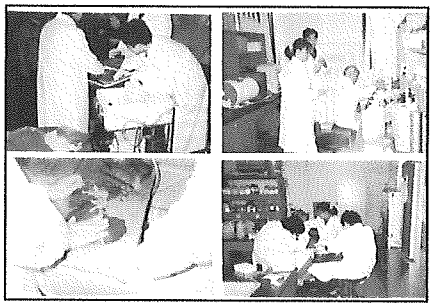
Preparation of Pig Primary Hepatocytes



Article 2

Donation means that the donor donates his/her whole body or part of it to clinical practice and research voluntarily. His/her close family members have right to make the decision of donation if the donor had not chance to present his/her willing.

Close family members means donor's parents, spouses, adult children.

Shanghai Donation Regulation

By Shanghai People's Representatives (City Congress)

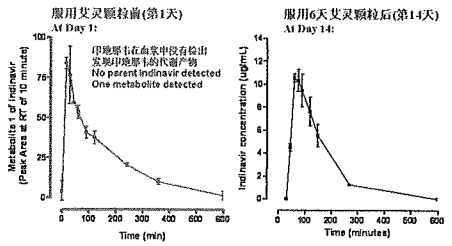
Effective date: March 1, 2001

Article 3

Donation must follow the principle of "voluntary" and "Free"

结果和讨论
Results & Discussion

4. 临床前研究-体内中药对抗HIV化学药的代谢性增效作用：论证体外结果
Preclinical confirmation: efficacy enhancement of antiHIV drugs by herbs



Article 5

Donor's willing and his/her body must be respected and protected by this regulation fully.

Shanghai Donation Regulation

By Shanghai People's Representatives (City Congress)

Effective date: March 1, 2001

Article 9

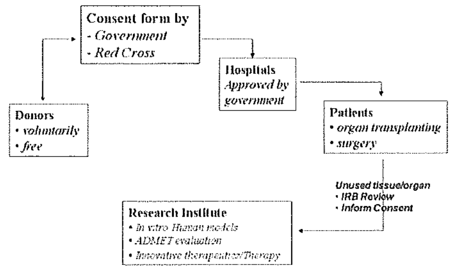
Hospital receiving donation must be qualified by the following requirements:

- ***Capability to conduct research and clinical practice***
- ***Specific department with trained staff***
- ***Necessary Equipment and suitable facility***

Article 10

Hospital receiving donation must get approval by Government and Red Cross

Donation Flow Chart



Article 11

Anyone violating this regulation is subject to criminal charge

【体外药物代谢和相互作用研究】知情同意书
Informed Consent Form for In Vitro Drug Interaction Study

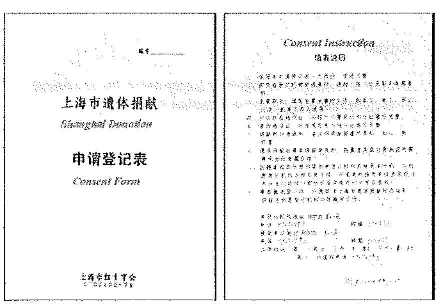
本知情同意书遵循"人体医学研究的伦理准则"(世界医学大会赫尔辛基宣言)

研究项目简介： 根据国家科技部创新基金项目（体外药物代谢和相互作用评价服务）（项目编号：[illegible]，起止时间：2003 - 2005），瑞德肝脏疾病研究(上海)正开展一个用体外生物技术研究药物进入体内后的吸收代谢分布排泄，该项目的成功将对同时服用多种药物的病人的临床药效和安全提供极其有益的参考数据。

Project Background: Research Institute for Liver Diseases (Shanghai) Co. Ltd (RILD) is conducting evaluation of ADMET of new drug candidates by using in vitro biotechnologies. The success of these evaluations will provide valuable infomation on efficacy and drug-drug interaction.

Sample Informed Consent

Consent Inform



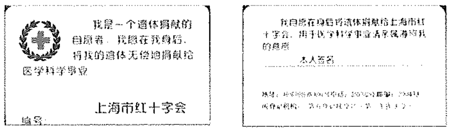
【体外药物代谢和相互作用研究】知情同意书
Informed Consent Form for In Vitro Drug Interaction Study

研究过程简介： 研究将使用您在手术中切除的废弃的部分组织(肾组织)，研究药物吸收/代谢/排泄的机制，受瑞德肝脏疾病研究(上海)委托，我们在您的同意下，将在临床检查之余，将废弃的组织，置于冰冻的培养液中，转送瑞德肝脏疾病研究(上海)，进行体外药物代谢和相互作用研究。

Procedures: We like to get your pemission to collect discard kidney tissues from your surgical operation. The discarded kidney tissue will be used in evaluation of drug metabolism and interactions at RILD.

Sample Informed Consent

Consent Inform



I am a volunteer for donating my body to clinical and scientific coursesunconditionally.

Shanghai Red Cross

No. ....

I voluntarily to donate my body to Shanghai Red Cross for clinical and scientific courses. My family members please respect my willing.

Signature.................

【体外药物代谢和相互作用研究】知情同意书
Informed Consent Form for In Vitro Drug Interaction Study

您的权利： 您同意使用您手术中切除的废弃组织完全自愿的，您可以拒绝使用您的手术切除废弃组织而无需任何理由，绝不会影响您和医务人员的关系及今后的诊治；您的所有个人资料均属保密，仅供本研究使用。

您在了解了本项目的目的、方法、可能获得的治疗利益和可能发生的不良反应后，同意该课题使用我在手术中切除的废弃组织。

Your right: It will be totally voluntary to you to make decision if donate the discard kidney tissue from your surgical operation or not. In any case that you do not make decision for donation, your treatment by clinicians and nurses won't be affected. Your personal information will be confidential to any third party.

You understand the objective, procedure, your benefits, and your risk clearly as above and agree to donate the discard kidney tissue from your surgical operation.

Sample Informed Consent

[体外药物代谢和相互作用研究]知情同意书
Informed Consent Form for In Vitro Drug Interaction Study

您的权利：您同意使用您手术中切除的废弃组织完全自愿的，您可以拒绝使用您的手术切除废弃组织而无需任何理由，绝不会影响您和医务人员的关系及今后的治疗；您的所有个人资料均属保密，仅供本研究使用。

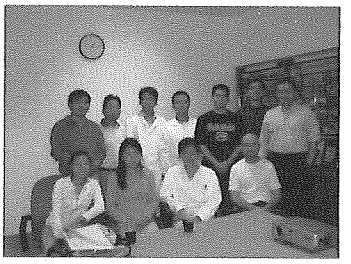
您在了解了本项目的目的、方法、可能获得的治疗利益和可能发生的不良反应后，同意该课题使用我在手术中切除的废弃组织。

Your right: It will be totally voluntary to you to make decision if donate the discard kidney tissue from your surgical operation or not. In any case that you do not make decision for donation, your treatment by clinicians and nurses won't be affected. Your personal information will be confidential to any third party.

You understand the objective, procedure, your benefits, and your risk clearly as above and agree to donate the discard kidney tissue from your surgical operation.

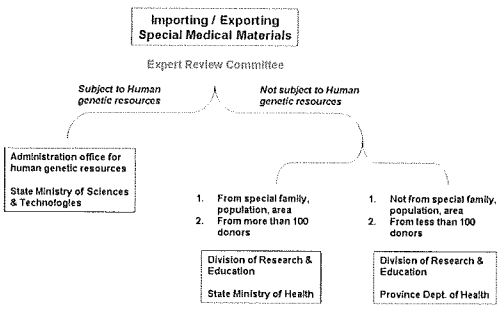
Sample Informed Consent

Acknowledgements

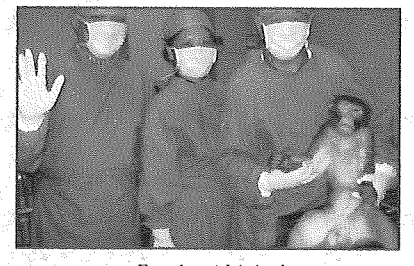


Graduate Students & Study Directors

医用特殊物品出入境管理
Administration for Importing and Exporting Special Medical Materials

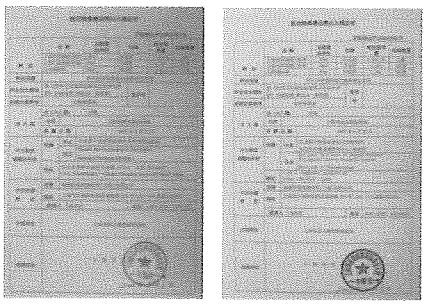


Remembrance



Experimental Animals

医用特殊物品出入境管理
Administration for Importing and Exporting Special Medical Materials



Remembrances

Shanghai Donation Memoriam

Acknowledgements

- 国家科技部科技型中小企业创新基金项目《体外药物代谢和相互作用评价服务》No. 16203C36213100901
  State Grant (16202C36213100901: *In vitro drug metabolism and interaction evaluation services*
- 国家"十五"科技攻关课题:中药对"鸡尾酒"疗法代谢性相互作用的增效机制研究.No. 2004BA719A09-04
  State Grant (2004BA719A09-04): *Efficacy National Grant: enhancement of anti-HIV therapeutics by metabolic interactions with herbs*
- 挪威科技大学项目基金
  Norwegian University of Science & Technology RD-2005-EU-001: *Drug-drug interaction by marketed herb products*

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Mizuguchi T, Mitaka T, Katsuramaki T, Hirata K	Hepatocyte transplantation for total liver repopulation (review)	Journal of Hepatobiliary Pancreatic Surgery			2005
Kikkawa Y, Mochizuki Y, Miner JH, Mitaka T	Transient expression of laminin α1 chain in regenerating murine liver: Restricted localization of laminin chanins and nidogen-1	Experimental Cell Research	305(1)	99-109	2005
Sugimoto S, Harada K, Shiotani T, Ikeda S, Katsura N, Ikai I, Mizuguchi T, Hirata K, Yamaoka Y, Mitaka T	Hepatic organoid formation in collagen sponge of cells isolated from human liver	Tissue Engineering	11(3-4)	626-633	2005
Mizuguchi T, Oshima H, Imaizumi H, Kohara H, Kawamoto M, Nobuoka T, Kawasaki H, Harada K, Masuda Y, Kikkawa Y, Mitaka T, Asai Y, Hirata K.	Hyperbaric oxygen stimulates cell proliferation and normalized MRP2 (anion transporter) protein localization in primary rat hepatocytes	Wound Repair and Regeneration	13(6)	551-557	2005
Sudo R, Kohara H, Mitaka T, Ikeda M, Tanishita K.	Coordination of Bile Canalicular Contraction in Hepatic Organoid Reconstructed by Rat Small Hepatocytes and Nonparenchymal Cells	Annals of Biomedical Engineering	33(5)	696-708	2005

Sudo R, Mitaka T, Ikeda M, Tanishita K.	Reconstruction of 3D stacked-up structures by rat small hepatocytes on microporous membranes	FASEB Journal	19(12)	1695-1697	2005
Miyamoto S, Hirata K, Sugimoto S, Harada K, Takeda H, Mitaka T	Expression of cytochrome P450 enzymes in hepatic organoid reconstructed by rat small hepatocytes	Journal of Gastroenterology and Hepatology	20(6)	865-872	2005
Shibata C, Muzuguchi T, Kikkawa Y, Nobuoka T, Oshima H, Kawasaki H, Kawamoto M, Katsuramaki T, Mitaka T, Hirata K.	Liver repopulation and long-term function of rat small hepatocyte transplantation as an alternative cell source for hepatocyte transplantation	Liver Transplantation	12(1)	78-87	2006
Kon J, Ooe H, Oshima H, Kikkawa Y, Mitaka T.	Expression of CD44 in rat hepatic progenitor cells	Journal of Hepatology		In press	2006
Nagaya M, Kubota S, Akashi K, Mitaka T.	Thermoreversible gelation polymer induces the emergence of hepatic stem cells in a partially injured rat liver	Hepatology		In press	2006
吉川大和、三高俊広	肝再生とラミニンα1鎖	Surgery Frontier		印刷中	2006

TISSUE ENGINEERING
Volume 11, Number 3/4, 2005

Hepatic Organoid Formation in Collagen Sponge of Cells Isolated from Human Liver Tissues

SHINICHI SUGIMOTO, M.D., Ph.D.,[1,2] KEISUKE HARADA, M.D., Ph.D.,[2,3]
TOMOHIRO SHIOTANI, M.D.,[1] SHINICHIRO IKEDA, M.D., Ph.D.,[2,3]
NAGATO KATSURA, M.D., Ph.D.,[1] IWAO IKAI, M.D., Ph.D.,[1]
TORU MIZUGUCHI, M.D., Ph.D.,[3] KOICHI HIRATA, M.D., Ph.D.,[3]
YOSHIO YAMAOKA, M.D., Ph.D.,[1] and TOSHIHIRO MITAKA, M.D., Ph.D.[2]

ABSTRACT

We examined whether small hepatocytes (SHs), which are hepatic progenitor cells, could be isolated from a normal human liver and whether human hepatic cells could form hepatic organoids in a collagen sponge. Normal liver tissues were obtained from resected specimens from nine patients who underwent hepatic resection. Isolated hepatic cells were plated on dishes and a collagen sponge. More than 1 month later, SH-like cells appeared and proliferated on the dishes, whereas cell aggregates were formed in the sponge and showed characteristic tissue architecture: columnar and/or cuboidal epithelial cells lined the surface of the sponge. Clusters of epithelial cells with a large cytoplasm and ductular structures were observed under the lining cells. The lining and ductular cells were positive for cytokeratins 7 and 19, which indicated they were biliary epithelial cells (BECs), and the epithelial cells forming clusters were positive for the anti-human hepatocyte antibody, identifying them as hepatocytes. Some lining cells were positive for both the hepatic marker and the BEC markers. The cells in the collagen sponge actively proliferated and the hepatocytes excreted albumin into the medium. Thus, hepatic organoids could be reconstructed in a collagen sponge by normal human liver cells.

INTRODUCTION

BIOARTIFICIAL LIVER (BAL) DEVICES supporting a large mass of metabolically active hepatocytes are thought to be necessary for the successful treatment of patients with severe acute liver failure. Many researchers have attempted to improve the differentiated hepatic functions of such devices.[1–5] In those experiments, primary hepatocytes, immortalized hepatic cells, or hepatoma-derived cells were used as bioreactors of BAL and, to maintain the hepatic functions, various materials and devices such as microcarrier beads, multiporous microcarriers, polyurethane foam, and porous resin have been produced and used as carriers of cells. Furthermore, devices for packing carriers have been developed such as hollow fibers, a multicompartment capillary fiber, a synthetic biodegradable polymer scaffold, and a three-dimensional perfused microarray bioreactor. Although the materials and devices have been improved, hepatic functions of the cells are not good enough to reach the level of clinical application. Therefore, the development of methods for maintaining highly differentiated functions of cells has

[1]Department of Gastroenterological Surgery, Kyoto University Graduate School of Medicine, Kyoto, Japan.
[2]Department of Pathophysiology, Cancer Research Institute, Sapporo Medical University School of Medicine, Sapporo, Japan.
[3]First Department of Surgery, Sapporo Medical University School of Medicine, Sapporo, Japan.

TABLE 1. PATIENT PROFILES

Patient no.	*Primary disease*	*Age (years)*	*Sex*	*Weight (g)*	*Yield ($\times 10^6$ cells/g)*
1	Liver metastasis (colon)	54	M	9.0	2.46
2	Hepatocellular carcinoma	73	M	5.8	3.03
3	Gallbladder carcinoma	23	F	12.0	3.20
4	Hepatocellular carcinoma	68	M	8.5	2.50
5	Liver metastasis (colon)	59	M	5.4	2.07
6	Liver metastasis (colon)	51	M	15.4	1.86
7	Hepatocellular carcinoma	57	M	11.2	1.89
8	Hepatocellular carcinoma	72	M	11.4	1.30
9	Liver metastasis (lung)	64	M	5.5	2.65

been required. Three-dimensional culture is well known to be a method by which hepatocytes can keep their differentiated functions well via spheroid formation[6,7] and coculture with fibroblasts.[8,9] The formation of hepatic organoids, which are reconstructed by the cells isolated from a normal adult rat liver, has been reported.[10,11] The tissues reported consisted of epithelial cells, which are both mature hepatocytes (MHs) and biliary epithelial cells (BECs), and connective tissues. Although Lázaro *et al.*[12] reported that a hepatic organoid could be formed by human fetal hepatocytes after long-term culture, there is no report that cells from a normal human adult liver reconstruct hepatic tissues *in vitro*.

We have reported that rat small hepatocytes (SHs), which are hepatic progenitor cells, appear and proliferate in a medium supplemented with nicotinamide and epidermal growth factor (EGF).[13,14] SHs have high growth ability compared with MHs and can reconstruct hepatic organoids interacting with hepatic nonparenchymal cells (NPCs) such as stellate (Ito) cells and liver epithelial cells (LECs).[15] The morphological changes of SHs are correlated with hepatic maturation and can be induced by extracellular matrix (ECM).[15,16] We reported that rat SHs could proliferate in a collagen sponge after isolated SH colonies were replated.[17] The cells could become large ones that were morphologically and functionally differentiated hepatocytes. In addition, some ductlike structures consisting of cytokeratin 19 (CK19)-positive cells were formed. In the present experiment we examined whether proliferating SHs could be observed in the cells isolated from a normal human liver and whether human hepatic cells could form hepatic organoids in a collagen sponge.

MATERIALS AND METHODS

Liver specimens from patients

Normal liver tissues were obtained from resected specimens from nine patients who underwent hepatic resections at Kyoto University Hospital (Kyoto, Japan) and Sapporo Medical University (Sapporo, Japan), under informed consent and with the approval of the Kyoto University and Sapporo Medical University Ethics Committees, respectively. As shown in Table 1, four patients had liver metastasis of colon or lung cancer, four had hepatocellular carcinoma, and one had gallbladder carcinoma. All tissues were obtained from the patients without any viral infections. The mean age of the patients was 57.9 ± 15.2 years old, and the mean wet weight of the liver tissues was 9.4 ± 3.5 g.

Isolation of human hepatocytes

Hepatocytes were isolated from liver tissues by the injection method, using a 10-mL disposal injector with a 27-gauge needle. The isolation procedure was previously described.[18] Briefly, the liver tissue was examined for small vessels and Hanks' balanced salt solution (HBSS; GIBCO-BRL Invitrogen, Grand Island, NY) supplemented with EGTA (Dojindo Chemical Laboratories, Kumamoto, Japan) was poured into the vessels. After the washout of blood, HBSS containing 0.05% collagenase (Wako Pure Chemical, Osaka, Japan) and 0.5% dispase (Godo Shyusei, Tokyo, Japan) was poured into the vessels. This procedure was repeated many times and the swollen tissue was soaked in the enzyme solution during the procedure. The isolated cells were collected into centrifugal tubes. The cell suspension was centrifuged 50 × *g* for 1 min and the pellet (hepatocyte fraction) was suspended in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO) supplemented with insulin (0.5 mg/mL), 10^{-7} M dexamethasone, and antibiotics. The cell density was adjusted to 1×10^5 viable cells/mL. Nondigested liver tissues in the supernatant, which was collected after the first 50 × *g* centrifugation of the cell suspension, were soaked and stirred for 30 min at 37°C. Digested cell suspension was subjected to 50 × *g* centrifugation for 5 min. After this procedure was repeated several times, cells were resuspended in the

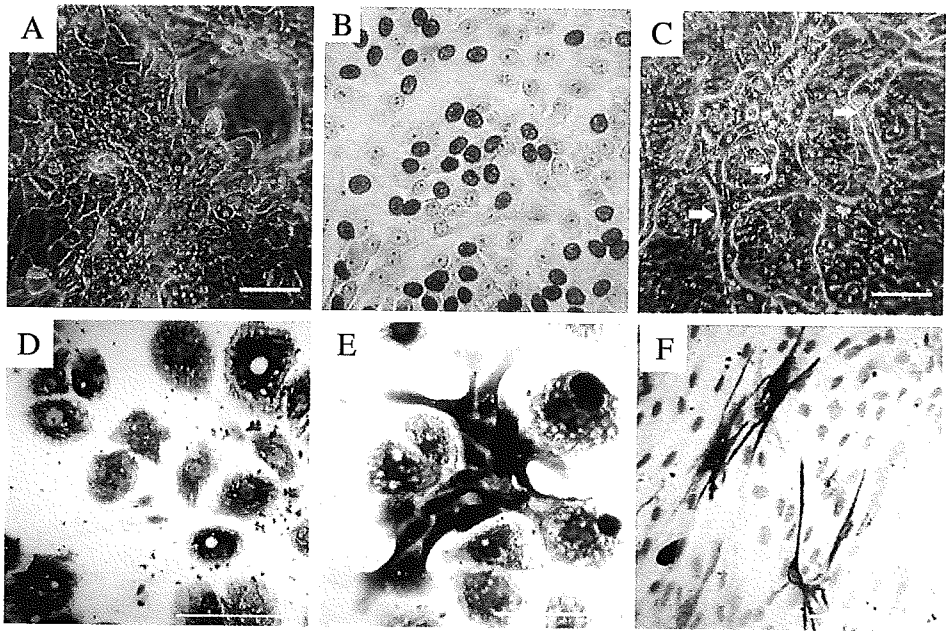


FIG. 1. Photographs of cells isolated from human liver tissues. Phase-contrast photographs of cells on dishes were taken on day 63 (**A**) and day 60 (**C**). (**B**) Cells were fixed with cold ethanol on day 60. Before fixation, 10 mM BrdU was added to the medium for 24 h and immunocytochemistry for BrdU was performed. Counterstaining was done with hematoxylin. Darkened nuclei were positive for BrdU. (**D–F**) A mixture of hepatocytes and hepatic NPCs was cultured on collagen-coated dishes and fixed on day 1 (**D** and **E**) and day 5 (**F**). Immunocytochemistry for anti-Hep (**D**), anti-CK19 (**E**), and anti-desmin antibodies (**F**) was carried out. Details are described in Materials and Methods. Scale bars: 50 μm.

medium. The cell density was adjusted to 1×10^5 viable cells/mL (NPC fraction).

An equal volume of each fraction (hepatocyte fraction:NPC fraction, 1:1) was suspended in a small volume of the medium and 1 mL of the cell suspension was slowly poured onto collagen sponge to avoid overflowing. Helistat (1×1.5 cm; COLLA-TEC, Plainsboro, NJ) was used as collagen sponge and directly placed on a 35-mm dish without any pretreatment. The cells were cultured in DMEM supplemented with 20 mM HEPES, 25 mM $NaHCO_3$, L-proline (30 mg/L), 10% human serum (HS), 5% fetal bovine serum (FBS; HyClone, Logan, UT), 10 mM nicotinamide (Katyama Chemical, Osaka, Japan), 1 mM ascorbic acid 2-phosphate (Asc2P; Wako Pure Chemical), hepatocyte growth factor (HGF, 20 ng/mL; Genzyme/Techne, Minneapolis, MN), EGF (10

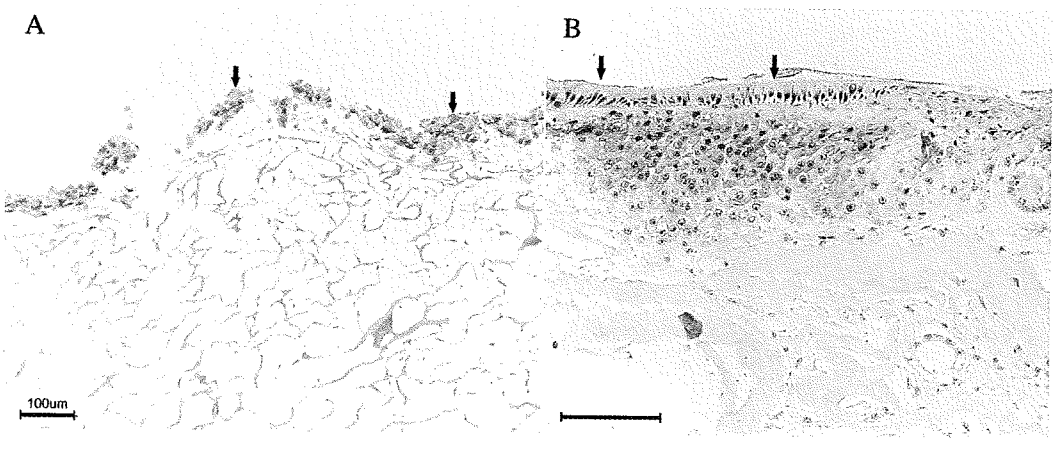


FIG. 2. Perpendicular sections of a collagen sponge on day 7 (**A**) and day 40 (**B**). Hematoxylin–eosin staining was carried out. Arrows in (**A**) show mature hepatocytes. Arrows in (**B**) show lining columnar epithelia. Scale bars: 100 μm.

ng/mL; Collaborative Research, Lexington, MA), insulin (0.5 mg/mL), 10^{-7} M dexamethasone, and antibiotics. HS was obtained from several healthy volunteers. The remnant was plated on dishes (Corning Glass Works, Corning, NY) coated with rat tail collagen. Three hours after plating, 2 mL of the medium was added to the dish for the purpose of preventing the sponge surface from becoming dry. Several days later, we confirmed that most cells attached to the collagen sponge and that a few cells were observed around the sponge. The medium was changed every other day.

Immunocytochemistry

Cells grown on the collagen sponge were fixed with cold ethanol or 4% paraformaldehyde (PFA; Merck, Darmastadt, Germany) in PBS. Tissues were paraffin embedded and 5-μm-thick sections were prepared for immunostaining. To retrieve antigen, tissues were microwaved in citrate buffer. Mouse monoclonal anti-proliferating cell nuclear antigen (PCNA), anti-bromodeoxyuridine (BrdU), anti-human hepatocyte (Hep), anti-human desmin, anti-human CD68, anti-human macrophage, anti-von Willebrand factor (vWF), anti-CK19, and anti-CK7/17 antibodies (Dako, Copenhagen, Denmark) were used as the primary antibodies, followed by the avidin–biotin peroxidase complex method (Vectastain ABC Elite kit; Vector Laboratories, Burlingame, CA). 3′-Diaminobenzidine (Tokyo Kasei Industries, Tokyo, Japan) was used as a substrate. The cells were then counterstained with hematoxylin.

Albumin synthesis of cultured human hepatocytes

Albumin secretion was measured by enzyme-linked immunosorbent assay (ELISA), using a commercial available kit (Albuwell II; ExoCell, Philadelphia, PA). The cultured cells were washed three times with medium without serum and cultured for 2 h, after which the tis-

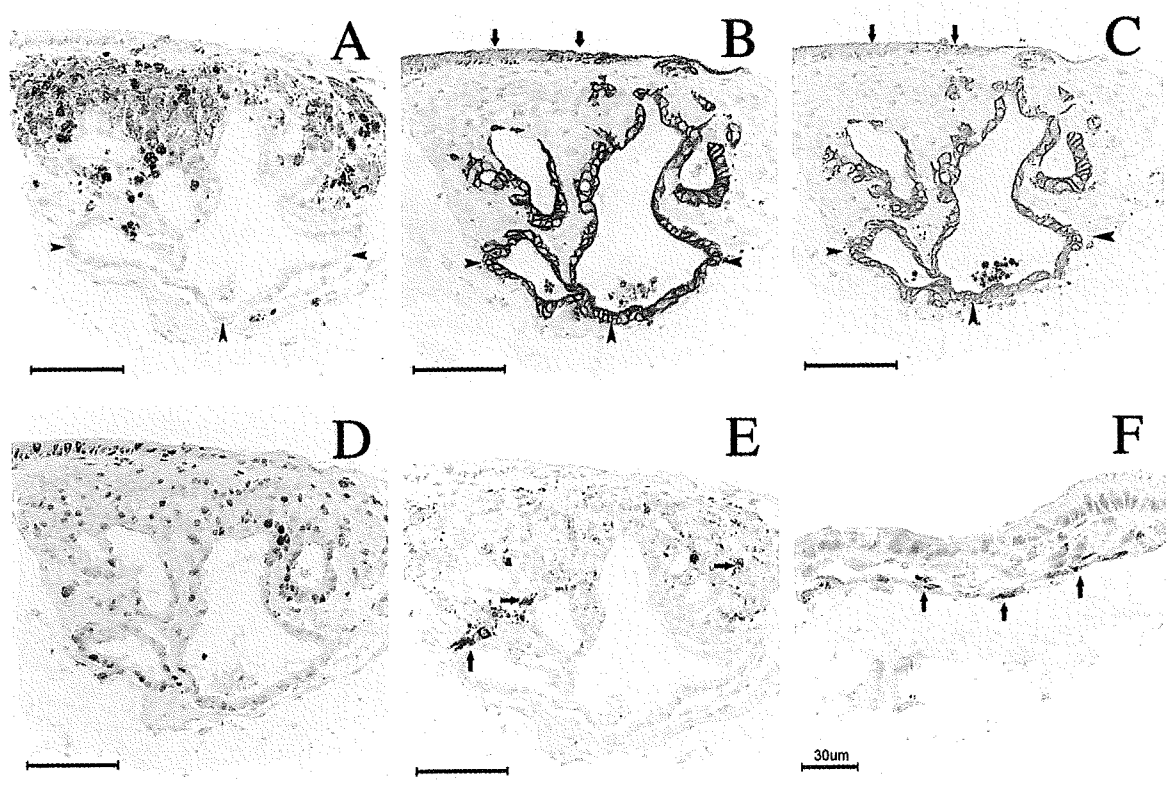


FIG. 3. Perpendicular sections of day 34 cell aggregates grown in collagen sponge. Immunocytochemistry for anti-Hep (**A**), CK7 (**B**), CK19 (**C**), PCNA (**D**), CD68 (**E**), and desmin (**F**) was carried out. Light brown staining is positive. All sections were prepared from the same cell aggregate, (**A–E**), adjacent sections.) (**A**) Hep was used as a marker for hepatocytes. Arrowheads show that ductular epithelia are negative. (**B** and **C**) CK7 and CK19 were used as markers for BECs. Arrows show the lining columnar epithelia and arrowheads show the ductular epithelia. All cells of both structures are positive. (**D**) PCNA was used as a marker for cell proliferation. (**E**) CD68 was used as a marker for Kupffer cells. Arrows show positive cells. (**F**) Desmin was used as a marker for stellate/myofibroblasts. Arrows show positive cells. Scale bars: (**A–E**) 100 μm; (**F**) 30 μm.

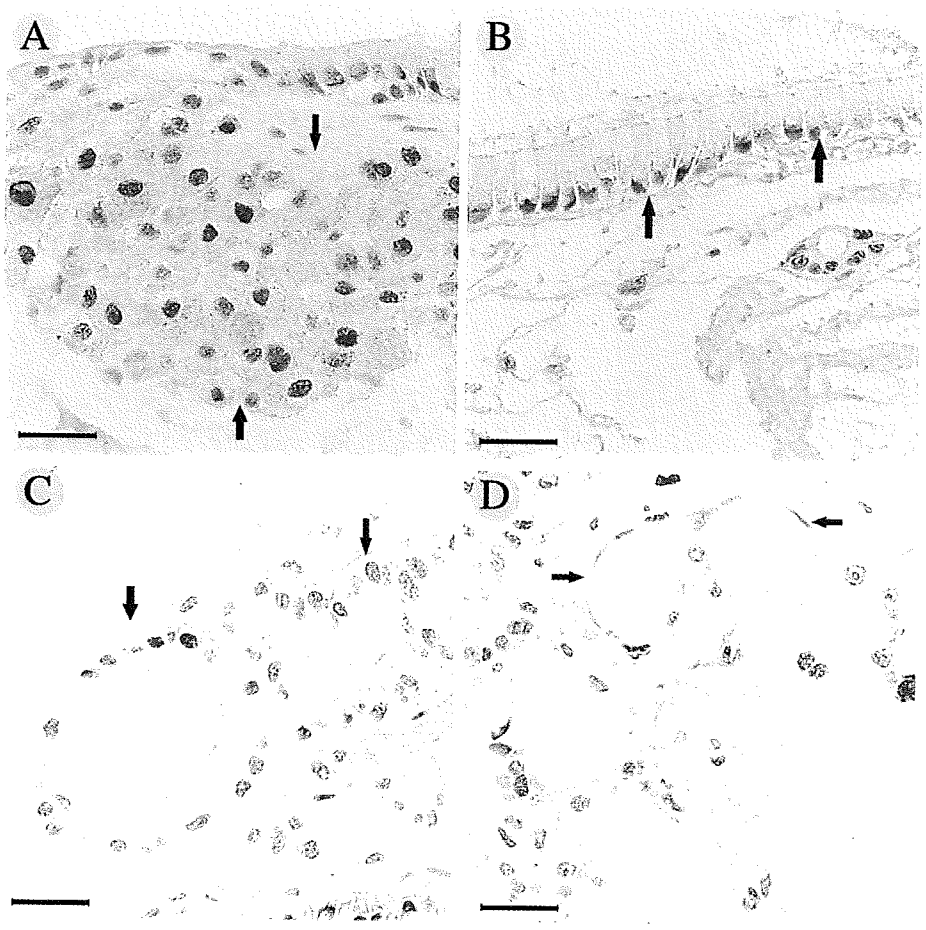


FIG. 4. Immunocytochemistry for PCNA of cells in collagen sponge. The cells were fixed with 4% PFA on day 34 (**A** and **B**) and day 40 (**C** and **D**). The nuclei of many hepatocytes (**A**; surrounded by arrows), columnar epithelia (**B**; arrows), cells forming ductlike structures (**C**; arrows), and cells forming vessels/capillaries (**D**; arrows) are positive for PCNA. Scale bars: 30 μm.

sue culture supernatant was collected and centrifuged at $1 \times 10^4 \times g$ for 10 min. The supernatant was kept at −30°C until use. Three dishes were used per experiment and three independent experiments were carried out.

RESULTS AND DISCUSSION

Normal hepatic cells were isolated from liver specimens of nine patients (Table 1) and cultured in DMEM supplemented with 10% HS, 5% FBS, 10 mM nicotinamide, 1 mM Asc2P, HGF (10 ng/mL), EGF (10 ng/mL), and hormones. The average yield of hepatocytes per 1 g wet weight of the tissue was 2.33×10^6 cells and the viability of the cells was more than 85%. When cells of the hepatocyte and NPC fractions were plated on dishes and cultured in medium supplemented with all additives, SH-like cells, the morphology of which was quite similar to that of rat SHs, appeared in many dishes al-

TABLE 2. PERCENTAGE OF PCNA-POSITIVE CELLS BY CELLS FORMING A SPECIFIC STRUCTURE

Cells	*PCNA-positive cells (%)*[a]
Hepatocytes[b]	67.3 ± 11.2
Biliary epithelial cells (duct formation)[b]	80.3 ± 15.3
Biliary epithelial cells (sheet formation)[b]	83.3 ± 9.3
Vessel or capillary-like structure[c]	71.3 ± 17.7

[a]Numbers indicate averages ± standard deviation.
[b]More than 100 cells were examined.
[c]About 50 cells were counted, as the number of structures was low.

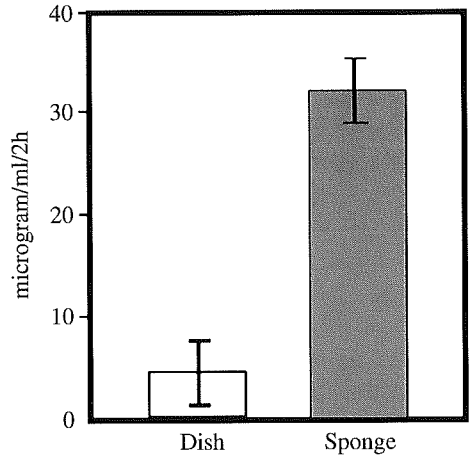


FIG. 5. Albumin secretion into culture medium of human cells cultured in collagen-coated dishes (open column) and collagen sponge (shaded column). On day 35 the medium of the cells was exchanged for serum-free medium and, 2 h later, the medium was collected. The amount of albumin was measured by ELISA. Four independent experiments were performed. Each column shows the average of three dishes and the error bars show standard deviations.

though the number of cells fluctuated between experiments (Fig. 1A). Even 2 months after plating, human SH-like cells could slowly proliferate as shown by the fact that many cells took bromodeoxyuridine (BrdU) into their nuclei (Fig. 1B). Without HS, nicotinamide, or HGF, SH-like cells did not appear. The mixture of 5% FBS and 10% HS was also reported to be necessary for the growth of human SH-like cells.[19] Although the authors of that report pointed out that SH-like cells required the conditioned medium of 3T3 cells for their proliferation, we found that SH-like cells expanded without the conditioned medium. When the cells were cultured for more than 2 months, piled-up cells could be observed (Fig. 1C). However, the frequency of appearance of the colonies with piled-up cells was low.

We reported that rat SHs could rapidly proliferate and differentiate into MHs within a collagen sponge.[17] As SH-like cells were known to exist in the human normal hepatic cells, we examined whether the cells could form hepatic organoid in the collagen sponge. In the present experiment we used Helistat, which is an absorbable collagen hemostatic sponge processed from bovine tendon that is usually used for surgical procedures to control bleeding. The identification of the attached cells was carried out on the dish by using antibodies specific to each cell type. On day 1 relatively large cells were positive for the anti-Hep antibody (Fig. 1D) and some clusters of small epithelial cells were positive for the anti-CK19 antibody. These were BECs (Fig. 1E). Cells with a little cytoplasm and long processes, which might have been stellate cells, were positive for anti-desmin (Fig. 1F). In the present experiment, hepatocytes (Hep^+), BECs ($CK19^+$), and Kupffer cells ($CD68^+$) accounted for about 70, 3.7, and 2% of the cells, respectively. Stellate and endothelial (vWF^+) cells were rarely observed. When the cells were plated on the collagen sponge, many MHs settled on the surface of the sponge on day 7 (Fig. 2A), but they gradually disappeared from the sponge (data not shown). Although it was difficult to identify each cell type on the sponge by phase-contrast microscopy, many cell aggregates were clearly found on the sponge about 1 month after plating. As shown in Fig. 2B, perpendicular sections of the portion of the aggregates showed that layers of cells were formed in the upper part of the sponge. On the surface facing the medium there was a continual monolayer of columnar/cuboidal epithelial cells, the nuclei of which were along the basal membrane (Fig. 2B). These cells morphologically resembled the epithelial cells of bile ducts and gallbladder and were positive for CK7 and CK19 (Fig. 3B and C), which may be BECs. Below the

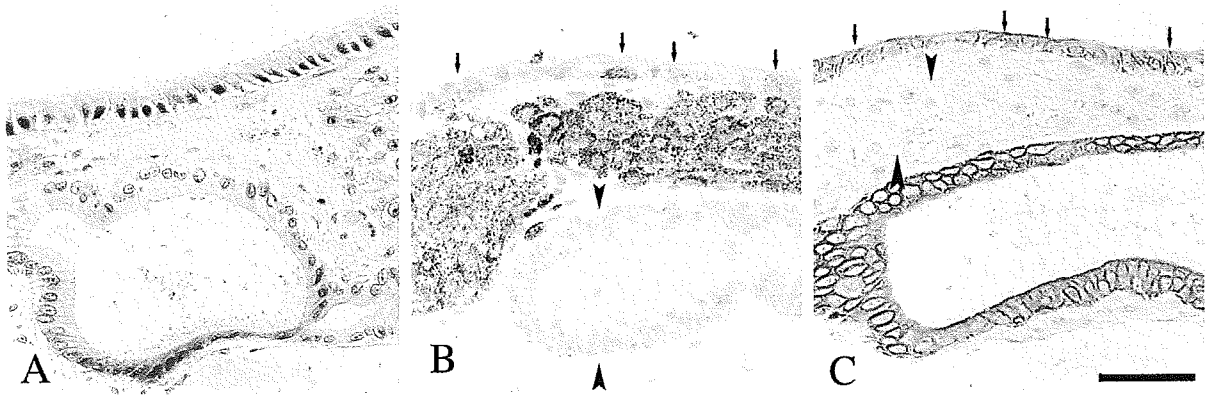


FIG. 6. Coexpression of hepatic and biliary markers in the cells. Shown are day 34 perpendicular sections of cell aggregates grown in collagen sponge. Adjacent sections were prepared and used for staining. Hematoxylin–eosin staining (**A**) and immunocytochemistry for Hep (**B**) and CK19 (**C**) were performed. Some lining BECs [arrows in (**B**) and (**C**) coexpress hepatic (**B**) and biliary (**C**) marker proteins, whereas BECs forming a ductlike structure do not express the hepatic marker (arrowheads, **B**) and hepatocytes are not stained with anti-CK19 antibody (**C**). Scale bar: 50 μm.

lining cells, there was a cluster of relatively large cells that were positive for anti-Hep antibody (Fig. 3A). Although most of the cells were mononucleate, some were binucleate cells. A thin layer of connective tissue usually separated the cluster of hepatocytes from lining BECs. Ductlike structures of various sizes consisting of cuboidal and/or flattened cells were formed in the sponge and they consisted of both CK7- and CK19-positive cells. Some vessel- or capillary-like structures that consisted of flattened thin cells were formed within the sponge (Fig. 4D). Although the morphology of the structure was similar to that of vessels or capillaries, CD31, CD34, vVW, CK7, and CK19 were not clearly stained (data not shown). Some Kupffer cells were scattered in and near hepatocyte clusters (Fig. 3E) and a few stellate cells (desmin$^+$) were observed under the clusters of hepatocytes (Fig. 3F).

The growth of cells was examined by PCNA staining (Figs. 3D and 4). Even more than 40 days after plating, nuclei of the epithelial cells were positive for PCNA. We measured the number of PCNA-positive cells by the type of cells; hepatocytes forming clusters (Fig. 4A), lining BECs (Fig. 4B), BECs forming ductlike structures (Fig. 4C), and cells forming capillary-like structures (Fig. 4D) accounted about 67, 80, 83, and 71% of the cells, respectively (Table 2). When MHs were plated on the sponge without NPC fractions, it was difficult to find the proliferating MHs, although many MHs survived in the sponge even after 1 month (data not shown). We also examined albumin secretion of the cells. Albumin secretion of the cells on the sponge decreased with time in culture and then, 2 to 3 weeks later, reversed to increase (data not shown). As shown in Fig. 5, on day 35, the amounts of albumin produced by the cells on the collagen-coated dish and the collagen sponge were 4.7 $\pm$ 3.2 and 32.8 $\pm$ 3.2 μg/mL per 2 h, respectively. This result may be coincident with the fact that MHs gradually disappeared from the sponge in the early culture period and that the clusters of hepatocytes appeared in the later culture period. We terminated most experiments within 2 months because the sponge gradually melted and could not support the three-dimensional structure.

It is of interest that coexpression of CK19 and Hep was found in some lining columnar/cuboidal cells on the surface of the sponge (Fig. 6). However, cells forming ductlike structures were negative for the anti-Hep antibody. Furthermore, hepatocytes forming clusters showed positivity for neither CK7 nor CK19. In the present experiment, although about 4% of the plating cells were BECs, ductlike structures were not observed on the collagen-coated dish. On the other hand, when rat SHs were plated on the sponge, some large CK19-positive cells were positive for albumin.[17] Michalopoulos *et al.*[10,11] reported that transdifferentiation of MHs into BECs might occur when primary rat hepatocytes were cultured in a pleated roller bottle with rotation. Under their culture conditions, CK19-positive cells with hepatic marker proteins appeared in the hepatic organoid. Thus, some CK19-positive cells that appeared in the sponge might have been derived from hepatocytes. In addition, although we do not have any direct proof, there is a possibility that both proliferating hepatocytes and BECs came from SHs, which were included in the isolated hepatic cells. Auth *et al.*[20] reported that isolated human BECs could develop three-dimensional structures in between collagen gels. When autologous human hepatocytes were cocultured with a small number of BECs in the collagen sandwich, the ductular formation of BECs was enhanced. In our culture system a small number of BECs was initially included in the isolated cells. Therefore, there is a possibility that the ductular formation of the BECs might have been enhanced by the coculture of MHs.

For the purpose of the reconstruction of functional hepatic organoids, the structure of the reformed organoids should be similar to liver lobules *in vivo*. In the present experiment, although cells derived from normal adult livers could proliferate and form hepatic organoids in the collagen sponge, the tissues were located mainly on the upper parts of the sponge and the sponge was never filled with cells. Therefore, to use collagen sponges as a scaffold, the size of the spaces between fibers and the thickness of the collagen should be considered and newly designed materials will be necessary to create a BAL. On the other hand, although vessel-like structures and bile ductlike structures were formed, tubelike structures were not found and the connection between bile canaliculi of hepatocytes and bile ductlike structures was not observed. For the enlargement of the organoids, the formation of vessels may be necessary to supply oxygen and nutrients inside the organoids. A method for growth factor-induced vascularization has been commonly used in the field of tissue engineering.[21,22] A scaffold and/or carrier combined with angiogenic factors such as vascular endothelial growth factor and/or fibroblast growth factor may improve *in vitro* organoid formation.

ACKNOWLEDGMENTS

We thank Drs. Shigeki Miyamoto, Hiroyuki Kawasaki, and Aki Nishikage, and Ms. M. Kuwano (Sapporo Medical University School of Medicine), for technical assistance. We also thank Mr. K. Barrymore for help with the manuscript. This work was supported by the Ministry of Education, Science, Sports, and Culture, Japan: 12670211 and 14370393 (T.M.), 14370353 (I.I.), and 12470265 and 13557107 (K.H.); and by a Health Sciences Research grant: Research on the Human Genome, Tissue Engineering Food Biotechnology, and the Science and Technology Incubation Program in Advanced Region, the Japan Science and Technology Agency.

REFERENCES

1. Takahashi, T., Malchesky, P.S., and Nose, Y. Artificial liver: State of the art. Dig. Dis. Sci. **36,** 1327, 1991.
2. Nyberg, S.L., Peshwa, M.V., Payne, W.D., Hu, W.-S., and Cerra, F.B. Evolution of the bioartificial liver: The need for randomized clinical trials. Am. J. Surg. **166,** 512, 1993.
3. Strain, A.J., and Neuberger, J.M. A bioartificial liver: State of the art. Science **195,** 1005, 2002.
4. Gerlach, J.C. Long-term liver cell cultures in bioreactors and possible application for liver support. Cell Biol. Toxicol. **13,** 349, 1997.
5. Kobayashi, N., Okitsu, T., Naji, S., and Tanaka, N. Hybrid bioartificial liver: Establishing a reversibly immortalized human hepatocyte line and developing a bioartificial liver for practical use. J. Artif. Organs **6,** 236, 2003.
6. Landry, J., Bernier, D., Ouellet, C., Goyette, R., and Marceau, N. Spheroidal aggregate culture of rat liver cells: Histotypic reorganization, biomatrix deposition, and maintenance of functional activities. J. Cell Biol. **101,** 914, 1985.
7. Koide, N., Shinji, T., Tanabe, T., Asano, K., Kawaguchi, M., Sakaguchi, K., Koide, Y., Mori, M., and Tsuji, T. Continued high albumin production by muticellular spheroids of adult rat hepatocytes formed in the presence of liver-derived proteoglycans. Biochem. Biophys. Res. Commun. **161,** 385, 1989.
8. Senoo, H., Tsukada, Y., Sato, T., and Hata, R. Co-culture of fibroblasts and hepatic parenchymal cells induces metabolic changes and formation of a three-dimensional structure. Cell Biol. Int. Rep. **13,** 197, 1989.
9. Takezawa, T., Yamazaki, M., Mori, Y., Yonaha, T., and Yoshizato, K. Morphological and immuno-cytochemical characterization of a hetero-spheroid composed of fibroblasts and hepatocytes. J. Cell Sci. **101,** 495, 1992.
10. Michalopoulos, G.K., Bowen, W.C., Mulè, K., and Stolz, D.B. Histological organization in hepatocyte organoid cultures. Am. J. Pathol. **159,** 1877, 2001.
11. Michalopoulos, G.K., Bowen, W.C., Mulè, K., Lopez-Talavera, J.C., and Mars, W. Hepatocytes undergo phenotypic transformation to biliary epithelium in organoid cultures. Hepatology **36,** 278, 2002.
12. Lázaro, C.A., Croager, E.J., Mitchell, C., Campbell, J.S., Yu, C., Foraker, J., Rhim, J.A., Yeoh, G.C.T., and Fausto, N. Establishment, characterization, and long-term maintenance of cultures of human fetal hepatocytes. Hepatology **38,** 1095, 2003.
13. Mitaka, T., Mikami, M., Sattler, G.L., Pitot, H.C., and Mochizuki, Y. Small cell colonies appear in the primary culture of adult rat hepatocytes in the presence of nicotinamide and epidermal growth factor. Hepatology **16,** 440, 1992.
14. Mitaka, T., Kojima, T., Mizuguchi, T., and Mochizuki, Y. Growth and maturation of small hepatocytes isolated from adult rat liver. Biochem. Biophys. Res. Commun. **214,** 310, 1995.
15. Mitaka, T., Sato, F., Mizuguchi, T., Yokono, T., and Mochizuki, Y. Reconstruction of hepatic organoid by rat small hepatocytes and hepatic nonparenchymal cells. Hepatology **29,** 111, 1999.
16. Sugimoto, S., Mitaka, T., Ikeda, S., Harada, K., Ikai, I., Yamaoka, Y., and Mochizuki, Y. Morphological changes induced by extracellular matrix are correlated with maturation of rat small hepatocytes. J. Cell. Biochem. **87,** 16, 2002.
17. Harada, K., Mitaka, T., Miyamoto, S., Sugimoto, S., Takeda, H., Mochizuki, Y., and Hirata, K. Rapid formation of hepatic organoid in collagen sponge by rat small hepatocytes and hepatic nonparenchymal cells. J. Hepatol. **39,** 716, 2003.
18. Katsura, N., Ikai, I., Mitaka, T., Shiotani, T, Matsushita, T., Yamanokuchi, S., Sugimoto, S., Kanazawa, A., Terajima, H., Mochizuki, Y., and Yamaoka, Y. Long-term culture of primary human hepatocytes with preservation of proliferative capacity and differentiated functions. J. Surg. Res. **106,** 115, 2002.
19. Hino, H., Tateno, C., Sato, H., Yamasaki, C., Katayama, S., Kohashi, T., Aratani, A., Asahara, T., Dohi, K., and Yoshizato, Y. A long-term culture of human hepatocytes which show a high growth potential and express their differentiated phenotypes. Biochem. Biophys. Res. Commun. **256,** 184, 1999.
20. Auth, M.K.H., Joplin, R.E., Okamoto, M., Ishida, Y., McMaster, P., Neuberger, J.M., Blaheta, R.A., Voit, T., and Strain, A.J. Morphogenesis of primary human biliary epithelial cells: induction in high-density culture or by coculture with autologous human hepatocytes. Hepatology **33,** 519, 2001.
21. Tabata, Y. The importance of drug delivery systems in tissue engineering. Pharm. Sci. Technol. Today **8,** 80, 2000.
22. Richardson, T.P., Peters, M.C., Ennett, A.B., and Moorny, D.J. Polymeric system for dual growth factor delivery. Nat. Biotechnol. **19,** 1029, 2001.

Address reprint requests to:
Toshihiro Mitaka, M.D., Ph.D.
Department of Pathophysiology
Cancer Research Institute
Sapporo Medical University School of Medicine
S-1, W-17, Chuo-Ku
Sapporo 060-8556, Japan

E-mail: tmitaka@sapmed.ac.jp

Available online at www.sciencedirect.com

SCIENCE DIRECT

Experimental Cell Research 305 (2005) 99–109

Experimental Cell Research

www.elsevier.com/locate/yexcr

Transient expression of laminin α1 chain in regenerating murine liver: Restricted localization of laminin chains and nidogen-1

Yamato Kikkawa[a,*], Yoichi Mochizuki[a], Jeffrey H. Miner[b], Toshihiro Mitaka[a]

[a]*Department of Pathophysiology, Cancer Research Institute, Sapporo Medical University School of Medicine, South 1, West 17, Chuo-ku, Sapporo 060-8556, Japan*

[b]*Renal Division, Department of Internal Medicine and Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, MO 63110, USA*

Received 3 November 2004, revised version received 30 December 2004

Abstract

Most interstitia between epithelial and endothelial cells contain basal laminae (BLs), as defined by electron microscopy. However, in liver, the sinusoidal interstitium (called space of Disse) between hepatocytes and sinusoidal endothelial cells (SECs) lacks BLs. Because laminins are major components of BLs throughout the body, whether laminins exist in sinusoids has been a controversial issue. Despite recent advances, the distribution and expression of laminin chains have not been well defined in mammalian liver. Here, using a panel of antibodies, we examined laminins in normal and regenerating mouse livers. Of α chains, α5 was widely observed in all BLs except for sinusoids, while the other α chains were variously expressed in Glisson's sheath and central veins. Laminin γ1 was also distributed to all BLs except for sinusoids. Although the β2 chain was observed in all BLs and sinusoids, the expression of β1 chain was restricted to Glisson's sheath. Detailed analysis of regenerating liver revealed that α1 and γ1 chains appeared in sinusoids and were produced by stellate cells. The staining of α1 and γ1 chains reached its maximum intensity at 6 days after two-thirds partial hepatectomy (PHx). Moreover, in vitro studies showed that α1-containing laminin promoted spreading of sinusoidal endothelial cells (SECs) isolated from normal liver, but not other hepatic cells. In addition, SECs isolated from regenerating liver elongated pseudopodia on α1-containing laminin more so than did cells from normal liver. The transient expression of laminin α1 may promote formation of sinusoids after PHx.

Keywords: Basal lamina; Laminin; Hepatic regeneration

Introduction

Liver plays a central role in metabolic homeostasis and is the major detoxifying organ in the body. After toxic or surgical injury, the liver exhibits unique properties of regeneration. Two-thirds partial hepatectomy (PHx) is a model that most clearly demonstrates the regenerative capacity of the liver. After PHx, the liver is capable of complete regeneration, restoring original size, architecture, and functions. Hepatic regeneration has multiple steps and involves many factors such as cytokines, growth factors, and extracellular matrix (ECM) [1–3]. Of multiple factors, ECM plays a potentially important role in maintaining and remodeling hepatic architecture.

The basal lamina (BL) is an extracellular matrix structure underlying many cell types, including epithelia, endothelia, muscle, fat, and peripheral nerve. BLs (also-called basement membranes) are defined by electron microscopy. Hepatic BLs are observed in bile ducts, arteries, and veins [1]. Interestingly, the sinusoidal interstitium (called space of Disse) between hepatocytes and SECs lacks BLs. BLs are formed by the complex interactions of its major compo-

Abbreviations: BL, basal lamina; PHx, two-thirds partial hepatectomy; SECs, sinusoidal endothelial cells; BECs, biliary epithelial cells; DMEM, Dulbecco's modified Eagle medium; PBS (−), Ca^{2+} and Mg^{2+}-free phosphate-buffered saline; BSA, bovine serum albumin; FBS, fetal bovine serum; EM, electron microscopy; PECAM, platelet endothelial cell adhesion molecule; CK19, cytokeratin 19.

* Corresponding author. Fax: +81 11 615 3099.
E-mail address: yamato@sapmed.ac.jp (Y. Kikkawa).

0014-4827/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.yexcr.2005.01.005

nents: laminins, collagen IV, perlecan, and nidogens [4]. Of these components, it is well known that laminins regulate various cellular functions such as adhesion, motility, growth, differentiation, and apoptosis through interactions with specific cell surface receptors [5,6]. All laminins are composed of three subunits, designated α, β, and γ chains. Laminin was initially isolated from tumor cells as a heterotrimer of A, B1, and B2 subunits [7,8], later renamed α1, β1, and γ1 [9]. Until now, five α, four β, and three γ chains have been identified. The existence of multiple chains provides a means to generate not only structural but also functional diversity within a common structural framework [10]. To date, 15 different laminin heterotrimers have been found to be synthesized and secreted by various cells [9,11,12]. More trimeric molecules are theoretically possible. Despite recent advances, limited information is available about the distribution of the laminin chains in a liver.

To investigate the roles of laminins in normal and regenerating livers, their spatiotemporal depositions were characterized by immunohistochemistry. In the present study, we found that laminin chains were variously distributed in Glisson's sheath, sinusoids, central veins, and mesothelium. They were expressed in overlapping but distinct patterns in liver, as observed in other tissues. Of laminin chains, we focused on laminin α1 that is transiently expressed in sinusoids during hepatic regeneration. In vitro studies also showed that the adhesion of SECs was promoted by α1-containing laminin. The SECs isolated from regenerating liver revealed active movement on α1-containing laminin more so than on fibronectin. Our results suggest that transient expression of laminin α1 is associated with reorganization of liver lobules.

Materials and methods

Animals and surgery

All animal studies were performed according to Sapporo Medical University guidelines, and the animals used in the experiments received humane care. ICR mice (20–24 weeks old) and Sprague–Dawley rats (8–10 weeks old) were purchased from Nihon SLC (Hamamatsu, Japan) and Nihon Charls River (Yokohama, Japan), respectively. For PHx, mice and rats were subjected to conventional PHx under ethyl ether anesthesia [13]. According to the standard method of Higgins and Anderson [13], two-thirds partial hepatectomy (PHx) was performed in adult mouse liver. At different time after PHx, the mice and rats were anesthetized with ethyl ether, and livers were processed for histology and cell isolation.

Proteins and antibodies

Mouse laminin-1 (α1-containing laminin) and human fibronectin were purchased from BD Biosciences (Bedford, MA). Human laminin-8 (α4-containing laminin) [14] was a gift from Dr. Kiyotoshi Sekiguchi (Osaka University, Osaka, Japan). Rat monoclonal antibody 8B3 to laminin α1 [15] was a gift from Dr. Dale Abrahamson (University of Kansas Medical Center, Kansas City, KS). Polyclonal antibodies against the following mouse laminins were gifts from Dr. Takako Sasaki (Max-Planck Institute, Martinsried, Germany): domain VI of laminin α1, domain IIIa of laminin α3, domain IIIa of mouse laminin α4, domain IV of laminin β1, domain IV of laminin β2, domain IV of laminin β3, and LE module 4–6 of laminin γ2 [16–19]. Polyclonal antibody against the globular (G) domain of laminin α2 [20] was a gift from Dr. Peter D. Yurchenco (Robert Wood Johnson Medical School, Piscataway, NJ). Rabbit antibody against domain IIIb/IVa of mouse laminin α5 was produced and characterized as described [11]. Rat monoclonal antibodies MAB1914 to laminin γ1 and MAB1946 to entactin/nidogen-1 were purchased from Chemicon International Inc. (Temecula, CA). Rat monoclonal antibodies MEC 13.3 to platelet endothelial cell adhesion molecule (PECAM) and CI:A3-1 to F4/80 antigen were purchased from BD Biosciences and Serotec (Oxford, UK), respectively. Mouse monoclonal antibodies D33 to desmin and RPN1165 to cytokeratin 19 (CK19) were purchased from Dako (Glostrup, Denmark) and Amersham Biosciences (Piscataway, NJ), respectively. Mouse monoclonal antibodies ED1 to CD68 and SE-1 to SECs were purchased from Serotec (Oxford, UK) and IBL (Fujinaka, Japan), respectively. Rabbit antibody to rat albumin was purchased from Cappel (Aurora, OH).

Immunohistochemistry

For immunohistochemistry, mouse livers were frozen in OCT compound and quick-frozen in 2-methylbutane cooled in a dry ice-ethanol bath. Sections were cut at 7 μm in a cryostat and air-dried. For staining, sections were blocked in 10% normal goat serum and then incubated with primary antibody. All antibody incubations were in PBS (−) containing 1% bovine serum albumin (BSA), and all washes were in PBS (−). Secondary antibodies were conjugated to Alexa488 or 594 (Invitrogen, Carlsbad, CA). After several washes, sections were mounted in 90% glycerol containing 0.1 × PBS (−) and 1 mg/ml *p*-phenylenediamine. Images captured using a Zeiss LSM510 confocal microscope (Carl Zeiss, Oberkochen, Germany) were imported into Adobe Photoshop CS and Adobe Illustrator CS (San Jose, CA) for processing and layout.

Transmission electron microscopy

The liver at 7 days after PHx was perfused with fixation buffer containing 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) followed by Hanks's balanced solution. The liver was postfixed in 2% osmium tetroxide

in the buffer, dehydrated by graded ethanol, and embedded in situ in Epon812. The details of the procedure have been described in our previous study [21].

Cell isolation and adhesion assay

Hepatic cells were isolated by two-step collagenase perfusion as described in Mitaka et al. [21]. After collagenase digestion, the liver was minced in L-15 medium. The cell suspension was filtered through a 70-μm mesh and centrifuged at 50 × *g* for 1 min. The supernatant was centrifuged at 150 × *g* for 5 min. To remove blood cells, the cell pellet was resuspended in PBS (−) supplemented with 0.9% NH_4Cl and incubated for 5 min at room temperature. Hepatic cells were collected with centrifugation at 150 × *g* for 5 min and resuspended in serum-free DMEM. Trypan blue exclusion test was conducted to count the number of living cells. For adhesion assays, cover glasses were coated with 10 μg/ml of laminin isoforms or fibronectin and blocked with PBS (−) containing 1% BSA. The isolated cells were plated on the substrata and incubated at 37°C for 3 h. Adherent cells were fixed with cold ethanol and stained with cell-type-specific antibodies. The stained cells were observed under the confocal microscope.

The SEC-enriched fraction was prepared by sequential centrifugation as described in Yamane et al. [22]. Briefly, after two-step collagenase perfusion, the cell suspensions were centrifuged at 50 × *g* for 1 min twice and then centrifuged at 50 × *g* for 5 min to remove hepatocytes. As described above, blood cells were removed from the cell fraction. The SEC-enriched fraction (~92%) was resuspended in serum-free DMEM. Quantitative adhesion assays were performed as described previously [23]. Briefly, 0–20 μg/ml of laminin-1 (α1-containing laminin) or laminin-8 (α4-containing laminin) was coated onto a 96-well plate (Nunc, Naperville, IL) at 37°C for 1 h. The wells were blocked with 1% BSA. 100 μl of the fractionated cells at 2 × 10^6 cells/ml in DMEM was added to the wells. After a 1-h incubation, the attached cells were stained with 0.2% crystal violet in 20% methanol for 10 min, and spread cells were counted under the microscope. Cells were considered to have a spread morphology when they had become flattened with the long axis more than twice the diameter of the nucleus. Open fenestration is a conspicuous feature of SECs. To exclude other hepatic cells, SECs were morphologically defined under the microscope.

Results

Distribution of laminin chains in normal liver

We first determined which laminin chains (of α1–5, β1–3, γ1, and γ2) were present in the hepatic basal laminae (BLs) and sinusoids of adult mouse liver. Frozen sections were doubly stained with antibodies to laminin α chains and with an antibody to CK19 to label biliary epithelial cells (BECs). Laminin α1 was not observed in adult mouse liver (Fig. 1A). Although laminin α1 was weakly observed in sinusoids of postnatal liver, it disappeared from this region of adult liver (data not shown). In portal veins, α5 and α4 chains were easily detected, and α2 was weakly expressed (Figs. 1D, E, and B). Laminin α5 was also detected in BLs underlying central veins and mesothelium (Figs. 1F and G). In hepatic arteries, the α2−5 chains were detected, but α1 was not (Figs. 1A–G). The antibody to α4 chain often stained central veins (data not shown). Laminins α3 and α5 were present in bile ducts of Glisson's sheath (Figs. 1C and E). Although laminin β1 is ubiquitously observed in BLs of other tissues, in liver it was restricted to portal veins and bile ducts (Fig. 1H). In contrast, β2 was deposited in all BLs and sinusoids (Fig. 1I). As shown in Fig. 1L, γ1 was detected in all BLs except for sinusoids. Similar to α3, laminin γ2 was present in bile ducts and hepatic arteries (Fig. 1K). Laminin β3 was only observed in bile ducts (Fig. 1J), indicating that laminin-5 (α3, β3, γ2) underlies BECs. In hepatic arteries, α3 seemed to be assembled into laminin-6/7 (α3, β1/2, γ1). On the other hand, since laminin γ2 is secreted as a single chain in tumor cells [68], it may be present as a monomer in hepatic arteries. Laminin chains other than β2 were not observed in sinusoids, suggesting that the β2 chain might also be secreted as a single subunit.

Laminin chains in hepatic regeneration

It has been described that the ECM is of critical importance in regulating hepatic regeneration [1,24]. We also examined the expression of laminin chains in regenerating liver after PHx (Fig. 1). Although no α chains were observed in sinusoids of normal liver, α1 and α4 chains appeared in those of regenerating liver (Figs. 1A′ and D′). Both a polyclonal and a monoclonal antibody to α1 stained the same regions in regenerating liver (data not shown). In comparison with the α1 chain, the expression of laminin α4 was weak. The other α chains were not observed (data not shown). Deposition of laminin β2, the only chain detected in sinusoids of normal liver, was not significantly changed in regenerating liver. Laminin γ1 also appeared in sinusoids after PHx and co-localized with the α1 chain, suggesting that sinusoidal laminin is composed of α1, β2, and γ1. The antibodies to α1 and γ1 chains intensively stained sinusoids around Glisson's sheath, but more weakly around central veins (Fig. 3 and unpublished data). In contrast, the antibody to β2 chain intensively stained adjacent to sinusoids (Fig. 1I and data not shown). Thus, the staining patterns of α1 and γ1 chains were not identical with that of β2 chain around central veins. The expression of the β1, β3, and γ2 chains was not significantly changed between normal and

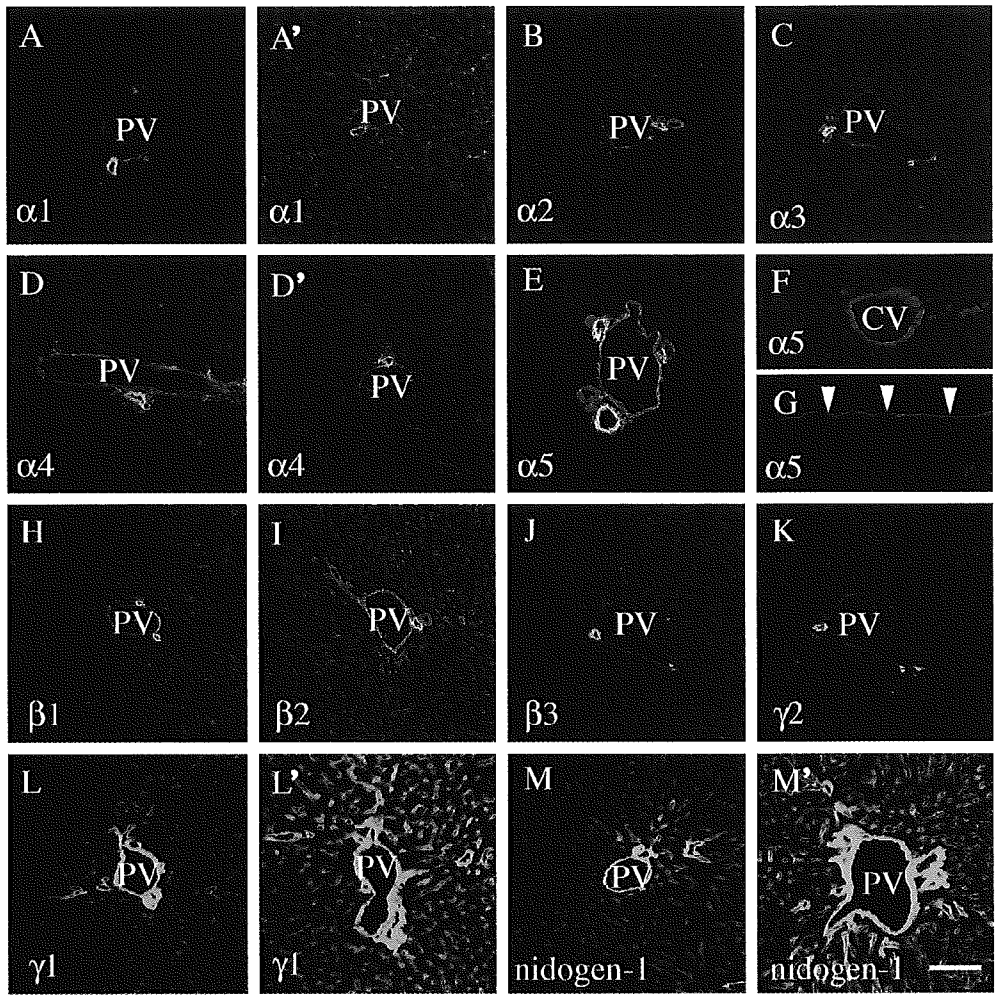


Fig. 1. Immunohistochemical analysis of laminin α, β, γ chains and nidogen-1 in normal and regenerating livers. Frozen sections of control (A–M) and regenerating (A′, D′, L′, M′) livers were doubly stained with rabbit antibodies to laminin α1–5, β1–3, γ2 chains (A–M, Red) and mouse monoclonal antibody to CK19 (A–M, A′, D′; Green). The CK19-positive cells indicate BECs. The regions of overlap are indicated by yellow. The sections were also stained with rat monoclonal antibodies to γ1 (L and L′) and nidogen-1 (M and M′). Arrowheads indicate hepatic mesothelium. PV, portal vein; CV, central vein. Scale bar, 100 μm.

regenerating livers (data not shown). Nidogen-1 binds to γ1III4 of laminin γ1 [25] and connects the laminin lattice to the sheets of collagen IV [26]. The expression of nidogen-1 increased in relation to that of laminin γ1 (Fig. 1M′), suggesting that it integrates the newly synthesized laminin into BLs in sinusoids. The staining patterns of laminin chains and nidogen-1 are summarized in Table 1.

After PHx in rodents, DNA synthesis reaches its maximum at about 24 h, followed by active cell division for about 2 days [27,28]. Following the proliferation of hepatocytes, the architecture of the liver lobule is reconstructed by 7–10 days after PHx [1,2]. The remodeling of ECM is critical for hepatic regeneration. Therefore, we examined the expression of laminin chains in regenerating livers at several time points (Fig. 2). Laminin α1 and γ1 chains began to be deposited in sinusoids at 3 days after PHx. The staining reached its maximum intensity at 6 days after PHx and decreased by 10 days after PHx. Although the intensity of laminin α5 staining increased in BLs, it did not appear in sinusoids. The expression of laminin α4 chain increased at 1 day after PHx and gradually decreased (data not shown). Our results suggest that sinusoidal laminin containing the α1 chain is associated with reorganization of sinusoids but not proliferation of hepatocytes.

Identification of cell-type-producing laminin α1 chain in sinusoids

Three types of cells populate the hepatic sinusoids: SECs, Kupffer cells, and stellate cells. To identify which cell type produced laminin α1 in sinusoids, we used cell-type-specific antibodies (Fig. 3). Frozen sections of regenerating liver were stained with antibodies to desmin, PECAM, and F4/80 to label stellate cells, endothelial cells, and Kupffer cells, respectively. Desmin staining increased at sinusoids near Glisson's sheath in regenerating liver. The localization of stellate cells was correlated with that of laminin α1, suggesting that stellate cells express laminin α1. On the

Table 1
Summary of the expression of laminin chains and nidogen-1 in normal and regenerating livers

	α1			α2			α3			α4			α5		
	Cont.	PHx	Changes	Cont.	PHx	Changes	Cont.	PHx	Changes	Cont.	PHx	Changes	Cont.	PHx	Changes
Glisson's sheath															
Portal vein	–	–	→	+/–	+/–	→	–	–	→	++	++	→	+++	+++	→
Bile duct	–	–	→	–	–	→	++	++	→	–	–	→	+++	+++	→
Hepatic artery	–	–	→	++	++	→	+	+	→	++	++	→	+++	+++	→
Sinusoid	+/–	+++	↑	–	–	→	–	–	→	+/–	+	↑	–	–	→
Central vein	–	–	→	–	–	→	–	–	→	+/–	+/–	→	+++	+++	→
Hepatic mesothelium	–	+/–	→	–	–	→	–	–	→	–	–	→	+++	+++	→

	β1			β2			β3		
	Cont.	PHx	Changes	Cont.	PHx	Changes	Cont.	PHx	Changes
Glisson's sheath									
Portal vein	++	++	→	+++	+++	→	–	–	→
Bile duct	++	++	→	+++	+++	→	+	+	→
Hepatic artery	+/–	+/–	→	+++	+++	→	–	–	→
Sinusoid	–	–	→	+++	+++	→	–	–	→
Central vein	–	–	→	+++	+++	→	–	–	→
Hepatic mesothelium	–	–	→	+++	+++	→	–	–	→

	γ1			γ2			nidogen-1		
	Cont.	PHx	Changes	Cont.	PHx	Changes	Cont.	PHx	Changes
Glisson's sheath									
Portal vein	+++	+++	→	–	–	→	+++	+++	→
Bile duct	+++	+++	→	++	++	→	+++	+++	→
Hepatic artery	+++	+++	→	+	+	→	+++	+++	→
Sinusoid	+/–	++	↑	–	–	→	+	+++	↑
Central vein	++	++	→	–	–	→	+++	+++	→
Hepatic mesothelium	+++	+++	→	–	–	→	+++	+++	→

other hand, the expression of PECAM was observed adjacent to sinusoids and venous vessels. The localization of SECs was correlated with that of laminin α1 near Glisson's sheath. However, SECs often lay on sinusoidal interstitium lacking α1 chain around the central vein, suggesting that SECs do not express laminin α1. F4/80-positive cells were randomly localized in the sinusoidal space. There was no difference in staining of PECAM and F4/80 between normal and regenerating liver (data not shown). Therefore, it was unlikely that SECs and Kupffer cells expressed laminin α1. The staining of γ1 chain was also correlated with the localization of stellate cells (data not shown). The expression of α4 chain was observed adjacent to sinusoids and venous vessels, indicating that SECs express laminin α4.

Ultrastructural analysis of sinusoidal interstitium in regenerating liver

BLs are defined as distinct structures in electron micrographs. For instance, in renal glomerulus and lung alveolus, the epithelial and endothelial cells are separated by BLs that are thin sheets composed of lamina densa and lamina lucida. All endothelial cells throughout the body are laid on a continuous BL except for that of sinusoids in normal liver [29]. However, discontinuous BLs containing laminin appeared in the sinusoidal interstitium (space of Disse) in cirrhotic livers [30]. Sinusoidal interstitium underlying SECs contained perlecan and collagen IV but not laminins or nidogens in adult normal liver (our unpublished data) [31–34]. Since all BLs contain four major components, laminin, nidogen, perlecan, and collagen IV, the missing components seem to be required for the assembly of a BL in the sinusoidal interstitium. As shown in Fig. 1, laminin α1, β2, γ1, and nidogen-1 were deposited in sinusoids of regenerating liver. This spurred us to examine the ultrastructure of sinusoidal interstitium in regenerating liver using transmission electron microscopy (Fig. 4). However, although all four major BL components were deposited at sinusoids of regenerating liver, they did not organize to form a distinct BL. The formation of BL was therefore not necessary for hepatic regeneration, suggesting that α1-

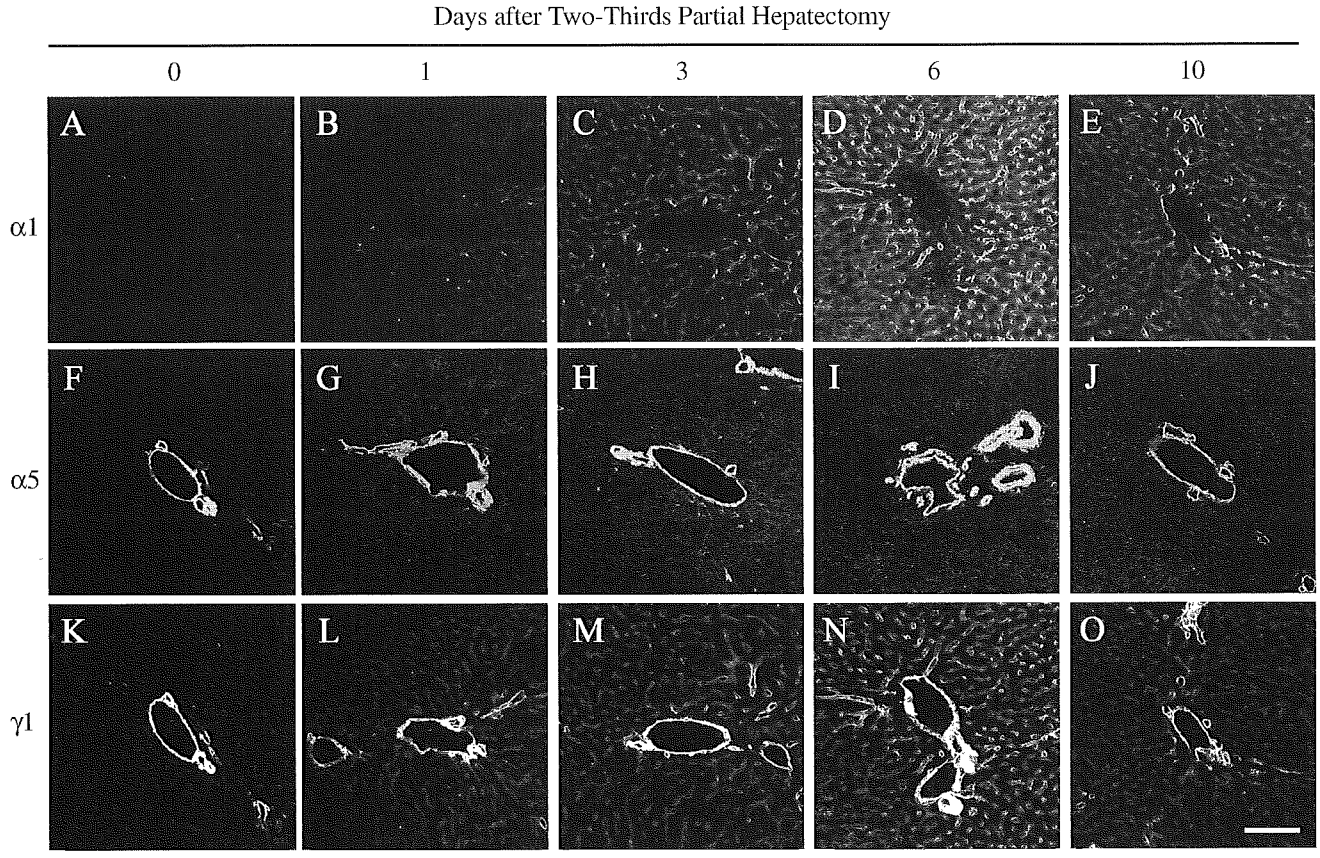


Fig. 2. Time course of laminin chains in regenerating liver. Frozen sections of control (0 day) and regenerating livers (1, 3, 6, 10 days) after PHx were stained with antibodies to α1 (A–E), α5 (F–J), and γ1 (K–O) chains. The staining of α1 and γ1 chains reached its maximum intensity at 6 days after PHx and decreased by 10 days. PV, portal vein. Scale bar, 100 μm.

containing laminin has a transient role in the formation of sinusoids.

Adhesion of hepatic cells to α1-containing laminin in vitro

To investigate the role of the laminin α1 that transiently appeared in sinusoids of regenerating liver, cell adhesion assays were performed using cells isolated from the liver after PHx. The isolated cells were a mixture of liver cells such as hepatocyte, SECs, stellate cells, and Kupffer cells. For cell adhesion assays, we chose rat rather than mouse liver because of the availability of a mouse monoclonal antibody (SE-1) that specifically recognizes SECs [35]. As shown in Fig. 3, the antibody against PECAM cannot distinguish between SECs and the other endothelial cells. Laminin α1 chain plays pivotal roles in laminin-mediated cellular functions [36], so we chose to use commercially available laminin-1 (composed of α1, β1 and γ1) as an α1-containing laminin. Human laminin-8 containing the α4 chain that appeared in sinusoids of regenerating liver was also used in adhesion assays. The hepatic cells were plated on the substrates and incubated at 37°C for 3 h. The attached cells were visualized by immunocytochemistry using cell-specific antibodies. Hepatocytes isolated from normal or regenerating liver did not spread on α1-containing laminin (Fig. 5). Hepatocytes isolated from normal or regenerating liver spread on fibronectin at the same coating concentration (data not shown). The SECs isolated from regenerating liver elongated pseudopodia on α1-containing laminin (Fig. 5A′) more than those from normal liver (Fig. 5A). The stellate cells isolated from regenerating liver also elongated pseudopodia on α1-containing laminin (Fig. 5C′). Kupffer cells attached to substrates but did not spread (Figs. 5E and E′). Although we also used a preparation of laminin-1 plus nidogen-1 in adhesion assays, there was no significant change in the adhesion of hepatic cells (data not shown). Adhesion activity of α4-containing laminin to hepatic cells was less than that of α1-containing laminin at the same coating concentration (Figs. 5B, D, F, B′, D′, and F′). The β and γ chains of laminin-8 (α4β1γ1) are the same as those of laminin-1 (α1β1γ1). We therefore conclude that the α1 chain promotes adhesion of SECs.

To furthermore examine the adhesion of SECs, quantitative adhesion assays were performed using the SEC-enriched fraction. Spread SECs were morphologically defined and counted under the microscope. Quantitative analysis revealed that the number of spread cells on α1-

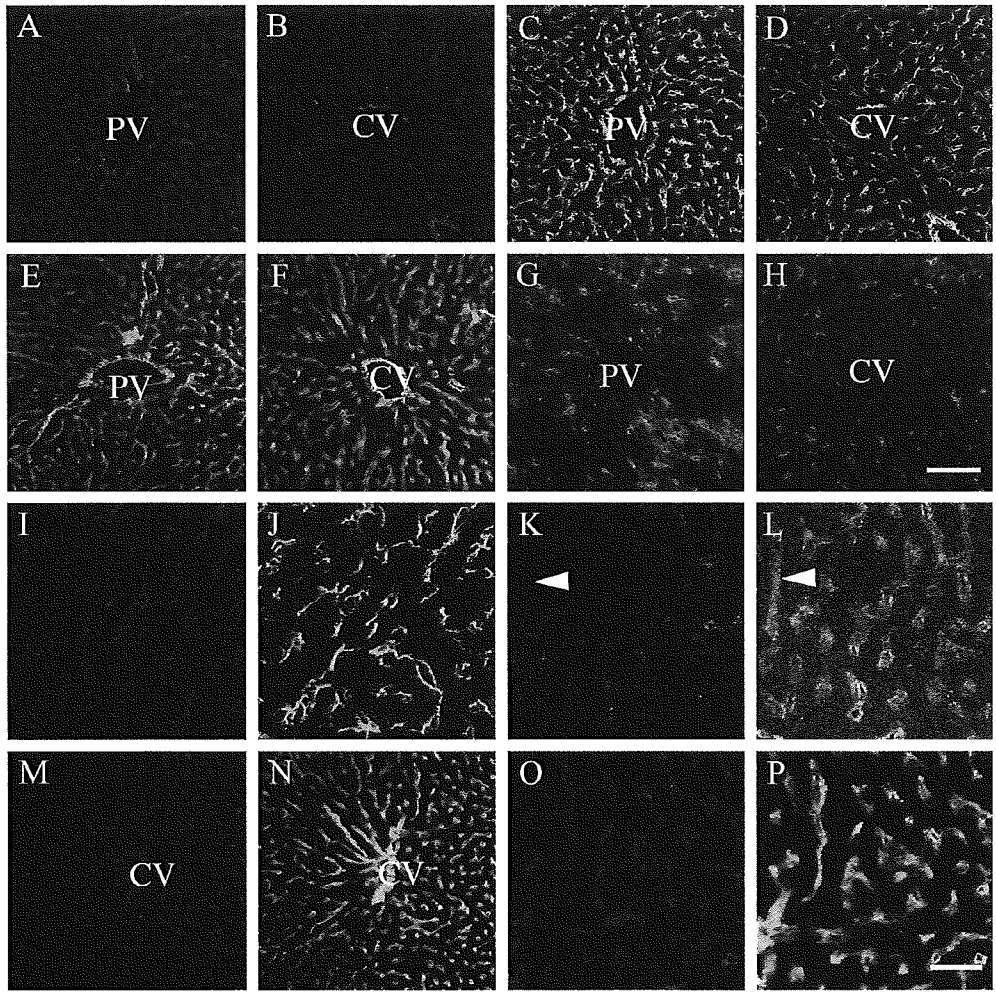


Fig. 3. Identification of cell types expressing laminin α1. Frozen sections of regenerating livers 6 days after PHx were doubly stained with rabbit antibody to α1 chain (A–L, Red) and monoclonal antibodies to desmin to label stellate cells (C, D, and J, Green), PECAM to label endothelial cells (E, F, and L, Green), and F4/80 to label Kupffer cells (G and H, Green). The localization of α1 was correlated with that of stellate cells. Arrowheads indicate that SECs lie on sinusoidal interstitium lacking laminin α1. The tissues were doubly stained with rabbit antibody to α4 chain (M–P, Red) and monoclonal antibody to PECAM (N and P, Green). The localization of α4 chain was correlated with that of SEC. PV, portal vein; CV, central vein. Scale bars in H and P represent 100 μm for A–H, M, and N; and 250 μm for I–L, O, and P.

containing laminin increased in a dose-dependent manner and reached a plateau (Fig. 6). The SECs isolated from regenerating liver spread better than did cells from normal liver, suggesting that SECs are activated in regenerating liver. SECs could attach to α4-conatining laminin but not spread at the higher concentration. Taken together, our results suggest that transient expression of laminin α1 in sinusoids may regulate the movement of SECs during hepatic regeneration.

Discussion

In many studies of the distribution of laminins, polyclonal antibodies against laminin-1 (composed of α1, β1, and γ1) have been used to demonstrate the presence of "laminin" in tissues, including liver. Since such polyclonal antibodies detect the ubiquitous β1 and γ1 chains, the observed distribution was a combination of the distinct localization of individual laminin chains. Moreover, since some laminin chains had not been identified, previous reports of the distribution of laminins have been inexact and incomplete. For example, the presence of laminins in hepatic basal lamina and sinusoidal interstitium was not consistent in previous studies [29,32,33,37–44]. Here, using a panel of well-characterized specific antibodies to individual laminin chains, we reevaluated the distribution and expression in normal and regenerating livers. Our results showed that laminins are expressed in overlapping but distinct patterns in the liver, similar to other tissues. Although the existence of sinusoidal laminin has been debated in previous studies, we found that laminin α1 transiently appeared in sinusoids of the regenerating liver. Recently, Sasaki et al. showed that laminin α1 was present in sinusoids of normal liver [44]. We also observed that the α1 chain is expressed in sinusoids of postnatal liver and