor peptide abolished staining (positive and negative control data not shown).

**Alveolar cell proliferation during injury with 85% oxygen.** Proliferation was minimal in control lung and lung exposed to hyperoxia for 1 and 2 days (Fig. 4A). PCNA expression was low, and there was little BrdU incorporated into lung cell DNA. Corner alveolar cells expressed PCNA and took up BrdU between 4 and 8 days of exposure to 85% oxygen (Fig. 4, B and C). Proliferation markers were reduced after 10 days of hyperoxia (Fig. 4D).

**Effect of PTHrP on BrdU incorporation during 85% oxygen.** Exogenous PTHrP-(1–34) was instilled into rat lungs after 4 days of hyperoxia to restore levels toward normal. Lung levels increased from 34 ± 3 ng PTHrP/g dry lung in PBS controls (\( n = 3 \)) to 64 ± 8 ng dry lung (\( n = 4 \)) 8 h after instillation (\( P < 0.05 \)). Eight hours after instillation, BrdU labeling in alveolar cells was reduced (\( n = 3 \)) compared with that in hyperoxic rats instilled with PBS (\( n = 2 \) (Fig. 5). Lungs instilled with PBS contained 3.2 ± 0.4 immunoreactive cells per high-power field compared with 0.5 ± 0.2 stained cells per high-power field in lungs instilled with PTHrP-(1–34) (\( P < 0.01 \)).

**Extent of lung injury produced by 85% oxygen.** Rats exposed to 85% oxygen progressively lost weight beginning at 4 days of hyperoxia (Table 1). Total lung weight and water-to-dry weight ratio increased with injury and was maximal at 4 and 6 days. Blood hemoglobin concentration increased in hyperoxic rats, but the changes were not statistically significant by ANOVA. After exposure to hyperoxia for 4 days, all rats had small pleural effusions (=2 ml) that resolved at 6 days.

DISCUSSION

In a previous study Hastings et al. (14) presented evidence that PTHrP-(1–34) is a type II cell growth inhibitor. Incubating cultured type II cells with neu-
tralizing antibodies to PTHrP stimulated the cells to proliferate, but the proliferation could be blocked with exogenous PTHrP-(1–34) (14). Treatment of the cells with exogenous PTHrP-(1–34) blocks this proliferation. Our interpretation for these findings was that endogenous PTHrP-(1–34) inhibited pneumocyte growth and that reducing its levels with antibody treatment released a block on type II cell division. PTHrP antibodies also induced expression of PCNA in type II cells when instilled into the lung (14). The discovery that PTHrP-(1–34) had growth-inhibitory actions on type II cells led us to develop the hypothesis we tested in this study. We conjectured that levels of PTHrP would decrease after lung injury, diminishing its growth-inhibitory effects and resulting in type II cell proliferation. To test this hypothesis, we measured lung PTHrP expression during the period of type II cell growth after lung injury, and we performed experiments to evaluate

Fig. 3. Expression of PTHrP receptor in hyperoxic lungs. PTHrP receptor immunoreactivity was present in control and hyperoxic lungs at all time points. A: control lung. B: lung exposed to 85% oxygen for 8 days. Both type I cells and type II cells were immunoreactive. Rat kidney, the positive control, also stained for PTHrP receptor, but sections in which antibody was preadsorbed with the PTHrP receptor peptide did not (data not shown). Magnification ×260.

Fig. 4. Proliferation markers in the lung before and after hyperoxic lung injury. Proliferating cell nuclear antigen (PCNA) immunoreactivity was not present in lungs of control rats (A) or rats exposed to 85% oxygen for 1 or 2 days (data not shown). PCNA immunoreactivity appeared at day 4 and was present through day 8 (B, day 6), predominantly in corner alveolar cells. A similar pattern was observed for uptake of 5-bromo-2′-deoxyuridine (BrdU). Corner cells incorporated BrdU from day 4 through day 8 (C, day 6), but BrdU was greatly diminished by day 10 (D). Magnification ×260.
the effect of changes in PTHrP levels on pneumocyte proliferation.

The first set of experiments demonstrated that lung levels of PTHrP decrease after lung injury caused by chronic exposure to 85% oxygen. We assayed whole lung rather than type II cells isolated from hyperoxic lungs because we wanted to measure the total level of PTHrP to which the cells were exposed. Any change in PTHrP levels, whether due to changes in expression by type II cells or changes from another source, might affect type II cell proliferation. Based on immunohistochemical staining, the fall in PTHrP was due, at least in part, to changes in expression by type II cells. PTHrP immunoreactivity in corner epithelial cells, cells that are likely to be type II cells, was decreased over the same period of time as decreases in lung PTHrP levels measured by immunoassay. We believe that type II cells are the predominant lung cell that expresses PTHrP. We have not observed expression in alveolar macrophages or lung fibroblasts (12), but we have not conducted an exhaustive study of all the cell types present in the lung. These experiments proved the first part of our hypothesis, that PTHrP levels would fall after hyperoxic lung injury. Our next step was to examine whether the fall in PTHrP levels overlapped the period when type II cells were proliferating.

We compared the time frames for proliferative activity and changes in PTHrP expression by examining growth markers immunohistochemically. We found that type II cell proliferation was indeed concomitant with the decrease in PTHrP expression. Type II cells exhibited few signs of growth during the first days of hyperoxic exposure. However, beginning on day 4 and continuing through day 14, cells expressed PCNA and incorporated BrdU into cellular DNA. PCNA, the DNA polymerase-δ accessory protein, is expressed only in cells progressing through the cell cycle (17). Other investigators have reported a similar time course for type II cell proliferation induced by injury with 85% oxygen in rat lungs. The proliferative phase lasts from approximately day 4 to day 14 (5). DNA synthesis increases after day 3 (2, 26), and the total number of type II cells in the lung and the average type II cell volume increases roughly twofold by day 7 (7, 11).

To investigate whether the fall in PTHrP regulated type II cell proliferation in hyperoxic lungs, we conducted a final experiment in which we treated hyperoxic lungs with PTHrP. We knew that type II cells in hyperoxic lungs expressed the receptor for PTHrP, so we expected that the cells would be able to respond to exogenous peptide. Exogenous PTHrP significantly reduced incorporation of BrdU into corner alveolar cells compared with the hyperoxic lungs treated with PBS. This result suggests that the reduction in lung PTHrP after exposure to 85% oxygen is a necessary condition for type II cell proliferation. The quantity of PTHrP

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Table 1. Lung injury induced by exposure to 85% oxygen

<table>
<thead>
<tr>
<th>Hypoxia Days</th>
<th>n</th>
<th>Weight Loss, %</th>
<th>Blood Hb, g%</th>
<th>Lung Weight, g</th>
<th>Water-to-Dry Weight Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4</td>
<td>0.0 ± 0.0</td>
<td>12.4 ± 0.3</td>
<td>1.30 ± 0.10</td>
<td>3.97 ± 0.35</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>0.4 ± 0.6</td>
<td>13.5 ± 0.9</td>
<td>1.37 ± 0.13</td>
<td>4.38 ± 0.31</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>7.9 ± 1.1*</td>
<td>13.4 ± 1.0</td>
<td>1.31 ± 0.05</td>
<td>4.45 ± 0.21</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>8.0 ± 2.1*</td>
<td>14.6 ± 0.8</td>
<td>2.03 ± 0.16*</td>
<td>5.89 ± 0.53*</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>14.1 ± 0.8*</td>
<td>13.1 ± 2.5</td>
<td>2.71 ± 0.31*</td>
<td>4.82 ± 0.12</td>
</tr>
<tr>
<td>8</td>
<td>6</td>
<td>15.0 ± 0.4</td>
<td>15.0 ± 0.4</td>
<td>2.42 ± 0.13*</td>
<td>3.48 ± 0.09</td>
</tr>
<tr>
<td>10</td>
<td>4</td>
<td>15.9 ± 2.6*</td>
<td>15.9 ± 0.5</td>
<td>1.97 ± 0.17*</td>
<td>3.61 ± 0.02</td>
</tr>
<tr>
<td>14</td>
<td>5</td>
<td>8.5 ± 2.0*</td>
<td>14.7 ± 0.6</td>
<td>1.82 ± 0.17*</td>
<td>4.06 ± 0.19</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, number of rats. *P < 0.05 vs. day 0.
instilled was high, some 200 times the amount present in normal lungs. Thus we cannot exclude the possibility that the growth inhibition might have been a pharmacological effect of high PTHrP levels. However, the levels of PTHrP in this experiment might not be supraphysiological compared with the concentrations that PTHrP receptors on type II cells normally experience. Because PTHrP is an autocrine factor secreted by type II cells, local concentrations of PTHrP at the receptor on the apical surface of type II cells may be normally much higher than levels one would calculate assuming that PTHrP is equally distributed throughout the lung.

Because lung injury causes stage-specific changes in expression of several growth factors, we believe that lack of PTHrP is not the sole reason for type II cell division after hyperoxic injury. For example, lung levels of TGF-β and expression of TGF-β by type II cells are decreased in the proliferative phase after hyperoxic or bleomycin lung injury (15). Lung expression of type II cell growth stimulators, such as HGF-α, IGF-I, basic fibroblast growth factor (FGF), and TGF-α, increases after various forms of experimental and environmental injury (3, 10, 20, 24). Changes in expression of some of these factors may be related to type II cell proliferation. For example, the nadir in TGF-β levels after hyperoxic injury occurs at the height of type II cell proliferation (4). In addition, the rate of DNA synthesis in pneumocytes recovered from lungs injured by hyperoxia or bleomycin is inversely related to the amount of TGF-β they produce (4, 15). The mitogenic activity for cultured type II cells in silicotic alveolar fluid or derived from silica-exposed alveolar macrophages is neutralized partially by antibodies against IGF-I (19), while blocking the action of TGF-α with tyrphostin B56 decreases DNA synthesis in hyperoxic type II cells in culture (20). However, to our knowledge, a direct causal relationship between type II cell proliferation and changes in expression of these growth factors has not been demonstrated. We have shown that a protein kinase C-dependent pathway stimulates PTHrP production by type II cells (12) but have not studied other factors. TGF-β, FGF, and IGF-I stimulate PTHrP expression in nonpulmonary tissue (28) and could potentially regulate PTHrP expression in lung.

A wide body of evidence demonstrates that PTHrP regulates growth and function of fetal and adult alveolar epithelium. PTHrP derived from fetal pneumocytes stimulates disaturated phosphatidylcholine synthesis in rat fetal lung explants and in pneumocytes maintained in mixed cultures with fibroblasts (23). Knockout of the PTHrP gene is fatal due to pulmonary insufficiency. PTHrP deficiency is associated with lung hypoplasia, arrested canaliculare development, impaired type II cell differentiation, and failure of septal remodeling (22). The regulatory effects of PTHrP for normal adult lung and adult pneumocytes have already been described. Finally, this study demonstrates that treatment with exogenous PTHrP reduces alveolar epithelial proliferation during chronic lung injury induced with 85% oxygen.

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