Isolation of functional polarized bile duct units from mouse liver

WON KYOO CHO, 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 HCO\(_3\)\(^{-}\) 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.

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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, DMSP, hydrochloric acid, DNase (DN-25), nigericin, amiloride, sodium gluconate, potassium gluconate, and hemicalcium gluconate were purchased from Sigma Chemical (St. Louis, MO). BCECF-AM was obtained from Molecular Probes (Eugene, OR). Vasovactive intestinal peptide (VIP) and secretin were purchased from Bachem (Torrance, CA). Matrigel was from Collaborative Biomedical (Bedford, MA), collagenase D was from Boehringer Mannheim Biochemicals (Indianapolis, IN), and Pronase was from Calbiochem (San Diego, CA). Liebovitz L-15 medium, MEM, α-MEM, l-glutamine, gentamicin, and FCS were from Gibco (Grand Island, NY). Monoclonal anti-cytokeratin-7 and -19 antibodies were from Amersham. All other chemicals were of the highest purity commercially available.

Solutions. The Krebs-Ringer bicarbonate (KRBB) HEPES, and gluconate buffer solutions were composed as described previously (2, 20). All peptides were made up in perfusion buffer with 1% (wt/vol) BSA carrier or 1% BSA alone via a precalibrated pump. Each IBDU served as its own internal control, while maintaining the same focal plane at the maximum luminal area. Secretory responses in IBDUs were determined by measuring cross-sectional luminal areas using a Zeiss IM 35 or Olympus IX-70 inverted microscope with a cooled CCD video camera (Hamamatsu Photonics Systems, Bridgewater, NJ) connected to a Power Mac computer with image analysis software (Improvision, Boston, MA). Transmission electron microscopy was performed on IBDU preparations after 48 h (12, 20). Coverslips containing IBDUs were fixed in 2.5% glutaraldehyde and 1.0% 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 KRBB 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 cross-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). After a 10-min prestimulation period with buffer alone, IBDUs were stimulated for 30–40 min with infusions of various peptides with 1% (wt/vol) BSA carrier or 1% BSA alone via a precalibrated syringe pump. Each IBDU served as its own internal control, and changes in luminal area are expressed as a percentage of baseline values at time 0. Viability of the IBDUs was assessed by additions of trypan blue to the specimen chamber after each experiment.

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

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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 mitochondria 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-
Substitution of Cl\(^-\) with gluconate in the perfusion solution significantly reduced secretin (100 nM)-stimulated secretion from an increase of \(43.0 \pm 4.4\%\) in the luminal area \((n = 12)\) in 30 min with gluconate to a \(5.1 \pm 1.9\%\) increase \((n = 11, P < 0.001)\) with Cl\(^-\) substitution. This indicates dependence of secretin-stimulated biliary secretion on the presence of Cl\(^-\) in mouse IBDU (Fig. 8). Likewise, substituting HCO\(_3^+\) with HEPES in the perfusion solution also resulted in a significant reduction in secretin (100 nM)-stimulated secretion from a \(43.0 \pm 4.4\%\) \((n = 12)\) increase in luminal area after 30 min to \(4.5 \pm 1.7\%\) \((n = 11, P < 0.001)\), respectively, indicating a dependence of secretion on HCO\(_3^+\) in mouse IBDU (Fig. 8). These findings are consistent with an involvement of a Cl\(^-\)/HCO\(_3^+\) 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-

![Dose-response of VIP-stimulated secretion in mouse IBDU.](image)

The lumen of the mouse IBDUs increased by 15% after 30-min stimulation with 1 nM VIP and by 56% with 100 nM VIP; values are means ± SE.

![Effect of Cl⁻ and HCO₃⁻ omission on the secretory response of IBDU to secretin.](image)

The secretory response to secretin is significantly inhibited by omitting Cl⁻ or HCO₃⁻ from the perfusion solution. KRB, Krebs-Ringer bicarbonate.
ders in knockout models now available in the mouse as described in several preliminary reports (8, 13).

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REFERENCES


