Isolation of functional polarized bile duct units from mouse liver

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Received 19 October 1999; accepted in final form 29 August 2000

Cho, Won Kyoo, Albert Mennone, and James L. Boyer. Isolation of functional polarized bile duct units from mouse liver. Am J Physiol Gastrointest Liver Physiol 280: G241–G246, 2001.—The development of genetically altered murine animals has generated a need for in vitro systems in the mouse. We have now characterized a novel isolated bile duct unit (IBDU) preparation from the mouse to facilitate such studies. The mouse IBDU is isolated by portal perfusion of collagenase, blunt dissection, further enzymatic digestions, filtering through sized mesh, and culturing on Matrigel for 16–72 h. This mouse IBDU forms a central, enclosed lumen lined by polarized cytokeratin-19-positive cholangiocytes with numerous microvilli on the apical membrane. The IBDU responds to secretory stimuli, including secretin, vasoactive intestinal peptide, IBMX, and forskolin, resulting in expansion of the central lumen from secretion as quantified by videomicroscopy. The secretory response to secretin is dependent on Cl− and HCO3− in the perfusate. These findings indicate that mouse IBDUs are intact, polarized, functional bile duct secretory units that permit quantitative measurements of fluid secretion from mouse bile duct epithelium for the first time. This method should facilitate studies of cholangiocyte secretion in genetically altered murine animal models. isolated bile duct unit; biliary secretion; quantitative videomicroscopy; enzymatic isolation

RECENT ADVANCES IN ISOLATION techniques for cholangiocytes and bile duct units have resulted in the rapid expansion of knowledge about bile duct epithelial cell biology and physiology (3, 6, 15, 19). However, most isolated cholangiocyte preparations have certain inherent limitations due to low cell yield and diminishing viability after isolation as well as the excessive labor and cost involved in the isolation procedures. Isolated cell preparations and established cholangiocyte cell lines also lose cell polarity and local cellular contacts, which are vital for various aspects of epithelial cell physiology such as ion transport and expression of cellular proteins. Cholangiocyte cell lines established from rats or humans also raise concerns about clonal selection and dedifferentiation, which may modify phenotypes and gene expression after multiple passages. Thus the use of these preparations raises questions about the validity of applying results to normal biliary physiology.

To overcome some of these problems, large intact polarized bile duct units were first isolated from pig (21) and rat (16) livers with microdissection techniques. These cell preparations were useful for studying intracellular pH regulation and permitted measurements of changes in luminal pH and Cl− concentration during secretin stimulation after microinjection of fluorescent pH and Cl− markers into the lumen (16). However, these microdissection methods are very labor intensive and time consuming and yield only a small number of isolated bile duct units (IBDUs). In addition, with these techniques it is difficult to isolate smaller interlobular IBDUs, which comprise a major portion of the biliary tree and are thought to represent the main “active” site for secretion as well as pathogenesis of various cholangiopathies (1, 4).

More recently, we (12) have developed from the rat liver a novel intact polarized IBDU, consisting of an enclosed lumen lined by polarized cholangiocytes. This isolation method eliminates the need for difficult and time-consuming microdissections and produces many functional and polarized IBDUs from small and medium (30–100 μm) rat liver bile ducts after several enzymatic digestion and mechanical separation steps (12). Previous studies (9, 10, 12) indicated that these IBDUs are an excellent functional in vitro model to study the effects of various agonists and inhibitors on biliary secretion from cholangiocytes. These novel IBDUs are the only intact, functional, polarized in vitro bile duct model that allows direct measurement of net fluid secretion with quantitative videomicroscopy in small-to-medium interlobular segments of the biliary tree (1, 12). Moreover, the lumen is large enough to be microinjected with cell-impermeant pH-sensitive 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF)-dextran to study changes in luminal pH (10, 12). Pop-
ulations of IBDU from different segments of the biliary tree with apparent functional and structural heterogeneities (1, 4) can also be studied by selecting different sizes of bile duct fragments during enzymatic digestion steps. However, for this IBDU model to be useful for the study of many genetically altered animals, an isolation method must be developed from mouse liver.

In this study, we now describe the first isolation method for preparation of IBDUs from mouse liver. This model should be useful in the study of various knockout mouse models now available that may affect cholangiocytes, including disorders such as cystic fibrosis.

MATERIALS AND METHODS

Materials. BSA, penicillin-streptomycin, EDTA, heparin, HEPES, D(+)-glucose, insulin, DMSO, hyaluronidase, DNase (DN-25), nigericin, amiloride, sodium glutconate, potassium glutconate, and hemicalcium glutconate were purchased from Sigma Chemical (St. Louis, MO). BCECF-AM was obtained from Molecular Probes (Eugene, OR). Vasoactive intestinal peptide (VIP) and secretin were purchased from Bachem (Torrance, CA). Collagenase D was from Boehringer Mannheim Biochemicals (Indianapolis, IN), and Pronase was from Calbiochem (San Diego, CA). Liebovitz L-15 medium, MEM, α-MEM, t-glutamine, gentamicin, and FCS were from GIBCO (Grand Island, NY). Monoclonal anticytokeratin-7 and -19 antibodies were from Amersham. All other chemicals were of the highest purity commercially available.

Solutions. The Krebs-Ringer bicarbonate (KRB), HEPES, and glutconate buffer solutions were composed as described previously (2, 20). All peptides were made up in perfusion buffer with 1% (wt/vol) BSA as a carrier.

Isolation of bile duct units. Male B6125 (Jackson Laboratory, Bar Harbor, ME) or C57BL6 (Harlan Laboratory, Indianapolis, IN) mice aged 4–8 wk were housed and allowed free access to water and Purina rodent chow (St. Louis, MO). Animal care and studies were performed in compliance with institutional animal care and use committee guidelines. Mice were anesthetized by intraperitoneal injection of pentobarbital sodium (50–100 mg/kg), as described previously in rats (2, 12). The portal vein was cannulated from Mission (St. Louis, MO). BCECF-AM was obtained from Molecular Probes (Eugene, OR). Vasoactive intestinal peptide (VIP) and secretin were purchased from Bachem (Torrance, CA). Collagenase D was from Boehringer Mannheim Biochemicals (Indianapolis, IN), and Pronase was from Calbiochem (San Diego, CA). Liebovitz L-15 medium, MEM, α-MEM, t-glutamine, gentamicin, and FCS were from GIBCO (Grand Island, NY). Monoclonal anticytokeratin-7 and -19 antibodies were from Amersham. All other chemicals were of the highest purity commercially available.

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Transmission electron microscopy was performed on IBDU preparations after 48 h (12, 20). Coverslips containing IBDUs were fixed in 2.5% glutaraldehyde and 1% paraformaldehyde in 0.1 M sodium cacodylate buffer for 4 h. After postfixation in 1% osmium tetroxide for 1 h, the mouse IBDUs were dehydrated in ethanol and embedded in Epon. Sections were stained with uranyl acetate and lead citrate before viewing and photographed with a Zeiss EM 910 electron microscope (Carl Zeiss, Thornwood, NY).

Quantitation of secretory response with videomicroscopy. IBDUs cultured overnight on Matrigel-coated glass coverslips were preincubated in KRB solution for 15–25 min before being placed in a thermostated specimen chamber on a microscope stage. Coverslips were scanned for 5–10 min to select relatively spherical IBDUs with sharp borders and without connections to other contiguous duct units. Video images of these IBDUs were obtained at 5-min intervals while maintaining the same focal plane at the maximum luminal area. Secretory responses in IBDUs were determined by measuring across-sectional luminal areas using a Zeiss IM 35 or Olympus IX-70 inverted microscope with Nomarski optics equipped with a cooled CCD video camera (Hamamatsu Photonics Systems) connected to a computer with image analysis software (Improvision, Boston, MA). Transmitted electron microscopy was performed on IBDU preparations after 48 h (12, 20). Coverslips containing IBDUs were fixed in 2.5% glutaraldehyde and 1% paraformaldehyde in 0.1 M sodium cacodylate buffer for 4 h. After post-fixation in 1% osmium tetroxide for 1 h, the mouse IBDUs were dehydrated in ethanol and embedded in Epon. Sections were stained with uranyl acetate and lead citrate before viewing and photographed with a Zeiss EM 910 electron microscope (Carl Zeiss, Thornwood, NY).

Statistical analysis. All data from videomicroscopy measurements are presented as means ± SE, and cell purity and viability are presented as means ± SD. Statistical differences were assessed by unpaired or paired Student’s t-tests using the INSTAT statistical computer program (GraphPad Software, San Diego, CA).

RESULTS

Morphological characterizations with light microscopy. As with rat tissue, bile duct fragments from mouse liver isolated immediately after the enzymatic
Digestions appeared as tubulelike structures, which formed spherical clusters of cells with defined lumen after 24–48 h in culture (Fig. 1). Viability of the IBDUs was >95% as assessed by trypan blue exclusion 24–72 h after culture. The average lumen diameter of mouse IBDUs determined by videomicroscopy after 48 h in culture was 50.3 ± 11.9 μm with a range of 26.9–77.6 μm (n = 24 from 4 preparations).

Morphological characterizations with electron microscopy. Electron micrographs of mouse IBDU preparations obtained after 48 h in culture demonstrated that the lumen was lined with polarized cholangiocytes with numerous apical microvilli (Fig. 2). These cholangiocytes had typical ultrastructural characteristics of bile duct epithelial cells demonstrated by a centrally located multilobulated nucleus, with a small cytoplasmic-to-nuclear ratio, a relatively low number of mitochondrion compared with hepatocytes, and many vesicular structures, especially at the subapical region of the cell. Tight junctions were well formed between adjacent cells, and the apical membrane of the cells had numerous microvilli, consistent with its role in solute transport and absorption.

Immunocytochemical characterizations. The cells lining the lumen were identified as bile duct epithelial cells by immunocytochemistry positive for cytokeratin-19 or both cytokeratin-7 and -19 (Fig. 3). Staining for γ-glutamyltranspeptidase (γ-GT) was not positive as previously reported (14) for mouse bile duct cell preparations, although controls with rat IBDUs strongly stained positive.

Functional characterizations with quantitative videomicroscopy. The mouse IBDU responded to stimulation with secretin (100 nM) with expansion of the central lumen by 31.1 ± 3.6% (n = 6) in 30 min (Fig. 4). The time course of this secretory response was followed and measured by quantitative videomicroscopy as summarized in Fig. 5. As shown in Fig. 5, the luminal area decreases after discontinuation of secretin administration, indicating the specific increase in the lumen area with secretin stimulation. Comparison of this secretory response to secretin (100 nM) with the same dose of VIP (100 nM) in the same mouse IBDU prepa-

Fig. 1. Nomarski image of 48-h cultured mouse isolated bile duct unit (IBDU). The IBDU from mouse liver has a central lumen lined with a thin bile duct cell layer.

Fig. 2. Electron micrograph of 48 h-cultured mouse IBDU. The IBDU from mouse liver has a central lumen lined with 1 layer of polarized bile duct epithelial cells with numerous microvilli on the luminal surface and tight junction complexes between the epithelial cells.

Fig. 3. Cytokeratin immunocytochemistry of 48-h cultured mouse IBDU. The IBDUs from mouse liver stained for α-cytokeratin-7 and -19 are brightly positive, whereas negative, unstained IBDUs (not shown) have a minimal background autofluorescence.
Substitution of Cl\textsuperscript{−} with gluconate in the perfusion solution significantly reduced secretin (100 nM)-stimulated secretion from an increase of 43.0 ± 4.4% in the luminal area (\(n = 12\)) in 30 min with gluconate to a 5.1 ± 1.9% increase (\(n = 11\), \(P < 0.001\)) with Cl\textsuperscript{−} substitution. This indicates dependence of secretin-stimulated biliary secretion on the presence of Cl\textsuperscript{−} in mouse IBDU (Fig. 8). Likewise, substituting HCO\textsubscript{3}{\textsuperscript{−}} with HEPES in the perfusion solution also resulted in a significant reduction in secretin (100 nM)-stimulated secretion from a 43.0 ± 4.4% (\(n = 12\)) increase in luminal area after 30 min to 4.5 ± 1.7% (\(n = 11\), \(P < 0.001\)), respectively, indicating a dependence of secretion on HCO\textsubscript{3}{\textsuperscript{−}} in mouse IBDU (Fig. 8). These findings are consistent with an involvement of a Cl\textsuperscript{−}/HCO\textsubscript{3}{\textsuperscript{−}} exchanger in the secretin-stimulated biliary secretion as previously established in rat cholangiocytes (2).

**DISCUSSION**

In this study, we report the first successful isolation of small, intact polarized bile duct units from mouse
liver and demonstrate that they can respond physiologically to secretory stimuli including secretin, VIP, and DBcAMP. Previously, IBDU preparations have been reported from the large bile ducts of pig (21) and rat (16), using labor-intensive microdissection techniques as well as enzymatic digestions (12). These models have provided invaluable tools for the study of biliary secretion in bile duct epithelia and have enhanced our understanding of ion transport mechanisms and their regulation. However, the paucity of suitable disease models affecting bile ducts in the pig or rat has limited their use.

To take advantage of various disease models now available in the mouse as a consequence of advances in genetic engineering, we now have developed isolation methods to prepare IBDUs from normal mouse liver. Although the isolation methods used are quite similar to those used for rat IBDU preparation described previously (12), several modifications in the isolation procedure were required to successfully isolate functional IBDUs from mouse livers. Because mouse liver is smaller than rat liver, we used smaller intravenous catheters with a slower rate of portal perfusion. To optimize liver perfusion, intermittent brief obstruction of the inferior vena cava was also used, which improved the IBDU yield. Furthermore, the amount of enzymes used in each enzymatic digestion step has been adjusted to optimize both IBDU yield and viability. With these modifications in technique, intact polarized IBDUs can be isolated easily without using labor-intensive microdissection methods.

As shown previously with rat IBDUs, these mouse IBDUs represent an intact polarized bile duct epithelial cell model. These isolated bile duct segments form a central enclosed lumen lined by a single layer of epithelial cells (Fig. 1) that demonstrate positive staining for cholangiocytes by cytokeratin-19 or cytokeratin-7 and -19 immunocytochemistry (Fig. 3). As previously reported for mouse bile duct epithelium (14), mouse IBDUs did not stain positive for γ-GT, whereas positive controls with rat IBDUs stained strongly positive. Unlike rat IBDUs, less connective tissue surrounds mouse IBDUs and the mouse cholangiocytes exhibit a more refractory pattern under light microscopy with Nomarski optics. Mouse IBDUs are lined by a single layer of intact polarized bile duct epithelial cells with large, lobulated, basally situated nuclei and sparse mitochondria and have intact tight junctions between adjacent cells sealing the lumen, as shown in electron microscopy images (Fig. 2). These findings resemble previous morphological descriptions of isolated rat cholangiocytes (5, 18) or rat IBDUs (12).

More importantly, these mouse IBDUs are functional units from intrahepatic bile ducts, which provide an invaluable tool to directly measure the secretory responses to various neuroendocrine peptides and chemicals. As in rat IBDUs (7, 10, 12), these IBDUs respond to neuroendocrine peptides such as secretin and VIP and to other chemical secretagogues such as IBMX and forskolin with expansion of their central enclosed lumen caused by fluid secretion (Fig. 4), which can be measured easily by quantitative videomicroscopy (Fig. 5). After 30-min stimulation with secretin (100 nM) or VIP (100 nM), the luminal areas of mouse IBDUs increase by 30–40% and 50–60%, respectively, as assessed by quantitative videomicroscopy. Similar studies in rat IBDUs (7, 10, 12) showed increases of ~60% and 90–100% after stimulation with secretin and VIP, respectively. However, the median effective dose of 4.5 nM in mouse IBDU for the VIP response is quite comparable to that of 3.2 nM in rat IBDU (7). These findings suggest that the secretory responses of mouse IBDUs to various secretagogues may be less robust compared with the rat, but the dose-response relationship to the secretagogues appears to be conserved. In summary, the present findings indicate that mouse IBDUs are a novel intact, polarized, functional in vitro cholangiocyte model suited to directly measure biliary secretion at the level of cholangiocytes from interlobular ducts. The ability to prepare IBDUs from mouse liver should facilitate the study of biliary disor-
ders in knockout models now available in the mouse as described in several preliminary reports (8, 13).

We thank Michelle Pate for technical assistance with portal perfusion.

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-25636 (J. L. Boyer), DK-34989 (the hepatocyte isolation, organ perfusion, and morphology cores of the Yale Liver Center), DK-07356 (W. K. Cho), and K08-DK-02613-01 (W. K. Cho), an Advanced Research Award from the American Gastroenterology Association (W. K. Cho), and an Indiana University Biomedical Research Grant (W. K. Cho).

A portion of this work was presented at the American Association for the Study of Liver Diseases meeting in Chicago, IL, in November 1997 and was published previously in abstract form (Hepatology 26: 398A, 1997).

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