can feasibly support liver functions; however, FHs are the exclusive cell source for clinical therapy because of safety issues. The use of FHs is also limited due to a shortage of donor cells. Therefore, we have been attempting to obtain sufficient numbers of donor cells for HT that can express and support liver functions just like FH.

Small hepatocytes (SHs) are hepatic progenitor cells that possess high proliferative activity and can generate mature differentiated hepatocytes in vitro.[6] The mature hepatocytes generated from SHs can express liver functions similar to those seen in vivo, including protein production and bilirubin metabolism. Unlike conventional hepatoyctes, which fail to express liver functions within a few days in vitro, SHs can proliferate and express liver functions in extended long-term culture.[7] SHs can be isolated not only from rodents but also from humans. The SH character in humans is very similar to that in rodents.[8] In addition, SHs tolerate cryopreservation well compared to FHs and can be stored for an extended period.[9] This suggests that enormous numbers of SHs can be obtained for clinical treatment. However, the functions of SHs in vivo have never been studied to determine if they can express and support liver functions as seen in vitro.

Liver repopulation by transplanted hepatocytes has been shown in a number of experiments. Hepatic toxins such as retrorsine,[10] carbon tetrachloride,[11] and 3,5-diethoxycarbonyl-1,4-dihydrocollidine,[12] and radiation[13-15] have been used for providing a space for cell repopulation and fatal damage to the host hepatocytes to block their proliferation. In addition, hepatectomy has been combined with these manipulations in order to stimulate the transplanted cells to proliferate and repopulate in the recipients. However, these strategies are difficult to apply to clinical treatment due to their toxicity and surgical invasiveness, except for hepatic irradiation, in which liver damage can be controlled by the energy. Treatment of metabolic disorders may be one of the best applications for HT. Total liver repopulation is the ideal therapy in any case; however, it may not be necessary to support or correct liver functions. Clinical therapy should be safe and minimally invasive for the patient. Therefore, we selected hepatic irradiation alone, even though it might not stimulate total liver repopulation, as it supported part of the liver function.

Several markers have been investigated to identify the donor cells in the recipient organ after HT, including congenital genetic abnormalities, genetically manipulated markers, and sex mismatch markers.[5] The combination of sex mismatch cells and other markers is a simple method to identify donor cells with double markers in the recipient organ, which allows us to detect cells by protein, and mRNA and DNA levels. We used female dipeptidyl peptidase IV$^-$ (DPPIV$^-$) Fisher 344 rats, which are spontaneously mutated animals without other abnormalities compared to male DPPIV$^+$ rats.[16] Although this model can be suitable to investigate cell physiology and morphology after HT, it is far from the clinical situation because of no immunogenicity. Therefore, we investigated further, using analbuminemic rats, to test if the donor cells could exhibit hepatocyte functions in an immunosuppressive environment in a situation similar to the clinical setting.

The purpose of this study was to see if cultured SHs could be used for liver repopulation or supporting liver function compared to primary FHs as an alternative cell source for HT with or without immunosuppression.

MATERIALS AND METHODS

Animals

Male dipeptidyl peptidase IV$^+$ (DPPIV$^+$) Fisher 344 rats were purchased from Japan SLC (Shizuoka, Japan). Japanese-strain female DPPIV$^-$ Fisher 344 rats, which possess a spontaneously mutated gene, were purchased from Charles River Japan (Kanagawa, Japan). Male Sprague-Dawley (SD) rats were purchased from Japan SLC. Female Nagase analbuminemic rats (NAR) were purchased from Japan SLC. All animals were 8 to 12 weeks old. All animals used in the experiments received humane care and the experimental protocol was approved by the Committee of Laboratory Animals according to Sapporo Medical University guidelines.

Primary Hepatocyte Preparation for Donor Cells

Primary donor cells were isolated from either male DPPIV+ F344 rats or male SD rats by the two-step collagenase liver perfusion method.[17] The cells were washed with Dulbecco's modified Eagle's medium (DMEM; GIBCO Laboratories, Grand Island, NY) supplemented with 20 mM HEPES, 30 mg/L L-proline, 10% fetal bovine serum (Hy-Clone, Logan, UT). The cell viability was determined by the trypan blue exclusion test to be more than 90%. These cells were used for experiments. The cells were resuspended at 4×10^7 viable cells/ml of saline for transplantation.

Small Hepatocyte Isolation and Culture

The details of isolation and culture of liver cells were previously described.[6] Finally, the cells were suspended in L-15 medium (GIBCO Laboratories) with 20 mmol/L HEPES (Dojindo, Kumamoto, Japan), 1.1 g/L galactose (Katayama Chemical Co., Osaka, Japan), 30 mg/L L-proline, 0.5 mg/L insulin, 10^{-7} mol/L dexamethasone (Sigma Chemical Co., St. Louis, MO), and antibiotics, and the number of viable cells was counted. Two $\times$ 10^6 viable cells were inoculated on 100 mm culture dishes (Corning Glass Works, Corning, NY) coated with rat tail collagen (50 μg of dried tendon/0.1% acetic acid) and placed in a 100% air incubator at 37°C. Two to three hours after plating, the medium was changed to DMEM with 20 mmol/L HEPES, 25 mmol/L $NaHCO_3$, 30 mg/L L-proline, 0.5 mg/L insulin, 10^{-7} mol/L dexamethasone, 10% fetal bovine serum (FBS; Hy-Clone), 10 mmol/L nicotinamide (Katayama Chemical Co.), 1 mmol/L ascorbic acid 2-phosphate (Asc2P; Wako Pure Chem., Tokyo, Japan), 10 ng/ml epidermal growth factor (EGF; Collaborative Research Inc., Lex-

ington, MA), and antibiotics. The cells were then placed in a humidified, 5% CO_2/95% air incubator at 37°C and the medium was subsequently changed every other day. From day 4 (96 hours after plating), 1% dimethyl sulfoxide (DMSO; Aldrich Chemical Co., Inc., Milwaukee, WI) was added to the culture medium and cells were cultured for 12 days.

Cultured SHs at day 12 were dissociated with solution (Cell Dissociation Solution; Sigma Chemical Co.) for 5 minutes after pretreatment with 0.02% EDTA/PBS for 1 minute. Two $\times$ 10^6 (cells/ml) viable SHs were resuspended with saline and used for HT.

Hepatic Irradiation

Hepatic irradiation was performed under open laparotomy. The recipient rats were placed in the supine position on a cork platform. Anesthesia was induced by intraperitoneal injection of 12.5 mg/ml/kg pentobarbital sodium (Dainippon Pharmaceutical Co., Osaka, Japan). Two-by-three centimeter lead shields were wedged under the liver to protect other organs. A Softex X-ray irradiation device (M-150WE; Softex, Kanagawa, Japan) was used (120 kVp, 6 mA, 437 mm SSD, dose rate 3.32 Gy/min). The dose delivered to the liver was verified by dosimetry with an exclusive probe into the abdominal cavity.

Hepatocyte Transplantation

Forty-eight hours after hepatic irradiation, intrasplenic HT was performed. Female DPPIV- F344 rats and NAR rats were anesthetized with ether inhalation and the lower pole of the spleen was exposed by a left flank incision. Then 0.5 ml of saline (control group), male primary FHs (2×10^7 cells/0.5 ml) or male cultured SHs (1×10^6 cells/0.5 ml) were injected into the splenic pulp of the female rats. The hole for the injection was tied with 4-0 silk to achieve hemostasis.

Immunosupressive Treatment

The recipient female NAR rats in all the groups received 10 mg/kg cyclosporine (Calbiochem, La Jolla, CA) to suppress immune rejection of allogenic donor cells, and 2 mg/kg tetracycline (Sigma Chemical Co.) to prevent opportunistic infection from daily subcutaneous injections.

Experimental Protocol

DPPIV Rat Experiment

The rats were divided into three groups (nine rats for the control group, 18 rats for the FH group, and 18 rats for the SH group). The animals were killed at 1, 4, and 12 weeks after either injection or cell transplantation. In the experimental groups, six rats were killed at each time point and samples were collected for DPPIV enzyme histochemistry and Sry PCR analysis.

NAR Rat Experiment

The rats were divided into three groups (five rats for the control group, six rats for the FH group, and six rats for the SH group). During the experiment, the blood was collected through the jugular vein at 1, 4, 8, and 12 weeks after either saline injection or cell transplantation to measure the serum albumin level. All animals were killed at 12 weeks and samples were collected to examine immunofluorescent histochemistry, albumin mRNA expression by RT-PCR, albumin protein by Western blot analysis, and for Sry PCR analysis.

SH Proliferative Activity

The proliferative activity in cultured SHs before transplantation was determined by measuring incorporation of 5-bromo-2′-deoxyuridine (BrdU), as described previously.[7] Briefly, 40 μM of BrdU was added to each 35 mm dish 24 hours before fixation of cells in cold absolute ethanol. A mouse anti-BrdU antibody (DAKO A/S, Copenhagen, Denmark) was used as the primary antibody. Labeled nuclei in the cells were counted in 20 fields/dish and three different dishes were examined. Data are presented as means ± SD from three independent experiments.

Enzyme Histochemistry for Dipeptidyl Peptidase IV

DPPIV enzyme activity was detected as previously described.[18] Briefly, 8 μm liver and spleen cryosections were fixed in cold acetone for 10 min and air-dried. The samples were incubated for 60 minutes in a substrate solution containing 0.5 mg/mL gly-pro-methoxy-β-naphthylamide (Sigma Chemical Co.), 1.0 mg/mL Fast Blue BB (Sigma Chemical Co.), 100 mmol/L phosphate-buffer (pH 6.5), and 100 mmol/L NaCl. All tissue sections were counterstained with hematoxylin and mounted in glycerol.

Confocal Laser Scanning Microscopic Analysis for CK8 and Albumin

Engrafted hepatocytes were detected by CK8 and albumin double immunofluorescence. Then 8 μm liver and spleen cryosections were fixed in 4% paraformaldehyde for 10 minutes. The samples were incubated for 60 minutes at 4°C with rabbit anti-rat albumin IgG (Cappel, Durham, NC) and monoclonal anti-cytokeratin-8 IgG (Amersham Biosciences Corp., NJ) as the primary antibodies after blocking with normal goat serum. The samples were washed twice with PBS and incubated with Alexa^{488}-conjugated anti-rabbit IgG (Molecular Probes, Eugene, OR) and Alexa^{594}-conjugated anti-mouse IgG (Molecular Probes) as the secondary antibodies. A confocal laser scanning microscope (MRC-1024ES; Bio-Rad Laboratories, Inc., Hercules, CA) was used to detect the signals.

Measurement of Serum Albumin Level

The sera collected in NAR rat experiments were sent to a commercial laboratory (SRL, Inc., Tokyo, Japan) for albumin analysis. The albumin levels were determined by the BCG method using an automated clinical microchemistry system (OLYMPUS AU-5400; Olympus Optical Co.) with its exclusive reagent (Alb-HR2; Wako Pure Chem.).

Polymerase Chain Reaction (PCR) and RT-PCR

For DNA analysis, genomic DNA from the liver and spleen was extracted using a GenElute Mammalian Genomic DNA kit (Sigma Chemical Co.). PCR for the rat *Sry* gene was performed using *Sry* primers (sense 5′-CAGAGATCAGCAAGCATCTGG-3′; anti-sense 5′-TCTGGTTCTTGGAGGACTGG-3′)[16] at 94°C for 30 seconds, 57°C for 30 seconds, and 72°C for 30 seconds with 28 cycles. Percentages of engrafted cells were calculated by dilution analyses of hepatocyte DNA between male and/or female cells. *Sry* DPPIV$^+$ male rat hepatocytes were serially diluted with DPPIV$^-$ female rat hepatocytes.

For RNA analysis, tRNA was extracted from the liver and spleen using TRIzol Reagent (Invitrogen Co., Carlsbad, CA). Reverse transcription was performed using an Omniscript Reverse Transcription kit (QIAGEN, Valencia, CA). PCR reaction was performed using *Albumin* primers (sense 5′-GACAAGTTATGCGCCATTCC-3′; anti-sense 5′-ACTGGGTCAGAACCTCATTG-3′)[19] at 94°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec with 26 cycles. Rat *G3PDH* was used as a control (sense 5′-ACCACAGTCCATGCCATCAC-3′; anti-sense 5′-TCCACCACCCTGTTGCTGTA-3′) (Clontech Laboratories, Inc., Palo Alto, CA) at 94°C for 30 seconds, 57°C for 30 seconds, and 72°C for 1 minute with 26 cycles. A TaKaRa Taq PCR Kit (TAKARA BIO Inc., Shiga, Japan) was used for all PCR reactions.

Western Blot Analysis

The samples were homogenized using a Polytron (Kinematica, Switzerland) with a buffer containing 10 mM Tris-HCl, pH 7.4, 0.1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.15 M NaCl, 1 mM EDTA, 1% Triton X-100, 0.1 mM phenylmethane sulfonyl fluoride, and 1% Nonidet P-40 to extract protein. A BCA Protein Assay Reagent Kit (Pierce Biotechnology, Inc., Rockford, IL) was used to determine the concentrations. The proteins (20 μg/lane) were separated by SDS-10% polyacrylamide gel electrophoresis and then transferred electrophoretically to a nylon membrane (Immobilon-P; Millipore Corp., Bedford, MA) with a semi-dry transfer cell (Bio-Rad Laboratories, Richmond, CA). A rabbit anti-albumin antibody (Cappel) was used. A horseradish peroxidase-conjugated anti-rabbit IgG antibody (DAKO) was applied and positive bands were detected by incubation in an ECL Western Blotting Detection System (Amersham, Arlington Heights, IL).

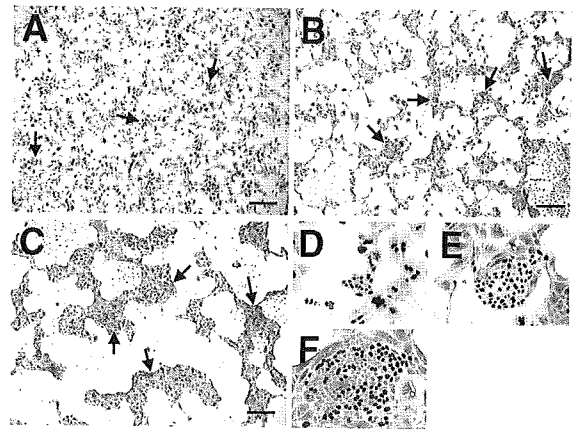

Figure 1. Immunocytochemistry of 5-bromo-2′-deoxyuridine (BrdU) for cultured small hepatocyte colonies at 4 days (A,D), 8 days (B,E), and 12 days (C,F) before hepatocyte transplantation. The cells were labeled with BrdU for 24 hours before harvesting. Arrows indicated the small hepatocyte colonies. The scale bar represents 200 μm.

Densitometric Analysis

Scanning densitometric analysis was performed as previously described.[7] The signals were quantified using the NIH Image 1.55 Densitometric Analysis Program.[20]

Statistical Analysis

Statistical analysis was performed using the StatView 4.5 software package (Abacus Concepts, Inc., Berkeley, CA). All data are expressed as mean ± standard deviation. Statistical significance in each experiment was calculated by either one-way analysis of variance (ANOVA) or Student's unpaired *t*-test when appropriate. A *P*-value < 0.05 was considered significant.

RESULTS

Character of SHs Before Transplantation

We isolated and cultured SHs for 12 days to activate the SH population. SHs could be identified after 4 to 6 days (Fig. 1A,D) in culture. SHs formed colonies and were well identified at day 8 (Fig. 1B,E) and day 12 (Fig. 1C,F). The numbers of SH colonies (Table 1) were 3.83 ± 1.47 (/6.25 mm^2) at day 8 and 4.03 ± 0.86 (/6.25 mm^2) at day 12. The number of SHs per colony increased with time in culture to 17.33 ± 5.92 at day 8 and 26.75 ± 17.31 at day 12, whereas the labeling index in SH colonies significantly decreased from 86.37 ± 8.74 at day 8 to 53.89 ± 5.18 at day 12 ($P < 0.01$). The estimated number of SHs used for HT was calculated to be about 1.0×10^6 (cells/animal), which was less than that of FHs used for HT (about 2.0×10^7 cells/animal).

Hepatocyte Engraftment in Liver and Spleen in the DPPIV Experiment

We used a semi-quantitative method for detection of engrafted cells in the recipients. Fragments of the Y-

chromosome were amplified and compared to control DNA from the mixture of male and female cells (Fig. 2A). In the liver, signals of the Y-chromosome in both groups increased with time in the experiment and the pattern of the increment was similar, although the average level of the signals in the FH group was greater than that in the SH group (Fig. 2B). On the other hand, in the spleen, the signals in both groups decreased with time in the experiments. The signals in the FH group decreased much more rapidly than in the SH group (Fig. 2C). Therefore, the signals of the groups were significantly different after 4 weeks.

Morphological Investigation in the DPPIV Rat Experiment

We confirmed cell engraftment by DPPIV enzyme staining. The engrafted cells were seen within the periportal region of the liver and formed island-like structures in both groups. Cell numbers expanded during the experiment. There was no morphological difference between the FH group (Fig. 3A-C) and the SH group (Fig. 3E-G).

TABLE 1. Characteristics of Small Hepatocyte Colonies at 8 And 12 Days Before Hepatocyte Transplantation

Days after plating	8 days	12 days
Number of SH colonies (/6.25 mm2)	3.83 ± 1.47	4.03 ± 0.86
Number of SHs (/colony)	17.33 ± 5.92	26.75 ± 17.31
BrdU Labeling Indices in SH colonies (%)	86.37 ± 8.74	53.89 ± 5.18*

The number of the colonies and small hepatocytes in the colonies were counted in 10 different independent fields from 3 different dishes.
Data shows means ± standard deviations.
*p < 0.01.

Two of the six rats in the FH group showed near total replacement of the liver. The engrafted cells aligned with the hepatic cord from the periportal region to the central vein region at 12 weeks after HT (Fig. 3D).

We also observed cell engraftment in the spleen. The cells in the FH group (Fig. 3H-O) were integrated in the liver parenchyma scatteringly; on the other hand, those in the SH group (Fig. 3L-N,P,Q) formed a mass. Moreover, engrafted cells in the SH group showed a hepatic organoid structure (Fig. 3P) and bile ductal structure (Fig. 3Q). The numbers of cells in both groups decreased with time in the experiment. However, massive cell engraftment in the spleen was observed in two of the six rats, which also showed near total hepatic repopulation (Fig. 3K).

Comparison of FHs and SHs as Cell Sources in the NAR Model

We observed much more hepatic cell engraftment in the FH group than in the SH group. On the other hand, there was much more splenic cell engraftment in the SH group than in the FH group without immunosuppression in a previous study. We therefore investigated albumin support in the NAR rat experiment with immunosuppression, which could be similar to the clinical situation of HT.

We measured serum albumin levels in the FH and SH groups periodically. Serum albumin levels in both groups increased with time in the experiment and there was no significant difference between the groups (Fig. 4).

Hepatocyte Engraftment in Liver and Spleen in the NAR Rat Experiment

We investigated hepatocyte engraftment at 12 weeks after HT in all groups (Fig. 5). In the liver, signals of Y-chromosomes were not significantly different between the groups (Table 2). On the other hand, in the spleen, the level of signals in the SH group was significantly higher than that in the FH group (2.9 ± 0.7 vs. 1.9 ± 0.4: $P = 0.013$).

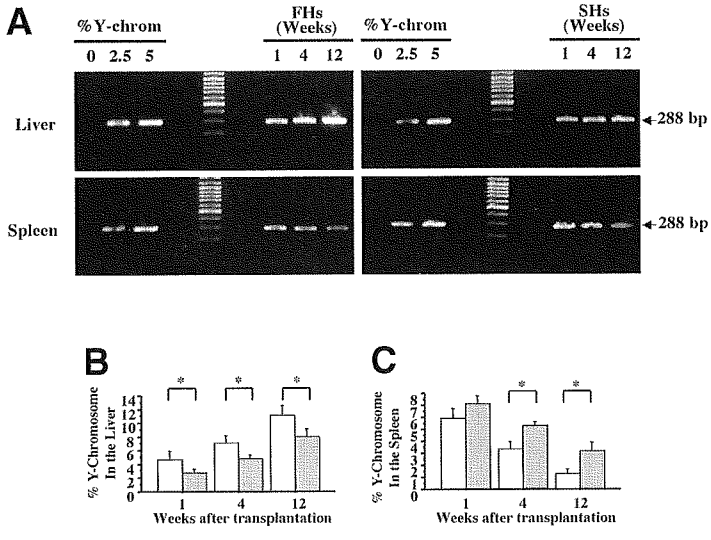

Figure 2. (A) PCR analysis for Y-chromosome gene in recipient organs. The genomic DNA was isolated from 6 different animals at three independent time points. Control amplification of the Y-chromosome was done using a mixture of male and female cells. (B) Densitometric analysis of Y-chromosome gene in the liver. Open columns represent the fresh hepatocyte (FH) group and closed columns represent the small hepatocyte (SH) group. (C) Densitometric analysis of Y-chromosome gene in the spleen. Open columns represent FH group and closed columns represent SH group. Asterisks represent significant differences between the FH group and SH group ($P < 0.05$).

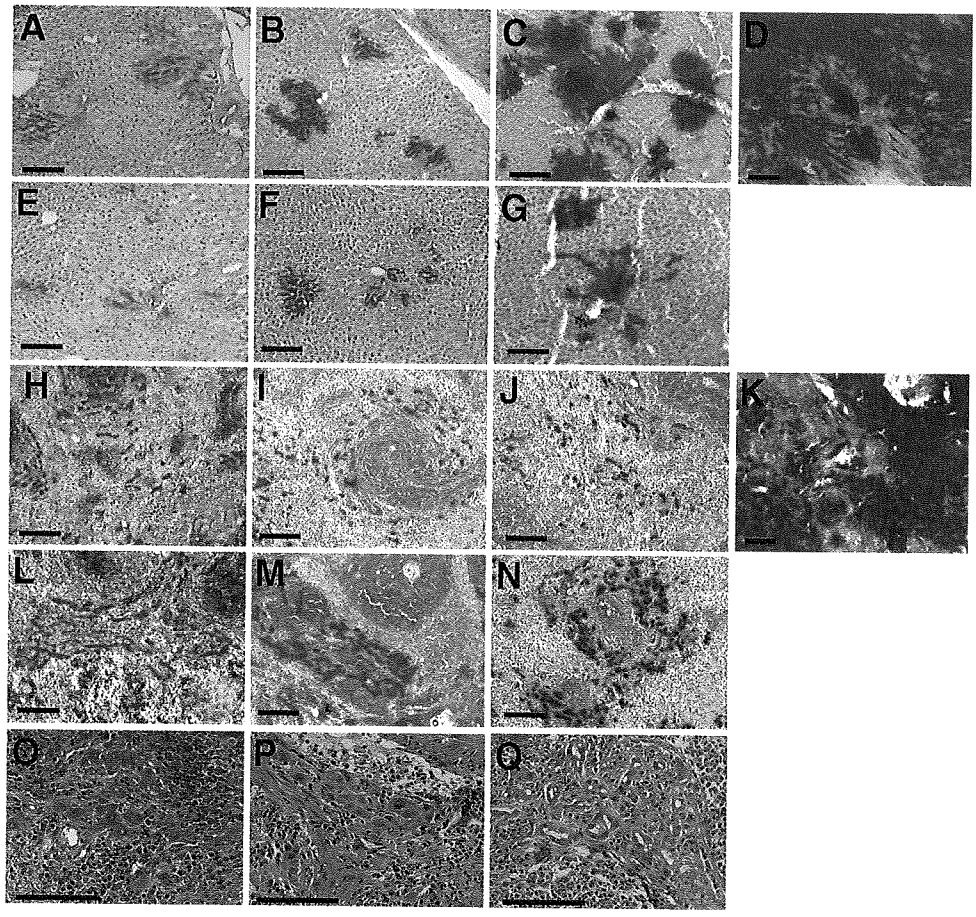

Figure 3. Enzyme histochemistry for dipeptidyl peptidase IV (DPPIV) in the liver (A-G) and in the spleen (H-Q). Hematoxylin-eosin staining of the spleen at 12 weeks in the SH group (O-Q). The specimens were obtained from the FH group (A-D,H-K,O) and SH group (E-G,L-N,P, Q) at 1 week (A,E,H,L), 4 weeks (B,F,I,M), and 12 weeks (C,D,G,J,K,N-Q). The brown staining represents the DPPIV activity. The scale bar represents 100 μm.

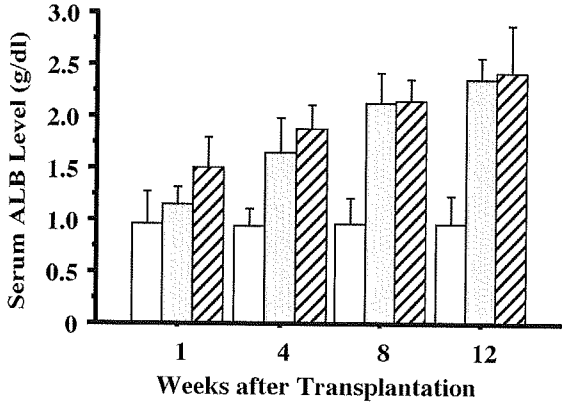

Figure 4. Serum albumin levels in the NAR experiments. Open columns represent the control group, which was injected with saline. Dotted columns represent fresh hepatocyte groups. Shaded columns represent small hepatocyte groups. The bars represent standard deviations.

Albumin mRNA and Protein in the Recipient Organs at 12 Weeks

We found that HT using both FHs and SHs could be effective to improve the serum albumin level in NAR recipients and the cells were detectable at 12 weeks after HT. We observed albumin mRNA expression (Fig. 6A) and protein (Fig. 6B) in the livers and the spleens from six different animals. There was no significant difference in the albumin mRNA expression and protein level between the groups either in the liver or in the spleen (Table 3).

Confocal Laser Scanning Microscopic Analysis of Albumin and CK8

We performed double immunofluorescence analysis of albumin for donor cells and CK8 for hepatocytes, including recipient cells. We detected albumin signals in both groups (Fig. 7). Albumin-positive hepatocytes in the FH group (Fig. 7A) showed a scattered pattern; on the other hand, in SH group (Fig. 7D), they showed colony formation as seen in vitro. All the cells were positive for CK8 (Fig. 7B,E), which is a hepatocyte marker, and all these cells were merged with ALB (Fig. 7C,F).

DISCUSSION

We investigated cell engraftment and the functions of cultured SHs in HT compared to FHs. The donor cell number in the SH group was much smaller than that in

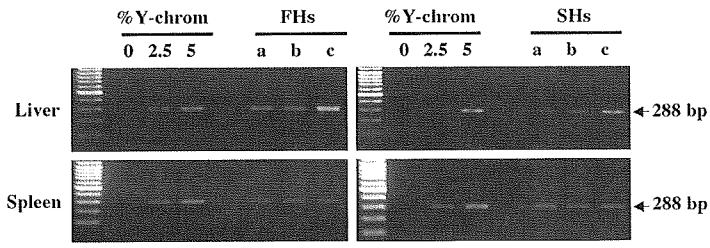

Figure 5. PCR analysis for Y-chromosome gene in female NAR at 12 weeks after hepatocyte transplantation in the fresh hepatocyte (FH) group and small hepatocyte (SH) group. The photographs show three representative data from 6 different animals at 12 weeks. Control amplification of the Y-chromosome was done using a mixture of male and female rat cells.

TABLE 2. Y-chromosome Gene Rate in the Recipient Organs at 12 Weeks After HT

	Y-chromosome (%)		
	FHs	SHs	P value
Liver	7.9 ± 4.4%	4.4 ± 3.4%	NS
Spleen	1.9 ± 0.4%	2.9 ± 0.7%	0.013

All data represent means ± standard deviations.
Organ samples were collected from 6 different animals and intensity of the PCR signals was measured by densitometric analysis as described in Materials and Methods.

the FH group. The cell engraftment in the SH group was smaller in the liver and larger in the spleen than in the FH group in the DPPIV rat experiments. The cell engraftment in the liver increased after HT; however, that in the spleen decreased after HT in both groups. HT using SHs supported the albumin level in the NAR rat experiments as did that using FHs. Thus, long-term cultured SHs could express and support hepatic functions in the repopulating model of the liver.

Potential for SH Proliferation

Although every mature hepatocyte may proliferate after partial hepatectomy,[21] it is uncertain if every one of them can proliferate forever and possess eternal activity. When hepatocytes are cultured for a long term, they do not proliferate equally. This suggests that a limited number of a small population, such as SHs, could exclusively possess high proliferative activity.

We found that the increment of cell engraftment was very similar in the FH and SH groups. This suggested that the 2×10^7 FHs contained similar numbers of cells that could proliferate and repopulate after HT compared to the 1×10^6 SHs. If the SH colony was generated from single cells, the numbers of colonies in the culture should be as same as the numbers of SHs in the initially inoculated cells in culture. This means that the 2×10^6 initial cells used for the culture (/10 mm dish) contained about 5×10^3 SHs and generated about 7.2×10^4 proliferating SHs at day 12. The L.I. of SHs decreased after day 8 in culture and the numbers of proliferating cells in the SH colony were almost same at day 8 (approximately 57.3 cells/6.25 mm^2) and at day 12 (approximately 58.1 cells/6.25 mm^2). Although the number of proliferating SHs could be limited after day 8 in culture, the number of proliferating SHs at day 12 reached 15 times more than that immediately after isolation. We do not know the exact reason why proliferating SHs in culture did not increase after day 8 in this experiment. A possible reason is that present culture conditions may be unsuitable for every SH to keep proliferating. The cultured SHs could proliferate for a long-term, but they always generated mature hepatocytes under the current protocol.6 Suitable conditions for culturing SHs, in which all SHs can maintain proliferative activity without differentiation, should be investigated in a future experiment. Another possible reason is the existence of a specific cell population, even in the SHs, which can keep proliferating. Such specific SHs may be the only cells that can proliferate and maintain the activity. Similar cells, such as SH-like progenitor cells, appear in vivo when rats are exposed to a chemical agent such as retrorsine.[22] It would be interesting to determine if there is any difference between SHs from culture and in vivo SH-like progenitor cells. Both types of cells could engraft and repopulate in the recipient liver. Unlike oval cells and other hepatic stem cells,[23-25] no specific marker for SHs is known. Therefore, it is very difficult to distinguish them only with a marker. A relation between cell size and proliferative activity of the hepatocytes, which is the only landmark for SHs, in a liver repopulating model has also been debated.[26,27] Small-sized hepatocytes from the adult liver do not always proliferate and repopulate in the recipient.[26] This observation and ours again suggest the existence of a specific cell population in SHs, which can proliferate for a prolonged time.

Liver Repopulation

The aim of HT is to support liver functions and it could be an alternative to LT.[3-5] The liver is the best place for hepatocytes to express their functions. However, a space for cell engraftment is necessary in the recipient. Otherwise, donor cells have no chance to proliferate or repopulate to support liver functions. Therefore, several strategies have been used for making a space in the recipient.

Pretreatment with retrorsine, a pyrrolizidine alkaloid, combined with partial hepatectomy[10,22,28] was shown to be effective to replace nearly the whole liver with transplanted hepatocytes. However, the carcinogenicity of retrorsine proscribes its clinical application in humans. Mild hepatic damage is preferable in clinical treatment. After radiation-induced liver damage, massive liver repopulation is accomplished by hepatocyte transplantation combined with partial hepatectomy.[13] A surgical procedure such as hepatectomy is considered to be necessary to promote proliferation of donor cells and repopulation in the recipient. However, surgical invasiveness should be avoided for clinical treatment as much as possible. Al-

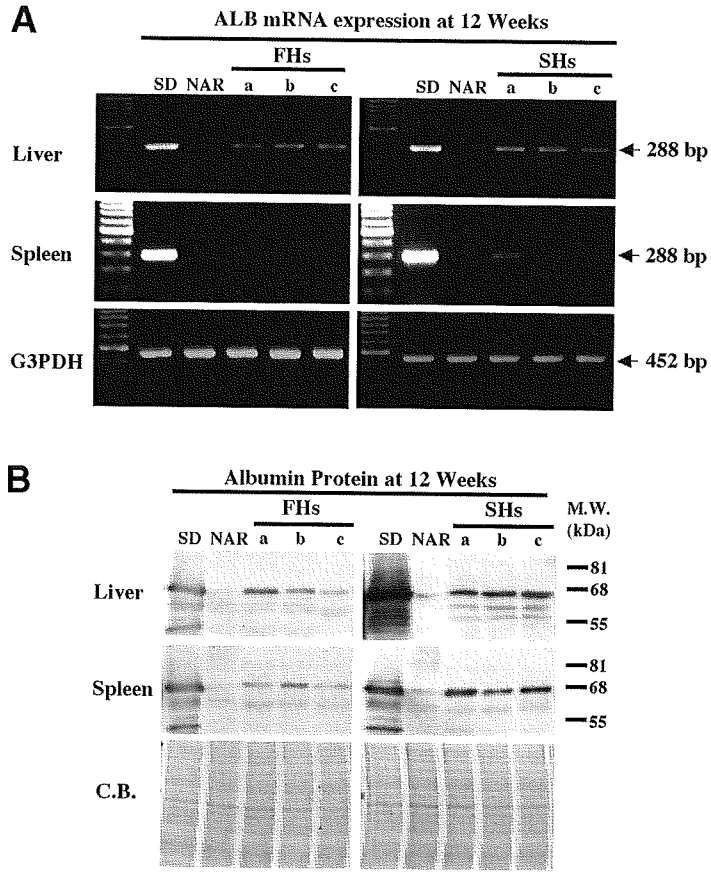

Figure 6. (A) RT-PCR analysis for albumin and G3PDH mRNA expression in NAR at 12 weeks after hepatocyte transplantation in the fresh hepatocyte (FH) group and small hepatocyte (SH) group. The photographs show three representative data from six different animals at 12 weeks. The control amplification of albumin mRNA was done using livers of male SD rats. Albumin mRNA was not detected in the spleen of the SD rat (data not shown). (B) Western blot analysis for albumin protein in NAR at 12 weeks after hepatocyte transplantation in the FH and SH groups. The photographs show three representative data from six different animals at 12 weeks. To each lane 10 mg of protein from the organ extracts was applied. The gels after protein transfer were stained with coomasie blue (C.B.).

though the efficacy of the hepatic repopulation was low in our protocol compared to previous reports,[13] it was high enough to support metabolic functions such as albumin production, as shown in this paper.

We observed that two rats showed near total hepatic repopulation at 12 weeks after HT in the FH group. This indicated that the adult liver could contain cells to repopulate the liver, which was consistent with other reports.[23,29,30] We do not know the exact reason why all rats did not show total hepatic repopulation. One possible reason could be that the damage due to hepatic irradiation was different in each animal. Because the rat liver is divided in nature, overlapping of the liver lobes is unavoidable. This suggests that the environment surrounding hepatic stem cells plays an important role in activating them to proliferate and repopulate. The activation of oval cells or SHs, which could be hepatic stem cells, and proliferation in vivo also depend on the environment where the proliferation of normal hepatocytes is inhibited.[22,24]

We do not know the reason why HT using SHs did not show total hepatic repopulation either. However, the trend of hepatic engraftment was similar in the FH and SH groups. This indicated that SHs could repopulate the liver. Our experiments might have been too short to observe total hepatic repopulation in the SH group. The ideal conditions for total hepatic repopulation should be investigated in future experiments.

We observed less engraftment in the NAR rat experiment than in the DPPIV rat experiment. The difference between the experiments was the use of immunosuppressive therapy. Controversial effects of cyclosporine on hepatocyte proliferation have been reported in vivo and in vitro.[31-33] We do not know the exact reason why we observed different results between the experiments; however, our findings suggested that immunosuppressive treatment might interfere with hepatocyte proliferation and repopulation.

Cell Source for HT

Primary FHs can be an ideal cell source for HT and liver repopulation. However, a large enough number of donor cells needs to be obtained for clinical treatment. Cells immortalized by gene transduction have been tested in experimental HT.[3] However, genetically manipulated cells are far from being available for clinical treatment because of safety issues. In addition, other stem cell sources[3] are unlikely to be candidates because of the time needed to express hepatic functions. SHs would be an ideal cell source as long as they could express liver functions as seen in vitro. We observed colony formation in the liver when SHs were transplanted into the NAR rat experiment. Cell engraftment in the SH group was less than in the FH group; however, the albumin production was similar. This suggested that cell-cell interaction between albumin-producing cells might play an important role in expression of albumin. Although SHs did not repopulate vigorously in vivo, the SH group achieved the same albumin pro-

TABLE 3. Albumin mRNA Expression and Protein in the Recipient Organs at 12 Weeks After HT

	FHs		SHs	
	mRNA	Protein	mRNA	Protein
Liver	32.37 ± 3.35%	16.75 ± 7.15%	35.57 ± 2.50%	19.97 ± 4.76%
Spleen	3.08 ± 1.14%	11.11 ± 3.22%	4.45 ± 2.98%	12.07 ± 3.49%

All data represent means ± standard deviations.
Organ samples were collected from 6 different animals and intensities of the PCR products and ECL signals were measured by densitometric analysis as described in Materials and Methods.

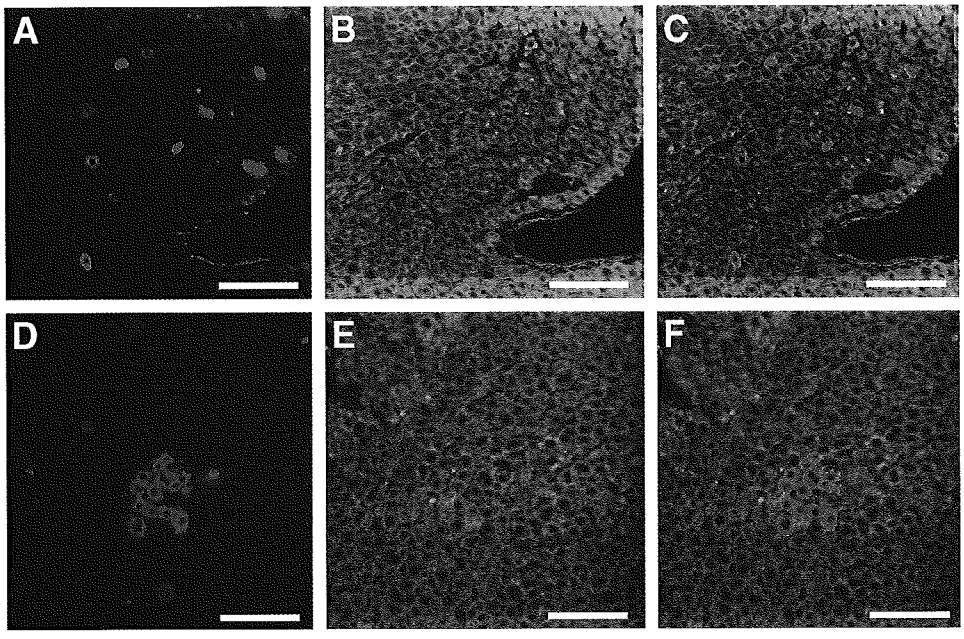

Figure 7. Confocal laser scanning microscopic analysis for ALB (red signals, A and D) and CK8 (green signals, B,E). The specimen was obtained from a female NAR liver at 12 weeks after transplantation using fresh hepatocytes (A-C) and small hepatocytes (D-F). All albumin-positive cells were consistent with CK8-positive cells (C,F). The scale bar represents 25 μm.

duction with fewer cells, which supports the idea that SHs could be an alternative cell source for HT.

The major advantage of SHs for HT could be the small number of cells, which could reduce the volume of the cells that need to be transplanted. A small volume of donor cells is preferable for any cell therapy because of the space needed for engraftment. In addition, another advantage of SHs could be their availability, as they are easily cryopreserved[9] and stored in comparison to FHs. However, the function of cryopreserved SHs in vivo needs to be investigated in a future study.

Fate of Transplanted Hepatocytes in the Spleen

Hepatocytes in the spleen were reported to disappear within a short period after HT.[34] Although the number of hepatocytes in the spleen decreased with time in our experiment, we detected albumin mRNA and protein in the spleen within a short period after HT at the different time points. This suggested that hepatocytes in the spleen after HT might support partial liver function until the time engrafted hepatocytes start to support it. HT using SHs may have advantages and disadvantages compared to HT using FHs. The mechanism of hepatic repopulation after HT through the spleen was explained by cell migration through the splenic vein to the recipient's liver.[35] When SHs were transplanted into the spleen, they could hardly escape from it because they tightly interacted and aggregated with each other. Therefore, hepatic engraftment using SHs is hard to achieve compared to using FHs, as seen in this paper. SHs in the spleen could support albumin production at a certain period, although the number of cells decreased gradually. They could not escape from the spleen to repopulate the liver; however, they stayed and engrafted in the spleen to express liver functions for a certain period. In any case, SHs could support liver functions in both the liver and spleen and could be an alternative to FHs as a cell source for HT.

In conclusion, HT using SHs showed hepatic repopulation similar to that using FHs. This suggested that both SHs and FHs contain hepatic cells that can repopulate the liver as if they were hepatic stem cells. In addition, HT using SHs supported liver functions such as albumin

correction at the same level as that using FHs. These observations support the hypothesis that SHs could be an alternative to FHs as a novel cell source for future HT.

ACKNOWLEDGMENTS

We thank Dr. Thomas Hui (McGill University, Montreal, Canada) and Dr. Yujo Kawashita (Nagasaki University, Nagasaki, Japan) for their valuable discussions, and Ms. Minako Kuwano for her technical assistance. We also thank to Dr. Hirohiko Ise (Shinshu University, Nagano, Japan) for his help in design of the Y-chromosome primers, and Mr. Kim Barrymore for his help in preparing this manuscript.

REFERENCES

1. Neuberger J. Developments in liver transplantation. Gut 2004;53:759-768.
2. Carithers RL, Jr. Liver transplantation: American Association for the Study of Liver Diseases. Liver Transpl 2000; 6:122-135.
3. Fox IJ, Roy-Chowdhury J. Hepatocyte transplantation. J Hepatol 2004;40:878-886.
4. Strom S, Fisher R. Hepatocyte transplantation: new possibilities for therapy. Gastroenterology 2003;124:568-571.
5. Horslen SP, Fox IJ. Hepatocyte transplantation. Transplantation 2004;77:1481-1486.
6. Mitaka T, Sato F, Mizuguchi T, Yokono T, Mochizuki Y. Reconstruction of hepatic organoid by rat small hepatocytes and hepatic nonparenchymal cells. Hepatology 1999;29:111-125.
7. Mizuguchi T, Mitaka T, Hirata K, Oda H, Mochizuki Y. Alteration of expression of liver-enriched transcription factors in the transition between growth and differentiation of primary cultured rat hepatocytes. J Cell Physiol 1998;174: 273-284.
8. Hino H, Tateno C, Sato H, Yamasaki C, Katayama S, Kohashi T, et al. A long-term culture of human hepatocytes which show a high growth potential and express their differentiated phenotypes. Biochem Biophys Res Commun 1999;256:184-191.
9. Ikeda S, Mitaka T, Harada K, Sugimoto S, Hirata K, Mochizuki Y. Proliferation of rat small hepatocytes after long-term cryopreservation. J Hepatol 2002;37:7-14.
10. Laconi E, Oren R, Mukhopadhyay DK, Hurston E, Laconi S, Pani P, et al. Long-term, near-total liver replacement by transplantation of isolated hepatocytes in rats treated with retrorsine. Am J Pathol 1998;153:319-329.
11. Gupta S, Rajvanshi P, Aragona E, Lee CD, Yerneni PR, Burk RD. Transplanted hepatocytes proliferate differently after CCl4 treatment and hepatocyte growth factor infusion. Am J Physiol 1999;276:G629-G638.
12. Wang X, Foster M, Al-Dhalimy M, Lagasse E, Finegold M, Grompe M. The origin and liver repopulating capacity of murine oval cells. Proc Natl Acad Sci U S A 2003;100:11881-11888.
13. Guha C, Sharma A, Gupta S, Alfieri A, Gorla GR, Gagandeep S, et al. Amelioration of radiation-induced liver damage in partially hepatectomized rats by hepatocyte transplantation. Cancer Res 1999;59:5871-5874.
14. Malhi H, Gorla GR, Irani AN, Annamaneni P, Gupta S. Cell transplantation after oxidative hepatic preconditioning with radiation and ischemia-reperfusion leads to extensive liver repopulation. Proc Natl Acad Sci U S A 2002;99: 13114-13119.
15. Takahashi M, Deb NJ, Kawashita Y, Lee SW, Furgueil J, Okuyama T, et al. A novel strategy for in vivo expansion of transplanted hepatocytes using preparative hepatic irradiation and FasL-induced hepatocellular apoptosis. Gene Ther 2003;10:304-313.
16. Ise H, Nikaido T, Negishi N, Sugihara N, Suzuki F, Akaike T, et al. Effective hepatocyte transplantation using rat hepatocytes with low asialoglycoprotein receptor expression. Am J Pathol 2004;165:501-510.
17. Seglen PO. Preparation of isolated rat liver cells. Methods Cell Biol 1976;13:29-83.
18. Lojda Z. Studies on dipeptidyl(amino)peptidase IV (glycyl-proline naphthylamidase). II. Blood vessels. Histochemistry 1979;59:153-166.
19. Miura K, Nagai H, Ueno Y, Goto T, Mikami K, Nakane K, et al. Epimorphin is involved in differentiation of rat hepatic stem-like cells through cell-cell contact. Biochem Biophys Res Commun 2003;311:415-423.
20. Masters DB, Griggs CT, Berde CB. High sensitivity quantification of RNA from gels and autoradiograms with affordable optical scanning. Biotechniques 1992; 12:902-906, 908-911.
21. Michalopoulos GK, DeFrances MC. Liver regeneration. Science 1997;276:60-66.
22. Gordon GJ, Butz GM, Grisham JW, Coleman WB. Isolation, short-term culture, and transplantation of small hepatocyte-like progenitor cells from retrorsine-exposed rats. Transplantation 2002;73:1236-1243.
23. Sell S. Heterogeneity and plasticity of hepatocyte lineage cells. Hepatology 2001;33:738-750.
24. Fausto N. Liver regeneration and repair: hepatocytes, progenitor cells, and stem cells. Hepatology 2004;39:1477-1487.
25. Laurson J, Selden C, Hodgson HJ. Hepatocyte progenitors in man and in rodents—multiple pathways, multiple candidates Int J Exp Pathol 2005;86:1-18.
26. Overturf K, Al-Dhalimy M, Finegold M, Grompe M. The repopulation potential of hepatocyte populations differing in size and prior mitotic expansion. Am J Pathol 1999; 155:2135-2143.
27. Katayama S, Tateno C, Asahara T, Yoshizato K. Size-dependent in vivo growth potential of adult rat hepatocytes. Am J Pathol 2001;158:97-105.
28. Oren R, Dabeva MD, Petkov PM, Hurston E, Laconi E, Shafritz DA. Restoration of serum albumin levels in Nagase analbuminemic rats by hepatocyte transplantation. Hepatology 1999;29:75-81.
29. Overturf K, al-Dhalimy M, Ou CN, Finegold M, Grompe M. Serial transplantation reveals the stem-cell-like regenerative potential of adult mouse hepatocytes. Am J Pathol 1997;151:1273-1280.
30. Wang J, Clark JB, Rhee GS, Fair JH, Reid LM, Gerber DA. Proliferation and hepatic differentiation of adult-derived progenitor cells. Cells Tissues Organs 2003;173:193-203.
31. Masuhara M, Ogasawara H, Katyal SL, Nakamura T, Shinozuka H. Cyclosporine stimulates hepatocyte proliferation and accelerates development of hepatocellular carcinomas in rats. Carcinogenesis 1993;14:1579-1584.
32. Lilja H, Blanc P, Demetriou AA, Rozga J. Response of cultured fetal and adult rat hepatocytes to growth factors and cyclosporine. Cell Transplant 1998;7:257-266.
33. Andres D, Diez-Fernandez C, Zaragoza A, Alvarez A, Cascales M. Induction of cell proliferation by cyclosporine A in primary cultures of rat hepatocytes. Biochem Pharmacol 2001;61:427-435.
34. Kusano M, Mito M. Observations on the fine structure of long-survived isolated hepatocytes inoculated into rat spleen. Gastroenterology 1982;82:616-628.
35. Lapidot T. Mechanism of human stem cell migration and repopulation of NOD/SCID and B2mnull NOD/SCID mice: the role of SDF-1/CXCR4 interactions. Ann N Y Acad Sci 2001;938:83-95.

ELSEVIER

Journal of Hepatology xx (xxxx) 1–9

Journal of Hepatology

www.elsevier.com/locate/jhep

Expression of CD44 in rat hepatic progenitor cells

Junko Kon, Hidekazu Ooe, Hideki Oshima, Yamato Kikkawa, Toshihiro Mitaka*

Department of Pathophysiology, Cancer Research Institute, Sapporo Medical University School of Medicine, South-1, West-17, Chuo-Ku, Sapporo 060-8556, Japan

MS539

***Background/Aims*: Small hepatocytes (SHs) are hepatic progenitor cells, but the phenotypical difference between SHs and mature hepatocytes (MHs) has never been demonstrated.**

***Methods*: The profile of gene expression was examined to clarify the difference between SHs and MHs by using a DNA microarray. Genes that were specifically expressed in SHs were identified and RT-PCR analysis of them was performed. Immunocytochemistry for CD44 standard form (CD44s) and variant form 6 (CD44v6) was performed using cultured SHs and the D-galactosamine (GalN)-injured rat liver. From the GalN-treated liver, $CD44s^+$ cells were obtained by sorting and RT-PCR analysis was performed.**

***Results*: Analysis using the DNA microarray and RT-PCR of them revealed restricted expression of CD44s and CD44v6 in SHs. In culture, CD44s appeared at day 3 and increased with the proliferation of SHs. CD44v6 expression was delayed compared to that of CD44s. With GalN-administration, $CD44^+$ hepatocytes appeared around periportal areas at days 3 and 4 and then decreased. Sorted $CD44s^+$ cells could form colonies and possessed hepatic markers.**

***Conclusions*: CD44 is a specific marker of SHs. The expression of CD44 mRNA and protein is restricted to SHs, and is up-regulated at the time that SHs start to proliferate both in vitro and in vivo.**

***Keywords*: CD44s; CD44v6; Small hepatocytes; Proliferation; Maturation**

1. Introduction

Small hepatocytes (SHs) are a subpopulation of hepatocytes that have high growth potential in culture [1–4]. The cells are less than half the size of mature hepatocytes (MHs), but they possess hepatic characteristics [5,6]. SHs can clonally proliferate to form colonies that survive for more than 5 months in defined medium [5,6] and can differentiate into MHs by interacting with hepatic nonparenchymal cells (NPCs) [7] or as a result of treatment with Engelbreth–Holm–Swarm gel [8]. Thus, we consider that SHs may be 'committed progenitor cells' that can further differentiate into MHs. Although SHs are primary cells that are freshly prepared from rat liver, they can also proliferate after cryopreservation [9].

The molecular mechanisms regulating the characteristics of SHs remain to be elucidated. In addition, their precise origin and location within the liver are not clear because the preparation of purified SHs is difficult and specific markers for SHs have never been identified. Therefore, it is important to identify specific genes and proteins expressed in SHs, especially cell membrane-integrated proteins, because it will be possible to clarify the characteristics of the cells by the methods of cell sorting.

The CD44 gene encodes for a family of alternatively spliced, multifunctional adhesion molecules that participate in lymphocyte–endothelial cell interactions as lymphocyte homing receptors [10–12], and in adhesion of cells to

Received 18 May 2005; received in revised form 10 January 2006; accepted 17 January 2006

* Corresponding author. Tel.: +81 11 611 2111x2390; fax: +81 11 615 3099.

E-mail address: tmitaka@sapmed.ac.jp (T. Mitaka).

Abbreviations: BECs, biliary epithelial cells; CD44s, CD44 standard form; CD44v, CD44 variant form; C/EBPα, CCAAT/enhancer binding protein α; CYP, cytochrome P450; GalN, D-galactosamine; HA, hyaluronic acid; LECs, liver epithelial cells; MH, mature hepatocyte; NPC, hepatic nonparenchymal cell; SH, small hepatocyte.

0168-8278/$30.00 © 2006 Published by Elsevier B.V. on behalf of the European Association for the Study of the Liver.
doi:10.1016/j.jhep.2006.01.029

DOCTOPIC: Molecular and Cell Biology

extracellular matrix [13], T cell activation and adherence [14], and metastasis formation [15]. CD44 standard form (CD44s) is composed of a short cytoplasmic tail, a transmembrane region and two extracellular domains. There are 10 additional exons (v1–v10). Although the expression of CD44 variant forms (CD44v) was initially considered to occur as a result of aberrant splicing in tumor cells, variant expression was subsequently detected in normal cells [16]. The expression of variant forms in hematopoietic cells has been reported [17–19].

In the present study, we found that both CD44s and CD44v6 were expressed in cultured SHs and their expression decreased with the maturation of the cells. Although biliary epithelial cells (BECs) also expressed CD44s, no other epithelial cells within the normal rat liver did. However, when the rat liver was severely injured by D-galactosamine (GalN) treatment, $CD44s^+$ epithelial cells appeared near Glisson's capsule in the liver lobules and, by using a specific antibody, the cells could sorted and thereafter cultured. These $CD44^+$ cells expressed hepatic marker genes and could proliferate to form colonies consisting of SH cells.

2. Materials and methods

2.1. Isolation and culture of SHs

Male F344 rats (Sankyo Lab Service Corporation, Inc., Tokyo, Japan) weighing 150–200 g were used to isolate hepatic cells by the two-step liver perfusion method of Seglen [20] with some modifications [2]. Briefly, suspensions of liver cells were centrifuged at $50\times g$ for 1 min. The supernatant was used to prepare SHs and the precipitate was used to prepare MHs. The details of the isolation and culture procedure were previously reported [7]. After the number of viable cells was counted, cells were plated on dishes (7.5×10^4 cells/35-mm, 10×10^5 cells/100-mm dish; Corning Glass Works, Corning, NY). SH colonies cultured in a 100-mm dish were collected at day 14 and cryopreserved at −80 (C. The details of the method were previously reported [9]. After cryopreservation, SHs were thawed and suspended in the culture medium. To induce the maturation of SHs, they were overlaid with growth factor-reduced Matrigel® (BD Biosciences, Bedford, MA) at day 14 after thawing and the cells were then cultured. The details of the method were previously reported [8].

2.2. DNA microarray

Differences of the expression profiles of SHs and MHs were analyzed using a microarray approach. A DNA microarray spotted with 14,815 cDNAs (Agilent rat cDNA microarray kit) was purchased from Agilent Technologies, Inc. (Palo Alto, CA). $Poly(A)^+$ RNAs were prepared using ISOGEN (Nippon Gene, Tokyo, Japan) and mRNAs were prepared using a GenElute™-mRNA miniprep kit (Sigma Chem Co, St Louis, MO). Prepared mRNAs were labeled with Cy5- and Cy3-dUTP by reverse transcription. Analysis of the microarray was performed by Hokkaido System Science (Sapporo, Japan).

2.3. RT-PCR

Total RNA was isolated using ISOGEN. Reverse transcription and PCR amplification (RT-PCR) were performed in a one-step reaction according to the manufacturer's instructions (Invitrogen, San Diego, CA). Sequences of forward and reverse primers used are listed in Table 1. The constitutively expressed gene glycerol 3-phosphate dehydrogenase (GPDH) was also reverse-transcribed in a separate reaction as a qualitative and quantitative control.

2.4. Northern blot analysis

Northern blot analysis was performed as previously reported [21]. Probe labeling and RNA detection were performed according to the manufacturer's instructions for the AlkPhos Direct Labelling and Detection System with CDP-Star (Amersham Biosciences, Piscataway, NJ). For probes, the full-length CD44s and partial 450 bp GPDH fragment were used.

2.5. Western blot analysis

After washing with PBS twice, the cells were dissolved in lysis solution (20 mM Tris–HCl [pH 7.4], 150 mM NaCl, 2.5 mM EDTA, 1% Triton-X100, 1% aprotinin, and 20 mg/ml leupeptin). The cells were kept on ice for 30 min and sonicated. After the sonication, the solution was centrifuged at $22{,}000\times g$ for 20 min. The supernatant was kept at −80 °C until use and the protein content was measured using a BCA assay kit (Pierce, Rockford, IL). Western blot analysis was carried out as previously described [7].

2.6. Immunostaining

Antibodies used for immunostaining are listed in Table 2. SHs in a 35-mm dish were used for immunocytochemistry. After washing with PBS, the cells were fixed in 70% cold ethanol. After blocking with BlockAce (Dainippon Pharmaceuticals Co., Osaka, Japan) for 30 min at RT, cells were incubated with the primary antibody for 60 min at RT. Dishes were rinsed with PBS and subsequently incubated with an $Alexa^{488}$-conjugated antibody (Molecular Probe, Eugene, OR) for 30 min at RT. In case of double staining, the secondary antibody was applied for 60 min. After washing with PBS, the $Alexa^{594}$-conjugated antibody (Molecular Probe) was applied for 30 min. Finally, cells were embedded with 90% glycerol including 0.01% *p*-phenylenediamine and 4,6-diamidino-2-phenylindole (DAPI).

For immunohistochemistry, the liver was frozen at −80 °C until use. Then 7-μm-thick sections were prepared and air-dried. The staining procedure used for the sections was the same as for immunocytochemistry. A confocal laser microscope (Zeiss, Jena, Germany) was used for observation.

2.7. D-galactosamine administration

GalN (Sigma; 75 mg/100 g body weight dissolved in PBS) was intraperitoneally given to male F344 rats weighing 150–200 g [22]. The animals were killed 1–5 days after the treatment and their livers were removed. Liver slices were prepared, immediately frozen in liquid nitrogen and kept at −80 °C until use.

2.8. Cell sorting and culture

Four days after GalN treatment, hepatic cells were isolated as described above. The isolated cells were centrifuged at $50\times g$ for 1 min. The supernatant was collected and then centrifuged again. After the same procedure was repeated, the supernatant was centrifuged at $150\times g$ for 5 min and the pellet was suspended in PBS containing 2 mM EDTA and 0.5% BSA. An anti-CD44 antibody (625 ng/ml) was added and, following incubation for 10 min, cells were washed with the modified PBS. After washing, anti-rat IgG microbeads for MACS (Miltenyi Biotec, Bergisch Gladbach, Germany) were added. Magnetic separation was done using a MidiMACS separation unit (Miltenyi Biotec) and the positive fraction was collected. This fraction was plated on dishes and cultured in modified DMEM. Some of the sorted and cultured cells were harvested and employed for analysis by RT-PCR.

Table 1
Primer sequences used for RT-PCR analysis

Primer name	Forward	Reverse	Size of amplicon (bp)
CD44	CCCGAATTCATGGA-CAAGGTTTGGTGGCA	CCCGAATTCCTACACCCCAATCTT-CATAT	1095
GPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA	450
CD44s	TCCCACTATGACACATATTGC	ACACCTTCTCCTACTGTTGAC	548
CD44v1	TCCCACTATGACACATATTGC	TTGTGAATTACCAAACCAAG	407
CD44v2	TCCCACTATGACACATATTGC	TGTATGAAGATGACTCTTGG	428
CD44v3	TCCCACTATGACACATATTGC	TCATTTGGCTTCCAGCCTGT	358
CD44v4	TCCCACTATGACACATATTGC	TTGTCTGAAGTAGTACTTCTG	424
CD44v5	TCCCACTATGACACATATTGC	ATGTGGGGTCTCCTCTTCAT	418
CD44v6	TCCCACTATGACACATATTGC	GGAGTCTTCACTTGGGGTGG	430
CD44v7	TCCCACTATGACACATATTGC	AATCGGTCCATGAAACATCCT	396
CD44v8	TCCCACTATGACACATATTGC	GTATTTGGAGCCGCAGTAGGC	373
CD44v9	TCCCACTATGACACATATTGC	GTCTTCGCCTTCTTCCAGCTC	362
CD44v10	TCCCACTATGACACATATTGC	TTCCATTGTGTCTGGATATTG	409
Albumin	AAGGCACCCCGATTACTCCG	TGCGAAGTCACCCATCACCG	608
TAT (tyrosine aminotransferase)	TACTCAGTTCTGCTGGAGCC	GCAAAGTCTCTAGAGAGGCC	471
CK8 (cytokeratin 8)	GGAGGTGGACCCCAACATCC	CCACAGACGTGTCTGAGATC	500
Transferrin	CCTGACAAAACGGT-CAAATGGTGC	TAAAAACTCTGCTGCCACAGGC	251
C/EBPα	CGGGGCCGGCGCGGGCAAGG	GGGGAATTCTCACGCGCAGTTGCC-CATGG	277
Cx32	ATGAACTGGACAGGTCTATA	TCAGCAGGCTGAGCATCGGT	854
HNF4	GGGGAATTCATGGACATGGCT-GACTACAG	GGGGAATTCCTA-GATGGCTTCCTGCTTGG	1368
CYP1A1	GATGCTGAGGACCAGGAA-GACCGC	CAGGAGGCTGGACGAGAATGC	679
CYP3A1	CAGCTCTCACACTG-GAAACCTGGG	TCGAGGATCTAAACAACCTGAC	689
CYP4A3	TCGAGGATCTAAACAACCTGAC	GGTTGTGATACCTTTGGGTATGG	573
c-kit	TCCGCTGCCCCCTGACAGAC	CTACATTTTCCCCATCAGTT	600
CD34	ATGCCGGTCCACAGGGGCGC	GACTCCCGAGGTAACCAATG	900
Thy1.1	ATGAACCCAGCCATCAGCGT	TGCCGCCACACTTGACCAGT	400
CK19	ATGACTTCCTATAGCTATCG	CACCTCCAGCTCGCCATTAG	340

3. Results

3.1. The expression of CD44 in SHs

Using a DNA microarray, the profile of gene expression was examined to clarify the difference between SHs and MHs. We selected 164 genes with much higher expression in SHs than in MHs. From these genes, we chose 16 that possessed the transmembrane sequence (Table 3) and RT-PCR analysis was performed to confirm whether the selected genes were specifically expressed in SHs. CD44 was one of the three genes (CD44, D6.1A, BRI3) restrictedly expressed in SHs (Fig. 1A). To examine the length of the mRNA of CD44, Northern blotting was carried out. Fig. 1B shows two bands of CD44 mRNA. As CD44 is known to have many variant forms, the expression of variant forms in SHs was examined by RT-PCR. As shown in Fig. 1C, both CD44s and CD44v6 were expressed in SHs. The lengths of mRNAs of CD44s and CD44v6 detected by Northern blotting corresponded to the 1384 and 1614 bp that were expected from the sequences, respectively.

Immunocytochemistry for CD44s showed that CD44s$^+$ cells, in which the cell membrane and cytoplasm were faintly stained, first appeared at day 3. The timing of

Table 2
Antibodies

Antibodies	Company or producer	Dilution
Mouse anti-rat CD44	BD Biosciences PharMingen	1:1000
Rabbit anti-rat C/EBPα	Santa Cruz	1:400
Rabbit anti-rat CK19	Generous gift from Prof. Atsushi Miyajima[a]	1:1000
Mouse anti-rat Thy1.1	Serotec	1:500
Mouse anti-rat CD44v6	Generous gift from Dr Jonathan Sleeman[b]	1:100

[a] Molecular and Cellular Biology Institute, University of Tokyo, Tokyo, Japan.
[b] Institute of Genetics, Forschungszentrum Karlsruhe, Karlsruhe, Germany.

Table 3
Data from microarray

Name of the clone	Ratio[a]
Rat mRNA for D6.1A protein	6.6
Rat CD44 protein mRNA, complete cds	15.8
Rat heat stable CD24 mRNA, complete cds	24.7
Mouse brain cDNA, clone MNCb-2630, similar to Mouse G_1	12.8
Mouse mRNA for myeloid associated differentiation protein	16.3
Rat mRNA for HB2, complete cds	8.3
Mouse tetraspan TM4SF (Tspan-6) mRNA, complete cds	6.9
Mouse mRNA for adhesion protein RA175N, complete cds	5.3
Mouse mRNA for integrin alpha6 subunit	4.5
Mouse oncostatinM specific receptor mRNA, complete cds	4.7
KDEL receptor	4.3
KIAA0404	6.9
Mouse BRI3 mRNA, complete cds	4.4
Rat mRNA for E-cadherin, complete cds	10.7
Rat mRNA for caveolin	4.1
Rat mRNA for ad1-antigen	13.2

[a] Ratio: SH/MH

the appearance corresponded to that of SH proliferation. As SHs proliferated and formed colonies, the distribution of the protein was restricted to the cell membrane and the staining became strong (Fig. 2D and F). Colonies that consisted of $CD44s^+$ cells showed a honeycomb-like appearance (Fig. 2F), but primary cultured MHs showed no CD44s positivity (Fig. 2A and B). NPCs such as liver epithelial

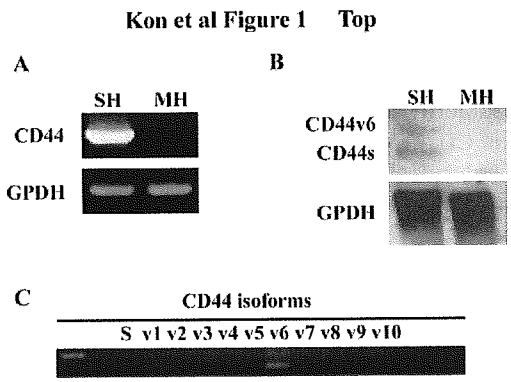

Fig. 1. Expression of CD44 in SHs cultured after cryopreservation. (A) Detection of transcript encoding CD44 by RT-PCR. Primer sets (nos 1 and 2 in Table 1) for CD44 and GPDH were used. Total RNA was extracted from SHs cultured for 2 weeks after cryopreservation. Total RNA of MHs was also prepared from normal rat liver. The expected sizes of PCR products corresponding to CD44 and GPDH were 1095 and 450 bp, respectively. (B) Northern analysis of CD44 in SHs. Twenty micrograms of total RNA was loaded into each lane. The filter was probed with alkaline phosphatase-linked DNA fragments specific for CD44 (upper) and GPDH (lower). Two bands of CD44 were observed in SHs. (C) Analysis of alternative spliced variants of CD44 expressed in SHs. Transcripts encoding CD44 were amplified by RT-PCR using primer sets (nos 1 and 3–13) listed in Table 2. Two spliced variants (S and v6) of CD44 were expressed in SHs.

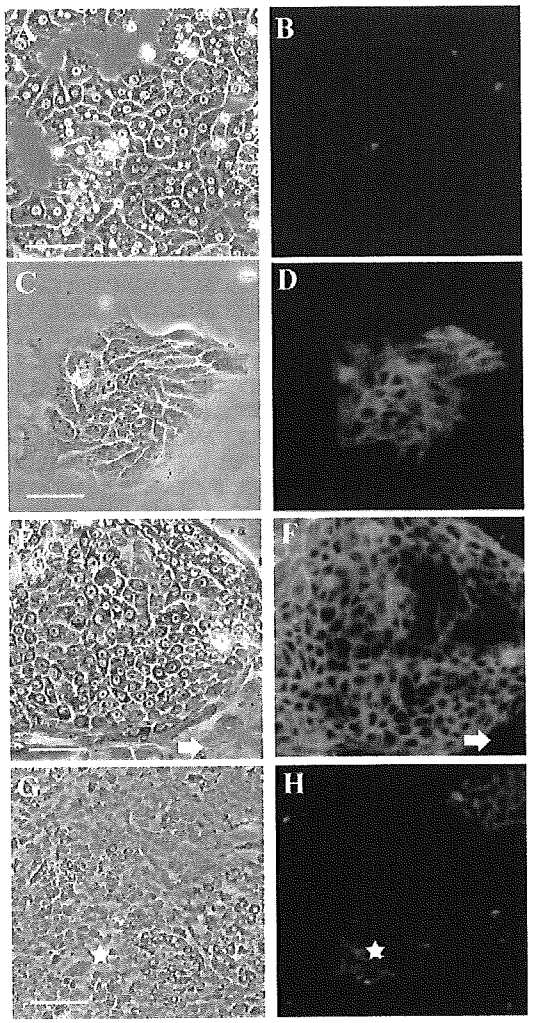

Fig. 2. Immunocytochemistry of CD44 on cultured SHs. Photographs show cell morphology under light microscopy (A, C, E, G) and immunostaining of CD44s (B, D, F, H; green). MHs isolated from a normal liver were cultured for 2 days (A and B). No expression of CD44s was observed in MHs. SHs were cultured for 5 (C and D), 18 (E and F) and 30 days (G and H). Expression of CD44 was initially observed at 5 days and reached the maximum around 18 days. CD44 disappeared from SHs by 30 days. Arrowheads indicate NPCs surrounding the SH colony (E and F). Asterisks also indicate the piled-up SHs (G and H). Expression of CD44s is faintly observed in a large, piled-up SH colony. Scale bars represent 140 μm for A, B, G, and H and 70 μm for C–F.

cells (LECs) and stellate (Ito) cells around SH colonies were not stained for CD44s either (Fig. 2F, arrow). As previously reported [7,8], large and piled-up cells sometimes appeared in SH colonies and were morphologically MHs. As the cells in a colony increased in size, CD44s-positivity gradually decreased and it was hard to detect the positivity in the piled-up cells (Fig. 2G and H, asterisks). The time course of CD44s mRNA expression is shown in Fig. 3A. CD44s expression was clearly detected from day 3 and maintained for about 4 weeks. We also examined the expression of CD44v6 mRNA and protein in SHs. In analysis by RT-PCR,

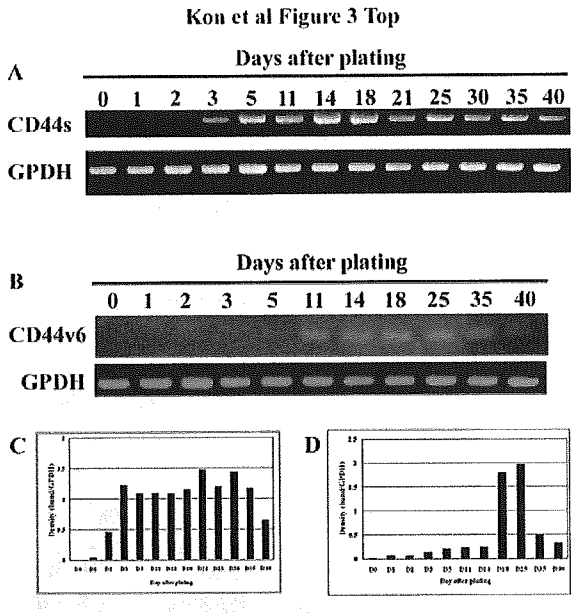

Fig. 3. Time course of CD44s expression in cultured SHs. (A) Detection of transcripts encoding CD44 by RT-PCR. SHs isolated from a normal liver were plated on culture dishes. Total RNA was extracted from cultured cells at each time point (0, 1, 2, 3, 5, 11, 14, 18, 21, 25, 30, 35, and 40 days). (B) Time course of CD44sv6 expression in cultured SHs. Total RNA was extracted from cultured cells at each time point (0, 1, 2, 3, 5, 11, 14, 18, 21, 25, 30, 35, and 40 days). Primer set (no. 9 in Table 1) was used to detect expression of CD44v6. The expected size of the PCR product corresponding to CD44 was 430 bp. (C) Quantitation of CD44s and (D) CD44v6 expression in cultured SHs. The intensity of CD44 bands was measured by NIH image. Figure shows the density of the band normalized by GPDH. The expression of CD44s reached the maximum at around 14 days. On the other hand, maximum expression of CD44v6 was delayed compared to CD44s.

the expression of CD44v6 mRNA was detected later than that of CD44s (Fig. 3B). Immunostaining for CD44v6 showed that, in clusters of cells which were larger than SHs but smaller than binucleate MHs, the cell membranes were sometimes positive (Fig. 4).

3.2. CD44 expression with the maturation of SHs

To investigate whether the expression of CD44 was related to the maturation of SHs, Matrigel® was used to induce maturation. As shown in Fig. 5, when the cells were treated with Matrigel®, the expression of CD44s protein rapidly decreased and no expression was observed at day 4, whereas the expression of C/EBPα in MHs increased at day 4. CD44v6 expression was enhanced at day 2 and then decreased (Fig. 5). Although the expression of CD44s decreased at day 4 after the Matrigel® treatment, that of CD44v6 was not so much decreased at that time. Double staining for CD44s and C/EBPα in cultured SHs revealed that the cells, in which CD44s was strongly expressed in the cell membrane, did not possess C/EBPα$^+$-nuclei (Fig. 6A). In contrast, the cells in which C/EBPα protein was well

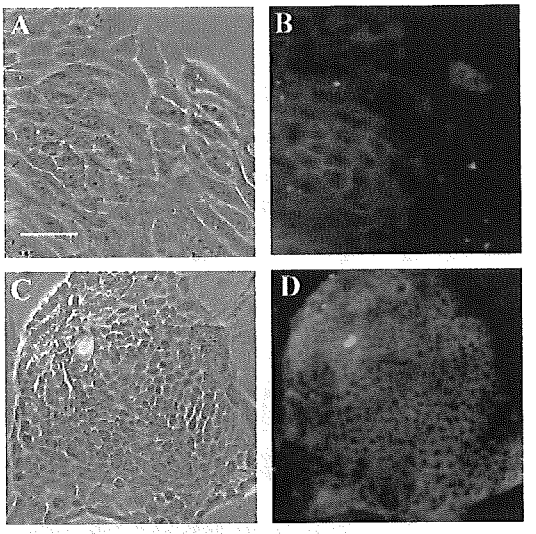

Fig. 4. Expression of spliced variant CD44v6 in cultured SHs. Photographs show cell morphology under light microscopy (A and C) and immunostaining of CD44v6 (B and D; green). SHs were cultured for 18 (A and B) and 30 days (C and D) after plating. CD44v6 appears on lateral surface of SHs.

expressed in the nuclei did not express CD44s in the cell membrane (Fig. 6B).

3.3. CD44 expression in vivo

Next, we examined the existence and distribution of CD44s$^+$ cells in the normal adult rat liver. BECs, lymphocytes, and fibroblastic cells in Glisson's capsule were positive for CD44s, whereas hepatocytes within hepatic lobules were not stained (Fig. 7A). As SHs were reported to appear in the rat liver severely injured by

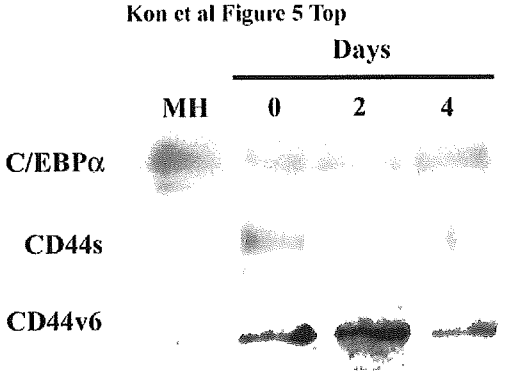

Fig. 5. Expression of C/EBPα CD44s, and CD44v6 in SHs treated with Matrigel. Immunoblotting of CD44s, CD44v6, and C/EBPα. Cell lysates were prepared from Matrigel-treated SHs and MHs. SHs were cultured for 0, 2, and 4 days after Matrigel treatment. Proteins separated by SDS-PAGE under reducing conditions were transferred onto nitrocellulose membranes followed by immunostaining with antibodies specific for C/EBPα (upper panel), CD44s (middle panel), and CD44v6 (lower panel). The expression of CD44s and CD44v6 disappeared as maturation occurred. However, the expression of CD44v6 was delayed compared to CD44s.

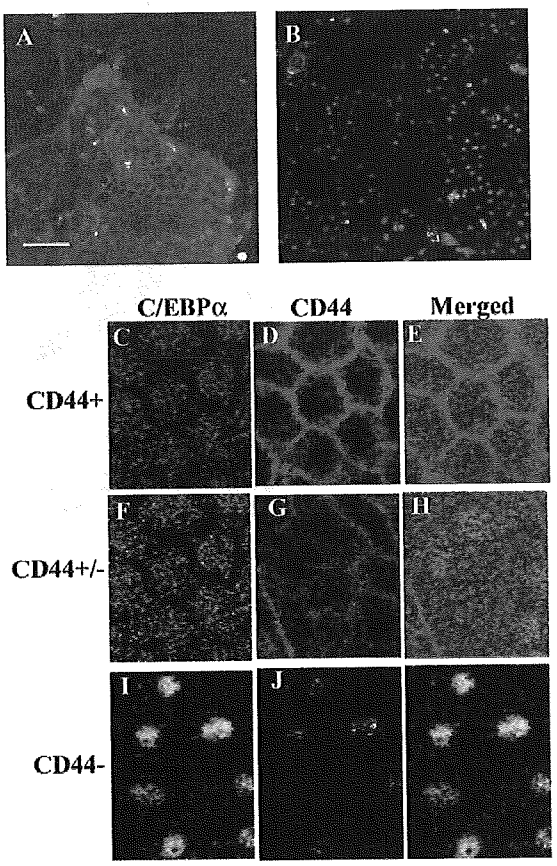

Fig. 6. Immunocytochemistry of CD44s and C/EBPα in cultured SHs. (A) Low-magnification photograph of SHs. Cells were doubly stained with a monoclonal antibody to CD44s (red) and polyclonal antibodies to C/EBPα (green). Expression of C/EBPα is only observed in matured SHs. (A) SHs were cultured for 21 and (B) 31 days after plating. Scale bars, 50 μm. (C–K) High-magnification photographs of SHs. Although CD44-positive cells did not express C/EBPα, negative cells had it in nuclei. Reciprocal expression of CD44s and C/EBPα is observed in cultured SHs.

GalN [22], we examined whether those cells in the liver treated with GalN might express CD44. To further investigate the cell types that positively expressed CD44s, we carried out immunostaining using cell type-specific markers. Frozen liver sections from day 0 (control), and days 1–5 after GalN administration were double stained with antibodies to CD44s and CK19, which is a marker of BECs, and CK19 and Thy1.1, which is a marker of oval cells. Oval cells are known to be hepatic progenitor cells that appear in the GalN injury model. As shown in Fig. 7A, CD44s was expressed around the periportal area at day 0, but at days 3 and 4 $CD44s^+$ cells increased and appeared within liver lobules (Fig. 7G and I). At day 5 the number of $CD44s^+$ hepatocytes decreased compared to that at day 4 (Fig. 7K). At day 2 Thy1.1-positive cells were increased between Glisson's capsule and hepatocytes (Fig. 7F).

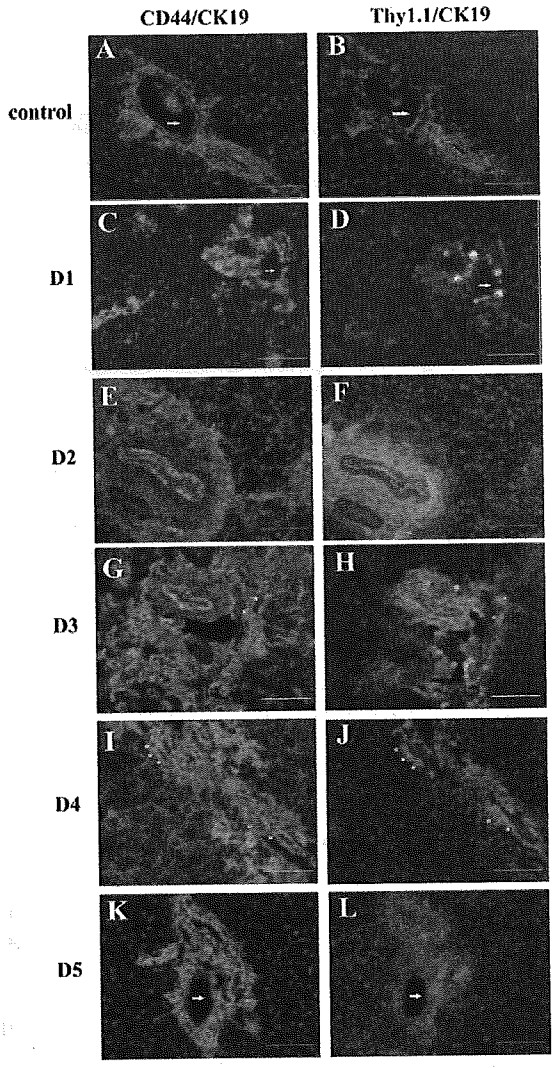

Fig. 7. Identification and localization of CD44s-positive cells in injured liver. Serial frozen sections of GalN-treated rat livers were doubly stained with antibodies to CD44s (green) and CK19 (red) (A, C, E, G, I and K) as well as Thy1.1 (green) and CK19 (red) (B, D, F, H, J and L). CK19 was used to identify BECs and Thy1.1 was used to identify oval cells. Nuclei were stained with DAPI (blue). No treatment: control rat liver; (A and B), D1; (C and D), D2; (E and F), D3; (G and H), D4; (I and J) and D5; (K and L): liver 1–5 days after treatment with GalN. Arrows show periportal region and asterisks show CD44s and Thy1.1 double-positive cells. Bile ducts are surrounded by CK-19-positive cells. Scale bars, 100 μm.

3.4. Sorting of $CD44s^+$ cells

As there were many $CD44s^+/CK19^-$ cells within liver lobules at day 4 after GalN treatment, we decided to isolate the $CD44s^+$ cells from the livers at that day. After $CD44s^+$ cells were sorted using MACS, the cells were cultured for 7 days. As shown in Table 4, about 1×10^6 $CD44s^+$ cells were separated from the GalN-treated rat, whereas few cells were separated from the normal rat. $CD44^+$ cells from the GalN-treated rat could form colonies, but the cells from

Table 4
Recovery rate of sorted CD44$^+$ cells from GalN-treated rat liver

Treatment	Number of rats	Initial number of cells[a] ($\times 10^6$)	Number of sorted cells ($\times 10^6$)	Recovery rate (%)
Control	3	179.0±1.2	>0.01	0
GalN	5	246.0±0.9	1.32±0.55	0.54

[a] MHs were depleted.

the normal rat could not form any colonies. Seven days after plating, most colonies formed in the dishes consisted of SHs (Fig. 8A). In Fig. 8C, the expression of mRNAs for hepatocytes, oval cells and BECs is analyzed by RT-PCR. Many hepatic markers such as albumin, transferrin, CK8, connexin32, HNF4, C/EBPα, cytochrome P450 (CYP) 1A1, and CYP4A3 were expressed in sorted CD44$^+$ cells, whereas there was no expression of markers for oval cells such as c-kit and Thy1.1, or for BECs such as CK19.

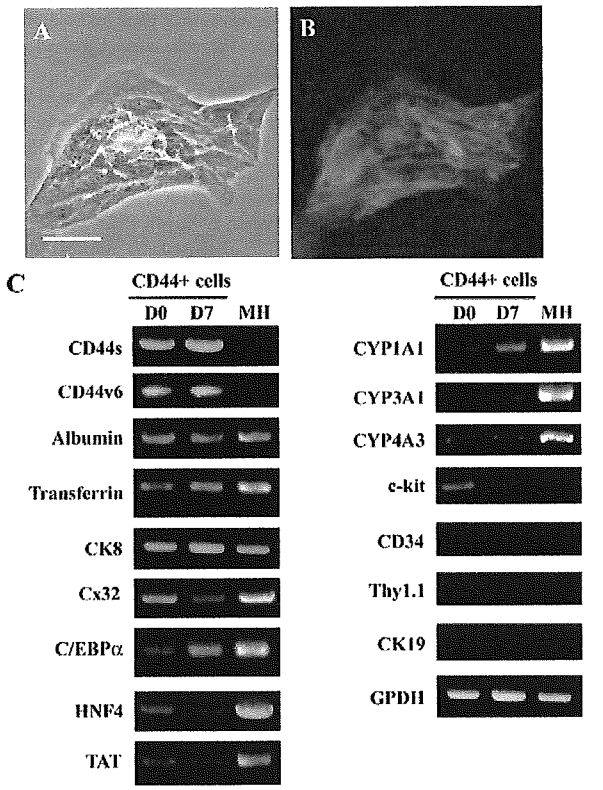

Fig. 8. Characterization of CD44-positive cells sorted from injured liver. (A) Expression of CD44s in the sorted cells. Photographs show cell morphology under light microscopy (left panel) and (B) immunostaining of CD44s in 7-day cultured cells. Scale bars, 70 μm. (C) Expression of hepatic markers in the sorted cells. Hepatic makers were detected by RT-PCR using primer sets (nos 1–3, 14–28). Total RNA was extracted from the sorted cells (day 0) and cultured CD44-positive cells (day 7). MHs were used as a positive control of hepatic maturation. Albumin, transferrin, CK8, Cx32, C/EBPα, HNF4, TAT, CYP1A1, CYP3A1, and CYP4A3 are markers for MHs; c-kit, CD34 for stem cells; Thy1.1 and CK19 for oval cells, and CK19 also for bile ductular cells.

4. Discussion

In the present study, we examined the gene expression profiles of SHs and MHs to find SH-specific proteins. From our analyses, we confirmed that CD44s and CD44v6 were specifically expressed in cultured SHs but not in MHs. In in vivo studies using the GalN-injury model, CD44s$^+$ hepatocytes were observed in the periportal region. The GalN-induced liver injury model was established for activation of facultative liver stem cells, i.e. oval cells. Administration of GalN causes massive necrosis of hepatic parenchymal cells in the pericentral lesion [23], which leads to the activation, proliferation, and differentiation of oval cells into hepatocytes [24–26]. As shown in Fig. 7, Thy1.1$^+$ oval cells appeared at days 2–4 in livers of GalN-treated rats. Although some Thy1.1$^+$ cells possessing relatively large cytoplasms appeared very close to Glisson's capsule at days 3 and 4, MHs with Thy1.1 expression were never observed within lobules. Furthermore, some sorted Thy1.1$^+$ cells expressed CD44s, but sorted CD44s$^+$ cells did not express Thy1.1 (data not shown). Nor could the sorted Thy1.1$^+$ cells from day 2 form any SH colonies, whereas some Thy1.1$^+$ cells from day 3 could form such colonies in our culture conditions (data not shown). These results suggested that some Thy1.1$^+$ oval cells could initially differentiate into SHs and then into MHs in regeneration of the GalN-treated liver. On the other hand, our preliminary results showed that CD44s expression occurred in the expanded SH-like progenitor cells that appeared in the retrorsine/PH-treated rat liver [27,28]. In this model, oval cells did not appear. Within the GalN-liver lobules at days 3 and 4, many CD44s$^+$/Thy1.1$^-$ cells appeared and most sorted CD44s$^+$ cells could form SH colonies in culture. Therefore, it is plausible that many CD44s$^+$ cells may come from MHs.

Expression of CD44s appeared at day 3 after plating of primary prepared SHs and increased at day 3 after GalN administration. These results suggested that CD44s expression could be up-regulated when the growth of SHs is stimulated. Therefore, although the mechanisms are at present unknown, situations where the replication of MHs is not sufficient to compensate for the loss of the cells or where SHs are free from the inhibitory signal(s) of MHs may act as a trigger for the cells, whose latent ability to be progenitors may be silenced.

A preliminary experiment provided a clue to the role of CD44 in SHs. Hyaluronic acid (HA) is a ligand for CD44. Although our data are preliminary, HA can induce the proliferation of SHs. When isolated hepatic cells including SHs and NPCs were plated on HA-coated dishes, only SHs could proliferate to form colonies and the cells expressed CD44s. Although SECs are known to have HA receptors [29], they could not survive more than 1 week. Furthermore, the proliferating SHs on HA could not mature and the CD44s expression was maintained. In SHs, further analyses of the mechanisms by which CD44s can be induced only

in SHs and how CD44 can signal the nucleus should be carried out.

The role of CD44v6 expression may be different from that of CD44s. CD44s may be related to the proliferation of SHs and to the maintenance of the capacity as progenitor cells, whereas CD44v6 may be related to their maturation because the expression of CD44v6 was delayed compared to that of CD44s in SHs and restricted to relatively large SHs. In addition, when SHs were treated with Matrigel®, the appearance of CD44v6 was delayed compared to that of CD44s. When SHs differentiated into MHs, the expression completely disappeared. CD44v6 was reported to be expressed in tumor cells, especially in metastasizing ones, and hematopoietic cells [30]. In the fetal rat liver, both CD44s and CD44v6 are expressed in hematopoietic precursor cells, including megakaryocytes [18]. In the present experiment, when SHs differentiated the cell morphology changed from small to large and piled-up and CD44v6 transiently appeared in the cells. Therefore, the expression of CD44v6 may be related to the initiation of maturation.

In the present experiment, we clarified that CD44 was a specific marker of SHs. The expression of CD44 mRNA and protein was restricted to SHs, and was up-regulated at the time that SHs started to proliferate both in vitro and in vivo. The sorted $CD44s^+$ cells possessed hepatic markers, but no BEC or oval cell markers, except c-kit, were detected. Considering the present and preliminary results, we suggest that SHs are hepatic progenitor cells derived from MHs and some oval cells. Further experiments will be necessary to clarify the exact mechanisms by which their capability as progenitor cells is hidden and how the activation happens.

Acknowledgements

We thank Dr Jonathan P. Sleeman (Forschungszentrum Karlsruhe, Germany) for the generous gift of the anti-rat CD44v6 antibody, and Dr Atsushi Miyajima (University of Tokyo, Tokyo, Japan) for the generous gift of the anti-rat CK19 antibody. We thank Dr Toshimitsu Uede (University of Hokkaido, Japan) and Dr Yan-Jun Jia for suggestive discussions, Ms Chieko Doi, Ms Fumie Saito and Ms Minako Kuwano for technical assistance. We also thank Mr K. Barrymore for help with the manuscript.

This study was supported by grants from the Science and Technology Incubation Program in Advanced Regions, the Japan Science and Technology Agency and the Ministry of Education, Culture, Sports, Science and Technology, Japan; 14370393 and 17390353 for T. Mitaka, and Ministry of Health, Labour and Welfare, Health and Labour Sciences Research Grants, Research on Advanced Medical Technology for T. Mitaka.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhep.2006.01.029

References

[1] Mitaka T, Sattler CA, Sargen LM, Pitot HC. Multiple cell cycles occur in rat hepatocytes cultured in the presence of nicotinamide and epidermal growth factor. Hepatology 1991;13:21–30.
[2] Mitaka T, Mikami M, Sattler GL, Pitot HC, Mochizuki Y. Small cell colonies appear in the primary culture of adult rat hepatocytes in the presence of nicotinamide and epidermal growth factor. Hepatology 1992;16:440–447.
[3] Mitaka T, Kojima T, Mizuguchi T, Mochizuki Y. Growth and maturation of small hepatocytes isolated from adult liver. Biochem Biophys Res Commun 1995;214:310–317.
[4] Tateno C, Yoshizato K. Long-term cultivation of adult rat hepatocytes that undergo multiple cell divisions and express normal parenchymal phenotypes. Am J Pathol 1996;148:383–392.
[5] Mitaka T, Sattler GL, Pitot HC, Mochizuki Y. Characteristics of small cell colonies developing in primary cultures of adult rat hepatocytes. Virchows Arch B Cell Pathol Incl Mol Pathol 1992;62:329–335.
[6] Tateno C, Yoshizato K. Growth and differentiation in culture of clonogenic hepatocytes that express both phenotypes of hepatocytes and biliary epithelial cells. Am J Pathol 1996;148:1593–1605.
[7] Mitaka T, Sato F, Mizuguchi T, Yokono T, Mochizuki Y. Reconstruction of hepatic organoid by rat small hepatocytes and hepatic nonparenchymal cells. Hepatology 1999;29:111–125.
[8] Sugimoto S, Mitaka T, Ikeda S, Harada K, Ikai I, Yamaoka Y, et al. Morphological changes induced by extracellular matrix are correlated with maturation of rat small hepatocytes. J Cell Biochem 2002;1:16–28.
[9] Ikeda S, Mitaka T, Harada K, Sugimoto S, Hirata K, Mochizuki Y. Proliferation of rat small hepatocytes after long-term cryopreservation. J Hepatol 2002;37:7–14.
[10] Jalkanen S, Bargatze RF, de los Toyos J, Butcher EC. Lymphocyte recognition of high endothelium: antibodies to distinct epitopes of an 85–95 kDa glycoprotein antigen differentiallly inhibit lymphocyte binding to lymph node, mucosal, or synovial endothelial cells. J Cell Biol 1987;105:983–990.
[11] Stamenkovic I, Amiot M, Pesando JM, Seed B. A lymphocyte molecule implicated in lymph node homing is a member of the cartilage link protein family. Cell 1989;56:1057–1062.
[12] DeGrendele CH, Estess P, Siegelmen MH. Requirement for CD44 in activated T cell extravasation into an inflammatory site. Science 1996;278:672–675.
[13] Aruffo A, Stamenkovic I, Melnik M, Underhill CB, Seed B. CD44 is the principal cell surface receptor for hyaluronate. Cell 1990;61:1303–1313.
[14] Arch RK, Wirth M, Hofmann H, Ponta S, Herrlich P, Zöller M. Participation in normal immune response of a metastasis-inducing splice variant of CD44. Science 1992;257:182–185.
[15] Gunthert U, Hofmann M, Rudy W, Reber S, Zöller M, Haussmann S, et al. A new variant of glycoprotein CD44 confers metastatic potential to rat carcinoma cells. Cell 1991;65:13–24.
[16] Mackay CR, Terpe HJ, Stauder R, Marston WL, Stark H, Gunthert U. Expression and modulation of CD44 variant isoforms in humans. J Cell Biol 1994;124:71–82.
[17] Stauder R, Eisterer W, Thaler J, Gunthert U. CD44 variant isoforms in non-Hodgkin's lymphoma: a new independent prognostic factor. Blood 1995;85:2885–2899.

[18] Weber B, Rosel M, Arch R, Möller P, Zöller M. Transient expression of CD44 variant isoforms in the ontogeny of the rat: Ectoderm-, endoderm-, and mesoderm-derived cells express different exon combinations. Differentiation 1996;60:17–229.
[19] Salles G, Zain M, Jiang WM, Boussiotis VA, Shipp MA. Alternatively spliced CD44 transcripts in diffuse large-cell lymphomas: characterization and comparison with normal activated B cells and epithelial malignancies. Blood 1993;82:3539–3547.
[20] Seglen PO. Preparation of isolated rat liver cells. Methods Cell Biol 1976;13:29–83.
[21] Iizuka J, Katagiri Y, Tada N, Murakami M, Ikeda T, Sato M, et al. Introduction of an osteopontin gene confers the increase on B1 cell population and the production of anti-DNA autoantibodies. Lab Invest 1998;12:1523–1533.
[22] Dabeva MD, Hurston E, Shafriz DA. Transcription factor and liver-specific mRNA expression in facultative epithelial progenitor cells of liver and pancreas. Am J Pathol 1995;147: 1633–1648.
[23] Decker K, Keppler D. Galactosamine hepatitis: key role of the nucleotide deficiency period in the pathogenesis of cell injury and cell death. Rev Physiol Biochem Pharmacol 1974;71:78–106.
[24] Lamire JM, Shiojiri N, Fausto N. Oval cell proliferation and the origin of small hepatocytes in liver injury induced by D-galactosamine. Am J Pathol 1991;139:535–552.
[25] Dabeva MD, Shafritz DA. Activation, proliferation, and differentiation of progenitor cells into hepatocytes in the D-galactosamine model of liver regeneration. Am J Pathol 1993;143:1606–1620.
[26] Dabeva MD, Alpini G, Hurston E, Shafritz DA. Models for hepatic progenitor cell activation. Proc Soc Exp Biol Med 1993;204:242–252.
[27] Gordon GJ, Coleman WB, Grisham JW. Temporal analysis of hepatocyte differentiation by small hepatocyte-like progenitor cells during liver regeneration in retrorsine-exposed rats. Am J Pathol 2000;157:771–786.
[28] Gordon GJ, Butz GM, Grisham JW, Coleman WB. Isolation, short-term culture, and transplantation of small hepatocyte-like progenitor cells from retrorsine-exposed rats. Transplantation 2002;73(8): 1236–1243.
[29] Goodison S, Urquidi V, Tarin D. CD44 cell adhesion molecules. J Clin Pathol Mol Pathol 1999;52:189–196.
[30] Smedsrød B, Pertoft H, Eriksson S, Fraser JRE, Laurent TC. Studies in vitro on the uptake and degradation of sodium hyaluronate in rat liver endothelial cells. Biochem J 1984;223:617–626.

Thermoreversible Gelation Polymer Induces the Emergence of Hepatic Stem Cells in the Partially Injured Rat Liver

Masaki Nagaya,[1,4] Sunao Kubota,[2] Noboru Suzuki,[3] Katsuya Akashi,[1] and Toshihiro Mitaka[4]

Focal injury of the adult liver causes formation of granulomatous tissue and fibrosis. When thermoreversible gelation polymer (TGP) was applied to such defects of the rat liver, complete recovery of hepatic tissues was observed without granulation. We analyzed the mechanism of the regeneration. TGP is a chemically synthesized biocompatible polymer material whose sol-gel transition is reversible by changing the temperature. Cooled TGP was poured into a penetration lesion of the rat liver. Immunohistochemistry and polymerase chain reaction were carried out using tissues and cultured cells isolated from ductular structures. Immunocytochemical and ultrastructural analyses were also conducted. Seven days after TGP treatment, ductular reactions were observed around the wound and ductules elongated to the injured area. Cells in the structures were alpha-fetoprotein (AFP) positive, albumin$^+$, CK19$^+$, c-Kit$^+$, and Thy1$^+$. Hepatocyte-like cells possessing glycogen appeared around the tips of the ductules from day 9. The defect was completely replaced with hepatocytes by day 28. Cells isolated from the ductules expressed Musashi-1, c-Kit, Thy1, AFP, albumin, transferrin, connexin 43, and CK19. When the cultured cells were covered by TGP, they rapidly proliferated to form colonies, whereas without TGP cells gradually died. Morphologically and ultrastructurally the cells were similar to hepatocytes. They expressed not only albumin and transferrin but TAT, CYP2E1, and CCAAT/enhancer binding protein α. Some cells formed bile canaliculus-like structures. In conclusion, TGP may trigger the initiation of hepatic stem cells in biliary ductules, and stem cell activation may occur even in the regeneration of the normal liver. (HEPATOLOGY 2006;43:000-000.)

The mechanisms of regeneration in focal liver injury are not well understood. We found that the defect made by focal injury was usually replaced by granulomatous tissue. When we used thermoreversible gelation polymer (TGP) as a sealing material for the injury, efficient regeneration occurred without any granulation.[1] TGP is a chemically synthesized biocompatible polymer material.[2] It is soluble below a lower critical solution temperature (LCST) and becomes solid above the LCST. When soluble TGP is applied, it can infiltrate into any part. Thereafter, it immediately changes into gel at body temperature and causes complete sealing of the defect, leading to efficient regeneration of the liver.[1] However, the mechanisms of the regeneration with TGP treatment have largely remained unknown.

The liver appears to have three distinct mechanisms of regeneration in response to loss of hepatocytes: (1) Mature hepatocyte (MH)s proliferate in case of partial hepatectomy and centrolobular injury by hepatotoxins.[3-5] Surviving hepatocytes immediately proliferate and restore the original mass[4]; (2) When the proliferation of hepatocytes is inhibited by some toxins such as 2-acetylaminofluorene, hepatic growth stimulation results in emergent ductules, and the cells in the ductules gradually migrate into the hepatic parenchyma.[3-5] "Oval cells" or "ductular hepatocytes" are involved in the ductular reaction in rodents.[5,6] These cells may be derived from putative stem cells and may differentiate into hepatocytes; (3) Bone

Abbreviations: TGP, thermoreversible gelation polymer; LCST, lower critical solution temperature; MH, mature hepatocyte; NIH, National Institutes of Health; HE, hematoxylin-eosin; PAS, periodic acid-Schiff; TEM, transmission electron microscopy; AFP, alpha-fetoprotein; CK19, cytokeratin 19; PCNA, proliferating cell nuclear antigen; C/EBPα, CCAAT/enhancer binding protein α; MRP2, multidrug-resistance associated protein 2; BEC, bile epithelial cell; BDL, common-bile-duct ligation; NT, no treatment; CG, collagen gel; FG, fibrin glue; POD, post operative day; BC, bile canaliculi.

From the [1]Department of Emergency and Critical Care Medicine; the [2]Department of General Surgery; the [3]Department of Immunology and Medicine; St. Marianna University, School of Medicine, Kawasaki, and the [4]Department of Pathophysiology, Cancer Research Institute, Sapporo Medical University School of Medicine, Sapporo, Japan.

Received October 14, 2005; accepted February 13, 2006.

Supported by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan (15790708 to M.N. and 14370393, 17390353 to T.M.) and the Marumo Fund (to M.N).

Address reprint requests to: Masaki Nagaya, M.D., Ph.D., Department of Emergency and Critical Care Medicine, St. Marianna University, School of Medicine, Kawasaki 261-8511, Japan. E-mail: m2nagaya@marianna-u.ac.jp; fax: (81) 44-979-1522.

Published online in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/hep.21153

Potential conflict of interest: Nothing to report.

marrow–derived cells may participate in hepatic regeneration.[4,5,7]

Here we report that, in the hepatic regeneration of focal injury by TGP, ductular reactions are initially induced, and hepatic stem cells involved in the ductules may differentiate into hepatocytes. In addition, cells isolated from the ductules induced by TGP treatment can rapidly proliferate and differentiate into hepatic cells when the cells are covered by TGP. Thus, hepatic stem cells may participate in hepatic regeneration even when no hepatotoxins are involved in the injury.

Materials and Methods

Animals and Surgery. Adult male Fisher 344 rats were obtained from Charles River (Atsugi, Kanagawa, Japan). All animals received humane care, and the experimental protocol was approved by the Committees of Laboratory Animals of St. Marianna and Sapporo Medical Universities and was in accordance with National Institutes of Health (NIH) guidelines. The total number of sacrificed rats was over 150, and at least five animals/point were examined. A penetrating, 4-mm-diameter defect was made in left middle lobe of the liver at a distance of 10 mm from lobular edges. The rats were then randomly assigned into three groups: penetration alone (control), penetration filled with fibrin glue (FG, Kaketsuken, Kumamoto, Japan), and penetration filled with TGP (TGP, Mebiol, Tokyo, Japan). FG and TGP (0.5 mL) were poured into the penetrated site.

Preparation of TGP. N-isopropylacrilamide, Eastman Kodak, New York, NY) was recrystallized from acetone and copolymerized with N-acryloxysuccinimide (Kokusan Chemical, Tokyo, Japan) to provide an activated form of Poly- N-isopropylacrilamide. Polymerization was carried out in $CHCl_3$, using azobisisobutyronitrile as the initiator. The activated copolymer was precipitated with diethylether and recovered. Then the copolymer and diamino-polyethylene glycol (Kawaken Fine Chemicals, Tokyo, Japan) were dissolved in $CHCl_3$ and reacted. The byproduct, N-hydroxylsuccinimide, was removed and the remaining solution was lyophilized to yield TGP. Saline was added to the TGP to adjust it to 5.5 % (wt/wt). The LCST was approximately 20°C[2], and toxicity of TGP was not observed.[1]

Histology. Liver tissues were obtained from the injured lobe, and other lobes were used as a control. Liver specimen (diameter 10 mm) surrounding the injured region was enucleated in a cylindrical shape. The specimen was cut in half, and both paraffin and frozen samples were prepared. The ventral halves of the specimens were fixed with 4% paraformaldehyde in phosphate-buffered saline and embedded in paraffin. To evaluate the size of the injury lesion, the area of fibrosis in hematoxylin-eosin (HE)-stained sections was measured and analyzed using a light microscope equipped with a CCD camera and AxioVision AC Rel. 4.1 software (Carl Zeiss, Jena, Germany). In addition, to evaluate the hepatic lobule size, the distance between portal veins was measured. The proximal portion less than 3 mm from the injured site and the distal portion farther than 5 mm from the injured site were evaluated. Periodic acid-Schiff (PAS)-staining was performed to examine the production of glycogen. Diastase was applied to test whether PAS-positive materials were glycogen.[8] The procedure used for transmission electron microscopy (TEM) was previously described.[9]

Immunostaining. Antibodies to c-Kit (Santa Cruz, CA), Thy1 (Pharmingen, Hamburg, Germany), α-fetoprotein (AFP; Dako Cytomation, Kyoto, Japan), albumin (Dako Cytomation), cytokeratin 19 (CK19; Novocastra Laboratories, Newcastle, UK and a gift of Dr. A. Miyajima, Tokyo University, Japan), proliferating cell nuclear antigen (PCNA; Dako Cytomation), Ki67 (Pharmingen), CCAAT/enhancer binding protein α (C/EBP α; Santa Cruz), and multidrug-resistance associated protein 2 (MRP2; Alexis Biochemichals, San Diego, CA) were used. The methods used for immunohistochemistry and immunocytochemistry were previously described.[9] 3, 3′-Diaminobenzidine and BCIP/NBT (Dako Cytomation) were used as a substrate for colorization. $Alexa^{488}$-conjugated and $Alexa^{594}$-conjugated antibodies (Molecular Probes, Eugene, OR) were used as secondary antibodies.

CK19 and Albumin Double-Positive Cells in the Ductules. Immunohistochemistry for CK19/albumin was carried out. The number of $CK19^+$/$albumin^+$ cells in the ductules in the upper right quadrant area was counted, which included part of the injured areas. Simultaneously, we measured the size of the area by using NIH image software.

Isolation and Culture of Ductular Cells. Intrahepatic biliary epithelial cell (BEC)s were separated from normal, common-bile-duct-ligated (BDL), and TGP-treated rat livers. To isolate the cells from the injury lesion, two-step liver perfusion[9] was initially carried out 1 week after operation. The tissue was enucleated in a cylindrical shape (diameter 10 mm) surrounding the injured site and transferred into a Petri dish. After hepatocytes were removed, the remnant tissues were collected, transferred into a flask, and then treated with collagenase, dispase (Godo Shyusei, Tokyo, Japan) and hyaluronidase (Sigma, St Louis, MO) for 30 minutes. The digested tissues were suspended in Dulbecco's modified Eagle's medium and centrifuged at 150*g* for 10 minutes. The pellet was resuspended in the medium, filtered