0022-1554/94/$3.30
The Journal of Histochemistry and Cytochemistry

Vol. 42, No. 9, pp. 1187–1199, 1994
Printed in U.S.A.

Original Article

Temporal–Spatial Distribution of SP-B and SP-C Proteins and mRNAs in Developing Respiratory Epithelium of Human Lung[1]

ANDRAS KHOOR, MILDRED T. STAHLMAN,[2] MARY E. GRAY, and JEFFREY A. WHITSETT

Departments of Pediatrics (MTS) and Pathology (AK,MEG), Vanderbilt University, Nashville, Tennessee, and Division of Pulmonary Biology, Children's Hospital, Cincinnati, Ohio (JAW).

Received for publication November 16, 1993 and in revised form April 15, 1994; accepted April 23, 1994 (3A3222).

We determined the temporal and spatial distribution of surfactant protein B (pro-SP-B) and C (pro-SP-C) mRNAs and proteins by immunohistochemistry and in situ hybridization in fetal, neonatal, and adult human lung. Pro-SP-B and SP-B mRNA were detected in bronchi and bronchioles by 15 weeks' gestation. After 25 weeks, pro-SP-B, active SP-B peptide, and SP-B mRNA were co-localized in bronchiolo–alveolar portal cells and in Type II epithelial cells. In adult lung, pro-SP-B and SP-B mRNA were detected primarily in non-ciliated bronchiolar epithelial cells and in Type II cells in the alveolus. Pro-SP-C and SP-C mRNA were detected in cells lining terminal airways from 15 weeks' gestation and thereafter. After 25 weeks, SP-C mRNA and precursor protein were detected in epithelial cells of the bronchiolo–alveolar portals and in Type II cells, where expression increased with advancing gestational age. Distinct cellular patterns of staining for pro-SP-B compared with SP-B active peptide support the concept that its proteolytic processing or cellular routing may be influenced by cell type and/or cell differentiation. SP-B and SP-C are expressed primarily in distal conducting and terminal airway epithelium of human fetal lung well in advance of surfactant lipid synthesis or physiologic requirements to produce pulmonary surfactant at the time of birth. (*J Histochem Cytochem 42:1187–1199, 1994*)

KEY WORDS: Human lung development; Surfactant-associated proteins; In situ hybridization; Immunohistochemistry.

Introduction

Pulmonary surfactant is a phospholipid-rich mixture that reduces the surface tension at the alveolar air–liquid interface, providing alveolar stability necessary for normal ventilation. Four distinct proteins isolated from pulmonary surfactant are now termed surfactant proteins A, B, C, and D. SP-A; (M_r 28,000–36,000) and SP-D (M_r 43,000) are collagenous carbohydrate-binding proteins, whereas SP-B (M_r 9000) and SP-C (M_r 4000) are non-collagenous hydrophobic proteins (1). SP-A, SP-B, and SP-C contribute to the surface-active properties of surfactant phospholipids. Purified SP-B and SP-C markedly enhance the rate of phospholipid film formation at an air–liquid interface in vitro; this activity is further enhanced by the addition of SP-A (2). Preparations of SP-B, SP-C, and surfactant lipids increase lung compliance and preserve the morphological integrity of the distal airways in prematurely delivered, ventilated fetal rabbits (3–5) and in surfactant-deficient isolated rat lungs (6). Surfactant lipid preparations containing SP-B and SP-C have been tested widely in clinical trials and shown to improve oxygenation and reduce the need for ventilatory support in infants with hyaline membrane disease (7–9). SP-A and SP-B are also thought to play an important role in the formation of tubular myelin, an intermediate form of surfactant (10). Furthermore, SP-A, SP-B, and SP-C enhance surfactant phospholipid uptake (11,12), but SP-A inhibits surfactant phospholipid secretion by isolated Type II cells (13–16). SP-A enhances phagocytosis of erythrocytes (17), bacteria (18), and viruses (19) by macrophages in vitro. The biological function of SP-D has not been elucidated.

It is generally accepted that surfactant proteins are synthesized in alveolar Type II cells, and SP-A has been shown recently to be present in bronchial and tracheal glands by in situ hybridization and by immunohistochemistry (20,21). SP-B mRNA has been demonstrated by in situ hybridization in Type II cells and in bronchiolar epithelial cells of adult human (22), mouse (23,24), rat (25,26), and rabbit (27) lung. SP-C mRNA has been detected in Type II cells in adult rat lung (25), mouse lung (28), and in pre-Type II and Type II cells in developing mouse (28) and rabbit lung (29). Alveolar macrophages lack SP-A, SP-B, and SP-C mRNAs (21,22,25,30). The cellular distribution of SP-B mRNA and SP-C mRNA in developing human lung has not yet been reported.

[1] Supported in part by HL 14214 and HL 35859.
[2] Correspondence to: Mildred T. Stahlman, MD, Dept. of Pediatrics, Vanderbilt U. School of Medicine, Nashville, TN 37232-2370.

Immunoreactive SP-B has been identified in lung explant cultures of 18- and 23-week-gestation human fetuses, where it was detected in granular inclusions in the apical region of Type II-like epithelial cells (31). Antiserum to a group of hydrophobic low molecular weight proteins, which probably contained both SP-B and SP-C, has been used to immunostain adult human lung, localizing these proteins within Type II alveolar epithelial cells and alveolar macrophages (30). We have recently demonstrated the presence and distribution of immunoreactive SP-B in human fetuses and neonates, localizing the fully processed active SP-B peptide in fetuses of <23 weeks' gestation to rare non-mucous cells of tracheal glands and to terminal airway lining cells. In live-born infants from 25 weeks to term, mature Type II cells and cells of the bronchiolo–alveolar portals of respiratory bronchioles were immunostained. In infants with chronic lung disease, occasional tracheal and bronchial gland cells, rare non-ciliated columnar cells in ciliated bronchioles, and relining Type II cells were immunostained (32).

In the present work we utilized both in situ hybridization and immunohistochemistry to determine the temporal–spatial distribution of SP-B and SP-C proteins and mRNAs in human tracheal and pulmonary tissue obtained from adults, normal human fetuses, and infants who died of non-pulmonary causes in the first 2 weeks after birth. The localization of pro-SP-B and pro-SP-C was assessed with antisera selective for the precursor protein and was compared with Immunohistochemical data previously generated for the active SP-B peptide in these same fetuses and infants (32).

Materials and Methods

Tissue Preparation. This study was approved by the Committee for the Protection of Human Subjects–Health Sciences, of Vanderbilt University Review Board. Lung tissue was available from 38 fetuses of 10–23 weeks' gestation and from eight neonates of 25–42 weeks' gestation who died of non-pulmonary causes. Tracheas were available from 18 fetuses and two neonates. Normal adult tissue was obtained from non-affected portions of five lung biopsy specimens taken at the time of lobectomy. Not all tissue was considered satisfactory for in situ hybridization studies. In situ hybridization for SP-B and SP-C mRNAs was analyzed in the adult lungs and in lung tissue from 27 of these fetuses and from four neonates. Thirteen fetal tracheas and two neonatal tracheas were used for in situ hybridization studies with both SP-B and SP-C mRNAs. Tissues were obtained with parental consent at hysterotomy or hysterectomy abortion, at postmortem biopsy, or at autopsy between 1979 and 1991. The source of these tissues from very immature fetuses is no longer available, limiting the numbers at early gestational ages. Tissues were fixed in 10% phosphate-buffered formalin, in most cases within 2 hr after death, dehydrated through graded ethanols, and embedded in paraffin. Four-μm thick sections were cut and mounted on Superfrost Plus (Fisher; Atlanta, GA) glass slides. A preliminary in situ hybridization study demonstrated that SP-B mRNA and SP-C mRNA were preserved in these tissue sections. This is in agreement with the data of Floros et al. (33) indicating that surfactant protein mRNAs are quite stable.

Probe Preparation. A 797-BP fragment of a human SP-B cDNA clone (SP-B 7.1) and an 800-BP fragment of a human SP-C cDNA clone (SP-C 2.1) (both from American Type Culture Collection; Rockville, MD) were subcloned separately into Bluescript II SK transcription vectors (Stratagene; La Jolla, CA). The cDNA clones hybridize with SP-B mRNA (2.0 KB) and SP-C mRNA (1.0 KB) in Northern blot analysis of human lung RNA (34). The orientation of the subcloned fragments with respect to T3 and T7 promoters was determined by restriction map analysis. Sense and anti-sense RNA probes were synthesized by in vitro transcription. The reaction mixture contained a linearized DNA template, 5'[a-^{35}S]-UTP (NEN, Boston, MA; 1000–1500 Ci/mM), reagents of a transcription kit (Riboprobe Gemini II Core System; Promega, Madison, WI), and T3 (Stratagene) or T7 (Promega) RNA polymerase. Radiolabeled probes were reduced to an average length of 100 bases by limited alkaline hydrolysis (35) and separated from unincorporated nucleotides by Sephadex G-50 column chromatography. Before hybridization, probes were sized on denaturing agarose gels to determine that the limited alkaline hydrolysis reduced the probe to the optimal length (100 bases).

In Situ Hybridization. In situ hybridization was performed as described by Pelton et al. (36), with modifications. Tissue sections were deparaffinized in xylene (twice for 10 min), rehydrated through a graded ethanol series (100 to 30%), rinsed in 1 × PBS (5 min), and post-fixed in a freshly prepared solution of 4% paraformaldehyde in 1 × PBS (20 min). Slides were then rinsed in 1 × PBS (twice for 5 min), and treated with a fresh solution of proteinase K (1 μg/ml) in 50 mM Tris-HCl, pH 8.0, 5 mM EDTA (30 min at 37°C). Slides were then rinsed in 1 × PBS (5 min), refixed in the same paraformaldehyde solution (5 min), quickly dipped in distilled water, and acetylated in freshly prepared 0.25% acetic anhydride in 0.1 M triethanolamine (twice for 10 min). Slides were subsequently rinsed in 1 × PBS (5 min), dehydrated through the ethanol series (30 to 100%), and dried in a slide drier (Oncor; Gaithersburg, MD). Tissue sections were covered with a hybridization solution that contained 2 × 10^4 cpm/μl sense or anti-sense probe, 50% formamide, 0.3 M NaCl, 10 mM Tris, pH 8.0, 10 mM $NaPO_4$, 0.5 mM EDTA, 1 × Denhardt's solution, 10% dextran sulfate, 0.2 mg/ml yeast RNA, and 10 mM dithiothreitol. Tissue and probe were covered with a siliconized coverslip and hybridized in a humid chamber (overnight at 55°C). After hybridization, coverslips were removed in 5 × SSC (1 × SSC = 150 mM NaCl, 15 mM sodium citrate), 20 mM β-mercaptoethanol (BME) (1 hr at 50°C). Slides were washed in 50% formamide, 2 × SSC, 200 mM BME (20 min at 65°C), rinsed in 1 × TEN (10 mM Tris, pH 7.5, 0.5 mM EDTA, 0.5 M NaCl) (twice for 10 min at 37°C), treated with a solution of RNAse A (20 μg/ml) and RNAse T_1 (1 U/ml) in 1 × TEN (30 min at 37°C), and rinsed again in 1 × TEN (twice for 10 min at 37°C). Slides were then washed in 50% formamide, 2 × SSC, 200 mM BME (20 min at 65°C), in 2 × SSC (twice for 15 min at 65°C), and in 0.1 × SSC (twice for 15 min at 65°C). Slides were dehydrated through graded ethanols containing 0.3 M ammonium acetate, dried, dipped in 50% Ilford K.5 emulsion in 1% glycerol, and dried again. After 4 (SP-B) or 6 (SP-C) days of exposure, autoradiographs were developed in Kodak D-19 and counterstained with 0.04% toluidine blue. The specificity of hybridization was established by sense probes which did not hybridize above the background levels observed with the anti-sense probes.

Antibody Preparation. Rabbit polyclonal antiserum was produced by repeated injection of the entire recombinant human SP-C precursor expressed in *E. coli* as previously described (37). The antisera reacts with recombinant human pro-SP-C expressed in Chinese hamster ovary cells or after in vitro translation of SP-C mRNA as assessed by immunoblot or immunoprecipitation assays, but does not recognize the active SP-C peptide (38). The antiserum precipitates [^{35}S]-methionine/cystine-labeled pro-SP-C M_r 22,000 and processing intermediates (M_r ~16,000) in fetal rat lung explant cultures and in immortalized mouse lung epithelial cells (MLE-12 cells) and does not crossreact with SP-B or its precursor protein (39).

Rabbit pro-SP-B antiserum was generated against recombinant pro-SP-B expressed in *E. coli* as previously described (37). This antiserum recognizes pro-SP-B and both amino and carboxyterminal portions of pro-SP-B but is less reactive with the active SP-B peptide (40). The antiserum used to identify the active SP-B peptide was generated against a synthetic peptide comprising amino acids 1–78 of the active SP-B peptide and has been described by us previously (32). Although this antiserum detects pro-SP-B and fully processed SP-B (M_r 8000) by immunoprecipitation assay, it is more

highly reactive for the active SP-B peptide and did not detect human pro-SP-B in immunohistochemical studies of human lung (40). This antisera immunoblots SP-B (M_r 9000 reduced and M_r 18,000–36,000 unreduced) and fails to react with either alveolar lavage surfactant or tissue extracts from patients with hereditary SP-B deficiency (41).

Immunohistochemistry. Localization of immunoreactive SP-B precursor and SP-C precursor was carried out with the peroxidase–anti-peroxidase (PAP) method of Sternberger (42). Sections were deparaffinized, hydrated, treated with 0.3% H_2O_2 in methanol, and exposed to 10% normal swine serum to eliminate nonspecific staining. The primary antibody was applied and allowed to remain overnight at room temperature. After washing, sections were exposed to a swine anti-rabbit immunoglobulin, washed again, and treated with horseradish peroxidase coupled to rabbit anti-peroxidase. The peroxidase activity was then localized by reaction with a solution containing 0.05% 3,3'-diaminobenzidine and 0.01% H_2O_2 and sections were counterstained with hematoxylin.

Specificity of immunohistochemical analysis was tested in several ways. Adjacent tissue sections were exposed to non-immune rabbit serum in place of the primary antiserum. Because of the insolubility of both pro-SP-B and pro-SP-C, the routine methods for antibody ablation could not be employed. Therefore, the recombinant antigens were separated by SDS-PAGE and transferred to nitrocellulose strips for adsorption of their respective antibodies. Antibodies were also exposed overnight at 4°C to identical nitrocellulose strips lacking the antigen as control. The immunizing recombinant antigen was absorbed through the nitrocellulose and the sections immunostained with the eluting antisera. The adsorbed antisera were then compared by immunohistochemistry with the antisera adsorbed to the blank nitrocellu-

Table 1. *SP-B mRNA and immunoreactive precursor in fetuses*[a]

Gestational age (wk)	Postnatal age	Trachea				Bronchi				Ciliated bronchioles		Cells lining terminal airways	
		Epithelium		Glands		Epithelium		Glands					
		IS	H	IS	H	IS	H	IS	H	IS	H	IS	H
10		–	P	–	0	–	P	–	0	–	P	–	P
11		na	na	na	na	na	0	na	0	na	P	na	P
12		na	na	na	na	na	0	na	0	na	P	na	+
12		–	P	0	0	–	P	0	0	–	P	–	P
13		–	P	–	–	–	0	–	0	–	P	–	+
14		na	na	na	na	0	0	0	0	–	P	–	P
14		na	na	na	na	na	P	na	–	na	P	na	+
14		na	na	na	na	na	0	na	0	na	P	na	P
15		na	P	–	–	na	0	na	0	na	+	na	+
15		na	na	na	na	+	0	0	0	–	P	–	+
16		na	P	na	–	+	+	–	–	+	P	+	+
16		–	+	–	–	+	P	–	0	+	P	+	P
16		–	–	–	–	0	+	0	0	+	P	+	+
17		na	–	na	–	+	P	0	0	+	P	+	+
17		na	na	na	na	0	0	0	0	+	+	+	+
18		–	–	–	–	+	+	–	–	+	+	+	+
18		–	+	–	+	+	+	–	+	–	+	–	+
18		na	na	na	na	na	0	na	0	na	+	na	+
19	SB	–	+	–	+	0	0	0	0	+	+	+	+
19	7 hr	–	+	–	+	0	+	0	–	+	+	+	+
20	1 hr	na	+	na	–	na	+	na	–	–	+	na	+
20	SB	–	–	–	–	0	+	0	–	+	+	+	+
20	SB	na	na	na	na	0	0	0	0	+	+	+	+
20	1 hr	na	na	na	na	na	0	na	0	na	+	na	+
20	SB	–	+	–	+	+	P	–	0	+	+	+	+
20	SB	na	na	na	na	0	+	na	0	na	+	na	+
20	SB	na	na	na	na	na	+	na	0	na	P	na	P
21	SB	na	na	na	na	+	+	–	0	+	+	+	+
21	SB	na	na	na	na	na	+	na	0	na	0	na	+
22	1.5 hr	–	–	–	–	0	+	0	–	+	+	+	+
22	0.5 hr	na	+	na	+	+	+	–	0	+	+	+	+
22	SB	na	+	na	–	na	+	na	+	na	+	na	+
22	1 hr	na	na	na	na	na	0	na	0	na	+	na	+
23	SB	–	+	–	+	+	+	–	–	+	+	+	+
23	SB	na	na	na	na	0	+	0	0	+	+	+	+
23	2 hr	na	na	na	na	+	+	–	–	+	+	+	+
23	2 hr	na	na	na	na	+	+	–	–	+	+	+	+
23	2.7 hr	na	na	na	na	+	+	0	0	+	+	+	+

[a] +, positively stained cells; –, no stained cells identified; 0, structure not present in section; na, not available; IS, in situ; H, immunohistochemistry; P, peripheral staining; SB, stillborn.

Table 2. *SP-B mRNA and immunoreactive precursor in neonates*[a]

Gestational age (wk)	Postnatal age	Trachea				Bronchi				Ciliated bronchioles		Respiratory bronchioles and B-A portals		Mature type II cells	
		Epithelium		Glands		Epithelium		Glands							
		IS	H	IS	H	IS	H	IS	H	IS	H	IS	H	IS	H
25	15 min	na	na	na	na	na	0	na	0	na	+	na	+	na	+
36	1.5 hr	na	na	na	na	+	+	−	+	+	+	+	+	+	+
37	1 day	na	na	na	na	na	0	na	0	na	+	na	+	na	+
38	24 hr	na	na	na	na	na	+	na	0	na	+	na	+	na	+
38	90 hr	na	na	na	na	na	+	na	+	na	+	na	+	na	+
40	10 hr	na	na	na	na	+	+	0	−	+	+	+	+	+	+
40	10 days	−	−	−	−	+	+	−	−	+	+	+	+	+	+
42	12 days	−	−	−	−	+	+	0	+	+	+	+	+	+	+

[a] +, positively stained cells; −, no stained cells identified; IS, in situ; H, immunohistochemistry; 0, structure not present in section; na, not available.

lose strips. The use of the nitrocellulose strips lacking the antigen in control sections diminished immunostaining of these controls in some instances, making interpretation of ablation in these instances difficult.

A number of non-pulmonary tissues were examined immunohistochemically for SP-B and SP-C precursors, including parotid and submandibular glands, tongue, esophagus, stomach, duodenum, large intestine, pancreas, gallbladder, liver, kidney, heart, and thyroid.

Results

SP-B Expression in Proximal Airways

The gestational and postnatal ages of the fetuses and neonates whose tracheas and lungs were studied for SP-B mRNA by in situ hybridization and for SP-B precursor by immunohistochemistry are shown in Tables 1 and 2. In five fetuses from 10–16 weeks' gestation, immunostaining for pro-SP-B was detected in non-ciliated epithelial cells of the trachea. This staining was localized to the outer edges of cells whose cytoplasm contained large pools of glycogen. This localization of staining is designated "P" for periphery in the tables. As gestation progressed and glycogen pools diminished in amount, immunostaining of tracheal cells became more generalized throughout the cell. In nine of 13 fetuses from 16–23 weeks' gestation, many non-ciliated tracheal cells were immunostained for pro-SP-B and stained epithelial cells were most numerous in the folds interpreted to be origins of tracheal glands. Rare non-mucous gland cells were immunostained in five of these tracheas. SP-B mRNA was not detected in fetal or neonatal tracheal lining epithelia or in tracheal glands as assessed by in situ hybridization. SP-B mRNA was detected in scattered cells in the bronchial epithelium of fetal lung beginning at 15 weeks' gestation and, with rare exception, was detected in bronchial epithelial cells throughout gestation and in newborn infants (Figure 1). In fetuses before 15 weeks' gestation, immunostaining of the peripheral margins of bronchial cells was similar to that in tracheas. From 15 weeks onward, scattered non-ciliated non-mucous cells immunostained for pro-SP-B were present in bronchi (Figure 2). SP-B mRNA was not detected in bronchial glands of fetuses or neonates. In the bronchial glands, rare non-mucous cells immunostained for pro-SP-B (five of the fetuses

Figure 1. SP-B mRNA is seen in scattered bronchial epithelial cells. Lung tissue from a fetus of 23 weeks' gestation hybridized in situ to an anti-sense SP-B probe and photographed with darkfield illumination. Original magnification × 90. Bar = 100 μm.

Figure 2. Bronchus from the lung of a fetus of 22 weeks' gestation immunostained for SP-B precursor. Non-ciliated cells in bronchial epithelium are immunolabeled, as well as cells lining terminal airways. Immunoperoxidase and hematoxylin. Original magnification × 100. Bar = 100 μm.

Figure 3. SP-B mRNA is detected in terminal airway epithelium and in bronchiolar epithelium. Lung of a fetus of 16 weeks' gestation hybridized in situ to an anti-sense SP-B probe and photographed with darkfield illumination. B, bronchiole. Original magnification × 110. Bar = 100 μm.

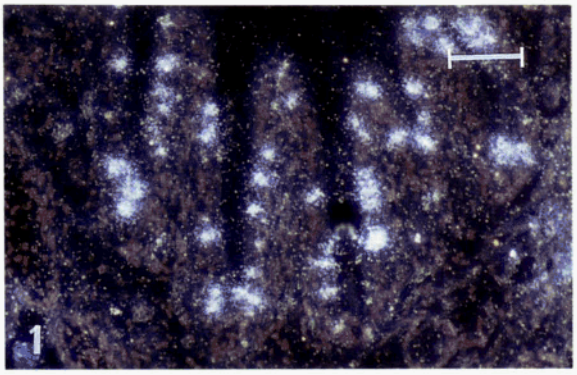
Figure 4. Lung of a fetus of 22 weeks' gestation hybridized in situ to an anti-sense SP-B probe and photographed with darkfield illumination. SP-B mRNA is expressed in both bronchiolar and in terminal airways lining cells. B, bronchiole. Original magnification × 150. Bar = 100 μm.

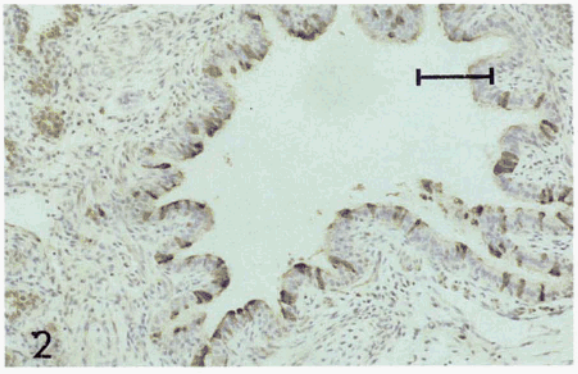
Figure 5. SP-B mRNA is localized in alveolar Type II cells and in bronchiolar epithelial cells, including those of the bronchiolo–alveolar portal. Lung of an infant of 40 weeks' gestation hybridized in situ to an anti-sense SP-B probe and photographed with darkfield illumination. B, bronchiole. Original magnification × 90. Bar = 100 μm.

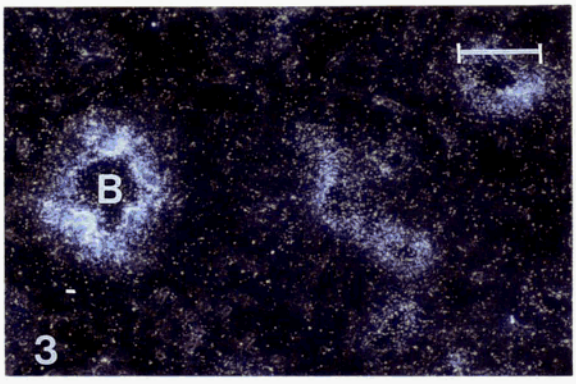
Figure 6. Lung of a fetus of 18 weeks' gestation immunostained for SP-B precursor. The more distal the airway, the more intense is the immunolabeling. Immunoperoxidase and hematoxylin. Original magnification × 100. Bar = 100 μm.

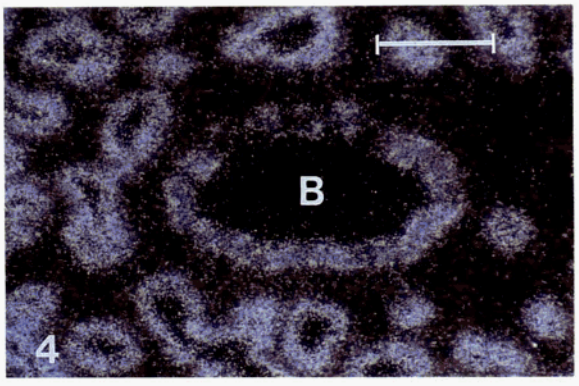
Figure 7. Lung of a fetus of 20 weeks' gestation showing many terminal airways lined with cells immunostained for SP-B precursor at the cell periphery. A few cells have a more diffuse cytoplasmic staining. Budding portions of airways have the most intense labeling. Immunoperoxidase and hematoxylin. Original magnification × 100. Bar = 100 μm.

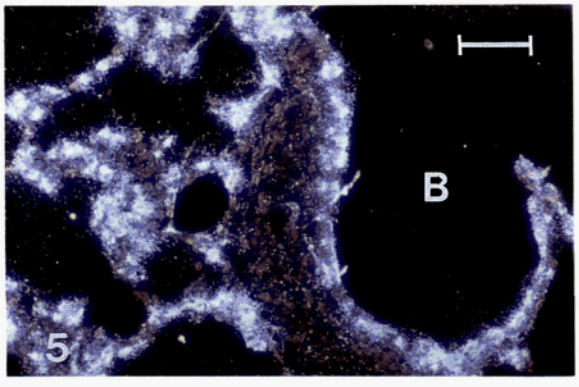
Figure 8. Large bronchiole and terminal airways from the lung of a live-born infant of 36 weeks' gestation immunostained for SP-B precursor. There is immunolabeling of non-ciliated bronchiolar cells and of many Type II cells in the terminal airways. Immunoperoxidase and hematoxylin. Original magnification × 175. Bar = 50 μm.

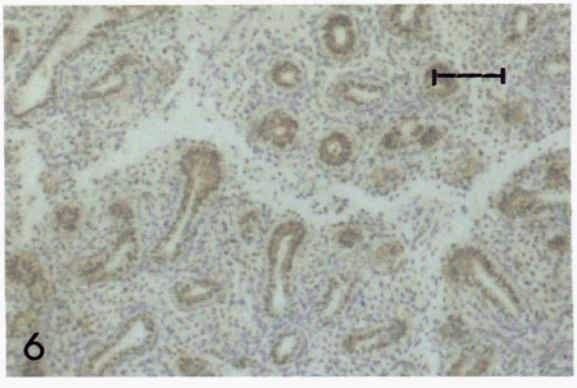

1

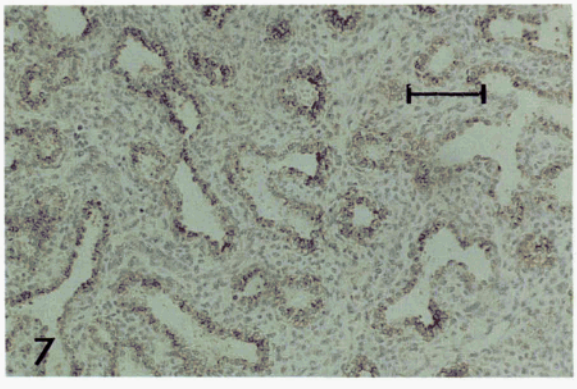

2

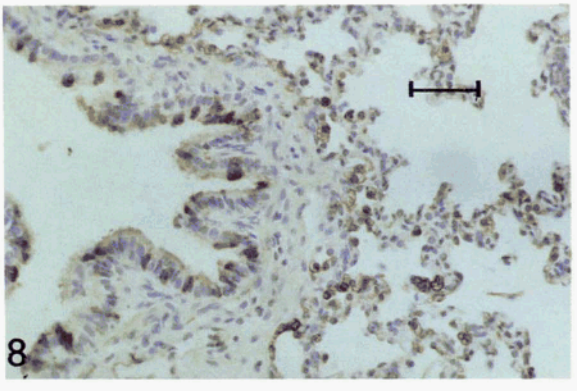

B
3

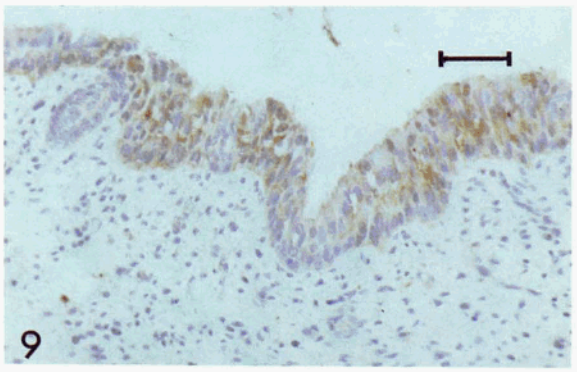

B
4

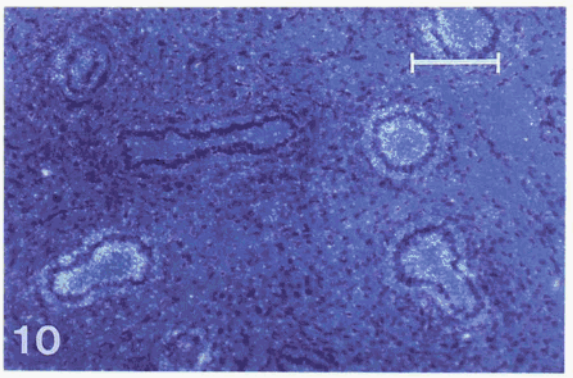

B
5

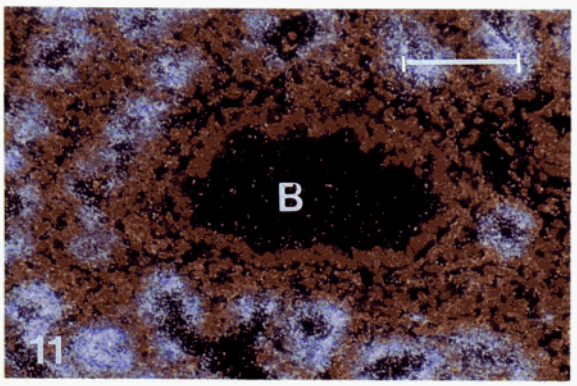

6

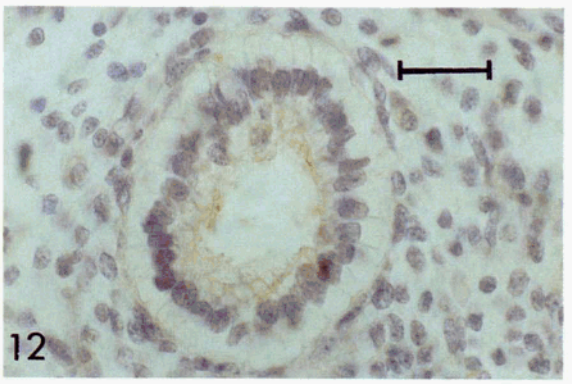

7

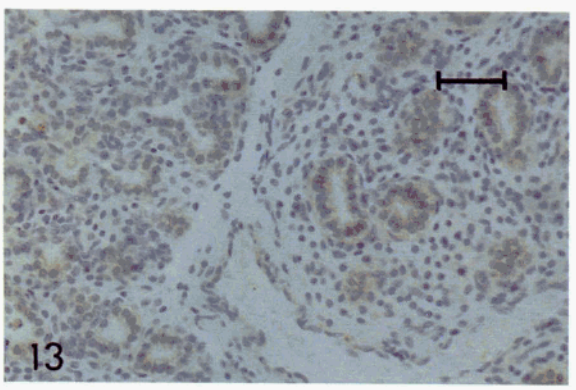

8

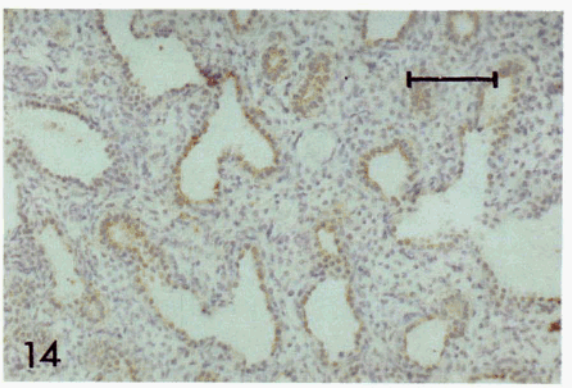

9

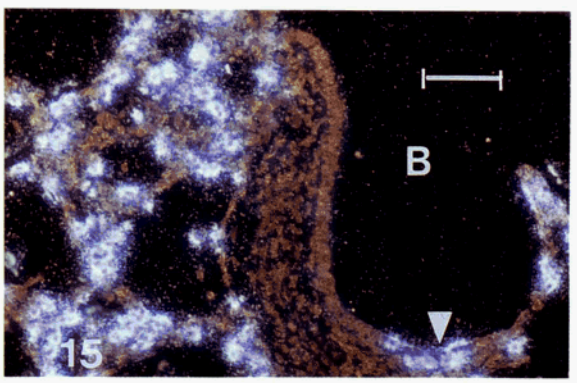

10

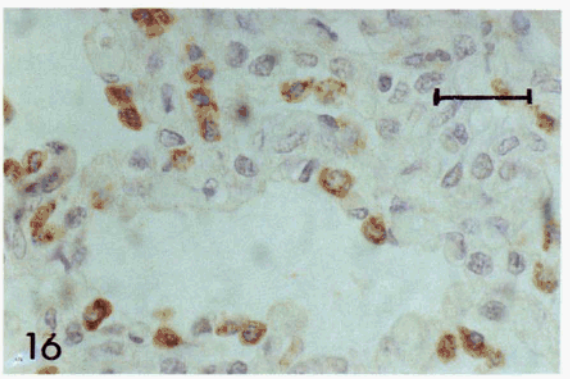

B
11


12


13


14


B
15


16

and neonates). Beginning at 16 weeks' gestation, and with one exception thereafter, in both fetuses and neonates studied some of the non-ciliated cells of bronchioles contained SP-B mRNA (Figure 3). Peripheral staining of the lining cells of ciliated bronchioles from fetuses was noted in early gestation and more generalized cytoplasmic staining for pro-SP-B in non-ciliated, non-mucous cells was noted in a pattern similar to that of SP-B mRNA. In the latter half of gestation and in neonates, pro-SP-B-immunostained cells in bronchi and bronchioles were less numerous in the more proximal airway epithelium. The widespread distribution of pro-SP-B in the developing conducting airways was distinct from that described previously for the active SP-B peptide, which was rarely seen in the conducting airways of the developing fetal lung (32).

SP-B Expression in Distal Airways

Terminal airways lined by cuboidal epithelial cells contained SP-B mRNA after 15 weeks' gestation (Figure 4). SP-B mRNA was detected readily in the cuboidal cell-lined bronchiolo–alveolar portals and in mature Type II cells, which are well developed at approximately 25 weeks' gestation (Figure 5). At all gestational ages before 25 weeks, the cells lining terminal airways immunostained for pro-SP-B; staining of the cell periphery was more common in the younger fetuses (Figures 6 and 7). Mature Type II cells and bronchiolo–alveolar portal cells in the mature infants were also immunolabeled by SP-B precursor antisera (Table 2) (Figure 8). When macrophages were present in live-born infants, they were usually immunostained for fully processed SP-B but never contained SP-B mRNA or pro-SP-B staining. Immunostaining for the active SP-B peptide correlated more closely with pro-SP-B and SP-B mRNA in the distal airway epithelium (32).

In the adult human lungs available for study, SP-B mRNA and pro-SP-B immunostaining were detected in non-ciliated non-mucous cells of ciliated bronchioles and in Type II cells in the alveoli. SP-B mRNA and pro-SP-B staining were rarely detected in the bronchial epithelium. Rows of Type II epithelial cells lining the edges of alveoli that abutted pleura, peribronchiolar, or peribronchial connective tissue, or adventitia around larger blood vessels, were characteristic localizations for SP-B mRNA and pro-SP-B protein in adult lung.

SP-C Expression in Proximal Airways

Analysis of SP-C mRNA and SP-C precursor in lungs and tracheas in the same population of fetuses and newborn infants as presented in Tables 1 and 2, is presented in Tables 3 and 4. Immunostaining for SP-C precursor was detected in the primitive tracheal epithelium of a 12-week fetus and at 16, 19, and 20 weeks, localized primarily to folds where tracheal glands arose (Figure 9). Necks of glands and rare non-mucous cells of differentiated glands stained with the SP-C precursor antiserum in five fetuses at 19–23 weeks' gestation. Neither tracheal epithelium nor tracheal glands contained pro-SP-C thereafter. SP-C mRNA was not detected in fetal or neonatal tracheas nor in tracheal glands, nor was SP-C mRNA detected in bronchi or bronchial glands. In contrast, the periphery of epithelial lining cells in bronchi was positive by immunohistochemistry for SP-C precursor in five fetuses at 12, 14, 15, 20, and 22 weeks. At 23 weeks a few bronchial cells were immunostained for the SP-C precursor. In these instances the rare positive cells were present in the folds near or at the origins of necks of submucosal glands. Rare non-mucous cells of bronchial glands immunostained for the SP-C precursor in five cases at 20, 22, 23, 38, and 42 weeks.

SP-C Expression in Distal Airways

SP-C mRNA was detected in cells lining terminal airways of fetuses and newborns beginning at 15 weeks and thereafter (Figures 10 and 11). Before 20 weeks' gestation, weak immunostaining for SP-C precursor was localized primarily at the basilar and apical cell periphery (designated as "P" in Table 3) (Figure 12). After 20 weeks'

Figure 9. Trachea from a fetus of 16 weeks' gestation immunostained for SP-C precursor. Scattered epithelial cells are immunolabeled. Immunoperoxidase and hematoxylin. Original magnification × 175. Bar = 50 μm.

Figure 10. Lung of a 15-week fetus hybridized in situ to an anti-sense SP-C probe and photographed with darkfield illumination. Terminal airways are the first to show SP-C mRNA. Original magnification × 110. Bar = 100 μm.

Figure 11. SP-C mRNA is seen in terminal airway epithelium but no SP-C mRNA is present in bronchiolar epithelium. Lung from the same fetus of 22 weeks' gestation as seen in Figure 3, hybridized in situ to an anti-sense SP-C probe and photographed with darkfield illumination. B, bronchiole. Original magnification × 150. Bar = 100 μm.

Figure 12. Terminal airway from the lung of a fetus of 15 weeks' gestation immunostained for SP-C precursor, showing light staining of cell periphery which is more intense at the luminal edge. Immunoperoxidase and hematoxylin. Original magnification × 450. Bar = 25 μm.

Figure 13. Lung of a fetus of 20 weeks' gestation immunolabeled for SP-C precursor in lining epithelial cells of terminal airways. Immunoperoxidase and hematoxylin. Original magnification × 175. Bar = 50 μm.

Figure 14. Lung of a fetus of 23 weeks' gestation immunostained for SP-C precursor. Only epithelial cells of the most distal airways are immunolabeled. Epithelial cells lining larger airways are unstained. Immunoperoxidase and hematoxylin. Original magnification × 100. Bar = 100 μm.

Figure 15. Near serial section of the same infant's lung as shown in Figure 5 hybridized in situ to an anti-sense SP-C probe and photographed with darkfield illumination. SP-C mRNA is localized in alveolar Type II cells and in cells of the bronchiolo–alveolar portal (arrowhead). B, bronchiole. Original magnification × 90. Bar = 100 μm.

Figure 16. Lung of a term gestation live-born infant immunostained for SP-C precursor. Type II cells in terminal airways are immunolabeled. Immunoperoxidase and hematoxylin. Original magnification × 450. Bar = 25 μm.

Table 3. *SP-C mRNA and immunoreactive precursor in fetuses*[a]

Gestational age (wk)	Postnatal age	Trachea				Bronchi				Ciliated bronchioles		Cells lining terminal airways	
		Epithelium		Glands		Epithelium		Glands					
		IS	H	IS	H	IS	H	IS	H	IS	H	IS	H
10		–	–	0	–	–	–	0	–	–	P	–	P
11		na	na	na	na	na	0	na	0	na	–	na	–
12		na	na	na	na	na	P	na	0	na	P	na	P
12		–	P	0	0	–	–	0	0	–	P	–	P
13		–	–	–	–	–	0	–	0	–	–	–	–
14		na	na	na	na	0	0	0	0	–	P	–	–
14		na	na	na	na	na	P	na	–	na	P	na	P
14		na	na	na	na	na	0	na	0	na	–	na	–
15		na	–	na	–	na	P	na	0	na	P	na	P
15		na	na	na	na	–	0	0	0	–	–	+	P
16		na	+	na	–	–	–	–	–	–	P	+	P
16		–	–	–	–	–	–	–	0	–	–	+	–
16		–	–	–	–	0	0	0	0	–	–	+	–
17		na	–	na	–	–	–	0	–	–	–	+	–
17		na	na	na	na	0	–	0	–	–	–	+	P
18		–	–	–	–	–	–	–	–	–	–	+	P
18		–	–	–	–	–	–	–	–	–	–	+	P
18		na	na	na	na	na	0	na	0	na	P	na	P
19	SB	–	+	–	+	0	0	0	0	–	P	+	P
19	0.7 hr	–	–	–	–	0	–	0	0	–	–	+	–
20	1 hr	na	–	na	+	na	–	na	+	na	–	na	+
20	SB	–	+	–	+	0	–	0	0	–	–	+	+
20	SB	na	na	na	na	0	0	0	0	–	+	+	+
20	1 hr	na	na	na	na	na	0	na	0	na	–	na	–
20	SB	–	–	–	–	–	0	–	0	–	–	+	P
20	SB	na	na	na	na	na	–	na	–	na	–	na	+
20	SB	na	na	na	na	na	P	na	0	na	P	na	+
21	SB	na	na	na	na	–	–	–	–	–	–	+	P
21	SB	na	na	na	na	na	0	na	0	na	–	na	–
22	1.5 hr	–	–	–	–	0	–	0	–	–	–	+	+
22	0.5 hr	na	na	na	na	–	P	–	–	–	P	+	+
22	SB	na	–	na	+	na	–	na	+	na	–	na	+
22	1 hr	na	na	na	na	0	0	0	0	–	–	+	+
23	SB	–	–	–	+	–	+	–	+	–	–	+	+
23	SB	na	na	na	na	0	0	0	0	–	–	+	+
23	2 hr	na	na	na	na	–	–	–	–	–	–	+	+
23	2 hr	na	na	na	na	–	–	–	–	–	–	+	–
23	2.7 hr	na	na	na	na	–	–	0	0	–	–	+	+

[a] +, positively stained cells; –, no stained cells identified; 0, structure not present in section; na, not available; IS, in situ; H, immunohistochemistry; P, peripheral staining; SB, stillborn.

gestation, immunoreactive SP-C precursor was noted within epithelial cells; the intensity of staining increasing with advancing gestational age (Figures 13 and 14). In live-born infants from 25 weeks onward, mature Type II cells contained SP-C mRNA and the SP-C precursor (Table 4). SP-C mRNA and immunolabeled SP-C precursor were also detected in cuboidal cells lining the bronchiolo–alveolar portals (Figures 15 and 16). When macrophages were present in airways of live-born infants, they were frequently immunostained with pro-SP-C antiserum but SP-C mRNA was not detected.

In adult lungs, SP-C mRNA and immunostaining for pro-SP-C were confined to alveolar Type II epithelial cells. As with SP-B precursor, rows of Type II cells immunostained for pro-SP-C lined the portion of alveoli that abutted pleura, peribronchiolar, or peribronchial connective tissue, or the adventitia surrounding larger blood vessels.

Ablation Studies

Ablation studies of pro-SP-B were carried out on tracheas of four fetuses at 13, 18, 19, and 20 weeks' gestation. In none of these was epithelial staining completely ablated, although it was qualitatively less intense in all four. Ablation studies of pro-SP-B were also carried out on the lungs of eight fetuses of 13, 14, 18, 19, 20, 21, 22, and 23 weeks' gestation. Staining of terminal airway cells was ab-

Table 4. *SP-C mRNA and immunoreactive precursor in neonates*[a]

Gestational age (wk)	Postnatal age	Trachea				Bronchi				Ciliated bronchioles		Respiratory bronchioles and B-A portals		Mature type II cells	
		Epithelium		Glands		Epithelium		Glands							
		IS	H	IS	H	IS	H	IS	H	IS	H	IS	H	IS	H
25	15 min	na	na	na	na	na	0	na	0	na	–	na	+	na	+
36	1.5 hr	na	na	na	na	–	–	–	–	–	–	+	+	+	+
37	1 day	na	na	na	na	na	–	na	–	na	–	na	–	na	+
38	24 hr	na	na	na	na	na	–	na	0	na	–	na	–	na	+
38	90 hr	na	na	na	na	na	+	na	+	na	–	na	+	na	+
40	10 hr	na	na	na	na	–	0	0	0	–	–	+	+	+	+
40	10 days	–	–	–	–	–	–	–	–	–	–	+	+	+	+
42	12 days	na	na	na	na	–	+	–	+	–	–	+	+	+	+

[a] +, positively stained cells; –, no stained cells identified; na, not available; IS, in situ; 0, structure not present in section; H, immunohistochemistry.

lated in fetuses of 18, 21, and 23 weeks' gestation and was less intense in those of 13, 14, 19, 20, and 22 weeks' gestation (Figures 17 and 18). Conducting airway immunostaining was ablated in fetuses of 18, 21, 22, and 23 weeks' gestation but was only diminished in those of 14 and 19 weeks' gestation. Similar ablation studies were carried out on lung tissue from four neonates. Type II cell immunostaining was ablated in three of the four and was almost ablated in a 36-week infant. Conducting airway immunostaining was ablated in all four neonates (Figures 19 and 20).

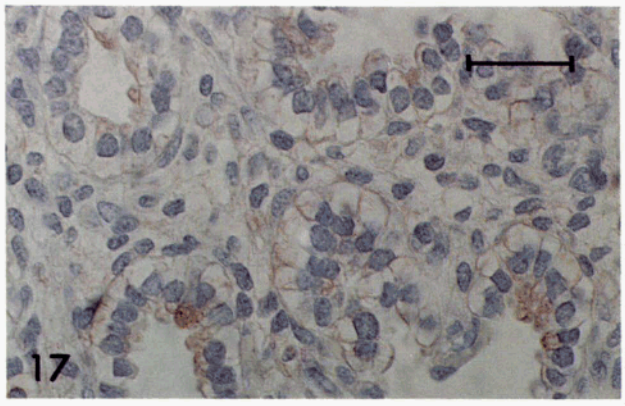
Ablation studies were carried out on two fetal tracheas at 16 and 19 weeks' gestation. Immunostaining for pro-SP-C was ablated in the 16-week trachea and, although immunostaining was diminished in the 19-week trachea, it was not completely ablated. Ablation studies were carried out on lungs of seven fetuses at 10, 12, 14, 15, 20, 21, and 23 weeks and of three live-born infants. Peripheral staining of epithelium of terminal airways was not ablated in five of the younger fetuses. In lungs of fetuses of 20, 21, and 22 weeks, peripheral staining of epithelial cells of terminal airways was diminished markedly over that of controls or ablated (Figures 21 and 22). In all three live-born infants, immunostaining of Type II cells was completely ablated (Figures 23 and 24).

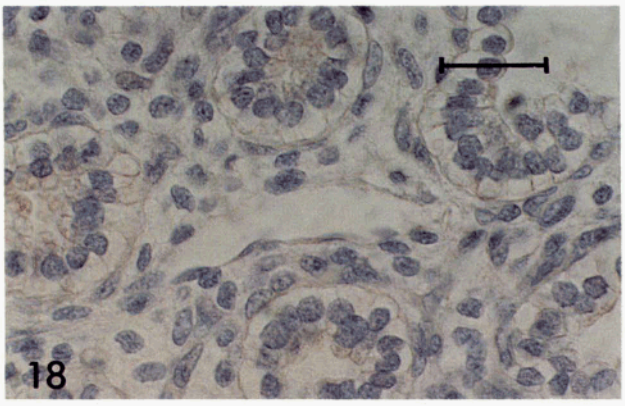
Discussion

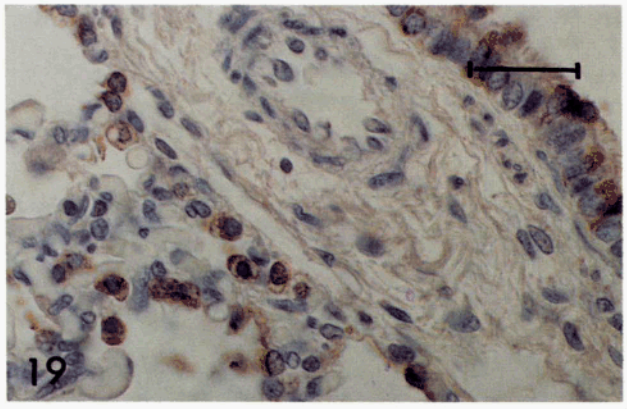
The ontogeny and distribution of SP-B and SP-C mRNAs and proteins were assessed in developing human lung. SP-B and SP-C mRNAs and pro-proteins were expressed in early fetal lung development, both being detected as early as 15 weeks' gestation, and were expressed in both conducting and terminal airway epithelial cells. Although neither mRNA was seen in tracheal epithelium or glands, immunostaining with SP-B precursor and SP-C precursor antisera was first detected in tracheal epithelium in early gestation and in rare tracheal and bronchial gland cells at mid-gestation. In the first trimester of gestation, "peripheral" immunostaining of epithelial cells was detected with SP-B and SP-C precursor antibodies in both bronchi and ciliated bronchioles. SP-B mRNA and SP-B precursor were detected in bronchial epithelial cells, occasionally in bronchial glands, and in ciliated bronchioles throughout the second and third trimesters and in neonates. Staining of bronchial and bronchiolar epithelial cells for pro-SP-C was detected less frequently than for pro-SP-B. Both SP-B and SP-C mRNAs were detected in terminal airways from 15 weeks onward and in bronchiolo-alveolar portals and mature Type II cells after 25 weeks' gestation. In terminal airways, staining for pro-SP-B and pro-SP-C was detected as early as 10–12 weeks' gestation and throughout the remainder of gestation and in neonates. The expression patterns of SP-B and SP-C are similar to but distinct from that of SP-A, the latter being more prominent in tracheal and bronchial glands, but sharing the expression in peripheral airways and in alveolar regions (21). The early and distinct patterns of expression of the surfactant proteins in fetal lung further delineate the cellular heterogeneity of the developing respiratory epithelium. The early expression of surfactant proteins, well in advance of the need for surfactant function at birth, supports the hypothesis that surfactant proteins may mark events in epithelial cell differentiation well before alveolarization and may play a role in lung homeostasis that is distinct from surfactant function.

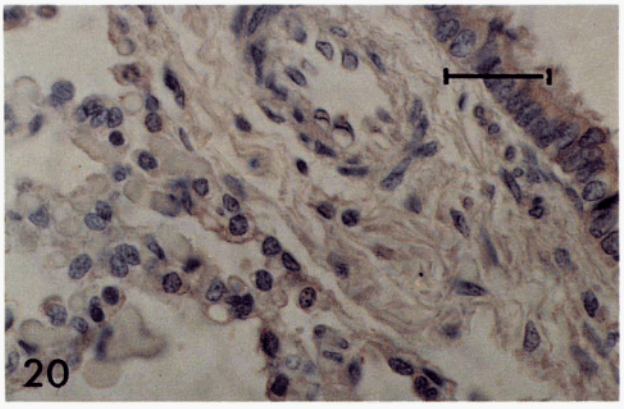
After 15 weeks' gestation, pro-SP-B and SP-B mRNA were distributed in a similar pattern in bronchial, bronchiolar, and alveolar regions of the lung. These findings are in contrast to those previously reported for immunostaining for the fully processed SP-B, in which trachea, tracheal glands, bronchi, bronchial glands, and ciliated bronchioles in normal fetuses and infants were rarely immunostained for the active SP-B peptide (32). The discrepancy between the detection of SP-B mRNA, pro-SP-B staining, and that of the SP-B peptide in the proximal airways supports the concept that the processing or presentation of the SP-B antigenic sites may be distinct in the proximal vs the distal respiratory epithelium. In the bronchiolo-alveolar portal and alveolus, immunostaining for pro-SP-B and the active SP-B peptide is similar. Because the SP-B antiserum is somewhat selective for the active peptide, it is possible that the lack of staining for the active SP-B peptide in the proximal regions of the lung is related to distinct processing of pro-SP-B and SP-B in bronchial/bronchiolar compared with alveolar regions of the lung. Alternatively, differences in intracellular storage may result in distinct reactivity of proximal airway cells with the antisera used in the present study.

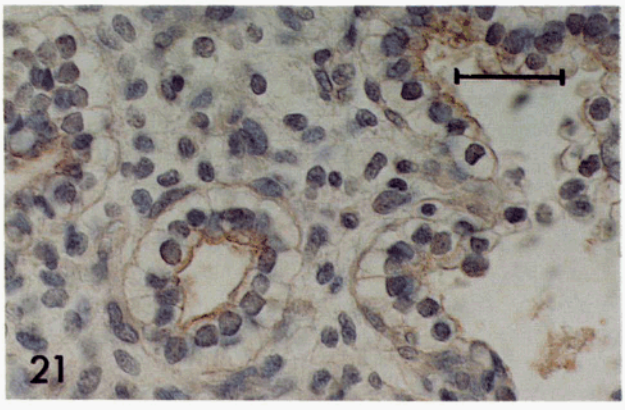
In early gestation, cell staining with pro-SP-B antiserum was distinct from that noted in tissue from mid-gestation fetal lung. At the early gestational ages, staining for pro-SP-B was noted in the

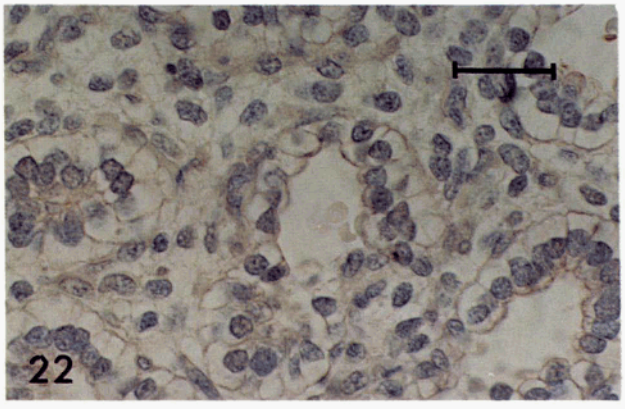

17

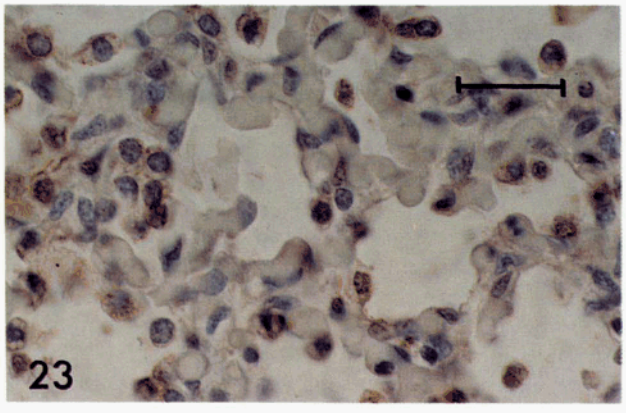

18

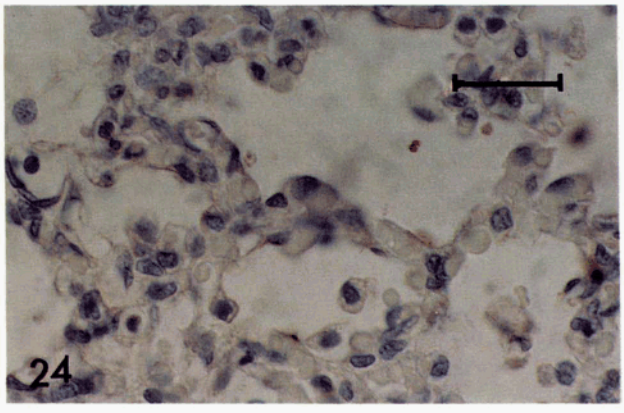

19


20


21


22


23


24

periphery of the airway cells rather than throughout the cytoplasm. Whereas pre-incubation of the antisera with pro-SP-B blocked bronchiolar and terminal airway staining in sections of the late fetal lung, ablation studies with samples in younger fetuses only partially ablated peripheral cellular immunostaining. It is therefore unclear whether the peripheral staining represents pro-SP-B or pro-SP-B antigenic epitopes that are distinct from that present in the periphery of the respiratory epithelium characteristic of early fetal lung. Electron microscopic analysis of the developing fetal lung offers a potential explanation for the distinct cellular staining pattern noted for SP-B with advancing gestation. Since large intracellular glycogen stores occupy the majority of the cytoplasmic compartment of airway epithelial cells, organelles such as endoplasmic reticulum and Golgi apparatus are relegated to the cell periphery and therefore may account for the peripheral pattern of staining noted at the earlier time points (Figure 25). These glycogen pools diminish in size and the cell organelles likely containing the pro-SP-B and SP-B are more evenly distributed in the more differentiated respiratory epithelial cells. Alternatively, the peripheral staining of SP-B detected at the earlier gestational ages may represent distinct antigenic epitopes either shared with SP-B or alternatively routed and processed forms of the SP-B precursor.

Staining for pro-SP-C in cells lining terminal airways was found in close association with the detection of SP-C mRNA by in situ hybridization, both occurring many weeks before the diffuse intracellular pro-SP-C staining was established in the more distal region of the respiratory epithelium. Pro-SP-C is palmitoylated and is associated closely with subcellular membranes during its biosynthesis (40,43). Pro-SP-C enters the endoplasmic reticulum as a transmembrane protein (38) that is probably anchored in the membrane by the hydrophobic region of the active SP-C peptide. Therefore, the detection of immunostaining for SP-C precursor in the peripheral region of cells at early gestational ages may reflect developmental differences in pro-SP-C trafficking or processing as described for pro-SP-B. Recombinant pro-SP-C ablated pro-SP-C antibody staining in the bronchiolo–alveolar portals and terminal airway cells in the more mature fetuses and in Type II cells of neonates but failed to ablate consistently the peripheral cell staining observed at the earlier gestational ages. It is therefore possible that this peripheral staining is related to pro-SP-C antigenic epitopes or cross-

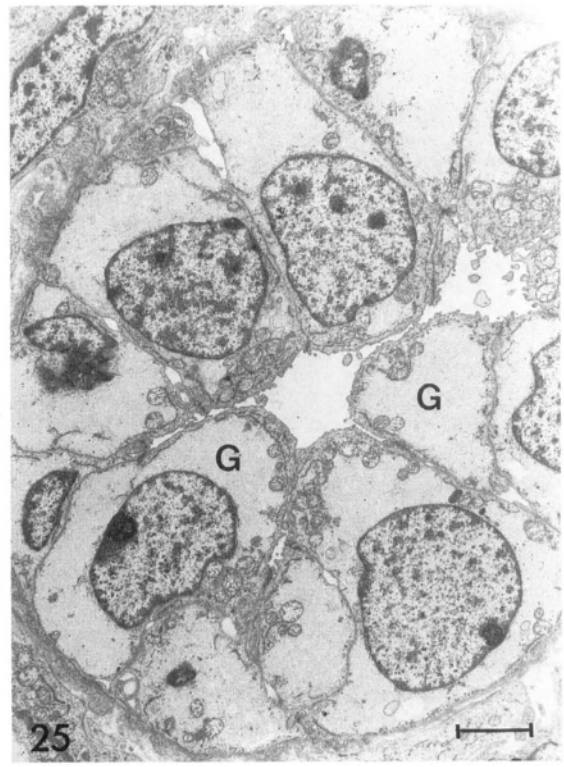


Figure 25. Electron micrograph of a terminal airway from the lung of a fetus of 18 weeks' gestation. Large pools of glycogen surround the nucleus. The organelle-containing cytoplasm is restricted to the perimeter of the cells. G, glycogen. Uranyl acetate and lead citrate. Original magnification × 4000. Bar = 2.5 μm.

Figure 17. Lung of a fetus of 21 weeks' gestation immunostained for SP-B precursor in the presence of a nitrocellulose strip. Many of the epithelial cells of the terminal airways are immunolabeled at the cell periphery; a few have more diffuse cytoplasmic staining. Immunoperoxidase and hematoxylin. Original magnification × 450. Bar = 25 μm.

Figure 18. Lung of the same fetus as that shown in Figure 17. Before use, the antibody to SP-B precursor was incubated with SP-B precursor adsorbed onto a nitrocellulose strip. The immunostaining seen in Figure 17 is almost ablated. Immunoperoxidase and hematoxylin. Original magnification × 450. Bar = 25 μm.

Figure 19. Lung of a term gestation live-born infant who survived for 10 days, immunostained for SP-B precursor. Alveolar Type II cells are well stained, as are scattered non-ciliated columnar epithelial cells in a bronchiole. Immunoperoxidase and hematoxylin. Original magnification × 450. Bar = 25 μm.

Figure 20. Lung of the same infant as that shown in Figure 19; same field from the adjacent section. Before use, the antibody to the SP-B precursor was incubated with SP-B precursor adsorbed onto a nitrocellulose strip. Immunolabeling of both Type II cells and non-ciliated columnar epithelial cells of the bronchiole has been ablated. Immunoperoxidase and hematoxylin. Original magnification × 450. Bar = 25 μm.

Figure 21. Lung of the same 21-week fetus as that seen in Figure 17, immunostained for SP-C precursor in the presence of a nitrocellulose strip. The lining cells of the terminal airways are immunolabeled at the cell periphery. Immunoperoxidase and hematoxylin. Original magnification × 450. Bar = 25 μm.

Figure 22. Lung of the same infant as that seen in Figure 21. Before use, the antibody to SP-C precursor was incubated with SP-C precursor adsorbed onto a nitrocellulose strip. Immunolabeling seen in Figure 21 is ablated. Immunoperoxidase and hematoxylin. Original magnification × 450. Bar = 25 μm.

Figure 23. Lung of the same live-born infant seen in Figure 19, immunostained for the SP-C precursor. The many Type II cells in alveolar walls are well stained. Immunoperoxidase and hematoxylin. Original magnification × 450. Bar = 25 μm.

Figure 24. Lung of the same infant seen in Figure 23. Before use, the antibody to SP-C precursor was incubated with SP-C precursor adsorbed onto a nitrocellulose strip. Immunolabeling of the Type II cells is ablated. Immunoperoxidase and hematoxylin. Original magnification × 450. Bar = 25 μm.

reacting epitopes that are distinct from those detected later in gestation. It is also unclear at present whether the peripheral staining is related to changes in routing, processing, or to potential differences in pro-SP-C or its intermediates that might occur with development.

SP-C mRNA and SP-B mRNA were detected in cells lining terminal airways at 15 and 16 weeks' gestation, respectively. This is in agreement with previous Northern blot analysis data showing that both SP-C mRNA and SP-B mRNA are present in human lung between 13 and 16 weeks' gestation (34,44). On the other hand, SP-A mRNA and lamellar bodies were not detected in immature Type II cells until 19 week's gestation in the same material as that used in the present study (21). In the rat, SP-C mRNA, SP-B mRNA, and lamellar bodies are readily detected on gestational days 17, 18, and 19, respectively (term = 22 days) (45). SP-C mRNA appears in the primordial lung bud as early as 11–12 days of gestation in the mouse (28). In the rabbit, SP-C mRNA has been detected as early as 19 days of gestation, whereas lamellar bodies have been observed at 26 days of gestation (term = 31 days) (29). Taken together, these data suggest that synthesis of SP-C mRNA and SP-B mRNA occurs well before differentiation of the Type II epithelial cell and the presence of lamellar bodies.

After their appearance early in the second trimester, SP-B mRNA and SP-C mRNA appeared to be present in all cells lining the developing pulmonary acinus. Similar SP-C mRNA distribution has been observed in fetal rabbit (29) and mouse lung (28). Cells of the developing pulmonary acinus have also been shown to react uniformly with a surfactant protein antibody (SALS–HuE) and share similar ultrastructural features (46).

After 25 weeks' gestation, both SP-B mRNA and SP-C mRNA were detected in cuboidal cells of the bronchiolo–alveolar portal. This observation is supported by the pattern of expression of chimeric genes containing the 5′ region of the human SP-C gene ligated to the bacterial chloramphenicol acetyltransferase gene, which is expressed in cuboidal cells of the most distal bronchioles in transgeneic mice (47). Cuboidal cells of the human respiratory bronchiole also contain other Type II cell markers, such as immunoreactive SP-A (21), immunoreactive SP-B (32), and immunoreactive SP-C precursor (48), but lack Clara cell-specific 10 KD protein immunoreactivity (49). These cuboidal cells lining the distal respiratory bronchiole and fetal pre-Type II cells share electron microscopic characteristics in humans (50) and in mice (51).

The structural relationships of the respiratory bronchiole and the developing acinus in human fetuses and neonates have been carefully described by Ten Have–Opbroek et al. (50). The distal portion of the respiratory bronchiole is lined with cuboidal cells whose terminal portion lines the bronchiolo–alveolar portal (50,52). This anatomic relationship is not present until late in fetal life. Cuboidal cells lining bronchiolo-alveolar portals are thought to be precursors of cells lining terminal airways. The terminal airways in the fetal lung are lined by cuboidal cells that may differentiate into mature Type II cells or into Type I cells as capillary invasion proceeds, producing the close association of capillaries and Type I cells at the alveolar–capillary interface.

The early appearance of both the mRNAs for SP-B and SP-C and their proteins before any possibility of extrauterine survival raises questions as to their intrauterine roles. The timing of their appearance also occurs before that of SP-A mRNA or its protein, or of lamellar bodies. In contrast to the dual role of SP-A in nonimmune host defense and in surfactant enhancement, SP-B and SP-C have been implicated only in the enhancement of surfactant biophysical properties. Further studies may indicate additional roles for these two small hydrophobic proteins.

Acknowledgments

We wish to thank Ms Sandra Olson for technical assistance, Mr Brent Weedman for photographic assistance, and Ms Shirley Hall for preparation of the manuscript.

Literature Cited

1. Hawgood S, Shiffer K. Structures and properties of the surfactant-associated proteins. Annu Rev Physiol 1991;53:375
2. Weaver TE, Whitsett JA. Function and regulation of expression of pulmonary surfactant-associated proteins. Biochem J 1991;273:249
3. Revak SD, Merritt TA, Degryse E, Stefani L, Courtney M, Hallman M, Cochrane CG. Use of human surfactant low molecular weight apoproteins in the reconstitution of surfactant biologic activity. J Clin Invest 1988;81:826
4. Grossmann G, Nilsson R, Robertson B. Scanning electron microscopy of epithelial lesions induced by artificial ventilation of the immature neonatal lung; the prophylactic effect of surfactant replacement. Eur J Pediatr 1986;145:361
5. Curstedt T, Jornvall H, Robertson B, Bergman T, Berggren P. Two hydrophobic low-molecular-mass protein fractions of pulmonary surfactant. Characterization and biophysical activity. Eur J Biochem 1987; 168:255
6. Sarin VK, Gupta S, Leung TK, Taylor VE, Ohning BL, Whitsett JA, Fox JL. Biophysical and biological activity of a synthetic 8.7-kDa hydrophobic pulmonary surfactant protein SP-B. Proc Natl Acad Sci USA 1990;87:2633
7. Collaborative European Multicenter Study Group. Surfactant replacement therapy for severe neonatal respiratory distress syndrome: an international randomized clinical trial. Pediatrics 1988;82:683
8. Hoekstra RE, Jackson JC, Myers TF, Frantz ID III, Stern ME, Powers WF, Maurer M, Raye JR, Carrier ST, Gunkel JH, Gold AJ. Improved neonatal survival following multiple doses of bovine surfactant in very premature neonates at risk for respiratory distress syndrome. Pediatrics 1991;88:10
9. Liechty EA, Donovan E, Purohit D, Gilhooly J, Feldman B, Noguchi A, Denson SE, Sehgal SS, Gross I, Stevens D, Ikegami M, Zachman RD, Carrier ST, Gunkel JH, Gold AJ. Reduction of neonatal mortality after multiple doses of bovine surfactant in low birth weight neonates with respiratory distress syndrome. Pediatrics 1991;88:19
10. Suzuki Y, Fujita Y, Kogishi K. Reconstitution of tubular myelin from synthetic lipids and proteins associated with pig pulmonary surfactant. Am Rev Respir Dis 1989;140:75
11. Wright JR, Wager RE, Hawgood S, Dobbs L, Clements JA. Surfactant apoprotein M_r = 26,000–36,000 enhances uptake of liposomes by Type II cells. J Biol Chem 1987;262:2888
12. Rice WR, Sarin VK, Fox JL, Baatz J, Wert S, Whitsett JA. Surfactant peptides stimulate uptake of phosphatidylcholine by isolated cells. Biochim Biophys Acta 1989;1006:237
13. Dobbs LG, Wright JR, Hawgood S, Gonzalez R, Venstrom K, Nellenbogen J. Pulmonary surfactant and its components inhibit secretion of phosphatidylcholine from cultured rat alveolar type II cells. Proc Natl Acad Sci USA 1987;84:1010
14. Rice WR, Ross GF, Singleton FM, Dingle S, Whitsett JA. Surfactant-associated protein inhibits phospholipid secretion from type II cells. J Appl Physiol 1987;63:692
15. Kuroki Y, Mason RJ, Voelker DR. Pulmonary surfactant apoprotein

A structure and modulation of surfactant secretion by rat alveolar type II cells. J Biol Chem 1988;263:3388

16. Kuroki Y, Mason RJ, Voelker DR. Chemical modification of surfactant protein A alters high affinity binding to rat alveolar type II cells and regulation of phospholipid secretion. J Biol Chem 1988;263:17596
17. Tenner AJ, Robinson SL, Borchelt J, Wright JR. Human pulmonary surfactant protein (SP-A), a protein structurally homologous to C1q, can enhance FcR- and CR1-mediated phagocytosis. J Biol Chem 1989;264:13923
18. van Iwaarden F, Welmers B, Verhoef J, Haagsman HP, van Golde LMG. Pulmonary surfactant protein A enhances the hot-defense mechanism of rat alveolar macrophages. Am J Respir Cell Mol Biol 1990;2:91
19. van Iwaarden JF, van Strijp JAG, Ebskamp MJM, Welmers AC, Verhoef J, van Golde LMG. Surfactant protein A is opsonin phagocytosis of Herpes simplex virus type 1 by rat alveolar macrophages. Am J Physiol 1991;261:L204
20. Endo H, Oka T. An immunohistochemical study of bronchial cells producing surfactant protein A in the developing human fetal lung. Early Hum Dev 1991;25:149
21. Khoor A, Gray ME, Hull WM, Whitsett JA, Stahlman MT. Developmental expression of SP-A and SP-A mRNA in the proximal and distal respiratory epithelium in the human fetus and newborn. J Histochem Cytochem 1993;41:1311
22. Phelps DS, Floros J. Localization of surfactant protein synthesis in human lung by in situ hybridization. Am Rev Respir Dis 1988;137:939
23. D'Amore-Bruno MA, Wikenheiser KA, Carter JE, Clark JC, Whitsett JA. Sequence, ontogeny, and cellular localization of murine surfactant protein B mRNA. Am J Physiol 1991;262:L40
24. Wikenheiser KA, Wert SE, Wispe JR, Stahlman M, D'Amore-Bruno M, Singh G, Katyal SL, Whitsett JA. Distinct effects of oxygen on surfactant protein B expression in bronchiolar and alveolar epithelium. Am J Physiol 1992;262:L32
25. Phelps DS, Floros J. Localization of pulmonary surfactant proteins using immunohistochemistry and tissue in situ hybridization. Exp Lung Res 1991;17:985
26. Kalina M, Mason RJ, Shannon JM. Surfactant protein C is expressed in alveolar Type II cells but not in Clara cells of rat lung. Am J Respir Cell Mol Biol 1992;6:594
27. Wohlford-Lenane CL, Snyder JM. Localization of surfactant-associated proteins SP-A and SP-B mRNA in fetal rabbit lung tissue by in situ hybridization. Am J Respir Cell Mol Biol 1992;7:335
28. Wert SE, Glasser SW, Korfhagen TR, Whitsett JA. Transcriptional elements from the human SP-C gene direct expression in the primordial respiratory epithelium of transgenic mice. Dev Biol 1993;156:426
29. Wohlford-Lenane CL, Durham PL, Snyder JM. Localization of surfactant-associated protein C (SP-C) mRNA in fetal rabbit lung tissue by in situ hybridization. Am J Respir Cell Mol Biol 1992;6:225
30. Phelps DS, Harding HP. Immunohistochemical localization of a low molecular weight surfactant-associated protein in human lung. J Histochem Cytochem 1987;35:1339
31. Weaver TE, Sarin VK, Sawtell N, Hull WM, Whitsett JA. Identification of surfactant proteolipid SP-B in human surfactant and fetal lung. J Appl Physiol 1988;65:982
32. Stahlman MT, Gray ME, Whitsett JA. The ontogeny and distribution of surfactant protein B in human fetuses and newborns. J Histochem Cytochem 1992;40:1471
33. Floros J, Phelps DS, deMello dE, Longmate J, Harding H, Benson B, White T. The utility of postmortem lung for RNA studies; variability and correlation of the expression of surfactant proteins in human lung. Exp Lung Res 1991;17:91
34. Whitsett JA, Weaver TE, Clark JC, Sawtell N, Glasser SW, Korfhagen TR, Hull WM. Glucocorticoid enhances surfactant proteolipid Phe and pVal synthesis and RNA in fetal lung. J Biol Chem 1987;262:15618
35. Cox KH, DeLeon DV, Angerer LM, Angerer RC. Detection of mRNAs in sea urchin embryos by in situ hybridization using asymmetric RNA probes. Dev Biol 1984;101:485
36. Pelton RW, Johnson MD, Perkett EA, Gold LI, Moses HL. Expression of transforming growth factor-β_1, -β_2, and -β_3 mRNA and protein in the murine lung. Am J Respir Cell Mol Biol 1991;5:522
37. O'Reilly MA, Gazdar AF, Clark JC, Pilot-Matias TJ, Wert SE, Hull WM, Whitsett JA. Glucocorticoids regulate surfactant protein synthesis in a pulmonary adenocarcinoma cell line. Am J Physiol 1989;257:L385
38. Vorbroker DK, Dey C, Weave TE, Whitsett JA. Surfactant protein C precursor is palmitoylated and associates with subcellular membranes. Biochim Biophys Acta 1992;1105:161
39. Wikenheiser KA, Vorbroker DK, Rice WR, Clark JC, Bachurski CJ, Oie HK, Whitsett JA. Production of immortalized distal respiratory epithelial cell lines from surfactant protein C/simian virus 40 large tumor antigen transgenic mice. Proc Natl Acad Sci USA 1993;90:11029
40. Voorhout WF, Veenendaal T, Haagsman HP, Weaver TE, Whitsett JA, Van Golde LMG, Geuze HJ. Intracellular processing of pulmonary surfactant protein B in an endosomal/lysosomal compartment. Am J Physiol 1992;263:L479
41. Nogee LM, deMello DE, Dehner LP, Colten HR. Brief report: deficiency of pulmonary surfactant protein B in congenital alveolar proteinosis. N Engl J Med 1993;328:406
42. Sternberger LA. The unlabeled antibody peroxidase-antiperoxidase (PAP) method. In Sternberger LA, ed. Immunocytochemistry. 2nd ed. New York: Wiley, 1979:104
43. Curstedt T, Johansson J, Persson P, Eklund A, Robertson B, Lowenadler B, Jornvall H. Hydrophic surfactant-associated polypeptides: SP-C is a lipopeptide with two palmitoylated cysteine residues, whereas SP-B lacks covalently linked fatty acyl groups. Proc Natl Acad Sci USA 1990;87:2985
44. Liley HG, White RT, Warr RG, Benson BJ, Hawgood S, Ballard PL. Regulation of messenger RNAs for the hydrophobic surfactant proteins in human lung. J Clin Invest 1989;83:1191
45. Schellhase DE, Emrie PA, Fisher JH, Shannon JM. Ontogency of surfactant apoproteins in the rat. Pediatr Res 1989;26:167
46. Otto-Verberne CJM, Ten Have-Opbroek AAW, Balkema JJ, Franken C. Detection of the type II cell or its precursor before week 20 of human gestation, using antibodies against surfactant-associated proteins. Anat Embryol 1988;178:29
47. Glasser SW, Korfhagen TR, Wert SE, Bruno MD, McWilliams KM, Vorbroker DK, Whitsett JA. Genetic element from human surfactant protein SP-C gene confers bronchiolar-alveolar cell specificity in transgenic mice. Am J Physiol 1991;261:L349
48. Stahlman MT, Gray ME, Whitsett JA. The ontogeny and distribution of surfactant protein C (SP-C) in human fetuses and newborns. Pediatr Res 1992;31:323A
49. Singh G, Singh J, Katyal SL, Brown WE, Kramps JA, Paradis IL, Dauber JH, Macpherson TA, Squeglia N. Identification, cellular localization, isolation, and characterization of human Clara cell-specific 10 KD protein. J Histochem Cytochem 1988;36:73
50. Ten Have-Opbroek AAW, Otto-Verberne CJM, Dubbeldam JA, Dykman JH. The proximal border of the human respiratory unit, as shown by scanning and transmission electron microscopy and light microscopical cytochemistry. Anat Rec 1991;229:339
51. Ten Have-Opbroek AAW. The structural composition of the pulmonary acinus in the mouse: a scanning electron microscopical and developmental-biological analysis. Anat Embryol 1986;174:49
52. Hoyt RF, Sorokin SP, Feldman H. Small-granule (neuro)endocrine cells in the infracardiac lobe of a hamster lung. Number, subtypes, and distribution. Exp Lung Res 1982;3:273