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Liver-specific extracellular matrix hydrogel promotes liver-specific functions of hepatocytes in vitro and survival of transplanted hepatocytes *in vivo*

Hiroyuki Ijima,[1,*] Shintaro Nakamura,[1] Ronald Perocho Bual,[1,2] and Kozue Yoshida[1]

Department of Chemical Engineering, Faculty of Engineering, Graduate School, Kyushu University, 744 Motooka, Nishi-ku, Fukuoka 819-0395, Japan[1] *and Department of Chemical Engineering & Technology, College of Engineering, Mindanao State University-Iligan Institute of Technology, A. Bonifacio Avenue, Tibanga, Iligan City 9200, Philippines*[2]

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A solubilized liver-specific extracellular matrix (L-ECM) substratum was obtained by decellularization of porcine liver using Triton X-100 and pepsin treatments. The L-ECM was able to immobilize hepatocyte growth factor at a high efficiency of 87%. L-ECM gelled spontaneously in a physiologically neutral environment. Primary hepatocytes embedded in the L-ECM gel showed a high albumin synthesis activity and ethoxyresorufin-O-deethylase (EROD) activity even at 3 weeks in culture. In addition, the L-ECM gel-embedded hepatocytes implanted subcutaneously into partial hepatectomized rats showed a high survival rate (18%) and formed a large liver tissue-like structure. Their efficiencies of EROD activity and large liver tissue-like structure formation were about twice those of collagen gel-embedded hepatocytes. Based on these results, we clarified the effectiveness of L-ECM gel as a substrate for hepatocyte culture and transplantation.

[**Key words:** Liver-specific extracellular matrix; Hydrogel; Hepatocyte; *In vitro* culture; *In vivo* transplantation]

Tissue engineering for regenerative medicine requires growth factors, cells, and a scaffold for optimal effectiveness. It is important to provide a microenvironment that ensures the greatest cellular capacity for realization of liver tissue engineering. Therefore, to provide an adequate microenvironment for cells, an optimal scaffold should be used.

As a scaffold, an organ-specific extracellular matrix (ECM) is desirable because of its physiological and physical properties. This prediction is supported by previous reports. In fact, matrices derived through decellularization and solubilization of native tissue are more effective as scaffolds for organ-specific functions, cellular growth and differentiation (1,2).

In addition, the micro-surrounding of a cell is a hydrogel derived from the ECM in cases of soft tissues such as the liver and brain. Moreover, for cell culture and tissue engineering, the ability to generate undefined forms of materials is important. Indeed, hepatocytes cultured in collagen gels also perform liver-specific functions (3). Therefore, it is expected that a hydrogel derived from an organ-specific ECM will be an appropriate scaffold for cells.

Growth factors are essential for growth, organ-specific functions, and increments of cell aggregates with multicellular layers (tissue-like structures) (4). However, growth factors are expensive and their biological activities can be unstable.

In recent years, studies have aimed at solving these problems by immobilizing growth factors on materials (5). Immobilization of growth factors may not only reduce consumption, but also improve their long-term stability. *In vivo*, many kinds of polysaccharides, such as heparin, and complexes of growth factors and polysaccharides form. Moreover, the bioactivities of growth factors are enhanced by interactions with ECM components (6).

In this study, we developed a scaffold that provides an adequate microenvironment for cells by focusing on growth factor immobilization to an organ-specific matrix gel for tissue regeneration. Specifically, solubilized liver-specific extracellular matrix (L-ECM) was obtained for the liver that plays an important metabolic function in our body. Furthermore, we demonstrated the effectiveness of L-ECM as a substrate for hepatocyte culture and transplantation.

MATERIALS AND METHODS

Decellularization of porcine liver Decellularized liver was obtained from pigs (Kyudo, Saga, Japan). All stages of the liver decellularization process were performed at 4 °C. Until the decellularization process was carried out, the porcine liver was stored at −80 °C. Then, the porcine liver was cut into small pieces and washed in calcium and magnesium-free phosphate-buffered saline (CMF-PBS) for 10 min. Triton X-100 (Sigma, St. Louis, MO, USA), a non-ionic surfactant, was then used to decellularize the organ. This process proceeded for 120 h in 1% Triton X-100/CMF-PBS. After cell components were removed, Triton X-100 was replaced by CMF-PBS, and the decellularized liver was obtained. These experiments were reviewed by the Ethics Committee on Animal Experiments of Kyushu University (A25-282-0, 21 Feb 2014).

Histological analysis For histological examination, tissue samples were fixed in 10% phosphate-buffered formalin (pH 7.4) for more than 3 days. The fixed samples were sectioned at 4 μm thicknesses and stained by a standard hematoxylin and eosin (H&E) protocol to validate the removal of cell nuclei. Stained samples were examined under a phase contrast microscope.

DNA isolation and quantification DNA content was used as an indicator of the presence of residual cells in decellularized tissue. Approximately 10 mg of each sample was placed in 1 ml of a proteinase K solution (Wako Pure Chemicals, Ltd., Osaka, Japan) (100 mg/ml in 0.01 M Tris–HCl and 0.01 M EDTA, pH 7.8) and digested

* Corresponding author. Tel./fax: +81 92 802 2748.
E-mail address: ijima@chem-eng.kyushu-u.ac.jp (H. Ijima).

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at 56 °C overnight. The DNA content in digested samples was assessed using a Fluorescent DNA Quantitation Kit (Bio-Rad Laboratories Inc., Hercules, CA, USA), according to the recommended protocol. The DNA concentration was calculated by fluorescence intensity measurements and expressed as micrograms per milligram of wet tissue weight.

Glycosaminoglycan quantification The sulfated glycosaminoglycan (GAG) content was used as an indicator of the alteration in structural composition of decellularized tissue. Native or decellularized tissue samples were lyophilized, minced, and weighed. The GAG amounts in native and decellularized livers were determined using a GAG quantitative kit (Euro Diagnostica AB, Malmö, Sweden), according to the manufacturer's instructions. Results are expressed as micrograms per milligram of wet tissue weight.

Preparation of hydrogel derived from decellularized porcine liver Decellularized tissue samples were lyophilized, minced, and weighed. Then, approximately 10 mg of each sample was placed in 1 ml of a pepsin solution (1 mg/ml in 0.1 N HCl; Sigma). L-ECM was obtained by solubilization in the pepsin solution at 4 °C for 72 h. The pH of L-ECM was adjusted to 3.0 by dialysis with Spectra/Por 6 (MWCO: 1000; Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA). The dialyzed L-ECM was mixed with concentrated minimum essential medium (×10 MEM) and buffer at a ratio of 8:1:1 (v/v) and kept on ice. The solution formed a gel after incubation at 37 °C for 30 min by self-assembly into a three-dimensional network.

Immobilization of growth factors to L-ECM The ability of L-ECM to immobilize growth factors was investigated using hepatocyte growth factor (HGF; R&D Systems, Minneapolis, MN, USA). A 0.03 mg/ml solution of L-ECM was prepared using 0.001 N HCl, and 200 μl of the L-ECM solution was poured into the well of a 48-well cell culture plate (bottom area: 1.1 cm^2, product No. CN-150687; Thermo Fisher Scientific, Kanagawa, Japan). The solution was air dried on a clean bench for approximately 2 days. After air drying, the formed film was crosslinked for stabilization by 1% glutaraldehyde (Kishida Chemical Co., Osaka, Japan) for 3 h. Residual glutaraldehyde was rinsed off by washing with CMF-PBS. Films of collagen (Cellmatrix Type I-C: 3 mg/ml) (Nitta Gelatin, Osaka, Japan) were also prepared by the same method. These films were treated using 0.1% bovine serum albumin (BSA; Wako Pure Chemical Industries) to inhibit physical adsorption of growth factors. The films were immersed in growth factors (200 μl) for 4 h at room temperature. The growth factor concentration was evaluated using a Duo Set ELISA Development kit (R&D Systems Inc.). The quantity of immobilized growth factor on the film was estimated by subtracting the non-immobilized growth factors from the total growth factors. In addition, the ratio of immobilized growth factor was calculated by dividing the amount of immobilized growth factors by the initial amount of growth factors.

Primary rat hepatocyte culture in an L-ECM gel Primary rat hepatocytes were isolated from 6–8-week-old male Wistar rats (Kyudo). Hepatocytes were prepared using a two-step collagenase perfusion method (7), and cell viability was estimated to be approximately 90% by trypan blue exclusion. Growth factor-containing, serum-free medium was used unless stated otherwise. The culture medium consisted of DMEM supplemented with 10 mg/l insulin from bovine pancreas (Sigma), 7.5 mg/l hydrocortisone (Sigma), and 60 mg/l L-proline (Sigma). Other supplements, except epidermal growth factor, were identical to those used in our previous study (8). This culture medium was named D-HDM. This experiment was reviewed by the Ethics Committee on Animal Experiments of the Kyushu University. Isolated hepatocytes were embedded in the L-ECM gel and a type I collagen gel (referred as I Col) at a density of 1×10^6 viable cells/ml. Embedded hepatocytes were cultured at 37 °C in a humidified atmosphere with 5% CO_2. The medium was changed at 4 h after cell inoculation and every 48 h thereafter.

Determination of cell number Hepatocytes cultured in L-ECM and collagen gels were harvested using collagenase (Wako) (9). Briefly, the hepatocytes embedded in each gel were dissociated by a collagenase solution (0.5 mg/ml). The cell lysate was produced by freeze (−80 °C)/thawing (25 °C) and stored at −80 °C. Total DNA was quantified using a commercially available Fluorescent DNA Quantitation Kit (Bio-Rad Laboratories Inc.), according to the manufacturer's instructions. For each group, a minimum of three samples were analyzed to determine total DNA, and each sample was analyzed in triplicate.

Hepatocyte functions Hepatocytes have a large number of functions in vivo, such as albumin secretion, urea synthesis, and drug metabolism. Consequently, we measured the protein composition, nitrogen metabolism, and drug metabolism that are representative of liver functions.

Albumin concentration in the culture medium was measured using the protein detector ELISA kit HRP/ABTS system (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) (10). Rat albumin standard and an anti-rat albumin antibody were purchased from ICN Pharmaceuticals (Aurora, OH, USA).

The urea synthesis rate was estimated by measuring the urea concentration in the medium. Briefly, medium was substituted with 1 mM ammonium chloride-supplemented D-HDM and incubated for 2 h. The urea concentration in the medium was measured by a urea nitrogen test (Wako Pure Chemical Industries).

Ethoxyresorufin-O-deethylase (EROD) activity is an indicator of cytochrome P450, family 1, subfamily A, polypeptide 1 (CYP1A1) activity and was estimated by measuring the intensity of resorufin fluorescence in the medium. In this assay, CYP1A1 was induced by 3-methylcholanthrene (Sigma). Briefly, the medium was substituted for 4 mM 3-methylcholanthrene (Sigma)-supplemented D-HDM and incubated for 24 h. The medium was replaced with 20 mM ethoxyresorufin (Wako Pure Chemical Industries)-supplemented D-HDM and incubated for 1 h. The intensity of resorufin fluorescence in the medium was measured using a fluorescent plate reader (1420 ARVOmx-L2 system; PerkinElmer, Waltham, MA, USA).

Each value was normalized to the cell number, and hepatocyte morphology on film was observed using a phase contrast microscope.

TABLE 1. Abbreviations of transplantation conditions.

Host rat	Carrier type	Abbreviation
PH treated rat	Type I collagen	PH-I
	L-ECM	PH-L
Non-treated rat	Type I collagen	Non-I
	L-ECM	Non-L

Preparation of hepatocytes embedded in an L-ECM gel-filled PUF scaffold Polyurethane foam (PUF; INOAC, Nagoya, Japan) is a biocompatible macroporous scaffold. It has a sponge-like macroporous structure with each pore consisting of smooth thin films and thick skeletons (8). The block of PUF was cut into round flat plates (1 cm diameter × 1 mm thickness). Before use, the PUF plates were submerged in ultrapure water, deaerated with a vacuum pump, and sterilized by autoclaving in ultrapure water. The sterilized PUF plates were coated with 1% BSA, and hydrophilic-treated PUF scaffolds were obtained by lyophilization (11). Primary rat hepatocytes were isolated as described above. Isolated hepatocytes were mixed in L-ECM and type I collagen sols at a density of 1×10^6 viable cells/ml and poured onto the PUF. These sols with hepatocytes formed a gel upon incubation at 37 °C for 30 min by self-assembly into a three-dimensional network.

Transplantation of hepatocytes embedded in an L-ECM gel-filled PUF scaffold All of the abovementioned scaffolds were transplanted in 6-week-old male Wistar rats. A 70% partial hepatectomy (PH) was performed at the same time. To minimize the influence of individual differences, each conditional sample was subcutaneously transplanted into one rat. To obtain reproducibility, similar transplantation operations were performed on three rats. Hepatocyte-embedded scaffolds were retrieved at 7 days after transplantation for subsequent analysis. Abbreviations of all conditions are defined in Table 1.

Evaluation of *in vivo* transplantation After subcutaneous transplantation of hepatocytes embedded in the L-ECM gel-filled PUF scaffold for 7 days, samples were retrieved for histological evaluation. The samples after transplantation experiments were soaked in formalin, embedded in paraffin, and sections for histological staining. The 4 μm-thick, paraffin-embedded sections were stained with hematoxylin–eosin (H&E). Transplanted hepatocytes were recognized by a previously reported method (12). In brief, hepatocytes were identified by their large size, large spherical nuclei, and distinct cytoplasmic staining by hematoxylin and eosin. All other cell types present in this tissue were distinguished by their much smaller, spindle-shaped morphology. The area of hepatocyte aggregation was analyzed by ImageJ and converted to the hepatocyte number per specimen by dividing the single hepatocyte area. The average area per hepatocyte was determined by calculating the mean of the measured area of 500 individual hepatocytes. Ten sections were analyzed per condition. These experiments were reviewed by the Ethics Committee on Animal Experiments of Kyushu University.

Statistical analysis Results are presented as the mean ± standard deviation. Statistical analysis was performed using a two-tailed unpaired Student's *t*-test. $p < 0.05$ was considered to be statistically significant.

RESULTS

Decellularization of porcine liver The color of porcine liver had changed from brown (Fig. 1A) to white (Fig. 1B) by treatment with the 1% Triton X-100 solution. To assess the efficiency of decellularization, H&E staining was used for histological evaluation. In the case of native porcine liver, cell components, nuclei, and cytoplasm were observed (Fig. 1C). However, in the decellularized liver, these components were not observed (Fig. 1D). Furthermore, the vascular structure was confirmed in native porcine liver by scanning electron microscopy (SEM) (Fig. 1E). A fibrous structure was observed in the decellularized liver (Fig. 1F). The dry weight of the decellularized liver ECM was 18.0 ± 0.2 g/g-wet weight of porcine liver.

DNA and glycosaminoglycan quantifications The DNA contents of native and decellularized livers were 286.2 and 22.5 ng/mg-wet weight, respectively (Fig. 2A). Therefore, 92.4% DNA

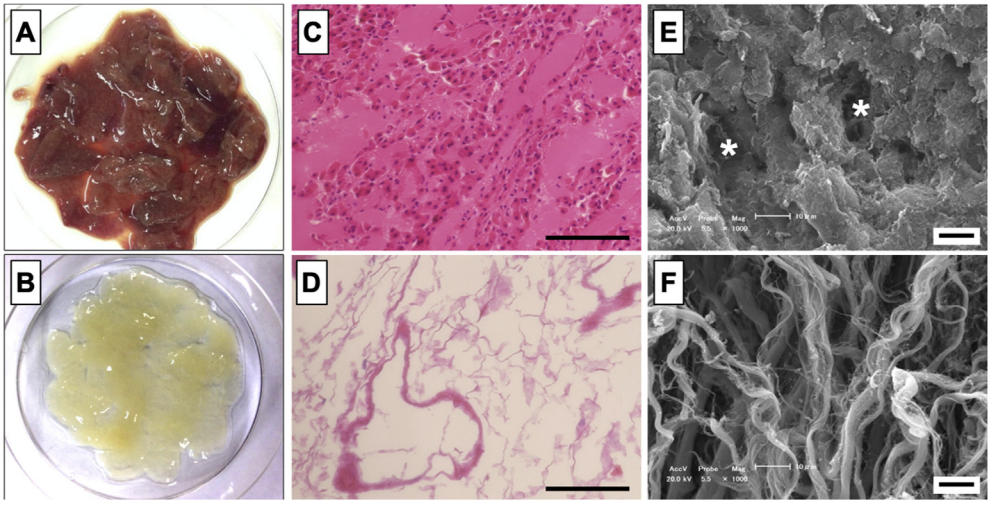

FIG. 1. Appearance of the decellularized porcine liver. Gross morphology of natural liver (A), and materials resulting from decellularization of liver (B). Histological evaluation of natural liver (C) and decellularized liver (D) by H&E staining. Purplish red is indicative of cell nuclei and red is indicative of cytoplasm (C). Pink is indicative of ECM (D). (C, D) Bars represent 100 μm. (E, F) SEM images of native porcine liver (E) and decellularized porcine liver (F). Asterisk (*) indicates blood vessels. SEM images show ×1000 magnification and scale bars represent 10 μm.

content was removed from the native liver. The GAG amounts in native and decellularized livers were 0.53 and 0.14 ng/mg-wet weight, respectively (Fig. 2B). Therefore, 26.4% GAGs had remained in the decellularized liver.

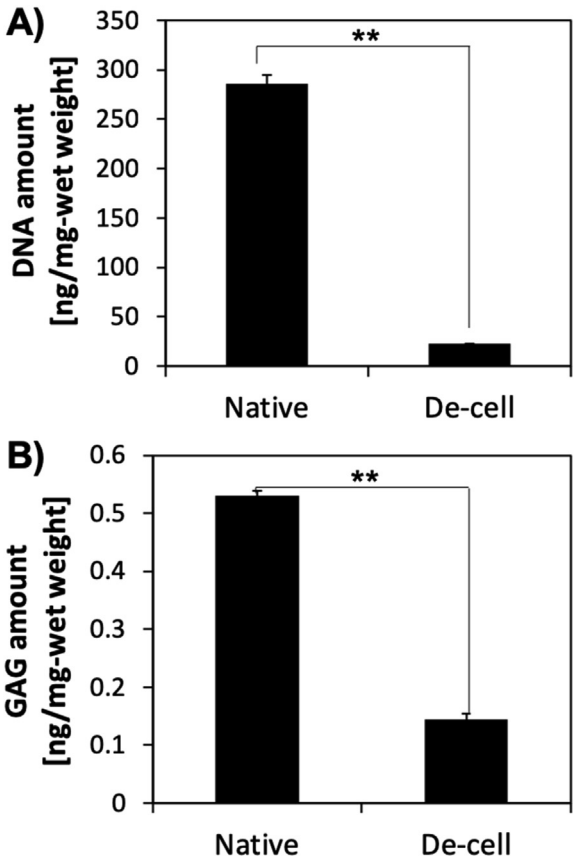

FIG. 2. DNA (A) and GAG (B) contents of liver ECM materials decellularized with Triton X-100. DNA content (A) and GAG contents (B) are shown. Samples prepared using Triton X-100 had lower DNA and GAG contents than native liver (**$p < 0.01$). n = 3, bars represent S.D.

Immobilization of growth factors to L-ECM and preparation of the L-ECM gel Hepatocyte growth factor (HGF) was immobilized on the L-ECM film to a greater extent than on the collagen film at a density of 10–100 ng/ml (Fig. 3A). The immobilized growth factor density increased linearly depending on the growth factor concentration applied to the films. In addition, the immobilization efficiencies of HGF on L-ECM and I col films were 86.9% and 17.6%, respectively (Fig. 3A). These data indicate that L-ECM has the ability to immobilize growth factors efficiently, even in the presence of albumin.

Upon adjusting the pH and temperature of the solubilized liver matrix (Fig. 3B) to physiological conditions (pH 7.4, 37 °C), the solution self-assembled into a gel (Fig. 3C). L-ECM and L-ECM gel were translucent.

Primary rat hepatocyte culture in the L-ECM gel For 3 weeks of culture, the spherical morphology of hepatocytes embedded in L-ECM and I col gels was confirmed by SEM (Fig. 4). Each gel has a fiber-like skeletal structure (Fig. 4). Additionally, it was observed that a cell could adhere within the fiber structure of the gel (Fig. 4). However, there was no difference in cell morphology between I col and L-ECM gels.

The measurement of viability is an important parameter that provides an accurate picture on how much percentage of viable hepatocytes (estimated by DNA amount) remained on the scaffold. The viability of hepatocytes was calculated by the cell number at different time interval (Fig. 5A) divided by the initial cell number. Results showed that the viabilities of hepatocytes cultured in L-ECM and I col gels were 65.5% and 71.3% at culture day 1, respectively. These viabilities decreased gradually with culture time. Finally, at culture day 21, the viabilities of hepatocytes cultured in L-ECM and I col gels were 51.0% and 41.1%, respectively.

Next, liver-specific functions were evaluated (Fig. 5B–D). The albumin secretion levels (pg/day) of hepatocytes cultured in L-ECM and I col gels were similar and were maintained for at least 3 weeks (data not shown). By calculating the albumin secretion rate (pg/day) divided by cell number at different time interval of Fig. 5A, the plot of Fig. 5B was obtained. However, considering the high cell number at day 3 (Fig. 5A), the albumin secretion rate per cell was expected to decrease. On the other hand, if we also consider the error bars (Fig. 5A), we can say that the liver function was perhaps maintained at similar level from culture day 1 to day 3

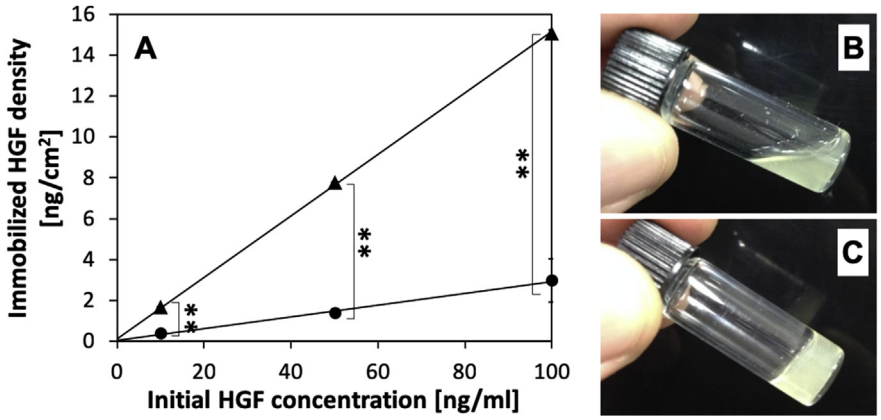

FIG. 3. HGF immobilizability of I col and L-ECM films (A). Triangles, L-ECM film; circles, I col film (n = 3, bars represent S.D.). Asterisk (*) indicates statistically significant differences from the I col film. $^{**}p < 0.01$. Gross morphology of L-ECM sol (B) and physically crosslinked L-ECM gel (C).

(p-value < 0.05, statistically significant difference). Moreover, the relative expression level of albumin was increased during day 5 and then maintained until day 21 of hepatocyte culture for both L-ECM and I Col gels.

On the other hand, the EROD activity of hepatocytes cultured in the L-ECM gel was similar to that of hepatocytes cultured in the I col gel until day 5 of culture (Fig. 5C). However, the level of EROD activity of hepatocytes cultured in the L-ECM gel was much higher than that of hepatocytes cultured in the I col gel after day 7 of culture (Fig. 5C). In contrast, the urea synthesis rates of hepatocyte cultured in both types of gels were similarly decreased with culture time (Fig. 5D).

Transplantation of hepatocytes embedded in the L-ECM gel-filled PUF scaffold The average area per hepatocyte was 250.9 ± 72.4 mm^2/single hepatocyte, and the average cell number per area before transplantation was 23.0 ± 2.2 cells/mm^2. Using these values, the distribution of the number of hepatocytes constituting the cell cluster was obtained (Fig. 6A–D). The largest cluster of hepatocytes was observed under the condition of an L-ECM gel transplanted into a PH-treated rat (PH-L) (Fig. 6B).

The largest areas of a hepatocyte cluster in an I col gel transplanted into PH-I, PH-L, Non-I, and Non-L were 1496, 3245, 871, and 1473 mm^2, respectively (Fig. 7A). The distribution of hepatocyte clusters indicated that the largest aggregation was formed in the case of PH-L among all conditions (Fig. 7A). These areas of clusters were converted to hepatocyte number per a cluster by dividing the single hepatocyte area. The number of hepatocytes forming the maximum cluster area in PH-I, PH-L, Non-I, and Non-L were estimated to 6, 13, 3.5, and 5.9, respectively (Fig. 7A). Moreover, clusters with more than 10 cells were only observed in the PH-L condition (Fig. 7A).

The numbers of clusters per specimen area of PH-I, PH-L, Non-I, and Non-L were 4.05, 6.31, 0.37, and 0.77 cluster/mm^2 (Fig. 7B). This evaluation indicated that the largest number of clusters was formed in the case of PH-L among all conditions (Fig. 7B). In addition, the viability of transplanted hepatocytes was calculated by the cluster number per area divided by the number of initial hepatocytes per area (Fig. 7C). The viabilities of transplanted hepatocytes of PH-I, PH-L, Non-I, and Non-L were 17.6%, 27.4%, 0.40% and 3.35%, respectively. This result indicates that the highest survival rate of transplanted hepatocytes was obtained in the case of PH-L among all conditions (Fig. 7C).

DISCUSSION

L-ECM gel culture The scaffold is one of the most important factors to consider in controlling cell behavior. Previous reports have shown the influence of a scaffold on a cell. In particular, matrices derived from decellularized tissue provide an appropriate environment for cell in terms of growth, differentiation, and organ-specific functions. In the present study, we developed a solubilized matrix derived from decellularized liver (L-ECM) to provide an appropriate microenvironment for hepatocytes. In addition, the gel consisting of L-ECM was developed to construct a three-dimensional scaffold, which was important to accomplish construction of a three-dimensional tissue with thickness. This gel was applied to cell culture and transplantation to evaluate the benefit for liver tissue engineering.

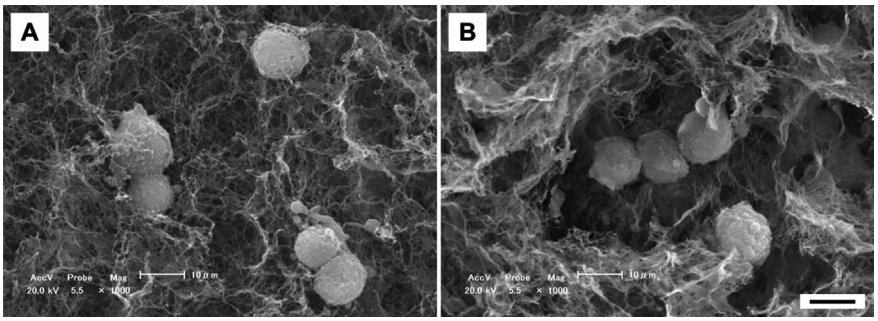

FIG. 4. Scanning electron micrographs of cells in I col gel (A) and L-ECM gel (B) at culture day 21. The hepatocyte morphology was round in both types of gel. Bars represent 10 μm.

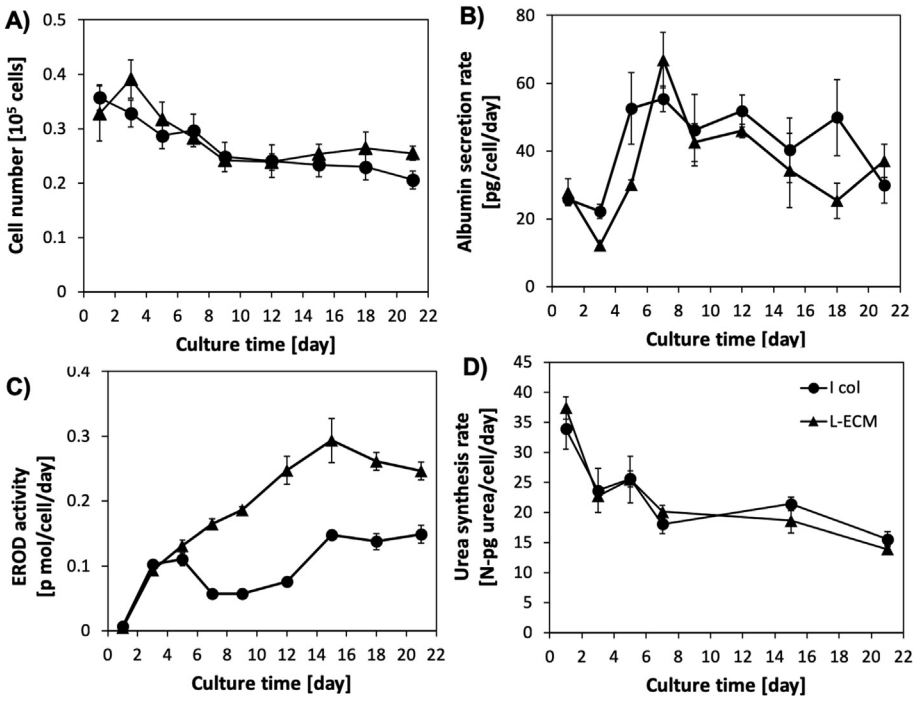

FIG. 5. Cell number and liver-specific functions of primary rat hepatocytes in I col and L-ECM gels. (A) Cell number; (B) albumin secretion rates; (C) EROD activity; (D) urea synthesis rates. n = 3, bars represent S.D.

In general, hepatocytes lose their liver-specific functions immediately after harvesting from the liver (13,14). In particular, expression of CYP enzyme is reduced to 20% within 24 h after harvest (15). Moreover, monolayer culture of hepatocyte causes deterioration of functions (16). Thus, maintenance of liver-specific functions is difficult even in the short term.

An organ-specific ECM is effective for cells to exhibit their phenotype (2,17–19). Moreover, a hydrogel can provide an adequate microenvironment for cells (2,20,21) and imbed a larger number of cells per volume than any other scaffold configuration (22). Because L-ECM is an organ-specific ECM with the ability to immobilize GFs, it was expected that a functional culture system of hepatocytes satisfying two major factors of cell-functional ECM contact and stimulation by GFs would be constructed. In this study, hepatocytes cultured in L-ECM and I col gels had similar levels of albumin secretion. This result indicated that hepatocytes maintain their albumin secretion activity for 3 weeks in these gels. Hepatocytes embedded in the L-ECM gel expressed particularly higher EROD activity than those in the I col gel from culture day 3. In the case of urea synthesis rates of hepatocytes embedded in both gels, these levels decreased with culture time.

Kim et al. (23) reported that albumin secretion and urea synthesis activities of hepatocytes cultured in a material developed by combining polyethylene glycol and heparin are maintained for 2 weeks. In addition, Ijima et al. (10) reported that hepatocyte spheroids cultured in polyurethane foam (PUF) exhibit albumin secretion and urea synthesis activities for more than 3 weeks. However, lidocaine metabolism, which is an index of drug metabolism in hepatocytes, was gradually decreased with culture time even in spheroid culture (10). Dunn et al. (20) reported that hepatocyte culture between two collagen gel layers (sandwich configuration) maintained transferrin synthesis activity for 3 weeks, although urea synthesis activity of hepatocytes was decreased from day 7 of culture. Therefore, the formation of an organoid structure with cell–cell contacts is effective but insufficient for exhaustive expression of an organ-specific phenotype.

Moghe et al. (24) reported that the albumin secretion activity of hepatocytes cultured using a sandwich configuration of type I collagen and cultured on Matrigel was maintained at a high cell density. The level of albumin secretion on Matrigel did not fluctuate despite the seeding cell density, although the level of albumin secretion from hepatocytes cultured using the sandwich configuration of type I collagen was decreased at a low cell density (24). Therefore, not only cell–cell interactions but also ECM components are important factors to maintain and improve cell functions.

To achieve long-term stable liver-specific functions of hepatocytes, it is necessary to construct a culture system that satisfies cell–cell interactions, cell-organ-specific ECM interactions, and stimulation by growth factors (25). Therefore, construction of the hepatocyte spheroid-embedded L-ECM gel culture system will be an important research goal in the future (26).

Hepatocyte-embedded L-ECM gel transplantation The largest aggregation, the largest number of hepatocytes, and the highest viability of transplanted hepatocytes were obtained in PH-L among all conditions. In addition, these values of PH-L were higher than those of Non-L. Based on these results, growth factors such as HGF secreted by PH treatment were effective for construction of large clusters and survival of transplanted hepatocytes. Moreover, in PH-L, it was expected that the secreted growth factors would be immobilized to the L-ECM gel and exert an effect on transplanted hepatocytes. Such a liver regeneration environment is formed in the patient's body. Therefore, L-ECM has a good potential to become an effective material for cell transplantation. In present study, the viability of hepatocytes in PH-L at 1 week was 27.4%. There are three possible factors that may have been responsible for this phenomenon: configuration, composition, and growth factor immobilizability of the scaffold.

The configuration of the scaffold affects cell behavior. According to a report by Mooney et al. (27), the viability of hepatocytes transplanted for 1 week with PLA or PLGA sponges was less than 6%. Likewise, Kneser et al. (28) reported that the viability of

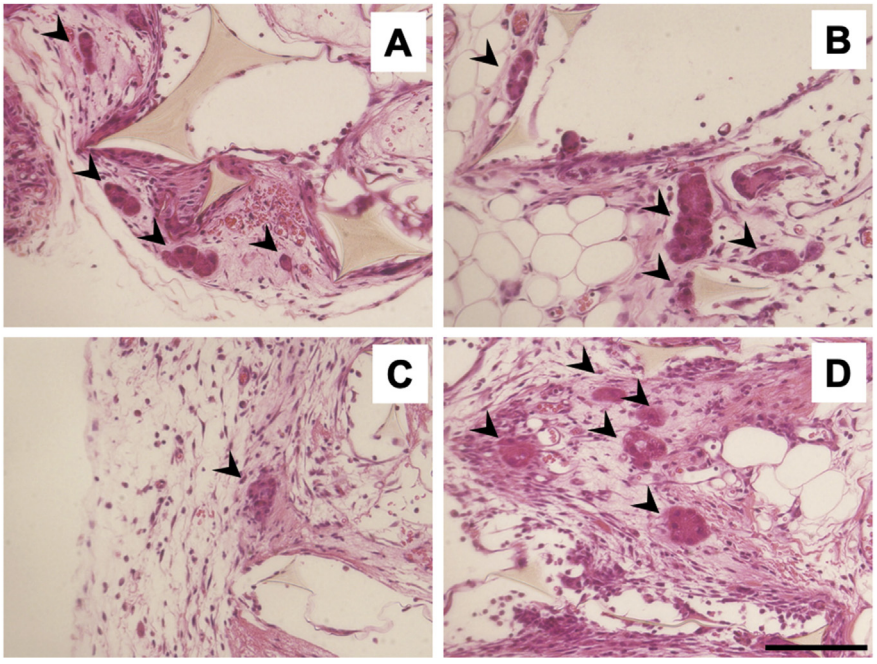

FIG. 6. Histological observation of a single hepatocyte-embedded in I col or L-ECM gels after transplantation into a rat treated by partial hepatectomy (PH-treated) and a normal rat (non-treated) at 7 days. (A) I col gel transplanted into a PH-treated rat; (B) L-ECM gel transplanted into a PH-treated rat; (C) I col gel transplanted into a non-treated rat; (D) L-ECM gel transplanted into a non-treated rat. Arrowheads indicate a cluster of hepatocytes. Bar represents 100 μm.

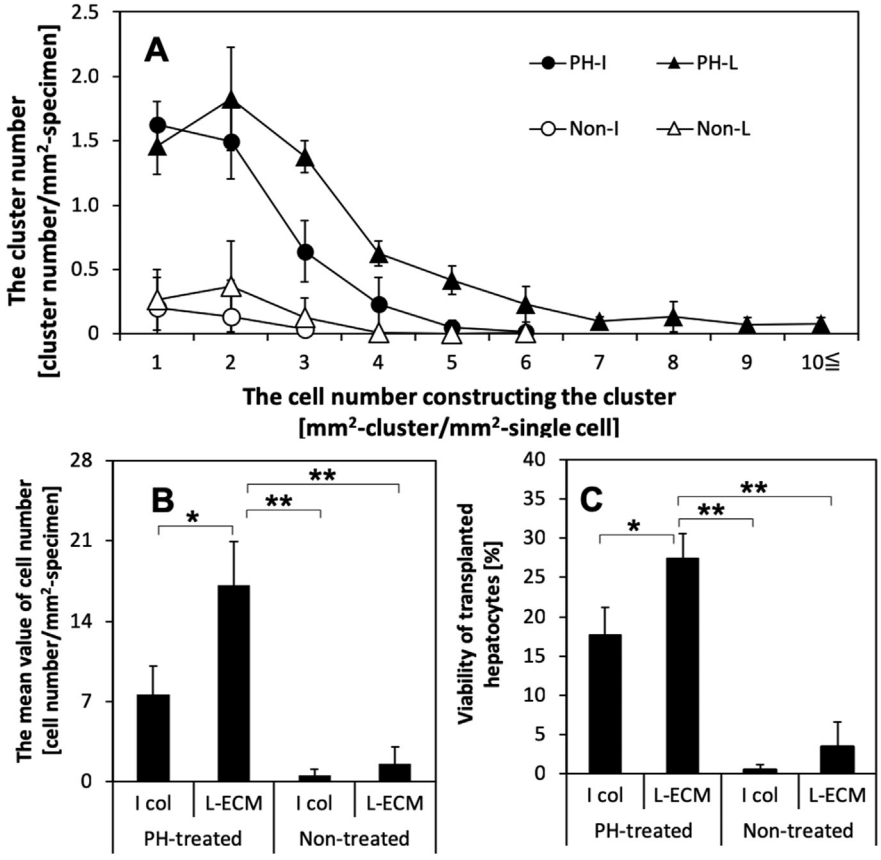

FIG. 7. Analysis of H&E staining images in Fig. 6 using ImageJ. Number of clusters classified by the constituent cell number (A). Samples were transplanted into non-treated and PH-treated rats. n = 3, bars represent S.D. Quantification of histological evaluation (B, C). Number of transplanted hepatocytes per square millimeter (B). Viability of transplanted hepatocytes (C). n = 3, bars represent S.D. Asterisk (*) indicates statistically significant differences from L-ECM samples in a PH-treated rat. $^{**}p < 0.01$, $^{*}p < 0.05$.

hepatocytes transplanted for 1 week with a PVA sponge was less than 2%. Bruns et al. (29) used a fibrin gel to transplant hepatocytes and found that survival of transplanted hepatocyte was improved. Based on these reports, the gel configuration is more effective for cell transplantation.

In terms of the scaffold composition, glycosaminoglycan, which is one of the components of L-ECM, plays an important role in cell functions and immobilization of growth factors. Hepatocytes cultured in glycosaminoglycan (dermatan sulfate, heparan sulfate, and heparin) formed spheroids, and the liver-specific functions of these hepatocytes were improved (30). Also, the composition of L-ECM was effective for hepatocytes, because degradation products of decellularized tissue stimulate angiogenesis (31) and cell proliferation (32). Moreover, Hou et al. (12) reported that heparin-conjugated-collagen, which can immobilize growth factors, was effective to improve transplanted hepatocyte viability and enhance angiogenesis. Another important component in L-ECM which constitutes its main structural element is Type I collagen. It provides tensile support, regulates cell adhesion and migration, and directs tissue development. Increasing the content of Type I collagen greatly contributes to the improvement of mechanical properties which are important for scaffold. In addition, porcine L-ECM contains types III, IV, V collagen and Laminin (33). Various effectiveness can be expected with the presence of these components.

Based on the above results, a hydrogel derived from a growth factor-immobilizable, liver-specific ECM has the potential to become an effective material to provide a suitable microenvironment for cells. However, for liver transplantation application, the use of L-ECM gel is not sufficient because it lacks the mechanical property necessary for maintaining the stability in vivo. That is why PUF was used as a support. The L-ECM gel filled-PUF scaffold configuration lasted for 3 weeks *in vivo* and facilitated the formation of tissue-like hepatocyte clusters. Having this kind of substrate that recreates the natural microenvironment for hepatocytes is of great advantage and a much-needed technology. Although the substrate system in this study was carefully designed to mimic the native ECM, there is still much work to be done. For instance, the use of spheroid cell morphology is effective to improve transplanted cell viability and angiogenesis (12). In addition, fetal liver cells (12) and hepatic progenitor cells (34) are promising cell sources to construct large scale hepatic tissues. Therefore, the combination of the L-ECM gel and spheroids of hepatic progenitor cells might be realized to establish hepatic tissue of a practical size. For the time being, having an L-ECM gel substrate that can be easily produced, stored, and applied *in vitro* and *in vivo* represents a step towards advancements of liver studies in tissue engineering.

In conclusion, the solubilized L-ECM derived from decellularized porcine liver was obtained by decellularization and solubilization with 1% Triton X-100 and 0.1 N HCl with pepsin. L-ECM has the ability to immobilize hepatocyte growth factor and self-assembled into a gel. EROD and albumin secretion activities of hepatocytes cultured in the L-ECM gel were maintained for 3 weeks, and EROD activity of hepatocytes in the L-ECM gel was higher than that of hepatocytes in the type I collagen gel. The largest aggregation, largest number of hepatocytes, and highest viability of transplanted hepatocytes were confirmed in the L-ECM gel transplanted into the PH-treated rat among all conditions. In conclusion, L-ECM has the potential to become an effective material in the field of liver tissue engineering.

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