Virchows Arch (2000) 436:140–146

ORIGINAL ARTICLE

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A method for culturing and transplanting biliary epithelial cell from syrian golden hamster

Received: 31 May 1999 / Accepted: 25 August 1999

Abstract The present paper describes the establishment of a method for simultaneous culturing of biliary epithelial cells (BECs) from the gall bladder (GB), extrahepatic bile duct (EBD) and intrahepatic bile duct (IBD) of the hamster. GB, EBD and IBD were cut from the biliary tree after collagenase perfusion of the liver. These biliary segments were minced into fragments. The fragments were embedded in collagen gel and cultured in Dulbecco's modified Eagle medium / HamF12 medium containing 10% fetal bovine serum. The various cells subsequently spread from the fragments and formed cellular sheets. After the fragments and flattened cells were removed with the aid of a Pasteur pipette under phase-contrast microscopy, the sheets remaining were found to be composed of cuboidal cells. These cuboidal cells were shown to express gamma glutamyl transpeptidase and cytokeratin 7, which are known to be specific markers of BECs. Ultrastructurally, a large number of microvilli were observed on the luminal surface and junctional complex and interdigitation was identifiable on the lateral surfaces. BEC cultures were subcultured by digestion with collagenase and dispase and then dissociated by subsequent digestion in trypsin and ethylenediaminetetraacetic acid and then maintained on collagen gel for up to 8 weeks. After several passages, the BECs in culture eventually increased in size and showed vacuoles in the cytoplasm. They demonstrated irreversible growth arrest at 9 weeks. The BECs tended to form cystic structures when the BECs with collagen gel were transplanted into the interscapular fat pads of syngeneic hamsters. We established a method for culturing and transplanting biliary cells from syrian golden hamsters. This method may help to clarify the mechanism of hepatobiliary diseases.

Key words Biliary epithelial cell · Culture on collagen gel · Hamster

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Introduction

Biliary epithelial cells (BECs) have phenotypes, structures and possibly functional heterogeneity varying with the different regions of the biliary tree [2, 14]. Pathologically, certain anatomical levels of the biliary tree are preferentially affected. For example, in primary biliary cirrhosis the intrahepatic small bile ducts are selectively damaged, while the extrahepatic and intrahepatic large bile ducts are preferentially involved in primary sclerosing cholangitis [13]. Such anatomical preferences in disease processes may be related to the functions and antigenicity of BECs and their microenvironments at the individual anatomical level. Up to now, BECs have been independently isolated and successfully cultured from different parts of the biliary tract in humans [1, 20] and rodents [9, 12, 15, 24]. Several studies have reported the importance for the establishment of long-term cultures of using collagen gel as the substrate for adherence [9, 12, 24]. Recently, the isolation and cultivation of pure BECs using an explant of the biliary tree on collagen gel was reported [9, 24]. All of these methods involve the isolation of BECs from a certain anatomical segment of the biliary tree or the isolation of certain pathologically proliferated BECs.

Syrian golden hamsters have been experimentally used for investigations of the pancreas, because the anatomical structure of the hamster's pancreaticobiliary ductal system is similar to that of humans [22]. Moreover, both the bile acid composition and the pancreatic juice component in this species also closely resemble those of humans [16, 17]. We previously reported the syrian golden hamster to be a useful animal for investigating biliary carcinogenesis [3, 6–8, 21, 23]. To our knowledge, no previous studies have been published on the successful culturing of biliary epithelial cells from the hamster.

We developed a direct method for the simultaneous isolation of intrahepatic, extrahepatic and gall bladder BECs in the normal hamster, followed by their cultivation and transplantation.

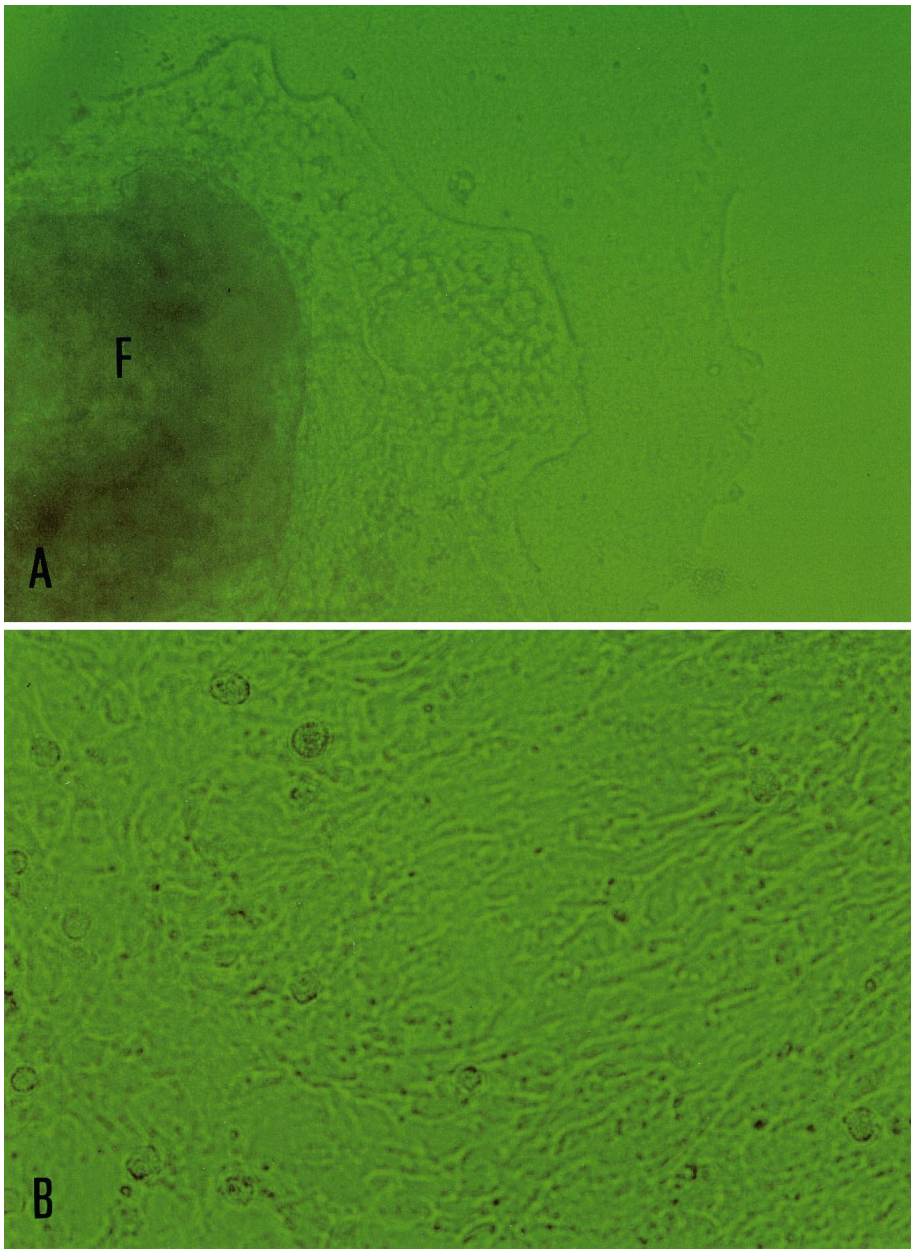

Fig. 1A, B The fragment (*F*) embedded in collagen gel and cultured for 7 days. Phase contrast microscopy **A** Varying amounts of cells spread from the fragment on collagen gel. ×60 **B** The peripheral region of the cells spreading from the fragment was made up of cuboidal cells. ×200

Materials and methods

Animals

Female syrian golden hamsters aged 5–6 weeks were supplied by the Shizuoka Laboratory Animal Center (Shizuoka, Japan) and were kept under standard laboratory conditions in the Laboratory Animal Center for Biochemical Research, Nagasaki University School of Medicine. Those animals were given a standard pellet diet and water ad libitum during the experiment. All experiments were done according to the Guidelines for Animal Experimentation of Nagasaki University.

The livers were perfused in situ with 100 ml Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (CMF-PBS) containing 10 mmol/l HEPES (pH 7.4), 1 mmol/l ethylene glucol-bis (E-aminoethylether) *N*,*N*,*N*',*N*'-tetraacetic acid (EGTA) via the inferior vena cava for 10 min at 37°C. The vein was clamped above the diaphragm, and the perfusate was allowed to drain through the portal vein, followed by perfusion with 100 ml Hanks' balanced salt solution containing 50 mmol/l HEPES, 0.02% soybean trypsin inhibitor (Sigma Chemical Co., St. Louis) and 0.04% collagenase (Wako Chemical Co., Tokyo) for 10 min at 37°C. Then the liver, gall bladder and extrahepatic bile duct were removed. After the capsule of the liver was removed with a comb and brush, the biliary tree was dissociated with scissors and separated into the intrahepatic bile duct (IBD), extrahepatic bile duct (EBD) and the gall bladder (GB) in CMF-PBS containing 0.1 mmol/l EGTA. These fragments were then incubated in culture medium composed of Dulbecco's modified Eagle medium (DMEM, Nissui Pharmaceutical Co., Tokyo) / HamF12 medium (HamF12, Nissui, Tokyo) containing 10% fetal bovine serum (GIBCO, Grand Island, N.Y.) for 12 h at 37°C in an incubator (5% CO_2).

In the present study, hamsters were used to obtain fragments of IBD, EBD and GB.

Table 1 The successful cultivation rate of biliary epithelial cells (BECs) from fragments differed depending on whether they were from extrahepatic bile duct, intrahepatic bile duct or gall bladder of 7 hamsters

Original site	No. of fragments	No. of isolations	Rate of isolation and cultivation (%)
Intrahepatic bile duct	52	17	33%
Extrahepatic bile duct	21	16	76%
Gall bladder	30	28	93%

Culture of biliary fragments and establishment of monolayer culture cells

A collagen gel plate was made by coating 60-mm petri dishes with 2 ml of an ice-cold mixture of collagen solution, 0.3% acid solution collagen (Cellmatrix Type I-A, Nittazeratin, Osaka), 10× Ham F12 and 0.8 N NaOH (8:1:1). The fragments of the biliary tree were embedded in the collagen gels. After incubation for 20–30 min at 37°C, collagen gels were overlaid with 5 ml of culture medium. After 7–10 days in culture, both these fragments and nonepithelial cells were collected under a phase-contrast microscope using a Pasteur pipette. The remnant culture cells were released from collagen gel by digestion for 40–50 min with 0.1% (wt./vol.) collagenase and 10 U/ml dispase (Gohdo Shusei, Chiba, Japan) in Hanks' solution and collected by centrifugation at 1000 rpm for 5 min. After a single wash through CMF-PBS by centrifugation at 1000 rpm for 5 min, the culture cells were dissociated in CMF-PBS containing 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA, Wako Chemical Co, Tokyo).

After incubation for 5 min at 37°C, the digestion was discontinued by the addition of the culture medium. These cuboidal cells were washed twice in CMF-PBS and were then resuspended at 1×10^5 cells/ml in the culture medium. Five milliliters of the cell suspension was plated on a plastic dish, a collagen-coated dish and on top of 2 ml collagen gels in 60-mm petri dishes. These culture cells were maintained at 37°C in an incubator, and the culture medium was changed every 3 days. At 80% confluence, the culture cells were released from collagen gel by digestion with collagenase and dispase and were then dissociated by subsequent digestion in 0.05% trypsin and 0.02% EDTA.

Morphological analysis

A morphological examination of the culture cells, including a histochemical and immunohistochemical study as well as an ultrastructural examination, was done at every passage.

The monolayer culture cells on the top of collagen gel were fixed in 10% buffered formalin. The cells on the collagen gel were then embedded in paraffin using standard techniques. Each 5-µm section of the paraffin block was stained with hematoxylin and eosin (H-E), periodic acid–Schiff (PAS) and mucicarmine stains.

The culture cells on collagen gel were fixed with paraformaldehyde 3 h at 4°C and then were embedded in Optimal Cutting Temperature (OCT) compounds (Miles, Elkhart, USA) and fixed at –80°C. Each 5-µm section of the OCT compound block was used for the immunohistochemical staining of cytokeratin 7 (Progen Biotechnik, Heidelberg, Germany) and the cytochemical demonstration of gamma glutamyl transpeptidase (GGT) as described by Rutenburg et al. [18]. Immunocytochemical staining was done using a Vectastain avidin–biotin peroxidase complex kit (Vector Laboratories, Burlingame, Calif.).

Transmission electron microscopy

To examine the ultrastructure of culture cells, the cultured cells on collagen gel were washed with 0.1 M cacodylate buffer and fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 at 37°C for 30 min. The cells were post-fixed in 1% osmium tetroxide at 4°C for 30 min. These fixed cells were dehydrated in ascending series of ethanol and then embedded in Epon mixture and polymerized at 60°C for 12 h. Thin sections were stained with uranyl acetate and lead tartrate, and examined with JEM-1200EX (Japan Electron Optics Laboratory, Tokyo) operated at 60–80 kV accelerating voltage.

Scanning electron microscopy

The cells were grown on collagen gel-coated 13-mm glass coverslips. They were washed, fixed and dehydrated by the same method for TEM and critical point drying necessary to prepare the cells for scanning electron microscopy. Each specimen was coated with gold-palladium and examined under a JSM-35CLaB6 (Japan Electron Optics Laboratory, Tokyo) with a 15-kV accelerating voltage.

Growth of the cells in vivo

In order to examine the growth pattern of these cells in vivo, the cultured cells were inoculated into the fad pads of syngeneic hamsters. When 90% confluence of the culture cells on 0.3 ml collagen gel/well was achieved (24-well plates, Falcon, Lincoln Park, N.J.), the culture cells were rinsed twice with CMF-PBS. The culture cells on collagen gel in wells were inoculated into the intrascapular fat pads of 6- to 7-week-old female syrian golden hamsters. After 3–4 weeks, the hamsters were sacrificed and then the portion of cell inoculation in the fad pad was fixed in 10% buffered formalin. Following appropriate processing, the fat pad tissue after transplantation was examined for H&E, PAS, mucicarmine, cytokeratin 7 and GGT stains.

Results

Culture of biliary epithelial cell

Varying a mounts of cells spread from the fragment (Fig. 1A). The distinction of epithelial cells from such nonepithelial cells as fibroblasts and endothelial cells was easy with a phase-contrast microscope. The spreading speed of the former was faster than that of the latter. The peripheral parts of the sheet were composed exclusively of cuboidal cells (Fig. 1B). The central parts were composed of epithelial cells and mesenchymal cells. The central region was released from collagen gel with a Pasture pipette. On the other hand, the subcultured cells formed cuboidal cells but few flattened cells were seen.

The successful isolation rate of epithelial cells differed depending on whether they had been taken from the gall bladder, the extrahepatic duct or the intrahepatic duct (Table 1). The composition of the substratum was also important for the growth, morphology and survival of cuboidal cells in culture. When a subculture cell suspension was plated on plastic, air-dried collagen or collagen gel, cuboidal cells attached and formed colonies with similar efficiencies. However, after 5–7 days in cul-

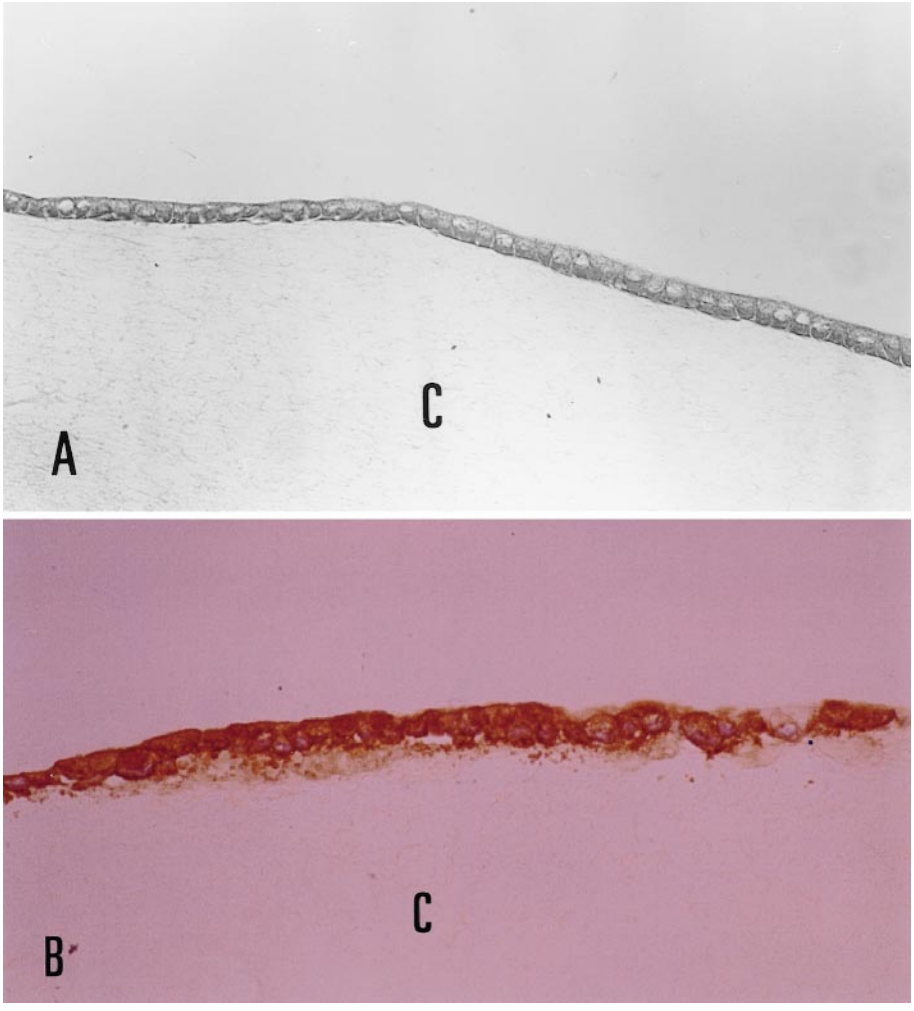

Fig. 2A, B Biliary epithelial cells cultured on collagen gel (*C*) after subculture. **A** The culture cells form a monolayer and are cuboidal or columnar cells. H-E, ×200 **B** The culture biliary epithelial layer is positive for cytokeratin 7. ABC method, ×400

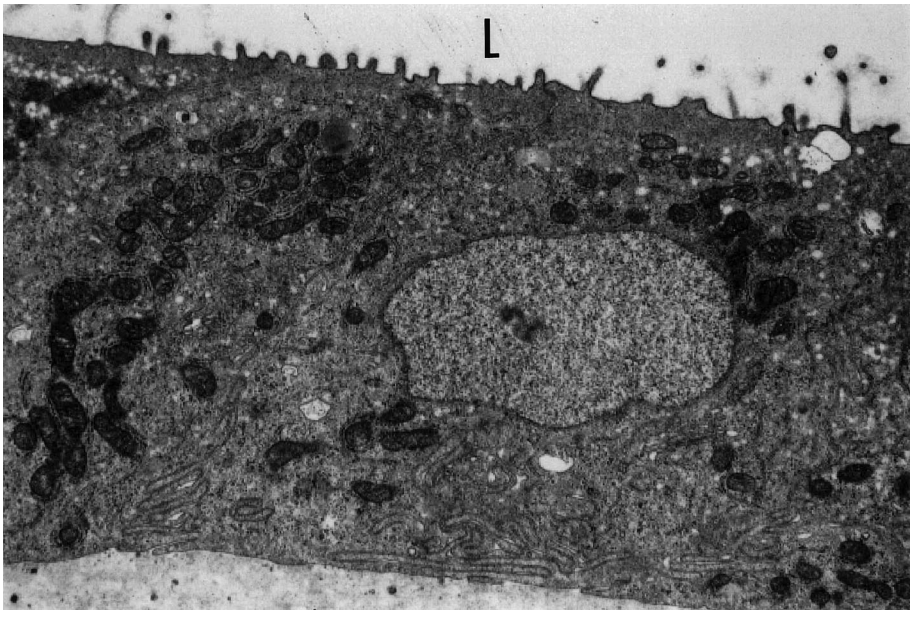

Fig. 3 Transmission electron microscopy of cultured cells on collagen gel after two passages. The monolayers of biliary epithelial cells were composed of polarized cells from the lateral membrane with well-defined junctional complexes and luminal membrane (*L*) covered with numerous short microvilli. The basement membrane of biliary epithelial cells lacked microvilli and showed evidence of basal lamina. ×4000

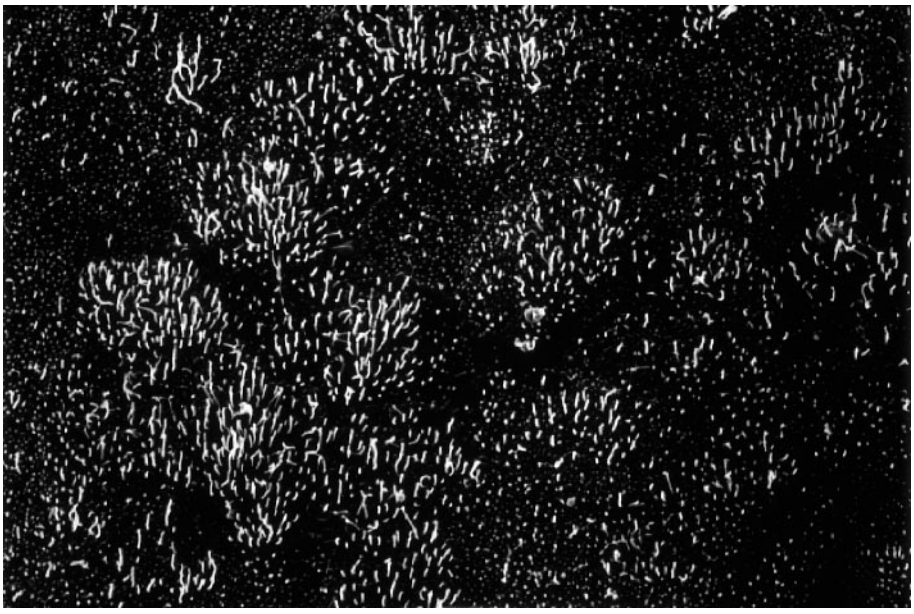

Fig. 4 Scanning electron microscopy of cultured cells on collagen gel after two passages. The apical membrane of the biliary epithelial cells demonstrates numerous microvilli. ×3000

ture, the cells growing on plastic or air-dried collagen-coated dishes began to detach from the dish. In contrast, under identical conditions cuboidal cells remained on top of the collagen gel while preserving an epithelial cell morphology and eventually formed a confluent monolayer by 8 weeks. Collagen gel rather than plastic dishes or collagen-coated dishes thus appeared to be most suitable for the growth and attachment of such culture cells. At 8 or 9 weeks, the culture cells began to detach from the collagen gel.

Characteristics of biliary epithelial cells

Light microscopy showed that the primary culture peripheral region was composed of cuboidal cells on collagen gel (Fig. 2A). At 8 weeks after primary culture, these cuboidal cells were positive for PAS and mucicarmine staining. In subculture, the culture cells exhibited a histochemical staining reaction for GGT activity. The cytoplasm of the culture cells reacted intensely with anti-cytokeratin 7 (Fig. 2B).

Transmission electron microscopy

Ultrastructurally, the cultured cells on collagen gel were covered with short stubby microvilli on the luminal surface and contained a golgi apparatus, abundant mitochondria and a rough endoplasmic reticulum in the cytoplasm. Junctional complexes such as desmosomes, tight junctions and interdigitations were formed between the intercellular spaces. The nuclei were located either centrally or basally (Fig. 3). At 8–9 weeks after primary culture the culture cells became attached to the collagen gel and maintained a junctional complex but demonstrated multiple vacuoles in the cytoplasm.

Scanning electron microscopy

SEM studies also revealed a regular lining of cells growing in one plane. No duct or gland-like structures were

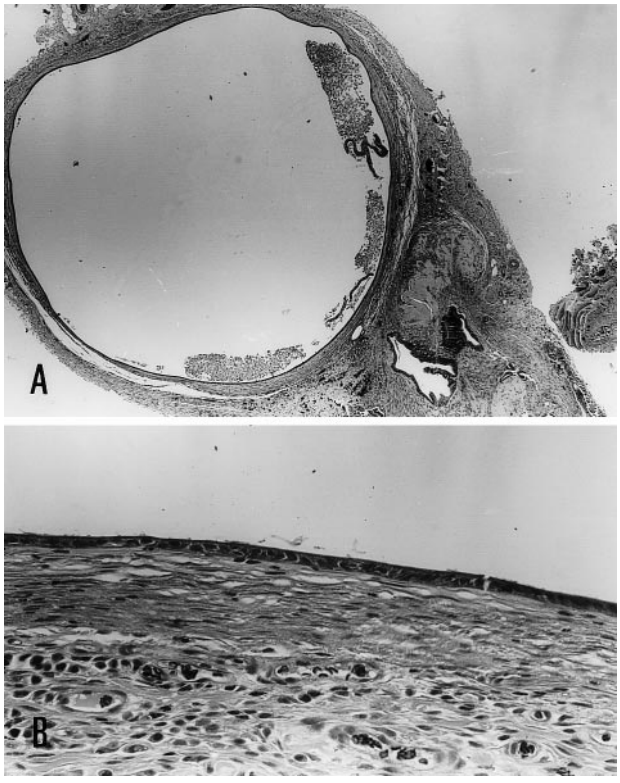

Fig. 5A, B Inoculation of cultured cells into the syngeneic recipient hamsters. **A** The cystic structure was formed 3 weeks after the inoculation of biliary epithelial cells. H-E, ×40 **B** The luminal surface is composed of cuboidal or low columnar cells. H-E, ×200

Table 2 The successful transplantation rate of BECs from 7 hamsters was no different from the original site. The BECs on collagen gel were obtained after two passages and inoculated into the interscapular fat pads of hamsters

Origin of BECs	No. of inoculated well	Cystic formation	Successful inoculation rate (%)
Intrahepatic bile duct	13	11	85%
Extrahepatic bile duct	15	13	87%
Gall bladder	21	18	86%

noted. The surface topography of the cultured cells at confluence was uniform and cell–cell junctions could be identified. The apical membranes by SEM were polygonal in shape and covered with numerous microvilli (Fig. 4). These features were very similar to those of normal BECs.

Transplantation of BECs

Transplanting culture cells into the interscapular fat pads of a syngeneic recipient hamster resulted in cystic growth. Normal BECs gave rise to cystic structures supported by a connective tissue stroma from 3 to 4 weeks after inoculation into the fat pads (Fig. 5A, B). These cells after transplantation were positive for PAS, mucicarmine, cytokeratin 7 and GGT.

The successful inoculation rate of BECs is shown in Table 2.

Discussion

In this study, we demonstrated a method for the simultaneous isolation, culture and transplantation of BECs from the IBD, EBD and GB in the normal hamster. Syrian golden hamsters have been used as an experimental model of the pancreas for both pancreatic carcinogenesis [4, 11] and cultivation of pancreatic duct [5]. Although we reported that the hamster was useful for studying biliary carcinoma, there has not yet been a report on the culture of normal BECs from the hamster.

Previously, the isolation of BECs has widely been used for isopyknic centrifugation, Percoll density gradient sedimentation, centrifugal elutriation and antibody sorting [19]. Those isolation methods require both surgical and pharmacological treatment to obtain higher yields of isolated BECs. The isolated cells were subsequently cultured on extracellular matrix materials. The culturing of some normal and neoplastic epithelial cells on specific extracellular matrix materials has been shown to greatly affect the organization and differentiation of these cells [12, 24]. Yang et al. [24] reported that the extracellular matrix has an important role in the maintenance of cell shape, proliferation and differentiation in the epithelial cells. The extracellular matrix was demonstrated to influence both the morphology and the differentiation of several different types of normal cells in culture [5, 12, 24]. However, collagen gel has recently been used for the isolation of BECs [9, 24].

In our studies, the fragment was embedded in collagen gel, BECs were grown on collagen gel, and fibroblasts could be observed in the collagen gel beneath the monolayer of BECs. The isolation used by collagen thus resulted in different growth patterns and rates. In addition, contaminant cells that were occasionally encountered in the BECs suspension were fibroblasts. They grew beneath the BEC monolayers on collagen gel, forming an environment similar to that in the bile duct in vivo. However, they never either interposed cultured BECs on collagen gel or broke any monolayers. By utilizing the feature of collagen gel, we used it to isolate the epithelial cells from normal hamster liver and thus culture the epithelial cells.

The different rates for the successful cultivation of BECs depending on the anatomical origin may be due to the presence of mechanical and enzymatic damage during the preparation of the biliary tree. In addition, more nonepithelial cells were observed around the IBD than around either the EBD or the GB. BECs can proliferate in culture and morphological findings, including ultrastructural and histochemical findings, can be maintained for up to at least 8 weeks. No differences were observed in the morphological findings, culture period or transplantation in the anatomical origin. Several reports have described the use of a primary culture as an in vitro model for studying carcinogenesis [10, 11]. As a result, the period of cultivation is thus considered to be sufficient for studying carcinogenesis in vitro.

This method is useful for analyzing the influence for BECs at different anatomical levels by immunologic or chemical treatment. Moreover, when BECs on collagen gel were inoculated into the interscapular fat pads of hamsters, BECs formed cystic structures with a luminal membrane of monolayer cells. These morphological findings have been shown to be characteristic of normal BECs in vivo. This transplantation method can be utilized for a pathological analysis of the culture cells.

Our results suggested the possibility of establishing an in vitro / transplantation model with these cells in order to study biliary carcinogenesis and the regulation of cell functions, proliferation, and modulation in various regions of the biliary tree under more controlled experimental conditions.

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