Regulation of human pulmonary surfactant protein gene expression by 1α,25-dihydroxyvitamin D₃

Sarabjit S. Phokela,¹ Sara Peleg,² Fernando R. Moya,¹ and Joseph L. Alcorn¹

¹Department of Pediatrics, Division of Neonatal-Perinatal Medicine, The University of Texas Health Science Center at Houston; and ²Department of Endocrine Disorders, The University of Texas, M. D. Anderson Cancer Center, Houston, Texas

Submitted 6 April 2004; accepted in final form 3 June 2005

PULMONARY SURFACTANT is a unique complex of phospholipids and proteins that maintains alveolar integrity by reducing surface tension at the alveolar air-liquid interface (15) and participates in host defense and the control of inflammation in the lung (16). Surfactant is composed of 90% lipids and 10% proteins by weight. Phosphatidylcholine, mostly dipalmitoyl phosphatidylcholine, constitutes 70 – 80% of the lipid portion of surfactant. Several distinct lung-specific proteins make up the protein component of surfactant, surfactant protein-A (SP-A), SP-B, SP-C, and SP-D (23). Whereas the lipid portion of surfactant is largely responsible for its surface activity, the protein component of surfactant, surfactant protein-A (SP-A), SP-B, SP-C, and SP-D (23). Whereas the lipid portion of surfactant is largely responsible for its surface activity, the highly hydrophobic proteins SP-B and SP-C also play a direct role in influencing the surface tension-reducing properties of surfactant (54). On the other hand, SP-A and SP-D are hydrophilic members of the collectin family of proteins (25) that play important roles in the control of host defense and inflammation in the lung (16). Pulmonary alveolar type II epithelial cells that are positioned in the corners of the alveoli carry out the highly specialized functions of synthesizing, secreting, and reutilizing surfactant (36, 49). Surfactant synthesis is initiated in alveolar type II cells only after 75% of gestation is complete (43). Infants born before this period of gestation lack sufficient surfactant and are predisposed to develop respiratory distress syndrome, one of the leading causes of neonatal morbidity and mortality (4).

The synthesis of surfactant by the fetal lung is regulated by a number of hormones, growth factors, and cytokines, some of which increase adenosine 3′,5′-cyclic monophosphate (cAMP) (36, 43). Steroid hormones, in particular, have varied effects on the regulation of expression of surfactant lipids and proteins in fetal lung both in vivo and in vitro. Glucocorticoids have been shown to accelerate fetal lung development and surfactant synthesis (31). In human fetal lung in organ culture, surfactant lipid synthesis and SP-B, SP-C (5, 8), and SP-D (17) mRNA expression are increased in the presence of glucocorticoids, whereas regulation of SP-A mRNA is more complex. Glucocorticoids increase transcription of SP-A mRNA and expression of SP-A protein at low concentrations, but SP-A mRNA and protein levels are decreased at high concentrations of glucocorticoids due to destabilization of SP-A mRNA (11, 26, 41). Retinoic acid increases expression of SP-B mRNA but decreases SP-A and SP-C mRNA in human fetal lung tissue in organ culture and in alveolar type II cells in culture (20, 21). These observations demonstrate that the regulation of fetal lung maturity and surfactant lipid and protein expression by hormones, and presumably their cognate receptors, is complex.

Another steroid hormone, the active form of 1α,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃], has been shown to affect fetal lung development (39). In fetal rat lung, 1,25(OH)₂D₃ increases the synthesis and secretion of surfactant lipids (34, 35) and accelerates the appearance of the morphological features of alveolar type II cells (34). Rat alveolar type II cells have been shown to contain the nuclear hormone receptor for 1,25(OH)₂D₃, which is responsible for many of its physiological effects (38). Recently, a report has suggested that a natural metabolite of 1,25(OH)₂D₃ increases surfactant phospholipid and SP-B mRNA and protein synthesis in human NCI-H441 cells (48). Nevertheless, the effects of 1,25(OH)₂D₃ on the expression of the various surfactant proteins in human fetal lung and epithelial cell lines have not been investigated. Therefore, we chose to determine the effects of 1,25(OH)₂D₃ on steady-state levels of SP-A, SP-B, and SP-C mRNA in human fetal...
lungs in organ culture, in isolated alveolar type II cells in primary culture, and in NCI-H441 cells. We hypothesized that 1,25(OH)2D3 would stimulate expression of surfactant protein mRNA in human fetal lung and epithelial cells.

**MATERIALS AND METHODS**

**Organ culture and alveolar type II isolation and primary culture.** Lung tissue from midtrimester human abortuses was obtained from Advanced Bioscience Resources (Alameda, CA) in accordance with protocols approved by The Committee for the Protection of Human Subjects of the University of Texas-Houston Health Science Center. The tissues were minced (1–2 mm3) and cultured on lens paper supported by stainless steel grids in serum-free RPMI-1640 medium (11875; Gibco-BRL, Gaithersburg, MD) in the presence of dibutyryl cAMP (Bt2cAMP, 1 mM, 104396; Boehringer Mannheim, Indianapolis, IN) as described previously (42). After 5 days of organ culture with daily medium changes, alveolar type II cells were isolated from the lung explants in organ culture (3). As described previously, the lung explants were dissociated by digestion with collagenase type I (0.5 mg/ml; Sigma Chemical, St. Louis, MO) and collagenase type IA (0.5 mg/ml, C-9891, Sigma Chemical) for 15 min at 37°C with vigorous pipetting. After collagenase digestion, the cells were treated with diethylaminoethyl-dextran hydrochloride (250 μg/ml, D9885, Sigma Chemical), to eliminate fibroblasts, and incubated for 45 min with shaking at 37°C. The cells were pelleted at 400 g and plated (2–5 × 104 cells per dish) onto 60-mm tissue culture dishes that were coated with extracellular matrix (ECM) derived from Madin-Darby canine kidney (MDCK) cells (ATCC CRL 6253). The ECM-coated dishes were prepared from confluent monolayers of MDCK cells that were treated with deoxycholate (1%) for 5 min. The ECM dishes were washed three times with Hank’s balanced salt solution and stored at 37°C until use. Plated epithelial cell-enriched cultures were incubated overnight in RPMI-1640 with 10% fetal bovine serum. Dishes were washed twice with medium to eliminate dead and nonadherent cells and then incubated in RPMI-1640 without fetal bovine serum and in the absence or presence of the various required effectors. All human cell and tissue culture incubations were performed in a humidified incubator at 37°C with 5% CO2.

**Cell culture of NCI-H441 cells.** The lung adenocarcinoma cell line NCI-H441 (19, 44) was cultured in RPMI-1640 containing fetal bovine serum (10% vol/vol). When the cells were to be used for experiments, they were incubated in RPMI-1640 containing charcoal-stripped dextran-treated fetal bovine serum (SH30068; Hyclone, Logan, UT) 24 h before treatment to eliminate possible effects from steroids in fetal bovine serum. All cell culture incubations were performed in a humidified incubator at 37°C with 5% CO2.

**Immunoblot analysis.** Proteins were isolated as described previously (2). In brief, cells were scraped from the plates and homogenized in ice-cold homogenization buffer [0.25 M sucrose, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF)]. The homogenized samples were centrifuged at 600 g for 5 min to sediment nuclei and debris, and the resulting supernatant was assayed for protein content. Cellular proteins were subjected to one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose by electrophoretic transfer. The proteins on the resulting blots were analyzed for the presence of vitamin D receptor (VDR) using specific antibodies (RP-710; Affinity Bioreagents, Golden, CO). The resulting immune complexes were visualized using the chemiluminescence ECL system (Amer sham Pharmacia Biotech, Piscataway, NJ). For SP-A immunoblot analysis, a rabbit anti-human SP-A antibody (1:1,000 dilution) (1) was used followed by peroxidase-conjugated goat anti-rabbit IgG (1:160,000 dilution, A0545; Sigma Chemical). For SP-B immunoblot analysis, a commercially available rabbit anti-SP-B antibody (AB3780; Chemicon International, Temecula, CA) was used followed by peroxidase-conjugated goat anti-rabbit IgG (1:16,000 dilution).

The immunoreactive proteins were detected with the chemiluminescence ECL system and recorded on high-performance chemiluminescence film for visualization of the resulting protein-antibody complexes.

**Northern analysis of surfactant mRNA.** Total RNA was extracted from the tissue and cells by homogenization in guanidinium isothiocyanate (4.0 M) using a tissue homogenizer. Cell extracts were centrifuged through a cesium chloride gradient (5.7 M), and the pelleted RNA was resuspended in water (13). Total RNA (15 μg) was electrophoresed, transferred to nitrocellulose, and probed using a 32P-labeled rabbit SP-A cDNA, rabbit SP-B cDNA, or rabbit SP-C cDNA as described in detail previously (9, 10, 33). Analysis of 18S rRNA was performed using an 80-bp 32P-labeled 18S fragment (7339; Ambion, Austin, TX). The specific surfactant protein mRNA species were visualized by autoradiography using Kodak X-OMAT radiographic film.

**Quantification of surfactant protein mRNA levels and SP-A protein levels.** Quantification of the surfactant protein mRNA bands and 18S RNA bands visualized by Northern analysis and SP-A protein and β-actin protein visualized by immunoblot analysis was performed using the imaging and quantitation abilities of the ChemiGenius imaging system (Syngene, Frederick, MD). The ChemiGenius is an analysis system that features a Peltier-cooled charge-coupled device camera and Total Recall feature, which creates every aspect of the capture of a particular image and ensures precise reproducibility of capture conditions from sample to sample. The system is linked to a computer for control and analysis via a fully integrated image-acquisition, archiving, and analysis package running in a Windows environment (InGenius Bioimaging software: Genetools version 3.04 and GeneSnap version 6.03). An automatic and full analysis of the saved image can be performed with a report of the analysis generated.

**Statistical analysis.** All results are expressed as average ± SE of three independent values. A nonparametric ANOVA method of analysis, Kruskal-Wallis, was used to determine the level of difference. P < 0.05 was considered to be significant. The analysis was performed using a software package by Number Cruncher Statistical Systems (Kaysville, UT).

**RESULTS**

VDR is detectable in human fetal lung in organ culture and alveolar type II cells in primary culture incubated in the presence of 1,25(OH)2D3. Although the presence of VDR in fetal rat lung and type II cells has been established (34, 38), its presence in human lung tissue and human alveolar type II cells has not been demonstrated. Human fetal lung and primary alveolar type II cells were incubated in the absence or presence of 1,25(OH)2D3 (10−7 M), and lysates were analyzed for the presence of the VDR by Western immunoblot analysis. Figure 1 shows the results of the analysis. Levels of VDR were barely detectable in human fetal lung and human type II cells cultured in the absence of 1,25(OH)2D3 (only visible when the blot was overexposed). However, when VDR was cultured in the presence of 1,25(OH)2D3, its levels increased dramatically in both human fetal lung tissues in organ culture and human alveolar type II cells in primary culture. These results suggest that 1,25(OH)2D3 increased the amount of VDR in human fetal lung and in human alveolar type II cells. Normally, vitamin D induces stabilization of the VDR; unoccupied VDR is cytoplasmic and proteasome sensitive, whereas binding of the ligand results in a rapid accumulation of VDR in the nucleus. On the other hand, Caco-2 cells possess proteasome-resistant VDR in the absence or presence of ligand (27, 45), making the lysate useful as a positive control for the presence of VDR.
1,25(OH)2D3 generally reduces expression of SP-A in human fetal lung tissue, human alveolar type II cells, and NCI-H441 cells. The effect of 1,25(OH)2D3 on SP-A gene expression was determined in human fetal lung in organ culture. Lung tissue was incubated in the absence or presence of 1,25(OH)2D3 (10-10 or 10-7 M) ± Bt2cAMP; and steady-state SP-A mRNA levels were determined. It is important to note that cAMP is added to the tissue and cells to both augment surfactant protein gene expression (36, 43) and to induce and maintain the differentiation of alveolar type II cells (3, 52).

To address the effect of 1,25(OH)2D3 on SP-A protein levels, we performed Western immunoblot analysis on cells incubated for 4 days in the absence or presence of Bt2cAMP and various concentrations of 1,25(OH)2D3. The results of these analyses are shown in Fig. 3B. Quantification of the SP-A protein signal indicates that the presence of 1,25(OH)2D3 had no significant change in SP-A protein levels in type II cells incubated in the absence of Bt2cAMP, but 1,25(OH)2D3 at 10-7 M significantly reduced SP-A protein in cells incubated in the presence of Bt2cAMP. These results indicate that 1,25(OH)2D3 reduces SP-A protein levels in type II cells in a dose-dependent manner only in the presence of Bt2cAMP.

We then investigated the effect of 1,25(OH)2D3 on SP-A mRNA levels in NCI-H441 cells, since these cells express SP-A mRNA (44). NCI-H441 were cultured in the absence or presence of various concentrations of 1,25(OH)2D3 (0, 10-10, 10-9, 10-8, and 10-7 M) ± Bt2cAMP. After 3 days in culture, the cells were harvested, and the RNA was isolated and subjected to Northern blot analysis. Figure 2D shows a quantification of the SP-A mRNA signal determined by scanning densitometry of three Northern analyses. As seen with the results using human alveolar type II cells in primary culture, Bt2cAMP caused an increase in the SP-A mRNA levels in NCI-H441 cells. In the absence of Bt2cAMP, lower concentrations of 1,25(OH)2D3 (10-10 to 10-9 M) seemed to increase SP-A mRNA levels, yet 1,25(OH)2D3 at concentrations of 10-8 to 10-7 M reduced the levels of SP-A mRNA in the cells below levels found in untreated cells. In cells incubated in the presence of Bt2cAMP, 1,25(OH)2D3 appeared to have a dose-dependent effect to antagonize the stimulatory effect of Bt2cAMP; higher concentrations of 1,25(OH)2D3 resulted in greater antagonism.

1,25(OH)2D3 increases steady-state SP-B mRNA levels in human alveolar type II cells in primary culture in a dose-dependent manner, but not in human fetal lung tissue in organ culture. The effect of 1,25(OH)2D3 on SP-B gene expression was determined in human fetal lung in organ culture. Lung tissue was incubated in the absence or presence of 1,25(OH)2D3 (10-10, 10-9, 10-8, and 10-7 M) ± Bt2cAMP. After 4 days in culture, the cells were harvested, and the RNA was isolated and subjected to Northern blot analysis. Figure 2A shows a representative Northern analysis of SP-A mRNA present in these cells. Quantification of the SP-A mRNA signal determined by scanning densitometry of three Northern analyses is shown in Fig. 2C. As seen in human fetal lung in organ culture, Bt2cAMP caused an increase in the SP-A mRNA levels in human alveolar type II cells in primary culture. In the absence of Bt2cAMP, lower concentrations of 1,25(OH)2D3 generally had little effect on SP-A mRNA levels, yet 1,25(OH)2D3 at a concentration of 10-7 M significantly reduced the levels of SP-A mRNA in the cells. In cells incubated in the presence of Bt2cAMP, 1,25(OH)2D3 appeared to have a dose-dependent effect to antagonize the stimulatory effect of Bt2cAMP; higher concentrations of 1,25(OH)2D3 resulted in greater antagonism.

The effect of 1,25(OH)2D3 on SP-A gene expression was determined in human fetal lung in organ culture. Lung tissue was incubated in the absence or presence of 1,25(OH)2D3 (10-10, 10-9, 10-8, and 10-7 M) ± Bt2cAMP; and steady-state SP-A mRNA levels were determined. It is important to note that cAMP is added to the tissue and cells to both augment surfactant protein gene expression (36, 43) and to induce and maintain the differentiation of alveolar type II cells (3, 52).

To address the effect of 1,25(OH)2D3 on SP-A protein levels, we performed Western immunoblot analysis on cells incubated for 4 days in the absence or presence of Bt2cAMP and various concentrations of 1,25(OH)2D3. The results of these analyses are shown in Fig. 3B. Quantification of the SP-A protein signal indicates that the presence of 1,25(OH)2D3 had no significant change in SP-A protein levels in type II cells incubated in the absence of Bt2cAMP, but 1,25(OH)2D3 at 10-7 M significantly reduced SP-A protein in cells incubated in the presence of Bt2cAMP. These results indicate that 1,25(OH)2D3 reduces SP-A protein levels in type II cells in a dose-dependent manner only in the presence of Bt2cAMP.

We then investigated the effect of 1,25(OH)2D3 on SP-A mRNA levels in NCI-H441 cells, since these cells express SP-A mRNA (44). NCI-H441 were cultured in the absence or presence of various concentrations of 1,25(OH)2D3 (0, 10-10, 10-9, 10-8, and 10-7 M) ± Bt2cAMP. After 3 days in culture, the cells were harvested, and the RNA was isolated and subjected to Northern blot analysis. Figure 2D shows a quantification of the SP-A mRNA signal determined by scanning densitometry of three Northern analyses. As seen with the results using human alveolar type II cells in primary culture, Bt2cAMP caused an increase in the SP-A mRNA levels in NCI-H441 cells. In the absence of Bt2cAMP, lower concentrations of 1,25(OH)2D3 (10-10 to 10-9 M) seemed to increase SP-A mRNA levels, yet 1,25(OH)2D3 at concentrations of 10-8 to 10-7 M reduced the levels of SP-A mRNA in the cells below levels found in untreated cells. In cells incubated in the presence of Bt2cAMP, 1,25(OH)2D3 appeared to have a dose-dependent effect to antagonize the stimulatory effect of Bt2cAMP; higher concentrations of 1,25(OH)2D3 resulted in greater antagonism.

1,25(OH)2D3 increases steady-state SP-B mRNA levels in human alveolar type II cells in primary culture in a dose-dependent manner, but not in human fetal lung tissue in organ culture. The effect of 1,25(OH)2D3 on SP-B gene expression was determined in human fetal lung in organ culture. Lung
The tissue was incubated in the absence or presence of 1,25(OH)_{2}D_{3} (10^{-10} or 10^{-7} M) \pm Bt2cAMP, and steady-state SP-B mRNA levels were determined. Figure 4A is a quantification of the SP-B mRNA signal determined by scanning densitometry of three Northern analyses (a representative Northern analysis is shown at the top). As expected, basal SP-B mRNA levels were low in human fetal lung tissue at 18–22 wk of gestation (day 0). The levels increased (ninefold) by day 2 and to 14-fold on day 4 of culture, indicating the expected differentiation of the tissue in organ culture. In tissue cultured in the presence of Bt2cAMP, the levels of SP-B mRNA were increased about two- to threefold over that of tissues incubated in the absence of the compound. SP-B mRNA levels were essentially unchanged in tissue cultured in the presence of 1,25(OH)_{2}D_{3} alone at either concentration for 2 or 4 days. When tissues were incubated in the presence of Bt2cAMP, 1,25(OH)_{2}D_{3} seemed to antagonize the stimulatory effect of Bt2cAMP at both concentrations. However, the apparent antagonism did not reach statistical significance, indicating that 1,25(OH)_{2}D_{3} has little effect on SP-B mRNA levels in human fetal lung tissue.

We then investigated the effect of various concentrations of 1,25(OH)_{2}D_{3} on SP-B mRNA levels in type II pneumocytes in primary culture. Human type II pneumocytes were isolated and incubated in primary culture in the absence or presence of 1,25(OH)_{2}D_{3} (0, 10^{-10}, 10^{-9}, 10^{-8}, and 10^{-7} M) ...
M) ± Bt2cAMP. After 4 days in primary culture, the cells were harvested, and the RNA was isolated and subjected to Northern blot analysis. Figure 4B is a quantification of the SP-B mRNA signal determined by scanning densitometry of three Northern analyses (a representative Northern analysis is shown at the top). SP-B mRNA levels were increased when the cells were incubated in the presence of Bt2cAMP. In cells incubated in the absence of Bt2cAMP, only the highest concentration of 1,25(OH)2D3 had an effect on SP-B mRNA levels; the presence of the hormone at 10^{-7} M increased the levels significantly. When type II cells were incubated in the presence of Bt2cAMP, a dose-dependent effect to increase SP-B mRNA levels was observed.

To address the effect of 1,25(OH)2D3 on SP-B protein levels, Western immunoblot analysis was performed on cells incubated for 4 days in the absence or presence of Bt2cAMP and various concentrations of 1,25(OH)2D3. The results of these analyses are shown in Fig. 4C. Quantification of the SP-B protein signal indicates that the presence of high concentrations of 1,25(OH)2D3 (10^{-7} M) significantly increased SP-B protein levels in type II cells incubated in the absence or presence of Bt2cAMP, but 1,25(OH)2D3 at 10^{-10} M had no significant effect. The effect of 1,25(OH)2D3 to increase SP-B protein levels is similar to the effect seen on SP-B mRNA levels in type II cells. These results indicate that while 1,25(OH)2D3 has no effect on SP-B mRNA levels in human fetal lung tissue, it increases SP-B protein and protein levels in type II cells. The latter is similar to published results indicating that 1,25(OH)2D3 increases SP-B mRNA and protein levels in NCI-H441 cells (48).

1,25(OH)2D3 has little effect on steady-state SP-C mRNA levels in human fetal lung tissue in organ culture. Finally, we investigated the effect of the presence 1,25(OH)2D3 on the levels of SP-C mRNA in human fetal lung tissue in organ culture. Figure 5 is a quantification of the SP-C mRNA signal determined by scanning densitometry of three Northern analyses (a representative analysis is shown at the top). Unlike that seen with SP-A and SP-B mRNA levels, SP-C mRNA levels in human fetal lung are relatively high at day 0. These levels decrease (10-fold) by day 2 of culture and show no change at day 4. This result is not completely unexpected, since detectable levels of SP-C mRNA in lung tissue appear to decrease during later development (3). The presence of 1,25(OH)2D3 had little effect on SP-C mRNA levels compared with tissue incubated in its absence. Again, as with SP-A and SP-B mRNA levels, the presence of Bt2cAMP increased the levels of SP-C mRNA. However, the presence of 1,25(OH)2D3 did not significantly change the levels of SP-C mRNA in these tissues. These results indicate that 1,25(OH)2D3 had little effect on SP-C mRNA levels in human fetal lung. We chose not to investigate the effects of 1,25(OH)2D3 on SP-C mRNA levels in human type II pneumonocytes in primary culture because we have found previously that detectable levels of SP-C mRNA had to be induced by the addition of dexamethasone (3). We felt that the presence of another steroid hormone in the culture medium would only complicate the phenomenon investigated.
DISCUSSION

The effects of steroid hormones, such as glucocorticoids (5, 8, 17, 31, 41), retinoids (20, 21), thyroid hormone (6, 47), estrogen (14), and testosterone (40, 53), on the synthesis of various surfactant components, have been studied at length over the last two decades. 1,25(OH)2D3 has been reported to accelerate fetal lung maturity and alveolar type II differentiation and to increase expression and secretion of surfactant lipids in rat lung tissue and rat type II cells (34, 35, 39). Despite these studies regarding the effect of 1,25(OH)2D3 on the
various components of surfactant and evidence that vitamin D receptors are present in rat alveolar type II cells (38), the consequences of 1,25(OH)2D3 on expression of the human surfactant protein genes have been investigated only in the context of SP-B mRNA and protein expression in a type II cell line, NCI-H441, where 1,25(OH)2D3 increased levels of SP-B mRNA and protein (48). We are interested in the effect of steroid hormones on human surfactant protein gene expression. We chose to investigate the heretofore unknown effects of 1,25(OH)2D3 on surfactant protein gene expression in human fetal lung tissue in organ culture and in alveolar type II cells in primary culture. We hypothesized that 1,25(OH)2D3 stimulates expression of surfactant protein mRNA in human fetal lung and epithelial cells since it has been reported to stimulate surfactant lipid synthesis and type II cell differentiation.

We found that human fetal lung in organ culture and isolated alveolar type II cells in primary culture contain very low levels of immunoreactive VDR, but VDR levels increase greatly in the tissue and cells when incubated in the presence of 1,25(OH)2D3. Although the results suggest that 1,25(OH)2D3 increased the expression of VDR in human fetal lung and in human alveolar type II cells, it probably represents stabilization of VDR in the cells. Unoccupied VDR is proteasome sensitive, whereas binding of the ligand 1,25(OH)2D3 results in a rapid accumulation of VDR in cells (27, 45). We have not performed experiments to distinguish either mode of accumulation of VDR in lung tissues and type II cells, but these results indicate that human lung tissue and type II cells do contain immunoreactive VDR, similar to published reports demonstrating the presence of VDR in rat lung tissue and type II cells (38).

To investigate the effect of 1,25(OH)2D3 on surfactant protein gene expression in human fetal lung tissue, we used a previously described organ culture system in which undifferentiated lung tissue is allowed to spontaneously differentiate in vitro and Bt2cAMP is added to accelerate differentiation and increase numbers of type II cells in the tissue (52). In human fetal lung, the major effect of 1,25(OH)2D3 on surfactant protein gene expression was to decrease expression of SP-A mRNA, whereas no significant effect of 1,25(OH)2D3 on SP-B and SP-C mRNA levels was observed. The inhibitory effect of 1,25(OH)2D3 on SP-A mRNA was most apparent when the tissue was incubated in the presence of Bt2cAMP, although the inhibitory effect of 1,25(OH)2D3 on SP-A mRNA levels also occurred in tissue incubated in the absence of Bt2cAMP. However, no significant effect of 1,25(OH)2D3 on SP-A protein levels was observed in the tissue. Thus it seems that the only significant effect of 1,25(OH)2D3 on surfactant protein gene expression in human fetal lung tissue is to reduce SP-A mRNA levels. This pattern of regulation of 1,25(OH)2D3 on SP-A mRNA levels is the same as that found in human fetal lung tissue in organ culture incubated with increasing concentrations of the synthetic glucocorticoid steroid hormone dexamethasone (11).

We then investigated the effect of 1,25(OH)2D3 on surfactant protein gene expression in isolated human alveolar type II cells in primary culture. In this system, alveolar type II cells are isolated from differentiated human fetal lung tissue in organ

---

Fig. 4. The effects of 1,25(OH)2D3 on steady-state SP-B mRNA levels in HFL and in alveolar epithelial cells. Representative Northern blots of RNA isolated from HFL tissue and isolated type II cells incubated in various concentrations of 1,25(OH)2D3 ± Bt2cAMP and probed for the presence of SP-B mRNA using radiolabeled human SP-B cDNA. Shown are the resulting autoradiographs (top) and the quantification of the mRNA signals (bottom). A: quantification of SP-B mRNA levels in human fetal lung tissue in organ culture incubated in the absence or presence of Bt2cAMP and/or 1,25(OH)2D3. The SP-B mRNA signal in autoradiographs was subjected to scanning densitometry, and the signal was normalized to the 18S RNA signal in each lane (not shown). Shown are the average levels ± SE of normalized SP-B mRNA signal relative to levels at day 0 of culture from 3 determinations. B: quantification of SP-B mRNA levels in human alveolar type II cells in primary culture incubated in the absence or presence of Bt2cAMP and/or 1,25(OH)2D3. Quantification of SP-B mRNA was determined as in A and is expressed relative to β-actin mRNA levels. *Significance (P < 0.05) compared with tissue incubated in the absence of 1,25(OH)2D3.

Fig. 5. The effects of 1,25(OH)2D3 on the expression of SP-C mRNA in HFL tissue in organ culture. Representative Northern blot of RNA isolated from HFL tissue incubated in various concentrations of 1,25(OH)2D3 ± Bt2cAMP and probed for the presence of SP-C mRNA using radiolabeled human SP-C cDNA. Shown is the resulting autoradiograph (top). Quantification of SP-C mRNA levels in human fetal tissue is shown at the bottom. The SP-C mRNA signal in autoradiographs was subjected to scanning densitometry, and the signal was normalized to the 18S RNA signal in each lane (not shown). Shown are the average levels ± SE of normalized SP-C mRNA signal relative to levels at day 0 of culture from 3 determinations.
culture and placed in primary culture (3). Bt2cAMP is added to maintain the type II cells in a differentiated state and to allow adequate expression levels of the surfactant genes for subsequent analysis. In these analyses, the effect of 1,25(OH)2D3 on SP-C gene expression was not investigated since adequate levels of SP-C gene expression can be attained only in the presence of glucocorticoids, whose presence would confound the interpretation of the results.

We found that the presence of 1,25(OH)2D3 reduced the levels of SP-A mRNA and protein in type II cells only when incubated in the presence of Bt2cAMP. There was no significant effect of the hormone on SP-A mRNA and protein levels in cells incubated without Bt2cAMP, but the levels of expression of SP-A in these cells were very low, and further reduction in the expression levels may not have been readily detected. 1,25(OH)2D3 antagonized the stimulatory effect of Bt2cAMP on SP-A mRNA levels in the cell in a dose-dependent manner much like that seen in human fetal tissue. In addition, a significant decrease in SP-A protein was detected in cells incubated with both Bt2cAMP and 1,25(OH)2D3 (10−7 M). 1,25(OH)2D3 has the same pattern of antagonism on SP-A mRNA levels as found with human alveolar type II cells incubated with increasing concentrations of dexamethasone (3, 5, 8, 11). Analysis on the effect of 1,25(OH)2D3 in NCI-H441, a lung adenocarcinoma cell line that expresses SP-A mRNA, indicates that low concentrations of the hormone (10−10 and 10−9 M) increased the levels of SP-A mRNA and higher concentrations reduced SP-A mRNA levels. In the absence or presence of cAMP, 1,25(OH)2D3 had a dose-dependent effect to decrease SP-A mRNA levels in the cell in a manner similar to that found with isolated alveolar type II cells. Thus it seems that one of the major effects of 1,25(OH)2D3 on surfactant protein gene expression in lung epithelial cells that express SP-A is to inhibit SP-A mRNA levels, and there is a pronounced antagonism of the stimulatory effect of Bt2cAMP on SP-A gene expression.

When we investigated the effect of 1,25(OH)2D3 on SP-B mRNA levels in isolated human alveolar type II cells in primary culture, we found that the presence of 1,25(OH)2D3 increases SP-B mRNA and protein levels in a dose-dependent manner when the cells are incubated in the absence or presence of Bt2cAMP. Although there is no significant effect of 1,25(OH)2D3 on SP-B mRNA levels in human fetal tissue, these results are consistent with previously reported results of 1,25(OH)2D3 on SP-B mRNA and protein levels in NCI-H441 cells (48). NCI-H441 are derived from Clara cells of the lung rather than alveolar type II cells; they express SP-A and SP-B, but not SP-C, and do not contain the distinguishing feature of type II cells, lamellar bodies (19). Despite the probable difference in origin between NCI-H441 cells and type II cells, the effect of 1,25(OH)2D3 on SP-B expression in the two cell types is similar, but both differ from the results with the fetal lung in which 1,25(OH)2D3 has no significant effect on SP-B mRNA expression.

The apparent incongruence between the effect of 1,25(OH)2D3 on expression of SP-B in fetal lung tissue in this study and in type II cells was quite unexpected in light of the fact that the regulation of SP-B expression by glucocorticoids in both tissue and cells is very similar (3, 11). However, it should be recognized that the regulation of the expression of specific surfactant protein genes may be different in isolated lung epithelial cells compared with human fetal lung tissue. It has been proposed that the effects of glucocorticoids on alveolar type II physiology are mediated through the action of the fibroblast pneumocyte factor (51), in which a secreted factor expressed by mesenchyme cells of the lung affects the synthesis of pulmonary surfactant components in alveolar type II epithelial cells. It is possible that some of the effects of 1,25(OH)2D3 on surfactant protein gene expression in the lung may be altered when cells are isolated and incubated in culture in a relatively purified state in which there is no interaction or communication with other cell types of the lung. 1,25(OH)2D3 may act directly on type II cells in primary culture to induce production of SP-B mRNA, whereas the compound may interact with many cell types of the lung that may produce factors...
that act on type II and Clara cells of the lung to elicit no net change in SP-B mRNA expression.

On the other hand, it has been shown that the effects of retinoic acid, the ligand of the heterodimer partner for VDR, depend on the stage of development of the lung. Retinoic acid signaling is ubiquitous in developing lung, but during branching morphogenesis, retinoic acid signaling is suppressed in the epithelium by various mechanisms (32). It has been shown that retinoic acid causes a dose-dependent inhibition of SP-A mRNA levels in human fetal lung in organ culture, and SP-B mRNA levels are increased in a dose-dependent manner, whereas SP-C gene expression is inhibited only at the highest levels of the agent (37). However, retinoids causes dose-dependent inhibition of SP-A, SP-B, and SP-C mRNA levels in cultured lung buds from 13.5-day gestational age rat embryos (12). Perhaps the potential retinoic acid receptor partner to the VDR complicates regulation of the surfactant proteins by 1,25(OH)2D3. The effects of retinoic acid on surfactant protein gene expression in our type II cell primary culture system conditions have not been defined and may be different from that reported in developing lung or that we see in our organ culture system.

Steroid hormones have been described as exerting their influence on cellular processes by activation of the hormone’s cognate receptor by binding to the receptor and altering transcription of a specific promoter by binding to DNA proximal to the promoter region (7, 18), although it is apparent that steroid hormones may have nongenomic effects on cellular physiology as well (50). The VDR influences transcription of a number of genes by the binding of VDR homodimers or vitamin D/retinoid X receptor (RXR) heterodimers to their cognate recognition sequences or vitamin D responsive elements (VDREs) that consist of an imperfect direct repeat of a core hexanucleotide sequence, (G/A)GGT(G/C)A, with a spacer region of three or six nucleotides separating each half-element (24, 28, 30). The mechanism of VDR binding to VDREs is reflected in the direct repeat nature of the element. Class I members of the nuclear receptor superfamily (e.g., glucocorticoid or progesterone receptor) bind to palindromic response elements as symmetrical homodimers. In contrast, the class II receptors (of which VDR is a member) generally bind to direct repeat elements as asymmetrical heterodimers with RRs. The direct repeat motif is asymmetrical, and the VDR-RXR heterodimer binds to the VDRE with a defined polarity. VDR and RXR bind to the DR-3 element with RXR occupying the 5′ half-site and VDR occupying the 3′ half-site. Because we were interested in the potential regulation of transcription of the surfactant protein genes by VDR, we scanned the available DNA sequences flanking the promoter regions of the human SP-A1 (accession number AJ19391), human SP-A2 [accession numbers: AF061969, M68519 (29)], SP-B [accession number: M24461 (46)], and SP-C [accession number: J03890 (22)] genes for sequences that correspond to the canonical binding site for the receptors. As shown in Fig. 6, we found that the DNA sequences surrounding the promoter regions of the three surfactant protein genes contain several perfect VDR-binding half-sites but no “perfect” direct repeats. However, seldom are perfect sites found in well-characterized 1,25(OH)2D3-regulated genes (28, 30), suggesting that perfect sites are not required for regulation. Whether VDR binds to these sequences surrounding the SP-A, SP-B, or SP-C promoters requires further study, but at least the possibility exists. We have observed that cAMP seems to make the cells more sensitive to the inhibitory effect of 1,25(OH)2D3 on expression of SP-A and SP-B, but not SP-C. The sequence of the 5′-flanking region of the human SP-A promoter indicates of the four potential VDR binding sites, two of them overlap sequence shown to be responsive to cAMP-regulated transcription factors (55), suggesting that interference between the VDR and cAMP-regulated transcription factors may play a part in the reduction of SP-A mRNA levels in the cells when incubated in the presence of Bt2cAMP and 1,25(OH)2D3. In the human SP-B promoter region, the only potential VDR binding site is separated from a potential cAMP protein binding site by 33 base pairs. Although it is not certain whether this region is of a size that is sufficient for interference, the proximity of the sites to each other suggests some type of communication between transcription factors.

The regulation of gene expression by steroid hormone receptors in relation to lung development, differentiation, and morphogenesis is quite complex. In particular, regulation of pulmonary surfactant protein gene expression by steroid hormones and their cognate receptors in fetal lung tissue depends greatly on gestational age, concentration of the steroid hormone, and the context of alveolar type II cells with other lung cell types. We have found that 1,25-(OH)2D3 in general appears to reduce SP-A mRNA levels in fetal lung tissue and reduce SP-A mRNA and protein in isolated type II cells while it increases SP-B mRNA levels in alveolar epithelial type II cells. The hormone appears to have no significant effect on SP-B and SP-C mRNA levels in human fetal lung tissue in organ culture. These results indicate that although 1,25(OH)2D3 has been reported to induce maturation of alveolar type II cells and pulmonary surfactant lipids, it does not induce expression of surfactant proteins in a coordinated manner.

ACKNOWLEDGMENTS

The authors thank Gaye N. Jenkins at the University of Texas-Houston Medical School for technical assistance with experiments performed in the manuscript and critical reading of the manuscript.

GRANTS

This work was supported in part by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-50583 (to S. Peleg).

REFERENCES

SURFACTANT PROTEIN REGULATION BY 1,25(OH)2D3


