Bile duct cells: a novel in vitro model for the study of lipid metabolism and bile acid production

MONIKA ZOLTOWSKA, EDGARD E. DELVIN, KHAZAL PARADIS, ERNEST SEIDMAN, AND EMILE LEVY
Departments of Nutrition, Biochemistry, and Pediatrics, Centre de Recherche, Hôpital Ste-Justine, Université de Montréal, Montreal, Quebec, Canada H3T 1C5

Zoltowska, Monika, Edgard E. Delvin, Khasal Paradis, Ernest Seidman, and Emile Levy. Bile duct cells: a novel in vitro model for the study of lipid metabolism and bile acid production. Am. J. Physiol. 276 (Gastrointest. Liver Physiol. 39): G407–G414, 1999.—Immortalized bile duct cells (BDC), derived from transgenic mice harboring the SV40 thermosensitive immortalizing mutant gene ts458, were utilized to investigate the role of the biliary epithelium in lipid and sterol metabolism. This cell model closely resembles the in vivo situation because it expresses the specific phenotypic marker cytokeratin 19 (CK-19), exhibits the formation of bile duct-like structures, and displays well-formed microvilli projected from the apical side to central lumen. The BDC were found to incorporate $^{14}$C-acetate suggesting the presence of hydroxymethylglutaryl-CoA (HMG-CoA) reductase, the rate-limiting enzyme in the cholesterol biosynthetic pathway. With the use of $^{3}$H-jabeled acetate and $^{3}$H-cholesterol as precursors, both tauro- and glycoconjugates of bile acids were synthesized, indicating the presence of cholesterol 7a- and 26R-hydroxylases, the key enzymes involved in bile acid formation. The transport of bile acids was not limited, as shown by their marked accumulation in the medium (>6-fold of cell content). HMG-CoA reductase (53.0 ± 6.7), cholesterol 7a-hydroxylase (15.5 ± 0.5), and acyl-CoA:cholesterol acyltransferase (ACAT; 201.7 ± 10.2) activities (in pmol·min$^{-0.5}$), and acyl-CoA:cholesterol acyltransferase (ACAT; 201.7 ± 10.2) activities (in pmol·min$^{-0.5}$) were present in the microsomal fractions. Our data show that biliary epithelial cells actively synthesize lipids and may directly contribute bile acids to the biliary fluid in vivo. This BDC line thus represents an efficient experimental tool to evaluate biliary epithelium sterol metabolism and to study biliary physiology.

The liver plays a central role in the metabolism of plasma lipids and lipoproteins, serving as the major site of synthesis of several apoprotein components and various lipid species (13, 40, 41). It also secretes nascent lipoproteins (very-low-density lipoproteins and high-density lipoproteins) and degrades chylomicron remnants, as well as low- and high-density lipoproteins, after their uptake (9, 11). Furthermore, the liver also serves as a delivery system for the regulatory enzymes and conversion factors that promote and control the metabolic relationships and remodeling of plasma lipoproteins (26, 45). Finally, hepatic biliary cholesterol secretion and bile acid synthesis constitute the main routes of cholesterol elimination from the body (24, 33, 48).

Disturbances of plasma lipid and lipoprotein composition frequently occur in patients with chronic cholestatic liver disease (14, 20, 38, 40, 41). Despite the association between primary biliary cirrhosis and other cholestatic syndromes with major lipid and lipoprotein derangements, little is known about the contribution of the biliary epithelium to the metabolic disturbances. Although they make up only 3–5% of the overall population of liver cells, bile duct cells (BDC) provide a large surface area for exchange between blood and bile. They play a key physiological role in the formation of bile, producing as much as 40% of the daily volume (32, 47). BDC, in close proximity to the hepatic arterioles that serve as their vascular supply, also display absorptive and secretory capabilities (47). Furthermore, increasing evidence has been put forth indicating that BDC can modify the composition of bile by secreting water, protein, and bicarbonate and reabsorbing glucose, glutamate, and anions (8, 43, 46, 47, 49, 50).

Although bile is a complex mixture of organic compounds, studies on biliary epithelium reported to date have been largely restricted to bile flow and ion permeability (8, 43, 46, 49, 50). It is noteworthy that bile flow is the result of plasma/bile transport of bile acids and other solutes, which create osmotic gradients that stimulate bile formation (4).

Progress in understanding the cellular and molecular basis of biliary epithelium metabolism and transport of sterols and lipoproteins has lagged behind studies on hepatocytes, largely because of technical problems in isolating pure BDC. However, the recent availability of immortalized BDC, originating from H-2Kb-ts458 transgenic mice (34), allows for more rigorous studies of the biology and function of the biliary epithelium. In the present study, we utilized this cell model to examine the synthesis of neutral lipid and phospholipid classes, as well as the production and conjugation of bile acids.
METHODS

Cell culture. The immortalized BDC line, originating from the transgenic mouse harboring the SV40 thermosensitive mutant gene ts458, was established as previously described (22). Cells were allowed to grow on Matrigel in 75-cm² ventilated flasks (Corning Costar, Cambridge, MA). The culture medium consisted of a 50:50 mix of Dulbecco's modified Eagle's medium with L-glutamine and D-glucose-Ham's nutrient mixture F-12 with L-glutamine (0.1 mmol/l), minimal essential medium nonessential amino acid solution, D-glucose (5.4 g/l), and HEPES (10 mmol) buffer adjusted at pH 7.40. The medium was supplemented with 100 µg/l penicillin G and 100 µg/ml streptomycin sulfate (both from GIBCO, Grand Island, NY); 10 µg/ml epidermal growth factor, 5 ng/ml each of insulin and transferrin, and 5 µg/ml selenium (all from Collaborative Biomedical Products, Bedford, MA); and 32 ng/ml thyroxin, 10 ng/ml prostaglandin E₁, 40 ng/ml hydrocortisone, and 10 µg/ml mouse recombinant interferon-γ (all from Boehringer Mannheim). Cells were grown at 33°C and were used for experimental studies after confluence (7–10 days). Their viability was assessed by trypan blue exclusion.

Immunofluorescence. Standard immunofluorescence microscopy techniques were used to detect cytokeratin-19 (CK-19), albumin, and macrophage F4/80 antigen. Briefly, cells were washed with PBS at pH 7.40, fixed in cold acetone for 10 min, and air-dried. After nonspecific antibody binding was blocked, the slides were incubated with primary mouse anti-CK-19 (Amersham, Oakville, ON, Canada), the macrophage-specific antibody F4/80, or anti-albumin (Cedarlane Laboratories, Hornby, ON, Canada). This was followed by incubation with fluorescein isothiocyanate-labeled sheep anti-mouse immunoglobulin G2b. Slides were washed with PBS, mounted, and photographed.

Electron microscopy. Cells with apparent ductlike structures were fixed in glutaraldehyde, embedded in Epon, and then sectioned using routine methods for electron microscopy (23).

Lipid synthesis. [¹⁴C]oleic acid (specific activity 53.9 mCi/mmol; Amersham, Montreal, PQ, Canada) was added to the medium (final specific activity 1,000,000 dpm/mol). Lipids were extracted from aliquots of cell homogenates and their respective incubation media with chloroform-methanol (2:1, vol/vol) (25). Tracer amounts of
lipid standards were added to the samples before separation of individual lipid classes by unidimensional thin-layer chromatography (TLC) (silica gel, Eastman-Kodak, Rochester, NY) as described previously (25). The apolar solvent system was hexane-diethyl ether-glacial acetic acid (80:23:3, vol/vol/vol) and the polar solvent was chloroform-methanol-water-acetic acid (65:25:4:1, vol/vol/vol/vol). After scraping, the radioactivity of the separated fractions was measured in a liquid scintillation counter (Beckman Instruments, Mississauga, ON, Canada). Quench correction was done using computerized curves generated with external standards. Proteins were measured according to Lowry et al. (27) using BSA as standard. Results are expressed as disintegrations per minute per milligram protein.

Determination of cholesterol and bile acid synthesis. BDC cholesterol biogenesis was evaluated employing [3H]acetate as precursor (53.4 Ci/mmol) after a 20-h incubation period. Bile acid synthesis was assessed by measuring the incorporation of [3H]acetate or the conversion of [14C]cholesterol (2 µCi) into acidic products that were extracted into H2O-acetic acid (65:25:4:1, vol/vol/vol/vol). Tracer amounts (12 ng) of bile acid standards (taurocholic acid, taurochenodeoxycholic acid, taurochenodeoxycholic acid, glycolithocholic acid, glycochenodeoxycholic acid, glycochenodeoxycholic acid, chenodeoxycholic acid, and cholic acid) were added to the extract before individual bile acid classes were separated by unidimensional TLC using two consecutive migration solvent systems. The apolar solvent system was composed of isocetane: isopropyl ether-isopropanol-acetic acid (1:1:1:1, vol/vol/vol/vol), and the polar solvent was chloroform-methanol-water-acetic acid (65:25:4:1, vol/vol/vol/vol). The radioactive spots corresponding to the migration of bile acid standards were visualized by iodine vapor, scraped, and counted.

Preparation of BDC microsomes. Cultured cells were removed and placed in ice-cold buffer (pH 7.4) containing (in mmol/l) 250 sucrose, 20 Tris·HCl, 1 EDTA, 5 glutathione, and 20 dithiothreitol. Cells were rinsed, homogenized, and centrifuged for 15 min at 12,000 g at 4°C. The supernatant fraction was then centrifuged for 60 min at 100,000 g at 4°C as described previously (21). The last step was repeated once. The purity of the microsomal fraction was assessed by the determination of glucose-6-phosphatase activity. The washed microsomal pellets were quick-frozen and stored at −80°C before use.

Enzyme activity assays. Microsomal activity of hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase, cholesterol 7α-hydroxylase, and acyl-CoA: cholesterol acyltransferase (ACAT) was determined as previously described (21).

Statistics. Statistical evaluation of the results was performed by the Student's two-tailed t-test.

RESULTS

Morphology and immunofluorescence studies. Morphological properties characteristic of bile duct epithelial cells were observed in our BDC line. The majority of BDC consisted of homogeneous populations of small round cells, readily distinguished from hepatocytes by their size and morphology. The epithelial origin of the immortalized cell line was confirmed by immunofluorescent microscopy technique showing the presence of CK-19 (Fig. 1, A and B). Negative staining using specific antibodies against F4/80 and albumin excluded contamination by macrophages and hepatocytes, respectively. After the first 24-h period in culture, BDC formed small islands on Matrigel, a basement membrane gel complex. Thereafter, they developed branched, ductlike structures (Fig. 1C). Electron microscopy revealed well-formed microvilli and apical tight intercellular junctions (Fig. 1D).

Lipid synthesis and secretion. To determine whether biliary epithelial cells had the ability to synthesize and secrete newly formed lipids, BDC were incubated with [14C]oleic acid. The incorporation of this radioactive substrate into BDC was linear over time for up to 20 h (results not shown). We observed substantial incorpora-

Table 1. Composition of lipids synthesized by immortalized BDC

<table>
<thead>
<tr>
<th></th>
<th>TG</th>
<th>PL + MG</th>
<th>CE</th>
<th>FC</th>
<th>DG</th>
</tr>
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<tbody>
<tr>
<td>Cells</td>
<td>60.48 ± 1.63</td>
<td>29.50 ± 1.44</td>
<td>8.06 ± 0.20</td>
<td>1.05 ± 0.12</td>
<td>0.91 ± 0.09</td>
</tr>
<tr>
<td>Media</td>
<td>68.51 ± 1.27*</td>
<td>22.77 ± 1.15†</td>
<td>4.88 ± 0.17*</td>
<td>2.08 ± 0.24†</td>
<td>1.77 ± 0.12*</td>
</tr>
</tbody>
</table>

Values are expressed as % of total 14C-labeled lipid distribution and represent means ± SE of 5 experiments. Bile duct cells (BDC) were incubated with [14C]oleic acid substrate for 20 h. Lipids of cell homogenates and media were then extracted with chloroform-methanol (2:1), isolated by thin-layer chromatography (TLC), and quantitated as described in METHODS. PL, phospholipids; MG, monoacylglycerol; DG, diacylglycerol; FC, free cholesterol; FFA, free fatty acids; TG, triacylglycerol; CE, cholesteryl ester. *P < 0.005, †P < 0.01, media vs. cells.

Fig. 2. Lipid synthesis by immortalized BDC. BDC were incubated with [14C]oleic acid substrate for 20 h. Newly synthesized lipids in cell homogenates (cells) and media were then extracted with chloroform-methanol (2:1), isolated by thin-layer chromatography (TLC), and quantitated as described in METHODS. Values represent means ± SE of 5 experiments. TG, triglycerides; PL, phospholipids; CE, cholesteryl esters. ***P < 0.005, media vs. cells.
tion into cellular triglycerides, phospholipids, and cholesteryl esters (Fig. 2). In all experiments, the amount of lipids was higher in cells than in the media, suggestive of a limited secretory capacity of BDC. Table 1 depicts the composition of total lipids analyzed by TLC. Triglycerides were the predominant lipids, followed by phospholipids and cholesteryl esters. The same profile was observed for both cells and media. Although the medium was slightly enriched in triacylglycerol, diacylglycerol, and free cholesterol, it had a low content of phospholipids plus monoacylglycerol and cholesterol ester.

Phospholipid profile. The BDC were also found to be capable of producing all major phospholipid classes. The absolute amount of phospholipids synthesized was higher in cells than in media, the latter accounting for <5% of cell phospholipids (Table 2). Phosphatidylcholine was the predominant form of 14C-labeled lipids elaborated. Major differences were noted in phospholipid composition between the cellular compartment and the media (Fig. 3). The percentage of sphingomyelin, phosphatidylserine, and phosphatidylinositol was higher in the medium, whereas the proportion of phosphatidylcholine and phosphatidylethanolamine was preponderant within the cells.

[14C]acetate incorporation into cholesterol. The BDC cholesterol biogenesis was assessed using [14C]acetate. As can be seen in Fig. 4, BDC incorporated substantial amounts of [14C]acetate into cholesterol and cholesteryl ester. These findings confirm the data (Table 1) obtained with [14C]oleic acid and suggest the presence, in BDC, of HMG-CoA reductase and ACAT, the two key enzymes involved in cholesterol metabolism.

Bile acid synthesis. BDC were evaluated for their ability to synthesize bile acids in the presence of [14C]acetate (Table 3). Several types of bile acids were produced, including taurocholate, taurocholate, glycocholate, lithocholate, and cholic acid (Fig. 5). Similar results were obtained using [14C]cholesterol as substrate (results not shown). The composition of the bile acids in the cells was quite different from their distribution in the culture medium. Intracellularly, a preponderance of taurocholate, taurocholate, and lithocholate was found, whereas glycocholate and glycochenocholate were dominant in the medium. Overall, the total incorporation of [14C]acetate into bile acids in

| Total Sphingomyelin Phosphatidylcholine Phosphatidylserine Phosphatidylinositol Phosphatidylethanolamine |
|---------------------------------------------------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| Cells   | 685.22 ± 49.40 | 24.09 ± 0.80     | 404.10 ± 27.38    | 39.26 ± 3.06      | 52.37 ± 4.25      | 165.40 ± 15.00    |
| Media   | 24.10 ± 2.19   | 5.71 ± 0.84†     | 9.46 ± 1.12†      | 1.76 ± 0.14†      | 2.51 ± 0.18†      | 4.66 ± 0.33†      |

Values are expressed in nmoles/mg protein x 10^-3 and represent means ± SE of 7 experiments. Phospholipids from cell homogenates and media were extracted, isolated by TLC, and quantitated. * P < 0.01, † P < 0.005, media vs. cells.
the medium consistently exceeded fivefold that in the cells, indicating an active bile acid secretory capacity.

Enzyme activity. We also determined the activity of the three sterol enzymes that regulate hepatic intracellular cholesterol homeostasis. The activity of HMG-CoA reductase, cholesterol 7α-hydroxylase, and ACAT was detected in microsomes isolated from BDC (Fig. 6). The mean activities measured (in pmol · min⁻¹ · mg protein⁻¹) were 53.0 ± 6.7 for HMG-CoA reductase, 15.5 ± 0.5 for cholesterol 7α-hydroxylase, and 201.7 ± 10.2 for ACAT.

DISCUSSION

Until recently, study of the cell biology and function of biliary epithelial cells has been hampered by their inaccessibility. Attempts to isolate BDC have shed light on many properties of BDC (1, 2, 12, 18, 30, 35, 51, 52). However, none of these techniques yield pure populations of homogeneous BDC in sufficient quantity. With one exception (35), they do not recreate the tubular orientation of polarized BDC in vitro. Strategies have thus been designed to develop immortalized BDC clones that would mimic the in vivo situation as closely as possible. In this study, we confirm that immortalized BDC display well-differentiated features of mature biliary epithelium, with definite cellular polarization and formation of ductlike structures, well-developed apical microvilli, and tight junctions. The immortalized BDC also express cytokeratin-19, a phenotypic marker normally found in BDC. This cell line thus provides us with the opportunity to verify the emerging concept that intrahepatic biliary epithelial cells are actively involved in lipid and bile acid metabolism.

In the present study, we demonstrate the ability of BDC to incorporate fatty acids for lipid esterification. The mechanism of cellular uptake of these lipophilic compounds is not yet elucidated. Fatty acids may enter cells by passive diffusion or may be absorbed via an energy-independent, facilitated diffusion mechanism (6, 7, 36). Recent studies have also suggested that a saturable process mediated by specific binding sites on the cell surface is involved in the uptake of the albumin-ligand complex (36). The relative importance of these potential routes depends on the tissue and organ involved and the concentration and properties of the ligand (5, 7, 36). Additional work is needed to define the uptake and translocation of fatty acids to the endoplasmic reticulum for lipid esterification.

Triglycerides are the dominant class of lipids elaborated by BDC when using [14C]oleic acid as precursor. In the liver, after acylation in the rough endoplasmic reticulum, the majority of fatty acids are incorporated into triglycerides via the phosphatidic acid pathway (10). The conversion of phosphatidate to 1,2-diacylglycerol by phosphatidate phosphatase is the rate-limiting reaction of triglyceride biosynthesis. Our data suggest that BDC possess this important anabolic step. Furthermore, the high de novo-formed phospholipid content points to the active intracellular participation of the enzymes required for their synthesis.

In our experiments, the medium lipid content approaches only 7% of intracellular production, indicating a limited lipid export capacity of BDC. In this respect, BDC do not contribute a significant movement of locally manufactured lipids into bile, in contrast to hepatocytes. However, it is possible that lipid reabsorptive mechanisms are also operative in BDC.

Our results provide evidence for the capacity of BDC to synthesize and secrete bile acids that, qualitatively, are similar to those produced by hepatocytes. They
differ, however, in their relative proportions. Indeed, in our model, newly synthesized bile acids mostly consist of lithocholic acid and its tauro- and glycoconjugates (83%). Cholic and chenodeoxycholic acid derivatives account for 11 and 6%, respectively. Javitt (17) have clearly established that there are two main pathways for bile acid synthesis. The first, which utilizes the classical 7α-hydroxylation pathway, yields cholic and chenodeoxycholic acids, which account for 95% of the bile acids and salts synthesized by Hep G2 liver cells in culture (15). It must be stressed at this point that 7α-hydroxylase activity has been measured in our cells (Fig. 6). The fact that its specific activity is lower than that observed in liver microsomes in other models (19) supports our [14C]acetate incorporation results. We may thus surmise that this pathway is a minor one in BDC. The C-26 hydroxylation of cholesterol is the preferential pathway for the biosynthesis of lithocholic acid (15). It can also be derived from the 7α-dehydroxylation of chenodeoxycholic acid by the intestinal bacterial flora (16). Because our culture model is devoid of such bacterial contamination and because the enzyme responsible for C-26 hydroxylation of cholesterol is present in a number of epithelial cells (37), it can legitimately be invoked as a major pathway in our model.

Under physiological conditions, the coordination of the microsomal enzymes HMG-CoA reductase, cholesterol 7α-hydroxylase, and ACAT is closely associated with the maintenance of liver cholesterol homeostasis (3). HMG-CoA reductase has been demonstrated to be the rate-limiting enzyme in cholesterol biosynthesis (39). Cholesterol 7α-hydroxylase, a specific microsomal cytochrome P-450 isoenzyme, is the initial and rate-determining enzyme in the bile acid biosynthesis pathway (31, 42). Based on the failure to observe detectable activity of the cytochrome P-450 system (28, 29), it was assumed that the biliary epithelium does not possess the de novo sterol-synthesizing enzymes, which have not been tested in these studies. However, our data unequivocally show that labeled bile acids are formed from a radioactive precursor and that BDC microsomes contain HMG-CoA reductase and cholesterol 7α-hydroxylase activities. Furthermore, our results demonstrated the presence of microsomal ACAT, which is not part of the cytochrome P-450 system. It is therefore tempting to speculate that the biliary epithelium, having the capacity to elaborate and conjugate bile acids, may modify the composition of bile acids secreted. It is important to emphasize that our BDC model is devoid of any hepatocytes and macrophages, potential sources of contamination. These BDC have furthermore been clearly characterized with regard to their enzyme profiles and do not contain albumin, an essential characteristic of hepatocytes.

ACAT is the enzyme responsible for the acylation of cholesterol to cholesterol esters, a transformation that strongly influences hepatic excretion of cholesterol (44). Activity of this enzyme is detected in BDC, suggesting a potential role in the biliary epithelium. Activation of ACAT would effectively result in an increment of cholesteryl ester, with deficient conversion to bile acids.

In conclusion, evidence has been presented that BDC synthesize various lipid classes albeit with a limited capacity of exporting them. The biogenesis and secretion of sterols, including bile acids, suggest an active role in the modification of biliary content by the biliary epithelium. This BDC line represents an experimental model to study biliary epithelial cell biology under normal and pathophysiological conditions.

The authors thank Danielle St-Cyr for expert secretarial assistance.

This work has been supported by an operating grant from Natural Sciences and Engineering Research Council of Canada, Merck Frosst Canada, and Research Scholarship Awards (E. Seidman, E. Levy) from the Fonds de la Recherche en Santé du Québec.

Address for reprint requests: E. Levy, Gastroenterology-Nutrition Unit, Hôpital Ste-Justine, 3175 Côte Ste-Catherine, Montreal, Quebec, Canada H3T 1C5.

Received 25 June 1998; accepted in final form 21 October 1998.
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