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Fetal liver cell-containing hybrid organoids improve cell viability and albumin production upon transplantation

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Cell transplantation is a potential alternative for orthotopic liver transplantation because of the chronic donor shortage. Functional liver tissue is needed for cell transplantations. However, large functional liver tissue is difficult to construct because of the high oxygen consumption of hepatocytes. In our previous study, we developed a novel method to generate hybrid organoids. In this study, we used fetal liver cells (FLCs) to construct a hybrid organoid. Nucleus numbers, angiogenesis, and albumin production were measured in transplanted samples. Higher cell viability and larger liver tissue was found in FLC-containing samples than in hepatocyte-containing samples. Furthermore, the therapeutic efficiency of FLC-containing samples was evaluated by transplantation into Nagase analbuminemia rats. As a result, an increase in albumin concentration was found in rat blood. In summary, transplantation of a FLC-containing hybrid organoid is a potential approach for cell transplantation.

[**Key words:** Growth factor immobilization; Tissue engineering; Organoid; Viability; Growth factor; Fetal liver cells]

Cell transplantation is an alternative therapy for orthotopic liver transplantation because of the chronic donor shortage (1–3). However, construction of large functional liver tissue is difficult because of the high oxygen consumption of hepatocytes (4). The following factors are considered necessary for construction of clinical scale tissue: (i) high viability of transplanted cells; (ii) maintenance of liver functions; (iii) sufficient angiogenesis of capillary networks after transplantation; (iv) proliferation of transplanted cells in vivo; (v) a vascular tree network-containing matrix such as decellularized liver tissue.

In our previous study, we developed a new cell transplantation technology using hepatocytes and heparin-conjugated gel particles termed a hybrid organoid. Cell viability, liver functions, and angiogenesis were enhanced in the hybrid organoid (5). However, mature hepatocytes are quiescent cells that are difficult to maintain and expand upon transplantation. In a previous study, we constructed functional liver tissue, but without cell proliferation (5), and proliferative cells are needed for clinical scale liver tissue.

Hepatic progenitor cells have the capacity for proliferation (6,7). Therefore, hepatic progenitor cells are considered to be a good cell source for transplantation. Some recent reviews discuss the embryonic development of liver cells. In rats, the morphology of hepatoblasts appears at embryonic day (ED) 10.5 (8,9). Hepatoblasts begin to differentiate into hepatocytes and cholangiocyte cells at ED15 (10).

Recent studies have investigated transplantation of fetal liver cells (FLCs) at ED14. Machimoto et al. (11) reported improvement in the survival rate of mice by transplantation of ED14 FLCs. Shirakigawa et al. (12) reported higher viability of transplanted FLCs than transplanted hepatocytes.

In this study, we combined ED14 FLCs and hybrid organoids and investigated the cell viability, liver functions, and cell maturation in comparison with mature hepatocytes. Using this approach, we expected to enhance the cell viability of FLCs in hybrid organoid transplantation. Furthermore, the albumin (ALB) concentration, which is an index of liver function, in Nagase analbuminemia rat (NAR) blood was measured after transplantation. Our aim was to generate functional liver tissue using FLC-containing hybrid organoids.

MATERIALS AND METHODS

Reagents, animals, and equipment Collagen (Cellmatrix Type 1-A, Type 1-C) was purchased from Nitta Gelatin (Osaka, Japan) and gelatin (Porcine skin type A; G2625) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Heparin sodium (100,000 U), *N*-hydroxysuccinimide (NHS), and 10% formalin solution were purchased from Wako Pure Chemicals (Osaka, Japan). 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) was purchased from Peptide Institute Inc. (Osaka, Japan). Dulbecco's modified Eagle's medium (DMEM), bovine pancreatic insulin, hydrocortisone, and L-proline were purchased from Sigma–Aldrich. Epidermal growth factor was purchased from Biomedical Technologies (Stoughton, MA, USA). The protein detector enzyme-linked immunoabsorbent assay (ELISA) kit, horseradish peroxidase/2, 20-azinobis (3-ethylbenzothiazoline-6-sulfonic acid ammonium salt) system, was purchased from Kirkegaard & Perry Laboratories (Gaithersburg, MD, USA). Rat ALB standard and anti-rat ALB antibody were purchased from ICN Pharmaceuticals (Aurora, OH, USA). Polyurethane foam (PUF) was kindly donated by Inoac (Nagoya, Japan). The Illustra RNA spin Mini RNA Isolation Kit was purchased from GE Healthcare (Berlin, Germany). The High Capacity cDNA Reverse Transcription kit was purchased from Applied Biosystems (Waltham, MA, USA). The sGAG quantification kit was purchased from Euro Diagnostica (Malmö, Sweden). The Human FGF basic quantikine HS ELISA kit was purchased from R&D Systems (Minneapolis, MN, USA), TaqMan Universal PCR Master Mix, Ultrapure distilled water, and

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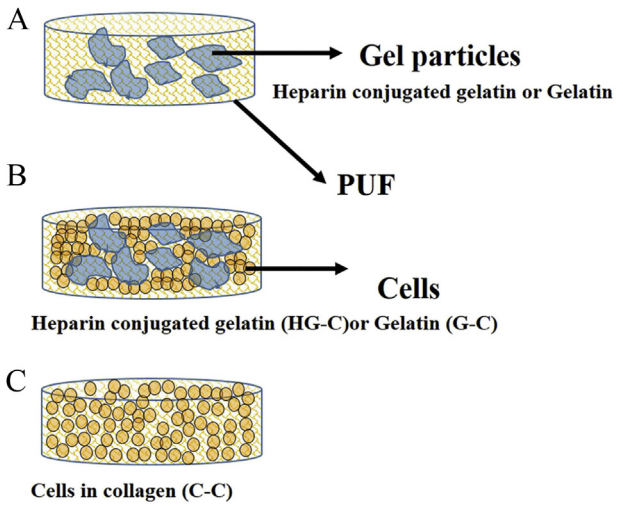

FIG. 1. Schematic of samples for transplantation. (A) Sample for evaluation of angiogenesis. (B, C) Samples for hybrid organoid transplantations.

TaqMan Gene Expression Assays [ALB (Rn00592480_m1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Rn01775763_g1)] were purchased from Life Technology (Carlsbad, CA, USA). Goat anti-rat ALB IgG (primary) and rabbit anti-goat IgG-FITC (secondary), rabbit anti-rat delta-like protein (DLK) IgG (primary), and goat anti-rabbit IgG-FITC (secondary) were purchased from Abcam (Tokyo, Japan). Hoechst 33342 solution was purchased from Dojindo (Kumamoto, Japan).

Male Wistar rats (6–8 weeks old) and pregnant Wistar rats at ED14 were purchased from Kyudo (Tosu, Japan). NARs (7 weeks old) were purchased from SLC (Shizuoka, Japan). Cell morphologies were observed under a phase-contrast microscope (Eclipse TE 300; Nikon, Tokyo, Japan). Absorbances were measured using a Multiskan FC Microplate Photometer (Thermo Scientific, MA, USA). The ratio of DLK^+ cells was measured by an SH800Z Cell Sorter (Sony Biotechnology Inc., Tokyo, Japan). The experimental protocol was reviewed and approved by the Ethics Committee on Animal Experiments of Kyushu University.

Heparin conjugation efficiency Heparin-conjugated gelatin and gelatin gels were formed using our previously reported method (5). Heparin (5 mg) was activated with 0.24 mg EDC (0.096 mg/ml) and 0.67 mg NHS (0.27 mg/ml) in 2.5 ml of 0.05 M 2-(*N*-morpholino) ethanesulfonic acid for 10 min at 37°C. Gelatin sol (20%) was mixed with the activated heparin solution (2.5 ml) at a ratio of 1:1, incubated at 37°C, and then allowed to cool for gelation. This heparin-conjugated gel (100 μl) was then immersed in an EDC (2 mg/ml)/NHS (8 mg/ml) solution for 3 h to permit cross-linking. Then, the gels were washed with 5M NaCl for 72 h. Water was removed from the gel by freeze drying to reduce the volume. In brief, washed gels were frozen at −20°C overnight and then freeze dried for 3 h. A 0.5% collagenase solution (400 μl/gel) was used to digest the gels. The quantity of heparin in the digested gels was measured by the sGAG quantification kit.

Growth factor immobilization and sustained release Heparin-conjugated gelatin and gelatin gels (50 μl) were prepared. Basic fibroblast growth factor (bFGF) promotes early morphological development of the fetal liver (13), which was used in the evaluation. To prevent adsorption of bFGF, gels were blocked in 1% bovine serum albumin (BSA) for 1 h. A bFGF solution (400 μl, 10 ng/ml) was added to gels and allowed to react overnight. bFGF concentrations were quantified by the Human FGF basic Quantikine HS ELISA kit. Immobilized bFGF concentrations were estimated by assaying the concentration of unbound bFGF in solution and subtracting it from the total bFGF concentration.

Gel digestion and bFGF sustained release in vivo were mimicked by collagenase digestion. Heparin-conjugated gelatin and gelatin gels (1 ml) were prepared and immersed in a bFGF solution (10 ml, 10 ng/ml) overnight. Then, the gels were removed from the bFGF solution and incubated in a collagenase solution (10 ml, 0.5 mg/ml) at 37°C. Gel weights and bFGF concentrations were measured at 0, 1, 2, 3, 3.5, and 4 h.

Induction of angiogenesis by heparin-conjugated gels PUF is a well-characterized biocompatible macroporous scaffold (14). A block of PUF was cut into round disks (1 cm in diameter; 2 mm thickness). Both heparin-conjugated gelatin particle- and gelatin particle-containing samples were embedded in PUF (Fig. 1A). Then, particle-containing PUF was transplanted subcutaneously into 70% partial hepatectomy-treated rats. After 7 days, the samples were retrieved from rats and evaluated by hematoxylin and eosin (H&E) staining to assess angiogenesis. Vessel numbers were counted and divided by the tissue area.

Cell morphology and flow cytometric analysis FLCs were obtained from ED14 rat fetuses by collagenase digestion and subsequent mesh filtration (40 μm pore size) (15,16). Cell viabilities were greater than 85% as assessed by the trypan blue exclusion method. FLCs were cultured in 96-well plates using DMEM-based hormonally defined medium at a density of 2×10^4 cells/cm^2. After 1 and 7 days of culture, the cells were analyzed by immunofluorescence staining. Goat anti-rat ALB IgG (primary) and rabbit anti-goat IgG (secondary) were diluted to 35 and 10 μg/ml, respectively. After fixation in 10% formalin and blocking with 1% BSA, the cells were incubated with goat anti-rat ALB IgG overnight followed by rabbit anti-goat IgG for 1 h. Nuclei were stained with Hoechst 33342 (5 μg/ml). The samples were examined under a fluorescence microscope. The ratio of ALB^+ cells to nuclei was calculated.

ED14 FLCs were fixed with 10% formalin and incubated with a rabbit anti-rat DLK antibody for at 4°C overnight. After washing with phosphate buffered saline (PBS), the cells were incubated with a goat anti-rabbit antibody for 1 h. Finally, DLK^+ cells were analyzed using a flow cytometer.

Transplantation of FLC-containing hybrid organoids Primary rat hepatocytes were obtained from 6–8-week-old male Wistar rats using a two-step collagenase perfusion method (17). To maintain the structure of the hybrid organoids, PUF was used during transplantation. Hybrid organoid samples, including 4×10^6 cell (hepatocyte or FLC) and heparin-immobilized gelatin gel particle-embedded PUF (HG-C) (Fig. 1B), cell (hepatocyte or FLC) and gelatin gel particle-embedded PUF (G-C) (Fig. 1B), and control cell (hepatocyte or FLC)-embedded collagen (C-C) (Fig. 1C), were prepared as reported in our previous study (5). Samples were transplanted subcutaneously into 70% partial hepatectomy-treated rats and retrieved at 7 days after transplantation for subsequent analyses. Periodic acid-Schiff (PAS) and H&E-stained sections were used to evaluate hepatocyte cluster sizes and cell numbers by counting nuclei. Frozen sections (8 μm thick) were prepared and fixed in 10% formalin. The sections were incubated with a goat anti-ALB antibody overnight. ALB immunostaining was visualized with a FITC-conjugated rabbit anti-goat antibody. Nuclei were counterstained with Hoechst 33342.

Real-time PCR Gene expression levels of ALB and GAPDH in the samples were evaluated after transplantation by RT-PCR. Total RNA was extracted using the RNA isolation kit according to the manufacturer's recommendations. Reverse transcription was performed with 0.45 μg total RNA using the High Capacity cDNA Reverse Transcription kit according to the manufacturer's instructions. cDNA synthesis was conducted at 37°C for 2 h and then terminated at 85°C for 5 min. Quantitative real-time PCR (QPCR) mixtures contained 12.5 μl TaqMan Universal PCR master mix (Applied Biosystems), 1.25 μl of each PCR primer, 8.75 μl Ultrapure distilled water, and 2.5 μl of 1:20 diluted cDNA in a total volume of 25 μl. PCR conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min in an Applied Biosystems 7300/7500 Real Time PCR System. QPCR results were analyzed by the comparative Ct method (18–20). The gene expression level of ALB was normalized to that of GAPDH. Cell viability was calculated by the following formula:

$$\text{cDNA mass of ALB (cALB)} = 2^{(-\text{Ct})} \times \text{dilution times} \quad (1)$$

$$\text{Viability (\%)} = \text{cALB after transplantation/cALB before transplantation} \quad (2)$$

To compare fetal and mature cell transplantations, the expression of ALB in adult cells and FLCs was calculated. The ratio of the ALB expression level [ALB (F/A)] and the GAPDH-normalized ALB expression level [ALB (F/A)/GAPDH (F/A)] between FLCs and adult cells was used in the evaluation.

Transplantation of FLCs into NARs FLC and heparin-immobilized gelatin gel particle-embedded PUF (HG-C) samples were subcutaneously transplanted into 70% partial hepatectomy-treated NARs. Rat blood was collected and ALB concentrations were evaluated at 0, 3, 5, 7 days by an ALB ELISA. Moreover, we investigated the ALB consumption rate in blood. In brief, an ALB solution was injected intravenously. Then, rat blood was collected at 3, 5, and 7 days and the concentration of ALB was measured by ELISA.

Statistical analysis Results are presented as means ± standard deviation (SD). Statistical analyses were performed using multiple comparisons with Tukey's test. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Characteristics of heparin-conjugated gels Immobilized heparin was confirmed by the sGAG quantification kit. A higher heparin concentration was found in heparin-conjugated gelatin gels than in gelatin gels. The immobilization efficiency of heparin was 59.5% at a density of 600 μg/ml of gel (Fig. 2A). Using a bFGF ELISA, we found more immobilized bFGF in heparin-conjugated gelatin gels than in gelatin gels. The efficiency of bFGF immobilization was 61.1% at a density of 27.3 ng/ml of gel (Fig. 2B).

Sustained release of growth factors was confirmed in heparin-conjugated gels. The quantity of bFGF released by collagenase