Journal of Bioscience and Bioengineering
VOL. 117 No. 1, 99–106, 2014

www.elsevier.com/locate/jbiosc

Hybrid sponge comprised of galactosylated chitosan and hyaluronic acid mediates the co-culture of hepatocytes and endothelial cells

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Received 10 June 2013; accepted 14 June 2013
Available online 27 July 2013

When constructing an *in vitro* model of liver tissue to mimic the *in vivo* liver microenvironment, the major challenge is to preserve and maintain the hepatocyte phenotype. The aim of this study was to develop a novel intelligent hybrid sponge for use in a dense co-culture system designed to simulate the liver microenvironment. We prepared a galactosylated chitosan (GCs)/hyaluronic acid (HA) hybrid sponge using a freeze-drying technique for the co-culture of primary hepatocytes and endothelial cells. Subsequently, we investigated the biocompatibility of the GCs/HA scaffold with primary hepatocytes and endothelial cells in terms of cell attachment, morphology, bioactivity, and maintenance of specific liver functions. The GCs/HA-hybrid sponge demonstrated good biocompatibility not only with primary hepatocytes, but also with endothelial cells. In our model, primary hepatocytes exhibited superior bioactivity and higher levels of liver-specific functions in terms of hepatocyte-specific gene expression, urea production, and testosterone metabolism as compared to a monoculture system. We succeeded in constructing a liver tissue-like model using the GCs/HA-hybrid sponge. Therefore, we anticipate that GCs/HA-hybrid sponges may be a promising matrix for the co-culture of hepatocytes and endothelial cells in liver tissue engineering, and might be employed as a novel co-culture model for applications in toxicology and drug metabolism.

[**Key words:** Liver; Tissue; Galactosylated chitosan; Hyaluronic acid; Primary hepatocyte; Endothelial cell]

The liver is an organ of considerable cellular heterogeneity with hepatic lobules consisting of parenchymal hepatocytes, non-parenchymal cells (e.g., sinusoidal endothelial, stellate, and Kupffer cells), and some extracellular matrices (ECMs) comprising proteins, glycosaminoglycans, glycoproteins, and proteoglycans. Hepatocytes observed *in situ* within the liver exhibit a polygonal morphology; however, hepatocytes grown *in vitro* as monolayers are flat. Hepatocytes are thought to be able to fully execute liver-specific functions only in the context of the proper structural microenvironment represented by intact liver tissue. Because primary hepatocytes cultured as monolayers quickly lose structural polarity, it is difficult to use those cultures for assays of hepatic functions, e.g., drug metabolism. Therefore, it remains a major challenge for tissue engineers to develop intelligent scaffold materials that can recapitulate the liver microenvironment in an *in vitro* model system. In this context, we focused on a very dense co-culture system of hepatocytes and endothelial cells that closely resembles the major cell populations found in the intact liver.

In 2005, we successfully achieved hepatic organogenesis from murine ES/iPS cells (1–3). This mouse ES cell–derived *in vitro* liver tissue model, IVL^{mES}, included both hepatocyte layers and a vascular-like endothelial cell network; the model was capable of recapitulating most hepatic functions. Furthermore, another *in vitro* liver model, $IVL^{PH\&HUVEC}$, using primary hepatocytes, human umbilical vein endothelial cells (HUVECs), and Engelbreth–Holm–Swarm (EHS) gel was also established for use in a drug metabolism assay (4). While the EHS gel contains a variety of extracellular matrix components secreted from the EHS tumor, an ideal *in vitro* liver model would use constituent-defined materials that would provide a uniform background for the development of pharmacokinetics assays and artificial liver systems. Hyaluronic acid (HA), a ligand for endothelial cell-expressed CD44, was expected to be useful for the co-culture of hepatocytes and endothelial cells (5–7). In this study, we designed a new galactosylated chitosan (GCs)/HA scaffold that was expected to bind to both hepatocytes (through GCs) and endothelial cells (through HA), as can be seen in Fig. 1, and succeeded to construct a liver tissue-like model consisting of hepatocytes and endothelial cells on a GCs/HA sponge.

MATERIALS AND METHODS

Materials Chitosan (Cs) (viscosity: 25 ~ 75 cps; molecular weight: 6×10^5; degree of deacetylation: 80%), 1-ethyl-(dimethylaminopropyl) carbodiimide (EDC), *N*′,*N*′,*N*′-tetramethylethylethylene diamine (TEMED), and Sulfo-*N*-

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1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2013.06.015

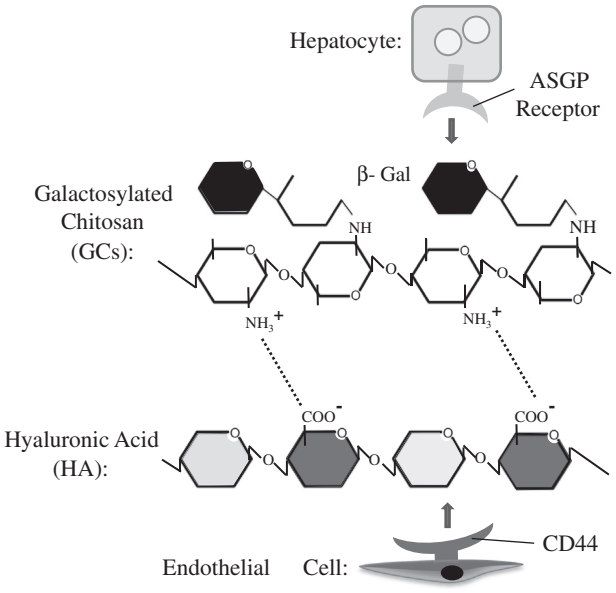

FIG. 1. The GCs/HA sponge is designed to bind both hepatocytes and endothelial cells by engaging specific cell-surface receptors on each cell type.

hydroxylsuccinimide (NHS) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). HA with viscous average molecular weight 8×10^5 was kindly gifted from Dr. Mitsuaki Goto, Molecular Engineering Institute, Kinki University (Fukuoka, Japan). Lactobionic acid (LA) was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Synthesis of GCs GCs was prepared from the reaction of Cs with LA by the method previously reported (8). Briefly, 12 mmol of LA was dissolved in 50 mL of 10 mM TEMED/HCl buffer (pH 4.7) and activated with a mixture of Sulfo-NHS (1.2 mmol) and EDC (12 mmol), and then reacted with Cs (24 mmol). The reaction was performed for 72 h at room temperature. The obtained GCs was purified by dialysis against Milli Q water for one week and then lyophilized.

Preparation of GCs/HA, GCs, and Cs sponges GCs/HA sponge was fabricated as follows: 2% GCs was dissolved in 0.5 M acetic acid aqueous solution by continuously stirring to form a homogenous solution, and mixed with 1% HA aqueous solution with a volume ratio of 1:1, followed by homogenizing for 20 min. Then, 0.2 mL of the obtained solution was poured into each well of 48-well polystyrene culture plates. The plates were frozen at −20°C, followed by freeze-drying to form a porous structure.

GCs and Cs sponges were prepared as described above. Briefly, 0.2 mL of 1% GCs and Cs solution was poured into each well of 48-well polystyrene culture plates and freeze-dried. Lyophilized sponges were treated by a gradient ethanol process. Before cell culture, the sponges were sterilized by ethylene oxide gas.

Scanning electron microscopy (SEM) The morphology of GCs/HA, GCs, and Cs sponges was observed by SEM (Topcon DS-720, Tokyo, Japan). The sponges were cut with a sharp scalpel, and then mounted onto an aluminum stub and sputter-coated with gold−palladium. Mean pore diameters were estimated by analysis of digital SEM images. Average pore sizes were determined based on the sizes of 40 pores for each sample.

Fourier transform infrared spectroscopy (FTIR) and ^{1}H nuclear magnetic resonance (^{1}H NMR) FTIR spectra were measured using a Nicolet 5700 FTIR spectrometer (Thermo Electron Co., WI, USA). Dried samples were ground with KBr powder and compressed into pellets for FTIR examination.

^{1}H NMR (600 MHz) spectra were recorded on an ECX-600 spectrometer (JEOL, Tokyo, Japan). The samples were dissolved in a 2/98 mixture of CD_3COOD/D_2O at a concentration of ca. 5 mg/mL.

Animals Male, 6−8 week old, DsRed2 C57BL/6 transgenic mice that constitutively and ubiquitously express the DsRed2 gene under the control of the CAG promoter (9) were used in this study. The experiments were conducted according to institutional ethical guidelines for animal experiments and safety guidelines for recombinant DNA experiments.

Isolation and culture of murine primary hepatocytes Red fluorescent hepatocytes were harvested from DsRed2 transgenic mice using a two-step *in situ* collagenase perfusion procedure (10) with slight modifications (4). Briefly, the liver was perfused with perfusion buffer and collagenase buffer by cannulation of the isolated portal vein with a 24-gauge catheter (Terumo, Tokyo, Japan). Then, the perfused liver was dissected, suspended in Hank's Buffered Salt Solution (HBSS), and filtered through a 100 μm pore mesh nylon cell strainer (BD Biosciences, Bedford, MA, USA). Hepatocytes were purified by density-gradient centrifugation (50 ×*g*, 10 min) using a 36% Percoll solution (GE Healthcare, Tokyo, Japan) at 4°C. Cell viability as measured by trypan blue exclusion was >90%. The primary hepatocytes were cultured with 10% FBS/William's medium E containing antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin).

Endothelial cells EAhy926 cells, generously provided by Dr. Cora-Jean S. Edgell, of the University of North Carolina, USA, were established by fusing primary human umbilical vein endothelial cells (HUVECs) with human lung adenocarcinoma epithelial cells (A549) (11). An enhanced green fluorescent protein (EGFP) gene-expressing EAhy926 cell line, GH7, was established by the introduction of the appropriate expression vector under control of the CAG promoter. The cells were cultured in DMEM (Invitrogen, Tokyo, Japan) supplemented with 10% FBS and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) in a humidified atmosphere of 5% CO_2/95% air at 37°C. The medium was replaced every other day. When confluent, the cells were detached by trypsinization (0.05% trypsin and 0.53 mM EDTA), resuspended in medium, and split for subculture. To block proliferation, GH7 cells were incubated in the presence of 10 μg/ml mitomycin C for 3 h before seeding on culture scaffolds.

Culture of primary hepatocytes and GH7 cells in sponges To culture primary hepatocytes and/or GH7 cells on three-dimensional Cs, GCs and GCs/HA-hybrid sponges, cells were seeded at $1–3 \times 10^5$ per sponge and cultured with William's E medium. The cells were observed using a fluorescence inverted microscope (Olympus, Tokyo, Japan).

Fluorescence intensity assay In order to quantitate the number of living cells, DsRed2 and EGFP fluorescence intensities were measured by using the LAS-4000 system (Fujifilm, Tokyo, Japan). Images were analyzed using the ImageJ Multi Gauge program version 3.1 (Fujifilm).

WST-8 assay Adherent cells were washed three times with PBS. Subsequently, fresh medium containing 10% (v/v) of WST-8 (Nacalai Tesque, Kyoto, Japan) was added, cells were incubated for 1 h, and 100 μL of the resulting supernatant was transferred to a 96-well microplate. The reduction of WST-8 was measured photometrically using an iMark Microplate Reader (Bio-Rad Laboratories, Hercules, CA, USA) at 450 nm.

Histological and immunofluorescence assessment The cell-incorporating sponges were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned (5 μm), and stained with hematoxylin and eosin (H&E) for histological evaluation. For immunofluorescence staining, sections were heated at 60°C for 1 min, deparaffinized in xylene, and rehydrated through graded ethanol to Milli Q water. The sections were washed three times with PBS and then incubated in Blocking One buffer (Nacalai Tesque) for 30 min at room temperature. Then, the slides were incubated with primary antibodies, including rabbit anti-red fluorescent protein IgG (1:500; Medical and Biological Laboratories, Nagoya, Japan) and goat anti-green fluorescent protein IgG (1:1000; Abcam, Tokyo, Japan) for 2 h at room temperature. Next, sections were incubated with 4′,6-diamidino-2-phenylindole (DAPI), donkey anti-rabbit IgG conjugated with Alexa Fluor 594 (1:2000; Invitrogen) and donkey anti-goat IgG conjugated with Alexa Fluor 488 (1:2000; Invitrogen) for 1 h at room temperature. Finally, the sections were mounted in Prolong Gold fluorescent mounting medium (Invitrogen).

Total RNA preparation and reverse transcriptase-polymerase chain reaction (RT-PCR) Total RNA was isolated from cells in the sponges using the cetyltrimethylammonium bromide (CTAB) method (12) because it was difficult to prepare RNA using the general method due to the contamination of polysaccharide scaffold materials. The purity and concentration of isolated RNA was assessed by UV absorbance, and RNA integrity was verified by 0.6% agarose gel electrophoresis in TAE buffer. First strand cDNA was prepared from the extracted total RNA in a reverse transcriptase reaction, using the SuperScript II Reverse Transcriptase kit and oligo dT primers (Invitrogen) according to the manufacturer's instructions. The cDNA corresponding to the genes of interest (Table 1) was amplified by PCR in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, Tokyo, Japan). After initial denaturation at 94°C for 1 min, PCR amplification was continued at 94°C for 30 s, at the annealing temperature for 30 s, and at 72°C for 30 s for a total 30−40 cycles, with a final extension at 72°C for 10 min. Amplified DNA fragments were separated by 1.5% (w/v) agarose gel electrophoresis with TBE buffer. The gels were stained with ethidium bromide (10 μg/mL) and photographed on a UV transilluminator (Bio-Rad Laboratories). For quantitative analysis of albumin *(Alb)* expression, real-time PCR was carried out by using the StepOnePlus Sequence Detection System (Applied Biosystems). Hypoxanthine-guanine phosphoribosyltransferase (*Hprt*) was used as an internal housekeeping reference. Gene expression was quantified using the ΔΔCt method.

Urea assay During the culture period, conditioned medium (CM) samples were collected every day and stored at −20°C until assayed using a QuantiChrom Urea Assay Kit (BioAssay Systems, Hayward, CA, USA). Briefly, 50 μL of CM were incubated with 200 μL of the reaction mixture for 20 min at room temperature. The urea-dependent chromogenic reaction was read using an iMark Microplate Reader (Bio-Rad Laboratories) at 490 nm. The urea concentration was determined using a standard curve.

Testosterone metabolism assay To examine the enzymatic activities of cytochrome P450s, each metabolite of testosterone in the culture medium was quantitatively detected using high performance liquid chromatography (HPLC) as