protects the liver from experimental cold ischemia-reperfusion injury. *Br J Surg* 2004; **91**: 184.
21. Ninomiya M, Shimada M, Harada N, *et al.* Beneficial effect of MCI-186 on hepatic warm ischemia-reperfusion in the rat. *Transplantation* 2002; **74**: 1470.
22. Abe T, Unno M, Takeuchi H, *et al.* A new free radical scavenger, edaravone, ameliorates oxidative liver damage because of ischemia-reperfusion *in vitro* and *in vivo*. *J Gastrointest Surg* 2004; **8**: 604.
23. Okatani Y, Wakatsuki A, Enzan H, Miyahara Y. Edaravone protects against ischemia/reperfusion-induced oxidative damage to mitochondria in rat liver. *Eur J Pharmacol* 2003; **465**: 163.
24. Soeda J, Miyagawa S, Sano K, Masumoto J, Taniguchi S, Kawasaki S. Cytochrome *c* release into cytosol with subsequent caspase activation during warm ischemia in rat liver. *Am J Physiol Gastrointest Liver Physiol* 2001; **281**: G1115.
25. Kawai H, Nakai H, Suga M, Yuki S, Watanabe T, Saito KI. Effects of a novel free radical scavenger, MCI-186, on ischemic brain damage in the rat distal middle cerebral artery occlusion model. *J Pharmacol Exp Ther* 1997; **281**: 921.
26. Yanagisawa A, Miyagawa M, Ishikawa K, Murota S. Cardioprotective effect of MCI-186 (3-methyl-1-phenyl-2 pyrazolin-5-one) during acute ischemia–reperfusion injury in rats. *Int J Angiol* 1994; **3**: 12.
27. Kono H, Asakawa M, Fujii H, *et al.* Edaravone, a novel free radical scavenger, prevents liver injury and mortality in rats administered endotoxin. *J Pharmacol Exp Ther* 2003; **307**: 74.
28. Suzuki S, Toledo-Pereyra LH, Rodriguez FJ, Cejalvo D. Neutrophil infiltration as an important factor in liver ischemia and reperfusion injury. Modulating effects of FK506 and cyclosporine. *Transplantation* 1993; **55**: 1265.
29. Wiezorek JS, Brown DH, Kupperman DE, Brass CA. Rapid conversion to high xanthine oxidase activity in viable Kupffer cells during hypoxia. *J Clin Invest* 1994; **94**: 2224.
30. Yokota R, Fukai M, Shimamura T, *et al.* A novel hydroxyl radical scavenger, nicaraven, protects the liver from warm ischemia and reperfusion injury. *Surgery* 2000; **127**: 661.
31. Zhong Z, Froh M, Connor HD, *et al.* Prevention of hepatic ischemia–reperfusion injury by green tea extract. *Am J Physiol Gastrointest Liver Physiol* 2002; **283**: G957.
32. Mizoe A, Kondo S, Azuma T, *et al.* Preventive effects of superoxide dismutase derivatives modified with monosaccharides on reperfusion injury in rat liver transplantation. *J Surg Res* 1997; **73**: 160.
33. Yabe Y, Kobayashi N, Nishihashi T, *et al.* Prevention of neutrophil-mediated hepatic ischemia/reperfusion injury by superoxide dismutase and catalase derivatives. *J Pharmacol Exp Ther* 2001; **298**: 894.
34. Okatani Y, Wakatsuki A, Reiter RJ, Enzan H, Miyahara Y. Protective effect of melatonin against mitochondrial injury induced by ischemia and reperfusion of rat liver. *Eur J Pharmacol* 2003; **469**: 145.
35. Kusumoto K, Morimoto T, Minor T, Uchino J, Isselhard W. Allopurinol effects in rat liver transplantation on recovery of energy metabolism and free radical-induced damage. *Eur Surg Res* 1995; **27**: 285.
36. Schreck R, Rieber P, Baeuerle PA. Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kappa B transcription factor and HIV-1. *EMBO J* 1991; **10**: 2247.
37. Satriano J, Schlondorff D. Activation and attenuation of transcription factor NF-κB in mouse glomerular mesangial cells in response to tumor necrosis factor-α, immunoglobulin G, and adenosine 3′:5′-cyclic monophosphate. Evidence for involvement of reactive oxygen species. *J Clin Invest* 1994; **94**: 1629.
38. Singh S, Aggarwal BB. Activation of transcription factor NF-κ B is suppressed by curcumin (diferuloylmethane). *J Biol Chem* 1995; **270**: 24995.
39. Shito M, Wakabayashi G, Ueda M, *et al.* Interleukin 1 receptor blockade reduces tumor necrosis factor production, tissue injury, and mortality after hepatic ischemia-reperfusion in the rat. *Transplantation* 1997; **63**: 143.
40. Shirasugi N, Wakabayashi G, Shimazu M, *et al.* Up-regulation of oxygen-derived free radicals by interleukin-1 in hepatic ischemia/reperfusion injury. *Transplantation* 1997; **64**: 1398.
41. Liang J, Yamaguchi Y, Matsumura F, *et al.* Calcium-channel blocker attenuates Kupffer cell production of cytokine-induced neutrophil chemoattractant following ischemia-reperfusion in rat liver. *Dig Dis Sci* 2000; **45**: 201.
42. Colletti LM, Kunkel SL, Walz A, *et al.* Chemokine expression during hepatic ischemia/reperfusion-induced lung injury in the rat. The role of epithelial neutrophil activating protein. *J Clin Invest* 1995; **95**: 134.
43. Colletti LM, Kunkel SL, Walz A, *et al.* The role of cytokine networks in the local liver injury following hepatic ischemia/reperfusion in the rat. *Hepatology* 1996; **23**: 506.
44. Yamaguchi Y, Matsumura F, Takeya M, *et al.* Monocyte chemoattractant protein-1 enhances expression of intercellular adhesion molecule-1 following ischemia-reperfusion of the liver in rats. *Hepatology* 1998; **27**: 727.
45. Martinez-Mier G, Toledo-Pereyra LH, Ward PA. Adhesion molecules in liver ischemia and reperfusion. *J Surg Res* 2000; **94**: 185.

STEM CELLS®
Original Article

Crucial Roles of Mesodermal Cell Lineages in a Murine Embryonic Stem Cell–Derived In Vitro Liver Organogenesis System

SHINICHIRO OGAWA,[a,b] YOH-ICHI TAGAWA,[a] AKIKO KAMIYOSHI,[a] AKIHIRO SUZUKI,[a] JUN NAKAYAMA,[c] YASUHIKO HASHIKURA,[b] SHINICHI MIYAGAWA[b]

[a]Division of Laboratory Animal Research, Research Center for Human and Environmental Sciences, Shinshu University;
[b]Department of Surgery, Shinshu University School of Medicine;
[c]Department of Pathology, Shinshu University School of Medicine, Shinshu, Japan

Key Words. Embryonic stem cell • Liver • Organogenesis • Cardiomyocyte • Endothelial cell • In vitro system

ABSTRACT

Recent studies in the field of regenerative medicine have exploited the pluripotency of embryonic stem (ES) cells to generate a variety of cell lineages. However, the target has always been only a single lineage, which was isolated from other differentiated cell populations. In the present study, we selected sublines with a high capability for differentiation to contracting cardiomyocytes and also produced germline chimeric mice from a parent ES line. We also succeed in establishing embryoid bodies prepared from the ES cells that differentiated into not only hepatocytes but also at least two mesodermal lineages: cardiomyocytes that supported liver development and endothelial cells corresponding to sinusoids. This allowed the development of an in vitro system using murine ES cells that approximated the events of liver development in vivo. The expression of *albumin* was significantly higher in cardiomyocytes that had arisen in differentiated ES cells than in those that had not. Our in vitro system for liver organogenesis consists of a blood/sinusoid vascular-like network and hepatocyte layers and shows higher levels of hepatic function, such as albumin production and ammonia degradation, than hepatic cell lines and primary cultures of murine adult hepatocytes. This innovative system will lead to the development of second-generation regenerative medicine techniques using ES cells and is expected to be useful for the development of bioartificial liver systems and drug-metabolism assays. STEM CELLS *2005;23:903–913*

INTRODUCTION

The liver develops from the ventral foregut in vertebrates, receiving multiple stimuli in the form of growth factors, cytokines, and hormonal factors, as well as intercellular and matrix cellular interactions [1–5]. In particular, the precardiac mesoderm produces factors that trigger hepatic development [6, 7], that is, cardiomyocytes support liver organogenesis (Fig. 7A). The signaling of fibroblast growth factor (FGF), produced in the cardiac mesoderm, induces the initial step of hepatogenesis in the ventral endoderm at E8.5–9.5 of mouse development, resulting in the activation of albumin and α-fetoprotein expressions [4, 6, 8]. As the hepatic precursor cells migrate into the septum transversum to form a liver bud [9], endothelial progenitor cells arise there simultaneously in close association with early developing hepatoblasts and hepatogenesis [10]. These endothelial cells develop a fenestrated morphology to form the hepatic sinusoids [11–13], and then finally the liver is completed, with its multiple and specific functions.

In the field of regenerative medicine, the pluripotency of embryonic stem (ES) cells has been applied to obtain a vari-

Correspondence: Yoh-ichi Tagawa, Ph.D., Department of Biomolecular Engineering, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, B-51 4259 Nagatsuta-cho, Midori-ku, Yokohama, Kanagawa, 226-8501 Japan. Telephone: 81-45-924-5791; Fax: 81-45-924-5815; e-mail: ytagawa@bio.titech.ac.jp Received October 23, 2004; accepted for publication March 20, 2005.
 1066-5099/2005/$12.00/0 doi: 10.1634/stemcells.2004-0295

ety of cell lineages. However, the targets of these systems have always been limited to only a single lineage, which was isolated from other differentiated cell populations. There have been a few reports on the differentiation of murine ES cells to hepatocyte-like or albumin-producing cells [14–19]. However, these studies focused only on hepatocytes as a single-cell lineage and did not refer to liver organogenesis. It also has been reported that hepatocyte-like cells spontaneously differentiate from human ES cells [20], as well as previous studies using murine ES cells. In particular, there has been no description about the roles of cardiomyocytes and endothelial cells in hepatocyte differentiation, although one previous study has detected albumin-positive cells adjacent to cardiomyocytes in teratoma-derived human ES cells in severe combined immunodeficiency mice [20]. Cardiac mesoderm has a strong capacity to induce liver organogenesis [4, 6, 8]. These cell lineages can also be obtained from ES cells. Therefore, we considered that emergence of cardiomyocytes would be necessary for liver morphogenesis from ES cells in vitro. Our purpose in the present study was to establish a system for in vitro hepatic morphogenesis consisting of not only hepatocytes but also cell lineages supporting hepatic differentiation, such as cardiomyocytes and endothelial cells, which correspond to those involved in liver organogenesis in vivo, from murine ES cells. We exploited the pluripotency of ES cells for differentiation of these cell lineages, which included hepatocytes, cardiomyocytes, and endothelial cells, and succeeded in establishing a novel system of hepatic morphogenesis from murine ES cells based on naturally occurring embryological events, that is, with contributions from cardiac mesoderm and endothelial cell lineages.

Materials and Methods

Cell Culture

E14-1 ES cells derived from 129/Ola were grown on mitomycin C–treated mouse embryonic fibroblast feeder layers to maintain them in an undifferentiated state in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Tokyo, http://www.invitrogen.com) containing 20% fetal bovine serum (FBS) (Hyclone, Logan, UT, http://www.hyclone.com), 1 mM sodium pyruvate (Invitrogen), 100 μM nonessential amino acids (Invitrogen), 100 μM 2-mercaptoethanol (Sigma, St. Louis, http://www.sigmaaldrich.com), and 10^3 U/ml leukemia inhibitory factor (LIF) (Chemicon, CA, http://www.chemicon.com). The cells were dissociated with 0.25% trypsin, 1% chicken serum (Invitrogen), and 1 mM EDTA in phosphate-buffered saline (PBS) and resuspended in Iscove's modified Dulbecco's medium (IMDM) (Invitrogen) containing 20% FBS, 1 mM sodium pyruvate, 100 μM nonessential amino acids, and 100 μM 2-mercaptoethanol without LIF and then formed into a hanging drop at a concentration of 1,000 cells per 50-μl drop. The hanging drop was cultured in an atmosphere of 5% CO_2 at 37°C for 5 days. An individual 5-day-old embryoid body (EB) was plated in each well of a gelatin-coated 96-well plate, and the growth factor was added to the culture medium. The day when the 5-day-old EBs were plated in the dish was denoted day 0 (A0). Human recombinant acidic fibroblast growth factor (aFGF) (Invitrogen) was added to the differentiation medium at a concentration of 100 ng/ml 2 days after plating of the 5-day-old EBs (A2), and 20 ng/ml human recombinant hepatocyte growth factor (HGF) (Genzyme/Techne, Minneapolis, http://www.g-tonline.com) was added at A4. Then, at A6, 10 ng/ml mouse recombinant oncostatin M (Genzyme/Techne), 100 nM dexamethasone (MP Biomedicals, Irvine, CA, http://www.mpbio.com), ITS (insulin 10 μg/ml, transferrin 5 μg/ml, selenium 5 ng/ml; Invitrogen), and 10 mM nicotinamide (Nakalai tesque, Kyoto, Japan, http://www.nacalai.co.jp) were added. The emergence frequency of contracting cells in the EB outgrowths, indicating cardiac muscle differentiation, was monitored daily. Emergence frequency was expressed as a percentage, where 100% meant detection of a contractile area in all wells containing EB outgrowths. Twenty recloned 5-day-old EBs were plated on a 6-cm dish coated with gelatin as a semi–large-scale culture.

To obtain fetal hepatocytes, mouse liver at E15 was minced and dissociated with collagenase II (Sigma) in Hanks' buffer (Invitrogen). The cells were seeded on a gelatin-coated dish in DMEM supplemented with 10% FBS, 100 μM nonessential amino acids, and 100 U/ml penicillin-100 μg/ml streptomycin-292 μg/ml glutamine for a few hours and washed once with the same medium. The medium was replaced every day.

Primary adult hepatocytes were isolated from male 129/SvJ mice by the two-step collagenase perfusion method. The hepatocytes were separated from the resulting cell suspension by centrifugation and then by centrifugation through a 50% Percoll (Sigma) gradient. Isolated hepatocytes were plated onto gelatin-coated dishes.

Production of Chimeric Mice

Chimeric mice were produced by the modified aggregation method [21]. This involved aggregation of 10 to 15 ES cells with two (BDF1 × C57BL) F_1 eight-cell-stage embryos, from which the zona pellucida had been removed with Tyrode's solution (Sigma), were placed in a hole on a plastic dish and cultured overnight. The ES cells and eight-cell-stage embryos became a single blastocyst, and the blastocysts were then transferred to the uterus of pseudopregnant female ICR mice. Male chimeric mice were then bred with C57BL/6 female mice, and germ-line transmission of the ES cells was checked by the agouti coat color of the offspring.

RNA Extraction and Reverse Transcription–Polymerase Chain Reaction Analysis

Total RNA was extracted from the outgrowths of the EBs using a MagEXtractor mRNA kit (Toyobo, Tokyo, http://www.toyobo.co.jp/e). Briefly, 2-μg aliquots of total RNA were reverse tran-

scribed to cDNAs using a Superscript II first-strand synthesis system with an oligo dT primer (Invitrogen). Semiquantitative reverse transcription–polymerase chain reaction (RT-PCR) was performed using Ex Taq DNA polymerase (Takara, Tokyo, http://www.takara-bio.com) with the following primer sets. For each experiment, a housekeeping gene, hypoxanthine phosphoribosyltransferase, was amplified with 25 cycles to normalize the expressions of other genes in the sample. The forward and reverse primers were located at different exons to discriminate the product from the targeted mRNA or its genomic DNA. The PCR primers used were as follows: albumin, GCTACGGCACAGTGCTTG and CAGGATTGCAGACAGATAGTC (product size, 265 bp; annealing temperature, 65°C); α-fetoprotein, TCGTATTCCAACAGGAGG and AGGCTTTTGCTTCACCAG (product size, 173 bp; annealing temperature, 65°C); transthyretin, CTCACCACAGATGAGAAG and GGCTGAGTCTCTCAATTC (product size, 223 bp; annealing temperature, 56°C); α1–anti-trypsin, AATGGAAGAAGCCATTCGAT and AAGACTGTAGCTGCTGCAGC (product size, 483 bp; annealing temperature, 50°C); Oct3/4, AGCACGAGTGGAAAGCACT and CTCATTGTTGTCGGCTTCCT (product size, 339 bp; annealing temperature, 60°C); tyrosine aminotransferase, ACCTTCAATCCCATCCGA and TCCCGACTGGATAGGTAG (product size, 205 bp; annealing temperature, 66°C); tryptophan 2,3-deoxygenase, TGCGCAAGAACTTCAGAGTGA and TGCGCAAGAACTTCAGAGTGA (product size, 419 bp; annealing temperature, 62°C); liver-specific organic anion transporter-1, TGCGCAAGAACTTCAGAGTGA and TGAGTTGGACCCCTTTTCAC (product size, 226 bp; annealing temperature, 65°C); asialoglycoprotein receptor-1, GCTGGAAAAACAGCAGAAGG and CTGTTCCATCCACCCATTTC (product size, 358 bp; annealing temperature, 65°C); asialoglycoprotein receptor-2, CGGACCCTGAAAGAAACCTT and ATGAAACTGGCTCCTGTGCT (product size, 410 bp; annealing temperature, 66°C); HGF, AGACACCACACCGGCACAGT and ATAGGGCAATAATCCCAAGG (product size, 484 bp; annealing temperature, 65°C); FGF-1, ACCGAGAGGTTCAACCTGCC and GCCATAGTGAGTCCGAGGACC (product size, 386 bp; annealing temperature, 66°C); vascular endothelial growth factor (VEGF), CAGGCTGCTGTAACGATGAA and AATGCTTTCTCCGCTCTGAA (product size, 206 bp; annealing temperature, 65°C); VEGFR1, TGTGGAGAAACTTGGTGACCT and TGGAGAACAGCAGGACTCCTT (product size, 504 bp; annealing temperature, 65°C); VEGFR2, TCTGTGGTTCTGCGTGGAGA and GTATCATTTCCAACCACCC (product size, 269 bp; annealing temperature, 55°C); platelet-endothelial cell adhesion molecule-1 (PECAM-1), GTCATGGCCATGGTCGAGTA and AGCAGGACAGGTCCAACAAC (product size, 168 bp; annealing temperature, 65°C); atrial natriuretic peptide, ATGGGCTCCTTCTCCCATCAC and TGTTGCAGCCTAGTCCACTC (product size, 541 bp; annealing temperature, 65°C); and hypoxanthine phosphoribosyltransferase, GTTGGATACAGGCCAGACTTTGTTG and GAGGGTAGGCTGGCCTATAGGCT (product size, 269 bp; annealing temperature, 65°C).

Immunohistochemical Analysis

Twenty EBs were cultured on gelatin-coated glass coverslips in a six-well plate. EB outgrowths on the coverslips were fixed with 4% paraformaldehyde/PBS for 20 minutes and then permeabilized with 0.1% Triton X for 10 minutes at room temperature. The fixed samples were incubated in blocking buffer containing 4% donkey serum (Jackson Immunoresearch, Baltimore, http://www.jacksonimmuno.com) for 10 minutes at room temperature. They were then incubated with the primary antibody overnight at 4°C and with the secondary antibody for 1 hour in a humidified chamber. The following antibodies were used: rabbit immunoglobulin (IgG) against mouse albumin (1:250; MP Biomedicals), goat IgG against mouse PECAM-1 (1:250; Santa Cruz Biotech, CA, http://www.scbt.com), tetramethylrhodamine isothiocyanate–conjugated swine anti-rabbit immunoglobulin (1:60; DakoCytomation A/S, Glostrup, Denmark, http://www.dakocytomation.com), and fluorescein-conjugated donkey anti-goat IgG (1:100; Jackson Immunoresearch, West Grove, PA). For nuclear staining, the cells were incubated for 5 minutes at room temperature with DAPI (4,6 diamidino-2-phenylindole). The samples were mounted in DakoCytomation fluorescent mounting medium and observed using a fluorescence microscope (BX 60; Olympus, Tokyo, http://www.olympus-global.com/en/global) and a confocal laser microscope (TCS SP2; Leica, Manheim, Germany, http://www.leica.com/index.html).

Western Blotting Analysis

ES cells and EBs were homogenized in buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 1% sodium deoxycholate, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 10 μg/ml leupeptin, and 5 μg/ml pepstatin, centrifuged at 12,000 rpm for 10 minutes at 4°C, and the supernatants were collected. Protein concentration was measured with a bicinchoninic acid protein assay (BCA protein assay kit; Pierce, Rockford, IL, http://www.piercenet.com). The same amounts (10 μg each lane) of proteins from cell homogenates were electrophoresed on 8% polyacrylamide gels. Proteins were transferred onto polyvinylidene difluoride membranes by electro blotting. The membranes were blocked for 1 hour at room temperature with 5% nonfat dried milk and 0.1% bovine serum albumin in tris-buffered saline (TBS) containing 0.1% (vol/vol) Tween 20 (TBS-T) and incubated for 1 hour with peroxidase-conjugated sheep anti-rabbit antibody (GE Healthcare, Piscataway, NJ, http://www.gehealthcare.com) diluted in TBS-T containing 5% FBS. After washing with TBS-T, the blots

were developed by enhanced chemiluminescence (GE Healthcare) and exposed to x-ray film (RX-U; Fuji, Kawasaki, Japan, http://home.fujifilm.com).

Treatment with Thalidomide or CBO-P11

Thalidomide (N-[2,6-dioxo-3-piperidinyl] phthalimide; Tocris Cookson, Ellisville, MO, http://www.tocris.com) was dissolved in dimethyl sulfoxide and added to the medium at the indicated concentration. CBO-P11 (cyclo-VEGI) (DFPQIMRIKPHQGQHIGE) (Calbiochem, San Diego, http://www.emdbiosciences.com/html/CBC/home.html), a cyclopeptidic vascular endothelial growth inhibitor, was dissolved in water and added to the medium at a concentration of 10 μM. These chemicals were added to the medium after 20 five-day-old EBs had been plated on the dish (A0). The medium was replaced every day.

Ammonia Modification Function Assay

To examine cellular ammonia degradation activity, 2 mM NH_4Cl was added to the serum-free culture medium of 30 or 50 EBs at days 10 and 18 after plating and further incubated for 24 hours. The concentration of NH_4Cl remaining in the medium was measured at various time points by a modified indophenol method using a commercial kit (Ammonia-test Wako; Wako, Osaka, Japan, http://www.wako-chem.co.jp).

Results

Roles of Embryonic Stem Cell–Derived Cardiomyocytes in Hepatic Differentiation from Murine Embryonic Stem Cells

As is the case in in vivo development, the emergence of cardiomyocytes is necessary for liver organogenesis in an in vitro differentiation system using ES cells. As an initial approach for inducing murine ES cells to undergo hepatic morphogenesis, we established a system for spontaneous differentiation to contracting cardiomyocytes with a high frequency of emergence. A single 5-day-old EB comprised of dissociated murine ES cells was plated onto gelatin-coated plates and allowed to adhere to the bottom of the plate. The EB outgrowths began to contract spontaneously within 5 days after plating. These ES cell–derived contracting cells were considered to be cardiomyocytes based on specific gene expression (Fig. 1B) and pharmacological responses. The outgrowths of EBs were cultured in the differentiation medium for 18 days (A18) after adhesion to the well bottom. The expression of *albumin* was compared in groups of EBs in which cardiomyocytes had and had not arisen at A10; the levels of albumin expression were significantly higher in those with outgrowths of contracting cardiomyocytes than in those without (Fig. 1B), suggesting that the ability to differentiate to cardiomyocytes in the ES cell population and the emergence of cardiomyocytes in the EB outgrowths are important for endodermal and hepatocyte differentiation.

To increase the efficiency of liver organogenesis from ES cells, it was considered important to increase the frequency of cardiomyocyte emergence in the EB outgrowths. The frequency of cardiomyocyte emergence at Ab-3 was less than 30% using the parental line of the E14-1 ES cells at passages 14 through 18, whereas the frequency was almost 100% using some sublines (My-1 and Ab-3) other than My-5, which were recloned from the parental line E14-1 (Fig. 1A). Ability for the production of chimeric mice was also compared in the parental line and these E14-1 sublines. The chimera-forming ability of the parental E14-1 was 2 germ-line/17 chimeric mice from

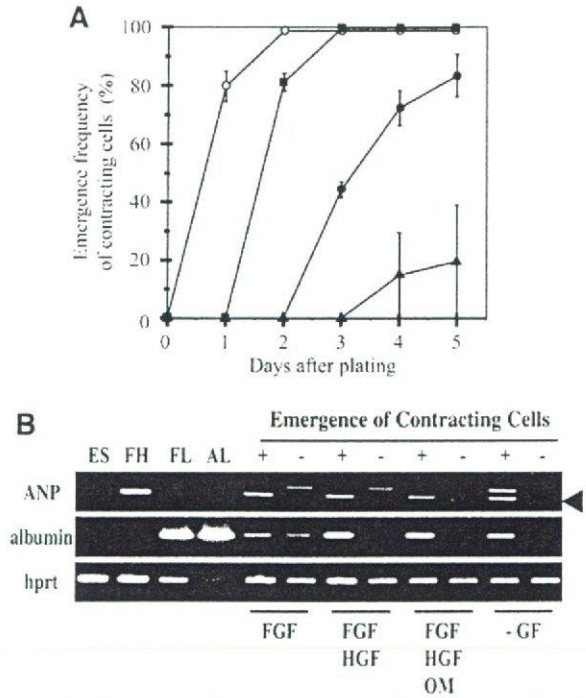

Figure 1. Establishment of a system for allowing differentiation of ES cells to cardiomyocytes at a frequency of almost 100% for hepatic differentiation. **(A):** Comparison of the time courses of the frequency of emergence contractile cells from outgrowths of EBs prepared from the parental line of E14-1 ES cells (●), subline My-1 (○), Ab-3 (■), and My-5 (▲). **(B):** Expression of albumin as a representative hepatic marker and of ANP as an atrial marker was determined by reverse transcription–PCR during differentiation of EBs. PCR amplication of ANP and albumin was carried out for 30 cycles. Human recombinant aFGF was added to the differentiation medium at a concentration of 100 ng/ml 2 days after plating of the 5-day-old EBs on the dish (A2), and then 20 ng/ml human recombinant HGF was added at A4. Then with 10 ng/ml mouse recombinant OSM, 100 nM dexamethasone, ITS (insulin 10 μg/ml, transferrin 5 μg/ ml, selenium 5 ng/ml), and 10 mM nicotinamide (Nacalai) were added at A6. Arrowhead indicates the expected band of ANP. Abbreviations: aFGF, acidic fibroblast growth factor; AL, mouse adult liver; ANP, atrial natriuretic peptide; EB, embryoid body; ES, embryonic stem; GF, growth factor; FH, mouse fetal heart at E15; FL, mouse fetal liver at E15; HGF, hepatocyte growth factor; OSM, oncostatin M; PCR, polymerase chain reaction.

171 ES-aggregated embryos, whereas that of a subline Ab-3 was 4 germ-line/18 chimeric mice from 155 ES-aggregated embryos. It is very important that undifferentiated and pluripotent ES cells should be present in these cultures for differentiation not only to cardiomyocytes but also to albumin-producing hepatocytes and endothelial cells corresponding to the developmental stages of the liver. For the following experiments, we used the selected subline, Ab-3, which showed high capability for differentiation to cardiomyocytes and also for production of germ-line chimeric mice, within six passages.

Expression and Function of Liver-Specific Gene Expressions and Functions in Murine Embryonic Stem Cell–Derived Hepatic Morphogenesis

Twenty 5-day-old EBs were placed together on gelatin-coated dishes in differentiation medium as a semi–large-scale system, because the absolute numbers of cells would be needed for hepatic development. Contracting cardiomyocytes emerged in the central area of EB outgrowth. A heterologous population was considered important for in vitro hepatic morphogenesis using murine ES cells. The expressions of endodermal/hepatocyte-specific genes, such as transthyretin, α-fetoprotein, α1-antitrypsin, and albumin, at the various stages of EB differentiation were examined (Fig. 2A). The levels of expression of these genes increased markedly as differentiation of the EBs proceeded, whereas that of *Oct-3/4*, a marker of undifferentiated ES cells, decreased. The levels of expression of liver-specific genes and *Oct-3/4* in the presence of growth factor were the same as those in the absence of growth factor, suggesting that cardiomyocytes were not induced in the presence of growth factor. We also confirmed that albumin protein was detectable in the differentiated outgrowths of EBs at A4 and increased gradually throughout differentiation (Fig. 2B), corresponding to the changes in mRNA levels (Fig. 2A).

As a second approach for inducing hepatic morphogenesis from ES cells, aFGF, HGF, and oncostatin M were added to the cultures to investigate the effects of growth factors. The levels of expression of albumin, tyrosine aminotransferase (*TAT*), and tryptophan oxygenase (*TO*) were significantly higher in the presence than in the absence of additional growth factors at A10, corresponding to the early differentiation stage of EBs. This suggests that the addition of growth factors artificially induces the expression of mature hepatocyte-specific genes or accelerates differentiation in the system at an early stage (Fig. 2C). On the other hand, at A18, corresponding to the late differentiation stage, the levels of expression of albumin, *TAT*, *TO*, and asialo glycoprotein receptors (*ASGR1*, *ASGR2*) in the EB were almost the same levels under conditions with and without

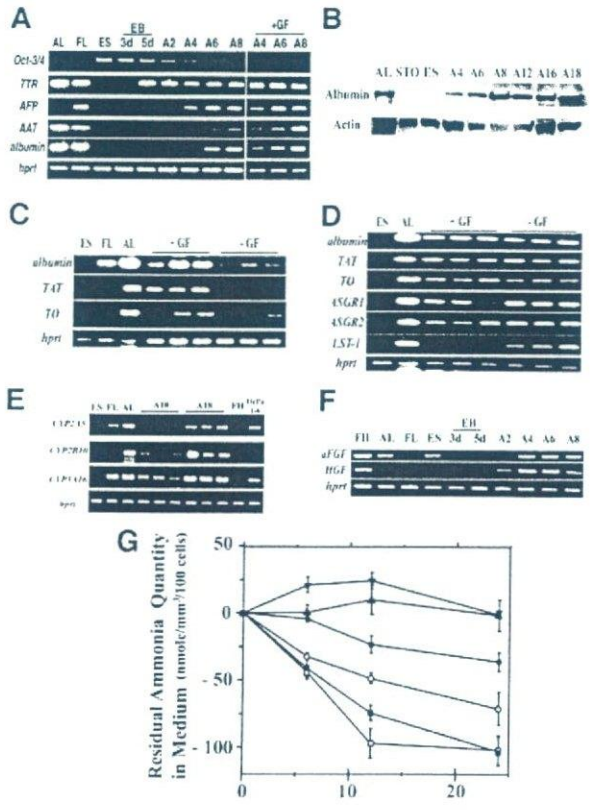

Figure 2. Expression by ES cell–derived hepatocytes of a variety of liver-specific genes, production of albumin protein, and ammonia modification function. **(A):** The expression of endodermal-specific genes was examined in the outgrowths of EBs from before plating (3- and 5-day-old EBs) to 8 days after plating (A8) by RT-PCR. PCR amplification of *Oct-3/4*, *TTR*, *AFP*, *AAT*, and *albumin* was carried out for 30 cycles. Five-day-old EBs were plated on gelatin-coated dishes and cultured in the absence of any growth factors. **(B):** The level of albumin protein was quantified by Western blotting analysis during hepatic differentiation of EBs. EBs were cultured in the absence of any growth factors for 18 days. **(C, D):** Expression of albumin and mature hepatocyte-specific genes expression was detected in the EB outgrowths at A10 **(C)** and A18 **(D)**. Comparison of cultures in the presence or absence of additional growth factors. PCR amplification of *albumin* was carried out for 25 cycles. Amplification of TAT, ASGR-1, ASGR-2, and LST-1 was carried out for 40 cycles. Amplification of TO was carried out for 30 cycles. **(E):** Expression of CYP family genes, such as Cyp2A5, 2B10, and 3A16, was detected by RT-PCR (40 cycles, respectively) in the EB outgrowths at A10 and A18 cultured in the absence of additional growth factors. **(G):** Ammonia modification function was measured in EB outgrowths at A18. The amounts of residual ammonia in 100 cells are indicated, which were calculated from the quantity of prepared genomic DNA. □, primary adult mouse hepatocyte culture; ■, outgrowth of 50 EBs at A18; ○, 30 EBs at A18; ●, 30 EBs at A10; ▼, mouse hepatoma cell line, HePa1–6; ▲, ES E14-1. **(F):** Endogenous aFGF and HGF gene expression was detected by RT-PCR (40 cycles, respectively) in the EB outgrowths during differentiation in the absence of these additional growth factors by RT-PCR. Arrowhead indicates the expected band of ANP. Abbreviations: aFGF, acidic fibroblast growth factor; AL, mouse adult liver; EB, embryoid body; ES, embryonic stem; GF, growth factor; FL, mouse fetal liver at E15; HGF, hepatocyte growth factor; RT-PCR, reverse transcription–polymerase chain reaction; TAT, tyrosine aminotransferase.

additional growth factors. Furthermore, *liver-specific transporter (LST-1)* mRNA was detected only in the absence of any growth factor (Fig. 2D), suggesting that these additional factors were not essential for hepatic differentiation and maturation from ES cells in our system. The expressions of some CYP genes were also detectable under conditions without growth factors (Fig. 2E).

To investigate whether the outgrowths of EBs could supply these growth factors themselves, the expressions of these genes were analyzed in EBs without these growth factors. Endogenous *aFGF* and *HGF* expression was detected in the outgrowths in the absence of the additional growth factors (Fig. 2F), suggesting that addition of these growth factors is not necessary for hepatic differentiation of EBs, as they are produced endogenously.

Assay of ammonia degradation, a representative hepatic function, was also carried out. Interestingly, the level of ammonia degradation was markedly higher in the differentiated EB outgrowths than in the hepatocyte cell line, HePa1-6, and in primary cultures of murine hepatocytes (Fig. 2E).

To investigate the distribution of albumin-positive cells in the EB outgrowths at A10 and A18, we performed immunohistochemical analyses using anti-mouse albumin antibody. The albumin-positive cells were visualized adjacent to the contracting cardiomyocytes, around the central area of the outgrowths, at A10 (Fig. 3A). These albumin-positive cells formed clusters and showed an islet-like morphology in the outgrowths (Figs. 3A, 3B). At A18, corresponding to the late stage of hepatic differentiation, these colonies had grown and were strongly detected, as can be seen in Figure 3C, suggesting that these cells were proliferating and expanding.

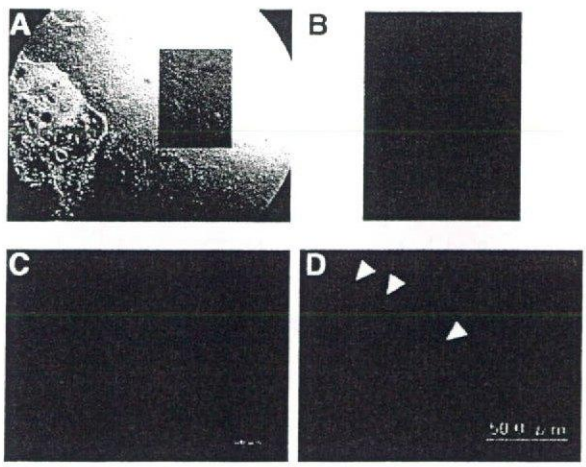

Figure 3. Albumin-positive cells observed as expanding colonies in the outgrowths of EBs. **(A):** Photomicrograph showing the contracting region and albumin-positive areas in the outgrowths of differentiated EBs at A10 in the absence of any growth factors. The shaded square is magnified in **(B)**. **(B–D):** Immunohistochemical analysis of the EB outgrowths at **(B)** A10 and **(C, D)** A18 using anti-albumin (red). White arrowheads indicate the binuclear cells in the outgrowths of the EB. These cultures were also carried out in the absence of any growth factors. Abbreviations: CA, contracting cardiomyocyte area; EB, embryoid body.

Morphology of Murine Embryonic Stem Cell–Derived Hepatocytes

Murine hepatocytes contained two morphologically distinct populations, a mononuclear population and a binuclear population. Hepatocytes in the resting liver consist predominantly of binuclear hepatocytes, whereas those in the regenerating liver are mainly mononuclear hepatocytes [22]. Some of these albumin-positive cells were binuclear, which is a characteristic of mature hepatocytes in mice (Fig. 3D). Thus, these cells were confirmed to be murine hepatocytes on the basis of morphology as well as by hepatic function and gene expression analysis. In addition to hepatic function and expression in our cultures, morphological evidence also suggested that the albumin-producing cells derived from ES cells in our system were hepatocytes.

Vasculogenesis in the System to Induce Murine Embryonic Stem Cells to Hepatic Morphogenesis

The contribution of the nonparenchymal hepatic cell population is necessary for hepatic in vitro morphogenesis from ES cells. Expression of *VEGF, VEGFR1,* and *VEGFR2* was detected from the EB in the period from before plating to the late stage of differentiated EB, and *CD31/PECAM-1,* a definitive endothelial cell–specific marker, began to be expressed at the stage of EB formation and continued to be detectable until the late stage of differentiation of the EB outgrowths (Fig. 4A), suggesting that vasculogenesis had been activated in this system. Indeed, CD31/PECAM-1–positive cells were shown to form network structures (Fig. 4C), indicating that CD31/PECAM-1–positive cells organized the formation of vascular networks in the EB outgrowths. In the presence of additional growth factors, few CD31/PECAM-1–positive cells were seen to be organizing capillary networks, which were twisted and slender (Fig. 4D), compared with those in the absence of additional growth factors.

To analyze the interactions between albumin-producing cells and these endothelial cells, the EB outgrowths at both the early and late stages after plating were stained with anti-albumin and anti-CD31/PECAM-1 antibodies. Using these antibodies discriminated parenchymal or nonparenchymal cells as anti-albumin–positive or anti-CD31/PECAM-1–positive cells, respectively, in mixed control cultures of cells prepared from mouse liver (Fig. 4B). Interestingly, the CD31/PECAM-1–positive cells were seen to be migrating in the albumin-positive areas of the EB outgrowths at A10 and made contact with the juxtapositions of the albumin-positive cells (Fig. 4E), similar to the situation liver organogenesis in the developing embryo [10]. As can be seen in Figures 4E and 4F, albumin-positive cells were proliferating from A10 to A18. The CD31/PECAM-1–positive cells were seen to be

proliferating and organizing networks with the spread of the albumin-positive area in the EB outgrowths at A18 (Fig. 4F), suggesting that these endothelial cells had a marked influence on formation of hepatic tissue within EBs.

To obtain conclusive evidence that vasculogenesis was necessary for hepatocytes to arise and grow in the EB outgrowth, vasculogenesis was inhibited by addition of thalidomide to the differentiation medium. First, the emergence frequency of contracting cardiomyocytes was significantly lower with the addition of thalidomide (26.7% ± 12.0% at 25 mg/L, 6.8% ± 4.2% at 100 mg/L) at A3 than without thalidomide (99.5% ± 0.5%), suggesting that thalidomide was able to inhibit the differentiation of ES cells to cardiomyocytes. The results of confocal microscopy analysis (Figs. 5A–5C) indicated that thalidomide could strongly inhibit the differentiation of CD31/PECAM-1–positive cells in the EB outgrowths compared with the control cultures without thalidomide. In addition, the albumin-positive area was significantly smaller in differentiated EB outgrowths exposed to thalidomide at A18 compared with the control culture in the absence of thalidomide (Fig. 5A), and the density and area of the vascular-like network consisting of ES-derived CD31/PECAM-1–positive endothelial cells were much lower in the thalidomide-treated EB outgrowths than in the absence of thalidomide. The expression of *VEGFR1*, *VEGFR2*, and *PECAM-1* was inhibited by thalidomide in a dose-dependent manner. These results of immunohistochem-

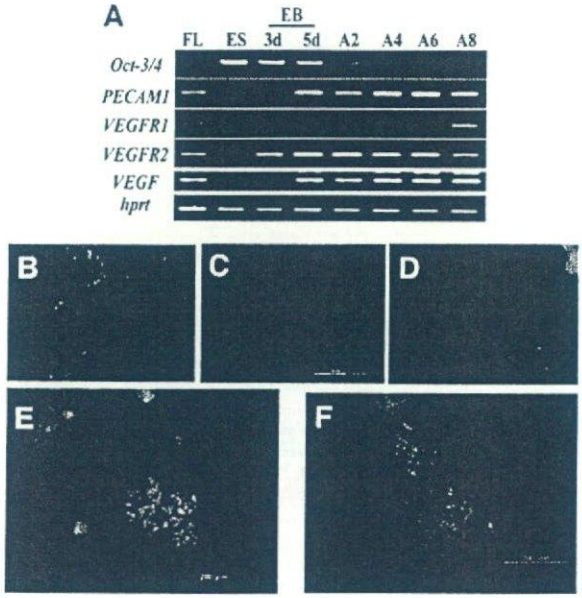

Figure 4. Hepatic morphogenesis derived from outgrowths of EBs consisting of albumin-positive hepatocytes and CD31/PECAM-1–positive endothelial cells expanding into vessel-network structures. **(A):** Endothelial development-associated gene expression was detected by reverse transcription–polymerase chain reaction analysis and activated during the differentiation of EBs. **(B):** Immunohistochemical analysis, with anti-albumin (red) and anti-CD31/PECAM-1 (green) antibodies, in mixed cultures of embryonic liver cells in vitro as a control. **(C, D):** CD31/PECAM-1–positive cells were shown to form a network structure in the presence of growth factors **(D)** and in the absence of growth factors **(C)**. The outgrowths of EBs at A18 were stained with CD31/PECAM-1 antibodies. Without exogenous growth factors, a part of the outgrowth EBs was shown as vessel-like formation. **(E, F):** Immunohistochemical analysis of the EB outgrowth at A10 **(E)** and A18 **(F)** using anti-albumin (red) and anti-CD31/PECAM-1 (green) antibodies in the absence of any growth factors. Abbreviations: EB, embryoid body; ES, embryonic stem; FL, mouse fetal liver at E15; PECAM-1, platelet-endothelial cell adhesion molecule-1; VEGFR, vascular endothelial growth factor receptor.

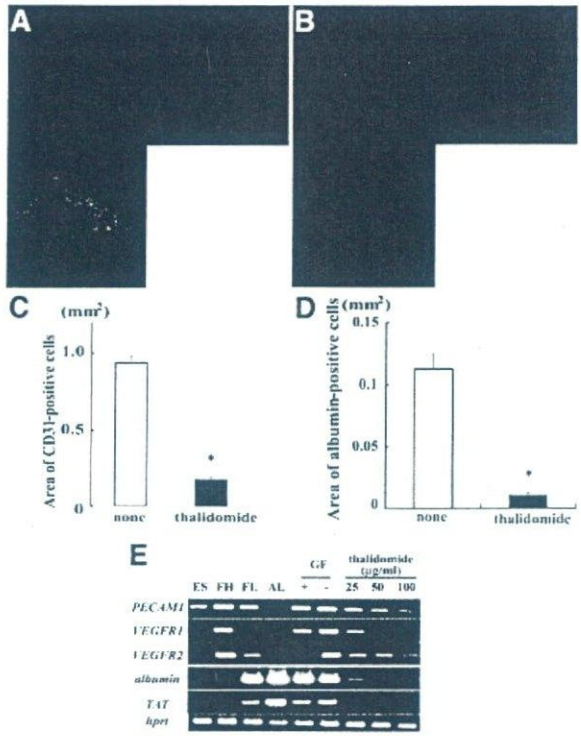

Figure 5. Dependence of hepatic morphogenesis depends on angiogenesis or vasculogenesis in the differentiated EBs. **(A, B):** Immunohistochemical analysis of the EB outgrowths at A18 using anti-CD31/PECAM-1 antibody in the presence of 100 μg/ml thalidomide for 18 days **(B)** and without thalidomide as a control **(A)**. **(C, D):** Quantitative analysis of the CD31/PECAM-1–positive area in the EB outgrowths at A18 was performed using the Scion image Beta 4.0.2 software in each of 15 selected individual fields from three independent experiments. Data are given as mean values ± standard error. Student's *t*-test for unpaired data was applied as appropriate. Difference of $p < .001$ was considered significant. **(E):** Endothelial cell and hepatocyte-associated gene expression was analyzed by reverse transcription–PCR in the thalidomide-treated EB outgrowths of the EBs at A18. PCR amplification of *PECAM-1*, *VEGFR1*, *VEGFR2*, and *TAT* was carried out for 40 cycles. Amplification of *albumin* was carried out for 30 cycles. Abbreviations: AL, mouse adult liver; ES, undifferentiated ES cells; FH, mouse fetal heart at E15; FL, mouse fetal liver at E15; GF, growth factor; PCR, polymerase chain reaction; PECAM-1, platelet-endothelial cell adhesion molecule-1; TAT, tyrosine aminotransferase; VEGFR, vascular endothelial growth factor receptor.

ical and RT-PCR analyses suggested that differentiation to endothelial cells was strongly inhibited by thalidomide. Expression of *albumin* and *TAT* was detected in the EBs at A18 in the absence, but not in the presence, of thalidomide (Fig. 5F).

Furthermore, to address the potential role of endothelial cell differentiation and proliferation in the growth of hepatocytes from ES cells, CBO-P11, a VEGF receptor–specific inhibitor, was added in this system. CD31/PECAM-1–positive cells were reduced by addition of CBO-P11 in the EB outgrowths at A18 compared with the control (Figs. 6A–6C). In the presence of CBO-P11, the morphology of the small CD31/PECAM-1–positive area was truncated and disconnected (Fig. 6B). Corresponding to the CD31/PECAM-1–positive cell populations, no albumin-positive cells were observed in the CBO-P11–treated EB outgrowths, whereas there were many albumin-positive cells in the control (Figs. 6A, 6B, 6D). The expression of *PECAM-1* was significantly lower in the CBO-P11–treated EB outgrowths than in the control without CBO-P11. Interestingly, no expression of albumin or TAT was detected in the EBs at A18 in the presence of CBO-P11 (Fig. 6F). The results of these experiments involving treatment with thalidomide and CBO-P11 suggest that CD31/PECAM-1–positive cells have a crucial role in the hepatic differentiation of ES cells.

Discussion

The purpose of this study was to devise an in vitro system that would allow liver morphogenesis from ES cells, reproducing the events of liver development in vivo. We first considered it necessary to promote the differentiation of outgrowths from EBs, derived from dissociated ES cells, to cardiomyocytes at high efficiency. Selected sublines from the parental ES cells showed a high frequency of emergence of cardiomyocytes and albumin-positive cells, whereas the frequency of cardiomyocyte emergence from the parental ES cells was not so high because of the heterogeneity of the ES cell culture in which albumin-positive cells failed to appear. The same effect was observed in our culture system, suggesting that the initial pluripotency of ES cells contributed to the differentiation of EBs to beating cardiomyocytes, and after induction to mesodermal lineages hepatic differentiation and maturation occurred in the EB outgrowths. Naggy et al. [23] described that recloned ES cell sublines could contribute significantly to the production of chimeric mice and for germ-line transmission in these mice compared with the parental line and could also become a complete body using ES cell–tetraploid aggregation [23, 24], because after a high number of passages, an ES cell culture is a heterogeneous mixture of undifferentiated and differentiated populations. It is very important for undifferentiated and pluripotent ES cells to be present in these cultures for differentiation not only to cardiomyocytes but also to albumin-producing hepatocytes and endothelial cells, corresponding to the various developmental stages of the liver. We tested several sublines of ES cells for their efficiency in the production of chimeric mice that would have the potential for germ-line transmission and a high frequency of cardiomyocyte emergence. It is interesting that the ES cell sublines that transmitted the germ line in chimeric mice at high efficiency corresponded to those with a frequency of cardiomyocyte emergence of almost 100%. The potential of ES cells to transmit the germ line in chimeric mice corresponded to the frequency of cardiomyocyte emergence in the EB outgrowths. We showed that albumin expression data in both emergence and non-

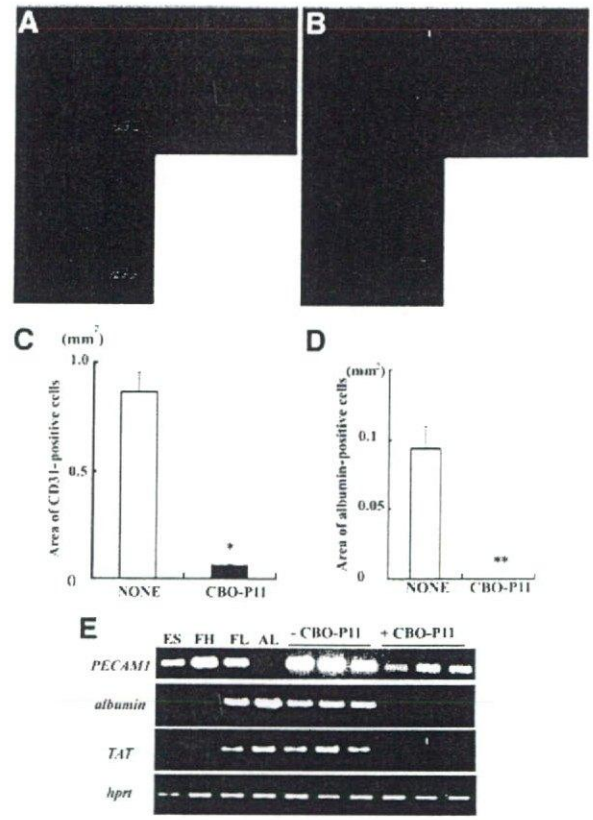

Figure 6. Lack of induction of hepatic morphogenesis by vascular endothelial growth factor inhibitor in the differentiated EBs. **(A, B):** Immunohistochemical analysis of the EB outgrowths at A18 using anti-CD31/PECAM-1 antibody in the presence of 10 μM CBO-P11 for 18 days **(B)** and without CBO-P11 as a control **(A)**. **(C, D):** Quantitative analysis of the CD31/PECAM-1–positive area in the EB outgrowths at A18 was performed using the same method as that for the thalidomide experiments in each of nine individual fields selected from three independent experiments. **(E):** Endothelial cell and hepatocyte-associated gene expression was analyzed by RT-PCR in the thalidomide-treated EB outgrowths at A18. PCR amplification of *PECAM-1* and *TAT* was carried out for 40 cycles. Amplification of albumin was carried out for 30 cycles. Abbreviations: AL, mouse adult liver; EB, embryoid body; ES, undifferentiated embryonic stem cells; FH, mouse fetal heart at E15; FL, mouse fetal liver at E15; PECAM-1, platelet-endothelial cell adhesion molecule-1; RT-PCR, reverse transcription–polymerase chain reaction; TAT, tyrosine aminotransferase.

emergence of cardiomyocytes, as shown in Figure 1B, although cardiomyocyte ablation and blocking cardiomyocyte differentiation experiments are very interesting. However, we consider that subclone screening for the frequency of emergence of cardiomyocytes from the parental murine ES cells would be useful for establishing an in vitro model of hepatic morphogenesis.

The number of albumin-producing cells increased cumulatively in the expanding vascular network area during differentiation to the late stage. It has been clarified that in vitro differentiation of murine ES cells within EBs leads to complex structures that can mimic the normal developmental process of the early embryo, in particular vasculogenesis and hematopoiesis, although no details of liver morphogenesis have been reported [25–27]. Our present results indicated that expansion of the endothelial cell network derived from ES cells plays an important role in the proliferation of hepatocytes and also liver morphogenesis in vitro, reproducing the events that occur in vivo. Furthermore, our liver morphogenesis system does not involve simple coculture of ES cells with endothelial cells prepared from liver sinusoids or blood vessels but is a novel system that utilizes the differentiation of pluripotent ES cells to cardiomyocytes to support subsequent differentiation to endothelial cells and hepatocytes. The interaction between hepatocytes and endothelial cells in liver organogenesis has already been reported by Matsumoto et al. [10]. Our in vitro system for the induction of liver morphogenesis from ES cells closely corresponds to the natural events of liver development that occur in vivo, as shown in Figure 7. Thalidomide, an inhibitor of angiogenesis, inhibited the differentiation and proliferation of CD31/PECAM-1–positive cells in EB outgrowths. However, there is a possibility that thalidomide may have directly suppressed the differentiation of hepatocytes. Therefore, to confirm the crucial role of endothelial cells in hepatic differentiation and maturation, we examined the effect of CBO-P11, a potent VEGF signal-specific inhibitor [28–30], in our culture system. CBO-P11 strongly suppressed the differentiation and proliferation of CD31/PECAM-1–positive cells, corresponding to endothelial cells, during EB differentiation, and consequently, the differentiation of albumin-positive cells, corresponding to hepatocytes, was completely blocked. These results were consistent with the data obtained from the thalidomide experiment.

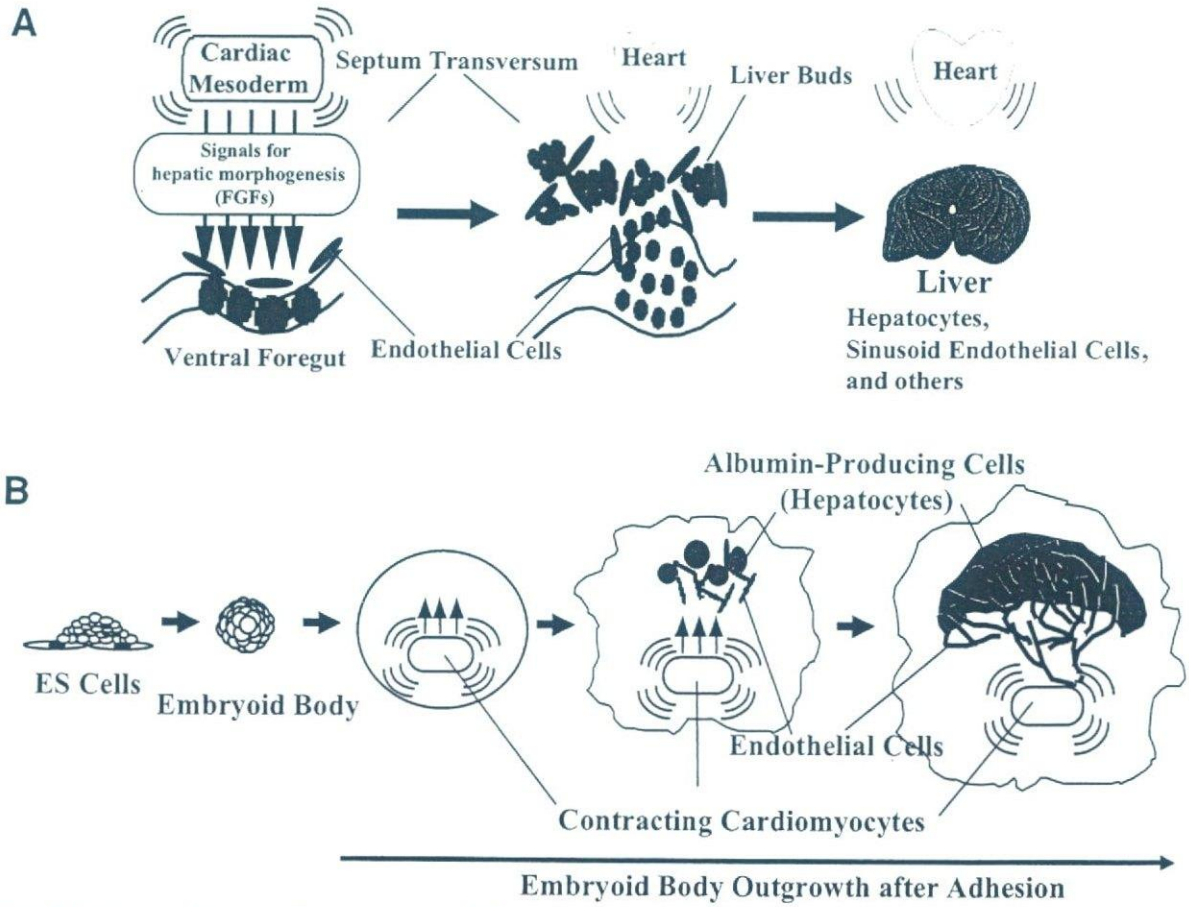

Figure 7. Illustrations of **(A)** in vivo liver development and **(B)** our in vitro system for the construction of hepatic organogenesis using murine ES cells. Abbreviations: ES, embryonic stem; FGF, fibroblast growth factor.

The process of organogenesis relies on the presence of specific microenvironments. However, it is difficult to reconstruct such microenvironments in vitro simply by addition of excess free molecules, such as FGF, HGF, and oncostatin M. Strategies for adding growth factors known as liver development inducers to the differentiation medium or for introducing an expression vector of a liver-specific master gene into ES cells can induce the expression of several specific genes in the area of cell interaction, which means that it is difficult to induce the differentiation of ES cell cultures to more than two cell lineages, such as hepatocytes and sinusoidal endothelial cells, through addition of these factors. These kinds of strategies make it impossible, or at least very difficult, to reconstruct target morphogenesis or to induce natural target cells with specific multiple functions. Therefore, no additional growth factors were used in our experiments. We succeeded in reconstructing in vitro the process of liver morphogenesis that involved at least three different types of cell populations: cardiomyocytes, endothelial cells, and hepatocytes. Initiation of liver organogenesis in vivo occurs in the septum transversum. The septum transversum mesenchyme also has important roles in liver organogenesis, such as production of HGF [9, 31]. In our present study, strong expression of HGF was detected in the ES differentiation system, as shown in Figure 2F. Although we detected many fibroblastic cells in this system, it was unclear whether some of these cells corresponded to the septum transversum. With regard to liver function, we were able to demonstrate ammonia degradation activity in this liver differentiation system (Fig. 2G). Unlike a primary hepatocyte culture, which contains almost 100% hepatocytes, our system contains a heterologous population including hepatocytes, endothelial cells, cardiomyocytes, and so on. However, the level of hepatic function, such as ammonia degradation, in each individual hepatocyte was higher in our differentiation system than in a primary culture, suggesting that hepatocyte–endothelial cell contact is very important for the generation of full hepatic function in hepatocytes, as well as for liver development and proliferation in the embryo. Generally, a hepatocyte primary culture has a short life span, shows no proliferation, and has low hepatic function. Therefore, we consider that a hepatocyte primary culture as a single-cell source is not sufficient for creation of a bioartificial liver system and that nonparenchymal cell populations are also necessary for reproducing the complex structure and polarity of the liver seen in vivo.

Here, we established a novel system for liver organogenesis from murine ES cells based on embryological events, i.e., with contributions from cardiac mesoderm and endothelial cell lineages, which were also derived from the ES cells. Furthermore, it has been difficult up to now to culture hepatocytes prepared from adult or embryonic liver in vitro for a long period, as well as to maintain the multiple functions characteristic of the liver. This is one of the major obstacles to the development of a bioartificial liver system. Our system makes it possible to culture ES cell–derived hepatocytes for a long period, at least for more than 30 days, and these cells are able maintain a high degree of hepatic function (Fig. 2F). This innovative system will be useful for creation of liver embryology and regeneration systems as well as for the development of a bioartificial liver system for bridging use in patients waiting for a liver donor and for drug-metabolism assays.

Acknowledgments

We are grateful to Prof. Nobuaki Yoshida and Prof. Yoichiro Iwakura (Institute of Medical Science, University of Tokyo, Toyko) for providing E14.1 ES cells, Prof. Hisato Kondo (Institute for Molecular and Cellular Biology, Osaka University, Osaka, Japan) for NHL7 cells, RIKEN Cell Bank for STO and HePa 1-6 cell lines, and General Research Laboratory, Shinshu University School of Medicine, Nagano, Japan, for technical assistance. This study was supported by grants from the Ministry of Education, Sports, Science and Technology of Japan (Tokyo) (15700314; 13470150, Grant-in-Aid for 21st Century COE program by the above ministry), Hokuto Foundation of Bioscience (Nagano, Japan), and Foundation of Shinshu Igakushinko (Matsumoto, Japan).

References

1 Zaret KS. Regulatory phases of early liver development: paradigms of organogenesis. Nat Rev Genet 2002;3:499–512.

2 Reif S, Terranova VP, el-Bendary M et al. Modulation of extracellular matrix proteins in rat liver during development. Hepatology 1990;12:519–525.

3 Kamiya A, Kojima N, Kinoshita T et al. Maturation of fetal hepatocytes in vitro by extracellular matrices and oncostatin M: induction of tryptophan oxygenase. Hepatology 2002;35:1351–1359.

4 Gualdi R, Bossard P, Zheng M et al. Hepatic specification of the gut endoderm in vitro: cell signaling and transcriptional control. Genes Dev 1996;10:1670–1682.

5 Couvelard A, Bringuier AF, Dauge MC et al. Expression of integrins during liver organogenesis in humans. Hepatology 1998;27:839–847.

6 Jung J, Zheng M, Goldfarb M et al. Initiation of mammalian liver development from endoderm by fibroblast growth factors. Science 1999;284:1998–2003.

7 Fukuda-Taira S. Hepatic induction in the avian embryo: specificity of reactive endoderm and inductive mesoderm. J Embryol Exp Morphol 1981;63:111–125.

8 Douarin NM. An experimental analysis of liver development. Med Biol 1975;53:427–455.

9 Rossi JM, Dunn NR, Hogan BL et al. Distinct mesodermal signals, including BMPs from the septum transversum mesenchyme, are required in combination for hepatogenesis from the endoderm. Genes Dev 2001;15:1998–2009.

10 Matsumoto K, Yoshitomi H, Rossant J et al. Liver organogenesis promoted by endothelial cells prior to vascular function. Science 2001;294:559–563.

11 Sherer GK. Tissue interaction in chick liver development: a reevaluation, I. Epithelial morphogenesis: the role of vascularity in mesenchymal specificity. Dev Biol 1975;46:281–295.

12 Bankston PW, Pino RM. The development of the sinusoids of fetal rat liver: morphology of endothelial cells, Kupffer cells, and the transmural migration of blood cells into the sinusoids. Am J Anat 1980;159:1–15.

13 Enzan H, Himeno H, Hiroi M et al. Development of hepatic sinusoidal structure with special reference to the Ito cells. Microsc Res Tech 1997;39:336–349.

14 Hamazaki T, Iiboshi Y, Oka M et al. Hepatic maturation in differentiating embryonic stem cells in vitro. FEBS Lett 2001;497:15–19.

15 Yamamoto H, Quinn G, Asari A et al. Differentiation of embryonic stem cells into hepatocytes: biological functions and therapeutic application. Hepatology 2003;37:983–993.

16 Yamada T, Yoshikawa M, Kanda S et al. In vitro differentiation of embryonic stem cells into hepatocyte-like cells identified by cellular uptake of indocyanine green. STEM CELLS 2002;20:146–154.

17 Yin Y, Lim YK, Salto-Tellez M et al. AFP(+), ESC-derived cells engraft and differentiate into hepatocytes in vivo. STEM CELLS 2002;20:338–346.

18 Jones EA, Tosh D, Wilson DI et al. Hepatic differentiation of murine embryonic stem cells. Exp Cell Res 2002;272:15–22.

19 Chinzei R, Tanaka Y, Shimizu-Saito K et al. Embryoid-body cells derived from a mouse embryonic stem cell line show differentiation into functional hepatocytes. Hepatology 2002;36:22–29.

20 Lavon N, Yanuka O, Benvenisty N. Differentiation and isolation of hepatic-like cells from human embryonic stem cells. Differentiation 2004;72:230–238.

21 Horai R, Asano M, Sudo K et al. Production of mice deficient in genes for interleukin (IL)-1α, IL-1β, IL-1α/β, and IL-1 receptor antagonist shows that IL-1β is crucial in turpentine-induced fever development and glucocorticoid secretion. J Exp Med 1998;187:1463–1475.

22 Itoh H, Abo T, Sugawara S et al. Age-related variation in the proportion and activity of murine liver natural killer cells and their cytotoxicity against regenerating hepatocytes. J Immunol 1988;141:315–323.

23 Nagy A, Rossant J, Nagy R et al. Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. Proc Natl Acad Sci U S A 1993;90:8424–8428.

24 Auerbach W, Dunmore JH, Fairchild-Huntress V et al. Establishment and chimera analysis of 129/SvEv- and C57BL/6-derived mouse embryonic stem cell lines. Biotechniques 2000;29:1024–1028, 1030, 1032.

25 Wartenberg M, Gunther J, Hescheler J et al. The embryoid body as a novel in vitro assay system for antiangiogenic agents. Lab Invest 1998;78:1301–1314.

26 Hirashima M, Kataoka H, Nishikawa S et al. Maturation of embryonic stem cells into endothelial cells in an in vitro model of vasculogenesis. Blood 1999;93:1253–1263.

27 Wang R, Clark R, Bautch VL. Embryonic stem cell-derived cystic embryoid bodies form vascular channels: an in vitro model of blood vessel development. Development 1992;114:303–316.

28 Bikfalvi A. Recent developments in the inhibition of angiogenesis: examples from studies on platelet factor-4 and the VEGF/VEGFR system. Biochem Pharmacol 2004;68:1017–1021.

29 Bello L, Lucini V, Costa F et al. Combinatorial administration of molecules that simultaneously inhibit angiogenesis and invasion leads to increased therapeutic efficacy in mouse models of malignant glioma. Clin Cancer Res 2004;10:4527–4537.

30 Zilberberg L, Shinkaruk S, Lequin O et al. Structure and inhibitory effects on angiogenesis and tumor development of a new vascular endothelial growth inhibitor. J Biol Chem 2003;278:35564–35573.

31 Andermarcher E, Surani MA, Gherardi E. Co-expression of the HGF/SF and c-met genes during early mouse embryogenesis precedes reciprocal expression in adjacent tissues during organogenesis. Dev Genet 1996;18:254–266.

第7回肝移植臨床検討会

生体肝移植後の再発C型肝炎の臨床病理学的検討

中澤勇一・佐近雅宏・三原基弘・三田篤義・浦田浩一・
池上俊彦・寺田 克・橋倉泰彦・宮川眞一[*1]，中山 淳[*2]

Clinicopathologic analysis of recurrent hepatitis C after living donor liver transplantation

key words：再発C型肝炎，生体肝移植，拒絶反応，抗ウイルス療法

肝移植術後においてC型肝炎ウイルス(HCV)の出現はほぼ必発であり，移植後患者の70～80％において肝機能異常を伴う再発C型肝炎を発症するとされる[1]．

当初，再発C型肝炎は，再発B型肝炎に比し予後が良好であるといわれてきた．しかし最近，術後5年で再発例のうち20～30％が肝硬変となり，さらに術後10年で50％が肝硬変となると報告されており，肝移植後の免疫抑制とそれによるHCVの著明な増殖がその原因と考えられている[2]．また，生体肝移植後の再発C型肝炎は，脳死全肝移植後に比し重症化するとの報告も散見され，これら移植後のC型肝炎の予後解析が急務となっている[3]．

今回，信州大学移植外科にて生体肝移植が施行されたC型肝炎症例の術後の再発肝炎を中心に，臨床病理学的に検討したので報告する．

対象と方法

1990年6月～2004年3月まで，当施設にて18例のC型肝炎・肝硬変症例に対して生体肝移植が施行された．これら18例の移植前の臨床・検査成績は，表1のとおりである．

18例全例で術後定期的にHCV-RNAが定量測定され，再発C型肝炎の診断は組織学的あるいは臨床的に行われた．臨床的再発は，生検は施行されないものの肝機能異常とHCV-RNA陽性所見が認められ，かつ他の肝機能異常の原因が臨床的に否定されたものとした．また，再発後複数回の組織学的検査が施行された症例において，組織所見の進行について検討した．再発と診断され，抗ウイルス療法が可能な状態と判断された症例でインターフェロンα-2b(300万単位，週3回投与)とリバビリン(600～800 mg/日，内服)による治療を施行した．

肝生検が施行された再発症例を，臨床的に急性拒絶反応が先行しステロイドパルス療法が施行され，その後に再発と診断された症例とそうでない症例とにわけ，臨床・病理学的に比較検討した．

C型肝炎症例18例の生体肝移植後生存率については，Kaplan-Meier法により検討した．また，患者生存に関与すると考えられる因子について考察した．

表1 C型肝炎症例18例の移植前所見

臨床・検査所見	18
1. 性別：male/female	14/4
2. 平均年齢(齢)	55(32～69)
3. HCC合併例	12(67％)
4. HCVジェノタイプ1b	18(100％)
5. 移植グラフト：left lobe/others	17/1
6. 平均GV/SV(％)	37(34～49)
7. 免疫抑制：FK/CsA	16/2

表2 再発C型肝炎の組織所見

初回再発診断時(8例)
- F0/A1-A2

肝生検による長期フォローアップ例(4例)
- F0/A1(POD 40) ⟹ F2/A2 (POD420)
- F0/A1(POD 52) ⟹ F1/A1(POD211)
- F0/A2(POD142) ⟹ F2/A1(POD1201)
- F0/A1(POD 50) ⟹ F3/A2(FCH?)(POD169)

[*1]信州大学医学部移植外科学教室，[*2]同 病理部

表3　急性拒絶反応→再発C型肝炎症例

	Case 1	Case 2	Case 3
Age (yr)	62	61	60
Timing ACR	POD 25	POD 126	POD 43
Timing hepatitis	POD 37	POD 142	POD 50
AST max (U/L)	278 (POD37)	607 (POD133)	263 (POD157)
T.Bil max (mg/dL)	4.5 (POD72)	26.6 (POD146)	55.2 (POD183)
IFN + RIBA	POD40	POD145	(−)
Prognosis	46 mo. Alive	47 mo. Alive	6 mo. Dead. FCH?

表4　急性拒絶反応→再発C型肝炎症例の組織所見

Case	Biopsy No.	POD	Eosinophils	Bile duct damage	Endotheliitis	Kupfer cell hypertrophy	Focal cell loss	Apoptosis count	Diagnosis
1.	6505	25	++	+	++		+	1.3	ACR
	6560	29	+		+	+		1.7	ACR
	6743	37		+			+	5.3	Hepatitis
2.	1544	126		++	++	+	+	0.6	ACR
	1852	133			+	+	+	1.3	ACR
	3885	142	+			+		0.3	Hepatitis
3.	1000	43		+	+	+	+	3.3	ACR
	1134	50		+		+	+	4.7	Hepatitis
	1270	57		+	+		+	2.1	ACR
	1540	75				+	+	2.5	Hepatitis

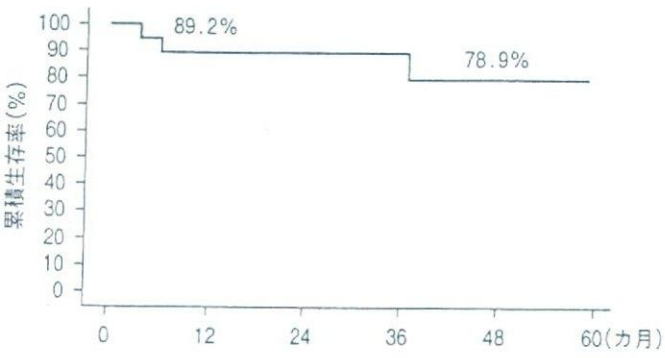

図1　C型肝炎症例の移植後生存率(18例)

結　果

移植後，18例全例で血清HCV-RNAは陽性化した．うち10例でHCV-RNAはアンプリコアモニター法にて定量測定され，その最高値は320～850以上KIU/mLであり，8例ではその定量のためにリアルタイムPCRが用いられ，その術後最高測定値は3.7×10^7～7.0×10^9copies/mLであった．

18例中10例(56%)でC型肝炎が再発した．8例の再発肝炎診断は術後89日(中央値31～132日)に組織学的に行われた．8例の再発初回診断時の組織学的所見はF0/A1-A2であり，うち4例でフォローアップの肝生検が施行され，これら4例はいずれの症例でも線維化の進行が認められた(表2)．

肝炎再発症例中6例(60%)でインターフェロン＋リバビリンによる抗ウイルス療法が導入された．しかし，2例でインターフェロンによる精神症状が出現し，また，拒絶反応と感染症によりそれぞれ1例でこれら治療が中止された．最終的には2例でのみ6カ月以上の長期投与が可能であったが，この2例においてもHCVは排除されず，肝機能の改善が認められたのみであった．

臨床的に拒絶反応が先行しない再発肝炎症例5例では，一過性のトランスアミナーゼ値の上昇が特徴的であり，診断後1カ月以内に抗ウイルス療法の施行にかかわらずトランスアミナーゼ値は正

表5 C型肝炎術後死亡症例

	症例1	症例2	症例3
年齢(歳)	69	64	60
HCC	(+)単発	(+)16個	(−)
術前MELD	21	9	15
術後合併症	胆管狭窄		拒絶反応
術後(カ月)	3	25	6
死因	アスペルギルス，肝不全	HCC再発(肺・脳)	アスペルギルス，FCH？

常化した．組織学上，異常所見が肝小葉内に限局するlobular hepatitisの像を呈し，C型肝炎(再発)の診断は容易であった．

一方，拒絶反応が先行し，その治療後再発C型肝炎と診断された3例では，トランスアミナーゼ，ビリルビンのピーク値が高値であり，2例ではトランスアミナーゼ値の正常化に5カ月以上を要し，1例は術後約6カ月に肝不全で死亡した．死亡症例の死亡直前の組織所見は，fibrosing cholestatic hepatitis(FCH)に類似した像を呈していた(表3)．

これら3例の初回3～4回の具体的肝生検所見を検討した(表4)．eosinophiliaとendotheliitisは，再発肝炎に比し拒絶反応に有意に高頻度に認められたが，胆管障害と肝小葉内の変化は再発肝炎と拒絶反応ともに認められた．しかし，小葉内のapoptotic bodyの数は，再発肝炎で高値の傾向があった．

C型肝炎症例18例の移植後フォローアップ期間は34カ月(中央値3.5～60カ月)であり，1，2年患者生存率は89.2%，5年患者生存率は78.9%であった．再移植が施行された症例はなく，グラフト生存率も同値であった(図1)．死亡例はそれぞれ，1例では高齢＋MELDスコア高値のリスクファクターを有し，1例では高度進行肝細胞がんを術前合併し，1例では移植後の難治性拒絶反応治療による重症感染症，重症再発C型肝炎が死因となった(表5)．

考　察

C型肝炎に対する肝移植の予後に影響する主な因子として，再発C型肝炎と再発肝炎以外の移植時年齢，術前状態などのレシピエント因子があげられる[2,4]．再発C型肝炎の進行の防止・発症予防には，HCVそのものに対する抗ウイルス療法が必須であるが，実際には今回の検討で示されたように，インターフェロンとリバビリンにはさまざまな副作用があり，このような副作用により治療導入後の中止・脱落が高頻度である[5]．

また，ペグインターフェロンとリバビリンによる治療にても，いまだ満足な治療成績が得られていないのが実情であり，このような状況下で，肝移植後に高ウイルス血症が認められるものの，肝機能が安定している際には抗ウイルス療法を導入しない施設も多い．今後は，より有効な治療法の開発，治療の開始時期，投与量，投与期間など，さらなる検討が望まれる．

拒絶反応の治療後に再発C型肝炎と診断された症例では，2例で肝機能回復に時間を要し，1例は肝不全へ進行した．これらの所見は，拒絶反応に対するステロイドパルス療法，OKT3投与は肝細胞内のHCV増殖を促進するため，重症・進行性の再発C型肝炎発症に寄与するという報告を支持するものと考えられる．

C型肝炎症例の肝移植後においては，拒絶反応，それに対する治療がその予後に影響を及ぼす可能性を常に留意すべきである．場合によりC型肝炎症例では，組織検査上，軽微な拒絶反応と診断される症例では，無治療のまま経過を観察するなどの非積極的な拒絶反応の治療も必要であるかもしれない．今回の検討では，拒絶反応と再発C型肝炎の組織学的所見のオーバーラップが明らかとなったが，不必要な免疫抑制強化を避けるためにも，正確な拒絶反応の組織診断，拒絶反応と

再発肝炎の鑑別はきわめて重要である．

死亡例2例では，1例は69歳と高齢かつ術前のMELDスコアが高値であり，1例では高度進行肝細胞がんを合併し，移植後再発肝細胞がんが直接の死因であった．一般に生体肝移植においては，脳死肝移植に比し移植の適応は拡大されているものと考えられるが，これら2例の術後予後は移植適応決定について示唆を与えるものと考えられる．

C型肝炎症例の生体肝移植後5年生存率は比較的良好であった．18例中移植後5年以内での再発C型肝炎による死亡は1例のみであり，再発肝炎の短期予後に及ぼす影響は明らかでなかった．しかし，近年，再発C型肝炎が予後に影響する時期は移植後5～10年とされており，さらに正確な予後の判定には長期の経過観察が必要であると考えられる．

文　献

1) Gane EJ, Portmann BC, Naoumov NV, Smith HM, Underhill JA, Donaldson PT et al.: Long-term outcome of hepatitis C infection after liver transplantation. N Engl J Med 334 : 815-820, 1996.
2) Berenguer M: Host and donor risk factors before and after liver transplantation that impact HCV recurrence. Liver Transpl 9 : S44-S47, 2003.
3) Gaglio PJ, Malireddy S, Levitt BS, Lapointe-Rudow D, Lefkowitch J, Kinkhabwala M et al.: Increased risk of cholestatic hepatitis C in recipients of grafts from living versus cadaveric liver donors. Liver Transpl 9 : 1028-1035, 2003.
4) Onaca NN, Levy MF, Sanchez EQ, Chinnakotla S, Fasola CG, Thomas MJ et al.: A correlation between the pretransplantation MELD score and mortality in the first two years after liver transplantation. Liver Transpl 9 : 117-123, 2003.
5) Lavezzo B, Franchello A, Smedile A, David E, Barbui A, Torrani M et al.: Treatment of recurrent hepatitis C in liver transplants: efficacy of a six versus a twelve month course of interferon alfa 2b with ribavirin. J Hepatol 37 : 247-252, 2002.

Steroid-Free Living-Donor Liver Transplantation in Adults

Shigeru Marubashi, Keizo Dono, Koji Amano, Naoki Hama, Kunihito Gotoh, Hidenori Takahashi, Kazuhiko Hashimoto, Atsushi Miyamoto, Yutaka Takeda, Hiroaki Nagano, Koji Umeshita, and Morito Monden

To examine the benefits of steroid avoidance in adult living donor liver transplantation, we compared the clinical courses of nine recipients receiving basiliximab or daclizumab and 13 historical patients who received steroids. The 1-year patient and graft survival and the incidence of acute cellular rejection were similar in both groups. The side effects of immunosuppression tended to be more frequent in the steroid group. Hepatitis C virus (HCV)-RNA levels measured early after transplantation remained suppressed in the steroid-free group. Steroid avoidance was beneficial in the recipients, as both steroid side effects and recurrence of HCV could be avoided.

Keywords: Living donor, Liver transplantation, Steroid free.

(*Transplantation* 2005;80: 704–706)

Steroid avoidance in liver transplantation could be beneficial in preventing side effects such as infection, posttransplant diabetes and hypertension, as well as recurrence of viral hepatitis. Although steroid-free immunosuppressive regimens (IS) have been reported to be safe in cadaveric or pediatric liver transplantation (*1*–*3*), no report has been published yet in relation to adult-to-adult living donor liver transplantation (LDLT).

This prospective pilot study was aimed at testing the hypothesis that steroid-free IS are safe and beneficial in adult-to-adult living donor liver transplantation.

Nine adult LDLT recipients with hepatitis B virus (HBV; n=2) or hepatitis C virus (HCV; n=7) were included in this intent-to-treat analysis. Immunosuppression was induced with a calcineurin inhibitor (tacrolimus or cyclosporin A) (CNI) and mycophenolate mofetil (MMF), and an anti-IL2 receptor monoclonal antibody (basiliximab or daclizumab), without prophylactic steroids. These patients were compared with 13 historical patients with HBV (n=9) or HCV (n=4) hepatitis who received traditional IS, namely, a CNI with a steroid (steroid group). Tacrolimus or cyclosporin A was administered orally (initial dose 0.3 mg/kg and 4 mg/kg, respectively) with the dose titrated according to the blood concentrations, aiming for 8-12 ng/ml or 200-250 ng/ml, respectively, during the first month, and 5–8 ng/ml or 150-200 ng/ml, respectively, thereafter in both groups. MMF was administered at the initial dose of 2000 mg/day, tapered, and discontinued by 3 months after LDLT in the steroid-free group and given as needed in the steroid group. Basiliximab, a mouse-human chimeric anti-CD25 monoclonal antibody (20 mg; days 0, 4) or daclizumab, a humanized anti-CD25 monoclonal antibody (25 mg; days 0, 6, 13, 20) was administered intravenously after LDLT. In the steroid group, methylprednisone or prednisolone was administered at the dose of 1000 mg intraoperatively and 100 mg on day 1, and tapered off by the end of three months after LDLT. Liver biopsy was performed whenever liver enzymes were elevated. Protocol biopsy was performed at 3 months and 1 year after LDLT in both groups. Acute cellular rejection (ACR) was treated with a steroid, which was rapidly tapered off in the steroid-free group. HBV prophylaxis consisted of 100 mg/day lamivudine, started 1 month prior to the LDLT, and human hepatitis B immunoglobulin (HBIG) to maintain the serum anti-HBs antibody at more than 200 IU/l. HCV prophylaxis, with 400 mg ribavirin and 3 MU interferon-alpha administered three times a week, was preemptively started 1–3 months after the LDLT according to the allograft function.

Peripheral blood HCV-RNA levels in the HCV-positive recipients were compared between the steroid-free group (n=7) and the steroid group (n=4) preoperatively, at 14 days, and at 6 months after LDLT.

The patient data in the two groups were similar, except for the initially used CNI (Table 1). The 1-year patient and graft survival were 100% in the steroid-free group and 74.1% in the steroid group (mean follow-up period, 12.1 and 17.9 months, respectively). There were no surgical complications related to the arterial, portal and hepatic venous, or bile-duct anastomoses in these patients, although two patients with HBV died at 2.0 and 4.6 months after LDLT due to primary nonfunction allograft and sepsis, respectively, and one patient with HCV died at 3.2 months after LDLT due to primary nonfunction allograft. The concentration of CNI was similar in both groups.

Protocol biopsies at 3 months and 1 year after the LDLT showed no ACR or hepatitis, except for one patient from the steroid-free group with early ACR (day 6) treated with steroids who developed HCV hepatitis after 1 year.

Seven patients (77.8%) in the steroid-free group were ACR-free, and steroids could be completely avoided after the LDLT (Fig. 1). The incidence of ACR was similar in both groups (steroid-free; 22.2%, steroid; 23.1%). None of the patients (n=4) in the steroid-free group receiving tacrolimus-based IS showed ACR.

The side effects of IS (hypertension, diabetes, infection, renal impairment) tended to be more frequent in the steroid group. No HBV recurrence was noted in either group. HCV

Department of Surgery and Clinical Oncology, Osaka University, Graduate School of Medicine, Suita, Osaka, Japan.

Address correspondence to: Keizo Dono, M.D., Department of Surgery and Clinical Oncology, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita City, Osaka 565-0871, Japan. E-mail: kdono@surg2.med.osaka-u.ac.jp.

Received 11 January 2005.

Accepted 22 March 2005.

ISSN 0041-1337/05/8005-704

DOI: 10.1097/01.tp.0000172187.28376.3b

TABLE 1. Patient characteristics

	Steroid-free group	Steroid group
n	9	13
Sex (M/F)	6/3	10/3
Pretransplant diagnosis (HBV/HCV)	2/7	9/4
Age at LDLT (range)	56 (42-66)	53 (27-61)
MELD score (range)	16 (7-33)	20 (8-41)
Graft type (left/right/post)	6/3/0	9/3/1
Graft weight (range) (g)	751 (355-884)	626 (469-738)
GW/SLV ratio (range) (%)	61 (28-70)	51 (41-65)
Warm ischemia time (min)	40 (28-51)	38 (25-80)
HLA mismatch (range)		
HLA-A	1 (0-1)	1 (0-2)
HLA-B	1 (1-2)	1 (1-2)
HLA-DR	1 (0–1)	1 (1-2)
Initial calcineurin inhibitor (tacrolimus/cyclosporine A)	4/5	12/1
Anti IL-2 receptor monoclonal antibody (basiliximab/daclizumab)	8/1	2/2
Mycophenolate mofetil on LDLT	9 (100 %)	10 (76.9 %)
Concentration of CNI (tacrolimus/cyclosporine A) (ng/ml)		
Day 14	10.9/333	10.8/204
Day 28	9.8/236	11.7/210
Day 90	7.0/192	7.2/190
Day 180	6.2/167	6.0/249
Side effect after LDLT		
Hypertension	1 (14%)	3 (23%)
Posttransplant Diabetes	0	3 (23%)
Diuretics required after 3 months	0	3 (23%)
Infection	0	2 (15%) (sepsis, peritonitis)
HCV patients (n = 1.1)		
HCV-RNA (pre OP)		
Undetected (<0.7 mEq/ml)	3	4
Positive (mEq/ml)	4 (0.8, 1.8, 2.5, 3.7)	0
HCV-RNA (14 days post OP)		
Undetected (<0.7 mEq/ml)	3	2
Positive (mEq/ml)	4 (4.4, 9.0, 9.4, 9.5)	2 (1.5, 2.1)
HCV-RNA (6 months post OP)[a]		
Undetected (<0.7 mEq/ml)	4[b]	2[c]
Positive (mEq/ml)	3 (4.8, 10[d], 45)	1 (38)

Data are medians.
[a] One patient died of primary non-function allograft in the steroid group by 6 months after LDLT.
[b] Inclues two patients receiving preemptive interferon/ribavirin therapy.
[c] Both received preemptive interferon/ribavirin therapy.
[d] Received steroid for acute cellular rejection.
LDLT, living donor liver transplantation; MELD, model of end-stage-liver-disease; GW, graft weight; SLV, standard liver volume; CNI, calcineurin inhibitor.

recurred in two patients; one in the steroid-free group at 12 months after the LDLT and one in the steroid group at 15 months after the LDLT.

HCV-RNA was undetectable (<0.7 mEq/ml) preoperatively in three of the seven patients in the steroid-free group, and in all of the four patients in the steroid group. At 14 days after the LDLT, although HCV-RNA remained undetectable in all the three patients in the steroid-free group, it became detectable at 1.5 and 2.1 mEq/ml in two patients in the steroid group. At 6 months after the LDLT, HCV-RNA was undetectable in four of the seven patients in the steroid-free group, and in two of the three patients in the steroid group, although two in the steroid-free group and both of them in the steroid group had been receiving preemptive HCV prophylaxis treatment. It should be noted that HCV-RNA remains negative at 6 and 13 months after the LDLT in the two patients in the steroid-free group even without any HCV prophylaxis.

Steroid avoidance in liver transplantation has been reported to be safe and equivalent to traditional IS in cadaveric and pediatric liver transplantation (*1–3*). However, the effects of complete steroid avoidance have yet to be reported in adult LDLT. The allograft needs to regenerate and its recovery is often delayed in LDLT as well as in cases of split liver, fatty liver, graft with extended ischemia time or from older donors. The importance of steroids has not been evaluated yet in these disadvantaged liver grafts. Our data suggest that steroid

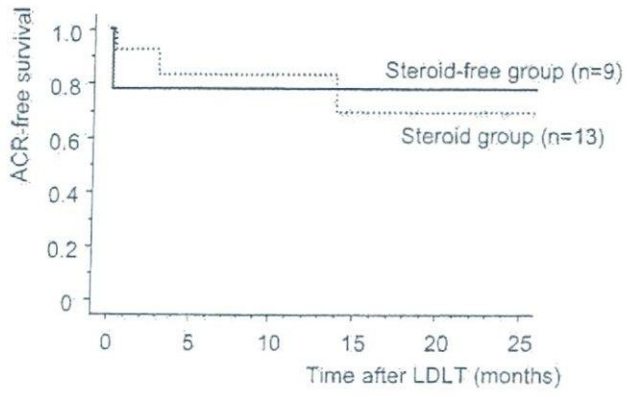

FIGURE 1. Acute cellular rejection (ACR)-free survival in living donor liver transplantation. The incidence of ACR was similar in both groups (steroid-free; 22.2%, steroid; 23.1%).

avoidance may not be harmful to LDLT patients, and administration of tacrolimus combined with MMF and basiliximab as a steroid substitute seemed to be a safe alternative in adult-to-adult LDLT. This is a pilot study with retrospective comparison, and findings should be confirmed on a larger number of cases and with more extended follow up.

HCV recurrence was more frequent in LDLT than in cadaveric liver transplantation in some reports, (*4*) although this is still under debate. The serum HCV-RNA level early after liver transplantation has been reported to be associated with severe HCV recurrence (*5*). Our data suggest that the levels in patients in the steroid-free group tend to remain suppressed. These results might represent the possible effects of complete steroid avoidance on HCV recurrence after LDLT.

REFERENCES

1. Reding R, Gras J, Sokal E, et al. Steroid-free liver transplantation in children. *Lancet* 2003; 362(9401): 2068.
2. Ringe B, Braun F, Schutz E, et al. A novel management strategy of steroid-free immunosuppression after liver transplantation: efficacy and safety of tacrolimus and mycophenolate mofetil. *Transplantation* 2001; 71(4): 508.
3. Eason JD, Loss GE, Blazek J, et al. Steroid-free liver transplantation using rabbit antithymocyte globulin induction: results of a prospective randomized trial. *Liver Transpl* 2001; 7(8): 693.
4. Garcia-Retortillo M, Forns X, Llovet JM, et al. Hepatitis C recurrence is more severe after living donor compared to cadaveric liver transplantation. *Hepatology* 2004; 40(3): 699.
5. Sreekumar R, Gonzalez-Koch A, Maor-Kendler Y, et al. Early identification of recipients with progressive histologic recurrence of hepatitis C after liver transplantation. *Hepatology* 2000; 32(5): 1125.

Portal Venous Reconstruction in a Living Liver Donor With an Anomalous Hepatic Arterial and Portal Venous Anatomy

Shigeru Marubashi, M.D., Keizo Dono, M.D., Ph.D., Masato Sakon, M.D., Ph.D., Kunihito Gotoh, M.D., Hidenori Takahashi, M.D., Kazuhiko Hashimoto, M.D., Masaru Kubota, M.D., Shinji Yamamoto, M.D., Atsushi Miyamoto, M.D., Ph.D., Hiroaki Nagano, M.D., Ph.D., Shoji Nakamori, M.D., Ph.D., Koji Umeshita, M.D., Ph.D., Morito Monden, M.D., Ph.D.

Hepatic arterial and portal venous anomalies in living liver donors are not uncommon. Modified surgical techniques may be required in such circumstances, although the safety of the living donor must always be given top priority. We describe here a successful portal venous reconstruction in a living donor with an anomalous hepatic arterial and portal venous anatomy in which the right anterior and posterior hepatic arteries encircled the main portal vein. Although such an anomaly of hepatic vessels was not frequently encountered, we should be able to alter the surgical strategy to deal with it. This case illustrates the importance of preoperative hepatic artery and portal venous evaluation in all living donors to identify the feasibility of modifying vessel anastomoses in living donors, as well as recipients, before living donor liver transplantation. (J GASTROINTEST SURG 2005;9:365–368)

KEY WORDS: Liver transplantation, living donor, portal vein, reconstruction

The safety of living liver donors has been emphasized, especially since the death of a living donor for the first time in Japan; several deaths have also occurred in Western countries.[1] Placing the lives of living donors in danger requires great courage. We report the case of a living right lobe liver donor with an unusual arterial anatomy in whom a portal venous reconstruction was inevitable to avoid arterial or portal venous complications in the recipient. This is the first report of a portal vein reconstruction performed in a living liver donor.

CASE REPORT

A 27-year-old man with acute fulminant hepatic failure resulting from a hepatitis B virus infection was transferred to our hospital for evaluation. The patient had been healthy until experiencing a general fatigue 2 weeks before hospital admission. His liver function progressively deteriorated, and he developed encephalopathy 1 week before admission to our hospital. He was intubated, and a sensor catheter for intracranial pressure monitoring was inserted because of a deep hepatic coma that occurred 3 days before admission to our hospital. His height was 170 cm and his weight was 97.1 kg at the time of admission. The health care team, which included hepatologists and transplant surgeons, discussed the case and concluded that an emergent liver transplantation was necessary to save the patient. A thorough explanation was presented to his family to identify a possible living liver donor. The patient had a younger brother who was 20 years old, but this family member decided not to donate part of his liver. As a result, the only volunteer donor candidate was the patient's mother. The mother was 54 years old and was of small stature. The three-dimensional image of a preoperative multi-detector-row computed tomography (MDCT) scan clearly revealed an anomalous hepatic arterial anatomy in which the right posterior hepatic artery passed behind the main portal vein while the right anterior hepatic artery passed in front of the main portal vein (Figs. 1, 2). The estimated volume of her right liver lobe [calculated using the following equation: 706.2 × body surface area (m^2) + 2.4^2)] was 580 mL (40.7% of the

From the Department of Surgery and Clinical Oncology, Osaka University, Graduate School of Medicine, Suita, Osaka, Japan.
Reprint requests: K. Dono, M.D., Department of Surgery and Clinical Oncology, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita City, Osaka 565-0871, Japan. e-mail: kdono@surg2.med.osaka-u.ac.jp

1091-255X/05/$—see front matter
doi:10.1016/j.gassur.2004.05.019