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THE CALCITRIOL ANALOGUE EB1089 IMPAIRS ALVEOLARIZATION AND INDUCES LOCALIZED REGIONS OF INCREASED FIBROBLAST DENSITY IN NEONATAL RAT LUNG

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Abstract

The active form of vitamin D₃, 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃, or calcitriol), is a potent mitogen for fibroblasts cultured from rat lungs at postnatal day 4 (P4), during the peak of septation (P3 to P7). In light of the key role of fibroblasts in alveolar septation, the authors conducted studies to measure the extent to which 1,25-(OH)₂D₃ affects lung maturation in vivo, as well as its ability to influence the stimulatory activity of all-*trans* retinoic acid (RA). To identify a calcitriol analogue with maximal mitogenic activity and low systemic toxicity, two compounds with reduced calcemic activity (EB1089 and CB1093) and a superagonist (MC1288) were evaluated in neonatal rat lung fibroblast cultures. All 3 analogues were more potent mitogens than 1,25-(OH)₂D₃ itself (MC1288 ~ CB1093 > EB1089 > 1,25-(OH)₂D₃). In addition, each was more effective than 1,25-(OH)₂D₃ (EB1089 > CB1093 > MC1288 > 1,25-(OH)₂D₃) in the activation of a vitamin D response element from the platelet-derived growth factor (PDGF)-A gene, whose expression is essential for normal alveolarization. Daily administration of EB1089 to rats 4 to 12 days of age caused an increase in mean alveolar chord length ($P < .0001$), and also elicited prominent regions of fibroblast hypercellularity, as defined in terms of a vimentin-positive, factor VIII-negative pheno-type. EB1089 and RA each induced the expression of 2 important lung structural proteins, collagen and elastin. Regions of fibroblast hypercellularity induced by EB1089 were strongly positive for expression of the alveolarization-relevant growth factors, PDGF-AA and vascular endothelial growth factor (VEGF). These studies demonstrate that 1,25-(OH)₂D₃ disrupts the overall alveolarization process in the neonatal lung, although it stimulates expression of some proteins associated with lung morphogenesis.

Keywords

alveolarization; calcitriol; 1,25-dihydroxyvitamin D₃; growth factor; lung development; retinoic acid

The basic gas exchange units of the lung are the alveoli, which are formed during neonatal life in a process known as alveolarization. Bronchopulmonary dysplasia (BPD) in the premature infant and emphysema in the adult are both diseases characterized by alveolar insufficiency and reduced gas exchange capacity. Impaired alveolarization in BPD arises after exposure to

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hyperoxia and anti-inflammatory glucocorticoid therapy [1–3], whereas emphysema represents a similar end point but is caused by damage to fully developed alveolar walls. Discovery of agents that can promote the alveolarization process and/or resist alveolar damage are of prime interest as potential therapies for these diseases. In this regard, the ability of all-*trans* retinoic acid (*at*-RA) to promote alveolarization in both neonatal and adult rats has provided optimism that such disorders can be treated pharmacologically. In neonatal rats, *at*-RA has been reported to increase the total number of alveoli formed, offset the alveolar deficit caused by dexamethasone treatment [4,5], and induce new alveolarization in emphysematous lungs of adult rats [6]. These effects are modest, however, and a recent study has shown they are not accompanied by improvement in pulmonary function [7]. Moreover, prospects for RA in treatment of BPD must be tempered by its well-documented teratogenic activity [8].

Because RA appears insufficient to promote all aspects of the alveolarization program, identification of other morphogenic agents to complement its beneficial actions is of interest. A potential candidate is the hormonally active form of vitamin D₃, 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃, or calcitriol), previously recognized for its actions in bone, intestine, and other tissues [9]. An association between vitamin D and lung development has been indicated by the high frequency of alveolar abnormalities and respiratory disorders in rat and human neonates born to vitamin D-deficient mothers [10,11]. Moreover, 1,25-(OH)₂D₃ is mitogenic for type II pneumocytes at the start of their differentiation and onset of surfactant secretion in the fetal rat [12], as well as for fibroblasts isolated from lungs of rat embryos and neonates [13]. 1,25-(OH)₂D₃ is produced locally in the lung fibroblast [14] and in alveolar macrophages [15], and its synthesis has also been detected in the human small cell lung carcinoma cell line H82 [16]. Finally, RA and 1,25-(OH)₂D₃ provide a cooperative mitogenic stimulus to neonatal rat lung fibroblasts (NRLFs) in culture, and this activity is mediated by activation of a platelet-derived growth factor (PDGF) autocrine loop [13]. Induction of the PDGF-A gene by these agents is of particular relevance in light of the phenotype exhibited by the PDGF-A-null mouse, which is characterized by a specific deficiency in alveolar myofibroblast number and an overall BPD-like phenotype [17]. The alveolar deficit has been attributed to absence of the fibroblast and its important instructive role in the initiation and development of alveolar septa [18]. In keeping with this hypothesis, the PDGF-A gene contains a complex transcriptional control region far upstream of the start site that contains overlapping response elements mediating induction by vitamin D [19] and retinoic acid [19a].

Taken together, these observations suggested that alveolarization is controlled in part by a complex growth axis involving RA and 1,25-(OH)₂D₃ as primary morphogens, with PDGF-AA serving as a critical effector of their actions. Moreover, they suggested that *in vivo* administration of 1,25-(OH)₂D₃ could facilitate the alveolarization-promoting actions of RA. To address these possibilities, the current study was undertaken to conduct a detailed analysis of the effects of 1,25-(OH)₂D₃ administration on alveolarization in the neonatal rat, and its interactions with RA and dexamethasone, other known modulators of alveolarization. We observe that 1,25-(OH)₂D₃ inhibits alveolar septation, probably via direct and nonphysiological stimulation of proliferation of interstitial fibroblasts, although in cooperation with RA, expression of such alveolarization-relevant proteins as collagen, elastin, and PDGF-AA is enhanced.

MATERIALS AND METHODS

Cell Culture, Transfections, and Luciferase Assays

Fibroblasts were isolated from lungs of neonatal Sprague-Dawley rats at postnatal day 4 (P4) and cultured according to the method of Al-Jumaily and Bruce [20] in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium, supplemented with 10% fetal bovine serum (FBS) (complete DMEM/F12). For treatment with RA and/or 1,25-

dihydroxyvitamin D₃ (1,25-(OH)₂D₃), cultures of neonatal rat lung fibroblasts (NRLFs) were made quiescent by preincubation for 24 hours in a serum-free medium formulation (keratinocyte SFM). CV-1 cells were obtained from the American Type Culture Collection and maintained as described previously [21] in DMEM supplemented with 5% FBS, 10 nM nonessential amino acids, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. All culture media and medium additions were obtained from GibcoBRL (Grand Island, NY).

Plasmids used in transient transfection experiments were purified by a batch ion-exchange chromatographic procedure (Qiagen, Valencia, CA). CV-1 cells were seeded in 24-well plates at 5×10^4 cells/well and transfected 16 to 18 hours later by calcium phosphate/DNA coprecipitation [22], as described [21]. For transfection, each dish received calcium phosphate precipitates containing 0.4 µg of firefly luciferase reporter plasmid, 0.04 µg of the *Renilla* plasmid pRL-TK, and 0.08 µg of VDR expression plasmid (pCMVVDR) or parent vector alone (pL2 [19]). Sixteen hours later, precipitates were removed and cells were fed with fresh DMEM supplemented with 5% charcoal-stripped FBS and 1,25-(OH)₂D₃ analogues (generously provided by Leo Pharmaceuticals, Ballerup, Denmark), the latter added as a solution in 95% ethanol (final concentration: 0.2%, v/v). Cells were harvested after 24 hours of treatment and lysed in 1 × Passive Buffer (Promega) for measurement of luciferase in a 96-well plate reader (TR-717, Applied Biosystems). Transfections were performed in 4 replicate wells/treatment group, with at least 3 independent experiments.

Mitogenesis Assays

Mitogenic activity of vitamin D analogues and RA in NRLFs was assessed by measuring rates of [³H]thymidine incorporation, as described [13,23]. Briefly, NRLFs were seeded in 96-well plates containing complete DMEM/F12 at 5×10^4 cells/well made quiescent in by preincubation for 24 hours in serum-free keratinocyte SFM, followed by addition of retinoic acid and/or vitamin D analogues for an additional 24 hours. [³H]thymidine (1 µCi/well, 20 Ci/mmol; Amersham) was added for the final 6 hours of the stimulation period. Cells were detached by trypsinization and harvested by vacuum filtration using a PHD harvester (Cambridge Biotechnologies) onto glass fiber mats (Reeves Angel 934AH; Brandel, Gaithersburg, MD). After extensive washing with H₂O, and successive rinses with 70%, 95%, and 100% ethanol, incorporated [³H]thymidine was quantified by liquid scintillation counting as described [24].

Animals and Treatments

Newborn Sprague-Dawley rat pups were randomly assigned to 1 of 6 treatment groups, consisting of daily intraperitoneal injections of the vitamin D analogue EB1089 (0.38 or 1.25 µg/kg body weight) alone, or in combination with all-*trans* retinoic acid (RA; Sigma; 500 µg/kg body weight) and/or dexamethasone (DEX; 0.25 µg/day) in a $3 \times 2 \times 2$ factorial design. EB1089 and RA injections were conducted on P3 through P12, whereas dexamethasone was administered on P4 through P12. EB1089 was prepared for injections in a quantity sufficient for all injections by dilution from a stock solution (4 mM in isopropanol) into the carrier Solutol HS 15 (BASF). Solutol-diluted EB1089 was stored as aliquots in sealed glass vials (ReactiVial; Pierce) under nitrogen gas at 4°C, with daily injections conducted with freshly unsealed aliquots of EB1089. Stock solutions of RA (50 mg/mL dimethyl sulfoxide [DMSO]) were stored under nitrogen at -80°C and prepared for injection by dilution of freshly thawed aliquots into cottonseed oil (2 µg/µL). A dexamethasone stock solution (10 mg/mL ethanol) was stored under nitrogen at 4°C and prepared fresh for daily injections in 0.9% NaCl (0.25 µg/µL). All rats received equivalent volumes of the 3 carrier solutions employed (solutol, cottonseed oil, 0.9% NaCl). EB1089 and RA were administered as a 2-phase solution via a 10-µL injection using a

20- μ L glass-barreled syringe and a 28-gauge needle (Hamilton), whereas dexamethasone was administered in a separate 10- μ L injection.

Lung Inflation, Tissue Preparation, and Histology

Tissue Harvesting—Upon termination of treatments, animals were administered a lethal dose of Nembutal solution (5 mg) via intraperitoneal injection. Lungs were inflated *in situ* with 10% phosphate-buffered formaldehyde using an intravenous catheter with an 18-gauge flexible tip (Becton-Dickinson). An inflation pressure of 18 cm H₂O was maintained by gravity for $\geq$ 30 minutes, followed by ligation of the trachea and excision of both lungs. Lungs were degassed under vacuum in 10% buffered formaldehyde for an additional 24 hours, then transferred to 70% ethanol for long-term storage. Lung volume (V_L) was measured in 50-mL polypropylene conical-bottom tubes by displacement of ethanol, as described [25].

Preparation of Mounted Sections—For histological analyses, the left lung was sliced sagittally into 3 portions, which were placed in embedding cassettes. The tissues were dehydrated by stepwise 5% increases in the ethanol concentration to 100%, then embedded in paraffin by standard procedures. Sagittal sections (8 μ m) were prepared with a cryostat microtome, then mounted on Superfrost Plus slides and dried for 2 hours at 55°C. Mounted sections of lung tissue were deparaffinized by extraction with 2 15-minute applications of the following solutions in succession at room temperature: xylene, then ethanol at concentrations of 100%, 95%, and 70%.

Morphometric, Histochemical, and Immunohistochemical Analyses—Treatment of lung sections with hematoxylin-eosin (H&E), Verhoeff-Van Gieson (elastin), and Masson's trichrome (collagen) stains was conducted by a commercial service (AML Laboratories, Rosedale, MD). All histochemical and immunostaining images were acquired using a Nikon Eclipse TE200 microscope (Nikon, Melville, NY) and a Spot Insight color camera (Diagnostic Instruments, Sterling Heights, MI) with accompanying operating software (Spot 3.7.4 for Windows). For lung morphometry, bright-field images of H&E-stained sections were obtained using a high-resolution black-and-white CCD camera (model 1300Y, 12 bit depth; Roper Scientific, Tucson, AZ). Image analysis was performed with the program Metamorph 6.0 (Molecular Devices, Sunnyvale, CA). For airspace measurements, images were acquired with an $\times 4$ lens, followed by background flattening and thresholding for airspaces based on image greyscale levels. All airspaces present in the tissue cross-sections were measured, with exclusion of objects touching the edge of the image or any areas greater than 100 μ m (i.e., airways, vessels and any nonairspace artifacts; Figure S1). For wall thickness measurements, images were acquired with an $\times 20$ lens and were flattened and thresholded as above. These images were then used to derive Euclidian distance and skeletonized images that were merged to provide the final measured image (for detailed description, see Figure S2). Internal surface area (ISA) was calculated using the formula $ISA = 4 (0.9) V_L/L_M$ [26].

For immunohistochemistry, sections were unmasked by a 25-minute incubation at 37°C with a solution containing 20 mM Tris, 0.1% trypsin, and 0.1% CaCl₂, pH 7.8. Prior to antibody incubations for analysis of PDGF-AA and vascular endothelial growth factor (VEGF), endogenous hydrogen peroxidase was quenched by incubation at room temperature (RT) for 30 minutes with a solution of 0.3% hydrogen peroxide in 100% methanol. Nonspecific signals were reduced by 15 minutes of incubation at RT with either 4% donkey serum for PDGF-AA or 4% goat serum for VEGF, followed by successive 15-minute blocking steps with streptavidin and biotin (Streptavidin Biotin Blocking Kit; Vector Laboratories, Burlingame, CA). To detect PDGF-AA, a primary goat anti-rat PDGF-AA polyclonal antibody (10 μ g/mL, immunoglobulin G [IgG] fraction; R&D Systems, Minneapolis, MN) was used in conjunction with a secondary donkey anti-goat IgG (2.8 μ g/mL, Biotin-SP-conjugated AffinPure; Jackson

ImmunoResearch, West Grove, PA) and a visualization kit using horseradish peroxidase (HRP)-conjugated streptavidin (ABC Staining System; Santa Cruz Biotechnology, Santa Cruz, CA). For detection of VEGF, a primary rabbit anti-VEGF-A IgG (2 $\mu\text{g/mL}$; Santa Cruz) cross-reactive with all known splice variants of the protein was employed. Binding of primary antibody was visualized by successive incubations with biotinylated goat anti-rabbit IgG (1 $\mu\text{g/mL}$; Santa Cruz), and the HRP conjugate kit cited above. Simultaneous detection of vimentin and factor VIII was obtained on the same section by successive antibody incubations. Sections were first unmasked by a 10-minute treatment at 95°C with 10 mM sodium citrate, pH 6.0, followed by cooling to RT for 20 minutes, followed by tissue permeabilization in 0.1% Triton X-100 for 5 minutes. For vimentin staining, sections were then subjected to successive blocking steps in 10% horse serum, avidin, and biotin as described above. Antibody incubations consisted of a primary goat anti-vimentin IgG (1.5 $\mu\text{g/mL}$; Santa Cruz) followed by a secondary biotinylated horse anti-goat IgG (5 $\mu\text{g/mL}$; Vector Laboratories). Bound antibodies were detected with fluorescein-conjugated streptavidin (10 $\mu\text{g/mL}$; Vector Laboratories). Factor VIII detection was then initiated on the same sections, first by repeating the blocking steps with 10% goat serum, avidin, and biotin. The primary antibody was rabbit anti-factor VIII (2 $\mu\text{g/mL}$; Dako, Carpinteria, CA), followed by incubations with biotinylated goat anti-rabbit IgG (5 $\mu\text{g/mL}$) and Texas Red-conjugated streptavidin (10 $\mu\text{g/mL}$), both from Vector Laboratories.

Statistical Analyses—Internal surface area, chord length, and wall thickness data were analyzed by analysis of variance (ANOVA) using the SigmaStat version 3.1 software package (Systat Software, San Jose), with mean separation determined either by the Holm-Sidak or Student-Newman-Keuls methods, as indicated in the text. Values were represented as means $\pm$ standard error of the mean (SEM). Alveolar chord length and wall thickness data were analyzed by 3-way factorial ANOVA using the Proc Mixed model (SAS, Cary NC), assuming a compound symmetric covariance matrix for the subjects. The Kenward-Roger method was used to estimate the degrees of freedom, with all estimates of fixed effects performed using least squared means [27]. A probability level of .05 or less was chosen to represent statistical significance for all analyses.

RESULTS

The Vitamin D Analogues EB1089, CB1093, and MC1288 Are Potent Mitogens in Cultured Neonatal Lung Fibroblasts and These Effects Are Synergistic with That of All-*trans* Retinoic Acid

The active metabolite of vitamin D, 1,25-(OH)₂D₃, is a potent mitogen for cultured neonatal rat lung fibroblasts (NRLFs) through a mechanism involving induction of a PDGF autocrine growth loop [13]. In addition, 1,25-(OH)₂D₃ and the alveolarization-promoting agent, all-*trans* retinoic acid (*at*-RA), act synergistically to promote mitogenesis of NRLFs, suggesting these 2 compounds may exert cooperativity in stimulating the alveolarization program in vivo as well. However, an examination of the potential applications of 1,25-(OH)₂D₃ to lung therapy is complicated by the tendency of 1,25-(OH)₂D₃ to induce severe hypercalcemia. A wide range of 1,25-(OH)₂D₃ analogues have been developed with the aim of reducing calcemic effects while retaining desired biological activities, such as the anti-tumor properties of the hormone [28–30]. The compound EB1089 (seocalcitol) has shown particular promise in this regard, possessing 50 to 200 times the growth- and differentiation-regulating activity of 1,25-(OH)₂D₃ while exhibiting significantly reduced calcemic activity in vivo (for a review, see [30]).

To identify the best possible vitamin analogue for these studies, 3 vitamin D analogues (EB1089, CB1093, and MC1288) were compared to 1,25-(OH)₂D₃ in terms of their ability to stimulate mitogenesis in cultured NRLFs. CB1093 has been reported to possess a profile of

increased potency and reduced calcemic activity similar to EB1089 [31], whereas MC1288 has been described as a “superagonist” [32] that exhibits increased biological activity but is also more calcemic [33]. As measured by [³H]thymidine incorporation assay, 1,25-(OH)₂D₃ elicited a potent, dose-responsive increase in mitogenic activity (ED₅₀ ~10 nM; Figure 1A), with a near-maximal induction of 12-fold achieved at a hormone concentration of 10 nM. The response was qualitatively similar to that reported previously in NRLFs isolated from 4-day-old pups [13], although the efficacy of the hormone was significantly more robust in these experiments. EB1089 was more potent as a mitogen than 1,25-(OH)₂D₃ (ED₅₀ ~ 3 nM), but the increase was less than that described for other biological indices [30]. CB1093 and MC1288 possessed the highest mitogenic potency, approximately 10 times greater than that of 1,25-(OH)₂D₃ (ED₅₀ ~ 1 nM). When the vitamin D analogues were coadministered with a dose of *at*-RA (10⁻⁶ M) shown previously to provide maximal induction of mitogenesis [13], greater-than-additive responses to the treatment combinations were observed (Figure 1B). Moreover, the same hierarchy of potency was seen when the vitamin D analogues were administered in combination with *at*-RA, as when they were given alone (MC1288 ~ CB1093 > EB1089 > 1,25-(OH)₂D₃). Taken together, these results show that the low calcemic vitamin D analogues EB1089 and CB1093, and the superagonist MC1288, each retained the ability of the parent hormone 1,25-(OH)₂D₃ to stimulate mitogenesis of NRLFs, both alone and cooperatively with *at*-RA.

Vitamin D Analogues Are Potent Inducers of a Vitamin D Response Element (VDRE) in the PDGF-A Gene

Vitamin D–induced stimulation of an autocrine PDGF growth loop in the lung fibroblast is likely to play an important role in development and persistence of lung alveolar structures. This conclusion is strongly suggested by the ability of vitamin D to stimulate expression of PDGF ligands and receptors, as well as the ability of anti-PDGF antibodies to block the mitogenic response [13]. Induction of PDGF-A gene transcription by vitamin D is mediated by a single prototypical vitamin D response element (A-VDRE) located within a complex enhanceosome far upstream of the promoter [19,34]. Having identified multiple vitamin D analogues as potent NRLF mitogens and, thus, potential alveolarization-promoting agents, it was considered important to measure their ability to activate the PDGF-A VDRE. Although VDRE-activating properties of these analogues have been analyzed to a limited extent, their relative activities are highly dependent on the specific VDRE chosen for study [35,36].

VDRE-inducing activity of the vitamin D analogues EB1089, CB1093, and MC1288 was assessed by transient transfection analyses in CV-1 cells, using a luciferase reporter construct with the A-VDRE inserted upstream of a minimal PDGF-A promoter fragment. 1,25-(OH)₂D₃ induced activity of the A-VDRE by approximately 8-fold at a maximal dose (ED₅₀ ~ 10 nM; Figure 2), consistent with previous studies [19]. MC1288 was slightly more potent than 1,25-(OH)₂D₃ (ED₅₀ ~ 3 nM), whereas EB1089 and CB1093 were strikingly more active in this assay (ED₅₀ ~ 0.05 nM) (Table 1). Vitamin D inducibility was targeted specifically to the A-VDRE, as a construct containing the minimal PDGF-A promoter alone was unresponsive to the vitamin D compounds (data not shown).

The Vitamin D Analogue EB1089 Inhibits Alveolarization While Inducing Pronounced Regions of Fibroblast Hypercellularity in the Neonatal Rat Lung

The ability of 1,25-(OH)₂D₃ and its synthetic analogues to activate aspects of the alveolarization program in cultured cells suggested these compounds could promote lung development *in vivo* as well. To test this possibility, the analogue EB1089 was chosen on the basis of its reduced toxicity and increased ability to induce PDGF-mediated proliferation of NRLFs. EB1089 was administered daily to postnatal rats from 4 to 12 days of age (P4 to P12) by intraperitoneal injection at either 0.38 or 1.25 μg/kg body weight (BW)/day. These dosages

were shown previously to be below the threshold for hypercalcemia in mice (2.5 $\mu\text{g/kg BW/day}$; [37–39]). In addition, EB1089 was administered in combination with daily injections of *at*-RA (500 $\mu\text{g/kg BW}$ [4]) and/or dexamethasone (0.25 $\mu\text{g/day}$ [7,40]) to assess its potential interactions with these known modulators of alveolar development. Only the highest dose of EB1089 (1.25 $\mu\text{g/kg BW}$) caused a significant reduction in weight gain when administered alone or in conjunction with dexamethasone, RA, or retinoic acid (Table 2). EB1089 alone had no significant effects on lung volume, although a significant reduction was observed when it was coadministered with retinoic acid.

To obtain assessments of alveolarization-relevant parameters, cross-sections of lung tissue were stained with hematoxylin-eosin and evaluated digitally over their entire areas through an adaptation of methods described by Escolar and colleagues [41,42]. Indices of alveolar integrity were derived from captured images of lung sections using the software program Metamorph (for a more detailed description of analytical procedures, see Figures S1A and B). Two of these indices, alveolar chord length (the distance between 2 walls of an alveolus) and wall thickness, were deemed particularly informative and are reported herein. Alveolar chord length is related inversely to the degree of alveolar septation and the available surface area for gaseous exchange, whereas alveolar wall thickness is an important factor contributing to proper alveolar inflation and elastic contraction of the alveolar walls [43]. Evident upon visual inspection of the stained sections were increases in alveolar volume in rats treated with either EB1089 or dexamethasone (Figure 3A), as well as conspicuous regions of increased tissue density associated with EB1089 treatment (Figure 3B). Although the EB1089-induced hypercellularity was manifested and possibly enhanced in the presence of *at*-RA, the effect was blocked by coadministration of dexamethasone (Table S1). Software-derived estimates of alveolar chord length and wall thickness (Table 3) were analyzed for treatment effects of EB1089 (0, 0.3, 1.0 $\mu\text{g/kg}$), *at*-RA and dexamethasone by a $3 \times 2 \times 2$ factorial analysis of variance (Tables S1 and S2). Significant increases in alveolar chord length in response to both dexamethasone and EB1089 treatment were verified ($P \leq .0001$), whereas *at*-RA administration alone had no effect (Tables 3 and S2). However, a significant interaction between *at*-RA and dexamethasone was detected ($P \leq .035$), reflecting a modest ability of *at*-RA to oppose the detrimental effects of dexamethasone, in accord with previous studies [4]. In contrast, *at*-RA failed to ameliorate the alveolarization-inhibiting effect of EB1089. No statistically significant interaction was detected among the 3 agents (i.e., EB1089 $\times$ *at*-RA $\times$ dexamethasone). All of the treatment effects on alveolar chord length were reflected in derived estimates for internal surface area (ISA) of the lungs, which takes lung volume (L_v) into account (Table 2). With respect to alveolar wall thickness (Tables 3 and S3), dexamethasone treatment caused a significant decrease ($P \leq .01$), whereas *at*-RA elicited a small increase that approached, but failed to reach statistical significance ($P \leq .06$); both trends are consistent with a recent report [7]. In addition, an interaction between EB1089 and *at*-RA approached, but also failed to achieve significance ($P \leq .06$). Additional studies demonstrated that a lower dose of EB1089 (0.125 $\mu\text{g/kg}$) had no effect on alveolar chord length and wall thickness (data not shown).

EB1089 Induces Proliferation of Vimentin-Expressing Fibroblasts and Endothelial Cell Expression of Factor VIII

To identify cell type(s) involved in the hypercellular regions induced by EB1089, lung sections were analyzed by immunohistochemistry for expression of vimentin and factor VIII. Vimentin is a useful marker of fibroblasts and, to a lesser extent, endothelial cells, whereas factor VIII is expressed exclusively by endothelial cells. Sections from control rats appeared to contain a modest number of vimentin-positive cells (Figure 4A), whereas vascular structures corresponding to endothelial cells did not express significant amounts of the protein, indicating that the vimentin-positive cells were primarily fibroblasts and not of endothelial origin. *at*-RA may have slightly induced the number of vimentin-positive fibroblasts, whereas

dexamethasone had no significant effect. However, EB1089 markedly induced the number of vimentin-positive fibroblasts, which constituted most, if not all, of the cells in regions of hypercellularity. Although the amount of immunoreactive vimentin appeared to be higher in fibroblasts of EB1089- and *at*-RA-treated rats, such an induction could not be verified quantitatively by immunoblot analysis in NRLF cultures treated with these agents (Figure S3). Factor VIII expression was restricted to endothelial cells lining the vessel walls, as expected (Figure 4B and C), with no obvious treatment-related effects on the number or average size of vessels visualized by factor VIII positivity.

Expression and Cellular Localization of Other Alveolarization-Relevant Protein Markers

The lung sections described above were evaluated further for expression of the alveolarization-relevant markers collagen, elastin, and the growth factors PDGF-AA and vascular endothelial growth factor (VEGF).

Collagen—Collagen expression is critical for alveolar septation [44] and is highly elevated at postnatal day 7 in the rat [45,46], coincident with the peak in septation [47,48]. Administration of dexamethasone and, to a lesser extent EB1089, resulted in modest up-regulation of collagen expression, whereas *at*-RA had no apparent effect (Figure 5A). Somewhat surprisingly, coadministration of dexamethasone and EB1089 failed to recapitulate the induction seen with these agents when administered alone, suggesting a possible negative cooperativity at the high doses employed. However, EB1089 and *at*-RA cooperatively induced collagen expression, and this increase was maintained when dexamethasone was added to the regimen.

Elastin—Elastin is also critical for alveolar integrity and overall lung elasticity, and its deposition at sites of primordial septal buds suggests an important instructive role in alveolar septation [44]. As described previously [49,50], dexamethasone appeared to induce overall elastin expression, with elastin fibers resembling those seen in control rats (Figure 5B). *at*-RA administration had little effect on overall elastin expression but did result in the appearance of tangled elastin fibers, particularly when coadministered with dexamethasone, in agreement with a previous report [7]. Abnormal deposition of elastin and/or morphology of elastin fibers are indicative of aberrant structural integrity and alveolar septation in lung [44a,44b], as well as pathology in other tissues [44c]. EB1089 administration induced overall elastin expression and, like *at*-RA, also appeared to elicit elastin fiber tangles. Concurrent administration of all 3 agents provided the highest ratio of tangled-to-normal elastin fibers.

PDGF-AA—PDGF-AA was expressed over a wide range of alveolar cell types in lung sections from control rats, although staining was highly concentrated in cells located at narrowed septal walls, most likely epithelial in origin, as well as in a small subset of parenchymal cells (Figure 6A). Dexamethasone did not appear to alter the PDGF-AA expression pattern while, unexpectedly, *at*-RA inhibited overall PDGF-AA expression; *at*-RA-dependent inhibition was dominant when dexamethasone was coadministered. Although EB1089 alone did not appear to have a significant effect on PDGF-AA expression, coadministration of EB1089 and *at*-RA resulted in a strong overall induction. PDGF-AA expression was also elevated in regions of fibroblast hypercellularity elicited by EB1089 alone (data not shown) or in cooperation with *at*-RA, suggesting a potential role for 1,25-(OH)₂D₃ in stimulation of a PDGF autocrine growth loop in these cells.

VEGF—VEGF was expressed in essentially all alveolar cell types in control lung sections (Figure 6B). Expression appeared to be induced by all 3 agents, with dexamethasone and EB1089 eliciting higher induction in cells located within narrowed septal walls and at junctional regions. Regions of myofibroblast hypercellularity induced by EB1089 (+/- *at*-RA)

continued to express VEGF, although the overall level of expression did not appear to be enhanced beyond that seen in control animals.

DISCUSSION

The current study represents the first detailed analysis of the effects of 1,25-(OH)₂D₃ and its synthetic analogues on the developing lung. A central finding was the ability of EB1089, a highly active analogue of 1,25-(OH)₂D₃, to induce regions of highly increased fibroblast density in the developing neonatal rat lung. The effect is most likely due to a direct mitogenic effect of 1,25-(OH)₂D₃ and its analogues, which we have observed both previously with NRLFs *ex vivo* [13] and in the current study, although apoptosis-inhibitory activity was not addressed as a possible contributory mechanism. Moreover, vitamin D-mediated mitogenesis in the lung fibroblast *ex vivo* has been shown to be mediated by induction of PDGF autocrine loop activity [13]. Consistent with such a mechanism, regions of fibroblast hyperproliferation induced by EB1089 were strongly positive for PDGF-AA, and in other lung cell types as well when administered in concert with RA (Figure 6A). These results are relevant to a current model of alveolarization in which PDGF-AA expression from epithelial cells at the septal bud provides a paracrine stimulus for fibroblast recruitment to the site [18]. This model is based in large part on observations in the PDGF-A-null mouse, which exhibits a lethal emphysema-like condition [51]. The lesion was attributed to an impairment of septal bud formation, secondary to an acute deficiency of fibroblasts at the site. Our findings suggest that autocrine PDGF activity promotes accumulation of a requisite mass of fibroblasts prior to their PDGF-mediated migration to the primitive septum (Figure 7). This is reminiscent of concomitant mitogenic and chemotactic activities demonstrated in the osteoblast [52]. By the same token, the alveolarization-inhibiting effects of EB1089 could be explained by hyperinduction of PDGF autocrine activity in the fibroblast, which might interfere with the PDGF-AA chemotactic gradient needed for fibroblast recruitment to the septal bud. Consistent with this notion, EB1089 treatment resulted in localized thickening of air sac walls with fibroblasts, but also an overall decrease in septal formation. Overall, these results suggest 1,25-(OH)₂D₃ might play an important role in the alveolarization process, but they also highlight difficulties associated with pharmacological induction of these events via systemic delivery of potential endogenous morphogens (e.g., 1,25-(OH)₂D₃, *at*-RA, etc.). Our studies also suggest the possibility that systemic delivery of 1,25-(OH)₂D₃ or its analogues may result in problematic effects on lung tissue in current therapeutic applications of this agent to such other disease states in humans as renal osteodystrophy and psoriasis. Further studies are indicated to address this possibility, and whether any detrimental effects of calcitriol analogues on the lung are reversible.

The vitamin D analogues chosen for this study exhibited different profiles in their induction of mitogenesis in cultured NRLFs, as well as to induce transcription through a vitamin D response element from the PDGF-A gene (A-VDRE). The ability of 1,25-(OH)₂D₃ analogues to induce cell division in lung fibroblasts represents a striking contrast with their antiproliferative, proapoptotic, and prodifferentiation activities in many different cell types (reviewed in [30]). CB1093 and MC1288 were much more potent NRLF mitogens than 1,25-(OH)₂D₃ or EB1089, consistent with their elevated growth inhibitory properties in other settings [33,53–58]. EB1089 was nearly equipotent with 1,25-(OH)₂D₃ as an NRLF mitogen, again consistent with the majority of reports of its antigrowth properties [59], although elevated antiproliferative activity has been described for this analogue in some systems as well [33, 55,60]. With respect to activation of the A-VDRE motif in the current study, EB1089 and CB1093 were much more potent (ED50 ~ 0.04–0.1 nM) than the superagonist MC1288 or the native hormone. Previous reports of relative potency of these analogues on VDRE activities have been rather divergent, depending on the particular VDRE motif and cellular context in which they were analyzed. The 20-*epi* analogue, 20EE-125D, exhibited a 200- to 500-fold

higher potency than 1,25-(OH)₂D₃ in its ability to induce a prototypical DR3 form of VDRE in Ros 12/2.8 osteoblast-like osteosarcoma cells. MC1288, another 20-epi analogue, was also more potent than the native hormone in inducing recruitment of DRIP coactivators [56]. In addition, EB1089 was shown in vitro to elicit significantly more binding than 1,25-(OH)₂D₃ of VDR/RXR heterodimers to the IP9 form of VDREs, a disparity not seen in the context of canonical DR3 motifs [61]. The molecular mechanisms underlying the marked variation in agonist properties of these vitamin D analogues is not well-understood at present. Different analogues may induce distinct VDR conformations with varying abilities to induce transcription via recruitment of coactivators and other components of the transcriptional machinery, analogous to the precedent of selective estrogen receptor modulators (SERMs). However, recent x-ray crystallographic studies of various vitamin D analogues in association with the ligand-binding domain (LBD) of VDR have revealed no differences in structure as compared to the native hormone [62,63]. It should be noted these structures were derived with the LBD in isolation and not with the intact receptor, as well as with proteins in crystallized rather than soluble form. Another factor potentially contributing to the agonist properties of these analogues is their bioavailability in vitro and in vivo, particularly in regard to their adsorption by the vitamin D binding protein (DBP), which is present at high levels in fetal bovine serum. A previous study suggests that decreased catabolism and DBP binding may account for the superagonist activity of MC1288 [64]. Overall, our results demonstrate important differences between vitamin D analogues in their relative abilities to stimulate certain alveolarization-relevant events, which in turn seem likely to impact upon the alveolarization process itself.

The apparent synergy between 1,25-(OH)₂D₃ and *at*-RA on mitogenesis of NRLFs in culture was not recapitulated in terms of lung development in vivo. On the whole, EB1089 inhibited alveolarization and interfered with the ability of *at*-RA to ameliorate the deleterious effects of dexamethasone. Pilot studies conducted with lower levels of EB1089 ($\leq 0.1 \mu\text{g/kg}$), alone or in conjunction with *at*-RA, were without significant effect on airspace volume or wall thickness (data not shown), indicating an optimal dosage was unlikely to have been overlooked in the current study. At higher levels, however, EB1089 induced regions of hypercellularity that were strongly positive for expression of vimentin and the alveolarization-relevant growth factors, PDGF-AA and VEGF. Interestingly, PDGF-AA expression persisted across both epithelial and fibroblast cell types in the presence of dexamethasone (Figure 6A), suggesting that the alveolarization-inhibiting effects of the agent are not mediated by blocking expression of this key growth factor. Both dexamethasone and EB1089 induced VEGF expression in numerous individual cells within alveolar tissue sections, although the specific cell types were not identified. Interestingly, *at*-RA inhibited PDGF-AA expression across all lung cell types in vivo when administered alone, an observation at odds with its ability to induce PDGF-AA expression in cultured NRLFs. In addition, EB1089 administration resulted in the formation of tangling of elastin fibers, a phenomenon described earlier in response to *at*-RA [7], and which appeared to be more severe when both agents were administered concurrently.

Administration of *at*-RA alone in the current study failed to stimulate alveolarization in the neonatal rat, in contrast with other reports [4,65]. However, our study recapitulated the protective effect of *at*-RA treatment against dexamethasone-induced toxicity ($P < .03$), as described previously [40], suggesting that the dose and delivery methods employed in this report were effective. Such a minimally effective *at*-RA dose should have been sufficient to observe interactions with EB1089 at some level and did, in fact, discriminate between its protection against dexamethasone-induced toxicity and the lack thereof against toxicity caused by EB1089. It should also be noted that the alveolarization-promoting activity of RA in other laboratories is rather subtle, and is not observed uniformly. For example, in studies of the detrimental effects of hyperoxia, RA alone does not elicit a significant induction of mean airspace volume in neonatal rats, but does provide protection against hyperoxia [5,66].

Moreover, *at*-RA does not promote alveolarization in neonatal sheep [67], a model admittedly different from the rat, in that alveolarization begins in utero.

Taken together, our observations suggest that 1,25-(OH)₂D₃ and *at*-RA act in concert to stimulate some, but not all, aspects of the alveolarization process. In turn, the partial but incomplete cooperativity between these agents would appear to indicate that other morphogens are probably required to drive the full alveolarization program, and that pharmacological approaches may require more complex combinatorial strategies. An intriguing possibility for testing in this regard is thyroid hormone, which has been shown to play a role in epithelial and mesenchymal differentiation in the early stages of lung development, particularly branching morphogenesis [68]. In light of the proposed importance of morphogen gradients in the development of the lung and other structures [18], however, it would appear induction of competent lung alveoli by systemic (or even localized) delivery of morphogenic agents may prove difficult to achieve. One hope lies in the identification of master regulators capable of eliciting the appropriate spatiotemporal characteristics of expression for downstream effectors, such as PDGF-AA, fibroblast growth factor (FGF)-10, and other alveolarization-relevant molecules.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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FIGURE 1.

Mitogenicity of vitamin D (vitD) analogues alone and in combination with all-*trans* retinoic acid (RA) in neonatal rat fibroblasts. Rates of [³H]thymidine incorporation were measured in first-passage cultures of lung fibroblasts derived from postnatal age 5 rats. Cultures were made quiescent for 24 hours in serum-free culture medium, then treated with the indicated concentrations of 1,25-(OH)₂-vitamin D₃ (1,25 vitD₃) or vitamin D analogues. Results represent means ± SEM for 3 independent experiments (*n* = 4 per experiment), and are expressed relative to those obtained with untreated cultures (relative mitogenicity), which were assigned a value of 1. (A) Vitamin D analogues. (B) Vitamin D analogues plus RA.

FIGURE 2.

The vitamin D analogues CB1093 and MC1288 are highly potent activators of a vitamin D response element (VDRE) in the PDGF-A gene. Activation of the PDGF-A VDRE by 1,25-(OH)₂D₃ and the synthetic analogues EB1089, CB1093, and MC1288 was assayed by transient transfection in CV-1 cells. Experiments were conducted by cotransfection of the reporter plasmid pAC261*luc*-ACE66, which contains the ACE66 and VDRE subfragment in fusion with a minimal PDGF-A promoter fragment (-261 to +8), and a VDR expression plasmid under the transcriptional control of the cytomegalovirus (CMV) promoter/enhancer. Displayed are relative luciferase activities, which represent raw luciferase values corrected for variation in transfection efficiency using a cotransfected *Renilla* luciferase expression plasmid, and expressed relative to activity obtained in the absence of vitamin D.

FIGURE 3.

Morphometric analysis of neonatal rat lung sections. (A) Representative sagittal cross-sections of the left lungs from treatment groups after staining with hematoxylin and eosin. Hi Vit D, a dosage of 1.25 μ EB1089/kg/day; Low Vit D, 0.38 μ EB1089/kg/day. Scale bar = 100 μ M. (B) Example of hypercellularity induced by EB1089 treatment (1.25 μ g/kg/day). Scale bar = 500 μ m for 4 $\times$ objective, 100 μ m for 20 $\times$, and 20 μ m for 100 $\times$ (right).

FIGURE 4.

Immunohistochemical staining of vimentin (A) and factor VIII (B) in representative lung sections from neonatal rats (P12) treated with EB1089 (vitD; 1.25 $\mu\text{g/kg}$ BW), dexamethasone (dex) and/or all-*trans* RA (RA). Highlighted with arrows within the mock-treated control (Con) section of A are regions of vimentin-positive staining (Vim), as well as autofluorescing erythrocytes (E). In B, regions of factor VIII positivity within endothelial cells of vessels (Ves) are identified with arrows. Included in both panels are representative staining patterns observed in thickened regions (Th) resulting from fibroblast hyperproliferation following EB1089 treatment. In C, costaining of vimentin and factor VIII is shown for the same section derived

from a rat treated with EB1089 (1.25 $\mu\text{g/kg}$). The image demonstrates that the thickened regions are composed of vimentin-positive, factor VIII-negative cells. Scale bars = 20 μm .

FIGURE 5.

Histochemical staining of lung sections for (A) collagen and (B) elastin. Staining for collagen was obtained using Masson's trichrome stain: collagen = blue; nuclei = black; cytoplasm and muscle = red. Elastin was visualized using Verhoeff-Van Gieson stain, as described in Materials and Methods. Green arrows identify collagen fibers in A and elastin fibers (E) in B; orange arrows in B identify elastin tangles (T). Scale bar inserted in lower left corner of panels 20 = μm . Shown to the right of each section of panel B is a 13 $\times$ expansion of the subregion outlined with black boxes.

FIGURE 6.

Immunohistochemical staining of the alveolarization-relevant growth factors, (A) platelet-derived growth factor (PDGF) and (B) vascular endothelial growth factor (VEGF), in representative lung sections following treatments with EB1089 (vitD), dexamethasone (Dex), and/or all-*trans* RA (RA). Shown in the upper left image of A was the result obtained with a lung section from a vehicle-treated rat and an irrelevant primary antibody (Irrelev. Ab; goat anti-GST, Santa Cruz). The upper left image in B represents immunoreactivity observed with a lung section from a vehicle-treated rat using rabbit anti-VEGF that had been preadsorbed with a VEGF peptide (anti-VEGF + peptide). Scale bars = 20 μ m.

FIGURE 7.

A potential mechanism through which calcitriol analogues induce fibroblast proliferation in the developing lung, while inhibiting the overall process of alveolarization.

TABLE 1

Activation of the PDGF-A VDRE by Vitamin D Analogues

Treatment	ED ₅₀ (nM)
1,25-(OH) ₂ D ₃	6
MC1288	8
EB1089	6
CB1093	4

TABLE 2

Body Weight, Lung Volume, and Internal Lung Surface Area

Treatment	<i>n</i>	Body Weight (g)	Lung Volume (cm ³)	Internal Surface Area (cm ²)
Control	6	28.2 ± 1.26	1.60 ± 0.078	1038 ± 77 ^a
Dexamethasone (DEX)	8	26.3 ± 1.10	1.42 ± 0.146	601 ± 71 ^e
Retinoic acid (RA)	6	29.6 ± 1.78	1.81 ± 0.104	1152 ± 105 ^a
EB1089 (1.25 µg/kg)	4	23.3 ± 3.12 [*]	1.49 ± 0.166	742 ± 120 ^{c,d}
EB1089 (0.38 µg/kg)	4	27.2 ± 1.23	1.66 ± 0.149	906 ± 233 ^{a-c}
DEX + RA	7	24.6 ± 1.14	1.58 ± 0.099	764 ± 53 ^{b-d}
DEX + EB1089 (1.25 µg/kg)	6	16.9 ± 1.64 [*]	1.47 ± 0.099	555 ± 41 ^e
DEX + EB1089 (0.38 µg/kg)	4	23.9 ± 1.61 [*]	1.66 ± 0.053	693 ± 31 ^{c,d}
RA + EB1089 (1.25 µg/kg)	5	18.5 ± 1.29 [*]	1.24 ± 0.089 [*]	566 ± 52 ^{d,e}
RA + EB1089 (0.38 µg/kg)	5	28.0 ± 1.57	1.63 ± 0.128	913 ± 84 ^{a-c}
DEX + RA + EB1089 (1.25 µg/kg)	7	16.4 ± 1.41 [*]	1.31 ± 0.073 [*]	543 ± 34 ^e
DEX + RA + EB1089 (0.38 µg/kg)	5	24.3 ± 1.19	1.59 ± 0.121	726 ± 59 ^{c,d}

Note. Results are mean values ± SEM; *n*, no. of rats.

^{*} $P \leq .05$ compared with rats treated with vehicle alone (control), using ANOVA and the Holm-Sidak method for mean separation.

^a Values not sharing a common superscript are significantly different ($P \leq .05$).

^b Values not sharing a common superscript are significantly different ($P \leq .05$).

^c Values not sharing a common superscript are significantly different ($P \leq .05$).

^d Values not sharing a common superscript are significantly different ($P \leq .05$).

^e Values not sharing a common superscript are significantly different ($P \leq .05$).

TABLE 3

Effects of the Vitamin D3 Analogue EB1089, Dexamethasone, and all-*trans* Retinoic Acid on Alveolar Airspace and Wall Thickness

Vit D ($\mu\text{g/kg}$)	RA	Dex	Chord Length (μm)	Wall Thickness (μm)
—	—	—	58.4 ± 3.05 (6) ^a	4.5 ± 0.37 (7)
0.38	—	—	64.5 ± 3.96 (5) ^{a,b}	3.7 ± 0.44 (6)
1.25	—	—	74.3 ± 4.33 (4) ^{b,c}	4.3 ± 0.46 (4)
—	+	—	57.3 ± 3.53 (6) ^a	4.2 ± 0.38 (6)
0.38	+	—	64.8 ± 3.90 (5) ^{a,b}	5.4 ± 0.40 (5)
1.25	+	—	80.5 ± 3.86 (5) ^{c,d}	4.4 ± 0.42 (5)
—	—	+	87.0 ± 3.12 (8) ^{d,e}	3.7 ± 0.35 (8)
0.38	—	+	84.6 ± 4.05 (5) ^{d,e}	3.8 ± 0.42 (5)
1.25	—	+	95.1 ± 3.65 (6) ^e	3.5 ± 0.40 (6)
—	+	+	74.7 ± 3.29 (7) ^{b-d}	3.6 ± 0.35 (7)
0.38	+	+	83.7 ± 3.81 (6) ^{d,e}	4.5 ± 0.46 (6)
1.25	+	+	87.3 ± 3.31 (7) ^{c-e}	4.0 ± 0.41 (7)

* Values represent mean $\pm$ SEM, with number of rats shown in parentheses.

^a Values not sharing a common superscript are significantly different ($P \leq .05$), as determined by the Student-Newman-Keuls procedure for mean separation. ANOVA tables summarizing overall treatment effects and interactions are displayed in Tables S2 and S3.

^b Values not sharing a common superscript are significantly different ($P \leq .05$), as determined by the Student-Newman-Keuls procedure for mean separation. ANOVA tables summarizing overall treatment effects and interactions are displayed in Tables S2 and S3.

^c Values not sharing a common superscript are significantly different ($P \leq .05$), as determined by the Student-Newman-Keuls procedure for mean separation. ANOVA tables summarizing overall treatment effects and interactions are displayed in Tables S2 and S3.

^d Values not sharing a common superscript are significantly different ($P \leq .05$), as determined by the Student-Newman-Keuls procedure for mean separation. ANOVA tables summarizing overall treatment effects and interactions are displayed in Tables S2 and S3.

^e Values not sharing a common superscript are significantly different ($P \leq .05$), as determined by the Student-Newman-Keuls procedure for mean separation. ANOVA tables summarizing overall treatment effects and interactions are displayed in Tables S2 and S3.