Development and characterization of secretin-stimulated secretion of cultured rat cholangiocytes

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Departments of 1Internal Medicine and 2Medical Biochemistry and Genetics, 3Division of Research and Education, 4Medical Physiology, Scott and White Hospital and Texas A&M University System, Health Science Center, College of Medicine and 5Central Texas Veterans Health Care System, Temple 76504; 6Department of Gastroenterology, University of Ancona, Ancona, Italy; and 7University of Texas, Houston, Texas 77030

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Alpini, Gianfranco, Jo Lynne Phinizy, Shannon Glaser, Heather Francis, Antonio Benedetti, Luca Marucci, and Gene LeSage. Development and characterization of secretin-stimulated secretion of cultured rat cholangiocytes. Am J Physiol Gastrointest Liver Physiol 284: G1066–G1073, 2003. First published January 22, 2003; 10.1152/ajpgi.00260.2002.—We sought to develop a cholangiocyte cell culture system that has preservation of receptors, transporters, and channels involved in secretin-induced secretion. Isolated bile duct fragments, obtained by enzyme perfusion of normal rat liver, were seeded on collagen and maintained in culture up to 18 wk. Cholangiocyte purity was assessed by staining for γ-glutamyl transpeptidase (γ-GT) and cytokeratin-19 (CK-19). We determined gene expression for secretin receptor (SR), cystic fibrosis transmembrane conductance regulator, Cl−/HCO3− exchanger, secretin-stimulated cAMP synthesis, Cl−/HCO3− exchanger activity, secretin-stimulated Cl− efflux, and apical membrane-directed secretion in polarized cells grown on tissue culture inserts. Cultured cholangiocytes were all γ-GT and CK-19 positive. The cells expressed SR and Cl−/HCO3− exchanger, and secretin-stimulated cAMP synthesis, Cl−/HCO3− exchanger activity, and Cl− efflux were similar to freshly isolated cholangiocytes. Forskolin (10 μM) induced fluid accumulation in the apical membrane of tissue culture inserts. In conclusion, we have developed a novel cholangiocyte line that has persistent HCO3−, Cl−, and fluid transport functions. This cell system should be useful to investigators who study cholangiocyte secretion.

bicarbonate secretion; bile flow; intrahepatic bile ducts; adenosine 3’,5’-cyclic monophosphate; secretin receptor

THE PRIMARY FUNCTION OF CHOLANGIOCYTES lining the intrahepatic bile ducts is to secrete a bicarbonate-rich bile in response to the hormone secretin (20, 39). Isolation of cholangiocytes or isolated intrahepatic bile duct units (IBDU) fragments from rats or mice has been used successfully by multiple investigators, but these techniques are cumbersome, due to high expense and low yield of cells (3, 9, 18, 19, 24, 30, 34, 38). Furthermore, in vitro studies (1, 5, 13, 28) with freshly isolated cells or IBDU fragments are limited by the lack of long-term (>24 h) viability of these cell preparations. Cholangiocarcinoma cell lines have been also employed successfully, but they suffer from the potential of undesired results due to study of undifferentiated cells (29, 31, 33, 35). Other investigators (1, 6, 7, 15) have successfully isolated and cultured normal rat intrahepatic bile duct cells, but secretory function characteristic of cholangiocytes (e.g., secretin-stimulated cAMP synthesis, Cl−/HCO3− exchanger activity, and functional chloride channels) and receptor complement (secretin receptors [SRs]) were not documented. Therefore, the aims of these studies were to develop a bile duct epithelial cultured cell system and to completely characterize the transporters involved in the secretin-stimulated ductal secretory phenomenon. The methods for establishing primary culture of cholangiocytes employed the use of cultured IBDU fragments, as previously reported (1). With multiple passages, pure populations of cholangiocytes were obtained, demonstrated by both protein and gene expression. Morphological evaluation of primary cultured cholangiocytes showed polarized cells with features typical of biliary epithelium. These cells were essentially identical to freshly isolated cholangiocytes regarding SR gene expression, secretin-stimulated cAMP synthesis, secretin-stimulated by Cl−/HCO3− exchanger activity, secretin-stimulated chloride flux, and secretin-stimulated fluid excretion across the apical membrane.

MATERIALS AND METHODS

Materials. Reagents were purchased from Sigma (St. Louis, MO) unless otherwise indicated. Porcine secretin was purchased from Peninsula (Belmont, CA). The substrate for γ-glutamyltranspeptidase (γ-GT; N-(γ-L-glutamyl)-4-methoxy-2-naphthylamide) was purchased form Polysciences (War- rington, PA).

Isolation of cholangiocytes for primary culture. IBDU fragments were obtained from male 344 Fisher rats (Charles River Laboratories, Wilmington, PA), as we previously reported (1). Rats were anesthetized with pentobarbital (50 mg/kg body wt) and the portal vein was cannulated and preperfused at 37°C with 250 ml of oxygenated buffer A [(in

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CULTURED RAT CHOLANGIOCYTES

We used the following \[^{32}P\]UTP-labeled single-stranded antisense riboprobes: a 345-bp riboprobe encoding for the sequence of the albumin gene was transcribed from pGEM4Z-albumin 345 (a gift of D. Shafritz, Albert Einstein Hospital, Bronx, NY); a 157-bp riboprobe encoding for the message for rat γ-GT was transcribed from rat pGEM4Z-γ-GT (M-N Chobert, Créteil, France); a 350-bp riboprobe encoding for the message of the rat CK-19 gene was generated from pBluescript CK-19 (a gift from A. Quaroni, Ithaca, NY); a probe 316-bases long, encoding sequences complementary to rat GAPDH mRNA, was obtained from Ambion; a 318-bp riboprobe encoding the message for SR was transcribed from pGEM4Z-SR (a gift of Dr. N. F. LaRusso, Mayo Clinic, Rochester, MN); and a 348-bp riboprobe encoding the message for Cl\(^{-}\)/HCO\(_3\)\(^{-}\) exchanger was transcribed from pGEM4Z-Cl\(^{-}\)/HCO\(_3\)\(^{-}\} exchanger (a gift of Dr. N. F. LaRusso, Mayo Clinic, Rochester, MN).

Intracellular cAMP levels. Spontaneous and secretin-stimulated intracellular cAMP levels in cultured cholangiocytes were determined as previously described (7). Cultured cholangiocytes were stimulated with 0.2% BSA (basal) or secretin (10 \(^{-8}\} M\) for 5 min at room temperature. After extraction with ethanol, cAMP levels were determined by a commercially available kit (Amersham) according to the instructions of the manufacturer. Intracellular cAMP levels were expressed as femtomoles per 100,000 cells.

Secretary activity of cultured cholangiocytes. Secretary activity was measured by 1) Cl\(^{-}\)/HCO\(_3\)\(^{-}\} exchanger activity, 2) rate of net apical Cl\(^{-}\} efflux and influx, and 3) accumulation of fluid secreted across the apical membrane in cultured cholangiocytes obtained from passages 10-20. The Cl\(^{-}\}/HCO\(_3\)\(^{-}\} exchanger activity was measured from the rate of intracellular alkalization in response to the removal of chloride from the media, as previously described (6). Cultured cells grown on coverslips were loaded with pH sensitive dye BCECF-acetoxymethyl ester (1 mM) for 10 min at 37°C and transferred to a perfusion chamber on the stage of a Nikon fluorescence microscope equipped with Omega optical quantitative fluorescence filter set. Intracellular pH of the cholangiocytes was measured by alternating excitations of 490 and 440 nm by a motor-driven rotating filter wheel (Ludh Electronic Products, Hawthorne, NY). The fluorescence over the cholangiocytes was measured by a single-photon-counting photo-multiplier tube (Hamamatsu, Hamatsu City, Japan). The cells were superfused with Krebs-Ringer-Henseleit buffer (pH 7.4) containing 0.7% (basal) albumin, secretin (10 \(^{-7}\} M\) with 0.2% albumin for 5 min. In some studies, cultured cholangiocytes were pretreated with 1 mM DIDS for 30 min before studies, which has previously been shown to inhibit cholangiocyte Cl\(^{-}\}/HCO\(_3\)\(^{-}\} exchanger activity (23). Chloride was then abruptly removed and replaced with equal amounts of gluconate. Cl\(^{-}\}/HCO\(_3\)\(^{-}\} exchanger activity was determined from both the overall increase in pH, and the rate of increase in pH as the gradient-induced chloride efflux from cells was exchanged for bicarbonate influx, resulting in the alkalization of the cells.

Cl\(^{-}\} permeability of apical membranes of confluent cholangiocytes monolayers cultured on tissue culture inserts was estimated by the measurement of intracellular N-ethoxycarbonylmethyl-6-methoxyquinolinium bromide (MQAE) fluorescence. MQAE, with its high Cl\(^{-}\} sensitivity, has been used successfully to measure intracellular Cl\(^{-}\} concentration ([Cl\(^{-}\}]) in various cell types (14, 25). Cells were loaded with 1 mM MQAE in a solution containing (in mM) 101 Cl\(^{-}\}, 5 HEPES, 0.8 MgSO\(_4\}, 1.0 NaHPO\(_4\}, 5.6 glucose, 1.8 Ca acetate, 96 NaCl, 5.3 KCl, 50 mannitol, and 22 NaHCO\(_3\}, plus 10% NCS at pH 7.4 for 2 h at 37°C. The cells were then washed...
three times with the same solution (without MQAE) to remove MQAE and the serum, and were then left for 10 min before measurements were started. MQAE fluorescence intensity was measured by using excitation and emission wavelengths of 360 and 460 nm, respectively. Cl− quenches MQAE in its excited state. Changes in MQAE fluorescence intensity, therefore, inversely reflect changes in [Cl−]. At the end of an experiment, the monolayer was perfused with KSCN (120 mM) solution (buffered with 10 mM HEPES-KOH, pH 7.2), which quenched MQAE fluorescence by >90% (14, 25). For data analysis, fluorescence (F) at each time interval was divided by the KSCN-quenched F value (F0). We quantitatively compared the effects of secretin (10−7 M) or BSA (control) on the rate of net apical Cl− efflux and influx.

Relative rates of Cl− influx and efflux were computed from the time course of intracellular fluorescence and were expressed as relative change in fluorescence by using the equation: (ΔF/Δt)/(F0·min−1), where ΔF/Δt is the initial rate of fluorescence change on the addition or removal of Cl−. Efflux or influx of Cl− across the apical membrane was assessed by the removal and addition of Cl− to the apical solutions, respectively. The cell monolayer was first perfused with NaCl solution in both apical and basolateral compartments. While MQAE fluorescence of the monolayer was recorded, the apical Cl− was replaced by equal molar gluconate solution and 5 min later was then replaced by an NaCl solution. In some studies (8), cultured cholangiocytes were pretreated for 20 min with 10 μM 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB), which has been shown to block Cl− channels in cholangiocytes.

Fluid secretion was measured in cultured monolayers by two independent techniques. In the first technique, the diameter of closed spaces between cholangiocytes cultured in monolayer was observed under a phase contrast microscope before and at 5-min intervals after the addition of either forskolin 10−4 M in 0.2% albumin or 0.2% albumin control. The change in volume of closed space was calculated as

\[ \frac{\Delta V}{\Delta t} \]

where \( \Delta V/\Delta t \) is the initial rate of volume change (J/min) and V is the volume of the closed space.

RESULTS

After 4 days, the IBDU fragments developed into cystic structures. Two weeks later, IBDU fragments were passed onto collagen cell culture plates. After 5–10 passages, the cholangiocytes began to proliferate to form a complete monolayer. At this point, the cells appeared morphologically homogeneous under phase contrast microscopy (Fig. 1) and absence of contaminating fibroblasts was also observed. The purity of cholangiocytes was assessed by immunohistochemistry for CK-19, a cholangiocyte-specific marker (27). One hundred percent of the cultured cells were CK-19-positive (Fig. 2), which is consistent with a cholangiocyte origin. In addition, the cultured cholangiocyte failed to stain for vimentin (Fig. 2), a marker for Kupffer cells (26).

Cholangiocytes, when seeded on collagen-coated cell inserts, proliferated to confluence over a 1-wk period. At confluence, the transepithelial resistance was 634 ± 124 Ω·cm². The ultrastructural morphological evaluation of cultured cholangiocytes grown on membranes show polarized epithelial cells with surface microvilli and nuclei closely adjacent to the membrane (Fig. 3). Golgi and submembrane vesicles were closely adjacent to the membrane containing microvilli (Fig. 2). Morphological features are consistent with polarized epithelial structure, with the cholangiocyte basolateral membrane adjacent to the culture membrane and the apical membrane opposite the culture membrane. The morphological features were very similar to cholangiocytes observed in situ.

SR gene expression and secretin-stimulated cAMP synthesis. The characteristic feature of cholangiocytes compared with the remainder of cells in the liver is the presence of SR, CFTR, Cl−/HCO3− exchanger, and secretin-stimulated cAMP synthesis (37). Cultured cholangiocytes express the message for SR, CFTR, and Cl−/HCO3− exchanger, and expression of the selected messages did not vary in passages 9, 13, and 25 (Fig. 4). Cultured cholangiocytes also express the transcript for γ-GT and CK-19 (two cholangiocyte-specific markers) and GAPDH (the housekeeping gene) but were negative for albumin mRNA (a marker for hepatocytes) (Fig. 4). Basal cAMP levels of cultured cholangiocytes were similar to that previously reported (5) in freshly isolated cholangiocytes (Fig. 5). When stimulated with secretin (10−7 M for 10 min), cAMP levels in cultured cholangiocytes increased by almost fivefold (Fig. 5). This increase is similar in magnitude to the secretin-stimulated cAMP synthesis we (5) previously observed in normal pooled or large cholangiocytes isolated from rat liver.
Cl⁻/HCO₃⁻ exchanger activity. Secretin stimulates a bicarbonate-rich ductal bile secretion. Bicarbonate secretion depends partly on the presence of a Cl⁻/HCO₃⁻ exchanger in cholangiocytes (10). We assessed Cl⁻/HCO₃⁻ exchanger activity in cultured cholangiocytes by measuring the change in intracellular pH in response to the removal of chloride. As shown in Fig. 6, when chloride is removed, there is intracellular alkalization due to the outward-directed movement of chloride in exchange for inward-directed bicarbonate. When culture cholangiocytes are stimulated with 10⁻⁷ M secretin for 10 min, the rate of alkalization in secretin-stimulated cholangiocytes is significantly greater than unstimulated cholangiocytes (0.43 ± 0.08 vs. 0.18 ± 0.04 pH U/min, P < 0.05). Similarly, the total magnitude of the increase in intracellular pH was greater in secretin-stimulated cholangiocytes compared with controls (0.44 ± 0.09 vs. 0.19 ± 0.05 pH units, P < 0.05).

Pretreatment with 1 mM DIDS inhibited (P < 0.05) the rate of alkalization in unstimulated and secretin-stimulated cholangiocytes (0.03 ± 0.08 and 0.05 ± 0.04 pH U/min, respectively). Data show the presence of a functioning secretin-stimulated Cl⁻/HCO₃⁻ exchanger in our cultured cholangiocyte system.

Secretin-stimulated chloride channel activity. Chloride channel activity appears to be required for the
generation of ductal secretion by cholangiocytes (32). Chloride influx and efflux across the apical membrane in cholangiocytes monolayer in cell culture inserts were assessed by measuring the rate of change of MQAE fluorescence during the removal and addition of chloride to the upper (apical) chamber. In cholangiocytes pretreated with secretin (10^{-7} M) for 5 min (compared to cholangiocytes treated with BSA control), there was an accelerated rate of increase of F/F_{0} after the removal of chloride and an accelerated rate of decreasing F/F_{0} after the restitution of chloride (Fig. 7). Data indicate that secretin increases the rate of both chloride influx and efflux across the apical membrane of cholangiocytes. In seven experiments, the rate of apical chloride influx was 0.08 ± 0.02 vs. 0.02 ± 0.01 fluorescence arbitrary units/min in secretin and BSA treated cholangiocytes, respectively (P < 0.05). Similarly, the rate of apical chloride influx was 0.07 ± 0.02 vs. 0.03 ± 0.01 fluorescence arbitrary units/min in secretin and BSA-treated cholangiocytes, respectively (P < 0.05). Pretreatment with the chloride channel inhibitor NPPB (10 μM) ablated the secretin-stimulated cholangiocyte apical membrane chloride efflux (NPPB; 0.02 ± 0.01 vs. control, 0.08 ± 0.02 arbitrary fluorescence units/min, P < 0.05) and the secretin-stimulated cholangiocyte apical membrane infl ux (NPPB; 0.02 ± 0.01 vs. control, 0.07 ± 0.02 arbitrary fluorescence units/min, P < 0.05).

Another characteristic of cholangiocytes is increased fluid secretion in response to increased intracellular
cAMP. As shown in Fig. 6, in monolayers of cholangiocytes, we occasionally observed cystic areas. When the cultured cholangiocytes were treated with forskolin, which directly stimulates cAMP synthesis, the average cystic area increased in diameter (34 ± 8.2 to 41 ± 6.1 μm), whereas in cultured cholangiocytes treated with BSA control, the average cystic area did not increase in diameter (29 ± 4.5 to 28 ± 6.1 μm) (Fig. 8).

In cholangiocytes on tissue culture inserts, as shown in Fig. 9, there was fluid secretion measured by the increase in weight of the insert after forskolin stimulation. The insert volume increased 9.7 ± 2.3 μl after stimulation with forskolin for 60 min at 37°C. In contrast, there was no increase in insert volume (loss of 4.4 ± 1.8 μl) with BSA control treatment (Fig. 9). There was a progressive increase in forskolin-treated insert volume at 2 and 3 h but no further increase at 24 h (Fig. 9). Findings of secretin-stimulated secretion in close spaces between cholangiocyte and forskolin-stimulated accumulation of fluid in cholangiocyte cell culture inserts are consistent with secretin-stimulated apical fluid secretion in our cholangiocyte cultured system.

DISCUSSION

Cholangiocytes are an important component of the liver, because they are major contributors to bile secretion (≤40% of the total bile flow in man and 10% in rats) (11). Cholangiocytes are the primary targets for specific liver diseases, such as primary biliary cirrhosis, primary sclerosing cholangitis, and rejection after liver transplantation (4). Understanding of the function and pathology of cholangiocytes has significantly increased in the last 15 yr, primarily due to the ability of investigators to isolate pure populations of cholangiocytes from experimental animals (2, 6, 13, 36). Further achievements have been partially impeded due to the difficulty and high expense to isolate cholangiocytes. Although other investigators (40) have previously established pure cultured cholangiocytes (referred to as normal rat cholangiocytes), the previous culture cholangiocyte systems have not been characterized by responses to secretin-induced secretion as we did in these studies. The present study expands the previous studies on cholangiocyte culture systems by the development of a new cultured system that expresses SR, CFTR, and secretin-stimulated cAMP synthesis, Cl⁻/HCO₃⁻ exchanger activity, chloride channel activity, and apical membrane-directed fluid secretion. These new culture cell systems should be useful to investigators who study secretory phenomenon in the biliary epithelium.

The primary culture of cholangiocytes utilized bile duct fragments isolated from liver by enzymatic digestion. It is likely that initiation of proliferating cholangiocytes in cell culture systems requires coculture with fibroblast (and/or other cell types) within the portal tract, because we have not had success in primary cultures of isolated cholangiocytes (data not shown). Similar to previous studies (40), noncholangiocyte cell populations are eliminated with successive passages. Cell purity was demonstrated, by using positive staining for CK-19, a cholangiocyte-specific marker and negative staining for vimentin and albumin. The latter would exclude even a small contamination from hepatocytes. In our cultured cholangiocyte cell system, SR, CFTR, and Cl⁻/HCO₃⁻ exchanger genetic expression did not vary among passages 8, 13, and 25, indicating that the cells do not become dedifferentiated with progressive passages. Because our cultured cholangiocytes are derived from >20 μm diameter IBDU frag-
ments, the cultured cells would be expected to originate from larger IBDUs, lined by large cholangiocytes, which we (5, 6) have previously shown to express SR, secretin-stimulated cAMP synthesis, Cl-\(^{-}/\)HCO\(_3^{-}\) exchanger activity, chloride channel activity, and proliferate after bile duct ligation in rats. Our cultured cells are unlikely contaminated with small cholangiocytes that lack SR, secretin-stimulated cAMP synthesis, Cl-\(^{-}/\)HCO\(_3^{-}\) exchanger activity, and chloride channel activity and do not respond the bile duct ligation (5, 6), because our primary culture material did not contain IBDU fragments smaller than 20 \(\mu\)m. Also consistent with a lack of cells derived from small cholangiocytes, morphological evaluation shows homogeneous cell size. Electron microscopy morphological analysis showed cultured cholangiocytes to be a polarized epithelium, essentially identical to the ultrastructure of cholangiocytes in situ when grown on collagen coated membrane inserts.

Secretin stimulation of cultured cholangiocytes secretion was demonstrated by multiple techniques and was found to be similar to that previously observed in freshly isolated cholangiocytes (5, 7). Each technique, however, evaluated a unique portion of cholangiocyte secretory function. It is currently believed that multiple transporters are responsible for hormone-stimulated ductal secretion (11, 17). The initial event of secretin stimulation of cholangiocyte secretion is increased intracellular cAMP synthesis (11, 17). SR expression, and increased secretin-stimulated intracellular cAMP levels in our cholangiocyte cultured system are quite similar to freshly isolated cholangiocytes (2). Increased cAMP is thought to activate apical chloride channels and Cl-\(^{-}/\)HCO\(_3^{-}\) exchanger in cholangiocytes (10). The Cl-\(^{-}/\)HCO\(_3^{-}\) exchanger is responsible for secretion of bicarbonate into bile and the chloride channels maintain exchanger activity by shunting cholangiocyte intracellular chloride back to bile (10). In our cholangiocyte system, secretin increased both Cl-\(^{-}/\)HCO\(_3^{-}\) exchanger activity and chloride channel activity (10). Our studies showed both secretin-stimulated apical chloride flux and Cl-\(^{-}/\)HCO\(_3^{-}\) exchanger activity in the cultured cholangiocytes. The secretin-induced increase in Cl-\(^{-}/\)HCO\(_3^{-}\) exchanger activity and chloride channel activity were ablated by specific chloride channel and Cl-\(^{-}/\)HCO\(_3^{-}\) exchanger inhibitors. Finally, consistent with fluid excretion by cultured cholangiocytes, we observed secretin-stimulated excretion into close spaces between cells in monolayer culture and increase in apical membrane-directed fluid excretion from polarized cholangiocytes cultured in collagen-coated cell inserts.

In summary, this study shows that intrahepatic cholangiocytes derived from large intrahepatic bile duct fragments isolated from normal rat liver can be successfully adapted to in vitro growth. The studies, for the first time, establish a cholangiocyte cell culture system that maintains all key elements of ductal secretion (SR expression, secretin-stimulated cAMP, chloride channel activity, Cl-\(^{-}/\)HCO\(_3^{-}\) exchanger activity, and apical membrane-directed fluid secretion. Development and characterization of cholangiocytes adapted to in vitro growth offers numerous advantages, including: 1) availability of unlimited number of cells, 2) ability to perform repeated experiments over long periods of time, 3) ability to manipulate cells in ways that is not possible in vitro, and 4) the ability to exchange cells among laboratories, allowing studies of identical materials. We are presently extending the methodologies described in this paper to the development of cholangiocytes culture derived from small intrahepatic bile ducts and establish a culture cholangiocytes derived system from models of hyperplastic bile ducts (e.g., bile duct ligated rats).

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