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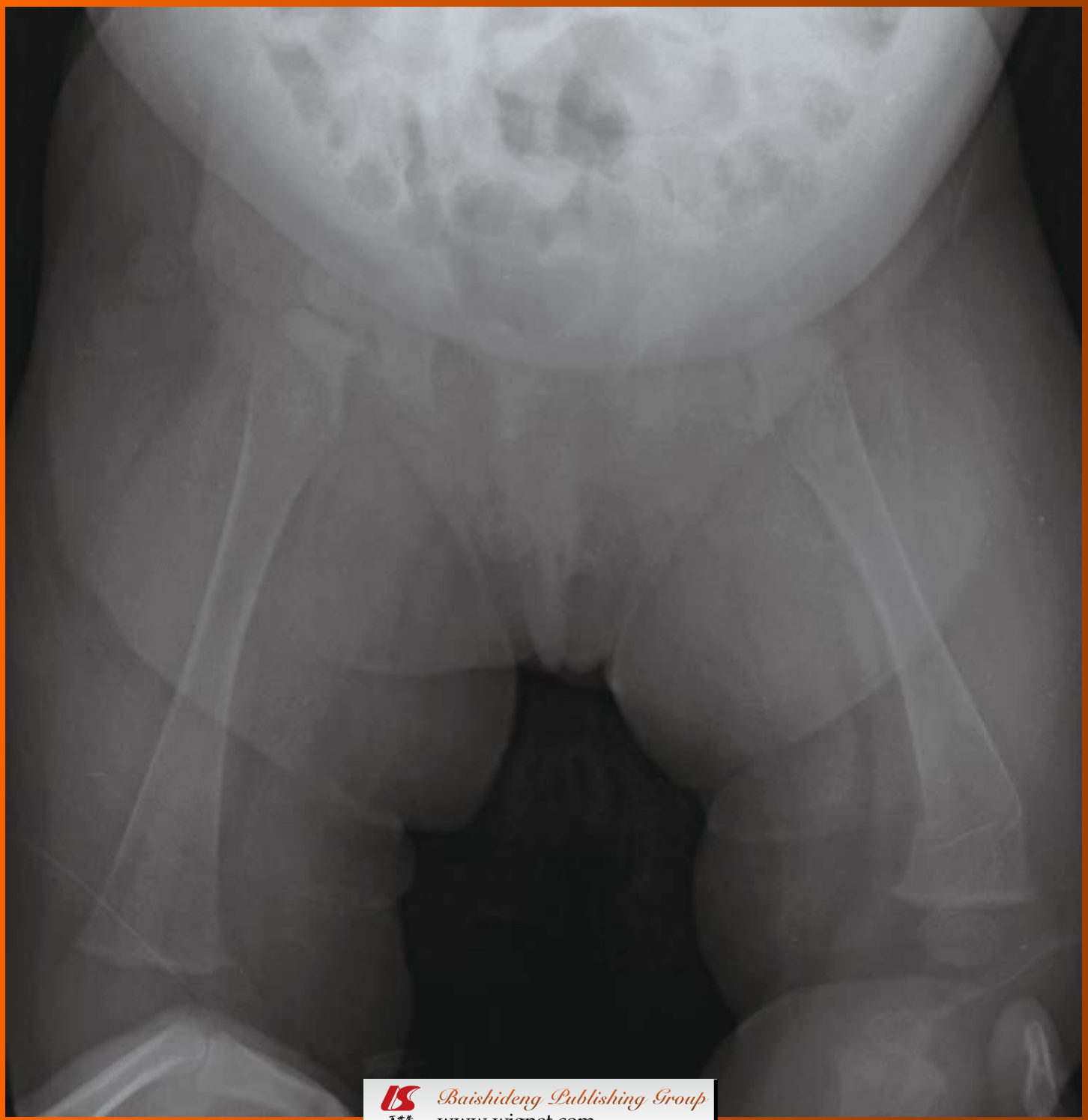

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ORIGINAL ARTICLE

Bone marrow progenitor cells do not contribute to liver fibrogenic cells

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Abstract

AIM: To investigate the contribution of bone marrow (BM) cells to hepatic fibrosis.

METHODS: To establish a model of chimerism, C57Bl/6 female mice were subjected to full-body irradiation (7 Gy) resulting in BM myeloablation. BM mononuclear cells obtained from male transgenic mice expressing enhanced green fluorescent protein (GFP) were used for reconstitution. Engraftment was confirmed by flow cytometry. To induce liver injury, chimeric animals received carbon tetrachloride (CCl_4) 0.5 mL/kg intraperitoneally twice a week for 30 d (CCl_4 30 d) and age-matched controls received saline (Saline 30 d). At the end of this period, animals were sacrificed for post mortem analysis. Liver samples were stained with hematoxylin and eosin to observe liver architectural changes and with Sirius red for collagen quantification by morphometric analysis. α-smooth muscle actin (α-SMA) was analyzed by confocal microscopy to identify GFP+ cells with myofibroblast (MF) characteristics. Liver tissue, BM and peripheral blood were collected and prepared for flow cytometric analysis using specific markers for detection of hepatic stellate cells (HSCs) and precursors from the BM.

RESULTS: Injury to the liver induced changes in the hepatic parenchymal architecture, as reflected by the presence of inflammatory infiltrate and an increase in collagen deposition (Saline 30 d = 11.10% ± 1.12% *vs* CCl_4 30 d = 12.60% ± 0.73%, $P = 0.0329$). Confocal microscopy revealed increased reactivity against α-SMA in CCl_4 30 d compared to Saline 30 d, but there was no co-localization with GFP+ cells, suggesting that cells from BM do not differentiate to MFs. Liver flow cytometric analysis showed a significant increase of CD45+/GFP+ cells in liver tissue (Saline 30 d = 3.2% ± 2.2% *vs* CCl_4 30 d = 5.8% ± 1.3%, $P = 0.0458$), suggesting that this increase was due to inflammatory cell infiltration (neutrophils and monocytes). There was also a significant increase of common myeloid progenitor cells (CD117+/CD45+) in the livers of CCl_4-treated animals

(Saline 30 d = 2.16% ± 1.80% *vs* CCl_4 30 d = 5.60% ± 1.30%, *P* = 0.0142). In addition the GFP-/CD38+/CD45- subpopulation was significantly increased in the CCl_4 30 d group compared to the Saline 30 d group (17.5% ± 3.9% *vs* 9.3% ± 2.4%, *P* = 0.004), indicating that the increase in the activated HSC subpopulation was not of BM origin.

CONCLUSION: BM progenitor cells do not contribute to fibrosis, but there is a high recruitment of inflammatory cells that stimulates HSCs and MFs of liver origin.

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Key words: Bone marrow; Liver; Fibrosis; Progenitor cells; Chimeric mice; Green fluorescent protein+ cells

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INTRODUCTION

In recent years, cell transplantation has emerged as a potential therapy to improve impaired liver function. In particular, bone marrow-derived stem cells (BMSCs) have been widely used in pre-clinical and clinical trials[1].

Interest in this particular cell type was kindled by the discovery of donor-derived cells in the livers of bone marrow (BM) transplant recipients[2]. This indicated a potential relationship between BM and the regenerating liver. However, results published so far show no consensus as to the role of BMSCs in liver repair, and the issue remains one of the most controversial in regenerative medicine.

Several studies using different experimental models of hepatic diseases have demonstrated functional recovery of the liver after cell therapy[3-6]. On the other hand, our group, in agreement with the results of several other studies[7-9], did not observe any benefits of using the bone marrow mononuclear cell fraction from normal[10] or cirrhotic rats[11] or BM-derived stromal cells[12] in a severe chronic hepatic injury model in rats.

Hepatic fibrosis and cirrhosis are the main causes of organ failure in chronic liver diseases of any etiology[13]. Persistent parenchymal cell injury leads to dysregulation of the normal processes of wound healing and to extensive deposition of extracellular matrix proteins[14], a dynamic process known as fibrogenesis. This pathology involves various cellular and molecular mechanisms, in which the myofibroblast (MF) and hepatic stellate cell (HSC) subpopulations play a major role when they become activated[15].

During fibrogenesis, activated HSCs, located mainly in the perisinusoidal space, go through a transdifferentiation process, changing to a MF-like morphology at the portal spaces[16]. MFs present at these sites display high rates of proliferation, excessive production of collagen fibers and decreased production of matrix metalloproteinases, causing an imbalance in matrix degradation and preventing the resolution of fibrosis[17]. Both the MF and activated HSC subpopulations express α-smooth muscle actin (α-SMA), a known marker of pro-fibrotic cells[18].

BMSCs may also play a role in generating liver fibrosis. Many authors reported that fibrocytes[19], a BM-derived CD45+/CD34+/procollagen type 1+ subpopulation, can contribute to fibrosis in different models such as chronic cystitis[20], ischemic cardiomyopathy[21] and asthma[22]. In addition, in a rat biliary duct ligation liver injury model, BM-derived fibrocytes [expressing collagen α1(I) promoter-green fluorescent protein (GFP)] were reported to comprise 5% of all collagen-producing cells[23], an impressive percentage considering the low frequency of this cell type under normal conditions (0.1%-1.0% in humans)[24].

In this scenario, where the use BM-derived cell therapies in liver disease is still controversial, it is important to investigate whether BM-derived cells can in fact contribute to liver fibrosis.

MATERIALS AND METHODS

Animals

All procedures were performed in accordance with the standards of the Guide for Care and Use of Laboratory Animals (DHHS Publication No. NIH 85-23, revised 1996, Office of Science and Health Reports, Bethesda, MD 20892). Female C57/BL6 mice and male mice transgenic for GFP- line C57/BL6-Tg (CAG-EGFP) C14-Y01-FM1310 (GFP+ mice), donated by Okabe *et al*[25], were obtained from the Carlos Chagas Filho Institute of Biophysics animal facility. Animals were housed at a controlled temperature (23 ℃) and 12-h light-dark cycle.

Experimental model: Total body irradiation and cell transplants

Female mice 6-8 wk of age were exposed to whole-body radiation (7 Gy) in a linear accelerator (Varian-Clinac 2100 CD) used for radiotherapy. BM cells were harvested from the femurs and tibiae of 8- to 12-wk-old male mice expressing enhanced GFP. Bones were inserted into adapted centrifuge tubes and centrifuged for 3 min at 1500 × *g* to collect the marrow. The contents were resuspended in Dulbecco's Modified Eagle's Medium (DMEM-Invitrogen) and layered on Histopaque 1083 (Sigma). The tubes were centrifuged at 400 × *g* for 30 min and the band corresponding to mononuclear cells

Table 1 Antibody panel for flow cytometrric analysis of specific cells

Tubes	Cell number	Antibodies	Dil.	Target cells (tissue)
Control	1×10^5	No antibodies	-	-
1	1×10^6	CD45-PE-Cy7 (BD Biosciences)	1:25	Hepatic stellate cells (liver)
		CD38-PE-Cy5 (eBioscience)	1:10	
2	1×10^6	CD45-PE-Cy7 (BD Biosciences)	1:25	Hematopoietic stem cells, fibrocytes and monocytes (BM, PB and liver)
		CD14-APC (eBioscience)	1:10	
		CD34-PE (eBioscience)	1:10	
3	1×10^6	CD45-PE-Cy7 (BD Biosciences)	1:25	Hematopoietic stem cells, fibrocytes and endothelial progenitor Cells (BM, PB and liver
		CD133-APC (eBioscience)	1:10	
		CD34-PE (eBioscience)	1:10	
4	1×10^6	CD45-PE-Cy7 (BD Biosciences)	1:25	Common progenitor cells (BM)
		CD117-APC (eBioscience)	1:10	
		CD34-PE (eBioscience)	1:10	

BM: Bone marrow; PB: Peripheral blood.

was collected. The cells were then washed and counted and their viability determined by Trypan blue exclusion. The mononuclear cells were resuspended in sterile saline (NaCl 0.9%) and injected through the orbital plexus in the female wild-type (WT) previously irradiated mice.

Experimental groups

Infused animals were maintained under observation for 21 d after transplantation. Then, peripheral blood (PB) samples were collected to verify transplant efficiency by flow cytometric analysis of GFP+ cells. Animals showing percentages above 80% GFP+ cells were considered useful chimeric mice. PB samples of WT C57/BL6 and GFP+ mice were used as negative and positive controls, respectively. Selected chimeric mice ($n = 10$) were divided into two groups: CCl_4 30 d ($n = 5$), which received injections of carbon tetrachloride (CCl_4; dose of 0.5 mL/kg) twice a week for 30 d, and Saline 30 d ($n = 5$) (experimental control), which received injections of saline solution during the same period.

Histology

Liver tissue slices were fixed for 5 h in Gendre's solution, then overnight in 10% buffered formalin solution (pH 7.2) and embedded in paraffin. Liver samples were sectioned (5 μm) and stained with hematoxylin-eosin (HE) and Sirius red. According to standard protocols, histomorphometry was performed using an imaging system consisting of a Q-Color 5 digital camera (Olympus, Japan) coupled to an epifluorescence Axiovert 100 microscope (Carl Zeiss, Thornwood, NY, United States). Randomly picked fields of Sirius red sections were captured from each animal, using a 20 × objective lens. Quantification was estimated by the percentage of stained area in comparison to the total area of the fields examined, using Image-Pro Plus 5.0 (Media Cybernetics, Bethesda, MD, United States) image analysis software.

Peripheral blood samples for flow cytometry analysis

First, 50 μL of PB samples from tail veins of irradiated animals were collected at day 21 to evaluate GFP+ BM engraftment efficiency. WT and GFP+ PB samples were used as negative and positive controls, respectively. Erythrocyte lysis-fixation solution (BD FACS Lysing Solution, Becton Dickinson) was added to the samples as recommended by the manufacturer and incubated for 15 min at room temperature. After this period, samples were washed with phosphate-buffered saline (PBS) and centrifuged at 300 × *g* for 3 min and resuspended in 300 μL of PBS for data acquisition by flow cytometry (BD FACSAria, Becton Dickinson).

After the injury induction protocol, 0.3 mL of blood was drawn from the tail veins of animals from the experimental and control groups. Samples and antibodies were prepared as described in Table 1 and incubated 20 min in the dark at 4 ℃.

Digested liver tissue for flow cytometry analysis

The left hepatic lobe was carefully harvested and mechanically and enzymatically digested with type Ⅱ collagenase (Worthington) solution 0.2% at 37 ℃. After centrifugation at 250 × *g*, the pellet was suspended in 1 mL PBS-bovine serum albumin (BSA) 1% solution and filtered through 30 μm nylon filters (Filcons, Consul-TS). The liver cells obtained were then resuspended in 300 μL PBS-BSA 1% and incubated in the dark for 20 min at 4 ℃ with anti CD45-PE-Cy7 (BD Biosciences, San Jose, CA), anti CD38-PE-Cy5, anti CD34-PE, anti CD133-APC (all from eBioscience, San Diego, CA) for analysis by flow cytometry. After antibody incubation, stained liver cells were washed twice with PBS by centrifugation at 300 × *g* and 300 μL of PBS was added to prepare the cell suspension. All assays were conducted using concentrations of antibodies recommended by the manufacturers.

All data were obtained using a BD FACSAria Ⅱ flow cytometer (BD Biosciences) and BD FACSDiva acquisition software (version 6). The exported FCS 3.0 data file was analyzed using FlowJo version 7.6.4.

Direct fluorescence

Liver samples were embedded in Optimal Cutting Tem-

perature (OCT) compound (Tissue-Tek., Sakura, Japan) and preserved at -70 ℃. Sections (6 μm) were obtained using a cryostat (Leica CM1850, Leica) at -20 ℃ and fixed in acetone at 4 ℃. Sections were incubated for 5 min in 5 μL of 4, 6-diamino-2-phenylindole (1 mg/mL) and 5 μL of an antifading medium (VectaShield, Vector Laboratories).

Fluorescence was observed on a Zeiss LSM 510 Meta confocal microscope (Carl Zeiss, Thornwood, NY, United States). The specificity of the immunofluorescent staining was assessed for each experimental condition by performing the reaction in the absence of primary antibodies. No staining was observed under such conditions.

Immunofluorescence

Liver tissue was embedded in OCT (Tissue-Tek., Sakura, Japan) and preserved at -70 ℃. Sections (6 μm) were obtained using a cryostat (Leica CM 1850, Leica) at -20 ℃ and fixed in acetone at 4 ℃. Subsequently, sections were incubated in blocking solution (5% normal goat serum, 5% BSA in PBS, 0.1% Triton X-100, 0.05% Tween 20, 0.01% gelatin, all from Sigma Chemical Co.) for 1 h. Endogenous biotin was inhibited by using streptavidin and biotin solutions from a Dako blocking kit (Dako) according to the manufacturer's instructions. Indirect immunofluorescence technique was used to detect activated HSCs and MFs, using a primary monoclonal anti-α-SMA antibody (Dako, dilution of 1:40) biotinylated with the Animal Research Kit. Sections were incubated overnight at 4 ℃ and then washed twice in PBS-Tween 0.25% and then, the sections were incubated for 1.5 h with a streptavidin-Alexa 586 (Molecular Probes, Eugene, OR, United States) and the dye TO-PRO 3, at 1 mmol/L in DMSO (Invitrogen). After two washes in PBS-Tween 0.25% for 10 min, sections were mounted with Vectashield. Anti-mouse IgG was used as an isotype control. Serial plane images of 1.0 mm thick sections were obtained using a Zeiss LSM 510 Meta laser scanning confocal microscope (Carl Zeiss, Thornwood, NY, United States).

Statistical analysis

Data were analyzed using analysis of variance (ANOVA) with Dunnett's post-test for multiple comparisons and the *t* test when comparing two groups. $P < 0.05$ was considered statistically significant. The data are presented as mean ± SD (for Dunnett) and median and 25%-75% percentile (for Mann-Whitney post-test). GraphPad Prism 5 software was used.

RESULTS

Abnormal histological findings are only present in livers of irradiated and CCl_4-intoxicated animals

Initially, HE and Sirius Red staining were performed to evaluate whether the radiation procedure used for BM ablation could induce hepatic lesions. Pre-experimental observations showed that livers from irradiated animals presented no histological changes, displaying regular hepatic architecture and no septa formation, as observed in non-irradiated WT. The same characteristics were observed in animals from the Saline 30 d group (Figure 1A and C).

After 30 d of intoxication, livers from the CCl_4 30 d group showed inflammatory infiltration in regions of the centrilobular and portal veins (Figure 1B), indicating hepatocellular damage. Also, the CCl_4 30 d group showed formation of initial thin septae originating from regions around the centrilobular veins (Figure 1D), indicating deposits of collagen replacing parenchymal cells (fibrosis). Morphometric analysis indicated a significant increase ($P = 0.0329$) in collagen content in CCl_4 30 d group animals compared to Saline 30 d group animals (Figure 2).

Analysis of GFP+ cells derived from bone marrow in peripheral blood of irradiated/CCl_4-intoxicated animals

Blood population analysis showed that samples from all chimeric animals contained a mean of 88% CD45+/GFP+ cells, while 12% were CD45+/GFP-. Among the CD45+/GFP+ population, there was a significant decrease in total lymphocytes and a significant increase in monocytes and neutrophils (Figure 3).

We also analyzed the presence of GFP+ BM-derived precursor cells in the blood, analyzing hematopoietic precursor cells (CD45+/CD34+) (Figure 4A) and endothelial precursor cells (CD45+/CD133+) (Figure 4B). There was no significant difference in these precursor cells between the Saline 30 d and CCl_4 30 d groups (Figure 4C and D).

CD117 (c-Kit) precursor cells were analyzed in the BM (Figure 5A). The findings show a significant increase ($P = 0.0142$) in CD45+/GFP+/CD117+ cells in the CCl_4 30 d group (5.2% ± 1.3%) compared to the Saline 30 d group (2.6% ± 1.8%) (Figure 5B).

Bone marrow-derived cells are present in liver tissue

We analyzed cells from digested liver tissue and observed four well-defined subpopulations (Figure 6A): (1) high frequencies of GFP+/CD45+ and GFP-/CD45+ and (2) low frequencies of GFP+/CD45- and GFP-/CD45-. To identify cells of BM origin in liver tissue, we analyzed the GFP+/CD45+ subpopulation and observed a significant increase in this subpopulation in intoxicated livers (Saline 30 d = 3.2% ± 2.2% *vs* CCl_4 30 d = 5.8% ± 1.3%; $P = 0.0458$) (Figure 6B).

To identify HSCs, we used the CD38 surface marker. This molecule is known to identify B lymphocytes and activated T cells and also labels HSCs[26], indicating their presence in liver tissue. We identified two subpopulations of CD38+ cells: GFP+/CD38+ and GFP-/CD38+ (Figure 7A). We analyzed the GFP-/CD38+ subpopulation and found two other subpopulations: CD45+ and CD45- (Figure 7B). We also found that the GFP+/CD38+ cells were 100% CD45+ (Figure 7C). Considering that HSCs do not express the CD45 marker, we evaluated the GFP-/CD38+/CD45- subpopulation. We found a significant increase in that subpopulation when

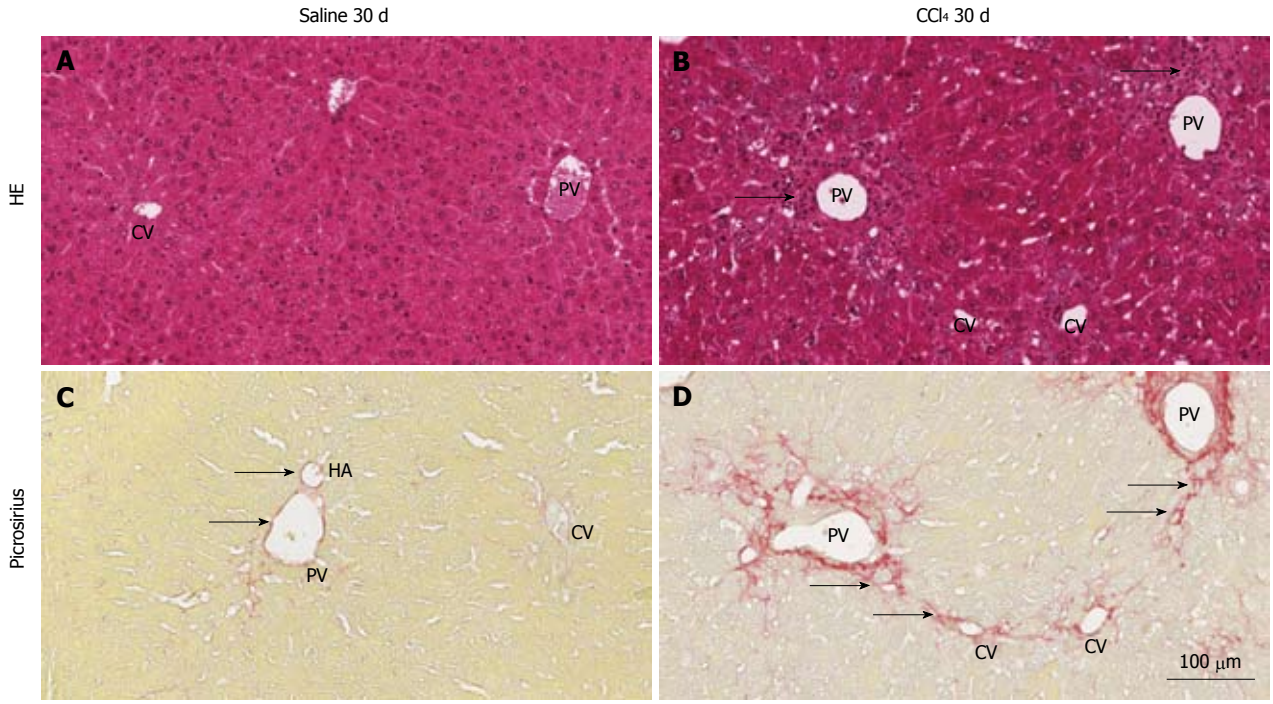

Figure 1 Representative images of histological sections of livers of normal and chimeric animals stained with hematoxylin and eosin or picrosirius. A: hematoxylin and eosin (HE): shows the central vein (CV), portal vein (PV) and regular hepatocyte plates, representing normal architecture of the liver; B: The PV and CV present inflammatory infiltrate (arrows) due to injury of hepatocytes in this region by CCl_4; C: Picrosirius: collagen (red) is present only in the perivascular region of the PV, hepatic artery (HA) (arrows) and lightly present surrounding the VC; D: a high deposition of collagen surrounding the PV and radiating fibers to the CV, indicating initiation of collagen septa (arrows) formation 30 d after injury induction.

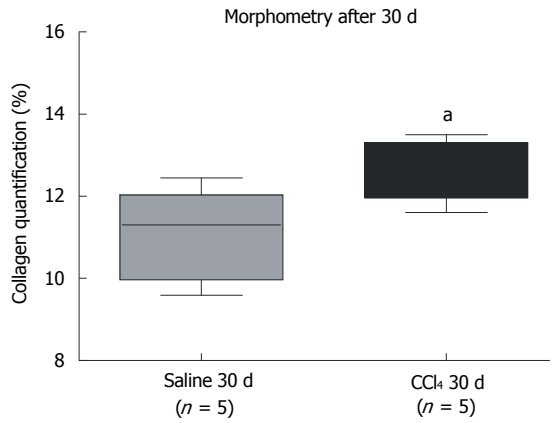

Figure 2 Quantification of collagen by morphometric analysis. A significant increase in collagen between the group that received CCl_4 compared to the group that received saline. The groups are represented by box–whisker diagrams in which the values in the boxes represent the medians of collagen content (n = 5) and the bars the 25%-75% range. The Mann-Whitney post-test was used to test for significance. ^aP = 0.0329 *vs* the Saline 30 d group.

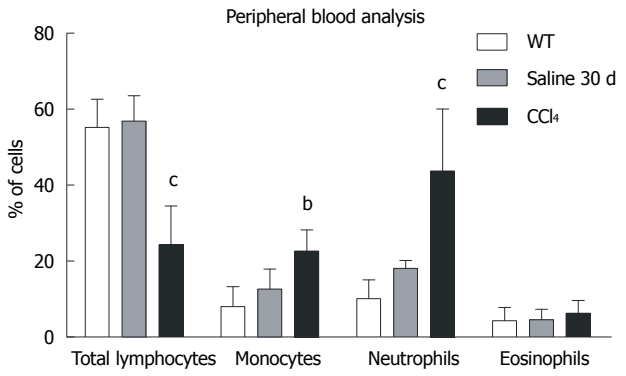

Figure 3 Graphical analysis of subpopulations of peripheral blood of the WT, Saline 30 d and CCl_4 30 d groups by flow cytometry. Only the animals in CCl_4 30 d group showed significant differences from wild-type (WT): Total lymphocytes (WT = 55.2% ± 7.4%; Saline 30 d = 56.9% ± 6.6%; CCl_4 30 d = 24.3% ± 10.2%); Monocytes (WT = 8.1% ± 5.3%; Saline 30 d = 12.7% ± 5.3%; CCl_4 30 d = 22.7% ± 5.6%); Neutrophils (WT = 10.1% ± 5.0%; Saline 30 d = 18.2% ± 2.1%, CCl_4 30 d = 43.8% ± 16.3%). One-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons was used. $^bP < 0.01$, $^cP < 0.001$ *vs* WT.

comparing the Saline 30 d group (9.3% ± 2.4%) to the CCl_4 30 d (17.5% ± 3.9%) group (P = 0.004) (Figure 7D), indicating that the increase in the HSC subpopulation was not of BM origin. We did not observe a significant increase in the GFP+/CD38+/CD45+ population between the experimental groups, suggesting that there is no increase in B and T mobilization after injury (data not shown).

No evidence of bone marrow contribution to liver fibrogenesis

The Saline 30 d group showed fusiform cells with green fluorescence, most of them situated in the sinusoidal space and a few in perivascular regions (Figure 8A), as found in GFP+ animals (data not shown). In contrast, the CCl_4 30 d group showed a higher number of GFP+ cells diffusely distributed in the parenchyma and perivas-

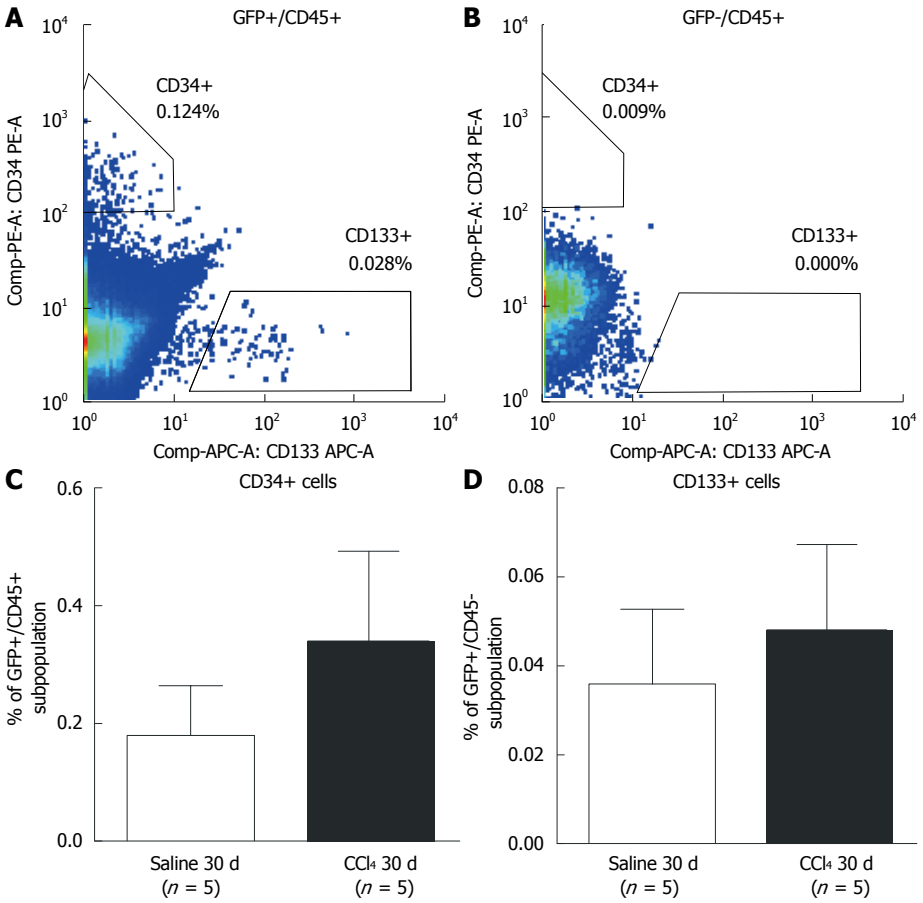

Figure 4 Flow cytometric analysis of subpopulations of hematopoietic and endothelial progenitor cells in bone marrow of chimeric mice. A: Representative results showing green fluorescent protein (GFP)+ precursor cells in both the Saline 30 d and CCl_4 30 d groups (side bar = ancestry gating). CD45+/CD34+ (hematopoietic) and CD45+/CD133+ (endothelial) cells were identified; B: Representative results showing GFP-subpopulation precursor cells in the Saline 30 d and CCl_4 30 d groups (side bar = ancestry gating). No progenitor cells were found in the GFP-subpopulation; C: Graphic of the percentage of GFP+/CD45+/CD34+ cells (hematopoietic progenitor cells); D: GFP+/CD45+/CD133+ (endothelial progenitor cells) in peripheral blood of the Saline 30 d and CCl_4 30 d groups. No significant difference was detected between the groups by Student's *t* test.

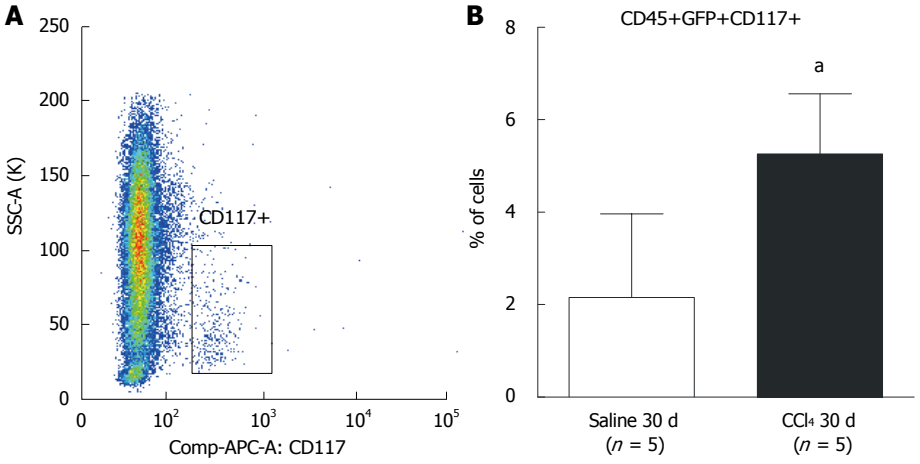

Figure 5 Flow cytometric analysis of subpopulations of CD117+ precursor cells in bone marrow of chimeric mice. A: Dot-plot analysis of CD117+ cells found in bone marrow. Side-bar dot-plots represent ancestry gating; B: A significantly greater population of CD117+ cells was observed in liver tissue from the CCl_4 30 d group compared to the Saline 30 d group (30 d saline = 2.6 ± 1.3%; CCl_4 30 d = 5.6 ± 1.8%) ($^aP = 0.0142$). GFP: green fluorescent protein.

cular region, presenting fusiform morphology, rounded morphology and also very small nuclei (Figure 8B).

As we confirmed the increased migration of BM GFP+ cells to the injured liver, we investigated if those cells were differentiating into activated HSCs and MF-like cells, which are responsible for the deposition of col-

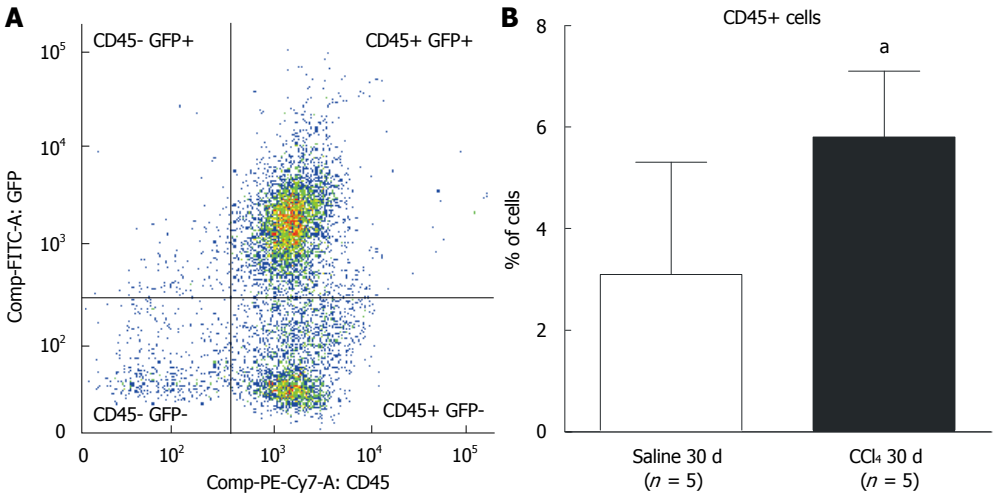

Figure 6 Flow cytometric analysis of bone marrow subpopulations in the liver of chimeric mice. A: Dot-plot analysis of cell populations in the liver; B: A significantly greater population of CD45+ cells was observed in liver tissue of animals from the CCl_4 30 d group compared to the Saline 30 d group (30 d saline = 3.1% ± 2.2%; CCl_4 30 d = 5.8% ± 1.3%) (^aP = 0.0458). GFP: green fluorescent protein.

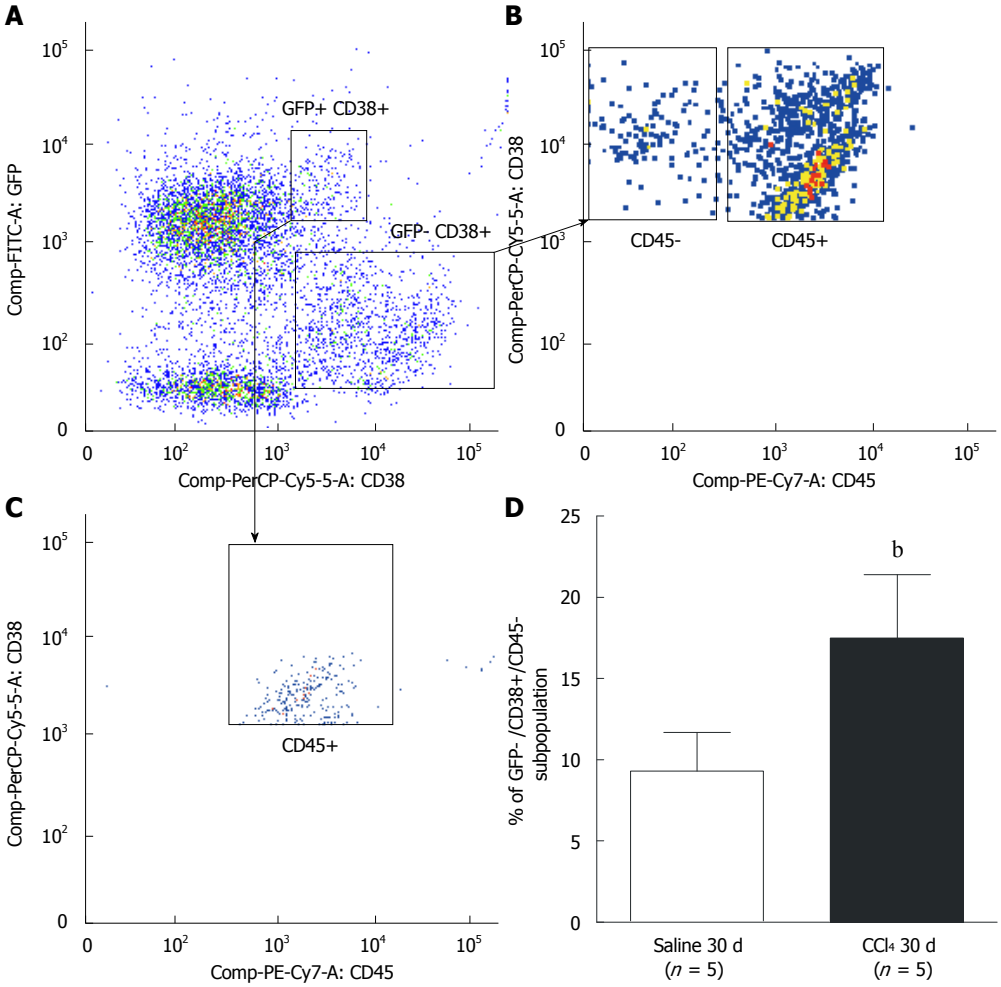

Figure 7 Representative analysis of CD38+ hepatic stellate cell subpopulations by flow cytometry. A: Two CD38+ subpopulations were observed: green fluorescent protein (GFP)+/CD38+ and GFP-/CD38+; B: Two subpopulations among GFP-/CD38+ cells were observed: GFP-/CD38+/CD45+ that identifies B lymphocytes, and GFP-/CD38+/CD45 that identifies hepatic stellate cells; C: Analysis of GFP+/CD38+ cells showed that all cells were of hematopoietic origin (CD45+); D: A significant increase of cells GFP/CD38+/CD45- was observed in liver tissue of the CCl_4 30 d group when compared to the Saline 30 d group (Saline 30 d = 9.3% ± 2.4%; CCl_4 30 d = 17.5% ± 3.9%, ^bP = 0.004). The statistical test used was Student's *t* test.

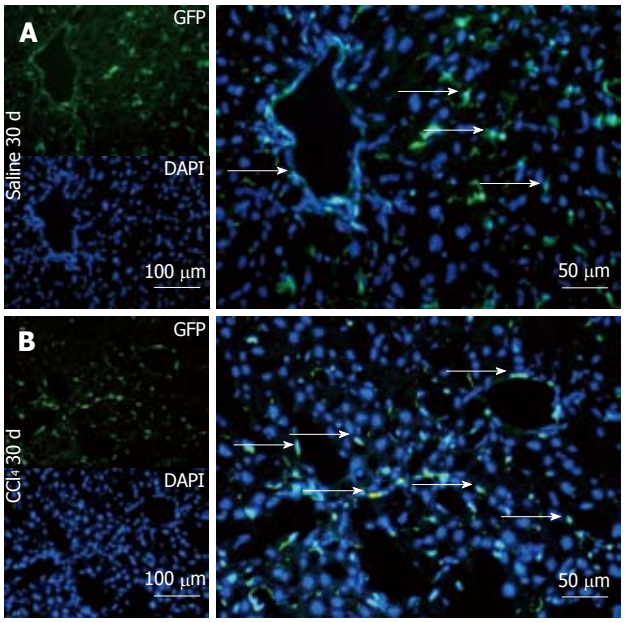

Figure 8 Representative direct fluorescence images of hepatic sections of chimeric animals after transplantation. Liver samples of chimeric animals after transplantation of green fluorescent protein (GFP)+ bone marrow- mononuclear cell, chimeric animals that received saline (Saline 30 d) and chimeric animals that received CCl_4 (CCl_4 30 d). In panel A (Salina 30 d), the GFP channel shows the presence of green fluorescence; in the 4, 6-diamino-2-phenylindole panel, nuclei are stained blue in the same field above; the merge panel superposes the two images, where GFP+ cells (arrows) can be seen distributed in major hepatic parenchyma and with fusiform morphology. In panel B (CCl_4 30 d), GFP+ cells can be seen with greater distribution in the hepatic parenchyma, some fusiform morphology (white arrows) and others with rounded morphology. DAPI: 4,6-diamino-2-phenyl indole.

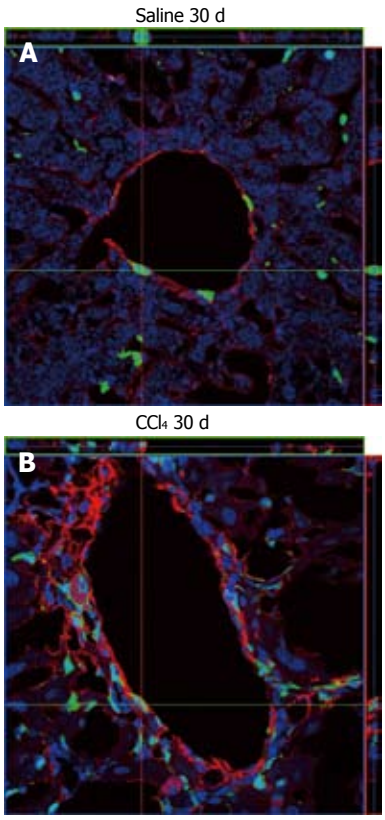

Figure 9 Immunostaining analysis of α-smooth muscle actin in liver samples from chimeric animals by confocal microscopy. A: α-smooth muscle actin (α-SMA)+ cells in the perivascular region were observed where green fluorescent protein (GFP)+ cells could also be seen, but the two cell types do not co-localize, as can be seen by the orientation of the red and green bars; B: An increase in reactivity against α-SMA around the perivascular region was observed, also with augmentation of GFP+ cells, but again these cell types do not co-localize. In both images the nuclei were stained with TO-PRO3 (blue).

lagen, by using an α-SMA antibody as a specific marker for these cell types. Our results demonstrate that α-SMA+ cells in normal and irradiated/non-intoxicated livers are mainly distributed surrounding periportal regions (Figure 9A), while in intoxicated animals there is an increase of α-SMA+ cells that spread from periportal regions to parenchyma, representing initial septa formation (Figure 9B). These results indicate proliferation of cells with MF characteristics when there is liver damage. However, in our confocal analysis, we found no co-localization of α-SMA+ with GFP+ cells either in normal or injured tissue, suggesting that cells of BM origin do not contribute to the population of MFs, even after induction of liver injury.

DISCUSSION

In the present study, we showed that BM-derived progenitor subpopulations do not contribute to the fibrogenic MF population after liver injury in mice.

It is already known that the BM is altered after chronic hepatic disease[27-29]. The presence of inflammatory cells in PB indirectly indicates alterations in the proliferation of cells in BM[30]. We observed a significant increase in mature hematopoietic cells in the blood of CCl_4-intoxicated animals (Figure 3), indicating a chronic inflammatory condition 30 d after liver injury induction. Because inflammatory cells are increased, it is expected that the progenitor compartment of BM should also be altered. In fact, we observed an increase in CD117 (c-Kit+) cells in BM of injured mice, which is a marker for BM progenitors such as common myeloid progenitors (CMPs)[31].

Among the progenitor cells in blood, we could identify a GFP+/CD45+/CD34+ subpopulation, a rare population that is more related to hematopoietic progenitor cells[32] but also to circulating fibrocytes[19]. We found this GFP+/CD45+/CD34+ population at a very low percentage in total PB and it did not increase even after 30 d of liver injury. Because CMPs were increased in BM, we believe that hematopoietic stem cells are quickly stimulated to differentiate into CMPs in response to signaling from inflammatory cells at the injury site, thus giving primitive hematopoietic stem cells a short life-span. Consequently, recruitment of hematopoietic stem cells to blood may not be efficient. Endothelial progenitor cells (CD133+) may be affected by the same behavior. Concerning fibrocytes, Scholten *et al*[33] have shown that fibrocytes are stimulated to migrate even in non-injured liver in older but not in younger mice. Therefore, detection of fibrocytes in blood may be difficult since we used young mice. We tried to evaluate whether hematopoietic progenitors could be identified in the liver by flow cytometry.

Studies report that hematopoietic stem cells can comprise 0.1% to 1.0% of total blood cells as well as fibrocytes[24]. Our samples presented a total hematopoietic cell frequency in liver of 6%, which is very low. For that reason, detection of rare cells in a low-frequency population is very difficult, and it will be necessary to have a

much larger sample to obtain reliable data to address this question. Although we could not detect if circulating fibroblasts were present in blood and liver samples, we suggest that this subpopulation did not contribute to liver fibrosis because no GFP+/α-SMA+ cells were detected in our liver tissue specimens. Moreover, even if they did transdifferentiate to an MF-like cell, they would be present at such a low frequency that they would be unlikely to exert a major influence on fibrogenesis.

On the other hand, HSCs do contribute importantly to the fibrogenetic process[34], but there is still controversy in the literature concerning the contribution of the BM to this liver subpopulation. Baba *et al*[35] reported co-localization of GFP and α-SMA in non-parenchymal cells in *in vitro* and *in vivo* models, whereas Higashiyama *et al*[36] showed a lack of co-localization of those markers after peak fibrosis. Our results corroborate the findings of Higashiyama *et al*[36], and suggest that BM-derived cells do not contribute to the HSC compartment. To confirm this hypothesis, we identified the HSCs by flow cytometry, using CD38 as a marker. The CD45-/CD38+ subpopulation was GFP negative, which agrees with our confocal microscopy results. Therefore, we suggest that the HSC population is not of BM hematopoietic origin, as it lacks both CD45 and GFP. Our results do not corroborate those reported by Miyata *et al*[9], who reported finding enhanced GFP+ HSCs in a longer injury induction experimental model, which may suggest that duration of injury could be a factor in determining the extent of BM cell contribution to fibrosis.

In conclusion, we found that BM progenitor cells do not contribute to fibrosis. However, there is a high response toward inflammatory cell recruitment that may contribute to fibrosis, by producing pro-inflammatory factors that stimulate HSCs and perivascular MFs of liver origin.

COMMENTS

Background

Bone marrow (BM) has been proposed as a source of cells for alternative therapy in liver diseases. Recent findings, however, suggest a potential deleterious effect of BM-derived cells in the regenerating liver.

Research frontiers

Results published so far show no consensus on the role of the BM in liver repair. Indeed, this issue remains one of the most controversial in the field of regenerative medicine. In this study, the authors demonstrate that BM stem cells do not participate in liver fibrogenesis.

Innovations and breakthroughs

Publications related to liver fibrosis and cell therapy have suggested that BM cells could contribute to liver fibrosis by differentiating into hepatic stellate cells (HSCs) and myofibroblasts (MFs), but have not identified which specific BM subpopulation participates in this process. In this study, the authors demonstrate that two BM subpopulations, the hematopoietic and endothelial progenitor cells, do not contribute to fibrosis by their differentiation to those fibrotic cells.

Applications

By understanding how BM cells function in an injured liver, this study is useful as a guide for strategies related to cell therapies using BM cells.

Terminology

Hematopoietic and endothelial progenitor cells are BM subpopulations which participate in hematopoiesis and vasculogenesis, respectively. HSCs are non-parenchymal liver cells found in the perisinusoidal space (Space of Disse) and are pivotal in liver fibrogenesis by producing fibrillar extracellular matrix when in an activated state.

Peer review

In this study, the findings suggest that the response toward inflammatory cell recruitment by producing pro-inflammatory factors that stimulate HSCs and perivascular MFs of liver origin may contribute to fibrosis, while no evidence in the study supports that BM-derived progenitor cells contribute to the population of α-smooth muscle actin-producing cells.

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CASE REPORT

Hepatic osteodystrophy complicated with bone fracture in early infants with biliary atresia

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Abstract

Biliary atresia (BA) is one of the major hepatobiliary abnormalities in infants and one of the causes of hepatic osteodystrophy. Bone disease may be caused by the malabsorption of calcium and magnesium by vitamin D in hepatobiliary diseases in which bile flow into the intestines is deficient or absent. Bone fracture before Kasai hepatic portoenterostomy or within one month after the procedure in an infant with BA is very rare. We herein report two infants: one infant with BA who initially presented with a bone fracture before Kasai hepatic portoenterostomy, and the other at 4 wk after Kasai hepatic portoenterostomy, and also provide a review of the literature. Moreover, we conclude that clinicians should consider BA in infants with bone fracture during early infancy.

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Key words: Biliary atresia; Bone fractur; Hepatic osteodystrophy; Kasai hepatic portoenterostomy; Vitamin D deficiency

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INTRODUCTION

Clinical findings in children with biliary atresia (BA) characteristically include jaundice and acholic stools at 1 or 2 mo after birth[1]. Osteodystrophy is a well-recognized complication of chronic liver disease. BA is one of the major hepatobiliary abnormalities in infants and one of the causes of hepatic osteodystrophy[1].

Vitamin D is hydroxylated at the carbon 25 position to form 25-hydroxy-vitamin D (25-OH-D)[2]. This occurs primarily in the liver[2]. Bile is important for the intestinal absorption of calcium and magnesium because it is necessary for the absorption of vitamin D[1].

In chronic liver disease, particularly where there is chronic cholestasis, generalized skeletal demineralization or rachitic change is seen[3]. Multiple spontaneous fractures of both the ribs and long bones have been reported in such infants. Furthermore, bone fractures are sometimes noted in patients with BA in the end-stage before liver transplantation[4]. However, bone fracture before Kasai hepatic portoenterostomy and within one month after the procedure in infants with BA is very rare.

We report two infants: firstly, a patient with BA who initially presented with bone fracture before Kasai he-

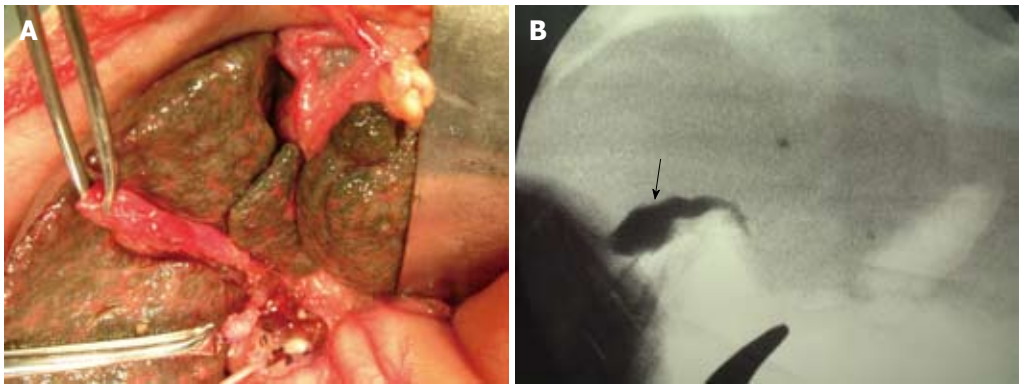

Figure 1 Intraoprative and imaging features. A: On laparotomy, the liver was brown and firm with a dull edge, suggesting cholestasis; B: Intraoperative cholangiography revealed a patent gallbladder (arrow) and no patency of the extrahepatic bile duct.

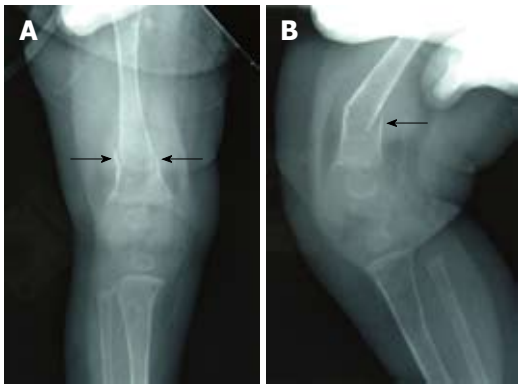

Figure 2 Plain skeletal radiographic features at the 7 d after hepaticojejunostomy in the case 1. Anteroposterior (A) and lateral (B) plain radiographs showing a displaced fracture (arrows) of the right distal femur.

patic portoenterostomy, and secondly, a patient with the onset of bone fracture within one month after Kasai hepatic portoenterostomy, and also provide a review of the literature.

CASE REPORT

Case 1

A girl was born vaginally at 39 wk gestation, weighing 2522 g. She presented with neither jaundice nor acholic stools. The infant was fed human milk. She was well nourished but was observed to have jaundice at a medical check-up at 1 mo of age. Abdominal ultrasonography (US) and computed tomography showed a sufficiently large gallbladder. Total and direct bilirubin (DB) decreased gradually at the follow-up checks. The patient presented with acholic stools and increased jaundice at the age of 5 mo, and was subsequently admitted to our institution for further examinations. Laboratory studies upon admission revealed the following: asparate aminotransferase (AST) 337 IU/L (normal range), alanine aminotransferase (ALT) 241 IU/L (normal range), total bilirubin (TB) 11.3 mg/dL, DB 7.4 mg/dL, alkaline phosphatase (ALP) 5,547 IU/L (normal range), γ-glutamyl transpeptidase (γ-GTP) 457 IU/L (normal range), choline esterase 192 IU/L (normal range), and serum calcium 8.1 mg/dL (normal range). There was severe jaundice noted at the conjunctiva. The findings on abdominal US were unevenness on the liver surface and an atrophic gallbladder which did not contract after the feeding of milk. Magnetic resonance cholangio-pancreatography (MRCP) revealed dilatation of neither the common bile duct nor intrahepatic bile duct. Therefore, BA was suspected based on these findings, and the infant underwent an exploratory laparotomy at 182 d of age. The patient started oral vitamin D at 173 d of age.

On laparotomy, the liver was brown and firm with a dull edge, suggesting cholestasis (Figure 1A). Intraoperative cholangiography revealed a patent gallbladder and no patency of the extrahepatic bile duct (Figure 1B). The macroscopic findings showed that the bilateral hepatic ducts and extrahepatic bile duct consisted of only remnants. The infant was diagnosed as BA (Ⅱb1γ)[5] based on cholangiographic and macroscopic findings. The remnants were totally removed en block and a Roux-en-Y hepaticojejunostomy was performed with a Roux loop of 60 cm applied antecolically. Microscopic findings of the liver biopsy specimen were pre-cirrhotic.

The patient could not move her right leg 1 d before the laparotomy, and a plain skeletal radiograph of the femur was performed 7 d after the HJ, when the general condition of the patient was stable. A displaced fracture of the right distal femur was shown by the plain radiograph (Figure 2A and B). Hepatic osteodystrophy was suspected based on the fact that there was no history of femur trauma and the patient suffered from chronic cholestasis. Child abuse by the family was not considered from the situation. Callus formation was seen 8 d after the application of an immobilizing plaster bandage (Figure 3A). The plaster bandage was removed after 20 d and the fracture of the right femur was cured at 6 mo post fracture (Figure 3B and C). The patient coughed up blood due to the perforation of esophageal varices and underwent a living-related liver transplantation at 10 mo of age. The postoperative course of living-related liver transplantation was uneventful and she is currently well at 4 years of age.

Case 2

A girl was born vaginally at 36 wk gestation, weighing 2310 g. She presented with neither jaundice nor acholic stools. She was well nourished but was observed to have

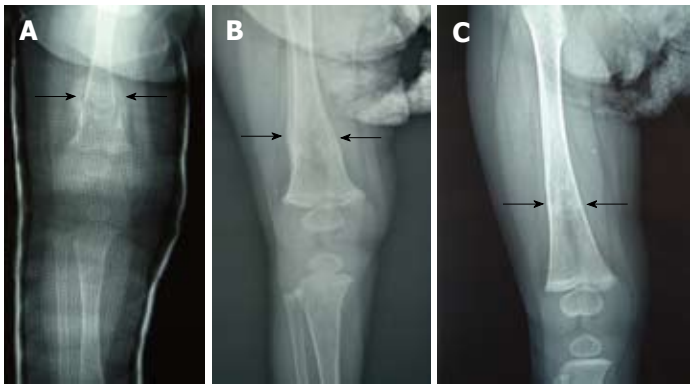

Figure 3 Plain skeletal radiographic features at the 8 d after the application of an immobilizing plaster bandage for the femur fracture in the case 1. Callus formation (arrows) was seen 8 d after the application of an immobilizing plaster bandage (A) in case 1. The plaster bandage was removed after 20 d (B) and the fracture of the right femur was cured 6 mo post-fracture (C).

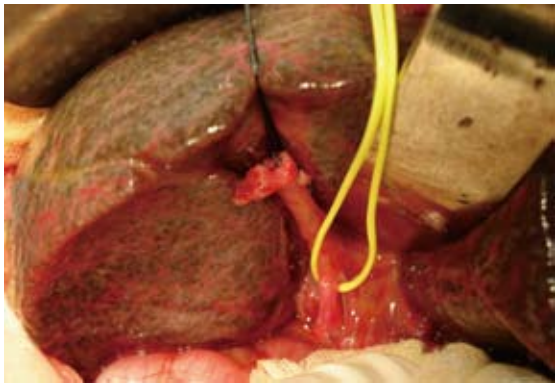

Figure 4 Intraoprative features. On laparotomy, the liver was brown and firm with a dull edge, suggesting cholestasis.

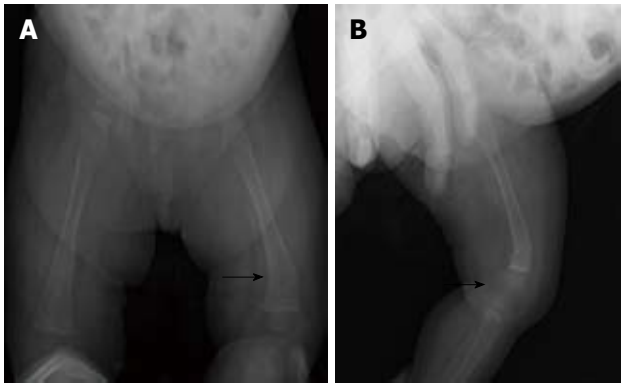

Figure 5 Plain skeletal radiographic features at the 28 d after hepaticojejunostomy in the case 2. Anteroposterior (A) and lateral (B) plain radiographs showing a displaced fracture (arrows) of the left distal femur.

jaundice at a medical check-up at 1 mo of age. The patient presented with acholic stools and increased jaundice at the age of 3 mo at a medical check-up, and was consequently admitted to our institution for further examinations. Laboratory studies upon admission revealed the following: AST 573 IU/L, ALT 377 IU/L, TB 6.6 mg/dL, DB 4.4 mg/dL, ALP 2248 IU/L, γ-GTP 666 IU/L, choline esterase 181 IU/L, and serum calcium 9.2 mg/dL. The findings on abdominal US and MRCP were just as same as those of the case 1. Therefore, BA was suspected, and the infant underwent an exploratory laparotomy at 113 d of age. The patient started oral vitamin D at 3 mo of age.

On laparotomy, the liver was brown and firm with a dull edge, suggesting cholestasis (Figure 4). Intraoperative cholangiography revealed a patent gallbladder and no patency of the extrahepatic bile duct. The infant was diagnosed as BA (Ⅱb1γ)[5] based on cholangiographic and macroscopic findings. The remnants were totally removed en block and a Roux-en-Y hepaticojejunostomy was performed with a Roux loop of 60 cm applied antecolically. Microscopic findings of the liver biopsy specimen were cirrhotic.

The patient could not move her left leg at 28 d post-laparotomy. A displaced fracture of the left distal femur was shown by plain skeletal radiograph (Figure 5A and B). Hepatic osteodystrophy was suspected based on the fact that there was no history of femur trauma and the patient suffered from chronic cholestasis. Child abuse by the family was not considered from the situation. Callus formation was seen 14 d after the application of an immobilizing plaster bandage. The plaster bandage was removed after 20 d and the fracture of the left femur was cured at 6 mo after post-fracture. Jaundice has been resolved and she is currently well at 11 mo of age.

DISCUSSION

BA is a rare disease with an incidence of approximately 1:10 000 live births in Japan and the Far East[6]. The most frequent symptom is prolonged jaundice. Several reports have shown that osteodystrophy was associated with severe chronic liver disease despite the administration of vitamin and mineral supplements[1]. Argao *et al*[7] suggested that the bone mineral content of patients with hepatic osteodystrophy did not improve despite successful normalization of the serum 25-OH vitamin D concentration by enhancing vitamin D absorption from the gastrointestinal tract. Chongsrisawat *et al*[8] reported that osteoporosis was recognized in up to 80% of a group of jaundiced BA patients in comparison with only 13.6% in a non-jaundiced group.

In BA, metabolic disturbance results from impairment of the passage of bile salts into the alimentary canal. As a consequence, the inadequate emulsification of fat results

in the incomplete absorption of vitamin D. Vitamin D is hydroxylated to 25-OH-D in the liver[2]. Additionally, over the course of the disease, liver cirrhosis develops and the hydroxylation of vitamin D is impaired. Vitamin D and hence calcium absorption are thus diminished. 25-OH-D is thought to be converted to more active forms, 125- or 2125-dehydro-OH-D. Rickets and osteoporosis were reported to be found in 23 of 39 patients (59%) with surgically unrepaired BA[1].

We herein report two infants: one infant with BA who initially presented with a bone fracture before Kasai hepatic portoenterostomy, and the other at 4 wk after Kasai hepatic portoenterostomy. There are a number of factors which may be important in the etiology of bone fractures in children, including trauma, metabolic bone disease, drugs, and immobilization[3]. However, the lack of significant trauma in the majority of cases (91%) is a notable feature in children with BA[3]. Hill *et al*[3] reported 12 (19%) children with fractures before and after transplantation out of 63 undergoing liver transplanatation. Eight of 12 children with fractures in BA had no identifiable trauma. The age at the time of fracture in BA ranged from 3 to 16 mo after birth, and the affected children suffered from osteopenia (generalized reduction in bone density). The fracture site was the ribs or long bones, and multiple fractures were seen in 2 children with BA (7 and 8 mo after birth). However, Hill *et al*[3] did not describe administering vitamin D supplements. BA patients with severe cholestasis have a risk of bone fracture despite the administration of essential vitamins and minerals such as our cases. In our cases, BA was diagnosed at 6 mo after birth in case 1 and at 3 mo after birth in case 2, with suspected severe cholestasis.

Conservative management such as immobilization using plaster bandages is generally effective for fractures in BA, and there were no complications related to fractures in our cases. In the literature, internal fixation was required in one case with oxalosis for a fractured neck of the femur[1]. The early diagnosis and treatment of BA before the occurrence of bone fracture is important. The measurement of reflected light from the surface of feces by near infrared reflectance spectroscopy was introduced by Akiyama *et al*[9] for the differential diagnosis of cholestatic diseases in infants. Another method, mass screening using color picture cards depicting normal and acholic stools, was carried out at 1 and 2 mo after birth in a Japanese prefecture[10]. Eight cases of BA were detected using this mass screening method during a 3-year period, with a specificity of 99.9% and a sensitivity of 80.0%. Such screening procedures could result in improved detection of BA in infants before bone disorders occur.

In summary, clinical awareness of BA should be maintained both in terms of careful handling to prevent possible bone fracturing and also in considering fractures as a possible diagnostic factor in children with reluctance to use a limb, even in the absence of previous trauma, before Kasai hepatic portoenterostomy. Radiological awareness is also important to avoid missing unsuspected fractures on radiographs.

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CASE REPORT

Peritoneal bleeding due to percutaneous transhepatic gallbladder drainage: An autopsy report

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Author contributions: Ihama Y, Fukazawa M and Ninomiya K performed the macroscopic and microscopic examination; Nagai T, Fuke C and Miyazaki T gave clinical and general advices.

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Abstract

A 77-year-old man underwent percutaneous transhepatic gallbladder drainage (PTGBD) for acute cholecystitis as a preoperative procedure; however, he suddenly suffered cardiopulmonary arrest 4 h after the PTGBD and died. There were three centesis scars for the PTGBD, and only one pathway from the most dorsal centesis scar reached the gallbladder. Microscopically, the PTGBD pathway crossed and injured the intrahepatic arterial wall, and hepatic parenchymal bleeding extended along the PTGBD pathway to the inferior surface of the liver. Blood flowed to the peritoneal cavity through a small gap between the liver and gallbladder. Consequently, the PTGBD caused lethal bleeding. When the percutaneous transhepatic cholangio drainage/PTGBD pathway runs close to vessels near the liver surface, it might be necessary to deal with the possibility of rapid and lethal peritoneal bleeding.

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Key words: Percutaneous transhepatic gallbladder drainage; Percutaneous transhepatic cholangio drainage; Lethal complication; Autopsy

INTRODUCTION

Percutaneous transhepatic cholangio drainage (PTCD) and percutaneous transhepatic gallbladder drainage (PTGBD) are effective procedures that promptly release congestive bile from the body. The safety and reliability of PTCD/PTGBD have been greatly increased by the use of ultrasonic guidance for centesis[1]. Recently, there have been very few reports of deaths directly resulting from PTCD/PTGBD in patients without severe preexisting disease[2]. Here we report a patient who died from bleeding shock resulting from PTGBD, and we discuss the causes of the lethal bleeding based on autopsy findings.

CASE REPORT

A man in his early seventies was admitted to the emergency department of a hospital with the chief complaint of abdominal pain that had been increasing for the past several days. Ultrasonography (US) showed a swelling gallbladder filled with debris and stones. Abdominal CT indicated an invagination of the stones into the neck of the gallbladder. The results of blood chemical tests administered at admission were as follows: white blood cell: 10 000 /L, hemoglobin: 14.5 g/dL, platelet (Plt): 17.6 ×

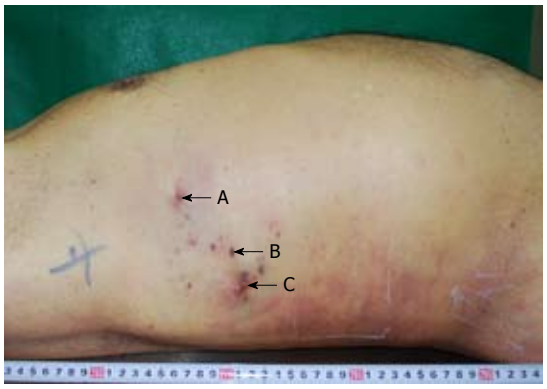

Figure 1 Three centesis scars on the right side of the chest. As shown in the figure, ventral, middle, and dorsal centeses were assigned as (A), (B) and (C), respectively.

10^4/L, total-bilirubin: 0.6 mg/mL, aspartate aminotransferase: 17 IU/L, alanine aminotransferase: 8 IU/L, lactate Dehydrogenase: 176 IU/L, alkaline phosphatase: 246 IU/L, Amylase: 129 IU/L, C-reactive protein: 7.04 mg/dL. The patient was diagnosed with acute cholecystitis due to bile stones. His general condition was stable. He had no bleeding disorder or coagulation abnormality, and he was not taking anticoagulant agents, such as warfarin or aspirin. Two years earlier, however, he was diagnosed with aortic dissection and from then on had been managed conservatively with antihypertensive agents. In the meantime, ultrasound-guided percutaneous transhepatic gallbladder aspiration or PTGBD was selected as a preoperative procedure. He had been complaining of severe abdominal pain despite the intravenous administration of pentazosine, an analgesic. Thus he could neither remain still on his back nor hold his breath. With the patient in the left lateral decubitus position, the third centesis finally reached the gallbladder, after two failures of centesis. Because the thick bile or debris in the gallbladder could not be adequately aspirated through a puncture needle, the operators changed their strategy and placed a catheter in the patient for PTGBD. The drainage catheter egested a thick bile, and no dye leakage or hemorrhage was revealed by post-PTGBD radiographic examination. Four hours after PTGBD, the patient suffered a sudden cardiopulmonary arrest in bed. At 14 h after PTGBD, he died despite undergoing intensive care.

Autopsy findings

The body was 165 cm in length and weighed 90 kg. The abdomen was tense with distension, and there were three puncture scars on the right side (Figure 1). The PTGBD drainage catheter had been removed in the hospital. As shown in Figure 1, the three puncture scars were labeled (A), (B) and (C). Volumes of blood with clots of 1050 mL and 1500 mL were present in the right thoracic and peritoneal cavities, respectively, and large numbers of clots were found around the gallbladder and inferior surface of the liver. The gallbladder wall was thick due to inflammation, and there were approximately 100

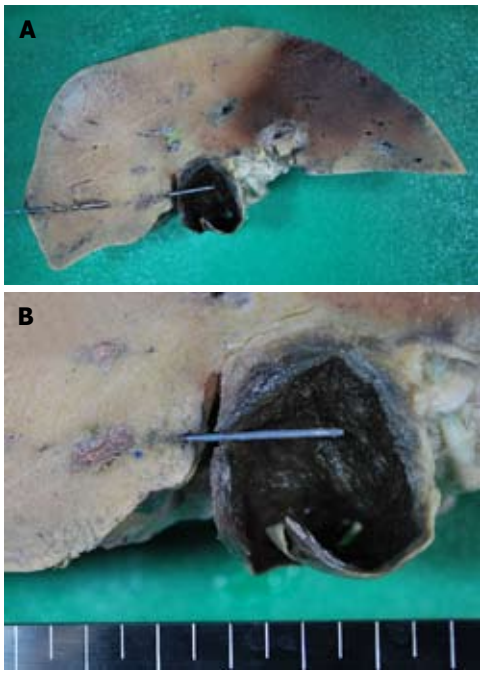

Figure 2 Frontal section of liver along pathway (C). A: Pathway (C) reaches the gallbladder transversely through the right hepatic lobe; B: Magnified photograph around the gallbladder. A small space between the liver and gallbladder is revealed, and the percutaneous transhepatic gallbladder drainage pathway is partially exposed into the peritoneal cavity. The pathway crosses the intrahepatic connective tissue area near the liver surface.

black stones in the gallbladder. Among the three pathways of centesis, only pathway (C) reached the gallbladder, transversely through the right hepatic lobe (Figure 2A). Macroscopically, pathway (C) crossed interlobular tissue near the liver surface and was exposed to the peritoneal cavity between the liver and gallbladder before entering the gallbladder (Figure 2B). Histological serial cross-sections of pathway (C) revealed that the pathway gradually came close to interlobular vessels and injured an intrahepatic artery (Figure 3). The bleeding from that artery extended along pathway (C) into the peritoneal cavity. The main organs were anemic, and there were no injuries to the other vessels and organs. The endothelial surface of the aorta showed severe atherosclerosis but no dissection.

We attributed the patient's death to abdominal and thoracic bleeding resulting from injury of the intrahepatic artery caused by PTGBD.

DISCUSSION

Guidelines on the diagnosis and treatment of acute cholangitis and cholecystitis issued in Japan in 2005 and based on scientific evidence, recommend emergency cholecystectomy for acute cholecystitis[3]. However, PTCD/PTGBD is an effective procedure for decompressing an obstructed biliary system. Thus preoperative PTCD/PTGBD for acute cholecystitis remains a frequent first-choice therapy[2]. According to a study published in 2008, the rates of the technical success of and clinical response

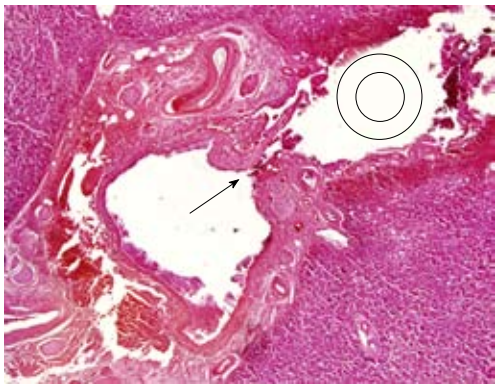

Figure 3 Microscopic specimen at the crossover site of intrahepatic connective tissue and the percutaneous transhepatic gallbladder drainage pathway (hematoxylin-eosin staining). There is a rupture of the arterial wall near the catheter pathway and intrahepatic bleeding along the percutaneous transhepatic gallbladder drainage pathway. The arrow dead shows the point where the intrahepatic artery is ruptured, and the double circle shows a cross section of the catheter on the pathway (C).

to this treatment were 100% and 90%, respectively[2]. Potential complications of PTCD/PTGBD can occur because of its invasiveness; the most serious complications are sepsis and bleeding[4]. The incidence of hemorrhage related with PTCD/PTGBD has been reported to be 2.5%; however, Burke *et al*[4] estimate that the actual incidence is twice as high as the recorded number. In recent randomized studies by Ito *et al*[2], the mortality rate after PTCD/PTGBD was found to be 2.2%, and all deaths were caused by preexisting disease, not by PTCD/PTGBD directly. Hemorrhage is not a rare complication of PTCD/PTGBD, but it is not likely to be fatal without there being a preexisting disease. We believe that the present case was an extremely rare and unfortunate accident.

In this case, the death was caused directly by bleeding due to PTGBD. We believe that this death was attributable to two factors: (1) an intrahepatic artery was injured near the surface of the liver; (2) a drainage pathway was partially opened into the peritoneal cavity between the liver and gallbladder. In the liver, many hepatic arteries, central veins and portal veins are entwined as in the mesh of a net; hence, there is always a risk of hemorrhage when performing the PTCD/PTGBD procedure. In this patient, no anatomical aberrations of the hepatic vessels were detected by US or in the autopsy examination. Generally, a PTGBD catheter is inserted into the gallbladder through the gallbladder bed under US[3]. Hence, the catheter never enters the peritoneal cavity. However, when the gallbladder is filled with congestive bile and stones, it is difficult to determine by US whether the pathway runs through the gallbladder bed or not. In this case, the increased tension and swelling of the gallbladder caused us to misread the ultrasound which seemed to show the catheter pathway running through the gallbladder bed. However, the bile drainage shrank the gallbladder, thereby creating a space between the gallbladder and liver. Textbooks recommend that PTGBD be performed from the patient's anterior direction, because following a longitudinal pathway against the gallbladder can reduce the risk that the drainage catheter will protrude from the liver. However, if the patient cannot remain still on his or her back because of the pain, the operator may need to puncture the patient's right side away from the recommended point.

It is not clear when the hepatic artery was ruptured. The ultrasound-guided PTGBD had been performed without vessel injury, and the post-PTGBD examination detected no bleeding in the liver or around the gallbladder. Therefore, we consider that the arterial injury occurred after the drainage catheter was placed. The PTGBD catheter running near the artery damaged the wall of the artery gradually by rubbing against it with slight movements. Since no vessel injury was detected during or soon after the PTCD/PTGBD, it is possible that the drainage catheter damaged the vessel wall after the procedure. In particular, when the PTCD/PTGBD pathway runs in close proximity to vessels near the liver surface, it might be necessary to deal with the possibility of rapid and lethal peritoneal bleeding, not only during but also after the procedure.

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San Francisco, CA, United States

January 20-21, 2012
AGA Clinical Congress of Gastroenterology and Hepatology: Practice, Evidence and Quality in 2012
Miami, FL, United States

January 27-28, 2012
28th Annual Meeting of the German Association for the Study of the Liver
Hamburg, Germany

January 30-31, 2012
5th International Conference on the Management of Patients with Viral Hepatitis
Paris, France

February 8-10, 2012
Stockholm Liver Week 2012
Stockholm, Sweden

February 16-19, 2012
22nd Conference of the Asian Pacific Association for the Study of the Liver
Taipei, Taiwan, China

March 16 -17, 2012
Hepatitis Single Topic Conference
Atlanta, GA, United States

March 16-17, 2012
ESGE - Workshop on Advanced Endoscopy with Live Demonstrations
Vienna, Austria

March 31-April 1, 2012
27th Annual New Treatments in Chronic Liver Disease
San Diego, CA, United States

April 18-22, 2012
The International Liver Congress by EASL
Barcelona, Spain

April 27-28, 2012
The European Society for Paediatric Gastroenterology, Hepatology and Nutrition
Stockholm, Sweden

May 16-19, 2012
International Liver Transplant Society 18th Annual International Congress 2012
San Francisco, CA, United States

May 19-22, 2012
Digestive Disease Week 2012
San Diego, CA, United States

June 22-23, 2012
EASL Monothematic Conference: Vascular Liver Diseases
Tallin, Estonia

July 1-5, 2012
10th World Congress of the International Hepato-Pancreato-Biliary Association 2012
Paris, France

September 5-8, 2012
International Congress of Pediatric Hepatology, Gastroenterology and Nutrition
Sharm El-Sheikh, Egypt

September 7-9, 2012
Viral Hepatitis Congress 2012
Macclesfield, United Kingdom

September 7-9, 2012
The Viral Hepatitis Congress
Frankfurt, Germany

September 14-16, 2012
The International Liver Cancer Association's 6th Annual Conference
Berlin, Germany

September 20-22, 2012
Prague Hepatology Meeting 2012
Prague, Czech Republic

September 20-22, 2012
1st World Congress on Controversies in the Management of Viral Hepatitis
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AASLD - The Liver Meeting 2012
Boston, MA, United States

November 9-13, 2012
The Liver Meeting - 63rd Annual Meeting and Postgraduate Course of the American Association for the Study of Liver Diseases
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3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

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5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1,274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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