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Purification and Monolayer Cell Culture of Murine Extrahepatic Biliary Epithelial Cells

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Summary. **The preparation of biliary epithelial cells is fundamental for their biologic and pathophysiologic evaluation. A new method for the isolation of extrahepatic biliary epithelial cells, using explants of murine extrahepatic bile ducts and gallbladder, is presented. First, small tissue pieces of the extrahepatic biliary tree and the gallbladder were plated as primary explants on collagen gel. The epithelial cells spread from the explants toward the periphery as a monolayer sheet, while the nonepithelial mesenchymal cells of the explants migrated into the gel below the epithelial layer. These different growth patterns created areas in epithelial cell sheets in which no mesenchymal cells were present. These areas were cut with scissors and then cultured on another collagen gel as secondary explants. From these explants, epithelial cells without nonepithelial mesenchymal cells spread out as a monolayer on the collagen gel. At the front and periphery of both the primary and secondary monolayer cell cultures, epithelial cells exhibited a polygonal flattened morphology, and BrdU-positive S-phase cells were densely distributed. In the central parts of both explants, the cells were cuboidal or low columnar with a luminal mucin positive layer and BrdU-positive S-phase cells were sparsely distributed, suggesting that the *in vivo* morphologic characteristics of the epithelial cells of the extrahepatic biliary tree were retained. This method provides purification and a monolayer cell culture or extrahepatic biliary epithelial cells that can be employed in various types of studies.**

Key words—biliary epithelium, collagen gel culture, cell culture, tissue culture, purification, explant culture.

INTRODUCTION

A number of neoplastic and nonneoplastic diseases affect the biliary epithelial cells lining the extrahepatic biliary tree.[1] For the pathologic and pathogenetic analysis of these diseases, an *in vitro* approach using cultured extrahepatic biliary epithelial cells can prove valuable. With such cultures, the morphologic and functional changes of biliary epithelial cells can be examined dynamically in an artificial environment or in conditions mimicking the *in vivo* biliary tract; the responses of such cells to the addition of biologically active substances, such as drugs, hormones, cytokines, and bile-derived substances, to the culture media can also be investigated. Two models for the culture of biliary epithelial cells, a monolayer and a three-dimensional cell culture, as well as living tissue cultures, are well known and have been employed for experimental studies.[2] However, with the biliary epithelial cell cultures reported thus far,[3-11] mesenchymal cell contamination and epithelial cell injury during preparation appear to be inevitable.

In this report, we present a simple and reproducible method for the preparation and subsequent culture of biliary epithelial cells from the rat extrahepatic bile duct and mouse gallbladder. These extrahepatic biliary tissues were chosen because of easy access and manipulation.

MATERIALS AND METHODS

Preparation of collagen gel and culture conditions

Equal volumes of Ham F12 (Ham's F-12 medium, Flow Laboratories, Irvine, Scotland) and DMEM (Dulbecco's modification of Eagle's medium, Flow Laboratories), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, NY, U.S.A.),

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served as the culture medium; 200 U/ml of penicillin G and 100 μg/ml of streptomycin (both from Meiji Seika Co., Tokyo, Japan) were added to prevent bacterial contamination. Immediately before the start of the culture, 0.3% acid-soluble type I collagen gel (Nitta Gelatin, Osaka, Japan), FBS, and a 10-fold concentrated culture medium without FBS were mixed at a ratio of 8 : 1 : 1 in an ice-water bath. This preparation was termed the gel mixture. Three ml of the gel mixture was then poured into each well (30-mm diameter) of 6-well plastic culture plates (Corning, NY, U.S.A.). These plates were incubated at 37℃ and the gel mixture rapidly became gelatinous (collagen gel). A primary or secondary explant was then plated on the collagen gel in each well, and the culture medium was added. The culture fluid was exchanged every 2 days thereafter. The cultures were maintained in an incubator under a 5% CO_2 atmosphere at 37°C.

Preparation of primary explants

Ten female mice (sIc/ICR: 6 to 10-weeks-old) and 10 adult male Sprague-Dawley rats (weighing 210-280 g) obtained from Japan SLC (Hamamatsu, Japan) were used. These animals were anesthetized with ether before being killed, according to the protocol of the Kanazawa University Animal Care Committee. Dissected pieces of gallbladder removed from the mice, and small tissue pieces, 2-3 mm in length, cut from the middle portion of the extrahepatic bile ducts of rats served as primary explants.

Preparation of biliary epithelial cells from primary explants and the secondary culture

In the primary culture, one small tissue piece (2-3 mm) (20 pieces of gallbladder pieces and 10 of extrahepatic bile duct) was gently set on the collagen gel in a well, and 3 ml of the culture medium was added at the start of the culture. The culture wells were observed every day under both a stereoscopic microscope (Olympus, Tokyo, Japan) and a phase-contrast microscope (Olympus) until the growth of the epithelial cells on the collagen gel was sufficient to be cut with scissors at about 2 to 4 weeks of culture (see below). These areas of the gel were cut into wedges 3 mm in length with special scissors (B-41; Natsume Seisakusho Co., Tokyo, Japan), to form explants consisting of epithelial cells for the secondary culture (secondary explants). Thirteen of these along with 5 secondary explants from the gallbladder and extrahepatic bile duct were available. Each secondary explant was set on another collagen gel (secondary culture), and these cultures were observed every day for up to 4 weeks.

Characteristics of biliary epithelial cells in primary and secondary cultures

The wells of the culture plates were fixed for 1 h in 10% neutral buffered formalin. Then the gels were detached from the plate, and 3-mm strips perpendicular to the cell surface were sliced and embedded in paraffin. After deparaffinization, thin sections (3-μm-thick) were then stained by H & E, periodic acid Schiff after diastase digestion (di-PAS), with alcian blue (AB) pH 2.5, or with mucicarmine.

For ultrastructural observations, both culture specimens were fixed in 2% glutaraldehyde for 30 min, post-fixed in 1% osmic acid and embedded in EPON 812. The specimens were then cut into ultra thin sections, double-stained with uranium acetate and lead citrate, and observed under a transmission electron microscope (Hitachi H-300, Tokyo, Japan).

Cell proliferation in primary and secondary cultures

S-phase (DNA-synthesizing stage) cells were analyzed by the 5-bromo-2′-deoxyuridine (BrdU) labeling method[12,13] and a routine avidin-biotin-peroxidase method. Breifly, BrdU (Sigma Chemical, St Louis, MO, USA) at a concentration of 10 μM/l was added to the culture media one week after the start of both the primary and the secondary cultures. The culture wells were incubated at 37°C for 30 min, and fixed in 10% neutral formalin for 30 min. Then the explants in collagen gel were cut into several strips perpendicular to the surface, and the strips embedded in paraffin. After deparaffinization, thin sections (4-μm-thick) were sequentially incubated in methanol with 0.3% H_2O_2 (for 30 min), 2 N-HCl (30 min), and 0.07 N-NaOH (2 min). The sections were then incubated first in 5% normal swine serum for 10 min, and then with monoclonal antibody to BrdU (diluted 1 : 100; Dakopatts, Santa Barbara, CA, USA) at room temperature for 30 min. Next, the sections were reacted with avidin-biotin-peroxidase complex (Vectastain ABC kit, Vector Laboratories, Burlingame, CA, USA) for 30 min and the reaction products visualized in 3, 3′ diaminobenzidine (DAB) (Sigma Chemical, St Louis, MO, USA) solution to which 0.03% H_2O_2 had been added. Positive reaction products were localized on nuclei. Such reaction products disappeared when the primary antibody was replaced by nonimmune serum.

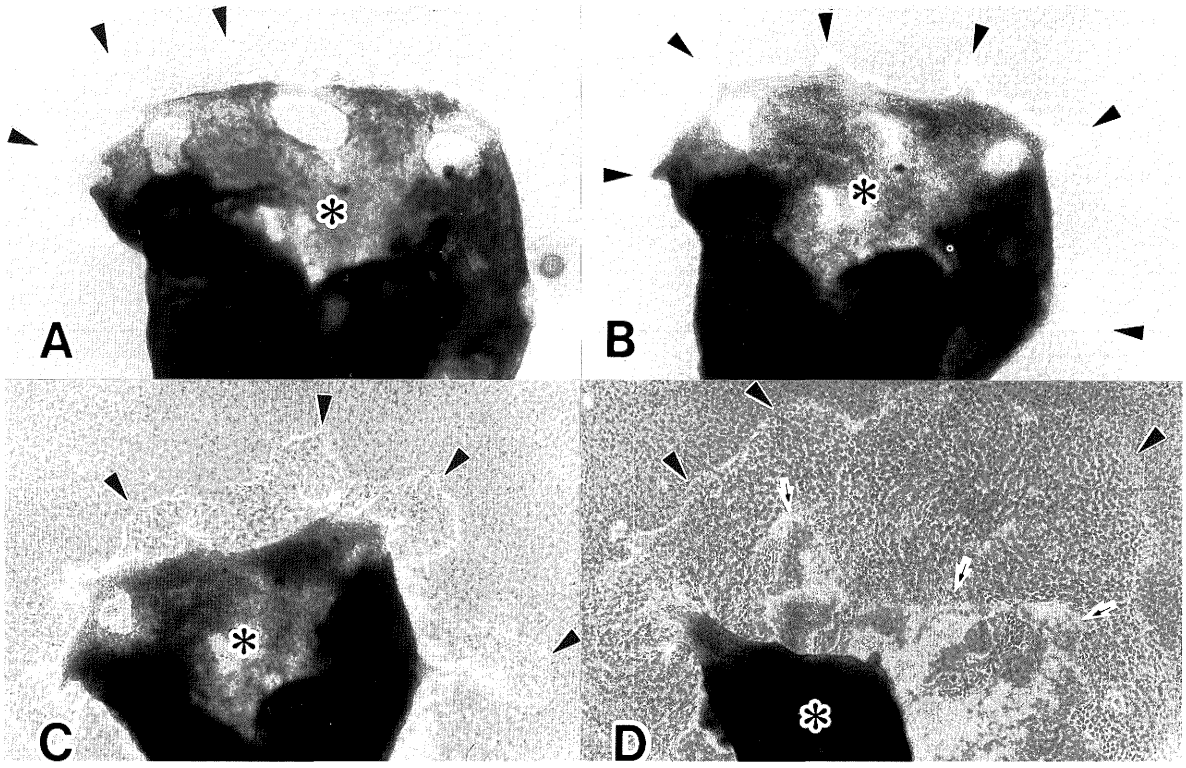

Fig. 1. Phase contrast photomicrograph of a primary explant (*) from murine gallbladder on collagen gel. Slight outgrowth of epithelial cell-like buds or leaves *(arrowheads)* from the explant (**A**. one day; and **B**. 2 days after cultivation) are observed, and these cells form epithelial sheets *(arrowheads)* around the explant (**C**. 3 days; and **D**. one week after cultivation).

Fig. 2. Histologic features of a tissue culture of a primary explant *(E)* from murine gallbladder 3 days after cultivation. Biliary epithelial cells are spreading and growing from the explant *(E)* as a monolayer, and the *arrowheads* show the proliferation of stromal cells. Note a monolayer of low columnar epithelium *(large arrows)* near the explant; these cells became flatter the further they were located from the explant *(small arrows)*. HE., ×107

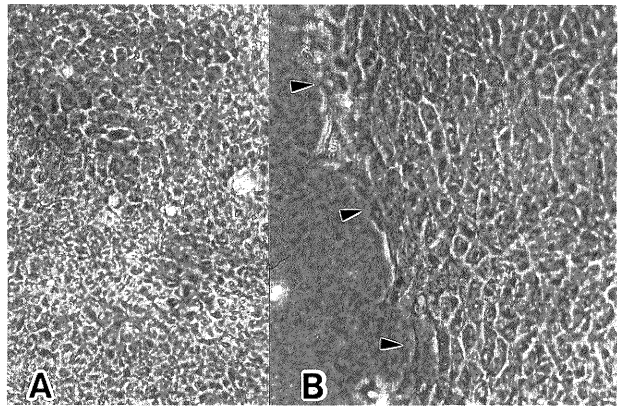

Fig. 3. Phase constrast photomicroscopy of growing and spreading epithelial cells forming a sheet-like configuration from a primary explant of murine gallbladder one week after cultivation. **A.** In the central part of the epithelial cell sheet, the growing epithelial cells are crowded and cell borders are indistinct. **B.** At the peripheral parts of the epithelial cell sheet, the epithelial cells are polyhedral, with cell borders relatively clear. *Arrows* denote the front of the outgrowing epithelial cell sheet.

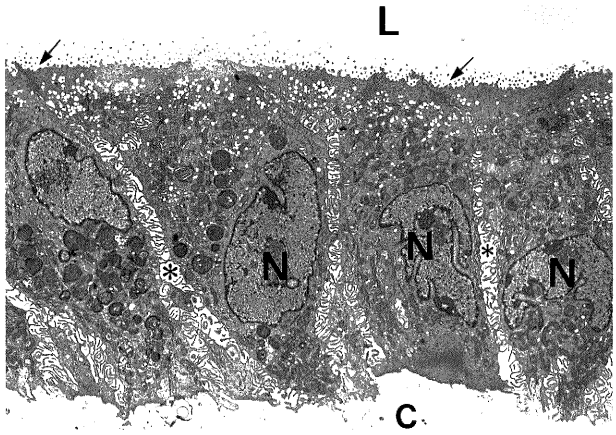

Fig. 4. Ultrastructural features of an epithelial cell sheet (monolayer) spreading from a primary explant of murine gallbladder. There are dense microvilli on the luminal surface, and intercellular digitations (*) and the subluminal junctional complex *(arrows)* are well-developed. *L*, luminal surface; *N*, nucleus; *C*, collagen gel. ×3,400

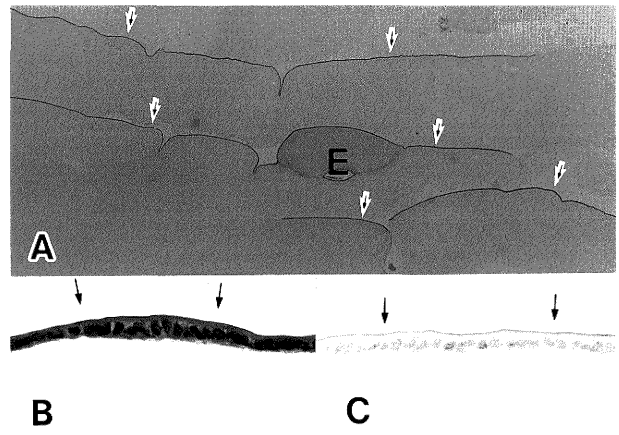

Fig. 5. Biliary epithelial cell sheets *(arrows)* cultured on collagen gel spread from a secondary purified explant of the extrahepatic bile duct of rat. **A.** Low power histologic features of several epithelial cell sheet specimens on collagen gel. *E*, secondary purified explant. **B.** In the central portion of the epithelial cell sheet, cells of the single epithelial cell layer cultured on collagen gel are low columnar in shape. *arrows*, luminal surface. HE., ×210 **C.** In the central portion of the epithelial cell sheet, the luminal surface of the epithelial cell layer cultured on collagen gel is faintly positive for alcian blue pH 2.5 *(arrows)*. ×210

RESULTS

Primary culture of biliary epithelial cells

Biliary epithelial cells of the gallbladders and extrahepatic bile ducts began to proliferate and spread like buddings or leaves on the collagen gel (Figs. 1, 2 and 3). These epithelial cell sheets spread toward the periphery at a rate of 0.3 mm/day (average) and continued to grow and spread for up to 4 weeks. Under the phase-contrast microscope, these cells were large and polygonal in shape and had recognizable intercellular borders at the edge of the epithelial sheet (Fig. 3A). In contrast, near the central parts of the explants, the epithelial cells were more compact or crowded (Fig. 3B). The manner and speed of biliary epithelial cell growth and spread were similar in the gallbladder and extrahepatic biliary tract explants, although the epithelial cells of the latter formed sheets that had a tendency not to extend.

Mesenchymal cells of the explants grew into the gel or under the epithelial cell layer. The front of the mesenchymal cells below the monolayer of epithelial cells were identifiable under the phase-contrast microscope (Fig. 1D). Histologically, these mesenchymal cells migrated into the collagen gel under the epithelial cell sheet (Fig. 2). At the periphery, the epithelial cells grew faster on the collagen gel than the mesenchymal cells, and so the front of the epithelial sheet contained no mesenchymal cells (Figs. 2, 3 and 4). After 1 to 4 weeks of culture, these areas were identifiable, both histologically and under the phase-contrast microscope, and were cut out with scissors and used for the secondary culture.

The epithelial cells in these areas were cuboidal or low columnar in shape in the central parts of the explants, and flattened at the periphery (Fig. 2). Ultrastructurally, these proliferating epithelial cells retained the *in vivo* features of the biliary epithelial cells, showing dense microvilli on the apical surface, mucus droplets in the supranuclear cytoplasm, and intercellular adhesion devices. Their apical surfaces showed slight bulging (Fig. 4).

Secondary culture of biliary epithelial cells

The epithelial cells extended in a monolayer as a sheet from the explants in half the explants of gallbladder and extrahepatic bile ducts, respectively (Fig. 5). Mesenchymal cell contamination was not evident. The epithelial cells from the gallbladder and extrahepatic bile ducts proliferated and spread in a similar fashion on the collagen gel at a rate of 0.3 mm/day (average) for the first 2 weeks. Thereafter, the speed of proliferation and extension was variable. These cells were cuboidal or short columnar in shape at the center of the sheet, and flattened at the periphery. Their apical surfaces were positive for AB 2.5, di-PAS, and mucicarmine staining. Ultrastructurally,

the cells near the center were characterized by flat apical borders with dense microvilli. The nuclei were located centrally or basally and the cell organelles were arranged toward the apex. Around the apex, intercellular attachment structures were developed, and lateral intercellular infoldings and interdigitations were found. The number of cellular organelles and cellular polarization in the secondary culture was small and insufficient when compared with the primary one.

After 4 weeks of culture, some parts of the epithelial cell sheets had detached focally from the collagen gel surface, causing several defects of epithelial cell sheets.

Proliferation of biliary epithelial cells

The flattened epithelial cells at the peripheral border of the epithelial layer in both explants were frequently positive for BrdU immunostaining, reflecting strong proliferative activity. In contrast, BrdU-positive cells in the central parts of the explant were distributed rather sparsely, suggesting that the proliferating zone was localized at the epithelial front.

DISCUSSION

Methods for the isolation of biliary epithelial cells from the intrahepatic biliary tree have been well established,[14-19] while various approaches have been tried for biliary epithelial cells from the extrahepatic biliary tree.[20-22] However, for all the methods for the latter reported to date, contamination of mesenchymal cells, particularly of fibroblasts, constitutes a major problem. In addition, the enzymatic, chemical, and mechanical methods employed for cell isolation and purification produce histologically detectable cellular damage.[15,24]

The present method of complete separation of epithelial cells from nonepithelial mesenchymal cells was besed on various reports of epithelial tissue cultures. Elhamady et al.[25] and Okumura[26] reported the biliary epithelial cells derived from gallbladder explants proliferated and spread out on culture-supporting matrices.[2,14,27] Since type I collagen gel promotes the proliferation as well as the functional and morphologic differentation of cultured epithelial cells,[28,29] it was used as the supporting matrix in this study. Consequently, the epithelial cells actively proliferated on the collagen gel, and the front of the cultured explants was wide enough to enable its removal with scissors. The successful removal of pure epithelial cell sheets was possible due to differences in the speed and direction of the proliferation of epithelial and mesenchymal cells in several places. Thus, there were several foci at the front of the epithelial cell sheets that had no mesenchymal cells beneath them. These areas were identifiable under a phase-contrast microscope. The cutting out of such foci allowed us to obtain explants composed exclusively of epithelial cells.

After plating these secondary explants on another collagen gel, we obtained a monolayer cell culture of purified biliary epithelial cells that lasted for up to 4 weeks. These cultured epithelial cells proliferated and retained the *in vivo* morphology and biological characteristics of epithelial cells. Meticulous processes for the separation of epithelial cells from other mesenchymal cells by mechanical, chemical or enzymatic means are not necessary with this method. However, the cultured cells showed variable necrotic changes and detachment from collagen gel after 4 weeks, indicating that examinations should be done within 4 weeks after the start of the culture.

Burwen et al.[30] reported that mammary epithelial cells that were dispersed and of squamous form on collagen gel showed active proliferation, while those that were differentiated and of cuboidal form failed to proliferate. This was also the case in our present study. The cells at the center of the sheet showed evidence of some morphologic differentiation and epithelial function such as mucus production, simulating the *in vivo* characteristics of biliary epithelial cells. That is, these findings suggest a relationship between the contact inhibition of epithelium and the functional differentiation of epithelial cells. The cultured epithelial cells are capable of simulating *in vivo* proliferation and functions.

In conclusion, this method makes it possible to culture pure epithelial cells of the biliary tree. We believe that this culture method may be applicable to other organs.

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