Mucin Production by SPOC1 Cells—An Immortalized Rat Tracheal Epithelial Cell Line

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An airway epithelial mucous goblet cell line would be useful towards understanding mechanisms underlying the common problem of respiratory mucus hypersecretion. SPOC1 is a novel rat tracheal epithelial (RTE) cell line that developed cytologic features suggestive of mucous goblet cells when grown in tracheal grafts *in vivo* (*Am. J. Respir. Cell Mol. Biol.* 1995; 12:385–395). Our aims were to determine whether SPOC1 cells were capable of mucin synthesis and to directly compare mucin production by SPOC1 cells and RTE cells. Towards this end, we validated the use of monoclonal antibody (mAb) RTE11 (*Exp. Lung Res.* 1992; 18:323–342) as an immunologic probe for rat airway secretory mucin. Our results strongly suggest that mAb RTE11 detects a carbohydrate antigen that is a sensitive and specific marker for rat tracheobronchial secretory mucin. SPOC1 cells in tracheal grafts *in vivo* contained granules with ultrastructural features similar to mucous granules in normal rat airway goblet cells and they were strongly stained by mAb RTE11. Retinoic acid (RA) and culture on porous supports are known to profoundly modify airway epithelial cell phenotype *in vitro*. Expression of several retinoid-responsive proteins was similar in cultured SPOC1 and primary RTE cells, but major differences in mucin production were noted. Primary RTE cells *in vitro* only made mucin when grown on porous supports in the presence of RA, whereas SPOC1 cells produced mucin when grown on plastic or glass surfaces and even in the absence of RA. Interestingly, RA enhanced mucin secretion by SPOC1 cells during the early plateau stage of culture but there were no differences due to RA late in the culture period. SPOC1 cells are capable of mucin production and will be a useful tool for studying select aspects of airway secretory cell differentiation and function.

Mucus hypersecretion is a hallmark of inflammatory airway diseases, including asthma, chronic bronchitis, and cystic fibrosis (1). Regulatory mechanisms governing secretory cell proliferation and differentiation or expression and release of characteristic secreted products remain poorly understood. A tracheobronchial epithelial cell line capable of mucin production would greatly facilitate analysis of airway epithelial secretory cell function. SPOC1 is a novel rat tracheal epithelial (RTE) cell line spontaneously derived from secondary RTE cell cultures (2). It is immortal but nontumorigenic and has a diploid karyotype. There are specific, nonrandom alterations in chromosomes 1, 3, and 6. SPOC1 demonstrates decreased requirements for peptide growth factors *in vitro* compared with primary RTE cells. In denuded tracheal grafts *in vivo*, SPOC1 cells generate a pseudostratified epithelium with gland-like invaginations into the submucosa. Like primary RTE cells (3), SPOC1 cells in tracheal grafts display time-dependent changes in differentiation state. As the grafts mature, the cytoplasm of most supra-basal cells in the surface layer and many cells in the gland-like structures become clear and frothy and Alcian blue-periodic acid–Schiff's (AB-PAS) positive, suggesting mucous goblet cell differentiation.

The purpose of the studies reported here was to determine whether SPOC1 cells synthesized mucin, both *in vivo* and *in vitro*. We directly compared the regulation of mucin production in SPOC1 cells and primary RTE cells. Towards this end, we performed a series of experiments to determine whether a previously described monoclonal antibody (mAb), RTE11 (4), was a suitable immunologic probe for rat airway

(Received in original form May 18, 1995 and in revised form September 15, 1995)

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Abbreviations: Alcian blue–periodic acid–Schiff's, AB-PAS; enzyme-linked immunosorbent assay, ELISA; electron microscopy, EM; monoclonal antibody, mAb; periodic acid–thicarbohydrazide–silver proteinate, PA-TCH-SP; phosphate-buffered saline, PBS; phosphate-buffered saline with 0.5% Tween-20, PBST; retinoic acid, RA; rat tracheal epithelial, RTE.

secretory mucin. Results herein strongly suggest that mAb RTE11 detects a rat secretory mucin-specific carbohydrate antigen, most likely present in the side chain region of the branched carbohydrate structure. SPOC1 cells in tracheal grafts *in vivo* contained granules with ultrastructural features similar to mucous granules in normal rat airway goblet cells and they were strongly stained by mAb RTE11. We evaluated the responses of SPOC1 cells and primary RTE cells to retinoic acid (RA) and culture on plastic versus permeable supports, manipulations known to dramatically modify the phenotype of airway epithelial cells (5). We then utilized mAb RTE11 to characterize mucin production by SPOC1 cells *in vitro*. SPOC1 cells differentiate to a mucin-producing phenotype and will be useful for studying select aspects of airway epithelial secretory cell function.

Materials and Methods

Cell Culture

Primary RTE cells (5) or passage 6 through 8 SPOC1 cells (2) were used. SPOC1 cells are a mixed clonal population and there may be differences between individual cells (2). Although later-passage SPOC1 cells may behave similarly, the results herein strictly apply only to passages 6 through 8. Cells were grown on Transwell-COL (Costar, Cambridge, MA) inserts as described previously (5), using a seeding density of 1.2×10^5 RTE cells or 0.3×10^5 SPOC1 cells per 24-mm Transwell. In some cases, Transwell membranes were precoated with a rat tail collagen gel or Matrigel according to the manufacturer's instructions (Collaborative Research, Bedford, MA). RTE cells and SPOC1 cells were also grown on six-well tissue culture plates using seeding densities of 3×10^5 and 1×10^5 cells per well, respectively. In selected experiments, SPOC1 cells were grown on eight-well glass chamber slides (Lab-Tek; Miles Scientific, Naperville, IL) using a seeding density of 1×10^4 cells per well. In experiments utilizing a rat tail collagen gel or Matrigel, cells were grown at 35°C in 97% air, 3% CO_2 to prevent dissolution of the gel (5). For all other experiments, the cells were maintained at 37°C in 95% air, 5% CO_2. On day 1 of culture, the media contained 3 mg/ml bovine serum albumin and 10% fetal bovine serum (FBS) (in the case of Transwells, only the basolateral chamber was supplemented with FBS). At 24 h, the cells were switched to serum-free medium and the concentration of bovine serum albumin was reduced to 0.5 mg/ml. As indicated, the medium was supplemented with 5×10^{-8} M RA. Prior to confluence, the medium was changed every 2 days, and daily thereafter. An air-liquid interface was established with cells grown on Transwell-COL inserts at day 8 by removing the apical medium and feeding from the basolateral compartment only. In some experiments, cells grown on Transwell-COL inserts were metabolically labeled with [^{3}H]glucosamine (15 μCi/ml) as described (5).

Column Chromatography

Apical secretions of [^{3}H]glucosamine-labeled Transwell cultures of RTE cells were collected by washing the apical surface with 1 ml of phosphate-buffered saline (PBS). The samples were digested with ovine testicular hyaluronidase and chromatographed over Sepharose CL4B as described (2). Radioactivity in each fraction was measured, and equal volumes of selected fractions were applied to nitrocellulose filters using a slot blot apparatus. The filters were stained with the periodic acid–Schiff's reaction (PAS) or were immunoblotted with mAb RTE11 (see below).

mAb RTE11 Slot Blot Assay

Conditioned culture medium, apical washings of Transwell-COL cultures, cell lysates, or aliquots of column fractions were applied to nitrocellulose filters using a slot blot apparatus. The membrane was blocked with 5% milk (from nonfat milk powder) in PBS with 0.5% Tween-20 (PBST) and incubated in a 1:50 dilution of mAb RTE11 hybridoma supernatant or an equal dilution of NS1 cell conditioned medium spiked with 50 μg/ml normal mouse serum IgG (negative control). Membranes were washed three times with 1% milk-PBST and incubated in peroxidase-conjugated secondary antibody diluted in 1% milk-PBST. After washing with PBST and 0.05 M Tris buffer, pH 7.6, peroxidase reaction product was developed using diaminobenzidine (0.2 mg/ml) and hydrogen peroxide (0.006%) in 0.05 M Tris buffer, pH 7.6. In some cases, enhanced chemiluminescence and fluorography (ECL; Amersham, Arlington Heights, IL) was used in place of diaminobenzidine. Band intensity was quantitated using NIH Image 1.41 software.

Enzyme Digestions

RTE cell mucin was prepared from pooled Sepharose CL4B void volume fractions as described below (mucin standard) and was incubated with Streptomyces hyaluronidase (Sigma Chemical Co., St. Louis, MO), chondroitinase ABC (Sigma), heparitinase (Sigma), neuraminindase (Sigma), endoglycosidase H (Boehringer Mannheim, Indianapolis, IN), α-galactosidase (Boehringer Mannheim), α-L-fucosidase (Boehringer Mannheim), *O*-glycanase (Genzyme, Cambridge, MA), endo-β-galactosidase (Boehringer Mannheim), or heparinase (Sigma) according to the manufacturers' instructions. Control incubations were performed in buffer alone. After the incubation, the specimens were applied to nitrocellulose filters and a slot blot assay was performed as described above.

Tracheal Grafts

A total of 1×10^4 passage 8 SPOC1 cells were seeded in denuded tracheal grafts and implanted in host rats as previously described for primary RTE cells (3). Grafts were harvested and studied on days 7 and 21 after inoculation. All experimental animals were utilized under the auspices of IACUC-approved protocols, in AAALAC-approved facilities.

Immunostaining, Electron Microscopy (EM), and Histology of Cultured Cells

Rat tracheal and bronchial specimens, SPOC1 cell-containing tracheal grafts, and cells grown on chamber slides were fixed in Omni-Fix II (An-Con Genetics, Melville, NY). Tissue and graft specimens were embedded in paraffin using conventional techniques. Normal rat tissues were also fixed with periodate-lysine-paraformaldehyde and embedded in Lowicryl for EM. Immunostaining of deparaffinized sec-

tions was performed using mAb RTE11 or control antibody and a peroxidase-conjugated second antibody technique as described previously (3–5). EM immunostaining was performed using a 12-nm colloidal gold–conjugated second antibody, and the sections were counterstained with uranyl acetate and lead citrate. Tracheal grafts containing SPOC1 cells and SPOC1 cell cultures, prepared as described above, were processed for EM using conventional techniques. Briefly, specimens were fixed with a formaldehyde-glutaraldehyde mixture, postfixed with osmium tetroxide, dehydrated with ethanol and propylene oxide, and embedded in epon or Spurr's resin. Ultrathin sections were obtained and were stained with periodic acid–thicarbohydrazide–silver proteinate (PA-TCH-SP) to localize complex carbohydrates (6) or with uranyl acetate and lead citrate. The sections were viewed in a Zeiss 10 electron microscope. SPOC1 cells grown on Transwell-COL inserts were prepared for histology by fixing with formalin or a formaldehyde-glutaraldehyde mixture and embedding in paraffin or soft plastic.

Preparation of a Mucin Standard and a Rabbit Anti-Rat Mucin Polyclonal Antibody

Apical washings of RTE cell Transwell cultures supplemented with RA, which displayed mucociliary differentiation, were used as a source of mucin. The samples were digested with ovine testicular hyaluronidase and chromatographed over a preparative Sepharose CL4B column (2.5 × 60 cm). Void volume fractions from multiple runs were pooled and extensively dialyzed against water. The samples were dried in a Speed Vac apparatus, weighed, and reconstituted in water at a concentration of 1 mg/ml, and aliquots were stored frozen at −20°C. To prepare a rabbit anti-rat mucin antiserum, 250 μg of mucin was emulsified in complete Freund's adjuvant and injected intradermally and subcutaneously in a New Zealand white rabbit. Four subcutaneous booster injections of 250 μg of mucin emulsified in incomplete Freund's adjuvant were subsequently given at regular intervals. The rabbit was anesthetized and exsanguinated 2 wk after the final boost. An IgG fraction was prepared from the above antiserum using protein A column chromatography.

Rat Mucin Sandwich Enzyme-linked Immunosorbent Assay (ELISA)

The hybridoma cell line RTE11 (4) was grown in media prepared with low-IgG FBS, and antibody was purified using a combination of ammonium sulfate precipitation, hydroxylapatite column chromatography, and ultrafiltration. IgG was purified from the rabbit anti-rat mucin antiserum as described above and was covalently linked to peroxidase. Flat-bottom, high-binding, 96-well plates were coated with mAb RTE11 and blocked with 5% milk-PBST solution. Samples or standards were added diluted in 1% milk-PBST. Bound mucin was reacted with peroxidase-conjugated rabbit anti-rat mucin diluted in 1% milk-PBST. Enzyme activity was detected using hydrogen peroxide and *O*-phenylenediamine, the reaction was stopped with HCl, and optical density at 490 nm was determined.

Western Blots

Protein extracts were prepared by washing cultured cells twice with PBS with proteinase inhibitors (1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 7 μm pepstatin A, and 6 μM leupeptin) and direct lysis with Laemmli sample buffer without reductant or loading dye. Samples were collected, sonicated, and centrifuged, and the protein content of the supernatant was measured using the BCA assay (Pierce, Rockford, IL). Reductant and loading dye were added, and appropriate amounts of protein (5 to 20 μg per lane) were electrophoresed on either 8% or 14% SDS-PAGE Laemmli minigels (Novex, San Diego, CA) and transferred to Immobilon P (Millipore, Bedford, MA) membranes. Blocking, washing, primary and secondary antibody incubations, and enhanced chemiluminescence visualization were performed as described above for the slot blot assay. The following primary antibodies were used: mAb BC.1 (transglutaminase I; Dr. S. M. Thacher, Texas A&M University, College Station, TX); SQ37 (cornifin; Dr. A. Jetten, NIEHS, Research Triangle Park, NC); AE8 (keratin 13; ICN Biochemicals, Costa Mesa, CA); CUB 7401 (transglutaminase II; Dr. P. J. Birchbickler, Samuel Roberts Noble Foundation, Ardmore, OK); CK18-2 (keratin 18; Dr. F. C. S. Ramaekers, University of Limburg, Maastricht, The Netherlands); RTE1 (keratin 19 [7]); and LL002 (keratin 14; Dr. E. B. Lane, Dundee, UK). Dilutions of 1:10, 1:1,000, and 1 μg/ml were utilized for hybridoma supernatants, antisera, and purified antibodies, respectively.

Results

Our main goals were to determine whether SPOC1 cells synthesized mucin and to compare the regulation of mucin production in SPOC1 cells and RTE cells. First, it was important to establish that mAb RTE11 (4) was a valid immunologic probe for rat airway secretory mucin. Previously published immunocytochemical studies demonstrated that the antigen detected by mAb RTE11 was present in mucous-type tracheobronchial epithelial cells (4) and that the antigen appeared in developing airways (7), regenerating tracheal grafts (3), and cultured RTE cells (5) commensurate with the appearance of morphologically defined mucous cells. In the present studies, we performed ultrastructural immunocytochemical localization of the mAb RTE11 antigen in normal rat bronchus (Figure 1). At our level of sensitivity, the antigen was principally localized to mucous goblet cell granules; signal was not detected in other organelles or on the plasma membrane and it was not found in different cell types or the extracellular matrix. The antigen was also present in the luminal space, associated with newly secreted mucous granule contents.

We evaluated retinoid and substratum regulation of mAb RTE11 antigen production by RTE cells *in vitro*. Apical secretions of differentiated, polarized RTE cells grown on permeable supports were an abundant source of the mAb RTE11 antigen. Omission of RA from the culture medium, which induced squamous metaplasia (5), resulted in only transient, low-level secretion of the mAb RTE11 antigen, and we did not detect the mAb RTE11 antigen in conditioned medium of RTE cells grown on plastic (not shown). Primary RTE cell secretions were used to determine the approximate molecular weight of the antigen. As shown in Figure 2, the RTE11 antigen eluted in the void volume of Sepharose CL4B columns, indicating a molecular mass of $> 1 \times 10^6$ kD and

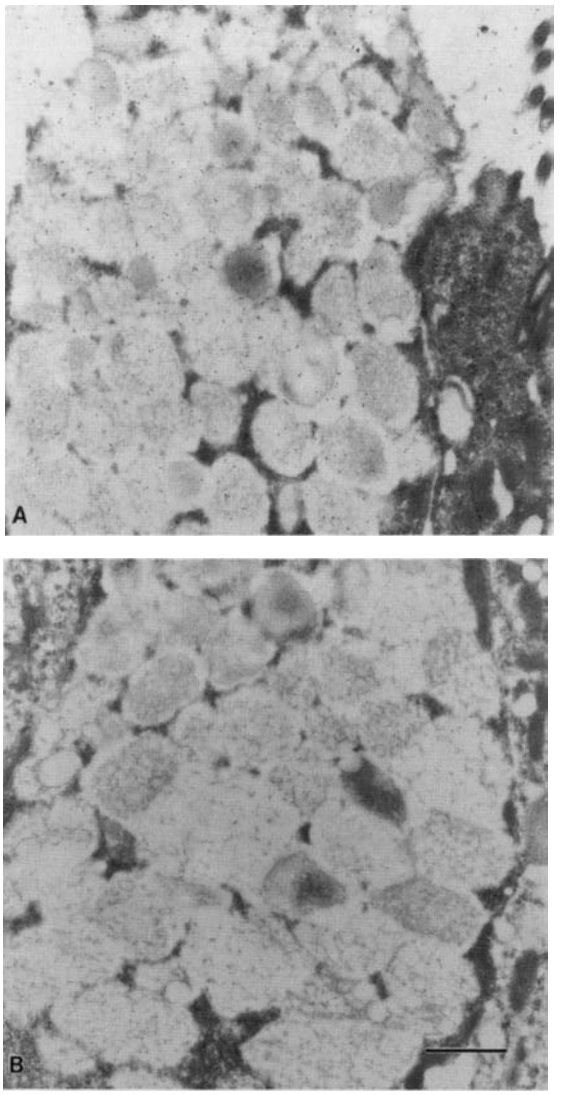

Figure 1. mAb RTE11-labeled mucous goblet cell granules. At our level of sensitivity, signal was not detected in other organelles or on the plasma membrane and it was not found in different cell types or the extracellular matrix. Electron micrographs of Lowicryl sections of rat bronchial goblet cells immunostained with mAb RTE11 (A) or a negative control mouse antibody (B); counterstained with uranyl acetate and lead citrate. Bar = 1.0 μm.

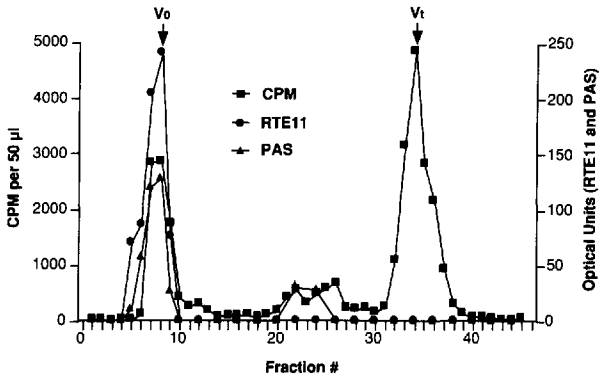

Figure 2. The RTE11 antigen eluted in the void volume of Sepharose CL4B columns, indicating a molecular mass of $> 1 \times 10^6$ kD, and coeluted with the major PAS-positive peak from the cultures. Sepharose CL4B elution profile of apical secretions of [^{3}H]glucosamine-labeled RTE cells cultured for 14 days on collagen gel–coated Transwell-COL inserts with media containing RA. Forty-five 1-ml fractions were collected, and radioactivity was measured in a 50-μl aliquot of each (*squares*). As empirically determined by the elution of blue dextran (molecular mass of 2×10^6 kD) and phenol red (molecular mass of 376), respectively, the void (Vo) and total (Vt) volumes of the column were 7 and 33 ml. Aliquots of selected fractions were applied to nitrocellulose and were stained with mAb RTE11 or the PAS reaction. The blots were digitized, and band intensity was measured and plotted (*circles*: RTE11; *triangles*: PAS).

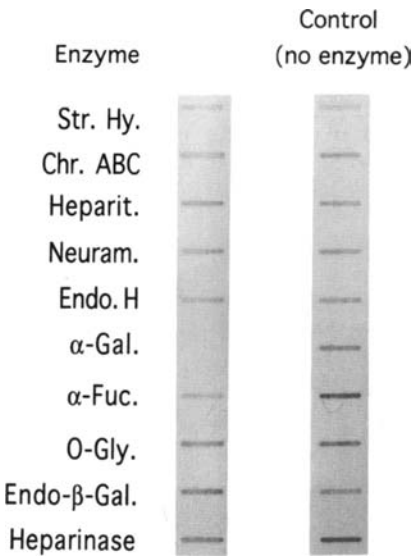

Figure 3. mAb RTE11 binding was relatively resistant to hyaluronidase and many other proteoglycan- and glycoconjugate-degrading enzymes but was sensitive to α-galactosidase treatment. Aliquots of purified mucin were treated with the indicated enzymes, blotted to nitrocellulose, and stained with mAb RTE11 as described in MATERIALS AND METHODS. Antibody binding was abolished only by α-galactosidase. Str. Hy. = Streptomyces hyaluronidase; Chr. ABC = chondroitinase ABC; Heparit. = heparitinase; Neuram. = neuraminindase; Endo. H = endoglycosidase H; α-Gal = α-galactosidase; α-Fuc = α-L-fucosidase; O-Gly = *O*-glycanase; Endo-β-Gal = endo-β-galactosidase.

it coeluted with the major PAS-positive peak from the cultures. We next examined the enzyme sensitivity of the antigen binding site. Aliquots of Sepharose CL4B void volume fractions were dialyzed against water, concentrated, treated with a series of glycoconjugate-degrading enzymes, blotted on nitrocellulose, and stained with mAb RTE11 as described in MATERIALS AND METHODS. As shown in Figure 3, mAb RTE11 binding to the putative mucin was relatively resistant to hyaluronidase and many other proteoglycan- and glycoconjugate-degrading enzymes but was completely destroyed by α-galactosidase treatment.

Collectively, these results strongly suggest that mAb RTE11 detects a rat secretory mucin-specific carbohydrate antigen,

most likely present in the side chain region of the branched carbohydrate structure. In conjunction with a standard mucin preparation (described in MATERIALS AND METHODS), mAb RTE11 serves as the basis for a slot blot assay useful for quantifying mucin. Preparation of a polyclonal rabbit antibody against rat mucin (described in MATERIALS AND METHODS) also enables it to be used in a sandwich ELISA.

Prior work suggested that SPOC1 cells were capable of mucous goblet cell differentiation (2). A main goal of our present studies was to test whether SPOC1 cells synthesized mucin, both *in vivo* and *in vitro*. We performed electron microscopy of SPOC1 cells seeded in tracheal grafts. As shown in Figure 4, most supra-basal SPOC1 cells from day 21 tracheal grafts contained membrane-bound granules. Many granule profiles displayed a variable pattern of electron lucency and some contained dense core structures, features typically present in mucous granules. The granules were PA-TCH-SP positive, indicating the presence of complex carbohydrates. SPOC1 cell granules were not identical to typical rat tracheobronchial mucous cell granules in that SPOC1 cell granules more consistently contained a prominent electron-lucent halo or cap. We next utilized mAb RTE11 to examine the differentiation dependence of mucin expression by SPOC1 cells. As shown in Figure 5, as SPOC1 cells in tracheal grafts differentiated between days 7 and 21, they displayed increased immunoreactivity with mAb RTE11. These results support the concept that SPOC1 cells underwent mucous goblet cell differentiation and produced mucin when grown in tracheal grafts *in vivo*.

We studied retinoid and substratum regulation of SPOC1 cells *in vitro*. We evaluated several proteins known to be regulated by retinoids and directly compared the responses of SPOC1 cells and RTE cells. Transglutaminase I, cornifin, keratin 13, transglutaminase II, keratin 18, and keratin 19 were measured by Western blot in protein extracts of post-confluent RTE and SPOC1 cells. Keratin 14 was included as a control. Cells were grown in the presence or absence of RA on plastic, submerged in medium, or on Transwell-COL inserts, at an air-liquid interface. For the most part, expression patterns of retinoid-responsive proteins were similar between RTE cells and SPOC1 cells (Figure 6). RTE cells on plastic strongly expressed transglutaminase I, which is normally suppressed by retinoids, even in the presence of RA. Transglutaminase I was only faintly detectable in SPOC1 cells under the same conditions. Interestingly, on a per

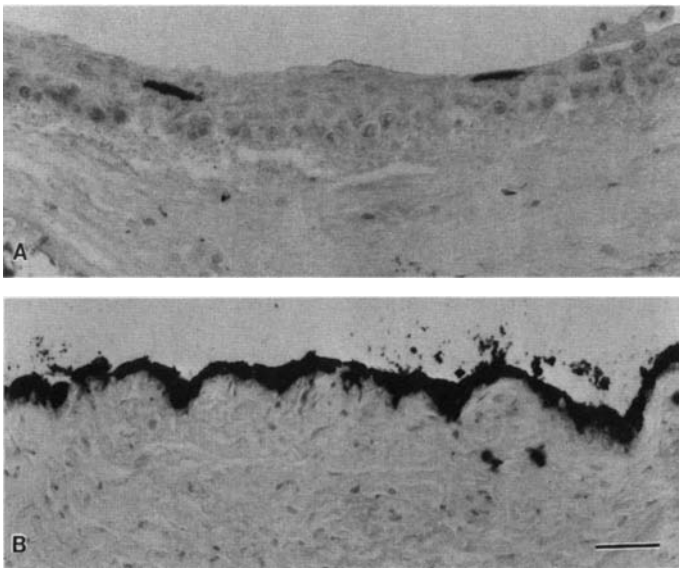

Figure 5. Expression of the antigen detected by the anti-mucin mAb, RTE11, increased as SPOC1 cells differentiated in tracheal grafts *in vivo*. Photomicrographs of mAb RTE11-stained paraffin sections of tracheal grafts that were inoculated with passage 8 SPOC1 cells 7 days (A) or 21 days (B) previously. Bar = 50 μm.

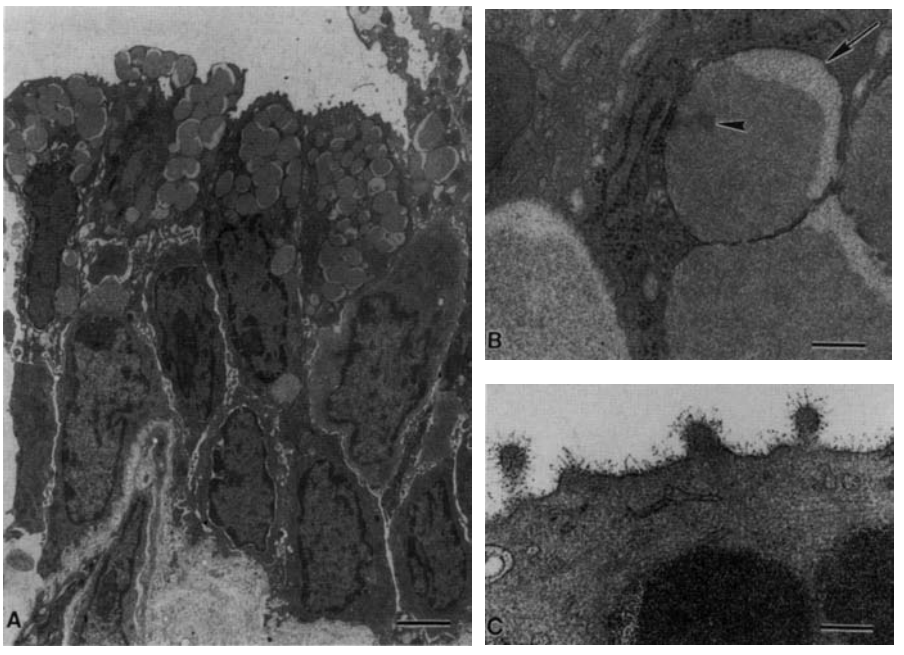

Figure 4. Electron microscopy demonstrated the presence of glycoconjugate-containing, membrane-bound granules in SPOC1 cells. Electron micrographs of uranyl acetate–lead citrate stained (A, B) or periodic acid-thicarbohydrazide–silver proteinate (PA-TCH-SP)-stained (C) SPOC1 cells from tracheal grafts that were inoculated with passage 8 SPOC1 cells 21 days previously. The supra-basal cells contain large, membrane-bound granules. Most granule profiles display an electron-lucent cap or rim (*arrow*) and many contain dense core structures (*arrowhead*). The apical plasma membrane, vesicular structures, and both the lucent and dense portions of the granules were PA-TCH-SP positive, indicating the presence of complex carbohydrates. Bar = 2.5 μm (A) or 0.25 μm (B and C).

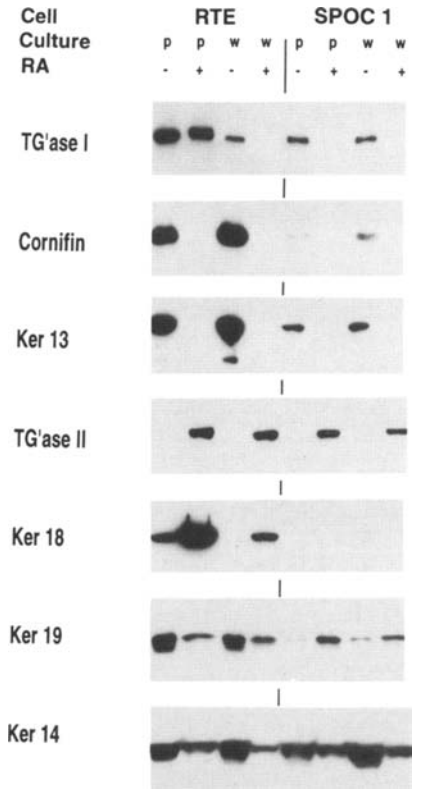

Figure 6. RA regulation of several genes in cultured SPOC1 cells resembled the pattern observed with primary RTE cells. The products of the retinoid-responsive genes transglutaminase I, cornifin, keratin 13, transglutaminase II, keratin 18, and keratin 19 were measured by Western blot in protein extracts of postconfluent RTE and SPOC1 cells grown in the presence or absence of RA on plastic (p) or Transwell-COL inserts (w). Keratin 14 was included as a control. For most proteins, the pattern of regulation was similar between RTE cells and SPOC1 cells. For greater discussion of the differences, see RESULTS.

microgram protein basis, cornifin and keratin 13, which were also suppressed by RA, were expressed to a much greater degree in retinoid-deficient RTE cells than in retinoid-deficient SPOC1 cells. Transglutaminase II was induced by RA in both RTE cells and SPOC1 cells. As noted in our prior study (2), SPOC1 cells were unable to express keratin 18 protein, which was induced by retinoids in RTE cells (5). Keratin 19 was induced by RA in SPOC1 cells. In RTE cells, keratin 19 expression more closely resembled the pattern of keratin 14, which was increased in retinoid-deficient cultures. These results demonstrate both similarities and differences in the retinoid responsiveness of primary RTE cells and SPOC1 cells and suggest that several aspects of retinoid regulatory pathways are functional in SPOC1 cells.

Histochemistry, immunostaining, and EM were used to examine the phenotype of SPOC1 cells *in vitro* (Figure 7). Postconfluent SPOC1 cultures grown on Transwell inserts with a collagen gel and media with RA contained cells with AB-PAS-positive granules. Somewhat greater amounts of AB-PAS-positive materials were present in RA-replete cultures and evidence of squamous differentiation was visible in the RA-deficient Transwell cultures, but the overall appearance was very similar regardless of collagen gel or retinoid status (not shown). SPOC1 cells on Transwells formed cysts and tubular structures, perhaps suggestive of gland formation. Ultrastructurally, granules similar to those found in tracheal grafts (described above) were visible in cultured SPOC1 cells. Matrigel coating of the Transwell resulted in the formation of gland-like structures. SPOC1 cells cultured on glass chamber slides generally grew as monolayers with occasional cysts, tubes, and multilayered foci. Scattered cells in the chamber slides were positive with the anti-mucin mAb, RTE11. Most typically, the mAb RTE11-positive cells were located within the cysts or were apically situated in multilayered foci.

The results above strongly suggested that SPOC1 cells produced mucin *in vitro* but also indicated that retinoids and substratum regulated mucin production differently in SPOC1 cells and primary RTE cells. A convenient biochemical mucin assay, suitable for use with SPOC1 cells, would facilitate in-depth analysis of mucin production. To validate the use of mAb RTE11 as a biochemical probe for SPOC1 mucin, we directly compared traditional biochemical methods for quantifying mucin (namely, hyaluronidase-resistant [^{3}H]glucosamine-labeled materials eluting in the void volume of Sepharose CL4B [8]) and the mAb RTE11 sandwich ELISA. SPOC1 or RTE cells were grown on Transwell-COL inserts in the presence of RA. The apical surface of [^{3}H]glucosamine-labeled interface cultures grown for 6 days after confluence was washed with 1 ml PBS. After 24 h, the surface was washed again and the mucin content of the apical washings was determined. Figure 8 demonstrates that measurement of mucin production by SPOC1 cells or primary RTE cells by two methods gave the same results. On a per culture basis, primary RTE cells secreted approximately sixfold more mucin than did SPOC1 cells.

Unlike RTE cells, SPOC1 cells promiscuously produced mucin on Transwell-COL inserts, irrespective of RA status. This suggested that perhaps SPOC1 cells could make mucin when grown on plastic or glass. We used mAb RTE11 immunostaining and the slot blot assay to explore the time course and retinoid dependence of SPOC1 cell mucin production on plastic or glass. Near-confluent SPOC1 cells were passaged and plated on sixwell tissue culture plates or glass chamber slides. One day later, and thereafter, half of the cells were provided media supplemented with RA. At various stages of growth, the chamber slides were fixed and immunostained with mAb RTE11 (not shown). Using the sixwell plates, cell number was measured and mucin was quantified both in the conditioned medium and in cell lysates (Figure 9). SPOC1 cells produced mucin when cultured on plastic or glass. Stored or adherent mucin associated with the freshly dissociated cells, or newly synthesized mucin, was evidently released as the cells attached on day 1. Interestingly, there was not a correspondingly high intracellular mucin content at 24 h. The precise cause of high mucin release during the attachment phase is unknown. Rapidly dividing, early log-stage cells produced relatively little mucin. Intracellular and extracellular mucin increased with time in culture in both RA-replete and -deficient cultures. Intracellular mucin content was similar in RA-supplemented and -deficient cultures. During the early plateau phase (days 5 and 7), secreted mu-

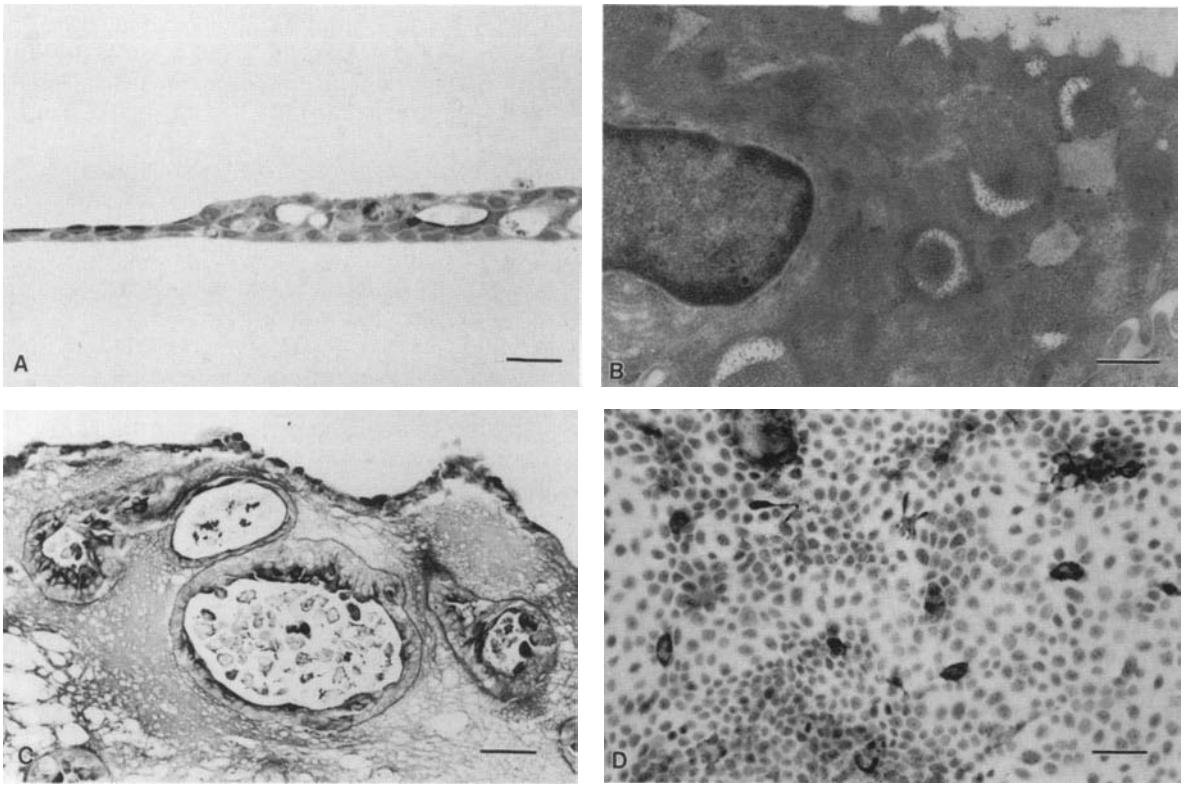

Figure 7. Histochemistry, EM, and immunostaining suggested mucin production by SPOC1 cells *in vitro*. A. Passage 6 SPOC1 cells grown on Transwell inserts with a collagen gel in the presence of RA for 6 days after confluence were prepared for histology. Apically situated cells and cells within cyst and tube structures contained AB-PAS–positive granules (AB-PAS-hematoxylin stain; bar = 25 μm). B. Ultrastructurally, the granules contained within cultured SPOC1 cells (grown precisely as described in panel A) displayed variable electron lucency and were similar to the granules observed in tracheal grafts (uranyl acetate–lead citrate stain; bar = 0.5 μm). C. SPOC1 cells grown in the presence of Matrigel for 14 days contained AB-PAS–positive granules and developed into gland-like structures (AB-PAS stain; bar = 50 μm). D. Passage 6 SPOC1 cells grown on glass chamber slides in the presence of RA for 9 days were immunostained with mAb RTE11. The dark reaction product indicates sites of mAb RTE11 binding (bar = 50 μm).

cin was greater in cultures containing RA. However, later in the plateau phase (day 13), differences in secreted mucin due to RA were minimal.

Discussion

Our main goals were to determine if SPOC1 cells synthesized mucin and to compare the regulation of mucin production in SPOC1 cells and primary RTE cells. To facilitate these studies, we determined whether mAb RTE11 (4) was a valid immunologic probe for rat airway secretory mucin. Anti-mucin mAbs have been increasingly utilized because they enable convenient quantitation. However, the mAb-based assays potentially lack specificity. Antibodies against carbohydrate determinants may detect broadly distributed cell surface, as well as secretory, mucins or might recognize the same carbohydrate modification on nonmucin molecules. Antibodies against apomucin do not usually detect the glycosylated product because of epitope masking (9). Although we have not precisely defined the epitope detected by mAb RTE11, our ultrastructural localization and biochemical results strongly suggest that it is a rat secretory mucin-specific carbohydrate antigen containing α-linked galactose. Blood group–related sugar structures containing α-linked galactose are typically present in the peripheral region of the branched carbohydrate structure of mucin (10). It is possible that the complex repertoire of secretory cell carbohydrate synthetic and modifying enzymes may change under certain circumstances, preventing the synthesis of, or perhaps masking, the epitope detected by mAb RTE11. The fact that the antigen appeared in developing airways (7), regenerating tracheal grafts (3), cultured RTE cells (5), and SPOC1 cells (this study) whenever morphologically defined mucous cells were present suggests that loss of synthetic capability or masking are not common and that mAb RTE11 is a sensitive marker. Restriction of the mAb RTE11 antigen to mucous granules and newly secreted mucosubstance suggests that it does not detect broadly distributed carbohydrate epitopes and is specific for secretory mucin. Most importantly, the results were nearly identical when we measured mucin production by primary RTE cells (high producers) and SPOC1 cells (low producers) using a conventional biochemical approach (hyaluronidase-resistant [^{3}H]glucosamine-labeled materials eluting in the void volume of Sepharose CL4B) and the mAb RTE11 sandwich ELISA. Thus, mAb RTE11 ap-

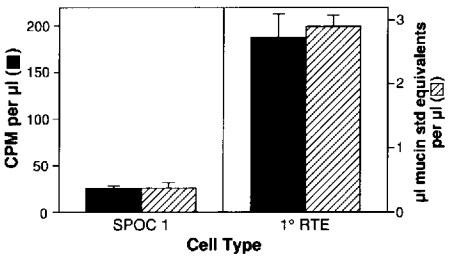

Figure 8. Measurement of mucin production by SPOC1 cells or primary RTE cells by two methods gave the same results—on a per culture basis, primary RTE cells secreted approximately sixfold more mucin than did SPOC1 cells. Mucin secretion by primary RTE or SPOC1 cells grown on Transwell-COL inserts in the presence of RA was measured by determining hyaluronidase-resistant [^{3}H]glucosamine-labeled materials eluting in the void volume of Sepharose CL4B (*solid bars*) or by mAb RTE11 ELISA (*hatched bars*). Cells were grown at an air-liquid interface on permeable supports for 6 days after confluence and the mucin content of apical washings was determined as described in MATERIALS AND METHODS.

pears to be a sensitive and specific reagent for measurement of rat tracheobronchial secretory mucin.

Our initial report (2) of SPOC1 cell differentiation in tracheal grafts showed that SPOC1 cells generated a pseudostratified epithelium with gland-like invaginations into the submucosa. As the grafts matured, the cytoplasm of most supra-basal cells in the surface layer and many cells in the gland-like structures became clear and frothy and AB-PAS positive, suggesting mucous goblet cell differentiation. The results of ultrastructural and immunostaining techniques used in the present study confirm and strengthen the notion that SPOC1 cells become mucous goblet cells in tracheal grafts.

The tracheal graft model reliably supports differentiation of mucous and ciliated primary airway epithelial cells but it is subject to the usual experimental limitations of *in vivo* studies. Several investigators have shown that a thick collagen gel or a permeable support and medium supplemented with an appropriate retinoid support mucociliary differentiation of primary airway epithelial cells *in vitro*. We directly compared SPOC1 and primary RTE cell responses to these profound modulators of differentiation. Except in the case of transglutaminase I, culture on plastic versus permeable supports, per se, had little effect on the retinoid-responsive proteins examined. A concentration of 5×10^{-8} M RA effectively suppressed transglutaminase I on permeable supports but not on plastic. The mechanism of this phenomena is unknown. Regardless of substratum, the overall effect of RA was similar in both cell types but there were notable exceptions. The present *in vitro* data confirm our previous finding in tracheal grafts that SPOC1 cells do not produce antibody-detectable keratin 18 (2), which is preferentially expressed in columnar cells in rat tracheas (11) and is induced by RA in RTE cells on both plastic and permeable supports (reference 5 and the present study). Perhaps this loss relates to the chromosomal abnormalities of SPOC1. As expected, transglutaminase I, cornifin, and keratin 13 were suppressed, and transglutaminase II was induced, by RA in both cell types.

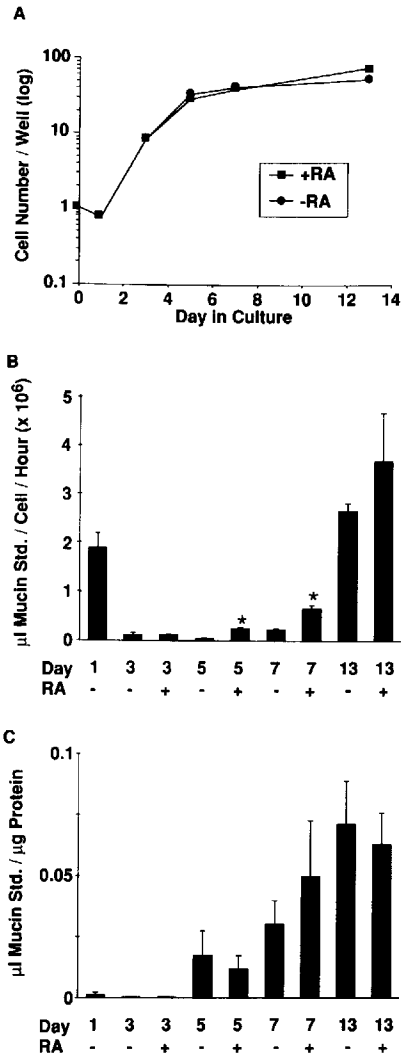

Figure 9. Quantitation of SPOC1 cell growth and mucin production on plastic. A. A total of 1×10^5 passage 6 SPOC1 cells were seeded in six-well tissue culture plates, grown with or without RA in the medium, and cell number per well was determined. B. Mucin in conditioned medium was measured using the mAb RTE11 slot blot assay and was expressed as a function of time and cell number. C. Mucin in cell lysates was measured using the mAb RTE11 slot blot assay and was normalized for cell protein content. Each point is the mean of three samples ± SD; the asterisk indicates a significant difference due to RA status when compared with the same time point ($P < 0.001$, t test).

However, cornifin and keratin 13 proteins, expressed per unit of cell total protein, were much more abundant in primary RTE cells than in SPOC1 cells. Keratin 19 was an interesting case—it behaved like a classic retinoid-inducible protein in SPOC1 but not in primary RTE cells. These results demonstrate that several aspects of retinoid regulation are intact and functional in SPOC1 cells but also suggest that other mechanisms operate to modulate ultimate protein expression of retinoid-responsive genes differently in SPOC1 and primary RTE cells.

Light and electron microscopy of postconfluent SPOC1 cells grown *in vitro* on permeable supports revealed mucous secretory granules. In contrast to primary RTE cells (5), RA or a collagen gel had little effect on the quantity of intracellular mucosubstance. This suggested that SPOC1 cells were subject to a rather fixed program for mucous goblet cell differentiation. In fact, we found that SPOC1 cells made mucin on plastic or glass and even without RA. Mucin production was directly related to time in culture. Exponentially growing SPOC1 cells made little mucin, and mucin content increased as the cells became confluent and postconfluent. Intracellular mucin was similar in both RA-supplemented and -deficient SPOC1 cells. RA upregulated mucin secretion during the early plateau stage of growth but similar amounts of secreted mucin were detected in postconfluent cells regardless of RA status. It is interesting that a degree of control was exerted by RA which was modulated or overridden by other factors operating during extended culture of SPOC1 cells. The differences between SPOC1 cells and RTE cells impose limitations on the validity of certain comparisons. Nonetheless, SPOC1 cells should be useful for studies of key cellular and molecular events regulating mucous production. That SPOC1 cells make mucin on plastic decreases the cost and increases the feasibility of such studies.

Expression of mucin genes would be an important measure of the secretory cell capabilities of SPOC1 cells. Only a few rat mucin genes have been cloned, and interspecies homology of mucin genes is generally poor. To date, we have been unable to detect a reliable signal from mucin-producing SPOC1 cells with Northern analysis using cDNA probes for RMUC176 (12), Ram7S (13), and a rat MUC2 homolog (14), despite good signals in positive control lanes (unpublished results). Reverse-transcriptase polymerase chain reaction of SPOC1 and primary RTE cells using primers for rat MUC2 uniformly gave bands of the expected size, but signal intensity did not correlate well with other indices of mucin production such as the histologic appearance of mucous goblet cells or the secretion of mAb RTE11 antigen (unpublished results obtained in collaboration with Dr. C. B. Basbaum, University of California, San Francisco). The precise reason we could not correlate mucin gene expression with mucin production is unknown. We speculate that either the most prevalent secretory mucin genes in cultured rat airway epithelial cells have not yet been cloned or that mucin production is not strictly regulated at the transcriptional level. These are important points for future investigations.

In summary, as judged by morphologic and biochemical criteria, SPOC1 cells produced mucin both *in vivo* and *in vitro*. Although clearly different than normal primary RTE cells, they will be a useful tool for studying select aspects of tracheobronchial epithelial secretory cell function.

Acknowledgments: The writers thank Dr. P. J. Birchbickler, Dr. A. Jetten, Dr. E. B. Lane, Dr. F. C. S. Ramaekers, and Dr. S. M. Thacher for providing antibodies and Dr. Carol B. Basbaum and Ms. Marianne Gallup for performing the rat MUC2 reverse-transcriptase polymerase chain reaction analysis. They also thank Dr. Karen Guzman for her critique of the manuscript.

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