Reconstruction of Ductular Structure by Rat Bile Ductular Epithelial Cells

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INTRODUCTION

Liver transplantation has become a popular approach to treatment of liver disease. Donor shortage has stimulated investigation of possibilities in regenerative medicine.

A recent study showed that small hepatocytes, thought of as hepatic progenitor cells in the adult rat liver, reconstruct into hepatic organoids within 2 weeks in co-culture with hepatic nonparenchymal cells^[1]. These hepatocytes developed into anastomosing bile canalicular (BC) networks. And the BC-like structure contracted coordinately^[2].

Although hepatocytes in these organoids were likely to secrete bile into the BC-like structures, the networks were filled within a few weeks, which seemed to disturb for hepatocytes. Since hepatocytes are hepatic parenchymal cells, studies of the regenerative medicine of the liver focused in many cases on the regulation of hepatocytes' growth and proliferation. Bile ductular morphogenesis is essential to the regeneration of the bile drainage systems *in vitro*. In the liver, bile canaliculi are connected to the ductules that transport bile to the gallbladder.

In this research, we isolated bile ductular epithelial cells (BECs) from the rat liver, and reconstructed the ductular structure *in vitro*. We then investigated the morphology and functions possessed by these cells.

MATERIALS AND METHODS

Bile Duct Ligation

Bile duct ligation (BDL) in rats stimulates massive bile ductular hyperplasia in the liver, and the isolated large numbers of hyperplastic BECs retain characteristics phenotypic and functional properties in primary monolayer culture^[3]. Fischer 344 male rats (range, 170-200 g) were subjected to BDL performed by double ligation of the common bile duct and then resection of the intervening segment under anesthetization^[4]. After more than 2 weeks, the BDL-treated rats were sacrificed for an isolation of BECs.

Isolation of BECs

At the first step, almost all the mature hepatocytes and hepatic nonparenchymal cells were removed (Fig. 1; bar = 1 cm) from bile ductular tree by two-step liver perfusion method of Seglen with some modification. The remaining tissues were cut well with scissors into small fragments (< 1-mm) in Leibovits L15 medium. This mix was then shaken for 40 min at 37 °C in L15 medium supplemented with 300 U/ml collagenase, 700 U/ml hyaluronidase (type III), 10⁻⁸M dexamethasone, antibiotics and 0.1 µM Insulin, and consecutively filtered

through 20 µm-mesh. The digested cells were centrifuged at 50g for 5 minutes and the pellet was resuspended in L15 medium and recentrifuged at 150g for 5 minutes. Thereafter, the pellet was suspended in the culture medium. This pellet mainly contains aggregates that were consisted of 3 - 15 BECs.

Primary BECs Culture

The obtained cells were seeded on untreated plastic culture dishes, plastic culture dishes coated with rat-tail collagen, or collagen gel. Culture medium was Dulbecco's modified Eagle's medium (DMEM) supplemented with 5 µg/ml transferrin, 10⁻⁸M dexamethasone, antibiotics, 0.1 µM Insulin, 10 mM nicotinamide, 1 mM ascorbic acid 2-phosphate, 10 ng/ml epidermal growth factor, 10% fetal bovine serum, and hepatocyte growth factor (HGF). In some of the experiments, BECs seeded on collagen gels were covered with collagen gel again within 5 days after seeding (collagen gel sandwich culture method).

Type I rat tail collagen was prepared from the dissected rat-tails.

Immunocytochemistry

Samples were fixed with ethanol, and immuno-histochemical staining was done using the primary antibodies against the markers of bile ductular epithelial cells (cytokeratin 19; CK19, Novacastra) with frozen sections.

Image Analysis

The movement of BECs cultured in a collagen gel sandwich method was followed using timelapse microscopy at the condition of 5% CO₂ and 37°C.

Fig. 1 Bile Ductular Tree

RESULTS AND DISCUSSION

The Effect of BDL

Frozen sections of BDL-rat and untreated rat liver were stained using anti-cytokeratin 19 (CK19), which is a marker of bile ducts. The 2-week BDL liver of massive bile ductular hyperplasia in the liver was seen (Fig. 2; bar = 100 μ m).

These results indicate that BDL method is a very useful tool to isolate hyperplastic BECs, which retain the phenotypes and functional properties of normal BECs.

Purity of BECs

We stained BECs 3h after seeding on plastic culture dishes coated with collagen. The results indicated that the purity of CK19-positive cells was about 90%. The CK19-negative population seemed like myofibroblasts, prevalent around the bile ductules in the liver.

The Effect of HGF on BECs

To investigate the effect of HGF on cultured BECs, we cultured the cells in the medium supplemented with 0, 1, 10, 50 ng/ml HGF. 10 ng/ml HGF was used to culture BECs (Fig. 3).

Fig. 2 CK19 staining of untreated (L) and BDL(R) rat liver

Fig. 3 The Effect of HGF on BECs Culture

Fig. 4 The Effect of substrate on BECs Culture

The Effects of Substrate on BECs

To investigate the effects of culture substrate on cultured BECs, we cultured the cells on untreated plastic culture dishes (nc), dishes coated with 6 μ g/cm² type I collagen (1xccc), dishes coated with 60 μ g/cm² collagen (10xccc) or 5mg/mL collagen gel. Although, on collagen gel, cells survived more than 2 weeks after seeding, many others were dead within 10 days (Fig. 4). Some signal from the collagen seemed to be very important for the cultured BECs to survive.

Covering the surface of BECs with the collagen gel sheet 4 days after seeding, no cell death was observed. The shape of BECs became more piled-up structure as observed by phase contrast microscopy. It suggested that when cultured in the 3-dimensional scaffolding, the BECs became 3-dimensional structures, which seemed to be very effective for cultured BECs to survive.

Ductular Structure Formation of BECs

Covering the surface of the colony with the thin collagen gel sheet, morphogenesis of BECs to ductular structure was achieved. Transmission electron microscopy showed a ductular structure, composed of 3 – 5 BECs (Fig. 5). These structures were surrounded by a basement membrane. Numerous microvilli (arrow) and tight-junctions indicated the cell polarity.

Timelapse movie shows that the ductular structure formed by BECs was contracting (Fig. 6 bar = 50 μ m). This ductular structure formation is an encouraging result to the feasibility of a tissue engineering of the liver.

Fig. 5 Transmission electron microscopy micrographs

Fig. 6 Contraction of Ductular Structure

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