Parathyroid hormone-related protein response to hyperoxic lung injury

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Parathyroid hormone-related protein (PTHrP) is a growth inhibitor for alveolar type II cells. Type II cell proliferation after lung injury from 85% oxygen is regulated, in part, by a fall in lung PTHrP. In this study, we investigated lung PTHrP after injury induced by >95% oxygen in rats and rabbits. In adult rats, lung PTHrP rose 10-fold over controls to 6,556 ± 710 pg/ml (mean ± SE) at 48 h of hyperoxia. Levels fell to 299 ± 78 pg/ml, and staining for PTHrP mRNA was greatly reduced at 60 h (P < 0.05), the point of most severe injury and greatest pneumocyte proliferation. In adult rabbits, lung PTHrP peaked at 3,239 ± 230 pg/ml after 64 h of hyperoxia with 24 h of normoxic recovery and then dropped to 1,629 ± 153 pg/ml at 48 h of recovery (P < 0.05). Type II cell proliferation peaked shortly after the fall in PTHrP. In newborn rabbits, lavage PTHrP increased by 50% during the first 8 days of hyperoxia, whereas type II cell growth decreased. PTHrP declined at the LD50, concurrent with increased type II cell division. In summary, lung PTHrP initially rises after injury with >95% hyperoxia and then falls near the peak of injury. Changes in PTHrP are temporally related to type II cell proliferation and may regulate repair of lung injury.

Our studies have demonstrated that alveolar type II epithelial cells produce and secrete PTHrP, that they express the PTHrP receptor, and that they respond to changes in ambient PTHrP concentration with alterations in function and growth (10, 13, 14). The amino-terminal portion of the molecule, PTHrP 1–34, stimulates characteristics that are typical of the differentiated type II cell phenotype, such as production of disaturated phosphatidylcholine and expression of alkaline phosphatase (13). Treating cultured type II cells with neutralizing PTHrP antibodies induces type II cell proliferation, whereas instillation of the same antibodies into normal lungs causes type II cell proliferation (14). Thus endogenous PTHrP 1–34 is an autocrine inhibitor of type II cell growth. PTHrP also appears to be involved in the epithelial response to injury. Lung PTHrP levels are decreased between 4 and 8 days of exposure to 85% oxygen in rats, concurrent with increased expression of proliferating cell nuclear antigen (PCNA) and increased incorporation of 5-bromo-2-deoxyuridine (BrdU) into DNA in alveolar type II cells (9). Instillation of PTHrP 1–34 into rat lungs on the fourth day of hyperoxia reduces BrdU incorporation. These data suggest that changes in lung PTHrP levels are involved in regulating type II cell growth in this model.

Parathyroid hormone-related protein (PTHrP) was originally discovered in a lung squamous cell carcinoma as the mediator of humoral hypercalcemia of malignancy. The protein is named for its similarity to parathyroid hormone. The amino-terminal portions of the two molecules have significant homology in primary and secondary structure (18). Thus PTHrP 1–34 binds to the classic type I PTH receptor and mimics all of the effects of PTH, including hypercalcemia, in tissues that bear the receptor (23). In contrast to PTH, PTHrP is not found in detectable levels in the circulation under normal conditions and does not act as a circulating hormone. PTHrP expression is distributed in a wide variety of normal tissues, including the lung, and many of its physiological functions are manifest as paracrine or autocrine effects on growth (27). PTHrP acts as a growth inhibitor in many tissues and cell types, including keratinocytes, vascular smooth muscle cells, and lung cancer cells (17, 19, 22).

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Type II cells divide, spread across a damaged alveolar surface, and differentiate into type I cells (1). However, changes in lung PTHrP levels have not been measured in other types of lung injury, in species other than rats, nor in neonatal animals. Alveolar type II cell proliferation is a common response to lung injury of many etiologies. Lung injury models may vary in severity, time course, and proliferative response. For example, 100% oxygen causes a more severe injury than 85% oxygen. Adult rats die within 72 h of exposure to 100% oxygen but can survive indefinitely at the lower concentration. Type II cell proliferation occurs throughout exposure to 85% oxygen, but becomes prominent with injury due to 100% oxygen when the animals are transferred from hyperoxia to room air (7, 25). Newborn animals are more tolerant of 100% oxygen than adults. They demonstrate inhibited type II cell proliferation during the acute injury phase and robust proliferation during the recovery phase (6, 8). Finally, the pattern of cell proliferation after lung injury may vary among different species in time course and magnitude (26). The goal of this study was to investigate the time course for changes in lung PTHrP levels relative to type II cell proliferation in adult rats exposed to and recovered from >95% oxygen. We compared the results in rats with the changes in PTHrP expression in hyperoxic adult and newborn rabbits.

METHODS

Animals

Male pathogen-free Sprague-Dawley rats (250–300 g) were ordered from Harlan Laboratories (San Diego, CA) for experiments at the University of California San Diego. Experiments were also performed on adult New Zealand White rabbits weighing 1.9–2.2 kg (Buffalo, NY) and newborn (NB) rabbits delivered vaginally at term (Rochester, NY). All animal protocols were reviewed and approved by the institutional animal care and use committee at the institution where the experiments were performed.

Hyperoxic Lung Injury in Adult Rats

Rats were housed two to a cage in a 50-l Plexiglas chamber ventilated with 6 l/min of >95% oxygen in nitrogen for 24, 48, or 60 h. Additional rats were exposed to oxygen for 48 h and then allowed to recover in the chamber ventilated with ambient air for 24, 48, or 72 h. These experimental groups will be labeled 24/0, 48/0, 60/0, 48/24, 48/48, and 48/72, with the first number referring to the hours of hyperoxia and the second number listing the recovery hours in air. The oxygen concentration and carbon dioxide tension of outflow gas were measured in line daily with an Ohmeda 5250 RGM gas analyzer (Englewood, CO). Oxygen concentrations were always >95% during the hyperoxic period and carbon dioxide tensions never exceeded 3 mmHg. Control rats were housed in the chamber ventilated with ambient air at the same flow rate for 48 and 96 h. Additional controls consisted of age-matched rats housed in the vivarium until death. Rats had free access to food and water. Cages were exchanged for clean cages every 2 days. The exchange took <1 min. At the end of the experimental period, rats were anesthetized with 80 mg/kg intraperitoneal pentobarbital sodium and exsanguinated by cutting the abdominal aorta. The thoracic cavity was opened, and pleural fluid was measured and saved for PTHrP assay. The right lung was removed, weighed, and homogenized distal to the primary bronchus for PTHrP assay. In five animals per time point in control, 48/0, and 60/0 groups, the lung was lavaged with four 5-ml aliquots of PBS plus protease inhibitors before homogenization (9). Bronchoalveolar lavage (BAL) was centrifuged at 1,200 g for 15 min to separate cells and supernatant for subsequent PTHrP assay. The left lung was fixed by tracheal instillation of 6 ml of 4% paraformaldehyde in 0.1 M phosphate buffer at 37°C, pH 7.4, and processed for histochemical staining or terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL).

Hyperoxic Injury in Adult Rabbits

Rabbits were allowed access to food and water ad libitum. Animals were exposed to 100% oxygen in individual Lucite chambers for 48 or 64 h (48/0 and 64/0 groups). After the 64-h exposure, some animals were exposed to room air for an additional 24–200 h (64/24, 64/48, 64/72, 64/96, 64/160, and 64/200 groups).

Lung preparation. At the end of an experiment, the rabbit was heparinized and administered an overdose of intravenous pentobarbital sodium (80 mg/kg). The chest was opened, and the lungs were perfused through a catheter placed in the pulmonary artery with ice-cold buffered saline until free of blood. Blocks of lung (0.3–1.5 g) were frozen in liquid nitrogen for later PTHrP assay. Lung PTHrP levels were measured in all groups from 48/0 through 64/72.

Type II cell preparation. In separate animals, the lungs were perfused as described above and then excised and lavaged with 400 ml of Ca2+-free balanced salt solution (BSS). The lungs were inflated with 50 ml of BSS containing 5 mg of BaSO4 particles and incubated for 10 min at 37°C. The lungs were then processed for type II cell isolation as described previously (16). Briefly, the lungs were digested with low concentrations of elastase, minced, filtered through Nitex nylon gauze filters, and washed free of protease. Type II cells were isolated from the crude cell preparation by centrifugation on a Ficoll discontinuous density gradient. Cell counts were determined with a hemocytometer, and cell viability was assessed by trypan blue exclusion. The purified type II cell population generally contained 85–90% type II cells with >95% viability. Purity was initially determined by the use of Papanicolaou staining of air-dried smears and later by a laser-flow cytometric determination of phosphine 3-1 fluorescence staining of lamellar bodies in individual type II cells. The yield, purity, and viability of type II cells from lungs of rabbits exposed to 100% oxygen for 64 h did not change compared with controls.

Cell cycle determinations. Cell cycle status based on DNA analysis was determined on isolated type II cells by flow cytometry. Pellets containing 5 × 106 cells were resuspended in a solution of 3.7 mM sodium citrate, 0.1% Triton X-100, and 1.74 mM propidium iodide and analyzed within 15 min on an EPICS-V flow cytometer (Coulter Electronics).

Hyperoxic Lung Injury in NB Rabbits

Samples were generated from a previously described NB rabbit hyperoxia model (6, 8). Briefly, New Zealand White term NB rabbit litters at <24 h of age (0 days of age) were placed in a large Plexiglas chamber (33 × 40 × 70 cm) and exposed to humidified oxygen or room air. Animals were exposed to 100% until 9 days of age and then recovered in 60% oxygen up to 14 days of age. Animals from these litters and room-air control age-matched animals were killed at 0, 2,
4, 8, 10, 12, and 14 days. Additional animals were studied after exposure to >95% oxygen to a point at which 50% of the pups died because of severe respiratory distress (LDso). The LDso was 8 days in one litter and 11 days in another. Data were collected from surviving animals. At least two litters (of six animals each) were exposed to each condition, and no litter contributed more than two animals per condition per time point (except for the LDso). Animals were killed by an intraperitoneal injection of 200 mg/kg of pentobarbital sodium. A total of 42 hyperoxia-exposed and 41 room air-exposed NB rabbits were examined.

Lung preparation. Paraffin sections were obtained from three to six animals per time point. Lungs were processed as previously described (6, 8). Briefly, a thoracotomy was performed immediately after the animal was killed, the right main stem bronchus was clamped, and the right lung was removed and flash-frozen in liquid nitrogen for other studies. The left lung was instilled in situ with phosphate-buffered 10% formalin under 25 cm Hg pressure for 30 min. It was then removed for 16–24 h of additional formalin fixation and preserved in 70% ethanol until embedded in paraffin for sectioning.

BAL. Separate animals were used for lavage, two to three animals per time point. Lungs were exposed by thoracotomy, perfused in situ with Hanks’ balanced salt solution (HBSS; Life Technologies, Gaithersburg, MD), and removed. Isolated lungs were lavaged with five aliquots of ice-cold HBSS with each aliquot fully distending the lung (and ranging from 5 to 40 ml, depending on the size of the animal, and equal for age-matched animals). Lavages were pooled, and cells were sedimented at 300 g for 6 min. Lavage supernatants were treated with 1 μM Pefabloc protease inhibitor (Roche, Indianapolis, IN) and stored at −80°C before PTHrP assay.

Radioimmunoassay of PTHrP

Lungs were homogenized in a standard 3:1 vol/wt of tissue lysis buffer with protease inhibitors. Homogenates were centrifuged at 16,000 g for 30 min (9). PTHrP levels were determined in the lung supernatants or BAL with a previously described radioimmunoassay (14, 24). Assays were performed in triplicate and using multiple dilutions that paralleled the corresponding standard curve. PTHrP values were reported from measurements that fell on the linear portion of the standard curve (fractional binding values between 0.25 and 0.75). In some cases, PTHrP values were normalized to DNA measured fluorometrically after reaction with Hoechst 33258 dye.

Control assay experiments were performed to test whether substances that might interfere with the PTHrP assay were present in homogenates of normal or injured lung. Homogenates from control lung, 48/0 lung, and 60/0 lung were added to the samples containing known quantities of PTHrP standards. The vol/vol ratio of homogenate to total assay volume was 1:5, matching the greatest proportion of homogenate in assays of any of the unknown samples. When measured PTHrP concentration was plotted vs. expected concentration, the points fell close to the line of identity and were indistinguishable for the control lung, 48/0, lung and 60/0 lung homogenates; these results indicate complete recovery of PTHrP and demonstrate that the assay measured changes in PTHrP levels in normal and injured lung homogenates with similar accuracy.

Immunohistology

Lung blocks were dehydrated through an ethanol/butanol series and embedded in paraffin. Sections (5 μm) were deparaffinized in xylene and hydrated through a graded ethanol series and embedded in paraffin. A total of 42 hyperoxia-exposed and 41 room air-exposed NB rabbits were examined.

TUNEL

TUNEL staining was performed with a kit from Kirkegaard and Perry Laboratories (Gaithersburg, MD). Cell nuclei in tissue sections were stripped of proteins by incubation with 20 μg/ml of proteinase K for 10 min before tissue was fixed in 4% paraformaldehyde at room temperature for 5 min. Slides were washed with 0.5% Triton X-100 in PBS for 5 min and then incubated in 1% glycine in PBS and rinsed in deionized water. Sections were covered in TDT (0.3 U/μl) and biotin-16-dUTP in TdT buffer (30 mM Tris at pH 7.2, 140 mM sodium cacodylate, and 1 mM cobalt chloride) and incubated in a humidified chamber at 37°C for 60 min. Slides were washed in PBS, incubated with streptavidin–horseradish peroxidase conjugate for 30 min at 37°C, and washed 1 min × 3 in PBS. Then they were immersed in buffer containing 3,3′-diaminobenzidine tetrahydrochloride and hydrogen peroxide for 30 min in the dark. Sections were lightly counterstained with methyl green. TUNEL staining was quantified as described in Immunohistochemistry.

In Situ Hybridization

Expression of PTHrP mRNA was investigated by nonradioactive in situ hybridization, as previously described (13). The probe was a digoxigenin-dUTP-labeled single-stranded cDNA fragment complementary to amino acids 15–120 of PTHrP. Sense strands were used as controls. Probes were generated by asymmetric polymerase chain reaction. Presence of the probe was visualized in lung sections after hybridization with an immunohistochemical technique using an alkaline phosphatase-conjugated anti-digoxigenin antibody (Boehringer Mannheim, Indianapolis, IN).
Statistics

Lung homogenate PTHrP levels, PTHrP immunoreactivity, PCNA immunoreactivity, and TUNEL staining were compared among groups by nonparametric ANOVA. Lung weights and BAL total protein contents were compared among groups by ANOVA. Hyperoxic treatment and time effects on NB rabbit BAL PTHrP levels were evaluated with two-way ANOVA. The Tukey and Dunn tests were used for post hoc pair-wise parametric and nonparametric comparisons, respectively (28). Data are reported as means ± SE. Significance was accepted if the probability of a type I error was <0.05.

RESULTS

Hyperoxic Lung Injury in Adult Rats

Extent of injury. Rats in the 48/0 and 48/48 hyperoxic groups showed an influx of inflammatory cells but near-normal lung architecture compared with normoxic control animals (Fig. 1, A, B, and D). Lungs from the 60/0 group contained thickened alveolar septa, alveolar exudate, and hemorrhage (Fig. 1C). Lung weight and BAL total protein levels varied significantly with exposure to hyperoxia (Table 1, P < 0.001). Rats in the 60/0 hyperoxic group had greater signs of injury than animals from the other groups. Average lung weight was 80% greater than control lungs, and BAL total protein was six times the control level. Lung weight normalized to body weight was 0.44 ± 0.03 mg/g in 60/0 rats compared with 0.22 to 0.29 in all other groups (P < 0.05). All of the 60/0 animals had pleural effusions, ranging from 2 to 7 ml in volume, whereas none of the other animals had effusions. Lung weight and BAL protein were significantly greater in 48/0 animals than control animals (P < 0.05).

Lung PTHrP content. Exposure to >95% oxygen caused significant changes in rat lung PTHrP levels with time (Fig. 2). PTHrP levels were 614 ± 18 pg/ml in the 24/0 group, unchanged from control lung, but then increased 10-fold to 6,356 ± 710 pg/ml in the 48/0 group (P < 0.05). Lung PTHrP content fell abruptly below control levels to 299 ± 78 pg/ml after an addi-

Table 1. Indexes of lung injury in adult rats

<table>
<thead>
<tr>
<th>Exposure, h</th>
<th>Recovery Period, h</th>
<th>n</th>
<th>Lung Weight, g</th>
<th>BAL Total Protein, mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>26</td>
<td>0.79 ± 0.02</td>
<td>82 ± 5</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>0</td>
<td>0.85 ± 0.06</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>0</td>
<td>0.92 ± 0.06</td>
<td>143 ± 34</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>0</td>
<td>1.43 ± 0.08</td>
<td>495 ± 99</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>24</td>
<td>1.14 ± 0.05</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>48</td>
<td>0.90 ± 0.08</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>72</td>
<td>0.99 ± 0.04</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

Lung weights are means ± SE. BAL, bronchoalveolar lavage; ND, not done. *P < 0.05 vs. controls; †P < 0.05 vs. all other groups.

Fig. 1. Rat lung histology after hyperoxic lung injury. Rats were exposed to >95% O2 for 24–60 h and then recovered in room air in an environmental chamber for various periods of time. Shown are lungs from rats treated with 48 h of normoxia in chamber (control, A), 48 h of hyperoxia/0 h of recovery (48/0, B), 60/0 (C), and 48/48 (D) groups. Hyperoxia caused an influx of inflammatory cells. Animals exposed to >95% O2 for up to 48 h showed otherwise normal lung structure as did animals from the recovery groups (B, D) compared with control lungs (A). In the 60/0 group, lungs contained thickened alveolar septa, alveolar exudate, and hemorrhage (arrows in C).

Fig. 2. Effects of hyperoxia and recovery on lung parathyroid hormone-related protein (PTHrP) levels in adult rats. Rats were exposed to >95% O2 and then recovered in room air in an environmental chamber for the times shown. Lungs were homogenized and centrifuged, and PTHrP was assayed in the supernatant by RIA. Lung PTHrP content (○, individual animals; ●, mean ± SE) varied significantly among experimental groups (P < 0.01). The greatest PTHrP levels, 6,356 ± 710 pg/ml, occurred during hyperoxic exposure at 48/0 (*P < 0.05 vs. 0/0, 24/0, 60/0, and 48/48 groups). Levels decreased to baseline values in all animals in the 60/0 group, only 12 h after the peak in PTHrP expression. After 48 h of hyperoxia and recovery in room air for 24–72 h, PTHrP levels returned to baseline in most rats but remained elevated in some. Lung PTHrP levels did not vary in rats exposed to only room air in the chamber for 48 and 96 h (data not shown). Mean lung levels for room air control animals at 96 h were 790 ± 81 pg/ml (n = 6). Type II cell proliferation, assessed by proliferating cell nuclear antigen (PCNA) expression, was present at 48 and 60 h of hyperoxia and in the recovery groups (heavy solid line).
tional 12 h of hyperoxic exposure in the 60/0 group ($P < 0.05$). Lung PTHrP concentrations returned to control levels in 21 of 31 rats allowed to recover in room air after 48 h of >95% oxygen, but remained elevated in 10 animals. To investigate whether the changes in PTHrP were due to the hyperoxia or to exposure to the chamber, we also studied control animals that were housed in the vivarium or in the environmental chamber ventilated with ambient air for 48 and 96 h. Lung PTHrP levels did not vary among the three control groups. The mean lung PTHrP levels were 691 ± 41, 757 ± 104, and 790 ± 81 pg/ml for vivarium (n = 25), 48-h chamber (n = 5), and 96-h chamber (n = 6) animals, respectively. Because of concerns that PTHrP levels should be normalized to a measure of cell number, we repeated the analysis, normalizing supernatant PTHrP levels to supernatant DNA content. The results of the analysis were unchanged (data not shown).

**PTHrP immunoreactivity.** PTHrP immunoreactivity was found in normoxic and hyperoxic lung in cells that were the size, shape, and location expected for type II epithelial cells (Fig. 3). Exposure to hyperoxia caused significant time-dependent changes in PTHrP expression (Table 2). Up to 50% of corner cells were immunoreactive in control lungs and lungs from the 24/0 and 48/0 groups. The fraction of type II cells containing PTHrP reaction product increased to 51–75% in the 60/0 groups ($P < 0.05$ vs. all other groups), even though lung PTHrP content measured by immunoassay fell below control levels. All animals at this time point

**Table 2. PTHrP immunoreactivity scores in alveolar corner cells of normoxic and hyperoxic rats**

<table>
<thead>
<tr>
<th>Experimental Group, hyperoxia/recovery hours</th>
<th>PTHrP Staining Scores by Rat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normoxic controls</td>
<td>2</td>
</tr>
<tr>
<td>24/0</td>
<td>0</td>
</tr>
<tr>
<td>48/0</td>
<td>1</td>
</tr>
<tr>
<td>60/0*</td>
<td>3</td>
</tr>
<tr>
<td>48/48</td>
<td>1</td>
</tr>
<tr>
<td>48/72†</td>
<td>0</td>
</tr>
</tbody>
</table>

Scores of 0-3 signify that <2%, 2-25%, 26-50% and 51-75% of the alveolar corner cells were immunoreactive for parathyroid hormone-related protein (PTHrP), respectively. Each score represents a separate animal. *$P < 0.05$ vs. all other groups. †$P < 0.05$ vs. normoxic control group.

**Fig. 3.** PTHrP immunoreactivity in hyperoxic and recovering rat lungs. Rats were treated as described in Figs. 1–2. Deparaffinized lung sections were stained for PTHrP with R759, a rabbit antibody directed against PTHrP 67–86; a biotinylated goat-anti-rabbit IgG secondary antibody; and an alkaline phosphatase-streptavidin technique. The reaction product was generated with fast blue substrate. Lungs are from animals treated as follows: room-air exposure in the chamber for 48 h (A), 48/0 (B), 60/0 (C), 48/48 (D), and 48/72 (E) groups. PTHrP immunoreactivity was more intense in all 3 rats in the 60/0 group (C) than in control animals or animals in the other experimental groups. Reaction product intensity was qualitatively diminished in the 48/48 and 48/72 recovery groups (D, E) compared with controls or earlier groups. We quantified differences in PTHrP staining by scoring the percentage of corner cells with PTHrP immunoreactivity and compared groups by Kruskal-Wallis ANOVA (Table 2). PTHrP immunoreactivity was present in a significantly greater number of corner cells (50–75%) in the rats of the 60/0 group than in control rats or animals in the other experimental groups ($P < 0.05$). Immunoreactivity was present in fewer cells in the 48/72 group than the control group ($P < 0.05$). Staining results were verified in 3 animals/group, and staining was repeated on 4 separate occasions. Reaction product was absent if the primary antibody was preadsorbed with a 100-fold molar excess of the antigenic peptide (data not shown).
in no immunoreactivity. The results are representative of 3 animals/group. Arrows denote representative immunoreactive cells.

Fig. 4. PTHrP mRNA expression in normoxic and hyperoxic lungs. Rats were exposed to hyperoxia for 48 or 60 h. Lung sections were stained for PTHrP mRNA by nonradioactive in situ hybridization as previously described (13). The digoxigenin-labeled antisense probe, complementary to bases coding for amino acid residues 15–120, was stained immunohistochemically using an anti-digoxigenin antibody–alkaline phosphatase conjugate. Blue reaction product was present in lungs of animals housed in room air chambers for 48 h (A) and in 48/0 hyperoxic animals (B). Reaction product was greatly diminished in the 60/0 group (C). Substituting sense probe for antisense resulted in no immunoreactivity (D). The results are representative of 3 animals/group. Arrows denote representative immunoreactive cells.

demonstrated a qualitative increase in the intensity of PTHrP staining (Fig. 3C). In the room-air recovery groups, the fraction of corner cells containing PTHrP reaction product diminished to <25% of cells in all animals (Fig. 3, D and E; \( P < 0.05 \) for 48/72 group vs. normoxic control group). The fraction of type II cells that was immunoreactive for PTHrP did not change with time in control animals exposed to room air in the environmental chamber (data not shown). Preadsorption of the antibody with the antigenic peptide abolished staining (not shown).

\textit{PTHrP in situ hybridization}. Reaction product for the digoxigenin-labeled PTHrP mRNA antisense probe was found in corner cells of normoxic and 48/0 hyperoxic lung (Fig. 4). Expression of PTHrP mRNA appeared to be greatly diminished in 60/0 lung. Reaction product was greatly reduced when sections were hybridized with the sense probe compared with the antisense probe. Results were verified in lung sections from three animals per group.

\textit{BAL PTHrP levels}. Because of the discordance between the changes in lung PTHrP content and lung cell PTHrP immunoreactivity at 60 h, we examined the distribution of PTHrP between lung and BAL in several rats. The percentage of total lung PTHrP found in BAL was 71 ± 4%, 71 ± 4%, and 50 ± 7% in age-matched control animals housed in the vivarium, room-air control animals housed in the chambers, and 48/0 hyperoxic animals, respectively (no significant difference). However, in 60/0 hyperoxic animals, BAL PTHrP accounted for only 9 ± 9% of the total (\( P < 0.05 \) vs. all other groups). Less than 0.1% of the total lung PTHrP was present in the BAL cell pellet at any time point. PTHrP was undetectable in the pleural fluid samples, all from animals in the 60/0 group.

\textit{Alveolar cell proliferation}. PCNA immunoreactivity was present in corner cells in rats from the 48/0, 60/0, and recovery groups but not at earlier time points (Fig. 5). The fraction of PCNA-positive corner cells was significantly greater in the 60/0 group than in the other groups (Table 3). The staining intensity was qualitatively greatest in the 60/0 group as well (Fig. 5C). The data in Table 3 have been incorporated into Fig. 2 to demonstrate the temporal relationship between changes in lung PTHrP expression and type II cell proliferation. The solid line beneath the graph in Fig. 2 marks the period of type II cell proliferation. The changes in PTHrP expression coincided with type II cell proliferation.

\textit{Alveolar apoptosis}. TUNEL-positive corner cells were found in animals from the 60/0, 48/0, 48/24, 48/48, and 48/72 groups but were absent from the lungs of control rats and rats in the 24/0 group (Fig. 6). The 60/0 group had a significantly greater incidence of TUNEL-positive corner cells than the other groups. The fraction of corner cells that were apoptotic was >25% in three out of three animals in this group but <25% in all other animals (\( P < 0.05 \)).

\textit{Hyperoxic Lung Injury in Adult Rabbits}

\textit{Lung PTHrP}. PTHrP levels in adult rabbit lung varied significantly with exposure to >95% oxygen and
subsequent recovery in room air (Fig. 7). The highest levels, 3,289 ± 230 pg/g lung, occurred in the 64/24 group, which had 24 h of room-air recovery. In the 64/48 group 24 h later, levels had dropped significantly to 1,629 ± 153 pg/mg lung \( (P < 0.05) \). PTHrP levels in control lung were 2,289 ± 181 pg/g lung.

**Type II cell proliferation.** No more than 2% of type II cells were in S phase in control rabbits and rabbits during hyperoxic exposure (Fig. 7, dashed line). The percentage increased to 36 ± 3% at 72 h of recovery \( (P < 0.05) \). The increase in S phase coincided with the onset of type II cell PCNA expression at 48 h of recovery (solid line beneath the graph), based on data from previous studies (6, 16). The greatest percentage of type II cells in G2/M phase was found in the 64/96 group, 24 h after the peak in S phase cells. G2/M phase represented 9 ± 2, 4 ± 0.3, 7 ± 2, 16 ± 2, * and 4 ± 0.3% of type II cells in control, 64/0, 64/72, 64/96, and 64/200 rabbits \( (* P < 0.05 \) vs. other groups). Data are presented for 5, 2, 7, 4, and 8 animals in the 0/0, 48/0, 64/0, 64/24, 64/48, and 64/72 groups, respectively. B: type II cell proliferation occurred in the room-air recovery period, shortly after the decrease in PTHrP. The percentage of cells in S phase peaked in the 64/72 group \( (** P < 0.05 \) vs. other groups), coincident with an increase in type II cell PCNA expression at 48 and 72 h of recovery (solid line above the graph) that we reported previously (6, 16).

**Hyperoxic Lung Injury in NB Rabbits**

**PTHrP in NB BAL.** BAL PTHrP concentrations in normoxic NB rabbits did not vary significantly from 2 to 14 days and averaged 396 ± 29 pg/ml (Fig. 8). Exposure to hyperoxia had a significant effect on BAL PTHrP concentrations \( (P < 0.01) \). PTHrP levels were increased ~150% compared with the air-breathing control animals in rabbits breathing 100% oxygen until 8 days and animals breathing 60% oxygen until 14 days \( (P < 0.05) \). In contrast, NB rabbits maintained in 100% oxygen past 8 days to the LD50 point showed a significant decrease in BAL PTHrP levels. PTHrP concentrations averaged 313 ± 53 pg/ml in LD50 animals, ~50% lower than levels in the other hyperoxic groups.
**Fig. 8. Bronchoalveolar lavage (BAL) PTHrP levels in normoxic and hyperoxic newborn (NB) rabbit lung.** Hyperoxic NB rabbits (solid bars) were housed in a chamber with an atmosphere of >95% O₂ for the first 9 days of life and then switched to 60% O₂ (stippled bars). Open bars represent age-matched animals exposed to room air in the same chamber. NB rabbits labeled “LD50” were maintained in >95% O₂ until half the animals in the litter had expired, 9–11 days. Hyperoxia had a significant effect on BAL PTHrP levels (P < 0.01). Average BAL PTHrP levels were greater at days 4, 8, 10, and 14 of hyperoxia than in the normoxic controls (P < 0.05). In contrast, exposure to >95% O₂ to the LD50 point resulted in a significant decrease in BAL PTHrP levels compared with other hyperoxic groups (P < 0.05 vs. hyperoxic days 4, 8, 10, and 14). The lines under the graph demonstrate type II cell proliferative activity in the different newborn rabbit groups (6). Normoxic NB rabbits demonstrate moderate type II cell proliferation through the first 14 days (solid line beneath the graph). Hyperoxic rabbits have decreased type II cell proliferation compared with control animals (dotted line). Proliferation increases markedly at the LD50 point and in animals transferred to 60% O₂ (heavy line).

(P < 0.05). The lines beneath the graph in Fig. 8 indicate the periods of type II cell proliferation in this model as determined in a previous study (6). In contrast to adult animals, NB rabbits demonstrate a moderate degree of type II cell proliferation under control conditions. Animals breathing >95% oxygen for 4 and 8 days show decreased type II cell proliferation compared with normoxic control animals (dotted line). Proliferation in LD50 rabbits and rabbits maintained in 60% oxygen from 8 until 14 days is greater than in the air-breathing control animals (heavy line).

**PTHrP immunoreactivity in NB rabbits.** PTHrP immunoreactivity was present in bronchial cells, corner alveolar cells (type II cells), and in noncorner alveolar cells (type I cells) in both normoxic and hyperoxic groups (Fig. 9). The fraction of type II cells that contained PTHrP reaction product was <25% in air-breathing NB rabbits at all time points (Table 4). PTHrP immunoreactivity was present in a significantly greater fraction of type II cells in hyperoxic rabbits, as high as 75% in some rabbits) at days 10 and 12 (P < 0.05). PTHrP immunoreactivity in type I cells and bronchial cells was not significantly affected by hyperoxia (data not shown).

**DISCUSSION**

**PTHrP Expression During Lung Injury in Adult Rats**

The studies described in this paper addressed the hypothesis that lung PTHrP expression changes as a general response to lung injury of various etiologies and that the changes are temporally related to periods of type II cell proliferation. A previous study from our laboratory found that lung PTHrP levels rose above control levels during the first 2 days of exposure to 85% oxygen in adult rats (9). PTHrP levels fell significantly below control values between days 4 and 8, approximately the same time that type II cells were dividing. In the current studies, we found that exposing adult rats to >95% oxygen caused a similar pattern of PTHrP expression relative to type II cell proliferation. Lung PTHrP peaked at 48 h of exposure (48/0 group) and fell dramatically at 60 h. PTHrP mRNA was decreased at 60 h as well. Pneumocyte PCNA expression was greatest in the 60/0 group. Thus lung PTHrP expression changes with the same temporal pattern in both the >95% oxygen and the 85% oxygen injury models in rats. PTHrP levels increase early after injury and then decrease during the period when type II cells are proliferating.

In addition to affecting overall levels of expression, lung injury altered the distribution of PTHrP between

**Table 4. PTHrP immunoreactivity scores in alveolar corner cells of newborn rabbits**

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>PTHrP Staining Scores by Rabbit</th>
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<tr>
<td>Days of Age</td>
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<td>Normoxic rabbits</td>
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<td>2</td>
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<td>4</td>
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<td>1 1 1 1 1 3</td>
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<tr>
<td>36</td>
<td>1 1 1 2 2 3</td>
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<td>Hyperoxic rabbits</td>
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Scores represent the fraction of cells that were immunoreactive for PTHrP, as described for Table 2. Each score represents a separate animal. *P < 0.05 vs. corresponding normoxic group.
the air spaces and the lung parenchyma. Approximately 70% of lung PTHrP was present in BAL (representing PTHrP secreted into the air spaces) in control adult rats and in hyperoxic rats after 48 h of exposure or less. However, BAL PTHrP levels fell dramatically at 60 h, the point of greatest injury and highest PCNA expression. In addition, no PTHrP was detected in pleural fluid at this time point. Thus secretion of PTHrP appears to be inhibited by severe hyperoxic lung injury. BAL PTHrP levels also decreased with severe lung injury in NB rabbits. We did not measure air space PTHrP in the adult rabbit experiment. Changes in PTHrP secretion may be significant because PTHrP can exert its effects through multiple pathways. When it is secreted, PTHrP can exert paracrine or autocrine effects at cell surface receptors. If it is not secreted, PTHrP may be transported to the nucleus or nucleolus where it can exert effects on cell function, a process termed “intracrine” signaling (15, 19). The PTHrP molecule contains clusters of multibasic residues in the 87–106 region that are similar in configuration to the bipartite nuclear/nucleolar localization sequences in viral transcription factors and growth factors, such as human immunodeficiency virus-1 transactivator of transcription and fibroblast growth factor (FGF)-2. This sequence mediates localization of PTHrP to the nucleus in several cell types (15). Interestingly, the intracrine and paracrine effects of PTHrP on growth may oppose each other. Massfelder and coworkers (19) showed that treatment with exogenous PTHrP inhibited proliferation in vascular smooth muscle cells. In contrast, introducing PTHrP into the cells by gene transfer augmented cell growth.

This study also identified a temporal relationship between changes in PTHrP expression and onset of apoptosis in lung cells in adult rats. Type II cell TUNEL staining was minimal in hyperoxic rat lungs until 60 h of exposure, the point at which PTHrP protein and mRNA levels were lowest. PTHrP has a role in inhibiting apoptosis in chondrocytes and cerebellar neurons (3, 15, 21). PTHrP increases expression of Bcl-2 in growth plate chondrocytes, leading to a delay in their maturation and apoptotic death (3). Overexpression of PTHrP in transgenic mice causes marked delays in skeletal development, whereas mice homozygous for PTHrP gene ablation exhibit skeletal deformities due to accelerated chondrocyte differentiation (2, 3). If PTHrP also inhibits apoptosis in lung epithelial cells, apoptosis after lung injury could be regulated by a fall in lung PTHrP levels. Preliminary reports suggest that PTHrP treatment could reduce type II cell apoptosis after silica lung injury (11, 12), but a definitive study has not been completed.

**PTHRP Expression During Lung Injury in Adult and Newborn Rabbits**

Hyperoxic adult rabbits showed an increase in lung PTHrP, followed by a decrease, just as in the adult rats. In both species, the changes were temporally related to alveolar epithelial proliferation. In the rabbits, PTHrP decreased at 48 h of recovery and remained below peak levels at 72 h, in close proximity to the onset of type II cell division. Type II cells express PCNA at 48 and 72 h of recovery (6), and the fraction of pneumocytes in S phase increases in adult rabbits at 72 h of recovery (64/72 group). These results are consistent with PTHrP playing a role in regulating type II cell proliferation. However, causality between the changes in PTHrP and type II cell growth has not been demonstrated.

Hyperoxic exposure in NB rabbits was associated with an initial increase in BAL PTHrP levels compared with air-breathing rabbits. BAL PTHrP levels were 50% greater in 4- and 8-day hyperoxic animals than in control animals. In previous studies, we observed decreased type II cell proliferation in hyperoxic animals at these time points compared with the normoxic controls (6). PTHrP levels decreased in NB rabbits taken to the LD50 point, just as they decreased in adult rats in the 60/0 group, the group with the most severe lung injury in that model. In both cases, the decrease in PTHrP level was associated with increased type II cell PCNA expression, consistent with our overall hypothesis that PTHrP inhibits type II cell growth. In our previous study with the NB rabbit model, we investigated changes in keratinocyte growth factor (KGF, FGF-7), a positive regulator of type II cell growth. The changes in KGF followed a pattern that was the mirror image found for PTHrP. KGF levels increased after 6–8 days of 100% oxygen, peaking when PTHrP levels were falling. KGF then fell dramatically after the rabbits were placed in 60% oxygen and at the LD50, when PTHrP levels were increased. The changes in expression of both PTHrP and KGF are consistent with a role in regulating pneumocyte proliferation after injury.

The demonstration that PTHrP is expressed in neonatal type I epithelial cells and Clara cells as well as type II cells was a new finding. PTHrP expression has not been observed in type I cells or Clara cells in adult rat lungs. The presence of PTHrP in other cell types in NB lung may be a residual of the distribution of PTHrP in the prenatal period. PTHrP immunoreactivity is present in bronchial epithelium at week 20 in human fetal lung and at day 18 in fetal rat lung (5, 20).

**Critique of Methods and Results**

Lung supernatant PTHrP levels fell after 60 h of hyperoxia whereas PTHrP immunoreactivity paradoxically increased. As discussed above, this finding might be produced by a decrease in total expression of PTHrP along with a decrease in secretion, resulting in less PTHrP in the lavage, less total PTHrP, but greater PTHrP immunoreactivity. The demonstration by in situ hybridization that PTHrP mRNA expression was
injury was induced by 85% or more oxygen. The pattern was similar whether the injury was caused by room air or 100% oxygen. These studies are consistent with our previous investigations, which show that lung PTHrP levels do not change during exposure to near 100% oxygen. Previous studies have found that total lung type II cell numbers do not change during exposure to near 100% oxygen. Pneumocyte numbers increase only after rats are returned to room air (7, 25). However, increases in type II cell numbers lag behind the onset of proliferation, especially if proliferation is also accompanied by cell death. Tryka and coworkers (26) found that the labeling index for lung cells incorporating thymidine was increased sevenfold in rats after 48 h of hyperoxia compared with control animals. Bui and colleagues (4) have observed that the number of rat type II cells in S phase and G2/M phase increases with 48 h of exposure to 100% oxygen. These findings are consistent with our results and indicate that type II cell proliferation begins during the hyperoxic period. We used PCNA staining and TUNEL as measures of cell proliferation and apoptosis, respectively. However, concurrent high PCNA and TUNEL staining could alternatively be explained by ongoing repair of DNA damage. This study only examined the temporal relationship between changes in PTHrP and type II cell proliferation. Additional studies in which lung PTHrP levels are manipulated will be necessary to test causal relationships.

Summary

Similar patterns were observed for PTHrP expression in adult rat, adult rabbit, and NB rabbit models of hyperoxic lung injury. PTHrP initially increases in a period with little type II cell proliferation. Levels then decrease near the time in which type II pneumocytes are dividing. The pattern was similar whether the injury was induced by 85% or >95% oxygen, although the time frame varied. Newborn rabbits demonstrated a short lag time between fall in PTHrP levels and onset of type II cell proliferation. Differences in temporal pattern might be related to species differences, differences in the experimental design, and differences in patterns of expression of other growth factors. Severe lung injury was accompanied by inhibition of PTHrP secretion, a situation in which paracrine stimulation would be reduced but the intracrine effect of PTHrP might continue. The importance of nuclear localization in mediating the effects of PTHrP and the nature of intracrine effects in the lung are unknown. The results of this study and our previous investigations support the hypothesis that PTHrP regulates alveolar cell proliferation and alveolar repair after injury. Further work is necessary to test whether changes in lung PTHrP expression bear a causal relationship with type II cell proliferation and apoptosis during lung injury caused by >95% oxygen.

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