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Expression and localization of surfactant proteins in human nasal epithelium

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Kim JK, Kim S-S, Rha KW, Kim C-H, Cho JH, Lee C-H, Lee J-G, Yoon J-H. Expression and localization of surfactant proteins in human nasal epithelium. *Am J Physiol Lung Cell Mol Physiol* 292: L879–L884, 2007. First published January 5, 2007; doi:10.1152/ajplung.00156.2006.—Surfactant proteins (SPs), designated SP-A, SP-B, SP-C, and SP-D, play an important role in surfactant metabolism and host defense mechanisms in the lung. This study investigates expression of the different SP types in human nasal mucosa and cultured normal human nasal epithelial (NHNE) cells and whether the expression of SP mRNA is influenced by the degree of mucociliary differentiation. RT-PCR was performed with mRNA from cultured NHNE cells and nasal mucosa. Immunohistochemical staining for SPs was performed on nasal mucosa specimens. Western blot analysis was performed on cell lysates from cultured NHNE cells. SP-A2, SP-B, and SP-D mRNAs were expressed in normal NHNE cells and human nasal mucosa. SPs were localized in ciliated cells of the surface epithelium and serous acini of the submucosal glands. SP-A, SP-B, and SP-D proteins were expressed in cultured NHNE cells. The degree of mucociliary differentiation influenced expression of the SP gene. We demonstrate that SP-A, SP-B, and SP-D are expressed in human nasal mucosa and cultured NHNE cells. Further study of the functional role of SPs in the upper airway is required.

surfactant proteins A, B, and D

SURFACTANT PROTEINS (SPs) are a complex mixture of lipids and proteins that play an important role in host defense mechanisms as well as surfactant metabolism. The surfactant-associated proteins are designated SP-A, SP-B, SP-C, and SP-D (9). SP-A and SP-D are sialoglycoproteins and members of the collectin family (7, 8, 9, 16). They are hydrophilic proteins and are considered to have a critical role in innate immunity (9, 16). SP-A and SP-D bind a variety of bacterial, viral, and fungal pathogens and are important in the initial phases of host defense (16). Human SP-A is encoded by two genes, SP-A1 and SP-A2 (3). In contrast, SP-B and SP-C are very hydrophobic proteins and are thought to be principally involved in surfactant function as well as the packaging and organizing of phospholipids (15). SPs were originally considered to be lung specific and to only be involved in breathing (1). The observation that a phospholipid-rich surface lining is not unique to the lung alveoli suggested that other organs might also express SPs. To date, the expression of SPs has been detected in the gastric and intestinal mucosa (1, 7), joints (1), peritoneum (1), pericardium (1, 7), skin (12), middle ear (2, 11), Eustachian tubes (13, 14), and maxillary sinus mucosa (2). In addition, surfactant-like materials have been identified in the tongue papillae and oral epithelium (1). However, the presence of SPs in human nasal mucosa has not been determined.

Recently, Goss et al. (3) reported the detection of SP-A1 and SP-A2 mRNA in adult human lung tissue and cultured human fetal lung explants. SP-A2 mRNA, but not SP-A1 mRNA, was also detected in human fetal trachea and bronchi. This same report further indicated that SP-A1 was expressed in the lung tissue including alveoli, but not in the trachea and bronchi, suggesting that the expression of certain surfactant genes could be different between the upper and lower airways. Gene expression could be influenced by the degree of mucociliary differentiation of the airway epithelium, which is greater in the upper airway.

The aims of this study were to determine the type of surfactant mRNA and proteins expressed in human nasal mucosa and cultured normal human nasal epithelial (NHNE) cells, to localize the SPs in human nasal mucosa, and to determine whether surfactant gene expression is influenced by the degree of mucociliary differentiation in human nasal epithelial cells.

MATERIALS AND METHODS

Materials. Inferior turbinate mucosa specimens were obtained from five patients who had undergone septoplasties without histories of chronic sinusitis, chronic asthma, aspirin sensitivity, or cystic fibrosis, and who had negative allergic skin-prick test results. Patients had not received an antibiotic regimen, oral steroids, or intranasal medication during the 3 mo before the study. Lung parenchymal specimens, including the terminal bronchi, were obtained from a lung cancer patient who had undergone a pneumonectomy. Informed consent was obtained from all patients, and the local Ethical Committee approved the use of all specimens. SP-A and SP-C were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). SP-B and SP-D antibodies were purchased from Abcam (Cambridge, UK).

Cell cultures. The epithelial cells from the nasal turbinate mucosa were isolated by treatment with 1% pronase (Type XIV protease; Sigma Chemical, St. Louis, MO) in a 1:1 mixture of DMEM/Ham's F-12 supplemented with penicillin G sodium (50 IU/ml) and streptomycin sulfate (50 μg/ml) for 18–20 h at 4°C. To remove fibroblasts, endothelial cells and myoepithelial cells by differential attachment to plastic, isolated cells were placed in a plastic dish and cultured for 1 h at 37°C. The nonadherent cells were mostly epithelial cells. Suspended epithelial cells were seeded at 3×10^4 cells/dish (500 cells/square cm) into 10-cm plastic tissue culture dishes. The culture medium used was bronchial epithelial growth medium (BEGM; Clonetics) containing all the supplements previously described (17). The culture medium was changed on *day 1* after seeding and every other day thereafter until the cultures reached 50–60% confluency (~6–7 days), at which time they were dissociated with 0.25% trypsin/EDTA

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(17). In brief, *passage 2* NHNE cells (2×10^5 cells/culture) were seeded in 0.5 ml of culture medium on Transwell clear culture inserts (24.5 mm, 0.45-μm pore size; Costar, Cambridge, MA). Cells were cultured in a 1:1 mixture of BEGM and DMEM containing all the supplements previously described (17). Cultures were grown while submerged for the first 9 days, during which time the culture medium was changed on *day 1* and every other day thereafter. The air-liquid interface was created on *day 9* by removing the apical medium and feeding the cultures only from the basal compartment. Culture medium was changed daily after interface creation. The cells were exposed to all-*trans* retinoic acid (10^{-7} mol/l) from the start of the culture to 28 days after confluence. To investigate differentiation in the cultured cells, total RNA was isolated from whole cells on 2, 7, 14, and 28 days after confluence using TRIzol according to the manufacturer's recommendations. Cell lysates were collected 2, 7, 14, and 28 days after confluence to investigate the presence of the proteins.

Immunohistochemistry. Paraffin-embedded sections (4 mm) were deparaffinized and rehydrated. After being washed and consecutive incubation with blocking medium, sections were incubated with primary antibodies overnight at 4°C (rabbit polyclonal SP-A antiserum, mouse monoclonal SP-D antiserum diluted 1:100, and mouse monoclonal SP-B antiserum diluted 1:50 in the dilution solution). The sections were then washed repeatedly in PBS. The reaction between the antigen and antibodies was detected using peroxidase-conjugated anti-rabbit secondary antibodies. Negative controls were stained with purified rabbit nonimmune IgG at the same concentration as the primary antibody.

RT-PCR. Oligonucleotide primers were designed according to published sequences and are shown in Table 1. Oligonucleotide amplimers for β_2-microglobulin (β_2M), used as a control gene for RT-PCR, were purchased from Clontech Laboratories (Palo Alto, CA) and generated a 335-bp PCR fragment. RT-PCR was performed. Three micrograms of RNA were used for the RT reaction, and then 1 μl of cDNA was used for PCR with a Perkin-Elmer Cetus DNA Thermal Cycler (Perkin-Elmer, Norwalk, CT) according to the manufacturer's recommendations. The same amount of RNA and cDNA was used for the lung, nasal turbinate, and NHNE cell samples. Comparative kinetic analysis was used to compare mRNA levels for each set of culture conditions. PCR products were separated by electrophoresis on a 2% Seakem agarose gel (FMC, Rockland, ME) containing 50 ng/ml ethidium bromide and photographed with Polaroid Type 55 film. The negatives were scanned on a Molecular Dynamics Densitometer (Sunnyvale, CA), and the signal was analyzed using ImageQuant software. To verify that the amplified products were from mRNA and not genomic DNA contamination, negative controls were performed omitting the reverse transcriptase; no PCR products were observed. Specific amplification of all target genes was confirmed by the sequencing of PCR products (dsDNA Cycle Sequencing System; GIBCO-BRL, Rockville, MD).

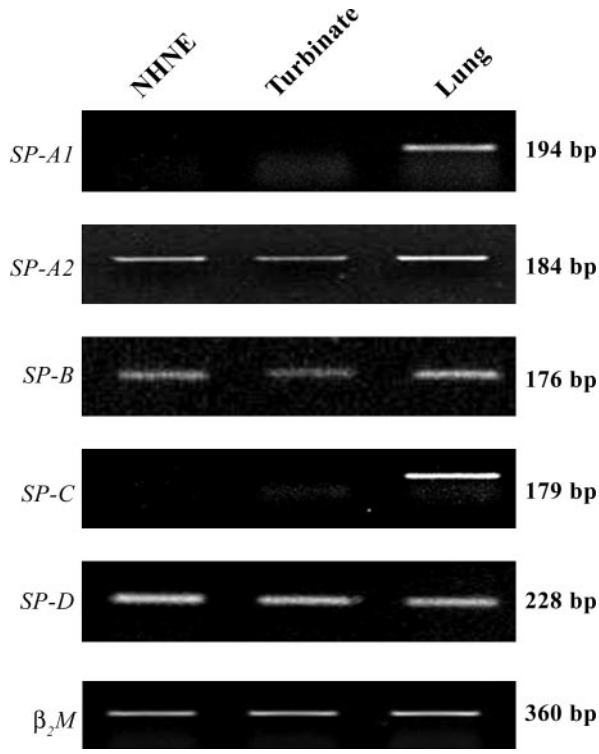

Fig. 1. Expression of surfactant protein (SP) mRNA in human nasal mucosa and cultured normal human nasal epithelial (NHNE) cells. Three micrograms of RNA and 1 μl of cDNA were used for all 3 samples. SP-A2, SP-B, and SP-D mRNA was expressed in human nasal mucosa and cultured NHNE cells. All 5 types of SP were expressed in human lung tissue used as a positive control. Shown is representative of 4 separate experiments. β_2M, β_2-microglobulin.

Table 1. *RT-PCR primers and reaction conditions*

Target Genes	Primer Sequences	Annealing Temperature, °C	Cycles	Size, bp
SP-A1	F-GTG TGG GTC GCT GAT TTC TT R-TCC AAC ACA AAC GTC CCT TCA	62	32	194
SP-A2	F-CAG TAC CGG CCA AGC ATA AT R-CAT GTC AGT CAC AGG GTT GG	62	32	184
SP-B	F-GGC CCT TGT CTG TCT AGC TC R-CTT CAA CCC CCT GTC CTG TA	62	32	176
SP-C	F-CTG GTT ACC ACT GCC ACC TT R-CTG GCC CAG CTT AGA CGT AG	55	32	179
SP-D	F-AAC CAT TTA CGG AGG CAC AG R-CTC TCC TGT GGG GTA GGT GA	55	32	228
Cornifin-α	F-CAT TCT GTC TCC CCC AAA AA R-ATG GGG GTA TAA GGG AGC TG	60	29	172
MUC5AC	F-TCC GGC TCC ATC TTC TCC R-ACT TGG GCA CTG GTG CTG	60	35	680
MUC8	F-ACA GGG TTT CTC CTC ATT G R-CGT TTA TTC CAG CAC TGT TC	55	35	239
β_2-microglobulin	F-TCG CGC TAC TCT CTC TTT CTG G R-GCT TAC ATG TCT CGA TCC CAC TTA A	55	28	360

SP, surfactant protein.

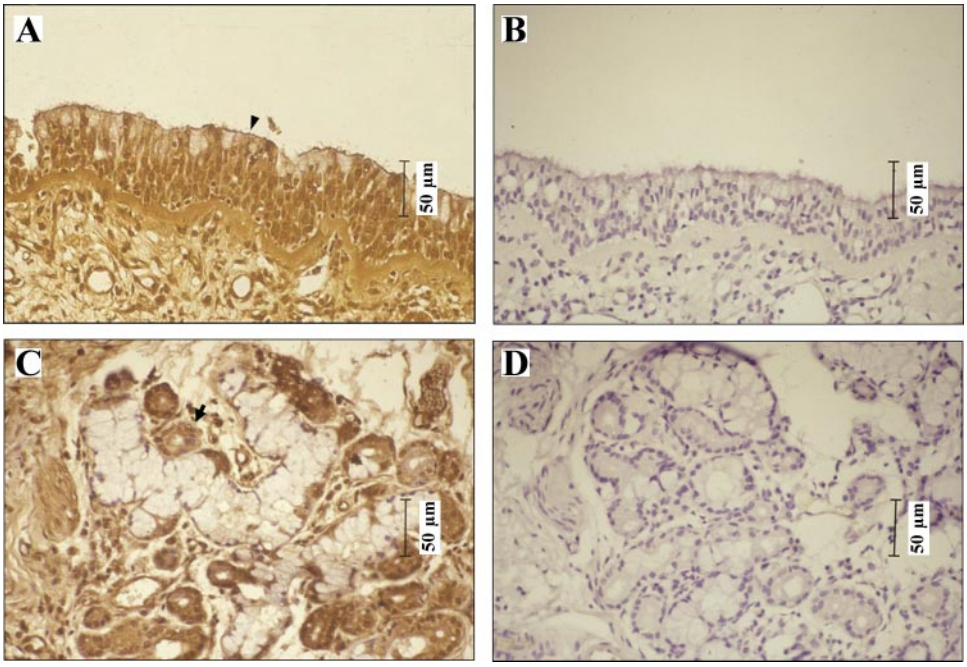

Fig. 2. Localization of SP-A in human nasal mucosa and cultured NHNE cells in immunohistochemical staining. *A* and *C*: SP-A was expressed in the cytoplasm of ciliated epithelial cells (arrowhead) and in serous acini (arrow) of the submucosal gland of human nasal mucosa. *B* and *D*: negative controls were stained with purified rabbit nonimmune IgG antibody.

Western blot analysis. Cells were lysed with 2× lysis buffer (250 mM Tris·HCl, pH 6.5, 2% SDS, 4% 2-mercaptoethanol, 0.02% bromphenol blue, 10% glycerol). Equal amounts (50 μg/25 μl) of whole cell lysates were resolved using 10% SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). Membranes were blocked with 5% skimmed milk in Tris-buffered saline (TBS; 50 mM Tris·HCl, pH 7.5, 150 mM NaCl) for 2 h at room temperature. The blot was then incubated overnight with primary antibody (rabbit polyclonal SP-A and SP-C antisera, mouse monoclonal SP-D antiserum diluted 1:1,000, and mouse monoclonal SP-B antiserum diluted 1:100 in the dilution solution) in 0.5% Tween 20 in TBS (TTBS). After being washed with TTBS, the blot was further incubated for 45 min at room temperature with anti-rabbit or anti-mouse antibody (Cell Signaling, Beverly, MA) in TTBS. Blots were visualized using the ECL system (Amersham-Pharmacia, Piscataway, NJ).

RESULTS

Expression of SP mRNA in human nasal mucosa and cultured NHNE cells. SP-A2, SP-B, and SP-D mRNAs were expressed in NHNE cells and inferior turbinate mucosa, but SP-A1 and SP-C mRNA were not. In contrast, all types of SP mRNA were expressed in the human lung tissue used as a positive control (Fig. 1). These results demonstrate that expression of SP mRNAs differs between the upper and the lower human airway mucosa. On the basis of these results, we

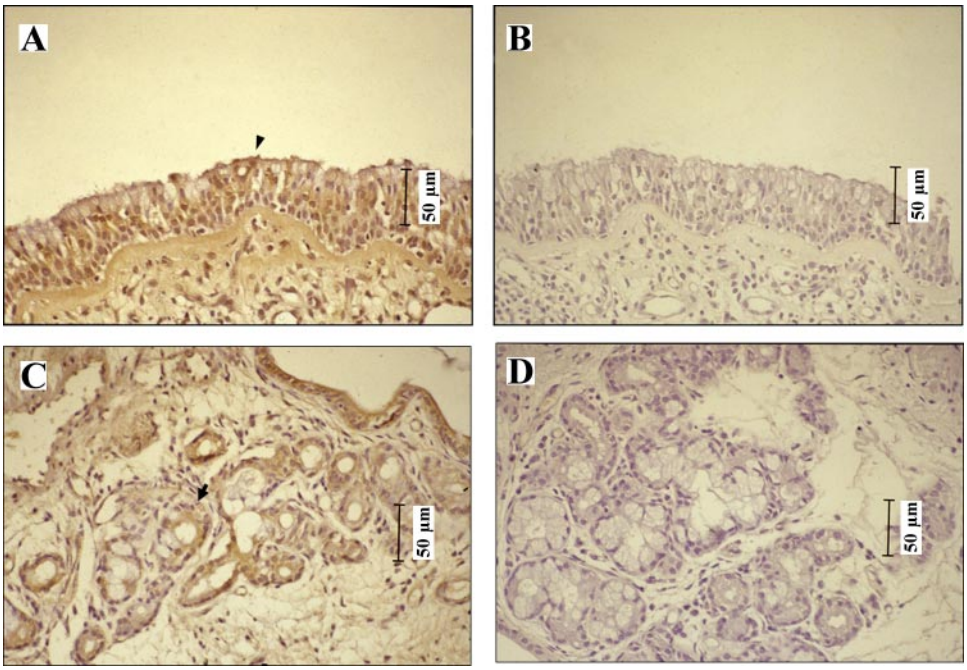

Fig. 3. Localization of SP-B in human nasal mucosa and cultured NHNE cells in immunohistochemical staining. *A* and *C*: SP-B was expressed in the cytoplasm of ciliated epithelial cells (arrow head) and in serous acini (arrow) of the submucosal gland of human nasal mucosa. *B* and *D*: negative controls were stained with purified rabbit nonimmune IgG antibody.

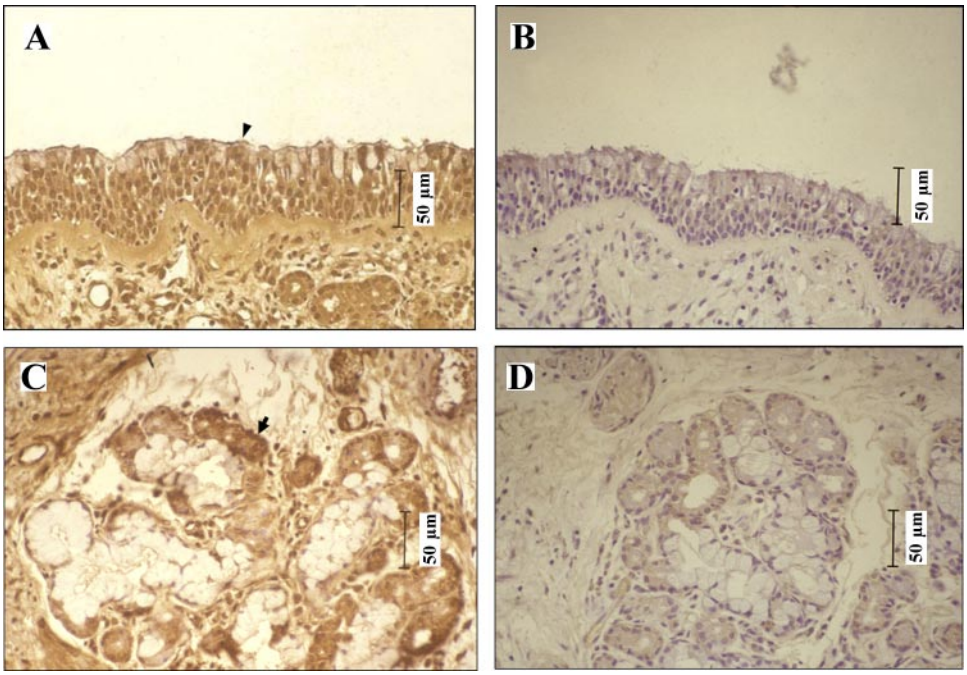

Fig. 4. Localization of SP-D in human nasal mucosa and cultured NHNE cells in immunohistochemical staining. *A* and *C*: SP-D was expressed in the cytoplasm of ciliated epithelial cells (arrow head) and in serous acini (arrow) of the submucosal gland of human nasal mucosa. *B* and *D*: negative controls were stained with purified rabbit nonimmune IgG antibody.

selected SP-A2, SP-B, and SP-D for all subsequent experiments.

Localization of SP proteins in human nasal mucosa. We investigated the localization of SP-A, SP-B, and SP-D proteins in human nasal mucosa. All three SP proteins showed the same pattern of expression; they were expressed in the ciliated cells of the surface epithelium but not the goblet cells. In the submucosal glands, the proteins were expressed in serous acini but not in mucous acini (Figs. 2, 3, 4). Some inflammatory cells were also stained. Negative controls using purified IgG showed no reactivity.

Expression of SP proteins in cultured NHNE cells. We next examined the expression of SP-A2, SP-B, and SP-D proteins in NHNE cells to confirm the RT-PCR data showing the presence of their mRNAs. Western immunoblot analysis revealed the characteristic molecular weight profiles of SP-A2 (35 kDa), SP-B (~10 kDa), and SP-D (43 kDa) proteins. SP-C protein (4–11 kDa) was identified in lung lysate but not in cultured NHNE cells. These results indicate that SP-A2, SP-B, and SP-D proteins are expressed in cultured NHNE cells (Fig. 5).

Induction of mucociliary differentiation of human nasal epithelial cells. Differentiation of mucociliary epithelium was induced by the addition of retinoic acid to the culture medium. Histological examination showed that the epithelium was monolayered on the day of confluence. Cuboidal epithelium was seen on the 7th day after confluence and columnar epithelium with some cilia on the 14th day after confluence. By the 28th day after confluence, columnar epithelium with many cilia could be observed (Fig. 6*A*). Expression of the MUC5AC gene, a marker of mucous differentiation (18), and MUC8, a ciliated cell marker (4), increased with time. In contrast, expression of cornifin-α, a marker of squamous cell differentiation (5), decreased (Fig. 6*B*). These results show that the epithelial cells differentiated into mucociliary epithelium in our in vitro cul-

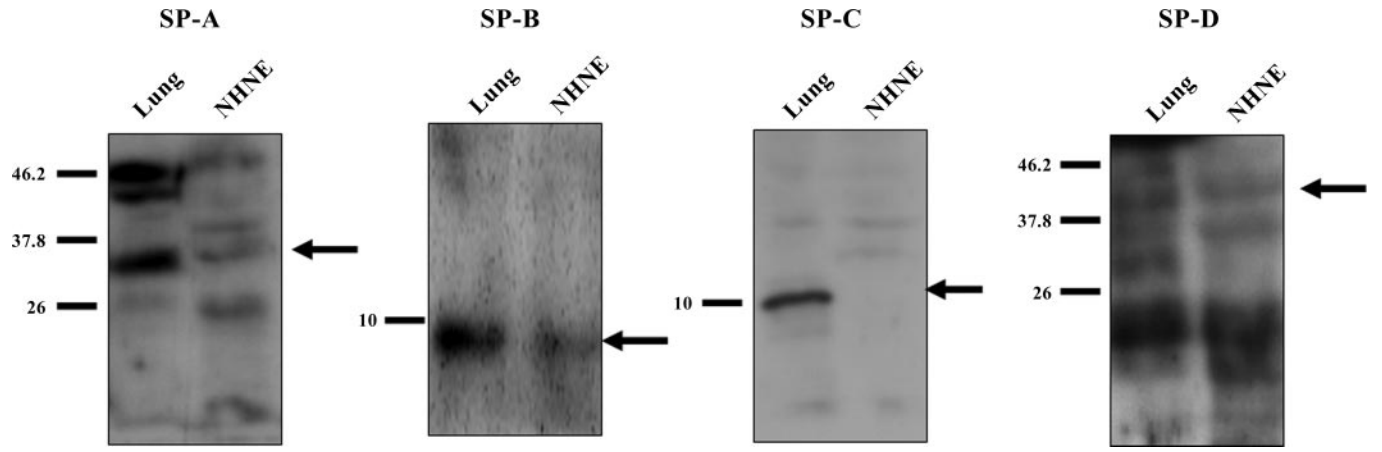

Fig. 5. Expression of SPs in cultured NHNE cells. Fifty micrograms of proteins were used for the lung sample and NHNE cells. SP-A, SP-B, and SP-D were detected in cultured NHNE cells by Western blot analysis, but SP-C was not. However, all 4 types of SP were expressed in the human lung tissue that was used as a positive control. Shown is representative of 4 separate experiments.

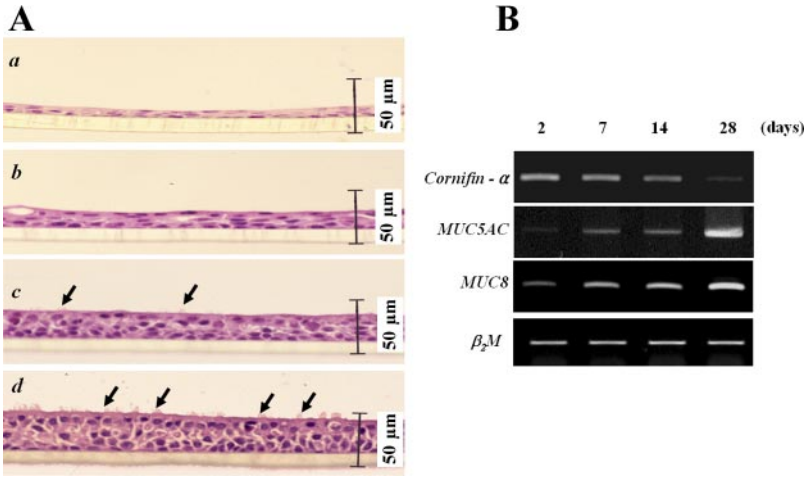

Fig. 6. Induction of mucociliary differentiation of human nasal epithelial cells. *A*: time-dependent changes in the histology of cultured NHNE cells. Cross-sections of intact culture were stained with hematoxylin and eosin. As culture duration increased, a multilayer epithelium was observed, and cilia (arrow) could be seen. *B*: there was greater expression of MUC5AC, a mucous differentiation marker, and MUC8, a ciliary differentiation marker, whereas expression of cornifin-α, a squamous epithelial marker, declined with increased culture duration. With increasing time, cultured NHNE cells were induced to differentiate into mucociliary epithelial cells. Shown is representative of 4 separate experiments.

ture system. The typical morphology of human airway epithelium is mucociliary epithelium.

SP mRNA expression as a function of mucociliary differentiation in NHNE cells. We wanted to examine whether the level of SP-A2, SP-B, and SP-D gene expression is influenced by the degree of mucociliary differentiation in human primary nasal epithelial cells. RT-PCR analysis revealed that SP-A2, SP-B, and SP-D gene expression increased as a function of mucociliary differentiation (Fig. 7, *A* and *B*). These results indicate that the degree of mucociliary differentiation influences the expression of SP-A2, SP-B, and SP-D mRNA in cultured human nasal epithelial cells. The expression of SP-A and SP-D proteins also increased during cell differentiation in a pattern similar to mRNA expression. SP-B, however, was not increased (Fig. 7*C*).

DISCUSSION

SPs are lipoprotein complexes composed of 90% phospholipids and 10% protein that are synthesized and secreted by alveolar type II epithelial cells and airway Clara cells into the thin liquid layer that lines the epithelium. Once in the extracellular space, SPs perform two distinct functions: they reduce surface tension at the air-liquid interface of the lung and they play a role in host defense against infection and inflammation (10, 16).

In this study, we found that all types of SP mRNA were expressed in human lung tissue, but only three types, SP-A2, SP-B, and SP-D, were expressed in human nasal mucosa. These results suggest that the pattern of SP mRNA expression is different between the upper and lower airways. SP-A and SP-D proteins and their transcripts have been found in a number of tissues besides airway mucosa, including rat and human gastric and intestinal mucosa, mesothelial tissues (mesentery, peritoneum, and pleura), synovial cells, adult rabbit Eustachian tube and sinus, and possibly human salivary glands, pancreas, and urinary tract (1). Initially, SP-B and SP-C appeared to be expressed in lung epithelium only (1), but Paananen et al. (14) have reported expression of SP-B mRNA

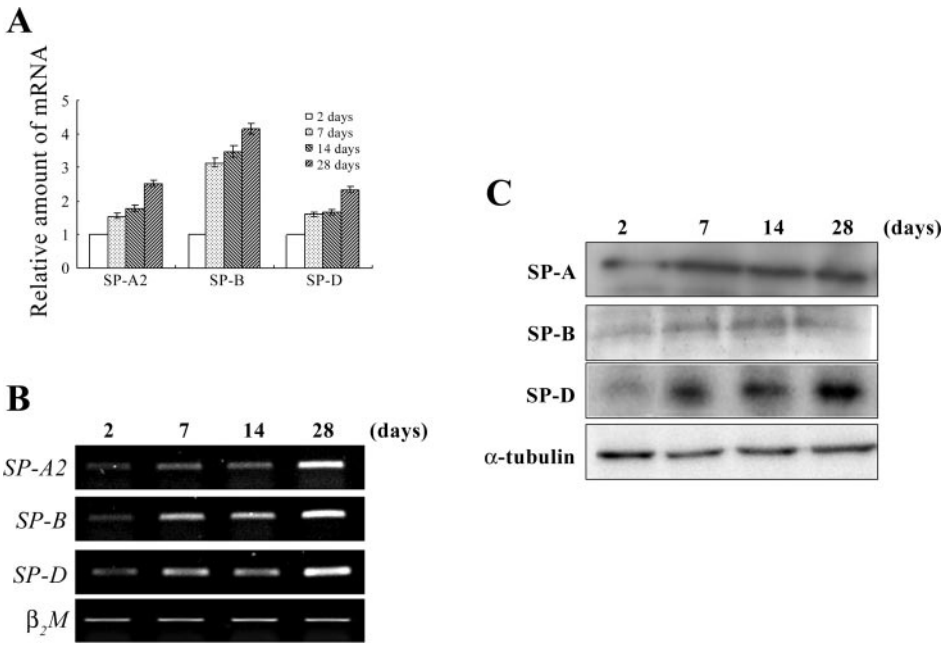

Fig. 7. Expression of SP genes and proteins as a function of mucociliary differentiation. *A*: densitometric analysis of PCR data obtained from 4 separate experiments. Data are expressed as means ± SE. *B*: SP-A2, SP-B, and SP-D gene expression increased at 7, 14, and 28 days after confluence. *C*: SP-A and SP-D protein expression increased with a parallel pattern, but SP-B levels did not change. Shown is representative of 4 separate experiments.

in extrapulmonary organs, especially the porcine Eustachian tube. These data suggest that surfactant mRNAs may be differentially expressed depending on organ type.

Concerning the localization of SPs, we found that SP-A2, SP-B, and SP-D were all expressed in the ciliated cells of the surface epithelium and serous acini of the submucosal gland in human nasal mucosa (Figs. 2, 3, 4). This result is in accordance with previous reports that SPs are expressed in surface epithelium and serous cells of submucosal glands (4, 6, 8, 13). Interestingly, SP-D has also been identified in mucus-secreting epithelium near the cardioesophageal junction (1). This discrepancy suggests that the cells secreting certain SPs may be different according to organ type.

We examined whether surfactant gene expression is influenced by the degree of mucociliary differentiation in human nasal epithelial cells. In the presence of retinoic acid, cultured NHNE cells were differentiated into mucociliary epithelium (17). Histological and molecular characterization of differentiated cells at the indicated time points confirmed that mucociliary differentiation occurred in our culture system. As mucociliary differentiation progressed, expression of MUC5AC, a mucous cell marker, and MUC8, a ciliated cell marker, increased. Expression of cornifin-α, a squamous cell marker, decreased. Expression of SP-A2, SP-B, and SP-D increased as a function of mucociliary differentiation. These results suggest that the level of SP increases as the epithelial cells differentiate into mucociliary epithelium. The expression of SP-A and SP-D proteins increased during cell differentiation to mucociliary epithelium. However, SP-B showed no change in four separate repeat experiments. Although we did not determine why SP-B expression did not increase, we postulate that SP-B protein is regulated at a posttranscriptional level.

In conclusion, we found that SP-A2, SP-B, and SP-D genes are expressed in the ciliated epithelial cells and serous acini of the submucosal glands of the human nasal mucosa. In addition, expression of these genes was influenced by the degree of mucociliary differentiation. Further studies are required to clarify the functions of SPs in human nasal mucosa.

GRANTS

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