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Contribution of bone marrow cells to liver regeneration after partial hepatectomy in mice

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***Background/Aims*: We examined whether bone marrow (BM) cells can commit to liver-consisting cells during liver regeneration after partial hepatectomy, using mice transplanted with green fluorescent protein (GFP) positive BM from GFP transgenic mice.**

***Methods*: Partial hepatectomy or sham operation was performed. Lineage marker analysis of GFP positive liver cells was by immunostaining and flow cytometry. DiI-labeled acetylated low-density lipoprotein uptake or microsphere phagocytosis was examined in vitro. Lineage marker expression in BM and peripheral blood (PB) cells, and the vascular endothelial growth factor (VEGF) concentration in the liver were also examined.**

***Results*: In hepatectomized mice, significantly more GFP positive cells participated in liver sinusoid than in sham-operated mice, expressing CD31 but not albumin. The percentage of cells that incorporated acetylated low-density lipoprotein but not microspheres was 69.5 ± 3.4%, while 28.3 ± 2.6% incorporated both, revealing sinusoidal endothelial and Kupffer cells, respectively. Increased expression of the CD31 and CD16/CD32 on GFP positive liver cells was also detected. The elevation of the VEGF concentration during liver regeneration and the increase in the CD34 and Flk-1 expression in the liver, BM, and PB cells suggested endothelial progenitor cell mobilization.**

***Conclusions*: GFP cell-marking provided direct evidence of the BM cells participation in liver regeneration after hepatectomy, where the majority was committed to sinusoidal endothelial cells probably through endothelial progenitor cell mobilization.**

***Keywords*: Liver regeneration; Bone marrow; Endothelial progenitor cell; Transplantation; Green fluorescent protein**

1. Introduction

Recent advances in stem cell research have revealed that bone marrow (BM) cells including hematopoietic stem cells can differentiate into cells in other lineages that consist of various tissues [1,2]. Related to the liver, a close relation between liver-consisting cells and hematopoietic cells has been reported. Fetal liver is a major site of hematopoiesis [3]. In addition, even in adults, the liver can support hematopoiesis in some situations [4]. In this respect, the possibility of BM cells committing to liver cells has been explored in several studies. One study reported that hepatic 'oval cells', which emerged in the injured liver, were of BM origin by in situ hybridization [5]. In another study, BM cells identified using the β-galactosidase gene-marking method were confirmed to become hepatocytes in fumarylacetoacetate hydrolase (FAH) deficient mice [6]. However, the behavior of BM cells after partial hepatectomy has not been investigated, although liver regeneration is an attractive biological problem long discussed and is often experienced clinically. To date, liver regeneration after partial hepatectomy has long been believed to depend only on

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replication of mature cells and not on stem characteristic cells [7]. Therefore, we intended to clarify if BM cells commit to liver cells during liver regeneration induced after partial hepatectomy. To directly evaluate and also to quantitatively analyze the BM cell commitment to liver cells, we employed a cell-marking method using green fluorescent protein (GFP) transgenic mice.

2. Materials and methods

2.1. Animals

C57BL/6J mice were provided from Nippon SLC (Hamamatsu, Japan). The syngenic GFP transgenic mice used were as described previously [8], which express GFP under CAG promoter. They were maintained according to the Animal Protection Guidelines of Kyoto University.

2.2. Reagents and antibodies

Phycoerythrin (PE)-conjugated anti-mouse platelet-endothelial cell adhesion molecule-1 (PECAM-1/CD31), CD16/CD32, and Flk-1, PE-conjugated rat IgG, and biotin-conjugated anti-rat IgG2a were obtained from BD PharMingen (San Diego, CA, USA). PE-conjugated anti-mouse CD34 was obtained from Caltag Lab. (Burlingame, CA, USA). Anti-mouse albumin was from Bethyl Lab. Inc., (Montgomery, TX, USA). Biotin-conjugated anti-goat IgG was from Chemicon International, Inc., (Temecula, CA, USA).

2.3. BM transplantation and 70% partial hepatectomy

GFP mice (8 weeks old, male) were sacrificed and their BM cells were obtained by flushing the tibiae and femora with RPMI 1640 medium. GFP positive BM cells were purified using Fluorescent Activated Cell Sorting (FACSVantage™, Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). A total of 1×10^5 sorted GFP positive BM cells were transplanted via a tail vein into age-matched recipient C57BL/6J mice that received whole body irradiation (12 Gy). Mice were then maintained with free access to food and water for 4 weeks until 70% partial hepatectomy ($n = 4$) or sham operation ($n = 4$) was performed under anesthesia with sodium pentobarbital.

2.4. Fluorescent microscopy and immunohistochemical staining

To evaluate cells included in the regenerating liver, passing hematopoietic cells were excluded by flushing with phosphate buffered saline (PBS) from the portal vein when liver specimens were collected 4 weeks after the operation. They were dissected to small pieces (2 or 3 mm thick), fixed with 4% paraformaldehyde for 7 h, and sectioned at 7-μm thickness. Either GFP expression or antigen stained by fluorescence-conjugated antibody was visualized by fluorescent microscopy Axiovert® 135 (Carl Zeiss, Oberkochen, Germany). In case of immunohistochemistry, liver tissues were incubated with anti-mouse PECAM-1 or albumin antibody for 16 h at 4°C after 2 h blocking followed by 1 h incubation with respective second antibodies at room temperature. Signals were amplified using streptavidin–horseradish peroxidase conjugates and tyramide–biotin (Life Science Products, Inc., Boston, MA, USA). Visualization was done using streptavidin conjugated Texas Red®-X (Molecular Probes, Inc., Eugene, OR, USA).

2.5. DiI-Ac LDL incorporation and phagocytosis

Four weeks after the operation, the livers of mice were perfused in situ via the inferior vena cava after ligation of the inferior vena cava above the diaphragm and dissection of the portal vein. Perfusion was performed using 50 ml Ca^{2+}-free Hanks' balanced solution containing 0.5 mM EGTA and 10 mM HEPES and then with 0.05% collagenase solution. After full digestion, cells were suspended in Hanks' balanced solution and filtered through a 71-μm nylon mesh to eliminate non-digested tissues. Parenchymal and non-parenchymal cells were separated using a low speed centrifuge method [9].

Freshly isolated non-parenchymal cells were cultured in Dulbecco's modified essential medium containing 10% fetal bovine serum on a collagen type I coated plate (Asahi Techno Glass Corp., Tokyo, Japan) at a density of 5×10^4 cells/cm^2. Two hours later, the cells were washed once to eliminate non-adherent cells. From 16 h after the beginning of culture, the cells were incubated with acetylated low-density lipoprotein labeled with dioctadecyl tetramethyl indocarbocyanine perchlorate (DiI-AcLDL) (Molecular Probes, Inc., Eugene, OR, USA) for another 8 h. In addition, 1.75-μm Bright Blue-latex microspheres (Fluoresbrite™ caboxylate microsphere, Polyscience, Inc., Warrington, PA, USA) were incubated for the last 1 h. DiI-AcLDL incorporation represents the character of the sinusoidal endothelial cells (SECs) or Kupffer cells, since DiI-AcLDL is incorporated in an LDL receptor-dependent manner, and the existence of LDL receptors is a marker for SECs or Kupffer cells [10]. In contrast, phagocytosis of latex microspheres solely represents Kupffer cell characterization [11]. Incorporation of DiI-AcLDL and latex microspheres among GFP positive cells were counted under fluorescent microscopy and analyzed.

2.6. Flow cytometry

After dissociation of the liver 4 weeks after the operation, 1×10^4 liver cells were analyzed by flow cytometry (FACSCalibur™, Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) for PECAM-1, CD16/CD32, CD34, and Flk-1 expression. In addition, CD34 and Flk-1 expressions were examined in 1×10^4 BM and peripheral blood (PB) cells obtained from C57BL/6J mice 5 days after either partial hepatectomy or sham operation. The cells were washed twice and incubated at 4°C for 30 min with each monoclonal antibody. They were then rinsed with PBS twice and analyzed. Propidium iodide positive dead cells were excluded from the analysis. Negative controls were carried out using PE-conjugated rat IgG.

2.7. Vascular endothelial growth factor (VEGF) levels in liver tissue

Mouse VEGF was quantified using a commercially available immunoassay kit (AN'ALYZA™; Genzyme Techne, Minneapolis, MN, USA). Liver tissue lysates were prepared at the indicated times after partial hepatectomy or sham operation by homogenization in lysis buffer (150 mM NaCl, 10 mM Tris (pH 7.5) supplemented with proteinase inhibitors (0.11 TIU aprotinin, 1 mM phenylmethylsulfonyl fluoride). Tissue lysates were adjusted to contain similar protein concentrations (5 mg/ml). Detection of VEGF was performed according to the supplier's recommendation.

2.8. Statistical analysis

All experiments were performed in four replications except in VEGF enzyme linked immunosorbent assay (ELISA) experiments in which results were determined in three replications. Statistical analysis was performed by Student's t test. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Fluorescent microscopic analysis of liver tissues

In 70% partial hepatectomized mice, a large number of GFP positive cells were detected in the liver surrounding the sinusoidal space (Fig. 1A). In contrast, only a few GFP

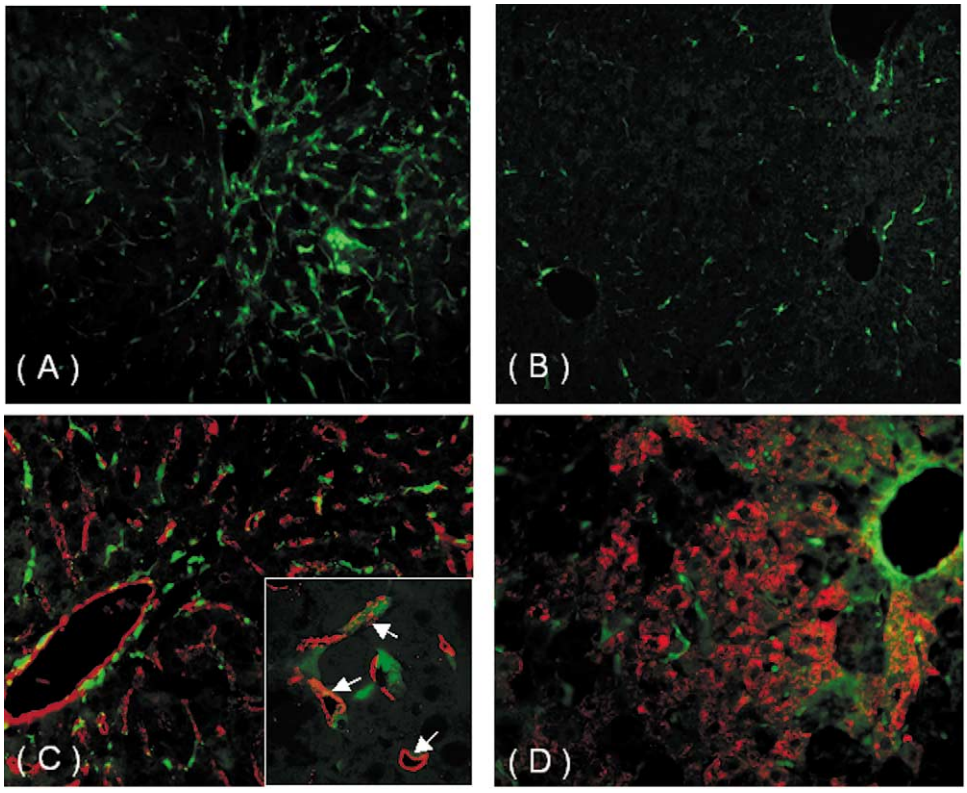

Fig. 1. Fluorescent microscopy of liver specimens after partial hepatectomy (A) or after sham operation (B). Fluorescent immunohistochemical staining against mouse PECAM-1 (C) and albumin (D) were merged with the GFP expression pattern. Arrows in the right lower quadrant square in C indicate both PECAM-1 and GFP positive cells. (A–D; original magnification ×200, the right lower quadrants square in C; original magnification ×400).

positive cells were detected in sham-operated mice (Fig. 1B). This localization of GFP positive cells suggested that these cells mainly consisted of non-parenchymal cells.

3.2. Immunohistochemical detection of marker genes

To further characterize GFP positive cells participating in regenerating liver, either PECAM-1 or albumin was immunostained (Fig. 1C, D). PECAM-1 is considered a marker for endothelial or Kupffer cells [4,12,13], while albumin is an endodermal marker [14]. Most GFP positive cells co-expressed PECAM-1 (Fig. 1C), but not albumin (Fig. 1D). This finding, together with the localization of GFP positive cells, suggested that the majority of GFP cells were either SECs or Kupffer cells.

3.3. In vitro analysis for receptor mediated AcLDL uptake and phagocytosis

To discriminate between SECs and Kupffer cells among GFP positive cells, we further confirmed their characterization by evaluating their incorporation of DiI-AcLDL and latex microspheres. When cultured in vitro, the majority of GFP positive cells in hepatectomized liver showed an endothelial morphology as shown in Fig. 2A. In addition, 94 ± 0.9% of GFP positive cells showed DiI-AcLDL incorporation, and 28.3 ± 2.6% of the cells showed phagocytosis of microspheres (Fig. 2B). Considering that DiI-AcLDL intake represents SEC or Kupffer cell characters and that phagocytosis solely represents Kupffer cell characters, at least about 70% of GFP cells can be considered SECs (Fig. 2C).

3.4. FACS analysis of liver cells

We confirmed the above results by evaluating surface marker expressions on GFP positive cells. Supporting the fluorescent microscopic observations of liver tissues, GFP positive cells in the liver appeared only in the non-parenchymal fraction (Fig. 3A), and 11.9 ± 2.3% of non-parenchymal cells become GFP positive in partial hepatectomized mice. The GFP positive cell number in partial hepatectomized mice was significantly higher than in sham-operated mice, again supporting the fluorescent microscopic results (Fig. 3B). Furthermore, we examined PECAM-1 and CD16/CD32 expression on GFP positive cells. CD16/CD32, a monocyte lineage marker was used for Kupffer cell characterization, since Kupffer cells were reported to differentiate from monocytes [15]. As expected, PECAM-1 and CD16/CD32 expression on GFP positive cells was significantly higher in partial hepatectomized mice compared with that in sham-operated mice (185.5 ± 32.9 vs. 986.5 ± 129.7, and 222.5 ± 17.8 vs. 326.8 ± 35.1 cells/1×10^4 cells,

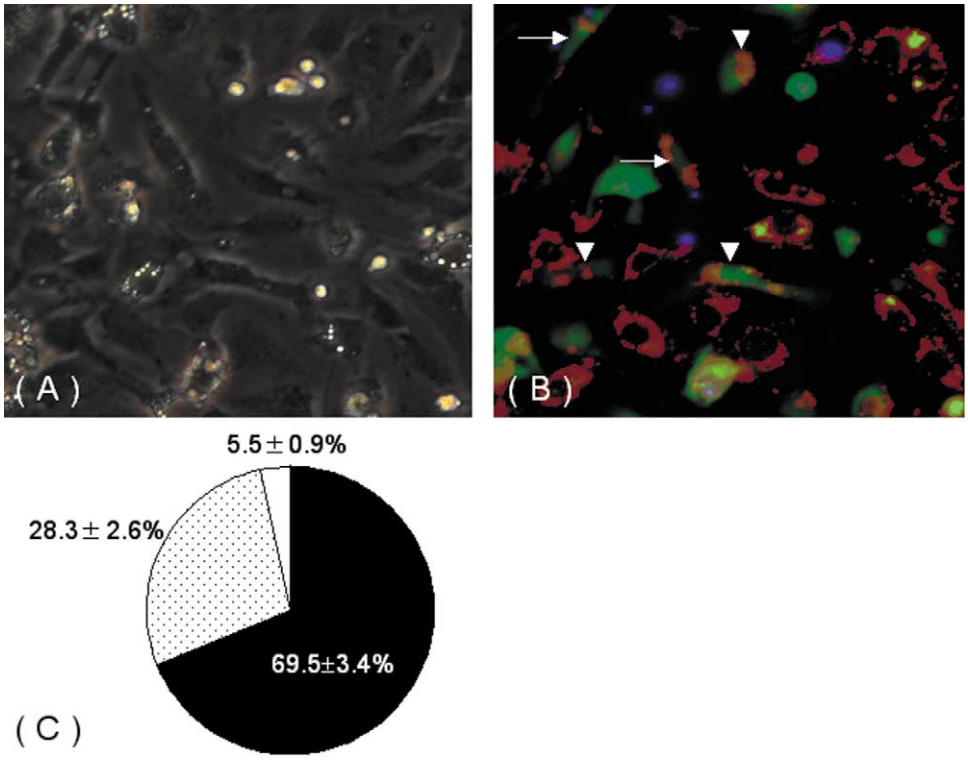

Fig. 2. (A) Phase contrast microscopy of isolated non-parenchymal cells from partial hepatectomized mice. (B) Merged image for GFP, DiI-AcLDL, and latex microspheres. Arrows indicate Kupffer cells positive for GFP incorporating both DiI-AcLDL and latex microspheres. Arrowheads indicate SECs positive for GFP incorporating DiI-AcLDL but not for latex microspheres. (A,B; original magnification × 200). These findings are summarized in C. Black, dotted, and white areas indicate the percentages of SECs, Kupffer cells, and the unidentified cell population among GFP positive cells, respectively ($n = 4$).

respectively, $P < 0.05$) (Fig. 3C). SECs became dominant in GFP positive cells after partial hepatectomy (48.2 ± 5.5 vs. 70.1 ± 4.9%, $P < 0.05$), which is compatible with the in vitro assay findings. Thus, the present findings suggest that BM cells can differentiate not only to Kupffer cells but also to hepatic SECs, and hepatic SECs differentiation is strongly induced after partial hepatectomy.

3.5. Expression of CD34 and Flk-1 in liver, BM, and PB cells

Since GFP positive cells were revealed to be SECs and Kupffer cells in regenerating liver, it is suggested that these cells have migrated from BM to the liver during regeneration. To support this hypothesis, we focused on the CD34, Flk-1 positive cell population since recent studies showed that CD34 positive, Flk-1 positive endothelial progenitor cells (EPCs) are mobilized from BM and incorporated into sites of vascular disorders for neovascularization [16–19]. In fact, CD34 and Flk-1 positive cells in the GFP positive population in regenerating livers were significantly higher compared with corresponding cells in the sham-operated livers (22.3 ± 7.4 vs. 119.3 ± 25.0, and 21.1 ± 11.7 vs. 88.3 ± 1.4 cells/1×10^4 cells, respectively, $P < 0.05$) (Fig. 4A). In partial hepatectomized mice, a significant increase in the monocyte fraction that was reported to include EPCs [20] was detected in BM cells as well as in PB cells (data not shown). In addition, the expressions of CD34 and Flk-1 in the monocyte fraction of BM cells as well as of PB cells after partial hepatectomy were significantly elevated (Fig. 4B, C).

3.6. Mouse VEGF concentration in liver tissue

From the above results, the EPC fraction of BM cells is suggested to migrate into the liver. Since VEGF is known to be one of the important stimuli for EPC activation [20], we next measured its concentration in the liver. After partial hepatectomy, VEGF protein concentrations in liver tissue were elevated to 268 ± 25 pg/ml at 7 days, although no significant change was seen in sham-operated mice (Fig. 5).

4. Discussion

Until recently, only Kupffer cells were suggested to be the cells in the liver derived from BM [15]. More recently, a new point of view has arisen. Petersen et al. showed that hepatic oval cells appearing in injured liver were of BM origin employing the in situ hybridization method. Furthermore, Lagasse et al. reported that BM cells can differentiate into hepatocytes in FAH deficient mice using the β-galactosidase gene-marking method. Although controversy

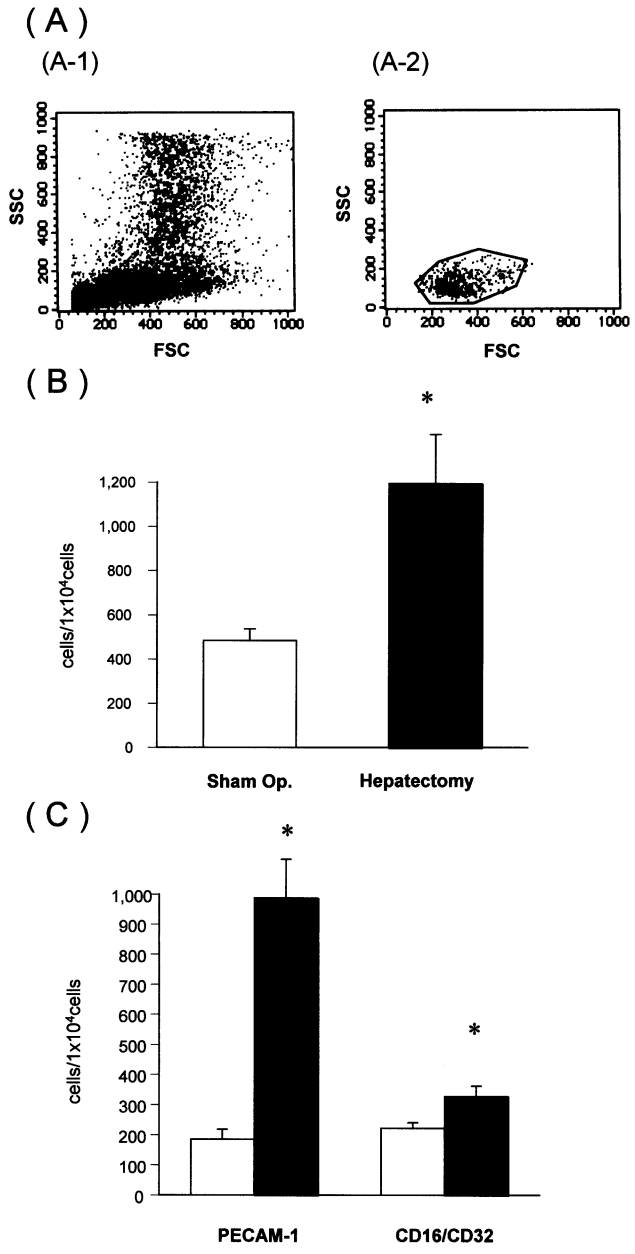

Fig. 3. Flow cytometric analysis of liver cells. (A-1) Dot blots of whole liver cell suspensions in partial hepatectomized mice. When GFP positive cells were gated, they were detected only in the non-parenchymal fraction depicted by the gate (A-2). (B) GFP positive cells in the liver after partial hepatectomy (closed column) and sham operation (open column). Values are expressed as means ± SEM. ($n = 4$) *: $P < 0.05$ vs. sham operation. Analysis was performed using Student's t test. (C) PECAM-1 and CD16/CD32 positive cells among GFP positive cells in liver after partial hepatectomy (closed column) and sham operation (open column). Values are expressed as means ± SEM. ($n = 4$) *: $P < 0.05$ vs. sham operation. Analysis was performed using Student's t test.

remains [21], these findings suggested that hepatic cells can be derived from BM. However, liver regeneration after partial hepatectomy was suggested to depend solely on the replication of mature cells in the liver. In the present study, to directly evaluate BM cells committing to liver cells after partial hepatectomy, we employed a GFP cell-marking system. As shown in the results, we could clearly show that BM cells migrated and contributed to the regenerating liver. To our knowledge, this is the first study directly showing that BM cells are involved in liver regeneration after partial hepatectomy.

In contrast to Lagasse's study using FAH deficient mice, GFP positive cells in the regenerating liver could only be detected in the liver surrounding the sinusoidal space. Further lineage marker studies together indicated that about 70% were SECs while 28% were Kupffer cells. In partial hepatectomy, SECs proliferate and migrate into avascular hepatic islands subsequent to the proliferation of parenchymal cells [22]. From the present findings, BM cells were shown to participate in this neovascularization by committing to SECs, which is also the first such evidence. Furthermore, from the detection of the EPC markers, CD34 and Flk-1, it was suggested that EPCs were mobilized from BM and differentiated to SECs during liver regeneration. This is compatible with the findings of Asahara et al. that showed EPC migration from BM to the site of vascular injury and their incorporation into neovascularization employing a different injury model [16,17,20]. In the present experiment, GFP positive SEC was also detected in non-hepatectomized liver. This finding is comparable with those of Gao et al.'s study [23] which showed replacement of liver venous endothelium by BM derived cells even in quiescent liver.

It is unclear what can be a trigger for EPC mobilization during liver regeneration. Shibuya and coworkers [24] reported that mRNA of VEGF is increased after hepatectomy and Taniguchi et al. [25] reported that VEGF expression after partial hepatectomy was mainly detected in periportal hepatocytes. Considering that VEGF is among the important stimuli for EPC activation [20], it should be a good candidate for stimulus inducing EPCs migration. As expected, the VEGF protein concentration in liver tissue was also increased at 5–7 days after partial hepatectomy, again supporting the proposed system of EPCs participation in the regenerating liver.

BM derived parenchymal cells were not apparent in the present study in contrast to previous studies [5,6,26]. To explain this discrepancy, three factors should be taken into consideration. One is the difference in the timing of observations. However, this explanation is not likely, since we could not detect BM cell differentiation to parenchymal cells even when we examined liver sections 2 months later, which is similar timing with Theise et al.'s study showing transdifferentiation of transplanted BM cells to hepatocytes [26]. Another factor is the difference in the detection system. Petersen et al. and Theise et al. showed their results by detecting BM derived Y-chromosome positive cells in the livers of female recipients using an in situ

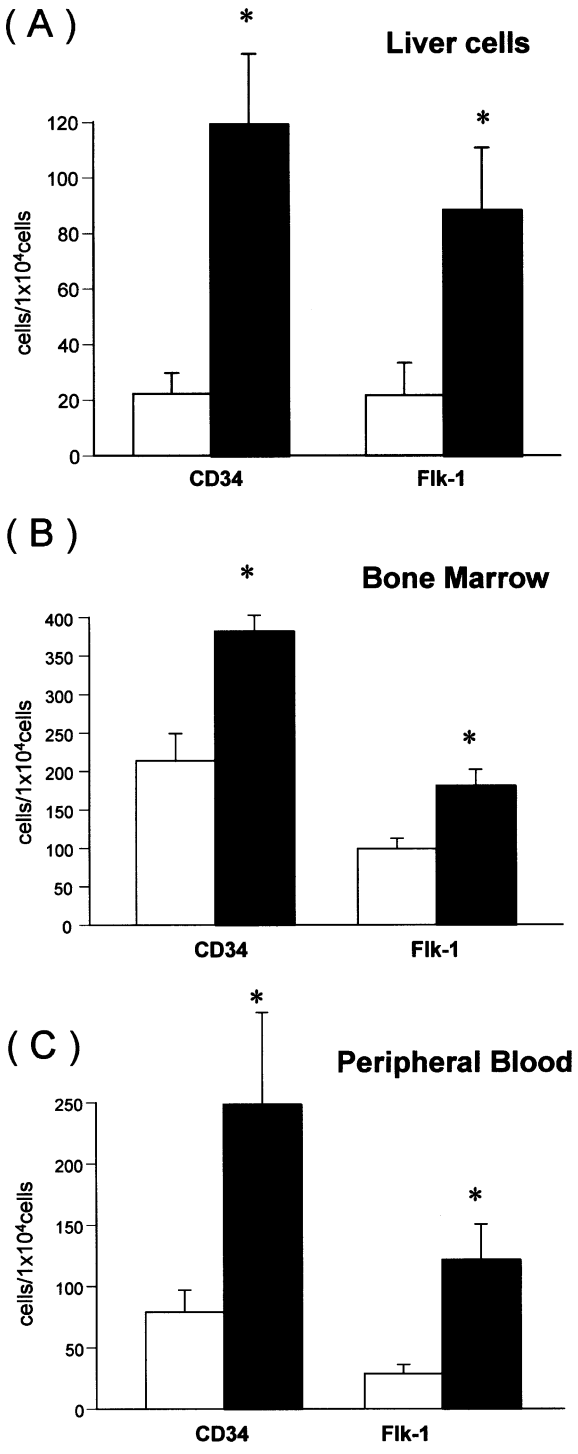

Fig. 4. Flow cytometric analysis of EPC markers in liver, BM, and PB cells. (A) CD34 and Flk-1 positive cells among GFP positive cells in liver after partial hepatectomy (closed column) and sham operation (open column). Values are expressed as means ± SEM. ($n = 4$) *: $P < 0.05$ vs. sham operation. Analysis was performed using Student's t test. Numbers of CD34 and Flk-1 positive cells in the monocyte fraction of BM (B) and PB (C) at 5 days after partial hepatectomy (closed column) and sham operation (open column). Values are expressed as means ± SEM. ($n = 4$) *: $P < 0.05$ vs. sham operation. Analysis was performed using Student's t test.

hybridization technique [5,6,27]. The findings obtained from the detection of the Y-chromosome requires cautious interpretation, since it has been reported that a Y-chromosome can be identified in liver tissues by polymerase chain reaction in about 70% of women who have been pregnant with male children [28]. In contrast, the GFP cell-marking technique provides direct and quantitative evidence for cell fate. However, the differences in the results again appear not to stem from differences in the detection systems, since GFP positive cells were likely to be observed in parenchymal tissue in our other preliminary studies using liver cirrhosis mice. The last but most important factor likely to explain the discrepancy is that it stems from differences in differentiation stimuli. In Petersen et al.'s study, liver damage was induced by 2-AAF followed by hepatectomy that causes fulminant liver failure, in which situation the emergence of oval cells is indispensable to replace the massive cell death of mature hepatocytes. Similarly, liver damage was so severe in FAH deficient mice that loss of liver function caused their neonatal death. In contrast, partial hepatectomy does not induce such critical damage. The residual liver maintains a normal function and simultaneously hepatocytes can proliferate. In such a case, the participation of BM cells in the regeneration of parenchyma may not be necessary. Furthermore, stimuli inducing SECs differentiation that persists during liver regeneration may interfere with the differentiation of BM cells into the parenchymal lineage.

A more recent study revealed that BM derived cholangiocytes can be detected even in quiescent situations [29]. This may be compatible with the present findings since a few GFP positive cells, which did not express endothelial or monocytic markers, were detected in the present system. Growing evidence including that of the present study will clarify the differentiation ability of BM cells to liver composing cells in various situations, further evaluation

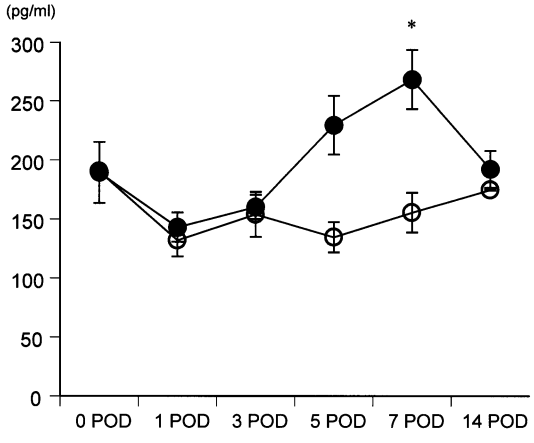

Fig. 5. Measurement of mouse VEGF protein concentrations in liver tissues. Closed circle (●) indicates partial hepatectomy and open circle (○) indicates sham operation. Values are expressed as means ± SEM. ($n = 3$) *: $P < 0.05$ vs. sham operation. Analysis was performed using Student's t test.

applying a direct system such as our GFP-marking-BM system in the case of critical liver damage would be ideal to clarify the mechanisms induced in transdifferentiation.

In conclusion, GFP cell-marking provided direct evidence of BM cells participation in regenerating liver after partial hepatectomy, the majority of cells committing to SECs. Not only helping clarification of aspects of liver regeneration but also of transdifferentiation, the present findings may provide a basis for therapy using BM cells as a vector for exogenous gene delivery, since BM cells may be isolated from patients themselves and be transfected ex vivo.

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