Regulation of mucin secretion from human bronchial epithelial cells grown in murine hosted xenografts

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Conway, Jason D., Tracy Bartolotta, Lubna H. Abdullah, and C. William Davis. Regulation of mucin secretion from human bronchial epithelial cells grown in murine hosted xenografts. Am J Physiol Lung Cell Mol Physiol 284:L945–L954, 2003—Studies of regulated mucin secretion from goblet cells in primary cultures of human bronchial epithelial (HBE) cells have suffered, generally, from poor signal-to-noise ratios, with reported secretory responses of <100% (less than onefold) relative to baseline. Using, instead, HBE cells grown as xenografts in the backs of nude mice, we found that UTP (100 μM) stimulated strong mucin secretory responses from isolated, luminally perfused preparations. The peak response (10 min) for 11 control experiments (37 xenografts) was 3.3 ± 0.5-fold relative to baseline, and the time-integrated response (60 min) was 23.4 ± 0.5-fold. Because responses to ATP and UTP were approximately equal, an apical membrane P2Y2-receptor (R) is suggested. Additionally, ADP activated mucin release from HBE xenografts, whereas UDP and 2-methylthio-ADP did not, a pattern of response inconsistent with known purinoceptors. Hence, either a novel receptor to ADP is suggested or there is significant conversion of ADP to ATP by ecto-adenylate kinase activity. Adenosine and a nitric oxide donor were without effect. Consistent with P2Y2-R coupling to phospholipase C, HBE xenografts responded to ionomycin and PMA; however, they were recalcitrant to forskolin and chlorophenylthio-cAMP, and to 8-bromo-cGMP. Hence, human airway goblet cells, like those of other species, appear to be regulated primarily via phospholipase C pathways, activated particularly by apical membrane P2Y2-R agonists.

mucus; purinergic regulation; purinoceptor; intracellular messengers

IMPROVEMENTS IN AIRWAY EPITHELIAL cell culture techniques over the past several years have enabled rigorous studies of regulated mucin secretion from superficial epithelial goblet cells in a variety of species (see Refs. 12, 30, 55). These efforts have led to the realization that ATP and UTP acting at apical membrane P2Y2 receptors provide a principal pathway for the regulation of mucin secretion from the superficial epithelium in the airways of all species studied to date; no other G protein-coupled receptor (GPCR) agonist has been implicated, consistently, in stimulating mucin secretion (13, 24, 30). Given the potential of artifact resulting from the use of cell culture models, however, it is vital that these results be verified in native tissue to ensure biological and clinical relevancy. On this point, the available data are sparse: the only studies testing the effects of purinergic agonists against goblet cells from native tissues are those from our laboratory, which employed explants of isolated superficial epithelium from canine trachea and human nasal turbinates, using video microscopy to assay for mucin secretory activity (14, 36).

Airway epithelial cells grown in denuded tracheas as xenografts have been used for many years, originally to study tumor induction (5) and to determine the differentiation potential of airway epithelial progenitor cells (9, 25). Additionally, xenografts have also proven useful more recently in providing human airway epithelia for studies related to cystic fibrosis and gene therapy (e.g., Refs. 16, 38). In this technique, the lumens of tracheas denuded of native cells by freeze-thawing are seeded with airway epithelial cells, and the trachea is implanted subcutaneously as a tracheal graft in syngeneic rats or mice or as a tracheal xenograft in an immune-compromised host, commonly a nude mouse. The grafted trachea is revascularized by the host, and the epithelial progenitor cells within multiply and develop into a mature epithelium under the influence of growth and differentiation factors provided by the host. In this study, we used tracheal xenografts bearing differentiated human bronchial epithelial cells to test the mucin secretory responses of human goblet cells to a variety of purinergic agonists and other secretagogues characteristically active in the airways. These data may be useful in establishing a surrogate “gold standard,” against which results from primary cultures of airway epithelial cells from humans and other species might be compared.

MATERIALS AND METHODS

Materials. Purinergic agonists were purchased from Boehringer-Mannheim (Indianapolis, IN), culture medium was purchased from Gibco-BRL (Gaithersburg, MD), and the supplements were from Collaborative Research (Bedford, MA). Ionomycin and PMA were purchased from Calbiochem...
REGULATION OF HUMAN AIRWAY GOBLET CELLS

Cell culture and tracheal xenografts. SPOC1 cells were grown as described previously (3). Human bronchial epithelial (HBE) cells were isolated under the auspices of Institutional Review Board-approved protocols as described in detail previously (40, 41) from freshly excised human bronchi from an individual with cystic fibrosis. The isolated cells were grown in primary culture on plastic in bronchial epithelial cell growth medium (BEGM) (20), harvested at 70–80% confluence, frozen in aliquots, and stored in liquid N₂. At intervals, vials were thawed, passage 1 cells were expanded on plastic in BEGM and harvested at 70–80% confluence, and passage 2 cells were seeded into denuded tracheas at ~1 × 10⁶ cells/graf in a volume of 50 μl. The tracheas were harvested from 5-day-old chickens and were denuded of indigent cells by three freeze-thaw cycles separated by luminal PBS washes. The tracheas were cannulated at each end with short lengths of polyethylene (PE) tubing and supported with a stint formed by tying a length of PE tubing to the cannulas. Batches of prepared tracheas were frozen until use. After removing the endothelium and the extrinsic staining patterns, the grafts subcutaneously into the backs of athymic nu/nu BALB/c mice (see Ref. 47) and harvested them after a 3-wk incubation. All animal procedures were performed under Institutional Animal Care and Use Committee-approved protocols.

At harvest, the xenograft lumens were gently flushed with 1 ml of PBS and mounted horizontally for perfusion in a bath of DMEM/F-12. The bath and the affluent tubing flowed in a water bath (37°C), and the xenografts (internal volume ~50 μl) were perfused with DMEM/F-12 at 50 μl/min. The organ and water baths were covered with a clear plastic sheet, and they, and the perfusion medium, were bubbled with 5% CO₂, 95% air. After a 2-h equilibration, the perfusates were collected with a fraction collector, and 5-min fractions were assessed for mucins.

Mucin assays. Mucins secreted by SPOC1 cells were detected in microtiter plates by an SBA ELLA as described previously (3).

Monoclonal antibody production. A monoclonal antibody (MAb), H6C5, was generated against human mucins purified from sputum collected from a patient with cystic fibrosis. From 9.65 g of sputum solubilized in 6 M guanidine·HCl (with EGTA and protease inhibitors), 22 mg of mucins were purified by CsCl density gradient centrifugation as described previously (3). The material was relatively free of DNA, as assessed for optical density (490 nm) and the mucin content/well was calculated and expressed, as above.

Histology. Paraffin blocks of human bronchi fixed in 4% paraformaldehyde (immunostaining) or 10% neutral-buffered formalin (PAS and periodic acid, biotin-hydrazide (PABH) staining) were cut in 8-μm sections and deparaffinized by standard techniques. For immunostaining, sections were blocked with 5% goat serum in PBS, incubated with or without (control) H6C5 MAb, washed, incubated with AutoProbe alkaline phosphatase-conjugated goat anti-mouse IgG secondary antibody (Biomedia, Foster City, CA), washed, counterstained, and coverslipped. For PABH staining, sections were oxidized for 30 min in 22 mM periodic acid, rinsed, incubated in sodium metabisulfite in acetate buffer, rinsed, blocked with avidin and biotin (Avidin/Biotin Blocking kit; Vector Laboratories, Burlingame, CA), rinsed, incubated 60 min with biotin hydrazide (33 mM) plus 1 μl of HRP streptavidin (Vector Laboratories), rinsed, reduced with sodium borohydrate, and rinsed. The stain was developed with diaminobenzidine tetrahydrochloride (Sigma), followed by a rinse, counterstaining, and coverslipping. Sections were also stained with PAS by standard techniques. All slides were counterstained with hematoxylin (Bluing Reagent; Richard Allen, Kalamazoo, MI).

RESULTS

Airway epithelial cells grown in xenografts. SPOC1 cells were used to test whether the higher degree of differentiation achieved by growing the cells in xenografts, versus culture (see Ref. 47), is accompanied by a more robust mucin secretory response. Figure 1 shows mucin standards were bound to 96-well, high-binding microtiter plates (Costar no. 3590) overnight at 4°C. After being washed with PBS containing 0.05% Tween 20 and 0.02% Thimerosal (PBS-T), the plates were blocked with 5% dry milk in PBS-T, incubated with H6C5 for 1 h at 37°C or overnight at 4°C, washed in PBS-T, incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1 h, 37°C), washed in PBS-T, and developed during a 15-min incubation in 0.04% wt/vol of the substrate O-phenylenediamine (OPD) in 0.0175 M citrate-phosphate buffer, pH 5.0, containing 0.01% hydrogen peroxide. The reaction was stopped with the addition of 4 M sulfuric acid, optical density at 490 nm determined in a microtiter plate reader (model MR5000; Dynatech, Chantilly, VA), and the mucin content (ng) of each well was calculated with a standard curve for purified human mucins constructed on each microtiter plate.

Periodic acid, biotin-hydrazide assay. To validate the results obtained with H6C5, we developed a periodate staining procedure suitable to small volumes in a microtiter plate format. Key to the reaction was the substitution of Schiff’s reagent, commonly used in periodic acid-Schiff (PAS) staining, and developed during a 15-min incubation with the aldehyde-reactive reagent hydrazide. One hundred-microliter samples of perfusate and mucin standards were bound to 96-well, high-binding microtiter plates as above. All remaining steps in the procedure were performed at room temperature. The plates were washed four times with PBST, oxidized with 1 mM periodic acid (100 μl) for 10 min in the dark, and then incubated for 45 min with an additional 50 μl biotin-conjugated hydrazide (0.1 mM) containing 1.0 mM sodium metabisulfite. After four 5-min washes in PBST, the plates were incubated with streptavidin-conjugated HRP (15,000 in PBST, 100 μl/well) for 20 min and then washed again with four changes of PBST. Lastly, the plates were developed with OPD substrate solution, the reaction was stopped with 4 M H₂SO₄, the reactions were assessed for optical density (490 nm), and the mucin content/well was calculated and expressed, as above.

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Fig. 1. Mucin secretory response to UTP (100 μM) in xenografts populated with SPOC1 cells. In this and subsequent mucin secretory time courses, each point represents the mucins released (means ± SE, n = 3) at the indicated times for the perfused xenografts. Mucin secretion for this and most such studies with HBE xenografts was normalized to the mean of the 30-min baseline values. Inset: mucin secretory response of SPOC1 cells grown in culture. Normalized data calculated from the original presented in Ref. 3 (n = 6).

The time course of mucin release elicited by UTP (100 μM) for three SPOC1 cell xenografts. For comparison, the figure also shows our original data derived from UTP-stimulated SPOC1 cells in culture (3), recalculated to normalize the data to baseline. The difference between the two response patterns is notable. The xenograft-grown SPOC1 cells exhibit a mucin secretory response that rose quickly to a peak and then declined in a manner we have recorded with native goblet cells of epithelial explants from canine trachea (13) and from human turbinates (36) and as we show below for HBE xenografts. The decline in mucin secretion likely reflects a receptor desensitization phenomenon and/or depletion of mucin stores. SPOC1 cells grown in culture, in contrast, are stimulated by UTP in an essentially undiminished pattern of release (with ATP, as shown in Fig. 7 of Ref. 3, there was literally no sign of a decline). In addition to an improved waveform, the magnitude of the xenograft-grown SPOC1 cell mucin secretory response was greater relative to cells grown in culture: respectively, the peak responses were 10.6- vs. 3.5-fold relative to baseline (onefold = 100% above baseline), and the integrated responses (1 h) were 59.3- vs. 34.2-fold. Hence, not only do SPOC1 cells grown in xenografts have a more robust goblet cell phenotype than those grown in culture, the time course and magnitude of their mucin secretory response to purinergic agonists are also superior.

HBE cells seeded into denuded tracheas repopulated the grafts and differentiated into a mature mucociliary epithelial phenotype over the 3-wk period of incubation in the backs of nude mice (Fig. 2). The epithelium was pseudostratified, and the columnar cells were predominantly ciliated, but as shown in Fig. 2, inset, there were also plentiful PAS-positive goblet cells. Notably, at harvest, the lumens of the HBE xenografts contained a compact mucous-like plug. After expression by syringe with 1 ml of PBS and solubilization in 6 M guanidinium hydrochloride, this material resolved as a single band of low mobility on agarose blots stained with PAS, which, retrospectively, also stained with mucin-specific antibodies to MUC5AC and MUC5B (data not shown).

H6C5/PABH staining and mucin assays. During its development, the H6C5 MAb was selected on the basis of its avid binding of mucin in microtiter plate screening assays. When applied to paraffin sections of human airways, the MAb intensely stained goblet cells, including their secretory granules, and the ciliary border (Fig. 3), and mucous cells in submucosal glands (data not shown). The heavy staining of cilia by MAbs generated against intact mucins has been noted previously e.g., for 17Q2 (50). In the case of H6C5, the ciliary staining was apparently due to an extracellular epitope, since positive staining was achieved by the luminal application of the MAb to fresh tissue before fixation (data not shown).

The procedure for PABH staining is based on the reaction of hydrazide with the dialdehyde produced in carbohydrates susceptible to periodate oxidation. In this regard, it is similar to Schiff’s reagent; however, the use of hydrazide has two advantages that makes the PABH assay more sensitive than PAS. First, each dialdehyde reacts with two molecules of hydrazide, as opposed to one molecule of Schiff’s reagent. Second, hydrazide can be coupled to many other reagents that might be useful as markers of periodate oxidation. In the PABH assay, the use of biotin-hydrazide allowed an enzymatic amplification of the signal with HRP-conjugated streptavidin. Figure 3, C and D, compares the staining patterns for PAS and PABH in HBE. Both procedures stained goblet cells intensely and with good resolution view of the epithelium stained with periodic acid-Schiff (PAS) to identify goblet cells.

Fig. 2. Human bronchial epithelial (HBE) xenograft histology. Larger image is a cross-sectional view of a hematoxylin-eosin-stained, 3-wk-old HBE xenograft, ~3 mm in diameter, after the lumen was flushed with PBS. Note the uniform pseudostratified epithelium with ciliated border. Inset: higher-magnification view of the epithelium stained with periodic acid-Schiff (PAS) to identify goblet cells.
selectivity. The heavily stained ciliated border in the PABH-treated preparation is the only readily discernible difference between the two staining patterns and one that likely reflects signal amplification.

Figure 3 shows the relative sensitivities of the mucin binding assays developed by PABH staining and the H6C5 ELISA. Both assays were approximately linear over much of their respective ranges, and both were very sensitive for mucin with lower detection limits in the low nanogram per milliliter mucin range.

Response of HBE xenografts to UTP and ATP. After a 2-h equilibration perfusion and a 30-min basal secretion period, HBE cell xenografts responded to 100 μM UTP added to the perfusate with a vigorous increase in mucin secretion (Fig. 4). In this experiment, the samples collected at each 5-min time point were assessed for mucins by both the H6C5 and PABH assays. As shown, the apparent baseline level of mucin secretion detected by H6C5 was substantially higher than that detected by PABH: at 1,526.9 ± 74 ng/fraction the H6C5-detected material was 40% higher than the 915 ± 36 ng/fraction detected by PABH. The time courses of the UTP secretory response reported by the two assays, however, were very similar. In both cases, the mucins released from the epithelium peaked 10 min after the agonist challenge and then slowly declined to values near baseline over the next 40 min. In fact, when the mean baseline rates of secreted materials were subtracted from their respective datasets, the two curves essentially overlaid one another (Fig. 4, inset). This observation most likely indicates that H6C5 detects other materials in addition to polymeric mucins. Because the release of these other materials appears to be constant, however, they may emanate from sources other than the regulated secretory pathway, e.g., they may be secreted from constitutive pathways and/or are shed from the luminal surface. Despite this drawback, we chose to use the H6C5 ELISA for the practical advantage of a simpler procedure in testing the large number of samples necessary for the routine monitoring of HBE xenograft mucin secretory responses.

Important to our strategy of secretagogue challenges with limited numbers of preparations was the testing of each HBE xenograft for a UTP-elicited mucin secretory response as an internal control. To this end, we examined the ability of HBE xenografts to mount a secretory response over time with two sets of perfused HBE xenografts. Both sets received a standard 2-h equilibration perfusion, and fractions were then collected at 5-min intervals for 3 h. After establishing a 30-min baseline, we gave the first set of xenografts a 100 μM UTP challenge for 60 min, following which UTP was removed for a 30-min washout period. The
ATP (100 µM) stimulated mucin secretion from HBE xenografts with a time course and magnitude similar to that for UTP (data not shown). The ATP-induced peak response, $4.4 \pm 0.4$-fold over baseline ($n = 3$), occurred 10–15 min following agonist challenge before declining toward baseline values. UTP, added to the perfusate with ATP 45 min after the peak response to ATP, had no additional effect.

Effects of nucleotide diphosphates. Of the six known P2Y purinoceptors (R), three are specific to diphosphate nucleotides (Table 1), P2Y$_{1}$-R, P2Y$_{2}$-R, and P2Y$_{12}$-R. Figure 6 shows that, of the hallmark agonists for these purinoceptors, only ADP elicited a response from HBE xenografts. In this case, the peak response was approximately one-half that elicited by UTP and ATP, and UTP applied subsequently elicited a second, higher maximal response than did ADP. The response to ADP, with the lack of response to 2-methylthio (2-MeS)-ADP, is interesting and may indicate an extracellular adenylate kinase activity or a novel receptor (see DISCUSSION).

Effects of adenosine and nitric oxide. Adenosine and nitric oxide (NO) are major, local regulators in many physiological systems, and recent reports have suggested that these agents are involved in the regulation of airway epithelial cells. Adenosine, acting through A$_{2B}$ receptors, has been shown recently to be a major regulator of ciliary activity in human nasal epithelial cells (43) and of CFTR channel activity and Cl$^{-}$ transport in Calu-3 cells (24). NO has been implicated in many lung functions (e.g., 18), including the regulation of mucin secretion (4, 17, 52). Neither adenosine nor an NO donor, however, had detectable effects on mucin secretion from HBE xenografts (Fig. 7), whereas subsequent exposures of the same tissues to UTP elicited typical, peak secretory responses of 4.6- and 3.1-fold over baseline.

Intracellular messengers. Permeant analogs of intracellular messengers, or agents that pharmacologically mimic or stimulate cellular messenger production or release, were tested for their effects on mucin secretion from HBE xenografts. To test the potential effects of cAMP on mucin secretion, we used both forskolin and 8-(4-chlorophenylthio)adenosine 3’-,5’-cyclic monophosphate (cpt-cAMP), and for cGMP we used 8-bromo-guanosine 3’-,5’-cyclic monophosphate (8-Br-cGMP). As shown in Fig. 8, none of these reagents elicited a

Table 1. Human P2Y receptor summary

<table>
<thead>
<tr>
<th>IUPHAR Name</th>
<th>Principal Agonists</th>
<th>Partial Agonists</th>
<th>Antagonists</th>
<th>Effector System</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2Y$_{1}$</td>
<td>2-MeS-ADP &gt; ADP &gt; 2-MeS-ATP</td>
<td>ATP</td>
<td>A3P5P PPADS</td>
<td>G$_{q}$, PLC</td>
</tr>
<tr>
<td>P2Y$_{2}$</td>
<td>UTP &gt;&gt; ATP = UTP_8S &gt;&gt; ATP_8S &gt;&gt; 2-MeS-ADP &gt; $\alpha_{2}$$\beta$ Me-ATP</td>
<td>ATP</td>
<td>G$_{q}$, PLC</td>
<td></td>
</tr>
<tr>
<td>P2Y$_{3}$</td>
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<td>G$_{q}$, PLC</td>
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<tr>
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<td>ARL67656</td>
<td>G$_{q}$, PLC</td>
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</tr>
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</table>

Modified from Refs. 45 and 51. IUPHAR, International Union of Pharmacology; PPADS, pyrodoxal-phosphate-6-azophenyl-2’,4’-disulfonic acid; 2-MeS-ADP, 2-methylthio-ADP; AC, adenylyl cyclase.

Fig. 5. Effects of UTP on mucin secretion from HBE cell xenografts. After equilibration and collection of fractions during an initial baseline period, one set of HBE xenografts received a perfusate containing UTP (100 µM) for 1 h. The UTP was then washed out for 30 min and reintroduced. The other set of xenografts was exposed to UTP only during the second exposure for the first set. The data are expressed relative to the mean level of mucin secretion recorded during the first baseline period ($n = 3$, each).

The second set was perfused with control medium for this 120-min period. After collecting fractions to establish a second baseline, we then challenged both sets of HBE xenografts with 100 µM UTP for 60 min. As shown in Fig. 5, the mucin secretory responses elicited by the first UTP challenge to each set of HBE xenografts were very similar, despite the fact that one had been perfused for 90 min longer than the other: peak responses were $3.1 \pm 0.3$ and $3.3 \pm 0.3$-fold higher than baseline, and the relative integrated responses were $26.3 \pm 2.4$ and $21.7 \pm 1.4$-fold higher than baseline, respectively. This result shows the HBE xenograft to be a robust preparation capable of mounting a full mucin secretory response after 4 h of perfusion.

For the HBE xenografts receiving a UTP exposure twice, the 30-min washout period allowed between the two challenges was apparently too brief to allow the tissue to recover from the initial challenge. The weak response to the second challenge could have been due to a receptor desensitization phenomenon and/or to depletion of mucin granules during the initial challenge.
detectible mucin secretory response from HBE xenografts, whereas subsequent exposures to UTP elicited normal responses in each case. Notably, cpt-cAMP used at the same concentration does stimulate ciliary activity in small explants of HBE cells (43), showing that this compound does permeate airway epithelial cells as expected.

Most P2Y receptors couple to Gq, which activates PLC to release diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (45, 51), and activating either limb of this pathway resulted in a stimulation of mucin secretion (Figs. 9 and 10). Figure 9, top, shows the time course of the effects of the Ca2+ ionophore ionomycin (3 μM) on mucin secretion, and the bottom panel shows its concentration-dependent effects on the integrated response. Mobilizing intracellular Ca2+ caused approximately a 2.5-fold increase in peak mucin secretion activity in small explants of HBE cells (43), showing that this compound does permeate airway epithelial cells as expected.

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from human goblet cells at 3 μM, similar to its effects in SPOC1 cells (2). The ionomycin secretory response was long lasting and was concentration dependent, saturating above 3 μM. UTP, added at the end of the ionomycin exposures, stimulated mucin secretion above the plateau level following ionomycin. The degree of postionomycin UTP stimulation decreased with increasing ionomycin concentrations.

PMA, the DAG mimic, also stimulated mucin secretion from HBE goblet cells. Figure 10, top, shows the time course of the concentration having maximal effects (300 μM) on mucin secretion, and Fig. 10, bottom, shows its concentration effects on the integrated response. Overall, the effects of PMA were similar to those of ionomycin: the stimulatory effect was concentration dependent up to 300 nM and was long lasting, and UTP added after the 1-h PMA exposure was stimulatory, especially at the lower PMA concentrations tested. Exposing HBE xenografts to 1 μM PMA, however, appeared to be deleterious to both phases of the secretory response, i.e., mucin secretion elicited by PMA and by PMA plus UTP were substantially reduced over the levels realized by 300 nM PMA.

DISCUSSION

Mucin secretion from HBE cultures vs. xenografts. Purinergic stimulation of mucin secretion from HBE-passaged primary cultures has been reported previously (11, 37, 54). Although statistically relevant, the mucin secretory responses reported for these cultures lacked rigor, exhibiting increases relative to baseline of only 0.6- to 0.75-fold over a period of 2 h. In our own hands with highly differentiated HBE cultures (e.g., Refs. 40, 41), UTP-induced mucin secretion has been equally poor; mucin secretion has been plentiful during these experiments, but the increases in secretion obtained during UTP challenges have been trivial to undetectable (data not shown). By way of comparison, ATP and UTP induce mucin secretion from SPOC1 cells grown in culture or in xenografts with peak responses ~3- to 10-fold, relative to baseline, and 1-h integrated responses of ~30- to 60-fold (Fig. 1). An additional concern with HBE cultures is that they appear to be refractory to PMA (37), an agent that has been shown to stimulate mucin secretion in every other airway goblet cell model so far tested (2, 13, 28, 30, 33). At this point, it is not clear whether the apparently universal problem with HBE cultures is caused by poor handling during experiments, inadequate mucin assays, and/or inappropriate culture conditions. In any case, the weak responses reported for HBE cultures to date make it difficult to understand even the basics of agonist-induced mucin secretion from human airways, let alone the more complicated phenomena associated with inflammatory agents. Hence, we sought a more robust experimental model that might be used to resolve these issues and to this end turned to xenograft cultures incubated in the backs of nude mice. In this situation, not only do the cells differentiate under the influence of growth and differentiation factors offered by a mammalian host, but the precannulated xenografts proved optimal for a gentle dissection from the host and preparation for perfusion. As shown in Fig. 1, SPOC1 cells grown in xenografts where they achieve a more robust goblet cell phenotype (see Ref. 47) also responded in a more physiological manner to agonist than do cells grown in culture: the magnitude of the response was greater, and the time course typified that expected for an agonist-induced response.

The HBE xenografts proved remarkably robust during experiments: grafts perfused for a total of 4 h responded equally well to UTP as grafts perfused for 2.5 h before the UTP challenge (Fig. 5). Furthermore, the mucin secretory responses mounted by HBE xenografts to UTP were substantial. In the control experiments (Figs. 4 and 5) and all the agonist challenge experiments in which there was no response to the primary secretagogue (e.g., Fig. 6, middle and bottom), the peak response to 100 μM UTP was 3.3 ± 0.05-fold and the integrated response over 60 min was 23.4 ± 0.5-fold relative to baseline (n = 11, taking the mean data from each experiment, over a total of 37 xenografts). These data indicate a preparation more suitable for a pharmacological characterization of agonist signaling in human goblet cells than previously reported models.

1 The study of Yerxa et al. (54) reported mucin secretion as raw data (OD units). Without a standard curve, the multiplicity of the mucin secretory response in this study could not be calculated.
Responses of HBE goblet cells to purinergic agonists. Of the nucleotide triphosphate agonists tested, goblet cells in the HBE xenografts were responsive to both UTP (Figs. 4 and 5) and ATP (RESULTS). These results are in accordance with our previous findings with video microscopy showing that goblet cells in isolated epithelial explants of human turbinate epithelium were stimulated to degranulate by these same agonists (36). For the three nucleotide triphosphate purinoceptors (P2Y2-R, P2Y1-R, and P2Y11-R), the approximate equality of the mucin secretory responses by HBE xenografts to ATP and UTP are most consistent with a P2Y2-R-mediated response to ATP and UTP (see Table 1). These nucleotide triphosphates are full agonists at P2Y2-R, whereas P2Y1-R is activated by ATP and antagonized by ADP, and P2Y11-R is activated by ATP but not UTP (45, 51). Hence, on pharmacological grounds there is no need to hypothesize more than the expression of P2Y2-R in HBE goblet cells.

Of the nucleotide diphosphate agonists tested, human goblet cells were responsive to ADP and unresponsive to UDP and 2-MeS-ADP (Fig. 6). ADP activates P2Y1-R and P2Y12-R; however, both of these purinoceptors are activated to an even greater degree by 2-MeS-ADP, an agonist to which the HBE xenografts were recalcitrant (Table 1). Interestingly, canine tracheal goblet cells observed by video microscopy underwent a partial degranulation in response to ADP but did not respond to 2-MeS-ATP, another full agonist at P2Y1-R (14). One explanation is that ADP may act as a partial agonist at a P2Y11, an ATP-selective purinoceptor that couples to both $G_q$ and $G_i$ (45, 51). Favoring this possibility is the rather low-grade response to ADP and the fact that ATP applied subsequently to HBE xenografts elicited an additional, sizable mucin secretory response (compare Figs. 5 and 6). Against the notion of P2Y11-R involvement in the ADP response, however, is its apparent basolateral localization in epithelial cells (44, 51). Another possibility is that the effects ascribed to ADP were in fact due to ATP generated from ADP plus phosphate by extracellular nucleotide/nucleoside metabolism. Support for this notion is offered by the recent finding of significant adenylate kinase activity in airway gland secretions and on epithelial cell surfaces (15). Hence, the mucin secretory response to ADP by both human and canine goblet cells is curious and needs to be examined further to distinguish between these two possibilities and that of a novel purinoceptor.

Given the lack of response by goblet cells of HBE xenografts (Fig. 7) and canine trachea (14) to adenosine, the cells appear to lack apical membrane adenosine receptors. As discussed below, these results are interesting in light of the strong response to adenosine we reported recently for ciliated cells (43). Adenosine and its analogs thus appear to be the only known purinergic agonists that stimulate ciliary activity (43) and fluid secretion into the airway lumen (24) without stimulating mucin secretion. This result therefore raises the possibility of an adenosine receptor-based therapy to stimulate mucociliary clearance in airway obstructive diseases. Unfortunately, it also has the limiting caveats that inappropriately high levels of adenosine can elicit airway bronchospasm and inflammatory responses, including goblet cell metaplasia (6, 22, 42).

Intracellular messenger systems in HBE goblet cells. The lack of response by HBE xenografts to permeant cyclic nucleotide analogs and forskolin (Fig. 8) suggests that mucin secretion from human goblet cells is not regulated by agonists or other factors whose effects are mediated by these cellular messenger systems. These results are consistent with the negative results obtained with adenosine and S-nitroso-N-acetyl-penicillamine, the NO donor (Fig. 7), since adenosine effects are generally mediated by adenylate cyclase and cAMP and since NO effects are mediated by soluble guanylate cyclase and cGMP. Additionally, the results are consistent with the lack of response by goblet cells in human turbinates and by SPOC1 cells to cyclic nucleotides (2).

Again, like human turbinate goblet cells and SPOC1 cells (2), HBE xenografts responded robustly to challenges with a Ca$^{2+}$ ionophore (ionomycin; Fig. 9) and a PKC activator (PMA; Fig. 10). Although the number of HBE xenografts available for these studies was limited, we were able to demonstrate a concentration dependency in the goblet cell secretory response for both ionomycin and PMA. Hence, these results suggest that mucin secretion in human goblet cells is regulated by cellular messengers generated by PLC.

The results with PMA deserve special comment in light of those recently derived from SPOC1 cells (1). This study showed that PMA activated PKC maximally at 30 nM, whereas mucin secretion was stimulated at levels up to 300 nM, suggesting a PKC-independent effect of PMA. PMA has been suggested to activate other C1 domain proteins in cells (29), a prime candidate for which is MUNC13, an obligate accessory protein to the exocytic complex (10) that we found was expressed in SPOC1 cells (1). Interestingly, HBE xenografts were stimulated nearly threefold more at 300 nM PMA than at 100 nM, the concentration chosen to ensure that PKC was activated maximally (Fig. 10). Hence, this result suggests that mucin secretion in human goblet cells is regulated by cellular messengers independent of PLC.

A variety of studies have implicated NO and/or cGMP in stimulating mucin secretion from primary cultures of guinea pig tracheal (4, 17, 52) and HBE cells (37). These results are at variance, however, with those presented herein with HBE xenografts (Figs. 7 and 8) and with our previous results from SPOC1 cells (2), both of which showed NO- and/or cGMP-active agents to be without effect. Mucin secretion from submucosal glands is also either unaffected or inhibited by NO (8). One general problem with the studies claiming NO/cGMP responsiveness is that the cultures used exhibited relatively low secretory responses (less than onefold relative to baseline; see above), which can easily hinder data analysis. A more specific problem was apparent in the studies with HBE cultures (37) in
which PMA (100 nM) and 8 Br-cGMP (1 mM) elicited a small, 2-h, integrated mucin secretory response (of \( \sim 1.1 \)-fold) only when used in combination. When used separately, neither reagent elicited mucin release. In the present study with HBE xenografs, by contrast, 100 nM PMA alone caused a large, 1-h, integrated mucin secretory response of 5.3 \( \pm 1.9 \)-fold \((n=4)\) relative to baseline, and the response increased to 14.4 \( \pm 4.2 \)-fold \((n=5)\) at 300 nM PMA (Fig. 10). Hence, activation of PKC by PMA, alone, appears sufficient to stimulate mucin secretion from human goblet cells. This result is consistent with the effects of PMA in eliciting mucin release from SPOC1 cells (2) from primary cultures of hamster (28) and feline (33) tracheal epithelial cells and from a variety of other mucin-secreting cells (e.g., 19, 23, 32). In light of these considerations, the roles of NO and cGMP in the mucin secretory responses of HBE and other airway cultures may need to be reconsidered.

**Goblet cells and purinergic signaling in the airways.** Two important questions raised by the original study indicate the importance of purinergic agonists in airway signaling (39): what is the source of ATP in the airway lumen, and how are the different cell types regulated differentially from one another? After some 12 years, it is now clear that cells in and out of the nervous system release ATP and UTP (7, 21) and metabolize it in the extracellular space (55), including airway epithelia (15, 35, 46). Outside of the nervous system, these agonists and their active metabolites generally work through P2Y purinoceptors and have local actions. In the lumen of the airways, they appear to regulate mucociliary clearance (31). P2Y\(_2\)-R appears to be a principal receptor on both ciliated and goblet cells, at which ATP and UTP stimulate Cl\(^-\) and fluid secretion (26, 39), ciliary activity (43), and mucin secretion from goblet cells (Ref. 36; Fig. 4). More pertinent to the discussion is the role of nonnucleotide triphosphate receptors. UDP (P2Y\(_{6}\)) and adenosine (A\(_2B\)) both stimulate increases in Cl\(^-\) and fluid secretion from ciliated cells (34), as well as ciliary activity (43), but these agonists have no effect on goblet cells (Figs. 6 and 7). For goblet cells, ADP may be a mucin secretagogue (Fig. 6), perhaps acting through a novel purinoceptor or in conjunction with an ecto-adenylate kinase, but the agonist has no apparent direct effects on ciliary cell function (43). Hence, the luminal metabolites of UTP and ATP may regulate ciliary and goblet cell function independently. These actions are likely to be controlled by the local rates of ATP and UTP secretion into the lumen, depth of airway surface liquid, rates of free and surface-active extracellular nucleotide metabolism and uptake mechanisms, and the availability of appropriate purinoceptors in the vicinity of agonists. Thus mucociliary clearance and its local regulation represent dynamic phenomena, for which continued investigations into specific signaling mechanisms and therapies promise to yield interesting academic and clinical possibilities.

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