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Regulation of pulmonary surfactant apoprotein SP 28–36 gene in fetal human lung

(dexamethasone/triiodothyronine/saturated phosphatidylcholine/RNA·cDNA hybridization/explant culture)

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ABSTRACT **Pulmonary surfactant stabilizes lung alveoli, preventing respiratory failure and hyaline membrane disease in premature infants. In addition to lipids, surfactant contains apoproteins that are thought to be critical for normal surfactant function. We have examined the ontogeny and regulation of the major surfactant-associated protein of molecular mass 28–36 kDa (SP 28–36) in human fetal lung. SP 28–36 was not detected in tissue from second trimester abortuses by either immunoblot analysis or enzyme-linked immunosorbent assay (<0.02 μg per mg of DNA). Levels of mRNA for SP 28–36, assayed by cDNA hybridization, were low or undetectable in all preculture specimens. The concentration of saturated phosphatidylcholine in lung tissue was 30% of the adult value with no apparent increase between 15 and 24 weeks gestation. SP 28–36 content increased during explant culture in the absence of serum and hormones, exceeding adult levels (3.2 ± 1.0 μg per mg of DNA) after 5 days. In cultures treated with triiodothyronine (2 nM) and dexamethasone (10 nM), hormones that regulate phosphatidylcholine synthesis, the increase in SP 28–36 was accelerated (treated/control ratio was 7.1 and 3.4 at 3 and 5 days, respectively). Levels of mRNA for SP 28–36 also increased during culture and were stimulated by hormones (treated/control = 8.6 and 1.9 at 3 and 5 days, respectively). SP 28–36 and its mRNA increased similarly in the presence of dexamethasone alone, whereas triiodothyronine alone had no apparent effect. The molecular weight and charge pattern was similar for SP 28–36 of adult and cultured fetal tissue. These findings indicate that expression of the SP 28–36 gene is low during the second trimester, increases during explant culture, and is accelerated by glucocorticoid treatment.**

Pulmonary surfactant lowers the surface tension within lung alveoli, allowing for normal respiration; a deficiency of surfactant at birth produces respiratory failure secondary to hyaline membrane disease (1, 2). In addition to surface-active saturated phosphatidylcholine and other phospholipids, surfactant contains protein species of relative molecular mass 28–36 kDa (SP 28–36) and 5–18 kDa (3–9) that are thought to be important for normal surfactant structure and function *in vivo*. Addition of purified SP 28–36 to surfactant lipids greatly enhances surface spreading of the mixture (10, 11).

Glucocorticoid treatment stimulates synthesis of surfactant phospholipids in lungs of fetal animals and reduces the incidence of hyaline membrane disease in premature human infants (2). When fetal lung is cultured as explants, there is an increase in the content of saturated phosphatidylcholine and appearance of lamellar bodies, the storage site of surfactant within alveolar type II cells (2, 12–16). Treatment with glucocorticoids accelerates these processes (2, 12), and addition of triiodothyronine (T_3) produces a synergistic response (2, 15–17). Recently, the gene for human SP 28–36 was cloned and found to contain a potential binding site for the glucocorticoid receptor (18).

The present report further examines regulation of the surfactant system by studying the major surfactant-associated protein, SP 28–36. We find that expression of the gene coding for SP 28–36 is low during the second trimester of human gestation and is increased by both culture in serum-free medium and by glucocorticoid treatment. A preliminary report of these results has appeared (19).

MATERIALS AND METHODS

Tissue. Adult lung tissue was obtained at pneumonectomy and fetal tissue from second trimester therapeutic abortuses under protocols approved by the University of California San Francisco Committee on Human Research. Fetal lung was studied freshly (preculture) or maintained in explant culture (15, 16) in serum-free Waymouth's medium and an atmosphere of 95% air/5% CO_2 for 1 to 5 days. Dexamethasone (10 nM) and/or T_3 (2 nM) were added to some cultures after 24 hr.

Preparation of SP 28–36 and Antibody. SP 28–36 was isolated from lavage of a patient with alveolar proteinosis as previously described (6, 11). This procedure involves sedimentation at 20,000 × *g*, extraction of lipids and very hydrophobic proteins with butanol, and removal of soluble serum proteins with buffered saline containing octyl glucopyranoside. The insoluble material is dialyzed against Tris·HCl buffer (pH 7.4) and clarified by centrifugation at 100,000 × *g* for 60 min. The supernatant, containing purified SP 28–36, was stored at −20°C and used both for raising antibodies and in an ELISA. With two-dimensional gel electrophoresis and silver staining this protein preparation contained multiple isoforms with pI values of 4.6–5.1 and molecular masses of 28–36 kDa, dimers (≈60 kDa), and higher order polymers.

The purified SP 28–36 was combined with Freund's complete adjuvant (final concentration, 0.5 mg/ml) and injected into rabbits (6). Immune rabbit serum was dialyzed against 0.1 M phosphate buffer (pH 8.1), applied to a DEAE-52 column (Whatman), and eluted with buffer containing concentrations of NaCl that increased in strength each elution. The IgG fraction was concentrated by ultrafiltration and absorbed against human serum conjugated to cyanogen bromide-activated Sepharose 4B. As previously reported, this antiserum detects proteins of alveolar proteinosis lavage (28–36 kDa and ≈60 kDa) on immunoblots but is not immunoreactive for proteins of human serum (6).

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Abbreviations: SP 28–36, surfactant-associated protein of molecular mass 28–36 kDa; T_3, triiodothyronine.

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ELISA. Multiwell plates were incubated with 10 ng of SP 28–36 in 0.1 ml of 0.1 M $NaHCO_3$ buffer (pH 9.6) overnight at 4°C. The plates were rinsed with phosphate-buffered saline (0.25 M K_2HPO_4, 1 M NaCl)/0.5% Tween 20, pH 7.4, and then exposed for 90 min at 37°C to antibody against SP 28–36 plus various amounts (0.1–10 ng) of SP 28–36 or lung sonicate diluted in phosphate-buffered saline/Tween 20/1% ovalbumin. The plates were rinsed 4 times in phosphate-buffered saline/Tween 20 and then incubated for 90 min at 37°C with goat anti-rabbit IgG conjugated with horseradish peroxidase. Absorbance at 490 nM was determined with a Bio-Tek EIA reader after color development with *o*-phenylenediamine and hydrogen peroxide.

Protein Electrophoresis. Fetal or adult lung tissue was sonicated in water containing protease inhibitors and two-dimensional isolectric focusing (pH 4 to 6) and polyacrylamide gel electrophoresis were done under reducing conditions using standard procedures (20). Proteins were transferred to nitrocellulose paper, and SP 28–36 was detected by immunostaining with primary antibody and secondary antibody conjugated with horseradish peroxidase (21).

Isolation of RNA and DNA Hybridization. RNA was isolated from lung tissue by the guanidinium isothiocyanate technique (22), and specific amounts were applied to nitrocellulose filters for dot blot analysis (23). Hybridization was carried out with ^{32}P-labeled cDNA probe (18) (pHs 10–5) for SP 28–36 mRNA prepared by nick-translation (22). The hybridization reaction used approximately 2×10^5 cpm/ml of labeled probe and was done for 16 hr at 40°C in 50% formamide/5 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate at pH 7.5)/0.05% $NaDodSO_4$/10 × Denhardt's solution (0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinylpyrrolidone)/50 μg per ml of yeast RNA/50 μg per ml of denatured and sheared salmon sperm DNA/0.005 M EDTA/0.05 M Hepes, pH 7. After washing the blots twice for 30 min at 50°C in 0.2 × SSC/0.1% $NaDodSO_4$, the paper was dried and applied to Kodak XAR film with Dupont Cronex intensifying screens for autoradiography. Developed films were scanned with a soft laser densitometer, and relative densities were calculated for linear portions of the dose–response curve of each RNA. For reprobing, papers were boiled in water for 10 min and hybridized with ^{32}P-labeled cDNA probe for actin (24) under the hybridization conditions described above.

Electrophoresis of RNA in the presence of glyoxal and $NaDodSO_4$ was done on 1.2% agarose gels, and major rRNA bands were visualized with ethidium bromide in 10× SSC. RNA was transferred to nitrocellulose paper by blotting and hybridized with ^{32}P-labeled cDNA for SP 28–36 under the conditions described.

Other Assays. Saturated phosphatidylcholine was assayed in sonicated lung tissue as described by Gonzales *et al.* (16). DNA was assayed by the fluorometric method of Setaro and Morley (25), and protein was determined according to Lowry *et al.* (26).

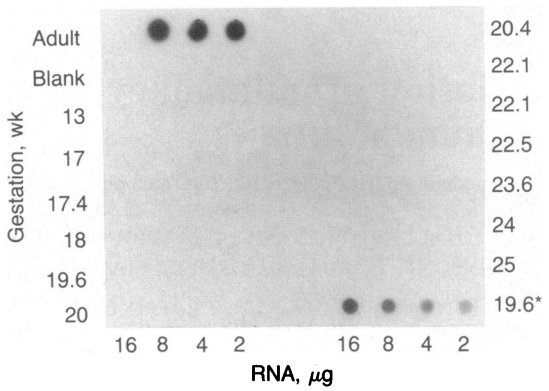

FIG. 1. Dot blot analysis of mRNA for SP 28–36 in preculture tissue. Total RNA was prepared from 13 fresh, preculture lung specimens from abortuses of 13–25 weeks gestation (as indicated by the numbers on the left and right sides of the figure) and probed with [^{32}P]cDNA for SP 28–36. Results with no RNA (blank), RNA prepared from fresh uncultured adult lung, and RNA from fetal lung cultured for 5 days with 4 days exposure to T_3 plus dexamethasone (19.6 weeks, treated) are also shown. The μg of RNA applied is shown at the bottom of the figure. *, Treated.

RESULTS

Ontogeny of SP 28–36 and Its mRNA. Experiments were done with human fetal lung of 13–25 weeks gestation. Prior to culture, SP 28–36 was not detected in lung homogenate by immunostaining of blots (not shown). Similarly, in the enzyme-linked immunosorbent assay (ELISA), SP 28–36 was undetectable (<0.02 μg/mg of DNA) in five lungs of 19.5–23.6 weeks of gestation. By comparison, homogenate of adult lung contained 3.2 ± 1.0 μg per mg of DNA (Table 1).

Total RNA was isolated from both fetal and adult human lung and mRNA for SP 28–36 was assayed by dot blot hybridization using radioactively labeled cDNA probe. In preculture lung specimens (13–25 weeks), mRNA for SP 28–36 was barely detectable in 13 of 18 RNA samples and not discernible in the others, after long exposure (14 days) of the blots to x-ray film (Fig. 1). Adult lungs were positive for SP 28–36 mRNA after only short exposure of the blot to film (12 hr).

Dot blots were washed free of SP 28–36 cDNA and reprobed with ^{32}P-labeled cDNA for actin. All of the preculture lung specimens contained mRNA for actin, and there was no apparent developmental change in the levels;

Table 1. SP 28–36 and its mRNA in human lung: Effect of culture and hormone treatment

	SP 28–36, μg/mg DNA		mRNA, % adult	
	Control	Ratio, treated/control	Control	Ratio, treated/control
Fetal				
Preculture	<0.02 (5)	—	<0.4 (18)	—
Day 1	0.03 ± 0.01 (5)	—	<0.4 (3)	—
Day 3	0.7 ± 0.3 (6)	7.1 ± 1.0	2.8 ± 1.6 (9)	8.6 ± 1.2
Day 5	6.9 ± 1.8* (8)	3.4 ± 0.7	30.5 ± 4.6 (12)	1.9 ± 0.2
Adult	3.2 ± 1.0* (7)	—	100	—

Fetal lung was examined by ELISA for content of SP 28–36 (18–24 wk) and by dot blot hybridization for SP 28–36 mRNA (13–25 wk) before (preculture) and after culture as explants for 1–5 days. Treated cultures were exposed to dexamethasone (10 nM) and T_3 (2 nM) beginning at 24 hr of culture (day 1). The content of both SP 28–36 and mRNA was higher in treated than control cultures in every comparison ($P < 0.05$ by paired Student's *t* test). Values are mean ± SEM with *n* in parentheses.
*$0.05 < P \leq 0.1$ by unpaired *t* test.

values ranged from 13 to 47% (mean 27%) of that for adult lung (one example is shown in Fig. 5). This finding suggests that degradation by RNase was not responsible for the very low levels of mRNA for SP 28–36. This conclusion was supported by the presence of the major ribosomal RNA bands after agarose gel electrophoresis of fetal lung RNA (data not shown).

Ontogeny of Saturated Phosphatidylcholine. The concentration of saturated phosphatidylcholine in preculture lung specimens ranged from 3.01 to 6.40 μg of P_i per mg of DNA (n = 15, mean = 3.95), with no apparent increase over the gestational age range of 15–24 weeks, compared with 12.94 μg P_i/mg of DNA (n = 4, $P < 0.01$) in adult lung (Fig. 2). These findings indicate that neither the major phospholipid nor the major apoprotein of surfactant increases during the second trimester of human gestation.

Effect of Culture and Hormones. To examine regulation of SP 28–36 *in vitro*, tissue was maintained in organ culture for up to 5 days in the presence or absence of dexamethasone (10 nM) plus T_3 (2 nM), concentrations which maximally stimulate saturated phosphatidylcholine synthesis (16). We examined SP 28–36 in cultured tissue by both immunoblot analysis and by ELISA. The content of SP 28–36 increased during organ culture in the absence of hormones, reaching a level after 5 days that was about twice that observed in homogenates of adult lung (P = 0.05–0.1, Table 1). In cultures treated with dexamethasone plus T_3, the increase in SP 28–36 content was accelerated, and the level after 5 days was about 8 times that found in adult lung (Table 1). These findings are consistent with results of immunofluorescence studies performed on lung tissue before and after organ culture (27).

The molecular mass and charge pattern of SP 28–36 from treated lung cultures is shown in Fig. 3. Comparable results were obtained with two-dimensional electrophoresis of proteins solubilized from adult lung (data not shown). In both fetal and adult tissue the antibody detected only SP 28–36. The findings are similar to those for SP 28–36 isolated from surfactant of normal adults (6–8) and from lung lavage of patients with alveolar proteinosis (6). The glycoprotein present in excess in these patients appears to be identical to SP 28–36 secreted by normal lungs (7, 18) and was used to prepare the antibody for these studies.

mRNA for SP 28–36 appeared as a single species of approximately 2.3 kilobases on RNA blot analysis of both adult and cultured fetal lung tissue (Fig. 4). This size is consistent with the length of the derived coding sequence

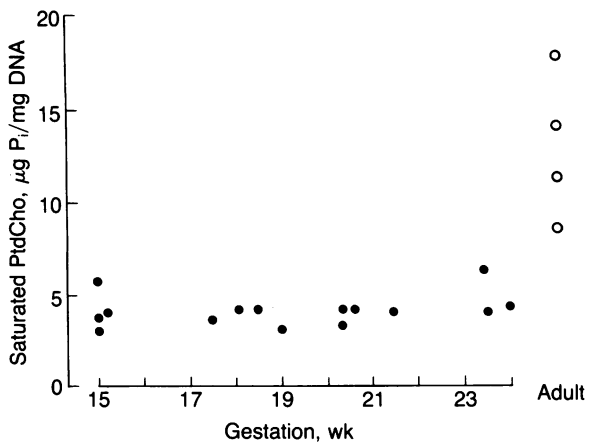

FIG. 2. Concentration of saturated phosphatidylcholine (PtdCho) in fetal and adult lung tissues. Each point represents a single specimen. The mean fetal value (3.95 ± 0.7) is significantly less than the adult level (12.94 ± 1.9, $P < 0.01$); P for slope of regression line >0.2.

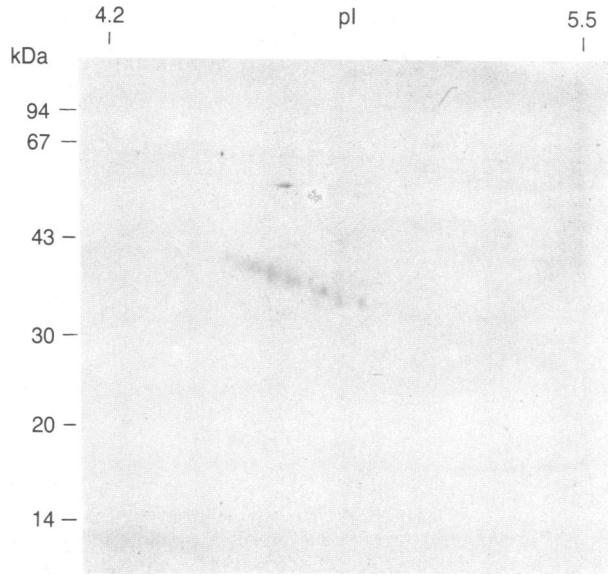

FIG. 3. Detection of surfactant protein (SP 28–36) in homogenate of hormone-treated fetal lung by two-dimensional gel electrophoresis and immunostaining. Seven hundred fifty micrograms of total lung protein was applied to the first dimension. Multiple isoforms of SP are observed with pI values of 4.6 to 5.1 (horizontal axis) and molecular masses of 32–36 kDa (vertical axis). In separate experiments no specific immunoreaction was observed with samples of preculture lung. Background levels of nonspecific staining of other lung proteins were related to their relative abundance and also occurred in the absence of the specific antibody to SP 28–36. With silver staining of similar two-dimensional gels, ≈1300 proteins are detected.

(≈2.1 kilobases) for human SP 28–36 (9, 18). SP 28–36 mRNA was not detected on RNA analysis of preculture fetal tissue (data not shown).

The content of SP 28–36 mRNA was determined by dot blot analysis. As illustrated in Fig. 5 and summarized in Table 1, levels of mRNA for SP 28–36 increased during explant culture. Message was detected (3% of adult levels) in fetal lung after 3 days of culture and was further increased (30% of adult) after 5 days. Tissue maintained with dexamethasone and T_3 accumulated mRNA at a faster rate. This effect was greatest after 3 days of culture when treated levels were 8 times more than control. After 5 days of culture with hormones, the level of SP 28–36 mRNA was twice the control level and approximately 50% of that found in adult lung. The

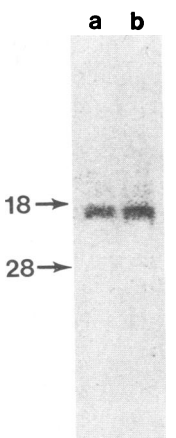

FIG. 4. RNA blot analysis of SP 28–36 mRNA. Lane a, adult lung RNA; lane b, RNA from fetal lung cultured for 5 days. The location of 18S and 28S rRNA as detected by ethidium bromide staining are indicated.

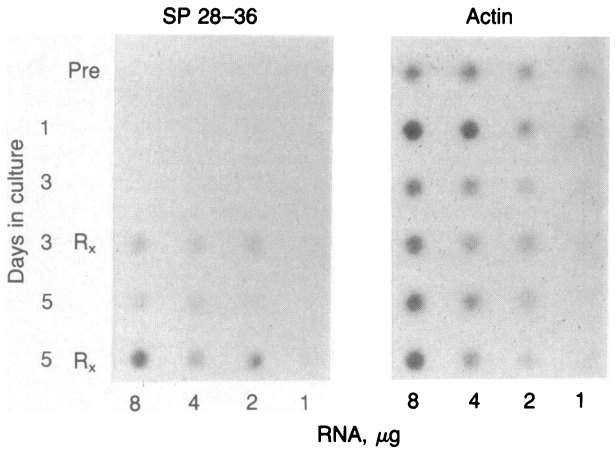

FIG. 5. Dot blot analysis of mRNA for SP 28–36 and actin in fetal lung during culture. Total RNA was prepared from lung of a 20-week human abortus before (Pre) and after explant culture for the time intervals in days (numbers at left) shown. Treated (R_x) specimens were exposed to dexamethasone (10 nM) and T_3 (2 nM) after 24 hours of culture (1 day). The blot was first probed with ^{32}P-cDNA for SP 28–36 (left) and subsequently washed and re-exposed to ^{32}P-cDNA for actin (right). The μg of RNA applied is shown.

decreased magnitude of hormonal stimulation with continued culture likely reflects the rapidly rising levels of both message and SP 28–36 in control cultures. By contrast, there was no consistent change in the amount of mRNA for actin during culture with or without hormones (range 43–62% of adult level).

We found considerable variability in the amount of both SP 28–36 and message between different specimens of cultured lung (range approximately 13-fold and 6-fold for SP 28–36 at 3 and 5 days, respectively). However, there was generally a correlation between levels of SP 28–36 and mRNA, and variability between samples for hormone stimulation was less (range 2- to 5-fold) than for the absolute content of SP 28–36. Over the relatively narrow range of gestation examined (19–24 weeks), there was no apparent correlation between the age of tissue and the level of SP 28–36 after either 3 or 5 days in explant culture. Differences in the time for initiation of the exponential increase in SP 28–36 during culture appear to account for much of the variability in content between samples.

The effect of adding dexamethasone and T_3 individually was examined after 3–4 days of culture when stimulation by combined treatment was greatest. As shown in Table 2, dexamethasone increased the content of SP 28–36 and its message to a level comparable to that with both hormones present. T_3 did not significantly stimulate accumulation of either SP 28–36 or its mRNA.

Table 2. Effect of dexamethasone vs. T_3 on SP 28–36 and its mRNA

Hormone treatment	SP 28–36, μg/mg DNA	mRNA, % of adult
None (control)	0.30 ± 0.09	1.1 ± 0.9
T_3 (2 nM)	0.53 ± 0.23	1.7 ± 1.4
Dexamethasone (10 nM)	4.43 ± 1.97*	24.0 ± 6.5*
T_3 + dexamethasone	4.46 ± 1.22*	30.0 ± 8.9*

Explants of fetal lung were maintained in culture for 3–4 days with addition of hormones as shown after 24 hr. The content of SP 28–36 and mRNA was increased by dexamethasone but not T_3 in each comparison. Values are mean ± SEM for seven experiments.
*$P < 0.05$ vs. control by paired Student's t test.

DISCUSSION

Differentiation of the surfactant system in the fetus is critical for normal lung function at birth. Previous studies in the human noted the appearance of lamellar bodies in type II cells and detectable surface-active material in lung tissue around 24 weeks of gestation (28–30). The data for content (this study) and synthetic rate (16) of saturated phosphatidylcholine are consistent with the morphologic data. In this study we find that the content of SP 28–36, the major apoprotein associated with surfactant, is very low or undetectable through 24 weeks of gestation. Previously, SP 28–36 was first detected in amniotic fluid at about 30 weeks gestation, and the concentration increased in parallel with surface-active lipid during the third trimester (31–33).

When human fetal lung is cultured as explants, lamellar bodies appear in epithelial cells and the content of saturated phosphatidylcholine increases markedly within 7 days (2, 13, 16). The time course for appearance and accumulation of SP 28–36 during culture closely parallels accumulation of phospholipid, and there is a similar response to hormone treatment. These results suggest that the genes for SP 28–36 and the developmentally regulated enzymes of phospholipid synthesis may be co-regulated. The apoprotein which appears during explant culture closely resembles SP 28–36 of adult lung on electrophoresis. Thus, it would appear that culture and hormone treatment cause precocious development of a fully-glycosylated, mature form of the apoprotein (6–8). Similar findings regarding the increase in SP 28–36 during explant culture have recently been reported by Weaver *et al.* (34).

The content of SP 28–36 in hormone-treated fetal lung was several-fold greater than in adult tissue, consistent with the percentage of type II cells in adult lung (8%) and treated explants (54%) (35, 36). By contrast, levels of SP 28–36 mRNA were less in treated explants than in adult lung. These results may indicate differences between fetal and adult tissue in translational efficiency or rate of degradation of SP 28–36. It is also possible that there is less recovery of solubilized SP 28–36 and/or rRNA, relative to mRNA, in adult lung compared to fetal tissue. Studies in progress with isolated type II cells will help to distinguish among these possibilities.

Glucocorticoids and thyroid hormones are known to affect transcription of specific genes in adult target tissues, often acting synergistically, but the mode of action of these hormones in differentiating fetal tissue such as the lung is largely unexplored (2). It is known that the stimulatory effects of glucocorticoids and T_3 on synthesis of surfactant phospholipids are mediated by receptors and are sensitive to inhibitors of both RNA and protein synthesis (12, 15, 16). A number of enzymes involved in pulmonary phospholipid synthesis are stimulated by glucocorticoid treatment of animals (2), but there is little information with regard to induced proteins in human tissue. Our findings indicate that at least one of the surfactant apoproteins is induced by glucocorticoids. T_3 had no effect on SP 28–36, either alone or in the presence of dexamethasone. It thus appears that synergy between glucocorticoid and thyroid hormone occurs for synthesis of surfactant lipid but not for the major surfactant-associated protein. A recent study in the fetal rabbit found induction of SP 28–36 by both cortisol and cAMP analog (37).

The content of SP 28–36 also increased during culture in the absence of hormones or serum. Thus, SP 28–36 appears to be regulated by at least two different processes *in vitro*; i.e., culture *per se* and hormones. The similar pattern of response for SP 28–36 and its mRNA suggests that both mechanisms involve, at least in part, pretranslational regulation. Additional experiments on the rate of transcription of

the SP 28–36 gene will be required to establish transcriptional control. Accumulation of surfactant phospholipids is also stimulated by culture alone (13, 16), suggesting a similar regulatory process for other genes. It is possible that the precocious differentiation of the pulmonary type II cell during explant culture reflects removal of an inhibitory serum factor.

In summary, we have found that the levels of the major surfactant-associated apoprotein (28–36 kDa) and its mRNA are very low in human lung throughout the second trimester. There is a precocious increase in SP 28–36 and its message during organ culture in the absence of serum or hormones, and these events are accelerated by treatment with dexamethasone. The size of the mRNA (≈2.3 kilobases) and the charge and molecular weight pattern of the protein are similar for adult and cultured fetal lung. Thus, both explant culture and glucocorticoid appear to activate expression of the SP 28–36 gene in human lung. We are presently examining effects of glucocorticoid and thyroid hormone on one of the lower molecular weight proteins of surfactant (SP 18) and other induced proteins of lung tissue. Results of this and earlier studies support the usefulness of hormone therapy for precocious induction of surfactant and prevention of hyaline membrane disease in premature infants.

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