Size-Dependent *in Vivo* Growth Potential of Adult Rat Hepatocytes

Shigeru Katayama,^{*†} Chise Tateno,^{*}
Toshimasa Asahara,[†] and Katsutoshi Yoshizato^{*‡}

From the Hiroshima Tissue Regeneration Project,^{*} Hiroshima Prefecture Joint-Research Project for Regional Intensive, Japan Science and Technology Corporation, Hiroshima Prefectural Institute of Industrial Science and Technology, Higashibiroshima; the Department of Second Surgery,[†] School of Medicine, Hiroshima University, Higashibiroshima; and the Developmental Biology Laboratory,[‡] Department of Biological Science, Graduate School of Science, Hiroshima University, Higashibiroshima, Hiroshima, Japan

The present study was performed to determine whether hepatocytes show a size-dependent growth *in vivo* using as a growth assay system, a retrorsine/partial hepatectomy model of dipeptidyl dipeptidase IV-deficient (DPPIV⁻) mutant Fischer rats. Nearly pure populations of small hepatocytes (SHs) and parenchymal hepatocytes (PHs) were prepared from DPPIV⁺ rats. The same number of these SHs and PHs was transplanted into the liver of retrorsine-treated and two-thirds partial hepatectomized DPPIV⁻ rats. At 21 days after transplantation, colonies derived from donor hepatocytes were detected as DPPIV⁺ cells by enzyme histochemistry. SHs were approximately three times more proliferative than PHs (673 ± 25 cells/colony *versus* 226 ± 10 cells/colony, mean $\pm$ SE). SHs were subfractionated by a fluorescence-activated cell sorter into SH-R2s and SH-R3s. SH-R3s showed a lower extent of granularity and autofluorescence, and a smaller size than SH-R2s that showed characteristics similar to PHs. The growth potential of SH-R3s assayed as above was approximately three times higher than that of SH-R2s ($1,101 \pm 46$ cells/colony *versus* 341 ± 13 cells). These results indicate that the *in vivo* growth potential of hepatocytes is heterogeneous and is correlated with their size, and the extent of their granularity and autofluorescence. (Am J Pathol 2001, 158:97-105)

Since a two-step collagenase perfusion method to isolate parenchymal hepatocytes (PHs) was established by Seglen,¹ extensive studies have been performed aiming at understanding the proliferation and differentiation of the hepatocytes using disaggregated and cultured rat hepatocytes. However, these cells cannot generally be maintained for a long term as replicating differentiated cells.

Recently, we have developed a culture method by which hepatocytes can grow forming clonal colonies for a long term.² This culture focused on small hepatocytes (SHs) present in the nonparenchymal cell fraction. We showed that the SHs have a higher growth potential than PHs.³ We further characterized SHs and PHs by subjecting them to fractionation by a fluorescent-activated cell sorter (FACS).³ SHs produced two cell populations, SH-R2s and SH-R3s.³ The former showed a higher granularity and autofluorescence than the latter.³ In contrast, PHs produced only one population (PH-R2s) whose FACS characteristics were similar to the SH-R2s.³ The size of hepatocytes of SH-R3s (17.1 ± 0.2 μ m, mean $\pm$ SE) as measured by diameter was smaller than those of SH-R2s (22.6 ± 0.5 μ m) and PH-R2s (24.1 ± 0.1 μ m), and there was not a significant overlap in the size distribution between the two groups.³ SH-R3s were highly replicative *in vitro*, their growth potential being four or five times higher than that of SH-R2s and PH-R2s.³ Apparently, the extent of cytoplasmic granularity and autofluorescence of hepatocytes was reversely related to their growth potential. Although little is known about the relationship between the extent of autofluorescence and the maturation of hepatocytes, there has been a report that related the increased granularity to the maturation of endoplasmic reticula, Golgi apparatus, lysosomes, and mitochondria of hepatocytes.⁴

Sigal and colleagues⁵ actually compared the extent of autofluorescence and granularity of hepatocytes among embryos, sucklings, and adults by FACS. Hepatocytes increased their granularity and autofluorescence as the liver developed from fetus to suckling, and to adult.⁵ Conversely the proportion of S-phase cells progressively declined during this developmental process.⁵ They also showed that the adult liver contains small and mononuclear hepatocytes whose granularity and autofluorescence were comparable to fetal hepatocytes.⁵ Our previous studies support the results obtained by the cited authors and suggest that not only the fetal but also the adult rat liver contains a population of hepatocytes that are small in size and highly replicative.^{2,3}

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Shigeru Katayama and Chise Tateno contributed equally to this work.

Address reprint requests to Katsutoshi Yoshizato, Ph.D., Developmental Biology Laboratory, Department of Biological Science, Graduate School of Science, Hiroshima University, 1-3-1, Kagamiyama, Higashihiroshima, Hiroshima 739-8526, Japan. E-mail: kyoshiz@hiroshima-u.ac.jp.

Laconi and co-workers⁶ described a retrorsine/partial hepatectomy rat model to assess the repopulation potential of transplanted hepatocytes in which exogenous hepatocytes can almost completely replace the host hepatocytes. The model is based on the mitoinhibitory effect of a retrorsine, pyrrolizidine alkaloid, on hepatocytes in the resident liver where transplanted hepatocytes can proliferate. The host and transplanted hepatocytes were distinguished by using dipeptidyl peptidase IV-deficient (DPPIV⁻) mutant Fischer rats and their wild-type counterparts (DPPIV⁺), respectively.⁶

The present study was performed to determine whether the growth potential of hepatocytes is heterogeneous and is correlated with their size, and the extent of their granularity and autofluorescence also *in vivo* as *in vitro*. The PHs, SHs, SH-R2s, and SH-R3s were prepared from DPPIV⁺ rats and were transplanted into the retrorsine/partial hepatectomy DPPIV⁻ rat model to assess their replication potential as an index of growth ability. This *in vivo* experiment clearly demonstrated that SH-R3 hepatocytes are highly proliferative also *in vivo* as compared to SH-R2s and PHs. The present study strongly suggests that the relationship between the size and growth potential of hepatocytes has some biological meaning in the physiological and pathological processes taking place in the liver.

Materials and Methods

Preparation of Hepatocytes

Fractions of SHs and PHs were prepared as described before³ with some modifications. Disaggregated liver cells were obtained from 10-week-old Fischer male rats by the two-step collagenase perfusion method¹ and were centrifuged at $50 \times g$ for 2 minutes. The pellet was centrifuged through 45% Percoll at $50 \times g$ for 24 minutes.³ The pellet thus obtained was further centrifuged at $50 \times g$ for 1 minute, and the pellet and supernatant were used as a fraction of PHs and SHs, respectively.

SHs and PHs were subfractionated by a cell sorter, FACS Vantage (Becton Dickinson, Mountain View, CA) with a 100- μ m nozzle.³ Fluorescence excited at 488 nm was measured through a 530-nm filter (FL1) and a 575-nm filter (FL2) with a 4-decade logarithmic amplification. To measure physical characteristics of the cells, a linear amplification was used for the forward scatter, which is a measure of cell size, and a 4-decade logarithmic amplification for the side scatter, which is a measure of cytoplasmic complexity. The optical bench was calibrated at a fixed amplitude and a photomultiplier voltage using fluorescent polystyrene beads (Fluorobrite Calibration Grade 6- μ m YG microspheres; Polysciences, Inc., Warrington, PA) and the instrument was used in the conditions in which these beads fell in the same peak channels. For all cell preparations tested, propidium iodide was added to cell suspensions at a concentration of 1 μ g/ml, and the cells excluding the dye (viable cells) were sorted for further investigations. Data obtained by FACS experiments were analyzed using a CELLQuest software (Becton Dickinson). Hepatocytes which had ex-

cluded dyes of trypan blue were photographed and their diameters were determined using a NIH Image version 1.62 (NIH, Bethesda, MD).

Hepatocyte Transplantation

Replicative potential of hepatocytes prepared from DPPIV⁺ male Fischer rats was determined as an index of growth ability by transplanting them into DPPIV⁻ Fischer female rats following the protocol described by Laconi.⁶ The DPPIV⁻ rats weighing 130 to 140 g were given two intraperitoneal injections of retrorsine of 30 mg/kg body weight, 2 weeks apart. Four weeks after the second injection, the animals were subjected to two-thirds partial hepatectomy. Hepatocytes prepared as above from DPPIV⁺ male rats were transplanted into the above DPPIV⁻ rats via the portal vein. The number of transplanted cells was 2×10^5 cells for PHs and SHs, and 1.5×10^5 cells for SH-R2s and SH-R3s. Each of these four fractions of hepatocytes was transplanted to five individual rats. All animals were harvested at 21 days after transplantation and the liver was examined for detecting the transplanted hepatocytes.^{7,8} Cryosections (10- μ m thick) were prepared from the liver, fixed in ice-cold acetone for 5 minutes, air-dried, and washed for 5 minutes in ice-cold 95% ethanol. The sections were air-dried and incubated for 40 to 60 minutes in a substrate reagent consisting of 0.5 mg/ml gly-pro-methoxy- β -naphthylamide (Sigma Chemical Co., St. Louis, MO), 1 mg/ml Fast Blue BB (Sigma Chemical Co.), 100 mmol/L Tris-maleate, pH 6.5, and 100 mmol/L NaCl. Then the sections were washed with phosphate-buffered saline and fixed in 10% formaldehyde. All tissue sections were counterstained with hematoxylin. An identical transplantation of DPPIV⁺ SHs was made as a control experiment, in which the host mutant animals did not receive the retrorsine exposure, but received partial hepatectomy. Only small colonies containing two or three transplanted SHs were formed in this transplantation experiment, indicating the usability of this retrorsine-hepatectomy animal model to assess the *in vivo* growth potential of hepatocytes.

Morphometric Analysis

Transplanted hepatocytes homed to the liver, started to replicate there, and formed small colonies at an early phase after transplantation. We made semiserial sections with a thickness of 10 μ m at intervals of 100 μ m from liver specimens. Different parts of a colony were seen in several different serial sections when the diameter of the colony was $>100 \mu$ m, which corresponds to 7,850 μ m² in area. We selected the section in which the longest diameter for the colony concerned was seen, and calculated the colony area using this diameter. For a colony whose diameter was $<100 \mu$ m, we assumed that the diameter measured in a section is the longest diameter of the colony and calculate the colony area using this diameter. We only measured the colonies that consisted of more than eight transplanted hepatocytes that were oval or round in shape. To quantitate the area of these colonies,

40 portal areas were examined in each animal at 21 days after transplantation. The volume of a colony was calculated from the area of the colony assuming that the colony was in a form of sphere and that the cross-section was made along the maximum diameter of the sphere. To calculate the mean diameter of cells in colonies formed from transplanted hepatocytes, the area and cell number of 10 colonies were measured. The mean cell number in a colony was calculated using parameters of the area or volume of the colony, and the mean cell diameter of hepatocytes.

Detection of Liver-Specific Markers

Cryosections with a thickness of 5 μm were prepared from the liver transplanted with hepatocytes, fixed in -20°C acetone for 5 minutes, and used for immunohistochemical detection of liver-related proteins. Primary antibodies used were: rabbit anti-rat albumin (Cappel, Durham, NC), mouse monoclonal antibody against cytokeratin 19 (CK19, Amersham), and rabbit anti-rat α -fetoprotein antiserum (α -FP, a gift from Dr. T. Mitaka, Sapporo Medical University). The antibodies were visualized by a Vectastain ABC kit (Vector Laboratories, Burlingame, CA) using diaminobenzidine as a substrate.

Engraftment Index of Transplanted Hepatocytes

PHs and SHs were prepared from DPPIV⁺ rats and 2×10^5 cells of each of them were transplanted into a retrorsine-treated and hepatectomized mutant DPPIV⁻ rat liver via the portal vein. We made liver cryosections after weighing liver mass at 48 hours after transplantation. The approximate numbers of total hepatocytes were calculated from the liver weight using the value of $(115 \pm 15) \times 10^6$ hepatocytes/g liver.⁹ We calculated a ratio of transplanted to host hepatocytes by counting their numbers on tissue sections. engraftment index was calculated by the ratio of transplanted to host hepatocytes, total number of hepatocytes of the liver, and the number of transplanted hepatocytes.

Statistical Analysis

The area of colonies formed from transplanted hepatocytes was measured with a NIH image version 1.62 software and the data were analyzed with Stat view version 5.0 software (SAS Institute Inc., Cary, NC). Results were expressed as mean $\pm$ SE, and the significance of difference was analyzed by Student's *t*-test when data were normally distributed and otherwise by Mann-Whitney rank sum test. A *P* value of <0.05 was considered significant.

Results

Size Characterization of Fractionated Hepatocytes

SHs and PHs were prepared and placed on nonadhesive dishes. Their diameter was determined as a measure of

Figure 1. FACS profiles of SHs. SHs were obtained from a 10-week-old male rat (250 g) and separated by FACS. SHs were analyzed on parameters of side scatter (SSC) and FL2 (A). The cells marked with propidium iodide (PI) were those that incorporated propidium iodide and were of a higher fluorescence. These cells were not viable. The cells marked with FR were agranular and less autofluorescent. These cells represented debris of fragmented cells. The R1 fractions of SHs (SH-R1s) were further analyzed using parameters of side scatter and FL2, and two cell populations (SH-R2s and SH-R3s) were identified (B). SH-R2s were relatively more granular and autofluorescent than SH-R3s.

cell size. SHs showed a diameter of 16.3 to 28.1 μm , and PHs showed a diameter of 22.9 to 34.2 μm , the average being $21.5 \pm 0.4 \mu\text{m}$ and $26.5 \pm 0.4 \mu\text{m}$, respectively, as reported previously.³ We showed that SHs could be subfractionated into SH-R2s and SH-R3s by FACS.³ The former showed a greater extent of granularity and autofluorescence than the latter (Figure 1A). In contrast, PHs produced only one population (PH-R2s) that corresponded to the SH-R2s with respect to their size, and the

Figure 2. Colonies derived from transplanted hepatocytes. SHs, PHs, SH-R2s, and SH-R3s were obtained from DPPIV⁺ male Fischer rats and transplanted into the retrorsine/partial hepatectomy-treated DPPIV⁻ female rats via the portal vein at 2×10^5 and 1.5×10^5 cells for PHs (A) and SHs (B), and SH-R2s (C) and SH-R3s (D), respectively. The animals were sacrificed and the livers were harvested at 21 days after transplantation. Cryosections of the livers were subjected to DPPIV histochemical staining. Recipient hepatocytes were negative for the staining, and the transplanted hepatocytes derived from DPPIV⁺ rats were stained brown-red. The size of the positive colonies formed from SHs and SH-R3s were larger than those by PHs and SH-R2s, respectively. Scale bar, 100 μ m.

extent of granularity and autofluorescence. SHs were fractionated into SH-R2s and SH-R3s by FACS (Figure 1B). Mean diameter of freshly isolated SH-R2s and SH-R3s was $22.5 \pm 0.13 \mu\text{m}$ ($n = 5$) and $17.7 \pm 0.10 \mu\text{m}$ ($n = 5$), respectively. The difference between SH-R2s and SH-R3s was statistically significant ($P < 0.0001$, Student's *t*-test). These size characteristics were in good agreement with the previously reported ones.³

Transplantation of Hepatocytes

PHs and SHs were prepared from DPPIV⁺ rats and 2×10^5 cells of each of them were transplanted into a retrorsine-treated and hepatectomized mutant DPPIV⁻ rat liver via the portal vein. Megalocytes appeared in the retrorsine-treated liver from 7 to 14 days after partial hepatectomy (see, for example, Figure 5), indicating that the retrorsine actually produced its effect on the liver in the present study as reported previously.⁶ Their engraftment index determined at 48 hours after transplantation was $11.0 \pm 6.4\%$ ($n = 3$) for PHs and $8.9 \pm 2.2\%$ ($n = 3$) for SHs.

Four fractions of hepatocytes, PHs, SHs, SH-R2s, and SH-R3s were prepared from wild rats, and 2×10^5 cells of each of PHs and SHs, and 1.5×10^5 cells of each of

SH-R2s and SH-R3s were transplanted into the mutant rats. DPPIV-positive colonies derived from PHs, SHs, SH-R2s, and SH-R3s were enzyme histochemically detected in the liver sections at 21 days after transplantation (Figure 2). Size of colonies formed from SHs, PHs, and SH-R3s was apparently heterogeneous as compared with that from SH-R2s, which was confirmed by quantitative analysis of colony areas as described in Figure 3. Apparently, colonies of SHs and SH-R3s were larger in their areas than those of PHs and SH-R2s, respectively. To quantitatively compare the colony size formed from each of transplanted hepatocytes, colony areas were measured in 40 portal areas of each recipient animal (Figure 3). The mean colony area was calculated from these measurements (Table 1). The colony area of SHs and SH-R3s was ~ 1.5 -fold to twofold larger than that of PHs and SH-R2s, respectively. The differences between PHs and SHs, and between SH-R2s and SH-R3s, were statistically significant ($P < 0.0001$, Mann-Whitney sum rank test). To obtain a more practical index of the growth potential of these hepatocytes we calculated the mean colony volume assuming that the colonies were clonally expanded and that each colony formed a sphere. The diameter of such a hypothetical sphere was measured from the semiserial sections with the thickness of 10 μm

Figure 3. Distribution of colony areas formed from transplanted hepatocytes. Forty portal regions were photographed for each animal and the area of colonies was measured using a NIH 1.62 soft on the photographs. Each colored line represents the incidence (%) of the area measured for each individual animal.

prepared from liver specimens at intervals of 100 μm as described in Materials and Methods section and as shown in Table 1.

The colony volume of SHs and SH-R3s was 1.7-fold and 2.8-fold larger than that for PHs and SH-R2s, respectively. From the mean area and volume of colonies developed by transplanted hepatocytes, we estimated the replication potential of a transplanted cell of each fraction. A hepatocyte is thought to be a cube which has eight to 12 sides.¹⁰ However, for making the calculation simple and easy, we assumed that transplanted hepatocytes are spheres and calculated the number of transplanted cells per area and volume of colonies using the hepatocyte's average diameter measured on the cross-sections of the transplant-derived colonies (Table 2). The

number per area formed from SHs and SH-R3s was twofold and 2.2-fold larger than that from PHs and SH-R2s, respectively. Likewise, the number per volume was threefold and 3.2-fold larger, respectively. The average cell division number of transplanted PHs, SHs, SH-R2s, and SH-R3s during 21 days was calculated as 7 to 8, 9 to 10, 8 to 9, and 10 to 11, respectively (Table 2). Thus, we concluded that the growth potential of hepatocytes is also heterogeneous *in vivo* and is correlated with their size.

Phenotype of Cells in Colonies Formed from Transplanted SH-R2s and SH-R3s

The cells in the colonies of SH-R2s and SH-R3s were characterized with respect to their expression of three lineage-specific markers of liver cells: albumin as a universal marker of hepatocytes, α -FP as a phenotype of immature or neoplastic hepatocytes, and CK19 as a marker of bile duct epithelial cells. The cells in colonies of SH-R2s and SH-R3s expressed albumin at a high level comparable to the surrounding host hepatocytes (Figure 4, C and D). There was no expression of α -FP (Figure 4, E and F) and CK19 (Figure 4, G and H) in the cells in colonies formed from both SH-R2s and SH-R3s. These results indicated that both SH-R2s and SH-R3s replicated retaining phenotypes of hepatocytes.

Recently, Gordon and colleagues¹¹ reported that a population of SH-like progenitor cells emerged in the liver of retrorsine-exposed and two-thirds hepatectomized rats, the same experimental animal model used in the

Table 1. The Mean Area and Volume of Colonies Formed from Transplanted Hepatocytes

	Area (μm^2)	Volume (μm^3)	Number*
PHs	14,984 $\pm$ 417 ^a	1,515,024 $\pm$ 65,145 ^e	584
SHs	21,598 $\pm$ 526 ^b	2,698,876 $\pm$ 100,503 ^f	622
SH-R2s	13,123 $\pm$ 277 ^c	1,284,874 $\pm$ 48,377 ^g	856
SH-R3s	25,637 $\pm$ 675 ^d	3,589,391 $\pm$ 148,474 ^h	679

Areas of colonies were measured for five different rats transplanted with hepatocytes in the identical experimental conditions as shown in Figure 3. The mean $\pm$ SE of areas and volumes of colonies was calculated from these measurements.

*Total number of colonies measured.

^a versus ^b; $P < 0.0001$; ^c versus ^d; $P < 0.0001$; ^e versus ^f; $P < 0.0001$; ^g versus ^h; $P < 0.0001$. P values were determined by Mann-Whitney rank sum test.

Table 2. Mean Cell Number in a Colony at 21 Days after Hepatocyte Transplantation and Number of Cell Divisions during 21 Days

	Cell diameter (μm)	Cell number per area of colony	Cell number per volume of colony	Cell divisions during 21 days
PHs	23.2 ± 0.5	36 ± 1	226 ± 10	7–8
SHs	19.7 ± 0.7	71 ± 2	673 ± 25	9–10
SH-R2s	19.3 ± 0.4	45 ± 1	342 ± 13	8–9
SH-R3s	18.4 ± 0.6	97 ± 3	$1,101 \pm 46$	10–11

Data are expressed as mean $\pm$ SE.

present study. These SHs expanded and finally replaced the entire liver mass. In the present study we also noticed colonies of such host-derived SHs (Figure 5B) on the same liver section where the SH-R3-derived colony was seen (Figure 5A). We measured the size of the hepatocytes in colonies of the host-derived SH-like progenitor cells and in colonies formed from SH-R3s at 7 days after transplantation. Their diameter was $12.8 \pm 0.46 \mu\text{m}$ for the former and $12.0 \pm 0.08 \mu\text{m}$ for the latter, indicating that these two populations of hepatocytes were indistinguishable in their size ($P = 0.1285$).

Discussion

Recently, several groups developed *in vivo* models to assess the growth potential of hepatocytes. In these models, isolated hepatocytes are transplanted into syngeneic or immunodeficient rodents whose hepatocytes are genetically or chemically impaired and unable to divide. The transplanted hepatocytes divide repeatedly in a recipient liver to replace the host hepatocytes. These model experiments showed that rat or mouse hepatocytes are able to replicate more than a dozen of times *in vivo*.^{6,12,13} But it is unclear whether a special population in the transplanted hepatocytes can multiply, or hepatocytes are generally proliferative. Our recent *in vitro* studies demonstrated the existence of a highly replicative population of SHs in rat and human livers.^{2,3,14} Their growth potential was heterogeneous, in the highest case a hepatocyte forming a colony consisting of more than 100 cells for 10 days, and in the lowest case a cell never dividing.² When a nearly pure fraction of PHs or SHs was co-cultured with a pure fraction of nonparenchymal cells, SHs increased 20-fold in cell number at 20 days, whereas the same culture conditions supported a ninefold increase in the number of PHs.³ These results suggested that the hepatocytes with a high growth potential exist in the SH fraction. The average size of SHs was smaller than that of PHs, but size distribution of SHs were half overlapped with that of PHs. To clearly demonstrate the relationship between the growth potential and the size of hepatocytes, SHs were further fractionated into SH-R2s and SH-R3s by FACS, SH-R2s being larger than the SH-R3s in size. There were no significant overlaps in the size between the two. SH-R3s showed four or five times higher growth ability than SH-R2s. Thus, we concluded that the *in vitro* growth potential of hepatocytes is heterogeneous and is correlated with their size.³

The present study was performed to investigate whether smaller hepatocytes exhibit a higher growth potential than larger ones also *in vivo*. SHs and PHs were isolated from DPPIV⁺ rats and were transplanted into retrorsine-treated and partial hepatectomized DPPIV⁻ rats. De Roos and colleagues¹⁵ investigated the engraftment index by transplanting BrdU-labeled rat hepatocytes into normal rat liver via the portal vein. Transplanted cells were primarily lost at 24 hours, and ~7% of the injected cells were engrafted.¹⁵ These hepatocytes migrated into the liver parenchyma from the portal venules at 48 hours.¹⁵ Gupta and co-workers¹⁶ reported that hepatocytes transplanted via spleen were translocated from sinusoids into liver plates between 16 and 20 hours after transplantation and were suggested to be engrafted into the liver parenchyma later than 24 hours. The engraftment index obtained in the present study at 48 hours after transplantation was $8.9 \pm 2.2\%$ for SHs and $11.0 \pm 6.4\%$ for PHs, which was comparable to that reported by De Roos et al.¹⁵ We noticed that the number of engrafted single hepatocytes observed at 48 hours was smaller than that of colonies formed from them at 21 days after transplantation. This difference might be explained as initially observed single hepatocytes in the periportal regions (zone 1) grew to form colonies, some of which split and formed new colonies in the mid-regions (zone 2) as Laconi et al⁶ suggested.

The colonies formed from transplanted cells at 21 days were examined in all residual livers of the recipients. Our present *in vivo* assay on growth potential of subfractionated hepatocytes showed that SH-R3s were most proliferative, followed by SHs, SH-R2s, and PHs in this sequence. This result was consistent with the previous *in vitro* study.³ Therefore, we concluded that the growth potential of hepatocytes is heterogeneous and is correlated with their size, and the extent of their granularity and autofluorescence *in vivo* as *in vitro*. It was suggested that the sex of host rats affects the growth of transplanted hepatocytes in the retrorsine/hepatectomy model.⁶ The transplants grew faster in the male hosts than in the female. The present study used female rats as hosts. A previous retrorsine/hepatectomy model using female hosts showed that the transplanted hepatocytes underwent ~12 to 13 cell divisions for 2 months,⁶ which was comparable to that obtained for PHs in the present study. Therefore, it can be said that our *in vivo* assay on the growth potential was done properly. Thus, we convincingly concluded that SHs is of higher growth ability than

Figure 4. Phenotypes of the cells in the colonies formed from SH-R2s and SH-R3s. SH-R2s (**A**, **C**, **E**, and **G**) and SH-R3s (**B**, **D**, **F**, and **H**) were transplanted into the retrorsine/partial hepatectomy-treated DPPIV⁺ rats. Serial liver cryosections were prepared at 21 days after transplantation and were subjected to DPPIV (**A** and **B**), albumin (**C** and **D**), α -FP (**E** and **F**), and CK19 (**G** and **H**) stainings. The region encircled by **broken lines** in **C** through **H** is the place where the major colony of the transplanted hepatocytes was formed as judged by DPPIV positivity shown in **A** and **B**. DPPIV-positive cells in colonies derived from both SH-R2s and SH-R3s were positive to albumin and negative to both α -FP and CK19. P indicates the portal vein. Scale bar, 100 μ m.

Figure 5. Colonies of transplanted SH-R3s and host-derived SH-like progenitor cells. DPPIV⁺ SH-R3s were transplanted into DPPIV⁻ retrorsine/hepatectomy rats, and the liver sections were stained for DPPIV as in Figure 2 at 7 days after transplantation. DPPIV-positive colonies (**A**) and colonies of SH-like progenitor cells (enclosed by **arrowheads in B**) were seen on the same section. Size and morphology of the cells in colonies derived from transplanted cells were similar to those in colonies derived from host cells. The **arrows** indicated the megalocytes that appeared after partial hepatectomy in the retrorsine-treated liver. Scale bar, 30 μ m.

PHs, and SH-R3s than SH-R2s. Overturf and colleagues¹⁷ performed experiments to relate the regenerative capacity of mouse hepatocytes with the hepatocyte's size in their serial transplantation study. The donor hepatocytes were fractionated into small (16 μ m)-, medium (21 μ m)-, and large (27 μ m)-sized cells using centrifugal elutriation, and separately transplanted into recipient mice. Their results were apparently against ours. The growth ability during the first round of transplantation was higher when the size was larger. The reason of this discrepancy is not clear at present, but might reside in the difference of species between rats and mice. In addition, it should be noted that the *in vivo* growth potential assessed by the colony formation of the transplanted hepatocytes actually represents the repopulation potential, which does not necessarily represent their replication potential, because there are several steps for the cells before the colony formation such as streaming in the blood, homing through sinusoids, engraftment into the liver plate, proliferation, and the colony formation.¹⁶ Many known and unknown factors might affect each of these steps, depending on the species of animals and the model of transplantation experiments.

Previously, we characterized the phenotypes of cells in colonies formed *in vitro* by SHs and PHs and found some cells became both CK19- and α 1-antitrypsin-positive,³ suggesting the phenotypic plasticity of hepatocytes *in vitro*. We asked if SH-R3s also show such plasticity *in vivo* by examining the expression of marker proteins specific to hepatocytes, immature hepatocytes, and bile duct epithelial cells. The cells in colonies formed from transplanted SH-R3s were positive to albumin, but negative to both CK19 and α -FP, which differed from the results of the *in vitro* study. It seems that there is a regulation *in vivo* under which hepatocytes stably express hepatocytic phenotypes, but not express the biliary ones.

Recently, the presence of a population of SH-like progenitor cells was demonstrated in the retrorsine-exposed rat.¹¹ These SHs emerged after the liver was partially hepatectomized, expanded, and replaced the entire liver mass. These cells expressed phenotypes characteristic

of fetal hepatoblasts, oval cells, and fully differentiated hepatocytes. The average diameter of these SH-like progenitor cells was estimated to be 13.1 μ m at 3 days after the operation.¹¹ In the present study we also noticed an emergence of the colony made of such host-derived SHs in the SH-R3s transplanted liver. These host-derived and transplant-derived colonies of SHs co-existed in the same liver. The diameter of hepatocytes in colonies derived from SH-R3s and the host-derived SH colonies was similar, 12.0 and 12.8 μ m, respectively. It is tempting to speculate that SH-R3s that we isolated from the normal liver play an important role(s) to repair the tissues when the liver is severely damaged. In this situation SH-R3s are activated and act as the SH-like progenitor cells that were identified by Gordon et al.¹¹ The identity between our SH-R3s and the SH-like progenitor cells is currently under investigation.

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