Retinoic acid stimulates immature lung fibroblast growth via a PDGF-mediated autocrine mechanism

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ALTHOUGH ALVEOLAR FORMATION in the developing lung is normally restricted to the late fetal and early postnatal periods, recent evidence suggests that treatment with all trans-retinoic acid (RA) reinitiates this process in emphysematous lungs of adult rats (19). Furthermore, when administered to neonatal rat pups, RA enhances ongoing alveolarization by increasing the total number of alveoli formed (18). Many of the known actions of RA during embryonic development are consistent with a role for RA in alveolar formation. RA influences cell proliferation, differentiation, migration, and fate (21) through its actions on RA-responsive genes encoding growth factors and their receptors (12) and on cell adhesion molecules (36). Inductive epithelial-epithelial and epithelial-mesenchymal interactions also have been attributed to RA (21, 33). Enthusiasm for the use of RA to ameliorate clinical disease states characterized by alveolar insufficiency is tempered, however, by a limited understanding of the mechanisms involved in the actions of excess, exogenous RA on the enhancement and reinitiation of alveolarization.

The effects of all trans-RA and 9-cis-RA are mediated by members of a superfamily of nuclear receptors for steroids and steroid-like molecules, which includes vitamin D3 receptors (VDR), thyroid receptors (T3R), RA receptors (RAR), and retinoid X receptors (RXR). RAR are activated by both all trans-RA and 9-cis-RA, whereas RXR are activated only by 9-cis-RA. RAR and RXR each contain three isotypes (α, β, and γ) encoded by separate genes with distinct patterns of expression during development (8). The association of RA with the ligand-binding domain of the RAR promotes binding to DNA at RA response elements (RARE) located in the promoter regions of specific genes.

Endogenous RA plays a critical role at specific embryologic stages of lung development. The congenital anomalies observed in RAR mutants suggest that RA influences axial patterning by regulating the expression of Hox homeobox genes (17). Altered bronchial branching and the absence of left lung budding were observed in RAR-α and RAR-β2 double mutants (21), implying the involvement of RA in these processes as well. Endogenous retinoids are also thought to participate in the formation of alveoli in the late fetal and early postnatal periods (20). Retinyl esters accumulate in the rat lung late in gestation on embryonic days (ED) 16–19 and then decrease substantially at birth. This rapid decline in the esterified storage form of RA is accompanied by an increase in more metabolically active forms, retinol and RA. A concomitant increase in mRNAs for RAR-β and RAR-γ occurs in lung interstitial fibroblasts and to a lesser extent in whole lung homogenate.

Many RA-responsive genes have been identified thus far, several of which are important for normal lung growth. In these studies we examined the effects of RA and 1,25-dihydroxyvitamin D3 on fibroblast growth via a PDGF-mediated autocrine mechanism. Am J Physiol Lung Cell Mol Physiol 279: L81–L90, 2000. —all trans-retinoic acid (RA) enhances alveolarization in neonates and reinitiates alveolarization in emphysematous adult rat lungs, suggesting that RA may stimulate cell proliferation by upregulating growth factor ligand and/or receptor expression either indirectly or directly by acting on RA-responsive genes encoding growth factors. We report that RA and 1,25-dihydroxyvitamin D3 (Vit D), alone and in combination, significantly increase [3H]thymidine incorporation in cultured fetal and postnatal rat lung fibroblasts (P < 0.05). The greatest increase (10-fold) was seen in 4-day cells treated with the two agents in combination (P < 0.0001). [3H]thymidine incorporation was age dependent. The greatest response to RA occurred in 4-day fibroblasts (P < 0.01), whereas the response to Vit D was greatest in embryonic day 20 fibroblasts (P < 0.001). Neutralizing antibody to platelet-derived growth factor (PDGF)-AB decreased [3H]thymidine incorporation in response to RA alone or in combination with Vit D, indicating a role for PDGF. Expression of mRNAs for PDGF-A and PDGF receptor (PDGFR)-α and -β was upregulated at the transcriptional level in an age- and treatment-dependent manner. Thus exogenous RA may influence alveolarization by stimulating fibroblast proliferation through a PDGF-mediated autocrine mechanism, which is enhanced when RA and Vit D are administered in combination.

1,25-dihydroxyvitamin D3; lung development; platelet-derived growth factor; retinoic acid; vitamin D; alveolarization; alveolar formation; lung development; emphysematous; PDGF; RAR; RXR

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RETINOIC ACID INCREASES PDGF-A AND PDGF RECEPTORS

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development. 9-cis-RA has been shown to increase transcriptional activity of the surfactant protein B (SP-B) gene in a lung epithelial cell line (MLE-15), and a RARE was identified that mediates the RA stimulation of the human SP-B promoter (38). In rat lung fibroblasts, endogenous RA increases the expression of mRNA for tropoelastin (20), the soluble precursor of cross-linked elastin, which in its fibrillar form provides structural support for emerging alveolar septa. In the late fetal and early postnatal lung, tropoelastin gene transcription (6) is temporally correlated with endogenous retinoid levels (20), both of which peak between late gestation (ED18–ED21) and postnatal day 2. RA-responsive genes expressing growth factors (transforming growth factor-βs, epidermal growth factor, and insulin-like growth factor) and growth factor receptors for epidermal growth factor and platelet-derived growth factor (PDGF) (12) are also candidates for involvement in the alveolarization process. One such example, PDGF, has been implicated recently in lung development. A potent chemotactant and mitogen for fetal lung epithelial cells and fibroblasts, PDGF is expressed early in the embryonic lung (3, 7, 28). The PDGF family consists of polypeptide monomers designated A chain and B chain that combine to form the homodimers PDGF-AA and PDGF-BB and the heterodimer PDGF-AB (13, 14). Two classes of PDGF receptors (PDGFR) have been identified; PDGFR-α binds PDGF-A and -B, whereas PDGFR-β binds PDGF-B. Ligand binding induces receptor dimerization and the subsequent activation of intrinsic tyrosine kinases. PDGF has been shown in recent targeted ablation studies to play a critical role in lung development during alveolar formation (5, 16). At 3 wk of age, the lungs of PDGF-A null mice contained few alveoli and virtually no interstitial elastic fibers, presumably due to the failure of a lung fibroblast subset expressing PDGFR-α to migrate distally in the fetal lung and to proliferate in the postnatal lung. Although upregulation of both PDGFR-α and -β mRNAs has been reported in other cell types treated with RA (1, 22, 34, 37), the effects of RA on expression of these receptors has not been investigated in either fetal or neonatal rat lung fibroblasts.

In the present study, we examined the effects of RA on proliferation and on PDGF ligand and receptor expression in fetal and postnatal lung fibroblasts to gain further insight into the mechanism(s) by which RA acts to enhance alveolarization of the immature lung. In addition, we examined the effects of the secosteroid hormone 1,25-dihydroxyvitamin D3 (Vit D) alone and in combination with RA on PDGF ligand and receptor expression. Because the VDR can form heterodimers with both RAR and RXR (32), these studies were undertaken to explore the possibility that administration of the two agents in combination might further alter expression of mRNAs for PDGF ligands and/or receptors.

METHODS

Isolation of Rat Lung Fibroblasts

Fibroblasts were isolated from the lungs of fetal and postnatal Sprague-Dawley rats (Harlan Sprague Dawley; Indianapolis, IN) at ED18 and ED20 of gestation (term 22 days) and at postnatal day 4 as described previously (6). In brief, lungs were removed, trimmed of extraneous tissue, minced, subjected to enzymatic digestion for 1 h, pelleted by centrifugation (1,200 rpm for 10 min), and resuspended in complete medium containing 1:1 (vol/vol) DMEM-Ham’s F-12 medium, 10% fetal bovine serum, penicillin (10,000 U/100 ml), streptomycin (10 mg/ml), and glucose (25.2 mg/ml). The cell suspension was plated for 1 h, and then the flasks were rinsed with calcium- and magnesium-free Hanks’ balanced salt solution (HBSS) to remove nonadherent cells and cultured in complete medium until 90–95% confluent. The isolated cells were determined to be predominantly fibroblasts (99%) based on their morphological appearance by phase-contrast microscopy and immunoreactivity with anti-vimentin (2). Adherent cells were not immunoreactive with anti-factor VIII, excluding the possibility of contamination with endothelial cells. Enzymes and tissue culture reagents were purchased from GIBCO BRL, Life Technologies (Grand Island, NY); antibodies were obtained from DAKO (Carpinteria, CA).

Proliferation of Fibroblasts Treated With RA and/or Vit D

[3H]thymidine incorporation. Confluent primary fibroblast cultures from ED18, ED20, and 4-day rat lungs were passaged to 96-well plates at a density of 5 × 104 cells/well, allowed to adhere for 5 h in complete medium, and then made quiescent by incubation for 24 h in defined keratinocyte serum-free medium (K-SFM) (GIBCO BRL). K-SFM contains hydrocortisone, ethanalamine, phosphoethanolamine, insulin, epidermal growth factor, and fibroblast growth factor. The relatively low calcium concentration (0.1 mM) in this culture medium limits fibroblast proliferation. K-SFM has been used successfully by other investigators to evaluate the mitogenic effects of specific growth factors on fibroblasts (4, 35).

The response of fetal and postnatal rat lung fibroblasts to RA and/or Vit D was initially assessed in DMEM-Ham’s F-12 medium containing 0.1% charcoal-stripped serum (GIBCO BRL). Under these culture conditions, significant cell loss was observed within 48 h in both treated and control cultures, in agreement with our previous observations (2). In contrast, there was no evidence of cell death in cultures maintained in K-SFM.

Quiescent cultures were treated with concentrations of all trans-RA ranging from 5 × 10−9 to 5 × 10−6 M, concentrations of Vit D ranging from 10−7 to 10−10 M, or with 10−6 M RA in combination with 10−7 M or 10−9 M Vit D in K-SFM for an additional 24 h. The stock solution of RA (Sigma; St. Louis, MO) was initially dissolved in dimethyl sulfoxide (Sigma) and then diluted in K-SFM. The final volume of dimethyl sulfoxide in cell cultures was <0.01% (vol/vol). Vit D (the generous gift of Lise Binderup, Leo Pharmaceuticals; Ballerup, Denmark) was dissolved in 100% ethanol before dilution in K-SFM. Both agents were added under a yellow light, and cell cultures were maintained in the dark throughout the remainder of the experiment to minimize the potential for photoisomerization. There were 10 replicates of each concentration of RA and/or Vit D in each of three or more experiments. [3H]thymidine (final concentration 1 µCi/ml) in 50 µl of medium containing RA, Vit D, or both agents was
then added to the initial 100 µl, and cells were cultured for 16 h. Next, cells were rinsed to remove unincorporated 
\(^3\text{H}\)thymidine, treated with trypsin, and stored at −20°C. 
\(^3\text{H}\)thymidine incorporation was quantitated as described previously (2).

Cell counts. Primary fibroblast cultures were passaged to 24-well plates at a density of 2.5 × 10^4 cells/well, allowed to adhere in complete medium for 5 h, made quiescent in K-SFM for 24 h, and then treated for 40 h with 10^{-6} M RA, 10^{-7} M Vit D, or 10^{-6} M RA plus 10^{-7} M Vit D. After aspiration of the medium, wells were rinsed two times with HBSS and stained with 0.1% crystal violet (Roboz Surgical Instrument; Lake Placid, NY). Nuclear counts were obtained with a hemocytometer.

\(^3\text{H}\)Thymidine Incorporation in Cells Treated With PDGF Ligand

Quiescent 4-day rat lung fibroblasts were treated for 24 h with 20, 50, 75, or 100 ng/ml human recombinant PDGF-AA or PDGF-AB (Boehringer Mannheim) and then cultured for an additional 16 h in the presence of \(^3\text{H}\)thymidine. Incorporation of \(^3\text{H}\)thymidine was quantitated as described above.

\(^3\text{H}\)Thymidine Incorporation in Cells Treated With all trans-RA and/or Vit D in the Presence of Neutralizing Anti-PDGF-AB Antibody

Four-day rat lung fibroblasts were made quiescent for 24 h and then treated for 24 h with 10^{-6} M RA, 10^{-7} M Vit D, or 10^{-6} M RA together with 10^{-7} M Vit D in the presence or absence of 25 µg/ml goat polyclonal anti-PDGF-AB, which blocks PDGF-AA, PDGF-BB, and PDGF-AB (Upstate Biotechnology; Lake Placid, NY). Goat IgG was used as an isotype control (Sigma). Medium containing \(^3\text{H}\)thymidine, neutralizing anti-PDGF-AB, RA, Vit D, or RA plus Vit D was then added, and the cells were cultured for an additional 16 h. Incorporation of \(^3\text{H}\)thymidine was quantitated as described above.

Extraction and Reverse Transcription of Total RNA

Fibroblasts. Primary fibroblast cultures were passaged to 25-cm² flasks, cultured until ~75% confluent, made quiescent in K-SFM for 24 h, and then treated with 10^{-6} M RA, 10^{-7} M Vit D, or 10^{-6} M RA plus Vit D for 3, 6, 20, or 40 h. Total RNA was extracted from the cell monolayer with Tri-Reagent-LS according to the manufacturer’s protocol (Molecular Research Center; Cincinnati, OH). Glycogen (100 µg/sample) was added as a carrier.

Whole lung homogenate. Four-day-old rat pups were given subcutaneous injections at the base of the neck with 500 µg/kg body wt of RA, 0.25 µg/kg of Vit D, or both agents on days 4 and 5 of life and killed on day 6, 48 h after the initial injection. RA was dissolved in cottonseed oil. Vit D was dissolved in propylene glycol-0.05 M Na_2HPO_4 (80:20); controls were treated with diluent. Lungs were removed and trimmed of extraneous tissue. Left lobes were frozen on dry ice and then homogenized in Tri-Reagent with a Polytron homogenizer (Brinkman Instruments; Westbury, NY). Total RNA was extracted according to the manufacturer’s recommended protocol (Molecular Research Center).

RT-PCR

RT-PCR was performed using a GeneAmp RNA PCR kit (Perkin-Elmer; Foster City, CA). The reverse transcription protocol recommended by the manufacturer was modified by the addition of 0.2 units of RNase-free DNase I (Warthington; Freehold, NJ) to remove genomic DNA before the RT was added (6). The initial reaction mix (19 µl) containing DNase I and 0.4 µg of input RNA was first incubated in the cycler (2400 Perkin-Elmer Cetus) for 30 min at 37°C to remove genomic DNA, heated at 75°C for 5 min to inactivate DNase I, and then cooled to 4°C. Next, 50 units of Moloney murine leukemia virus RT and 20 units of RNase inhibitor were added to the reaction mix, which was then incubated at room temperature for 10 min, followed by a 30-min incubation at 42°C. The reverse transcription reaction was terminated by heating at 90°C for 5 min and cooling at 4°C for 5 min. Parallel no-RT reactions in which 1 µl of diethyl pyrocarbonate-treated water was substituted for 1 µl of RT were run for each sample and primer pair combination. The PCR amplification stock solution was prepared on ice and divided into aliquots into separate tubes. Each 49 µl of stock solution contained 2 mM MgCl₂, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.08 mM each dGTP, dATP, dCTP, and dTTP, 1.25 units of AmpliTag DNA polymerase, and 20 pmol of each primer. After the addition of 1.0 µl of sample cDNA, tubes were placed in a cycler heated to 95°C and maintained at 95°C for 1 min 45 s to inactivate RT, denatured at 94°C for 30 s, annealed at 55°C (60°C for PDGF-B and cyclophilin) for 30 s, and extended at 72°C for 1.5 min. The PCR products were resolved by electrophoresis on 12.5% polyacrylamide gels and stained with SYBR-Gold (Molecular Probes; Eugene, OR). Fluorescent PCR products were quantitated on a STORM 840 using ImageQuant software (Molecular Dynamics; Sunnyvale, CA). Identities of the amplified cDNAs were confirmed on an automatic sequencer (Perkin-Elmer) located in the Macromolecular Structure Analysis Facility at the University of Kentucky. The PCR primers are listed in Table 1.

Statistical Analysis of Data

Significant differences in the incorporation of \(^3\text{H}\)thymidine between treated and control cells and among treatment groups were calculated by using a two-way analysis of variance (ANOVA). The factors of treatment and age and the interactions between these main effects were also evaluated by a two-way ANOVA. Post hoc analyses were based on Fishers’ least significant difference procedure for pairwise

Table 1. PCR primers

<table>
<thead>
<tr>
<th>5' Primer</th>
<th>3' Primer</th>
<th>Size, bp</th>
</tr>
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<tbody>
<tr>
<td>Cyclophilin</td>
<td>ATGGTGAAACCCCGAGGGTTT</td>
<td>GCCGTGAGAAGTCACACTT</td>
</tr>
<tr>
<td>PDGF-A</td>
<td>AGCCCATCTCCGAGTGGGCGAGAC</td>
<td>GCGTGGAGTGGGAGGGCAGTG</td>
</tr>
<tr>
<td>PDGF-B</td>
<td>AGTGAGGGATCTGGGATCTGG</td>
<td>TAGGGAGCTGCAAGGGCGAGAG</td>
</tr>
<tr>
<td>PDGF-α</td>
<td>ATGGTGACCCGAGTGGTCGC</td>
<td>CGCGGCGAGCACGGTCGCC</td>
</tr>
<tr>
<td>PDGF-β</td>
<td>CTGGGCGAGCACGGTCGCC</td>
<td>248</td>
</tr>
</tbody>
</table>

PDGF, platelet-derived growth factor; PDGF, PDGF receptor.
comparisons and on Dunnett’s test for comparison of treatment vs. control values. Statistical analyses were performed using the statistical analysis system (SAS) release 6.12 for Windows 1996 (SAS Institute; Cary, NC). Differences were considered statistically significant at \( P < 0.05 \). Data are presented as means ± SD.

**RESULTS**

Concentration-Dependent Effects of all trans-RA and Vit D on \([^{3}H]\)Thymidine Incorporation

Incorporation of \([^{3}H]\)thymidine was assessed as a measure of cell proliferation in ED18, ED20, and 4-day rat lung fibroblasts treated with a range of concentrations of RA and Vit D alone and in combination. Relative to same-age controls, a significant increase in incorporation of \([^{3}H]\)thymidine was seen at each age in response to treatment with RA (5 \( \times \) 10\(^{-6}\), 10\(^{-6}\), and 5 \( \times \) 10\(^{-7}\) M), 10\(^{-7}\) M Vit D, and 10\(^{-6}\) M RA plus 10\(^{-7}\) M or 10\(^{-9}\) M Vit D (\( P < 0.05 \)). RA (10\(^{-6}\) M) doubled \([^{3}H]\)thymidine incorporation in ED18 fibroblasts, whereas a somewhat greater stimulation of \([^{3}H]\)thymidine incorporation was seen at ED20 (5 times control) and at day 4 (6 times control) (Fig. 1A). The RA effect on 4-day fibroblasts was significantly greater than that seen at ED18 or ED20 (\( P < 0.01 \)). Treatment with 10\(^{-7}\) M Vit D also increased \([^{3}H]\)thymidine incorporation significantly, albeit to a lesser extent than RA. The greatest response to Vit D was seen in ED20 fibroblasts (\( P < 0.001 \); Fig. 1B). The most striking effect, however, was seen in response to treatment with RA in combination with 10\(^{-7}\) M Vit D, which increased \([^{3}H]\)thymidine incorporation in 4-day cells to 12 times control values (\( P = 0.0001 \); Fig. 1C). The response to the two agents combined was significantly greater in 4-day than in ED18 cells (\( P = 0.006 \)).

Comparison of Effects of Treatment With all trans-RA and Vit D Alone and in Combination on \([^{3}H]\)Thymidine Incorporation: Variation With Age

Responses to the concentrations of agents that caused the most significant increases in \([^{3}H]\)thymidine incorporation relative to controls (10\(^{-6}\) M RA and 10\(^{-7}\) M Vit D, alone and in combination) presented in Fig. 1 were then reevaluated by a two-way ANOVA to determine the overall effects of age and treatment with RA and Vit D, alone and in combination. Both age and treatment were found to be significant (\( P = 0.0001 \)) as was the interaction between these two factors (\( P = 0.009 \)). As shown in Fig. 2A, mean values for \([^{3}H]\)thymidine incorporation were consistently higher in fibroblasts treated with RA than in those treated with Vit D. Only in the 4-day fibroblasts, however, was \([^{3}H]\)thymidine incorporation significantly greater in response to RA than to Vit D (\( P = 0.007 \)). When compared with the response to Vit D alone, treatment with both...
agents in combination increased $[^{3}H]$thymidine incorporation significantly in both ED20 ($P = 0.008$) and 4-day fibroblasts ($P = 0.001$). Treatment of 4-day cells with both agents in combination also resulted in a significant increase in $[^{3}H]$thymidine incorporation relative to that seen in response to RA alone ($P = 0.005$).

A significant age effect was also seen in response to treatment with RA (Fig. 2B). $[^{3}H]$thymidine incorporation was significantly greater in ED20 than in ED18 cells ($P = 0.033$) and in 4-day vs. ED18 cells ($P = 0.005$) after RA treatment. When treated with the two agents in combination, the response in ED18 cells was significantly lower than that in ED20 ($P = 0.002$) or in 4-day ($P = 0.0001$) cells. In addition, the response to the combination of agents was significantly greater in 4-day than in ED20 fibroblasts ($P = 0.023$).

In separate studies, changes in cell number in response to treatment with these agents alone and in combination were similar to the results of the $[^{3}H]$thymidine incorporation studies in that there was a significant effect of treatment ($P < 0.0001$) on the increase in cell number (Fig. 3). The proliferative response to RA was significantly greater than the response to Vit D ($P < 0.0001$) as was the proliferative response to both agents in combination vs. Vit D alone ($P < 0.003$). However, the age effect was not significant.

### Upregulation of PDGF Ligand and Receptor mRNA Expression in Lung Fibroblasts After Treatment With RA and/or Vit D

Quiescent first-passage fetal and postnatal lung fibroblasts were treated with RA and Vit D alone and in combination for 40 h. Expression of mRNAs for PDGF-A and -B chains and for PDGFR-$\alpha$ and -$\beta$ was evaluated by RT-PCR. Values normalized to cyclophilin were expressed as a percent of control values (Fig. 4). Increased expression of mRNAs for PDGF-A and for PDGFR-$\alpha$ and -$\beta$ relative to same-age controls was observed in fibroblasts treated with RA and Vit D alone and in combination. PDGF-B mRNA was not quantifiable after amplification for 31 cycles in mRNA extracted from either treated or control cells (data not shown). Upregulation of PDGF-A mRNA varied significantly with treatment ($P = 0.007$; Fig. 4A); the greatest increase was seen in response to treatment with RA and Vit D in combination. However, expression of PDGFR mRNAs did not vary significantly among the treatment groups. Consistent with the observed changes in $[^{3}H]$thymidine incorporation, Vit D in-
creased the expression of PDGF-A, PDGFR-α, and PDGFR-β mRNAs to a greater extent in ED20 than in either ED18 or 4-day fibroblasts. The response to both agents administered in combination consistently exceeded the response to either agent administered alone in ED18 and 4-day fibroblasts but not in ED20 fibroblasts.

The time course of upregulation of PDGF-A mRNA expression, normalized to cyclophilin, was then evaluated in both ED20 and 4-day fibroblasts treated with RA and Vit D alone and in combination (Fig. 5). Cyclophilin expression remained constant throughout the 40-h incubation period (data not shown). In both fetal and postnatal fibroblasts, the RA-induced increase in mRNA encoding PDGF-A peaked at 20–40 h. After treatment with Vit D, PDGF-A mRNA levels peaked at 20 h. The maximal response to RA and Vit D in combination was seen at 20–40 h.
RA and Vit D Upregulate PDGF Ligand and Receptor mRNA Expression In Vivo

The separate and combined effects of RA and Vit D on PDGF ligand and receptor mRNA expression in vivo were evaluated in rat pups treated with the two agents alone and in combination on days 4 and 5 of life and killed on day 6. Lungs were removed, frozen at −80°C, and homogenized, total RNA was extracted, and PDGF ligand and receptor mRNAs were evaluated by RT-PCR as described in Methods. Values are the means ± SD of 2 animals at each treatment. Values were normalized to cyclophilin and are expressed as a percent of control values.

Influence of Anti-PDGF Neutralizing Antibody on [3H]Thymidine Incorporation

The proliferative response to PDGF ligand was determined in 4-day fibroblasts. After treatment for 40 h with 20, 50, 75, and 100 ng/ml PDGF-AA, incorporation of [3H]thymidine was 1.5, 4, 9, and 9 times control values, respectively. When treated with 50, 75, and 100 ng/ml PDGF-AB, incorporation of [3H]thymidine was 6, 14, and 19 times control values, respectively (data not shown).

The contribution of PDGF ligands (AA, AB, BB) to the proliferative responses to RA and Vit D alone and in combination was assessed in 4-day lung fibroblasts treated with these agents in the presence of neutralizing antibody to PDGF-AB. In the absence of RA and/or Vit D, the antibody alone had no significant effect on [3H]thymidine incorporation, suggesting that PDGF production by quiescent 4-day fibroblasts is minimal under these conditions. The presence of neutralizing antibody reduced the RA effect on [3H]thymidine incorporation by 50% (P = 0.065) and reduced the effect of both agents in combination by 61% (P = 0.029; Fig. 7). However, the 18% reduction in cells treated with Vit D plus antibody was not significant. Treatment with an isotype control was without effect on either the control or the RA plus Vit D-treated cells. These observations are consistent with a proliferative effect of PDGF mediated through an autocrine loop that is stimulated to a greater extent by the two agents administered in combination. However, the extent to which [3H]thymidine incorporation was diminished in the presence of neutralizing antibody may well underestimate the stimulatory effects of these agents on PDGF production. Antibodies are often relatively inefficient in blocking internal autocrine-mediated activities of growth factors. In addition, PDGF bound to the cell surface or to extracellular matrix proteoglycans is likely to be inaccessible to the neutralizing antibody.

DISCUSSION

We have shown that RA and Vit D alone and in combination increase DNA synthesis in immature rat lung fibroblasts. A critical role for PDGF was estab-
lished using a neutralizing anti-PDGF antibody, the presence of which substantially decreased [3H]thymidine incorporation in lung fibroblasts treated with RA alone or in combination with Vit D. These results suggest that exogenous RA may enhance and/or reinitiate alveolarization in part by stimulating lung fibroblast proliferation through a PDGF-mediated autocrine mechanism. In the rat lung, fibroblasts increase in number by 2.5-fold, from 7.5 to 17.5 × 10⁷ during alveolarization, an increase greater than that seen in any other lung cell type (15). Thus fibroblast proliferation is likely to be a key event in the enhancement or reinitiation of alveolarization. RA also has been shown to increase proliferation of type II cells in vitro, apparently because of decreased levels of insulin-like growth factor (IGF) binding protein-2, IGF-II, and IGF-II receptor (24), each of which has been reported to inhibit type II cell proliferation. The role of PDGF was not addressed by these authors, however.

In the embryo, PDGF-A is expressed primarily by epithelial cells, whereas PDGFR-α receptor expression occurs primarily in mesenchymal cells (3, 28). This ligand-receptor pair is thought to contribute to the epithelial-mesenchymal signaling in the lung during development (3). In the PDGF-A null mouse, postnatal alveolar formation does not occur, presumably due to the failure of PDGFR-α plus mesenchymal cells to proliferate and migrate distally (5, 16). In the absence of adequate numbers of mesenchymal cells, extracellular matrix production is markedly diminished, an event that may interfere with the involution of epithelial sheets that normally occurs during the formation of secondary septa in the lung.

PDGF ligands and receptors are also expressed in the postnatal rat lung. Expression of mRNAs for PDGF-A, and PDGFR-α and -β peaks on postnatal day 4 in fibroblasts (unpublished data), whereas PDGF-B mRNA is reported to peak on day 7 in whole lung homogenate (9). Postseptation, PDGF-A and -B and PDGFR-α mRNAs decrease to adult levels. This temporal correlation between alveolar formation and the expression of mRNAs for PDGF ligands and receptors in the postnatal rat lung suggests a role for this growth factor in normal lung development, further supporting the concept that RA could enhance and reinitiate alveolarization by upregulation of PDGF ligands and receptors.

A potential role for Vit D in normal lung development is suggested by observations that deficiency of this vitamin is associated with impaired lung development in both human infants and animal models (10, 11). Although the bioavailability of this steroid hormone is in large part a function of the metabolic activation of 25-hydroxyvitamin D in the kidney, the active form, Vit D, is also produced in the lung by alveolar macrophages (30). In addition, fetal lung fibroblasts contain Vit D (27), and both IMR-90 fetal lung fibroblasts and fetal type II epithelial cells express VDRs (25, 26). Relatively little is known regarding the effects of Vit D on growth factor production. This vitamin has recently been shown to induce nerve growth factor production in L929 mouse fibroblasts (23), vascular endothelial growth factor (VEGF) expression in osteoblastic cells (31), and PDGF production in keratinocytes but not in fibroblasts (39). One of us (Kaetzel, unpublished data) has recently identified a cell type-specific element of the PDGF-A promoter containing a Vit D binding site and response element that has a potent effect on the Vit D inducibility of the PDGF-A gene.

RA has been shown by others to alter the expression of PDGF ligand and receptors, primarily in teratocarcinoma cells. PDGF-B and -A chain transcripts were reported to decrease after 3 and 6 days, respectively, in F9 cells induced to undergo differentiation by treatment with RA and dibutylryl-cAMP (22). The results of the present study represent the first report of an RA-induced upregulation of PDGF-A mRNA expression, however. The time course of upregulation by RA and/or Vit D was inconsistent with a direct effect on PDGF-A gene transcription. The delayed increase in PDGF-A mRNA expression 20–40 h after the initiation of treatment implies that the effects of these agents on PDGF-A upregulation are indirect.

Although RA also has been shown to upregulate mRNAs encoding PDGFR-α (1, 22, 34, 37) and PDGFR-β (22, 34, 37) in teratocarcinoma cell lines (Tera-2 and F9) and in osteoblast-like cells (MC3T3-E1), the results presented herein represent the first report of an RA-induced upregulation of these receptors in fetal and neonatal fibroblasts. Treatment with RA upregulated PDGF-A and PDGFR-β, and to a lesser extent PDGFR-α, at the transcriptional level in cultured fetal and postnatal lung fibroblasts. A more modest upregulation of PDGF ligand and receptors was seen in whole lung homogenate, suggesting a minimal effect on PDGF mRNA expression in other lung cell types. The observed upregulation of mRNA expression for both PDGF-A and PDGFR-α and -β suggests a mechanism whereby these agents alone and in combination could amplify the PDGF autocrine loop to an even greater extent than would be the case if ligand or receptor alone were upregulated.

Upregulation of PDGF ligand and receptor by RA was greatest in 4-day fibroblasts, but the response to Vit D was substantially greater in ED20 than in ED18 or 4-day cells. Age-specific differences in lung cell responsiveness to Vit D have been reported in other cell types as well. Relative to the minimal effect of Vit D on [3H]thymidine incorporation by type II cells obtained from late fetal and early postnatal rat lungs, a substantial increase in [3H]thymidine incorporation was seen at postnatal day 18 (9). Although it is possible that this age dependence is due to cell-specific variability in VDR expression, there is no evidence to substantiate this possibility at present.

Although DNA synthesis, cell proliferation, and PDGF ligand and receptor mRNA expression were increased to variable degrees by treatment with RA or Vit D alone, the response to these agents administered in combination was often additive and possibly synergistic. There are several possible explanations for this
enhanced response. Both RA and Vit D act as transcriptional regulators by first binding with their specific nuclear receptors. Both all trans-RA and cis-9-RA bind RAR, whereas only 9-cis-RA binds RXR. RAR, RXR, VDR, and T3R form a subgroup within the nuclear receptor superfamily. RAR and VDR generally react with their respective response elements, RARE and Vit D response element (VDRE), through heterodimeric complexes with RXR. The presence of both VDRE and RARE on PDGF-A or PDGFR promoters could explain the additive (or synergistic) upregulation of transcription in cells treated with RA and Vit D in combination.

Alternatively, RA and Vit D could act at the same response element to upregulate PDGF gene transcription. Interactions between RA and Vit D signaling pathways have in fact been reported in other systems (29, 32). In addition to forming heterodimers with RXR, RAR can also form heterodimers with T3R and VDR. Using Drosophila SL-3 cells, which lack endogenous RARs, VDRs, or RXRs, Schrader et al. (32) transfected combinations of receptor expression plasmids and found that VDR-RAR forms strong binding complexes to the osteocalcin response element. Although there is as yet no evidence that the PDGF promoter contains a response element that binds the RAR-VDR heterodimer, the presence of a VDR response element in the PDGF promoter is consistent with this possibility. A third explanation for the enhanced response to RA and Vit D administered in combination is that all trans-RA may isomerize in vitro to the cis-9 form, which avidly binds RXR. RXR-VDR heterodimers would be even more likely to bind VDRE and/or RARE on the PDGF or other genes than would VDR homodimers.

Taken together, these results provide insight into one mechanism by which RA might act to enhance alveolarization in the neonatal lung. The RA-induced transcriptional upregulation of PDGF ligand and receptors and the autocrine-mediated proliferative stimulus described herein would appear to be an essential component for increased alveolar growth. In addition, the known participation of PDGF ligands and receptors in epithelial-mesenchymal interactions, e.g., those involving directed migration, suggest the possible involvement of paracrine stimulation in the lung as well. Although Vit D alone appeared to have little effect on fibroblast proliferation, when administered in combination with RA, the effects of these agents were at least additive. Mechanistic studies directed at delineating the combined effects of these agents in vivo may suggest an alternative therapeutic approach to enhancing or reinitiating the alveolarization process in the lung.

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REFERENCES


