Parathyroid hormone-related protein-(38–64) regulates lung cell proliferation after silica injury

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Parathyroid hormone-related protein-(38–64) regulates lung cell proliferation after silica injury. Am J Physiol Lung Cell Mol Physiol 283: L12–L21, 2002. First published January 4, 2002; 10.1152/ajplung.00308.2001.—Inhalation of silica leads to acute lung injury and alveolar type II cell proliferation. Type II cell proliferation after hyperoxic lung injury is regulated, in part, by parathyroid hormone-related protein (PTHRP). In this study, we investigated lung PTHrP and its effects on epithelial proliferation after injury induced by silica. Lung PTHrP decreased modestly 4 days after we instilled 10 mg of silica into rat lungs and then recovered from 4 to 28 days. The number of proliferating cell nuclear antigen (PCNA)-positive type II cells was increased threefold in silica-injured lungs compared with controls. Subsequently, rats were treated with four exogenous PTHrP peptides in the silica instillate, which were administered subcutaneously daily. One peptide, PTHrP-(38–64), had consistent and significant effects on cell proliferation. PTHrP-(38–64) increased the median number of PCNA-positive cells/field nearly fourfold above controls, 380 vs. 109 (P < 0.05). Thymidine incorporation was 2.5 times higher in type II cells isolated from rats treated with PTHrP-(38–64) compared with PBS. PTHrP-(38–64) significantly increased the number of cells expressing alkaline phosphatase, a type II cell marker. This study indicates that PTHrP-(38–64) stimulates type II cell growth and may have a role in lung repair in silica-injured rats.
adult respiratory distress syndrome; growth substances; parathyroid hormones; silicosis; type II pneumocyte

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those induced by hyperoxia. Furthermore, portions of the PTHrP molecule besides PTHrP-(1–34) are known to exert biological effects. For example, PTHrP-(38–95) activates intracellular calcium signaling pathways in several cell types (33). PTHrP-(67–86) inhibits mitogenesis in 8701-BC breast carcinoma cells and stimulates placental calcium transport in sheep (3, 17), whereas a sequence contained within PTHrP-(107–139) inhibits osteoclastic bone resorption (36), and PTHrP-(140–173) regulates chondrocyte function (7). No studies have investigated the pulmonary effects of peptides derived from PTHrP other than PTHrP-(1–34). Thus the goals of this study were to investigate changes in lung PTHrP levels after silica-induced lung injury and to evaluate the effects of several PTHrP-derived peptides on type II cell proliferation after silica injury.

METHODS

Animals

Male pathogen-free Sprague-Dawley rats were ordered from Charles River (Hollister, CA). The institutional animal care and use committee approved these studies.

Silica Lung Injury

Silica (Min-U-Sil; Pennsylvania Glass Sand, Los Angeles, CA) was boiled in 1 N HCl, washed, dried, suspended in PBS, and autoclaved. Suspensions were sonicated for 10 min before use (4). Rats (250-350 g) were anesthetized with 2–4% isoflurane, intubated orally with a #14 plastic intravenous catheter, intubated with 10 mg of silica in 1 ml of suspension, recovered, and exsanguinated. Control animals received PBS alone. At the end of the experimental period (0, 4, 7, 14, and 28 days), we anesthetized rats with 80 mg/kg intraperitoneal pentobarbital sodium and exsanguinated them by cutting the renal artery. Normal saline was perfused through the pulmonary artery to clear the lungs of blood, and the lungs were removed. The lungs were weighed and homogenized in PBS with protease inhibitors (3:1 vol/wt) as described previously (9). The homogenate was centrifuged at 16,000 g for 1 h at 4°C, and the supernatant was frozen at −70°C for subsequent assay.

PTHrP peptide treatment. After establishing the time course for changes in PTHrP expression after silica injury, we instilled additional rats with silica and treated them with PTHrP peptide. The duration of these experiments was 4 days. The instillate consisted of 10 mg of silica and 15 μg of PTHrP-(1–34), PTHrP-(38–64), PTHrP-(67–86), or PTHrP-(107–138) (Peninsula Labs, Torrance, CA) in 1 ml of 0.05% BSA in PBS. Rats also received subcutaneous injections of 5 μg of the same peptide in 100 μl of BSA-PBS at the time of instillation and daily for 3 days. As controls, rats received silica in BSA-PBS as the instillate and daily injections of BSA-PBS. In a second control group, rats received the PTHrP peptide in BSA-PBS without silica followed by daily injections of the peptide. Four days after instillation, rats were anesthetized and killed as described above.

Lung preparation. The right lung was removed and lavaged with five 4-ml aliquots of PBS before homogenization. Inflammatory cells were separated from BAL by centrifugation and resuspended in 1 ml for cell counts. Cytospin preparations were stained with xanthene and thiazine dyes (Newcomer Supply, Middleton, WI), washed, air-dried, and mounted for differential counts. The cell-free BAL was frozen at −70°C for subsequent 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, embedded in paraffin, and processed for hematoxylin and cosin staining, cytotoxic staining for alkaline phosphatase, or immunocytochemical staining. In some animals, the left lung was fixed by instillation with 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, embedded in Epon, postfixed in osmium tetroxide, stained 5 min in 4% ethanolic uranyl acetate, and briefly contrasted with bismuth lead citrate. Thin sections (80 nm) were examined and recorded using a Zeiss electron microscope.

Type II cell preparation. Type II cells were isolated from silica-injured and uninjured rat lungs by the method of Dobbs as described previously (5, 10). Cell harvest occurred 1 day after silica or PBS instillation. Briefly, the lungs were perfused and lavaged to remove airway inflammatory cells, digested with low concentrations of elastase, minced, filtered through gauze and nylon screen filters, and washed free of protease. Type II cells were isolated from the crude cell preparation by panning on IgG-coated plates. Cell counts were determined with a hemocytometer, and cell viability was assessed by trypan blue exclusion. Purity was determined by the use of Papanicolaou staining of air-dried smears. The purified type II cell population contained 65 ± 4% type II cells with >95% viability. The yield, purity, and viability of type II cells did not vary among PTHrP-(38–64)-treated and control silica-injured rats.

rTI40 Dot Blots

BAL levels of rTI40, a 40-kDa rat alveolar type I cell-specific membrane, increase in association with morphological injury to type I cells due to hyperoxia, nitrogen dioxide exposure, or bacterial pneumonia (19–21). BAL rTI40 levels were measured with an enzyme-linked immunosorbent assay-based assay described by McElroy and colleagues (19–21). The monoclonal antibody to rTI40 was kindly supplied by Leland Dobbs (San Francisco, CA). BAL samples were applied to Immobilon-P membranes (Millipore, Bedford, MA) using a dot-blot apparatus (1–10 μg protein/well). Membranes were then blocked for 2 h at room temperature with 0.4% nonfat milk powder in tris(hydroxymethyl)aminomethane-buffered saline (TBS [20 mM Tris base, 154 mM NaCl, pH 8.2]), washed with TBS containing 0.05% Tween-20 (TBS-T) five times, incubated with monoclonal antibody against rTI40 diluted 1:10 in 20 mM NaHCO3 for 2 h, and then washed again with TBS-T before incubation with sheep anti-mouse secondary antibody conjugated to horseradish peroxidase (1: 25,000 dilution; Calbiochem, La Jolla, CA). Membrane blots were once again washed with TBS-T five times before being developed with enhanced chemiluminescence reagents (NEN Life Sciences) and exposed to X-OMAT AR-5 film (NEN Life Sciences) for 2–3 min. Semiquantitative data from the dot blots were obtained by densitometry readings. The readings were normalized to a positive control consisting of homogenates of normal rat lung. Results were expressed as relative densitometry units per milligram of protein.

Radioimmunoassay of PTHrP

PTHrP levels were determined in the lung supernatants with a previously described radioimmunoassay using an antibody to PTHrP-(109–141) (12, 30).
Immunohistology

Lung sections (5 μm) were deparaffinized in xylene and hydrated through a graded ethanol series. The process to stain prosurfactant apoprotein C (SP-C) began by heating sections at 90°C in 10 mM sodium citrate buffer, pH 6.0, for antigen retrieval. Nonspecific protein binding was blocked with 10% goat serum. Sections were incubated with primary antibody for PCNA (1:20, PC10; Dako, Carpinteria, CA) or human pro-SP-C (1:1,000; Chemicon, Temecula, CA) diluted in 0.5% BSA in PBS overnight at 4°C. Immunoreactivity was absent when primary antibody was omitted on sections (not shown). Biotinylated goat anti-mouse antibody (Calbiochem), diluted at 1:33 with 0.01% goat serum in PBS, was applied for 25 min. Staining was completed by incubation with streptavidin-alkaline phosphatase or horseradish peroxidase conjugates. Chromogens were fast blue or vector red alkaline phosphatase substrates (Vector Laboratories, Burlingame, CA) or 3,3′-diaminobenzidine tetrahydrochloride. Staining experiments were performed on lung sections from at least three animals per time point. Staining was repeated at least once for each animal. PCNA immunoreactivity was quantified as a function of experimental manipulation by a blinded observer who counted the number of stained corner cells in 75 high-power fields (hpf, ×100) per section.

Cell Cycle Determinations

Cell cycle status determination based on DNA analysis was performed on type II cells and BAL cells isolated one day after silica injury. Pellets containing 3 × 10⁶ cells were fixed in an equal volume of 100% ethanol, resuspended in a solution of 50 μg/ml propidium iodide and 1.3 mg/ml RNase in PBS, and analyzed on a Coulter Elite flow cytometer (Beckman Coulter Electronics, Miami, FL). Additional cells were fixed in 1% paraformaldehyde and then stained by fluorescent terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) using a kit from Promega (Madison, WI). TUNEL-positive cells were quantified by flow cytometry.

[³H]Thymidine Incorporation

Type II cells were plated at 4 × 10⁵ cells/well in 24-well plates and allowed to adhere in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) for 24 h. The number of viable adherent cells, quantified by colorimetric assay of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, did not vary between rats treated with PTHrP-(38–64) and rats treated with PBS (data not shown). Cells were washed in PBS and incubated in fresh DMEM-10% FBS with 0.5 μCi of [³H]thymidine/well for 24 h. The supernatant was removed, and cells were washed three times with ice-cold PBS, three times with 1 ml/well of cold 10% trichloroacetic acid, and then lysed in 0.5 ml 1 N NaOH. The cell lysate was counted in scintillation fluid in a Beckman scintillation counter (Beckman Coulter, Fullerton, CA).

Alkaline Phosphatase Cytochemical Stain

Endogenous alkaline phosphatase activity was evaluated as a marker for type II cells as described for silica-injured lungs by Miller and colleagues (23). Lung sections were deparaffinized, washed in TBS, and incubated for 30 min with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium in 100 mM Tris-HCl, pH 9.5, using the alkaline phosphatase substrate kit IV from Vector Laboratories. Sections were washed in TBS and deionized water, dehydrated, and coverslipped. Sections from three random blocks were analyzed, and the results were combined for each animal. Alkaline phosphatase staining was quantified as a function of experimental manipulation by evaluating the number of stained corner cells/alveolus. This number, expressed as a percentage, ranged from <100% (signifying that not all alveoli had stained cells) to almost 200% (signifying two alkaline phosphatase-stained cells, on average, per alveolus). The percentage of alkaline phosphatase-positive cells/alveolus was scored on an ordinal scale, with < 2, 2–25, 26–50, 51–75, 76–100, 101–125, 126–150, 151–175, and 176–200% corresponding to scores of 0–8, respectively. Alkaline phosphatase expression in type II cells and lung inflammatory cells was compared by staining cytospin preparations of freshly isolated type II cells and BAL cells from silica-injured rats.

Statistics

Lung homogenate PTHrP levels, BAL rTI40 levels, PCNA immunoreactivity, and alkaline phosphatase staining were compared among groups by nonparametric ANOVA. Lung weights, BAL total protein contents, cell counts, thymidine incorporation, and percentage of S phase cells were compared among groups by ANOVA. The Tukey and Dunn tests were used for post hoc pair-wise parametric and nonparametric comparisons, respectively (35). Data are reported as means ± SE. Significance was accepted if the probability of a type I error was < 0.05.

RESULTS

Lung Injury and Inflammation After Silica

Comparison of untreated control and silica-injured lungs. Lung weights rose progressively with time after silica instillation and were significantly greater than in control lungs instilled with PBS (Table 1). BAL total protein was significantly increased after 4 days of silica injury, 280 ± 40 μg/ml (n = 12), compared with lungs treated with PBS, 170 ± 40 μg/ml (n = 16, P < 0.01). Silica treatment increased BAL macrophage and neutrophil counts above control values from 1.7 ± 0.1 to 2.8 ± 1.3 × 10⁶ cells/ml and from 0.1 ± 0.1 to 5.0 ± 2 × 10⁵ cells/ml, respectively (P < 0.05). BAL levels of rTI40 were greater 4 days after silica than in control lungs, 201 (140, 240) [median (interquartile gap)] vs. 49 (8, 165) relative units, but the difference did not reach the level for statistical significance (P = 0.06). Lungs 4 days after silica treatment showed a mixed histiocytic infiltration with predominant macrophages and occasional neutrophils (Fig. 1).

Table 1. Effect of silica instillation on wet lung weights

<table>
<thead>
<tr>
<th>Instillate</th>
<th>Day 0</th>
<th>Day 4</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>0.82 ± 0.03</td>
<td>0.78 ± 0.03</td>
<td>0.81 ± 0.02</td>
<td>0.83 ± 0.02</td>
<td>0.89 ± 0.03</td>
</tr>
<tr>
<td>Silica</td>
<td>0.96 ± 0.19</td>
<td>1.07 ± 0.08</td>
<td>1.47 ± 0.07*</td>
<td>1.54 ± 0.08*</td>
<td>2.04 ± 0.10*</td>
</tr>
</tbody>
</table>

Data are means ± SE of wet lung weight measured in grams. *P < 0.05 vs. PBS control group.

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Effects of PTHrP treatment. Table 2 summarizes several indexes of lung injury and inflammation as a function of lung injury and PTHrP peptide treatment. The effects of PTHrP peptide treatment combined with silica lung injury were analyzed by two-way ANOVA. Injury had a significant effect on lung weight, BAL total protein concentration, and BAL neutrophil counts ($P < 0.01$), but PTHrP treatment did not, and interaction effects were not significant. Lung injury and PTHrP treatment both had significant effects on macrophage counts with a significant interaction effect ($P < 0.01$). In silica-injured animals, PTHrP-(38–64) and PTHrP-(107–138) increased macrophage counts compared with the PBS treatment (Table 2). All indexes of injury and inflammation were significantly greater in silica-injured lungs than in control lungs (Table 2). rTI40 levels did not vary among treatment groups in either uninjured control lungs or silica-injured lungs (Fig. 2). rTI40 in silica-injured animals treated with PTHrP-(107–138) was significantly greater than in uninjured animals treated with PTHrP-(107–138) ($P < 0.05$). Body weights did not vary among the groups. PTHrP peptide treatment did not cause significant differences in any of the indexes of injury or inflammation in uninjured control lungs.

**Lung PTHrP Levels After Silica Injury**

Lung PTHrP levels decreased from 43,111 ± 9,685 pg/ml in control animals to 16,468 ± 3,281 pg/ml 4 days after silica, a 60% decline ($P < 0.05$). Levels rose progressively after 4 days and were not significantly different from control at 7, 14, and 28 days (Fig. 3).

![Fig. 1. Lung histology after silica instillation. Four days after the instillation of 10 mg silica, hematoxylin and eosin-stained rat lungs demonstrated a mixed histiocytic inflammatory cell influx, predominantly macrophages with a smaller number of neutrophils.](#)

**Table 2. Indexes of lung injury and inflammation**

<table>
<thead>
<tr>
<th>Condition/Treatment</th>
<th>$n$</th>
<th>Lung Wt, g</th>
<th>BAL Protein, μg/ml</th>
<th>BAL Macrophages, millions</th>
<th>BAL PMN, millions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Uninjured</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>16</td>
<td>0.71 ± 0.02$^*$</td>
<td>170 ± 40$^*$</td>
<td>1.7 ± 0.1$^*$</td>
<td>0.01 ± 0.01$^*$</td>
</tr>
<tr>
<td>PTHrP-(1–34)</td>
<td>7</td>
<td>0.70 ± 0.04$^*$</td>
<td>230 ± 70$^*$</td>
<td>0.7 ± 0.1$^*$</td>
<td>0.01 ± 0.00$^*$</td>
</tr>
<tr>
<td>PTHrP-(38–64)</td>
<td>7</td>
<td>0.78 ± 0.01$^*$</td>
<td>270 ± 60$^*$</td>
<td>1.1 ± 0.1$^*$</td>
<td>0.01 ± 0.00$^*$</td>
</tr>
<tr>
<td>PTHrP-(67–86)</td>
<td>7</td>
<td>0.79 ± 0.02$^*$</td>
<td>280 ± 80$^*$</td>
<td>1.5 ± 0.3$^*$</td>
<td>0.05 ± 0.01$^*$</td>
</tr>
<tr>
<td>PTHrP-(107–138)</td>
<td>6</td>
<td>0.72 ± 0.02$^*$</td>
<td>290 ± 70$^*$</td>
<td>1.2 ± 0.3$^*$</td>
<td>0.04 ± 0.03$^*$</td>
</tr>
<tr>
<td>Silica Injured</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>12</td>
<td>1.07 ± 0.08$^*$</td>
<td>280 ± 40$^*$</td>
<td>2.8 ± 1.3$^*$</td>
<td>0.5 ± 0.2$^*$</td>
</tr>
<tr>
<td>PTHrP-(1–34)</td>
<td>8</td>
<td>1.13 ± 0.12$^*$</td>
<td>290 ± 50$^*$</td>
<td>4.9 ± 1.0$^*$</td>
<td>1.3 ± 0.4$^*$</td>
</tr>
<tr>
<td>PTHrP-(38–64)</td>
<td>8</td>
<td>1.15 ± 0.04$^*$</td>
<td>430 ± 70$^*$</td>
<td>6.9 ± 1.0$^*$</td>
<td>2.6 ± 0.8$^*$</td>
</tr>
<tr>
<td>PTHrP-(67–86)</td>
<td>8</td>
<td>1.04 ± 0.09$^*$</td>
<td>330 ± 60$^*$</td>
<td>4.3 ± 1.2$^*$</td>
<td>0.9 ± 0.3$^*$</td>
</tr>
<tr>
<td>PTHrP-(107–138)</td>
<td>8</td>
<td>1.11 ± 0.05$^*$</td>
<td>390 ± 60$^*$</td>
<td>7.4 ± 1.1$^*$</td>
<td>1.2 ± 0.5$^*$</td>
</tr>
</tbody>
</table>

Values are means ± SE. $^*$P < 0.001, control vs. silica groups; †P < 0.05 vs. control PBS group; ‡P < 0.05 vs. silica PBS group. BAL, bronchoalveolar lavage; PMN, polymorphonuclear neutrophils; PTHrP, parathyroid hormone-related protein.
Lung PTHrP levels did not change over this same period in animals instilled with PBS. Because of questions about how PTHrP levels should be normalized in the setting of lung injury, we also analyzed total lung PTHrP normalized to lung weight. The results of the analysis were unchanged by normalizing total lung PTHrP to lung weight. At 4 days, the silica-injured lungs contained 62,613 ± 12,690 pg PTHrP/g lung compared with 126,526 ± 17,253 in control uninjured lung (*P < 0.05).

Effect of PTHrP Peptide on Cell Proliferation in Silica-Injured Lungs

**PCNA expression in injured lung.** PCNA immunoreactivity was present in corner cells in rats at 4 days (Fig. 4A) and longer after silica instillation. At 4 days, the fraction of PCNA-positive corner cells was significantly greater in silica-injured animals treated with PTHrP-(38–64) than in the other groups (Figs. 4B and 5). Other than the group treated with PTHrP-(38–64), the silica-injured groups did not display greater lung PCNA expression than the control, uninjured animals. Because PTHrP-(38–64) increased PCNA staining in silica-injured lungs, we carried out additional experiments to examine its effects on cell proliferation. We performed cell cycle analysis and [3H]thymidine incorporation assays on type II cells isolated from injured lungs treated with PTHrP-(38–64) or PBS. We also investigated type II cell numbers in silica-injured, PTHrP-treated lungs.

**Cell cycle distributions.** Silica injury increased the percentage of type II cells in S phase to 11.9 ± 1.7%, eightfold greater than in cells from uninjured lungs (Table 3, *P < 0.05*). PTHrP-(38–64) treatment nearly doubled that figure, a significant increase compared with both uninjured lungs and injured, PBS-treated lungs. The percentage of cells in G2/M phase did not vary significantly among uninjured, injured PBS-treated, and injured PTHrP-(38–64)-treated rats, respectively. The fraction of proliferating BAL inflammatory cells was much lower than for type II cells. Only 3.1 ± 1.1% and 1.4 ± 0.6% of BAL cells from injured lungs were in S phase and G2/M, respectively, with no significant difference between PBS and PTHrP treatment groups.

**Fluorescent TUNEL.** The percentages of freshly isolated type II cells that were TUNEL positive were...
similar in injured lungs treated with PBS and PTHrP-(38–64), 11.9 ± 4.6% and 11.1 ± 2.5%, respectively.

\( ^{3}H \)/Thymidine incorporation. Type II cells from silica-injured lungs treated with PTHrP-(38–64) incorporated thymidine into DNA 2.5-fold faster than cells from PBS-treated injured lungs (P < 0.05, Fig. 6).

Lung morphology. Injured lungs treated with PTHrP-(38–64) for 4 days contained clusters of cells in the alveolar corners (Fig. 7A). The appearance was similar to the knobby proliferation of type II cells seen after intratracheal keratinocyte growth factor treatment (31). Electron microscopy of thin sections in lungs after intratracheal keratinocyte growth factor treatment (Fig. 7B). We also stained silica-injured lung for pro-SP-C as a specific type II cell marker. Clusters of SP-C-positive type II cells were present after 4 days of treatment with PTHrP-(38–64) (Fig. 7, C and D), but lungs treated with PBS contained predominantly solitary type II cells (Fig. 7E).

Type II cell numbers. We enumerated type II cells in silica-injured lungs stained for endogenous alkaline phosphatase activity (6). Alkaline phosphatase was chosen as a marker because the cytochemical stain produces an intense, well-defined reaction product (Fig. 8) that has been used effectively as a type II cell marker after silica injury (23). The alkaline phosphatase-positive cells in injured lungs treated with PBS were usually solitary corner cells, but clusters of alkaline phosphatase-positive cells were found in lungs treated with PTHrP-(38–64). Lungs treated with PTHrP-(38–64) contained a significantly greater number of alkaline phosphatase-positive cells than lungs treated with PBS (Fig. 9). PTHrP-(38–64) animals contained roughly 25% more alkaline phosphatase-positive cells/alveolus [median score 1.25 (1, 1.5 interquartile gap), n = 8] than PBS animals [1.0 (0.75, 1.0), n = 7] (P < 0.05). Treatment with PTHrP-(1–34), PTHrP-(67–86), and PTHrP-(107–138) had no significant effect on the number of alkaline phosphatase-positive cells in silica-injured lung (data not shown). To test whether the alkaline phosphatase technique also stained inflammatory cells, we compared the staining in cytospins of freshly isolated type II cells and BAL cells from silica-injured lungs. A significantly greater percentage of type II cells than BAL cells was alkaline phosphatase positive. Only 8 ± 4% of macrophages was alkaline phosphatase positive compared with 50 ± 8% and 60 ± 10% of type II cells from injured lungs treated with PBS and PTHrP-(38–64), respectively (n = 6 animals/group, P < 0.01). As an additional test, we compared the distribution of alkaline phosphatase staining and pro-SP-C expression in sections of silica-injured lung that underwent dual staining for both markers (Fig. 10). The two markers colocalized to the same corner cells.

Effect of PTHrP-(38–64) on Cell Proliferation in Uninjured Lungs

In uninjured animals instilled with PTHrP-(38–64) and treated with subcutaneous PTHrP-(38–64) for 4 days, lungs contained 121 ± 22 PCNA-positive cells/75 hpf (n = 7). PCNA immunoreactivity was significantly less in uninjured animals treated with PBS, 45 ± 14 cells/75 hpf (n = 6, P < 0.05). However, type II cells isolated from normal lungs were not stimulated to increase DNA synthesis by treatment with PTHrP-(38–64) in vitro. Thymidine uptake in cells treated with 100 nM PTHrP-(38–64) for 24 h was 7,800 ± 920 counts/min (cpm) compared with 8,120 ± 920 cpm in untreated cells.

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**Table 3. Cell cycle data for type II cells and BAL cells after silica injury and PTHrP treatment**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Silica Injury</th>
<th>Treatment</th>
<th>n</th>
<th>S Phase, %</th>
<th>G2/M Phase, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type II cells</td>
<td>no</td>
<td>none</td>
<td>4</td>
<td>1.5 ± 0.2</td>
<td>2.2 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>yes</td>
<td>PBS</td>
<td>4</td>
<td>11.9 ± 1.7*</td>
<td>4.0 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>yes</td>
<td>PTHrP-(38–64)</td>
<td>5</td>
<td>20.3 ± 2.9†</td>
<td>4.7 ± 1.0</td>
</tr>
<tr>
<td>BAL cells</td>
<td>yes</td>
<td>PBS</td>
<td>3</td>
<td>2.2 ± 0.5†</td>
<td>1.2 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>yes</td>
<td>PTHrP-(38–64)</td>
<td>4</td>
<td>3.8 ± 1.9±</td>
<td>1.6 ± 1.0</td>
</tr>
</tbody>
</table>

Cell phase values are means ± SE. *P < 0.05 vs. uninjured type II cells; †P < 0.05 vs. other type II cell groups; ‡P < 0.01 vs. type II cells.

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**Fig. 6. Thymidine incorporation in type II cells from silica-injured lungs.** Type II pneumocytes were isolated from rats 1 day after silica instillation, allowed to adhere in culture for 1 day, and then assayed for incorporation of \( ^{3}H \)/thymidine for 24 h. Type II cells isolated from rats treated with intratracheal and subcutaneous PTHrP-(38–64) incorporated thymidine into their DNA 2.5 times faster than cells isolated from injured rats treated with PBS as controls (\( ^{2}P < 0.05 \)). The data represent results from 3 animals/group.
DISCUSSION

The data presented in this paper indicate that treatment with PTHrP-(38–64) increases the proliferation of alveolar type II cells after injury caused by a single instillation of silica particles. Expression of PCNA by alveolar corner cells is increased; a greater percentage of type II cells is in S phase; isolated type II cells incorporate greater levels of tritiated thymidine into cellular DNA; clusters of type II cells are present in the corners of alveoli in a pattern of knobby proliferation; and the number of alkaline phosphatase-positive cells increases in silica-injured lungs treated with PTHrP-(38–64) compared with injured lungs treated with PBS. The cells expressing PCNA and alkaline phosphatase could conceivably be inflammatory cells, which are present in increased numbers in lungs treated with PTHrP-(38–64). However, PTHrP-(107–138) treatment also increased the influx of inflammatory cells into injured lungs but did not result in increased PCNA expression. Furthermore, PTHrP-(38–64) increased DNA synthesis, as indicated by flow cytometric cell cycle analysis and [3H]thymidine uptake, in type II cells but not in the inflammatory cells from the same lungs. Only a few percent of the inflammatory cells were in S phase in silica-injured lungs, suggesting a lower level of proliferation than in type II cells, irre-

Fig. 7. Clusters of type II cells after PTHrP-(38–64) treatment. A: injured lung section stained with hematoxylin and eosin. B: electron micrograph of silica injured-lung treated with PTHrP-(38–64). C–E: sections of injured lung stained for prosurfactant protein (SP)-C. Lungs from animals treated with PTHrP-(38–64) contained clusters of cells in the alveolar corners (A, arrows). These clusters resembled the knobby proliferation of type II cells observed in lungs treated with keratinocyte growth factor (31). Electron microscopy (B) demonstrated that the lungs treated with PTHrP-(38–64) contained clusters of cuboidal alveolar cells with microvilli (mv). Lungs treated with PTHrP-(38–64) also contained clusters of cells expressing SP-C (C, D, arrows), whereas lungs treated with PBS generally had isolated SP-C-positive cells (E).

Fig. 8. Endogenous alkaline phosphatase staining in silica-injured lungs. Sections of lung obtained 4 days after silica injury were stained for endogenous alkaline phosphatase activity with fast blue substrate as a marker for type II cells (6). Alkaline phosphatase activity appeared in a qualitatively greater number of cells after 4 days of PTHrP-(38–64) (B) treatment compared with PBS treatment (A), often in clusters of cells (arrows). Inset: a higher-magnification view of an alkaline phosphatase-positive cell cluster.
For type II cells. In cytospin preparations, we performed two control stud-
iments, the histological appearance consistent with
oxygen (1). Lung PTHrP levels are decreased between 4 and 8
days of exposure to 85% oxygen and 64 h of exposure to
100% oxygen in rats and rabbits (9, 28). We hypothe-
sized that a fall in lung PTHrP was a general response to injuries of many etiologies, but the decrease in pulmonary PTHrP after silica was modest and greatly abbrevied compared with the changes seen after hyperoxic injury. During exposure to 85% oxygen, lung PTHrP levels are significantly decreased from 4 to 8 days, and the maximal decline is >80% (9). The difference might be due to differences in the pattern of expression of other growth factors in the two injury models. For example, transforming growth factor (TGF)-β expression increases dramatically in silica lung injury but is unchanged between 4 and 8 days of 85% oxygen and decreases during exposure to 100% oxygen (1, 15, 26, 32). TGF-β is a positive regulator of PTHrP expression (34) and may maintain higher levels of PTHrP during silica lung injury. Interleukin-1 and TGF-α are upregulated after silica lung injury in rats (15) and are also known to stimulate PTHrP expression (2, 14).

As described in the introduction, PTHrP-(1–34) is an autocrine inhibitor of type II cell division (12), and reduction in lung PTHrP-(1–34) levels appears to contribute to the type II cell growth after injury due to 85% oxygen (9). In the current study, however, PTHrP-(1–34) treatment did not reduce lung cell PCNA expression after silica injury. Other growth factors, such

reported previously.

Although PCNA is expressed in proliferating cells and has been used as a marker for proliferation, it may also be present in cells repairing DNA damage. We cannot exclude this possibility in our model, but the cell cycle analysis, the thymidine incorporation experiments, the histological appearance consistent with type II cell division, and the alkaline phosphatase marker studies indicate that the increase in PCNA expression was accompanied by increased type II cell proliferation. The increased number of alkaline phosphatase-expressing cells suggests that the increased proliferation after PTHrP-(38–64) treatment resulted in an increase in lung type II cell numbers as early as 4 days after the silica injury. More quantitative studies will be necessary to test this hypothesis and to evaluate whether type II cell numbers are altered over a

longer period of time. Type II cell numbers peak at ~14 days after silica injury in the absence of other experimental manipulations (24, 25). A decrease in cell death could result in increased type II cell numbers, but PTHrP-(38–64) had no effect on apoptosis of type II cells from injured lungs as measured by fluorescent TUNEL.

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sections of injured lung, alkaline phosphatase colocal-
ized with pro-SP-C, a specific marker for type II cells, with a high degree of fidelity (Fig. 9). Thus several lines of evidence suggest that inflammatory cells are not responsible for the increased cell proliferation after PTHrP-(38–64) treatment. To our knowledge, mito-
genic effects of PTHrP-(38–64) have not been reported previously.

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as IGF-I, epidermal growth factor, or acidic FGF (16, 22), have been proposed to regulate pneumocyte proliferation in silica injury and may predominate over PTHrP. Alternatively, the relatively low level of type II cell proliferation in the injured lungs may have hampered our ability to detect a change due to PTHrP-(1–34). Because silica injury at 4 days did not significantly increase PCNA expression over the uninjured state in the absence of exogenous PTHrP treatment, showing a decrease with PTHrP-(1–34) treatment would have been difficult. A longer time course is needed to evaluate adequately the potential inhibitory effects of PTHrP-(1–34). Other investigators have shown that type II cell proliferation peaks later in the course of silica injury, between 14 and 28 days (24, 27).

Peptides other than PTHrP-(1–34) had not been studied in the lung previously, and the growth-stimulatory effects of PTHrP-(38–64) were a new finding. Midmolecule PTHrP peptides are known to have effects in other cell types and organ systems, as discussed in the introduction (17, 33). The type I PTH/PTHrP receptor is specific for PTH and PTHrP species containing PTH or PTHrP-(1–34) and does not bind PTHrP-(38–64). Midmolecule PTHrP species are thought to act through yet unidentified receptor(s), receptors that our work suggests should be present on pulmonary cells. Further studies will be needed to determine the mechanism for the effects of PTHrP-(38–64) after silica lung injury. Because PTHrP-(38–64) did not stimulate thymidine incorporation in isolated type II cells, the peptide might be acting in concert with other growth factors released after silica injury, or it could be acting in a paracrine fashion to stimulate other types of cells to express factors regulating type II cell growth. The increased inflammatory cell influx seen after PTHrP-(38–64) treatment could result in increased lung levels of type II cell growth factors derived from those cells. However, growth factor expression by inflammatory cells is unlikely to be the sole mechanism for the effects of PTHrP-(38–64), because all of the PTHrP peptides increased the inflammatory cell population. PTHrP-(107–138) had an effect comparable with PTHrP-(38–64) on cell influx, yet only PTHrP-(38–64) stimulated PCNA expression. An agent might have an indirect effect on type II cell proliferation after lung injury by increasing the severity of the injury. The silica-injured animals treated with PTHrP-(38–64) showed the highest values of lung weight and BAL total protein, suggesting that it might increase the permeability of the alveolocapillary barrier and possibly worsen injury. However, lung weights and protein concentrations were not statistically different from other injured groups, and the high average protein concentration was due, in part, to one outlier value of 800 μg/ml. PTHrP-(38–64) did not appear to cause worse lung injury in histological sections, nor did it increase BAL rTL40 levels, a marker for epithelial damage. PTHrP-(107–138) treatment increased rTL40 in silica-injured animals treated compared with uninjured animals but did not increase lung PCNA expression, arguing against a potential link between worsened injury and increased proliferation after PTHrP treatment.

The physiological significance of the growth stimulatory effect of PTHrP-(38–64) after silica is unclear. Because lung PTHrP levels fall initially, it would appear unlikely that PTHrP-(38–64) plays an role in increased type II cell proliferation in the first 4 days after injury. While levels of PTHrP measured by an assay specific for PTHrP-(109–141) declined, endogenous PTHrP-(38–64) peptide might have increased in this period. The current study did not investigate PTHrP processing. Further studies are needed using sizing methods to investigate PTHrP processing with and without injury. Because PTHrP levels increase from 4 to 14 days, PTHrP-(38–64) might have a role in stimulating pneumocyte growth in this period. This hypothesis could be tested in future studies by reducing PTHrP levels over this period with neutralizing PTHrP antibodies, as described previously (11).

In summary, silica lung injury causes modest decreases in lung PTHrP levels. Treating silica-injured lungs with PTHrP-(38–64) increases type II cell proliferation, as evidenced by PCNA expression, formation of clusters of alveolar corner cells, increases in SP-C-positive and alkaline phosphatase-positive cells, and increases in S phase fraction and thymidine incorporation of type II cells isolated from injured lungs. The increased type II cell proliferation appears to be associated with an increase in type II cell numbers at 4 days after injury and treatment. The mechanisms responsible for the increase in pneumocyte growth and the physiological significance remain to be established.

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