ELSEVIER

Journal of Hepatology 45 (2006) 429–438

Journal of Hepatology

www.elsevier.com/locate/jhep

Bone marrow-derived fibrocytes participate in pathogenesis of liver fibrosis☆

Tatiana Kisseleva[1,*], Hiroshi Uchinami[1], Nikki Feirt[2], Oscar Quintana-Bustamante[3], Jose Carlos Segovia[3], Robert F. Schwabe[1], David A. Brenner[1]

[1]*Department of Medicine, College of Physicians and Surgeons, Columbia University, New York, NY 10032, USA*
[2]*Department of Pathology, College of Physicians and Surgeons, Columbia University, New York, NY 10032, USA*
[3]*Hematopoietic Gene Therapy Division, CIEMAT/M, Botin Foundation, Madrid, Spain*

***Background/Aims*: Hepatic stellate cells (HSCs) play a key role in hepatic fibrogenesis. However, their origin is still unknown. We tested the hypothesis that bone marrow (BM) contributes to the population of HSCs.**

***Methods*: Chimeric mice transplanted with donor BM from collagen α1(I)-GFP$^+$ reporter mice were subjected to the bile duct ligation (BDL)-induced liver injury.**

***Results*: In response to injury, BM-derived collagen-expressing GFP$^+$ cells were detected in liver tissues of chimeric mice. However, these cells were not activated HSCs in that they did not express α-smooth muscle actin or desmin and could not be isolated with the HSC fraction. Meanwhile, the majority of these BM-derived cells co-expressed collagen-GFP$^+$ and CD45$^+$, suggesting that these cells represent a unique population of fibrocytes. Consistent with their lymphoid origin, the number of GFP$^+$CD45$^+$ fibrocytes found in BM and spleen of chimeric mice increased in response to injury. Fibrocytes cultured in the presence of TGF-β1 differentiated into SMA$^+$desmin$^+$ collagen-producing myofibroblasts, potentially contributing to liver fibrosis.**

***Conclusions*: In response to the BDL-induced liver injury: (i) HSCs do not originate in the BM; (ii) collagen-producing fibrocytes are recruited from the BM to damaged liver.**

***Keywords*: Hepatic stellate cells; Collagen α1(I); Bone marrow transplantation; Bile duct ligation; Fibrocytes**

1. Introduction

Liver fibrosis, an outcome of many chronic liver diseases [1], is characterized by extensive deposition of extracellular matrix, mainly type I collagen [2]. Hepatic stellate cells (HSCs) are believed to play a major role in the pathogenesis of liver fibrosis [3]. In response to injury, quiescent HSCs undergo morphological and functional changes to become activated HSC with a myofibroblastic phenotype that express high amount of type I collagen [4]. However, it is still unknown whether hepatic HSCs proliferate in the liver or originate in the BM (bone marrow) and migrate to liver in response to injury. Recently, conversion of BM cells into several hepatic cell populations, e.g. hepatic oval cells, hepatocytes, sinusoidal endothelial cells, etc., has been intensively studied [5–9]. Conversion of BM progenitors into highly specialized cells of distinct origin occurs either due to transdifferentiation [6] or cellular fusion in the target organs [6,10]. However, differentiation without "change of the cell

Received 28 October 2005; received in revised form 7 April 2006; accepted 18 April 2006; available online 9 June 2006

☆ The authors who have taken part in the research of this paper have no relationship with the manufacturers of the drug involved either in the past or present. The authors state that they did not receive funding from the manufacturers to carry out their research. The authors received funding from NIH which enabled them to carry out their study.

* Corresponding author. Tel.: +1 212 342 0288; fax: +1 212 305 9822.

E-mail address: tk335@columbia.edu (T. Kisseleva).

Abbreviations: HSC, hepatic stellate cells; BDL, bile duct ligation; Col, transgenic mice expressing GFP under collagen α1(I) promoter; PBL, peripheral blood lymphocytes.

0168-8278/$32.00
doi:10.1016/j.jhep.2006.04.014

fate'' has been described for a subset of collagen-producing cells designated as fibrocytes, a unique population of collagen producing cells identified in peripheral blood [11–14]. This study tested the hypothesis that HSCs and/or other collagen-producing cells [15,16] arise from a population of cells originating in the bone marrow. Using BM chimeric mice expressing GFP under control of the collagen α1(I) promoter [17,18], we specifically investigated the BM contribution to the population of HSCs and non-HSC collagen-producing cells in response to bile duct ligation (BDL)-induced hepatic injury.

2. Experimental procedures

2.1. Generation of BM chimeric mice and induction of liver injury

Wild type C57BL/6 (B6) mice (wt) were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA). Transgenic mice, expressing GFP driven by collagen α1(I) promoter (Col), were previously characterized [17,18]. BM chimeric mice were generated as previously described [5,19]. Mice were allowed to recuperate for 2 months prior to induction of liver fibrosis. BM engraftment in Col-into-wt mice was confirmed by Southern blot analysis of BM-derived genomic DNA using ^{32}P-radiolabeled *gfp* specific probe. The percent of BM engraftment was evaluated in mice transplanted with β-actin-GFP by FACS analysis of peripheral blood lymphocytes (PBL) and constituted ≈95%. Using BMT, we generated 51 Col-into-wt mice, 7 Wt-into-Col mice, 3 wt-into-wt and 3 Col-into-Col. Liver injury was induced for 14–28 days by ligation of the common bile duct (BDL) as described [20]. All animal studies have been approved by the University Committee on Use and Care of Animals at Columbia University.

2.2. Characterization of the BMT mice by fluorescent microscopy

Livers and spleen were examined for GFP signal by fluorescent microscopy. Images were obtained on a Nikon ECLIPSE E 800 microscope and analyzed using Image-Pro Plus and Photoshop programs.

2.3. Immunohistochemistry

Fluorescent immunohistochemistry was performed on cryosections [21] using polyclonal anti-desmin (1:400, NeoMarkers, Fremont, CA), monoclonal anti-CD45 antibody (1:200, Serotec, Raleigh, NC) and anti-α-SMA (1:400; Dako, Carpinteria, CA) primary antibodies and analyzed using confocal microscope (LSM 510 NLO, Zeiss, Germany). Cryosections, stained with anti-α-SMA antibody, were pre-treated with the M.O.M. Kit (Vector Laboratories, Burlingame, CA).

2.4. Isolation of primary HSCs

Primary HSCs were isolated as previously described [22,23]. Freshly isolated HSCs were used for FACS analysis or cultured on uncoated plastic tissue culture dishes in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Grand Island, NY) supplemented with 10% fetal calf serum, 2 mM L-glutamine and standard antibiotics [23].

2.5. Isolation of GFP$^+$ cells from the whole liver

Livers were mashed and digested with collagenase D (500 μg/ml; Roche Diagnostics, Indianapolis, IN) and pronase E (200 μg/ml, EMD, Gibbstown, NJ) in solution (140 mM NaCl, 5 mM KCl, 0.7 mM NaH_2PO_4; 0.8 mM Na_2PO_4, 10 mM Hepes, 3 mM $NaCO_3$, 4 mM $CaCl_2$, pH 7.4) at 37 °C for 20 min, followed by filtration on a sterile gauze. Whole liver fractions were used for FACS or attached to collagen coated (2% in 0.1% acetic acid) glass slides by brief spinning and fixed in 4% formalin.

2.6. Single cell suspensions from lymphoid tissues

Isolated BM cells and splenocytes were used for FACS analysis or cultured for 10–14 days on uncoated plastic tissue culture dishes. Differentiation of BM cells and splenocytes was induced by TGF-β1 (2 ng/ml; RD systems, Minneapolis, MN) in DMEM supplemented with 10% FCS.

2.7. Flow cytometric analysis

GFP$^+$ cells, isolated from liver, spleen and BM, were analyzed using a FACScan flow cytometer (Becton–Dickinson, San Jose, CA) as described [24]. For co-expression of lymphoid markers on GFP$^+$ cells, cells were stained in PBS containing 2.5% BSA solution with PE-conjugated anti-CD45, anti-CD34 and anti-CD14 antibodies (BD Biosciences, San Diego, CA).

2.8. Fluorescence in situ hybridization

Liver cryosections were hybridized for Y chromosome identification according to the STARFISH manufacturer's protocol (Cambio, Cambridge, UK) described previously [25]. GFP+ cells were photographed prior to hybridization. Images were aligned using DAPI staining.

3. Results

3.1. Collagen producing cells of BM origin are present in fibrotic liver

We performed a series of bone marrow transplantations (BMT) in mice to determine whether hepatic stellate cells (HSCs) or other collagen producing cells originated in the BM in response to bile duct ligation (BDL), a well-established model of liver injury (Fig. 1A). To monitor migration of BM-derived collagen-expressing cells to injured liver, reporter mice expressing GFP under the collagen α1(I) promoter (Col mice) were used [17,18]. In a first set of experiments, BM from Col mice was transplanted into recipient wild type mice (Col-into-wt chimera). Successful engraftment of donor BM in chimeric mice was confirmed by Southern blot analysis (Fig. 1B). Liver injury was induced in these mice for two weeks after which livers were examined by fluorescent microscopy for BM-derived GFP$^+$ cells. No GFP$^+$ cells were present in sham-operated mice (Fig. S1). In contrast, GFP$^+$ cells were detected in livers of BDL-operated Col-into-wt mice (Fig. 2A, left panel). The majority of these cells were present in portal tracts, although single GFP$^+$ cells were also scattered throughout the hepatic parenchyma. Immunostaining with anti-GFP antibody confirmed the specificity of fluorescent GFP signal (Fig. 2A, right panel). Liver tissues from sham-operated

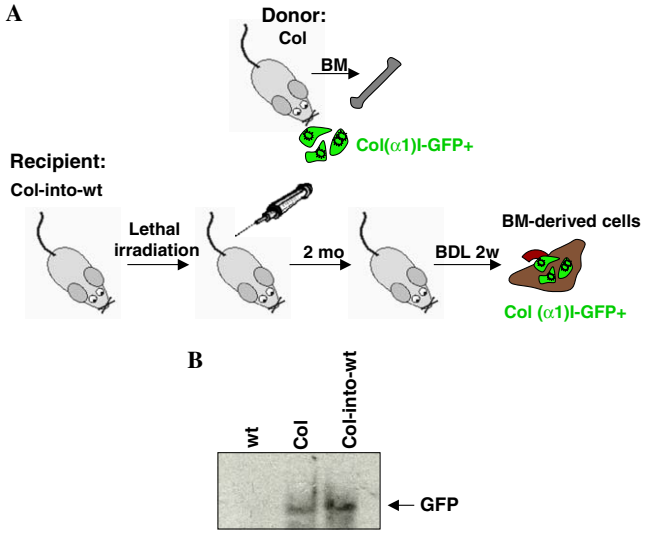

Fig. 1. Generation of Col-into-wt chimera to study BM contribution to population of collagen producing cells in fibrotic livers. (A) Study design. Lethally irradiated recipient wild type mice were transplanted with reporter collagen α1(I)-GFP$^+$ BM. Liver injury was induced in these mice by bile duct ligation (BDL). Liver tissue was analyzed for GFP$^+$ BM-derived cells. (B) BM engraftment in Col-into-wt was confirmed by Southern blot of genomic DNA, hybridized to the radiolabeled *gfp* probe. Specific *gfp* signal was detected in samples derived from Col-into-wt and Col mice (positive control). [This figure appears in colour on the web.]

or BDL-operated reporter Col mice served as controls (Fig. S1).

The role of BM-derived cells in BDL-induced collagen production was further studied using a "reverse" approach: Wild type BM was transplanted into Col reporter mice (wt-into-Col chimera) followed by BDL (Fig. 2B). If the BM made a major contribution to collagen expressing cells in the injured liver, then we would expect collagen α1(I)-GFP$^+$ expression to be reduced in these mice. In addition, Col mice were also transplanted with "self" BM (Col-into-Col chimera), to exclude effect of irradiation on liver fibrosis. Assessed by histological examination of trichrome stained liver tissues, all mice developed similar level of ECM deposition in response to BDL, suggesting that radiation has no or minimal effect on BDL-induced liver fibrosis (Fig. 2B, upper panel). BDL-operated chimera Col-into-Col reporter Col mice exhibited similar levels of GFP expression in response to BDL (Fig. 2B). Meanwhile, image analysis of collagen α1(I)-GFP$^+$ expression in wt-into-Col and Col-into-wt mice revealed that the contribution of BM to liver fibrogenesis was similar in both mice and ranged ≈5–10% in comparison with Col-into-Col (100%). Thus, a $9.7 \pm 4\%$ reduction of GFP expression was observed in wt-into-Col-BDL (Fig. 2C, $p < 0.05$). Likewise, a $5.7 \pm 0.7\%$ of GFP expression was detected in Col-into-wt chimera ($p < 0.005$).

3.2. BM cells did not contribute to HSC population in a BDL-induced model of liver fibrosis

To determine whether these BM-derived collagen α1(I)-GFP$^+$ producing cells belong to the HSC population, HSCs were isolated using a standard procedure and analyzed by FACS for GFP$^+$ cells (Table 1, [24]. Surprisingly, we detected no GFP$^+$ cells in the HSC fraction from BDL-operated Col-into-wt mice (Table 1). In contrast, 72% of HSCs from BDL-operated Col mice expressed GFP$^+$(Fig. 3A, Table 1). Meanwhile, cultured for 24 h HSCs from both Col-BDL and Col-into-wt-BDL mice exhibited similar levels of vitamin A expression. Of note, expression of GFP by isolated HSCs derived from Col-into-wt-BDL mice could not be detected even after several days in culture, suggesting that these HSCs do not originate from BM (Fig. 3A). Moreover, immunostaining of liver tissues from Coll-into-wt mice with anti-SMA and anti-desmin antibodies revealed that BM-derived GFP$^+$ cells do not express typical HSCs markers (Fig. 3B). Liver tissues from Col mice prepared under the same conditions served as a positive control. Taken together, based on three independent approaches (fluorescent microscopy, FACS analysis of isolated HSCs and immunohistochemistry) we concluded that BM cells do not contribute to the population of activated HSCs in BDL-induced liver injury.

3.3. BM-derived GFP$^+$ cells possess characteristics of fibrocytes

To further characterize BM-derived GFP$^+$ cells, an alternative method (mild enzymatic digestion without perfusion) was used to isolate GFP$^+$ cells from fibrotic livers. GFP$^+$ BM-derived cells were detected in the whole liver fraction of the BDL-operated Col-into-wt mice and represented $4.2 \pm 1.3\%$ of all viable cells (Table 2). The number of GFP$^+$ cells increased with the time after BDL (Fig. 4A).

Previous studies suggest that cells of BM-origin often retain expression of CD45, a common lymphoid marker [26]. Therefore, freshly isolated liver cells from BDL-operated Col-into-wt mice were stained with anti-CD45 and analyzed for GFP co-staining. Immunofluorescent expression of CD45$^+$ was detected in 70% of the GFP$^+$ population (Fig. 4C and D). CD34, an early hematopoietic marker, and CD14, a monocytic marker, were also expressed by ≈1% of the GFP$^+$ BM-derived cells. (Of note, FACS analysis failed to determine the correct number of GFP$^+$CD45$^+$ cells in the whole liver due to high autofluorescence which projected to the FL2 channel and interfered with detection of CD45 with anti-CD45-PE-conjugated antibody.)

BM-derived lymphoid cells may transdifferentiate into collagen-producing fibroblasts. Alternatively, a

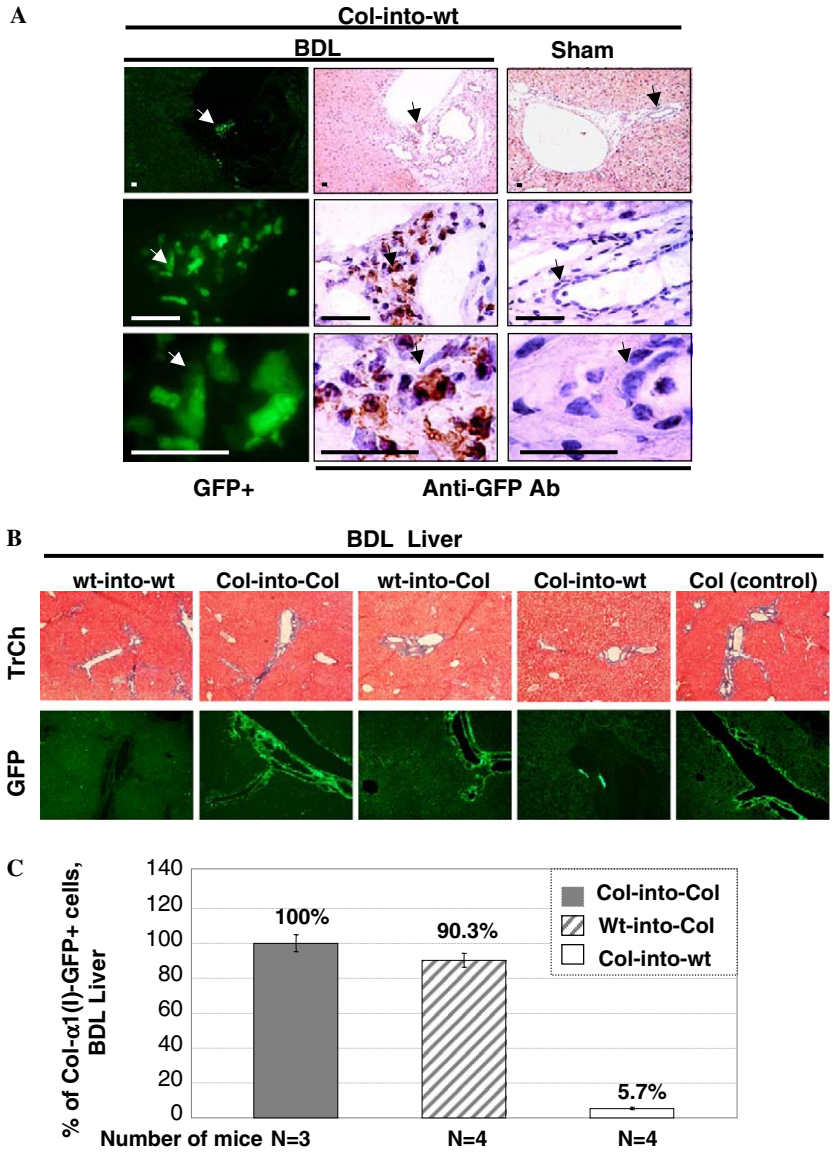

Fig. 2. Transplantation of the wild type BM into Col reporter mice decreased BDL-induced GFP expression in the liver. (A) BM-derived collagen α1(I)-GFP$^+$ cells populated Col-into-wt liver tissues two weeks after BDL. Immunostaining with anti-GFP antibody confirmed specificity of the GFP expression. Images, representing the same field, were captured using Fluorescent and BF Filters and placed in rows. (B) Radiation had no significant effect on liver fibrogenesis in chimeric mice compared to wild type mice two weeks after BDL (upper panel). Collagen deposition in these mice was assessed by Masson trichrome staining of the liver tissues (TriChr, blue staining). Collagen α1(I)-GFP$^+$ expression in these mice was analyzed by fluorescent microscopy of liver tissues (bottom panel). (C) Comparison of total collagen α1(I)-GFP$^+$deposition in BDL-livers from Col mice (100%) and chimeric mice (5.7% in Col-into-wt mice, $p < 0.005$; and 90.3% in wt-into-Col, $p < 0.05$) mice. GFP$^+$ expression was analyzed by fluorescent microscopy in at least 10 fields/mouse using Image-Pro Plus program. The data represent percent of GFP$^+$ areas in μm/liver tissue.

population of collagen-producing cells may already be present in the BM and colonize the liver in response to injury. To test this hypothesis, we analyzed BM cells for collagen-GFP$^+$ cells. Indeed, using FACS analysis GFP$^+$ cells were detected in the BM of BDL-operated Col-into-wt and Col mice (Fig. 4A). Surprisingly, GFP$^+$ BM-derived cells were also present in the spleen (Fig. 5A), but not in lungs or kidneys (data not shown), and looked morphologically identical to the liver-derived GFP$^+$ cells of BM origin (Fig. 4B). In response to liver injury, the number of GFP$^+$ cells in spleen and BM of Col-into-wt increased from 1% to 3.5% and 0.8% to 1.6% ($p < 0.005$), respectively, in a time-dependent manner (Fig. 5A). A similar effect was detected in BDL-operated Col mice. Interestingly, a small number of the GFP$^+$ splenocytes and BM cells were also found in these mice prior to BDL (Fig. 4A).

Akin to hepatic GFP$^+$ cells, GFP$^+$ cells isolated from the BM and spleen expressed hematopoietic markers (Fig. 4D). FACS analysis revealed that $65 \pm 11\%$ of GFP$^+$ splenocytes and $77 \pm 7\%$ of GFP$^+$ BM cells from

Table 1
FACS analysis of HSC fraction for BM-derived GFP$^+$ cells

Mice	HSC	
	Sham (%)	BDL (%)
Col	1.00 ± 0.5	72 ± 2.5
Col-into-wt	0.28 ± 0.6	0.8 ± 0.6
wt	0.0	0.9 ± 0.2
wt-into-wt	0.54 ± 0.1	0.7 ± 0.4

HSCs were isolated using standard procedure [22,23] from Col and chimeric mice four weeks after sham- or BDL-operation and analyzed by FACS for GFP$^+$ cells. $72 \pm 2.5\%$ of HSCs from Col-BDL mice expressed GFP ($p < 0.001$ compared to sham). No GFP$^+$ cells over the background were detected in Col-into-wt mice ($p < 0.005$ compared to HSCs isolated from Col-BDL mice). The data represent percent of GFP$^+$ cells detected in at least three independent experiments.

Table 2
FACS analysis of whole liver cells fraction for BM-derived GFP$^+$ cells

Mice	Total liver cells	
	Sham (%)	BDL (%)
Col	0.8 ± 0.5	15 ± 3.5
Col-into-wt	0.37 ± 0.4	4.2 ± 1.3
wt	0.0	1.6 ± 0.15
wt-into-wt	0.0 ± 0.1	1.1 ± 0.1

Whole liver cells were isolated from mice four weeks after sham- and BDL-operation and analyzed for GFP$^+$ cells by FACS. $4.2 \pm 1.3\%$ of whole liver cells from BDL-operated Col-into-wt mice expressed GFP ($p < 0.001$ compared to cells isolated from Col-BDL mice). Whole liver cells isolated from Col mice constituted $15 \pm 5.5\%$ ($p < 0.01$ compared to the sham). The data are presented as percent of GFP$^+$ cells detected in at least three independent experiments.

BDL-operated Col mice expressed CD45. $67 \pm 3\%$ of GFP$^+$ splenocytes and $52 \pm 13\%$ of GFP$^+$ BM cells from BDL-operated Col-into-wt mice were also CD45$^+$. However, only 1–3% of GFP$^+$ cells from BM and spleen expressed CD34 or CD14.

A similar phenotype of CD45$^+$ColI$^+$ has been previously described in hematopoietic cells designated as fibrocytes. Fibrocytes originate in the BM and migrate to the sites of injury and differentiate into myofibroblast in response to TGF-β1 [11–14] To test whether BM-derived GFP$^+$ collagen producing cells represent fibrocytes, GFP$^+$ splenocytes were isolated from BDL-operated Col mice and cultured in the presence of TGF-β1. Freshly isolated GFP$^+$ splenocytes expressed the lymphoid marker CD45 (Fig. 5A). Following 7 days of treatment with TGF-β1, GFP$^+$ splenocytes differentiated into myofibroblasts and upregulated α-SMA and desmin. Thus, both GFP$^+$ BM and splenocytes exhibited

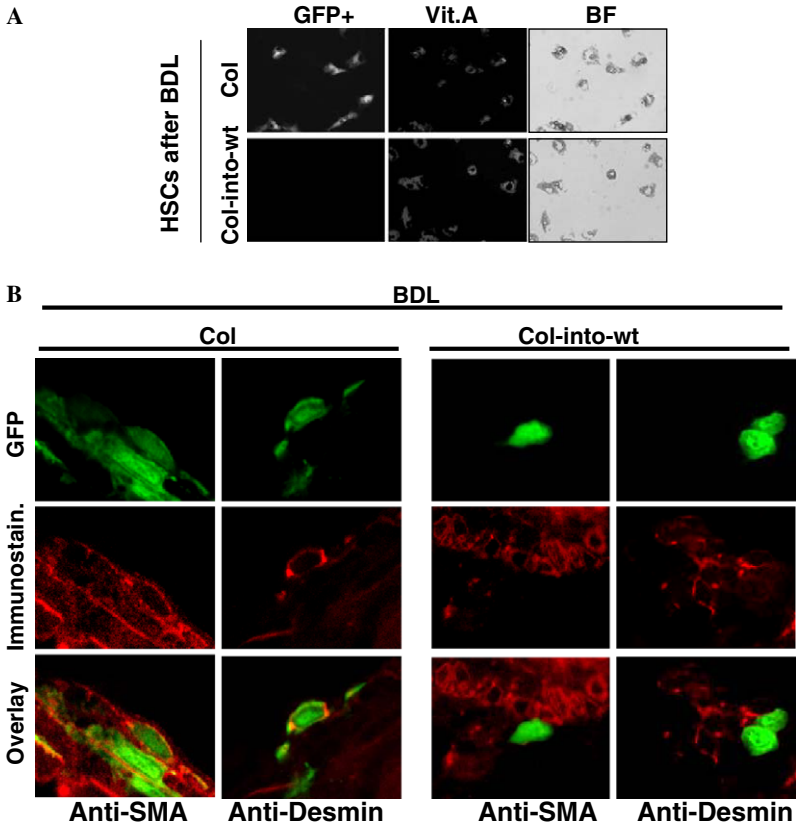

Fig. 3. HSCs did not originate from the BM in two weeks after BDL-induced liver injury. (A) Expression of collagen α1(I)-GFP was detected only in HSCs isolated from Col mice but Col-into-wt mice. All HSCs exhibited similar levels of vitamin A expression and displayed a myofibroblastic phenotype when cultured for 24 h. (B) GFP$^+$ cells from BDL-operated did not express markers of HSCs. Immunostaining with anti-SMA and anti-desmin antibodies revealed co-expression of HSCs-specific markers only in liver tissues from Col-BDL mice but Col-into-wt mice. Images were analyzed by confocal microscopy.

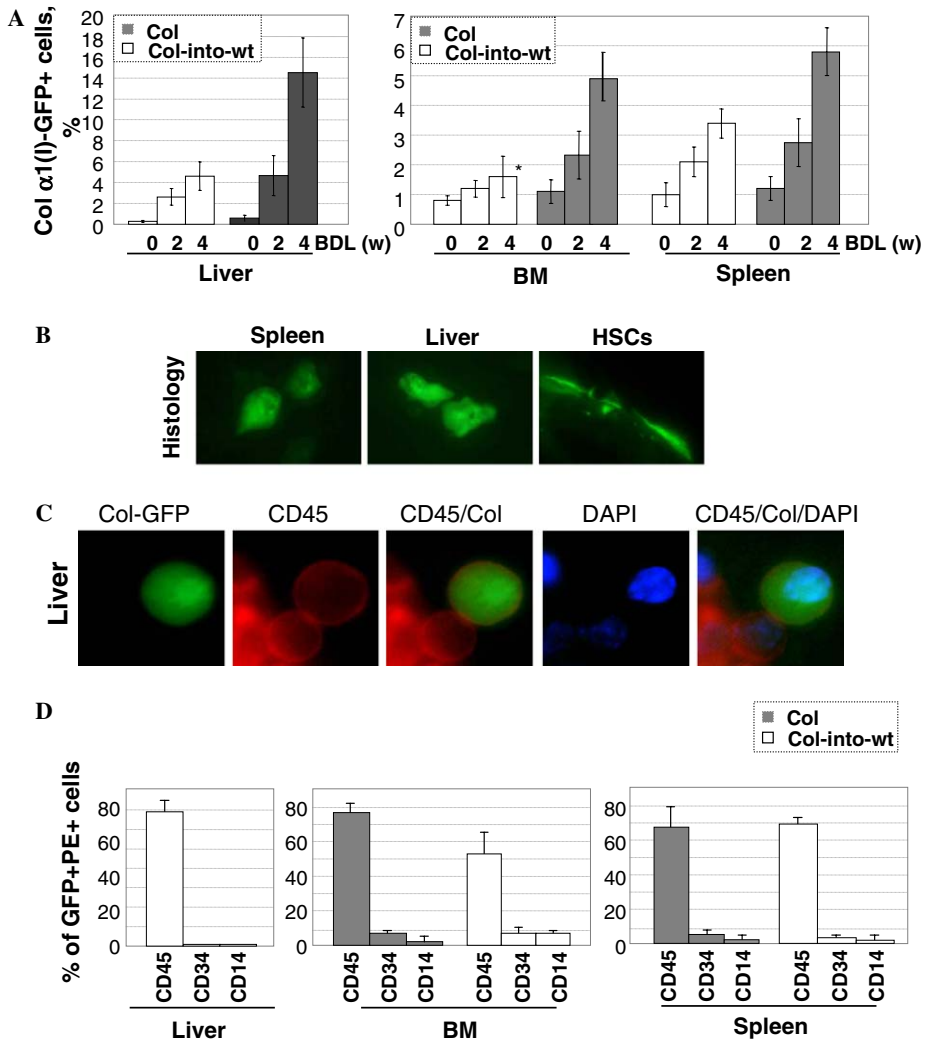

Fig. 4. (A) GFP$^+$ collagen-expressing cells detected in liver tissues of Col-into-wt mice are represented by fibrocytes. (A) FACS analysis of the whole liver fractions isolated from Col-into-wt mice revealed that increase in numbers of BM-derived collagen α1(I)-GFP$^+$ cell numbers (left panel) was dependent on time after BDL and correlated with increase in BM cells and total splenocytes (right panel). Similar effect was observed in Col mice. The data are representative of six independent experiments. (B) Immunofluorescent analysis of tissue sections identified that GFP$^+$ cells from spleen and liver of Col-into-wt-BDL mice are morphologically similar but different from HSCs observed in Col-BDL mice. (C) Detected by immunostaining with anti-CD45-PE$^+$ Ab, GFP$^+$ liver-derived collagen-expressing cells from Col-into-wt mice co-expressed CD45-PE$^+$. Cells were stained with DAPI to visualize nuclei. (D) Statistical analysis based on immunocytochemistry of liver-derived GFP$^+$CD45-PE$^+$ cells from Col-into-wt-BDL mice revealed that 70% of GFP$^+$ cells co-expressed CD45-PE$^+$. Meanwhile, detected by FACS analysis, 52-77% of BM- and spleen-derived GFP$^+$ cells co-expressed CD45.

features of fibrocytes. After leaving the bone marrow, fibrocytes enter the peripheral blood in which they can be commonly found [11,13]. We detected an increase in GFP$^+$ fibrocyte-like cells in the peripheral blood after BDL in Col mice ($0.37 \pm 0.2\%$ before BDL versus $1.4 \pm 0.5\%$ after BDL, $p < 0.01$), suggesting that liver injury enhances the migration of fibrocytes from BM to the peripheral blood (Fig. 5B) and to sites of injury.

3.4. BM-derived fibrocytes do not fuse with liver cells

Our findings suggest that fibrocytes originate in the BM in response to the liver injury and do not derive by cellular fusion with the recipient cells. Ninety percent of GFP$^+$ fibrocytes had one nucleus. Cells with two nuclei (10%) had identically structured nuclei and most likely were undergoing cell division (Fig. 6A). To prove this hypothesis, liver sections from BDL-operated Col-into-wt chimeric mice, transplanted with the sex mismatched bone marrow (female BM into male recipient mice), were analyzed by FISH (Fig. 6B). Unlike the Y+ recipient hepatocytes, donor-originated GFP$^+$ fibrocytes did not contain any Y chromosome. Therefore, fibrocytes were derived from the BM without fusion with recipient cells.

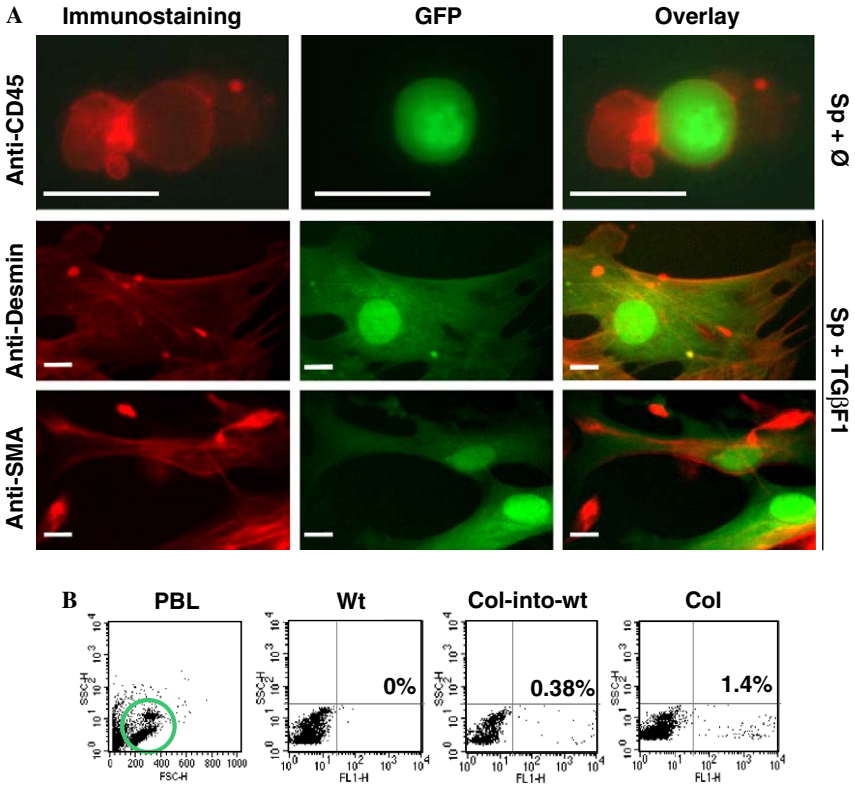

Fig. 5. Collagen expressing BM-derived GFP$^+$ splenocytes express markers characteristic of fibrocytes. (A) CD45$^+$ GFP$^+$ splenocytes, cultured in the presence of TGF-β1, upregulated myofibroblastic markers SMA, Desmin. (B) GFP$^+$ cells were detected in PBLs of Col-into-wt-BDL and Coll-BDL mice using FACS analysis. 0.37% and 1.4% of PBL were detected in Col-into-wt and Col mice after BDL.

4. Discussion

Hepatic stellate cells are the main producers of collagen in the injured liver. However, it has been suggested that fibroblasts in the liver also contribute to collagen-production after injury. The origins of HSCs and hepatic fibroblasts remain unresolved. It has been suggested that HSCs are bone marrow-derived [27]. Even though

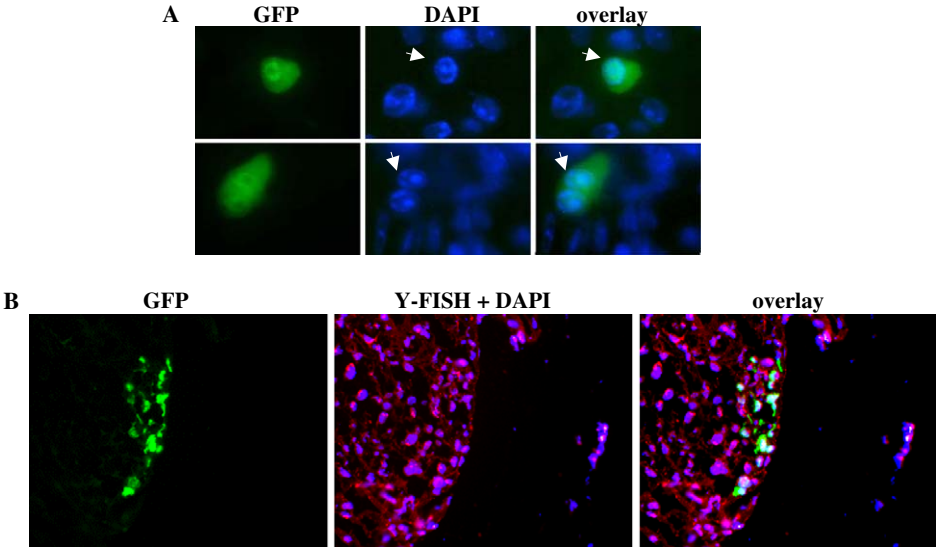

Fig. 6. BM-derived fibrocytes do not fuse with the recipient cells. (A) Nuclei of BM-derived fibrocytes were visualized with DAPI on liver sections (showed with arrows). 90% of BM-derived GFP$^+$ cells had one nucleus and 10% had two nuclei. The data are representative of four different BMT mice, in which over 100 cells were evaluated. (B) FISH analysis of GFP$^+$ BM-derived cells showed no fusion with the recipient cells. GFP$^+$ cells (green) contained no Y chromosome (pink). Over 50 cells were analyzed, lack of Y$^+$ chromosome specific staining in GFP$^+$ fibrocytes was confirmed using high power magnification. DAPI (blue) was used to visualize nuclei (blue).

HSCs express several markers that are typically found on BM-derived cells such as ICAM, CCR5 and CD40, they also display neural crest markers such as synaptophysin and glial fibrillar acidic protein arguing against their origin from BM [18,23,28]. Although our study detected a significant increase in cells expressing GFP under the control of the collagen α1(I) promoter in the liver of chimeric mice transplanted with BM from the Col mice, we did not detect α-SMA or desmin expression in these GFP^+ cells (Fig. 3B). Moreover, BM-derived GFP^+ cells were not isolated with the HSCs fraction (Table 1, Fig. 3). Based on these 2 lines of evidence, we can exclude that BM contributes to the pool of HSC in response to BDL. These findings are in contrast to reports which proposed that BM cells differentiate into HSCs in response to a different liver injury model [27]. Thus, BM-derived collagen α1(I)-GFP^+ expressing cells do not posses features of HSCs or myofibroblasts, and we propose that these cells represent a novel collagen producing cell type in the liver and constitute about 5% of collagen α1(I)-producing population in the liver after BDL.

Based on immunocytochemistry, ≈70% of BM-derived GFP^+ cells in the liver expressed $CD45^+$, while CD34/CD14 expression constituted ≈1% (Fig. 4). Our data are in concordance with the previously described phenotype of fibrocytes [29]. Surprisingly, BM cells and splenocytes of the BDL-operated chimera and Col mice were also GFP^+ and clearly expressed CD45 (Fig. 5). Similar to that, GFP^+CD45^+ fibrocytes constituted ∼1% of the BM cells in the sham-operated mice, indicating that fibrocytes originated in the BM and expanded in response to injury (Fig. 4A).

Fibrocytes are unique cells, initially identified a decade ago from PBL, that express hematopoietic and myeloid markers together with collagen [30,31]. Most of these studies suggested that co-expression of $Coll^+CD45^+$ can be considered a major characteristic of fibrocytes. Other markers such as CD34, CD14, CD13, HLA-DR, CCR4 and CCR7 have been reported to be expressed by fibrocytes but seem to vary between studies and depend on the examined organ [11,14,29,32]. Fibrocytes have been demonstrated in scarring skin [33,34], in nephrogenic fibrosing dermopathy [35], in subepithelial fibrotic lesions from patients with asthma [30], and pulmonary fibrosis in bleomycin treated mice [11,36], and may be involved in wound healing and repair [37]. The present study demonstrated for the first time that fibrocytes are involved in hepatic fibrogenesis.

The exact function of fibrocytes at the site of injury is still unclear. It was shown that fibrocytes can differentiate *in vitro* into myofibroblasts when stimulated with TGFβ1 [13,38], but to date only one study succeeded to demonstrate transition of fibrocytes into myofibroblasts *in vivo* [30]. Similar to that, we showed that fibrocytes residing in the liver and spleen lack α-SMA expression, but promptly differentiate into myofibroblasts *in vitro* (Fig. 5A). Moreover, Barth and colleagues, who focused their research on cellular repopulation of stroma in invasive squamous cell carcinomas, confirmed that fibrocytes and α-SMA^+ myofibroblasts represent two different cell types, which exhibit different localization and function [39].

Fibrocytes have been implicated in the scarring of lung and skin, where they comprise ≈15–25% of collagen producing cells [11,33,34,36]. Tissue injury is commonly caused in these studies by massive cellular death. For example, bleomycin-induced lung injury is induced by alveolar epithelial apoptosis [36]. Similar mechanism underlies fibrogenesis in burnt skin. Recently, Baba and colleagues reported that ≈33% of HSCs are derived from the BM during carbon tetrachloride (CCl_4)-induced liver damage. Moreover, mechanism of carbon tetrachloride (CCl_4)-induced liver injury, caused by severe hepatotoxicity and massive apoptosis of hepatocytes, is similar to bleomycin. Therefore, recruitment of BM cells to bleomycin or CCL_4-injured organs might be similar. In contrast, here we demonstrate that BM-derived fibrocytes contributed to 5–10% of all collagen α1(I) expressing cells in the BDL-operated liver (Fig. 2C). It is possible that the more gradual onset of hepatic injury and fibrogenesis after BDL, which more closely resembles the gradual fibrosis of clinical chronic liver disease, accounts for this modest recruitment of fibrocytes to BDL-injured liver.

In addition to their contribution to collagen production, fibrocytes may be required for other aspects of wound healing such as the activation or proliferation of surrounding cells and angiogenesis (reviewed in [32,40]). Fibrocytes express immunological markers typical for antigen presenting cells, and similarly to dendritic cells (DC) are functionally capable for antigen presentation to T cells and secrete a number of inflammatory cytokines and growth factors [37,41,42]. Thus, in response to liver injury fibrocytes were found in fibrotic regions in close proximity to HSCs. It is possible that fibrocytes provide important intercellular signals, which orchestrate a cross-talk between inflammatory, biliary epithelial and collagen-producing cells. In the present study, GFP^+ cells were detected in BM, PBL and spleen of BMT mice (Fig. 4A). Consistent with our findings, it has been reported that fibrocytes exist in avian species, are present in the circulation of the chicken embryo, and contribute to the organogenesis of the spleen [43]. Moreover, unlike BM-derived hepatocytes resulted from cellular fusion with recipient cells [25], BM-derived GFP^+ fibrocytes did not contain Y chromosome as the rest of liver cells from BMT chimeric mice, providing a compelling evidence that tissue and circulating fibrocytes originate in the BM.

Acknowledgements

We wish to thank Lin Yang and the Molecular Pathology Facility of the Herbert Irving Cancer Center of Columbia University provided superb histology service. We acknowledge Stella Stefanova for excellent technical assistance.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhep.2006.04.014.

References

[1] Bataller R, Brenner DA. Liver fibrosis. J Clin Invest 2005;115:209–218.
[2] Friedman SL. Liver fibrosis – from bench to bedside. J Hepatol 2003;38 (Suppl. 1):S38–S53.
[3] Friedman SL, Roll FJ, Boyles J, Bissell DM. Hepatic lipocytes: the principal collagen-producing cells of normal rat liver. Proc Natl Acad Sci USA 1985;82:8681–8685.
[4] Friedman SL. Molecular regulation of hepatic fibrosis, an integrated cellular response to tissue injury. J Biol Chem 2000;275:2247–2250.
[5] Jang YY, Collector MI, Baylin SB, Diehl AM, Sharkis SJ. Hematopoietic stem cells convert into liver cells within days without fusion. Nat Cell Biol 2004;6:532–539.
[6] Newsome PN, Johannessen I, Boyle S, Dalakas E, McAulay KA, Samuel K, et al. Human cord blood-derived cells can differentiate into hepatocytes in the mouse liver with no evidence of cellular fusion. Gastroenterology 2003;124:1891–1900.
[7] Lagasse E, Connors H, Al-Dhalimy M, Reitsma M, Dohse M, Osborne L, et al. Purified hematopoietic stem cells can differentiate into hepatocytes in vivo. Nat Med 2000;6:1229–1234.
[8] Wang X, Willenbring H, Akkari Y, Torimaru Y, Foster M, Al-Dhalimy M, et al. Cell fusion is the principal source of bone-marrow-derived hepatocytes. Nature 2003;422:897–901.
[9] Willenbring H, Bailey AS, Foster M, Akkari Y, Dorrell C, Olson S, et al. Myelomonocytic cells are sufficient for therapeutic cell fusion in liver. Nat Med 2004;10:744–748.
[10] Terada N, Hamazaki T, Oka M, Hoki M, Mastalerz DM, Nakano Y, et al. Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. Nature 2002;416:542–545.
[11] Phillips RJ, Burdick MD, Hong K, Lutz MA, Murray LA, Xue YY, et al. Circulating fibrocytes traffic to the lungs in response to CXCL12 and mediate fibrosis. J Clin Invest 2004;114:438–446.
[12] Direkze NC, Forbes SJ, Brittan M, Hunt T, Jeffery R, Preston SL, et al. Multiple organ engraftment by bone-marrow-derived myofibroblasts and fibroblasts in bone-marrow-transplanted mice. Stem Cells 2003;21:514–520.
[13] Abe R, Donnelly SC, Peng T, Bucala R, Metz CN. Peripheral blood fibrocytes: differentiation pathway and migration to wound sites. J Immunol 2001;166:7556–7562.
[14] Hashimoto N, Jin H, Liu T, Chensue SW, Phan SH. Bone marrow-derived progenitor cells in pulmonary fibrosis. J Clin Invest 2004;113:243–252.
[15] Ramadori G, Saile B. Portal tract fibrogenesis in the liver. Lab Invest 2004;84:153–159.
[16] Forbes SJ, Russo FP, Rey V, Burra P, Rugge M, Wright NA, et al. A significant proportion of myofibroblasts are of bone marrow origin in human liver fibrosis. Gastroenterology 2004;126:955–963.
[17] Yata Y, Scanga A, Gillan A, Yang L, Reif S, Breindl M, et al. DNase I-hypersensitive sites enhance alpha1(I) collagen gene expression in hepatic stellate cells. Hepatology 2003;37:267–276.
[18] Magness ST, Bataller R, Yang L, Brenner DA. A dual reporter gene transgenic mouse demonstrates heterogeneity in hepatic fibrogenic cell populations. Hepatology 2004;40:1151–1159.
[19] Yu L, Huang Z, Mariani J, Wang Y, Moskowitz M, Chen JF. Selective inactivation or reconstitution of adenosine A2A receptors in bone marrow cells reveals their significant contribution to the development of ischemic brain injury. Nat Med 2004;10:1081–1087.
[20] Hellerbrand C, Wang SC, Tsukamoto H, Brenner DA, Rippe RA. Expression of intracellular adhesion molecule 1 by activated hepatic stellate cells. Hepatology 1996;24:670–676.
[21] Vorontchikhina MA, Zimmermann RC, Shawber CJ, Tang H, Kitajewski J. Unique patterns of Notch1, Notch4 and Jagged1 expression in ovarian vessels during folliculogenesis and corpus luteum formation. Gene Expr Patterns 2005;5:701–709.
[22] Hellerbrand C, Jobin C, Iimuro Y, Licato L, Sartor RB, Brenner DA. Inhibition of NFkappaB in activated rat hepatic stellate cells by proteasome inhibitors and an IkappaB super-repressor. Hepatology 1998;27:1285–1295.
[23] Schwabe RF, Schnabl B, Kweon YO, Brenner DA. CD40 activates NF-kappa B and c-Jun N-terminal kinase and enhances chemokine secretion on activated human hepatic stellate cells. J Immunol 2001;166:6812–6819.
[24] Magness ST, Brenner DA. Targeted disruption of the mouse ferrochelatase gene producing an exon 10 deletion. Biochim Biophys Acta 1999;1453:161–174.
[25] Quintana-Bustamante O, Alvarez-Barrientos A, Kofman AV, Fabregat I, Bueren JA, Theise ND, et al. Hematopoietic mobilization in mice increases the presence of bone marrow-derived hepatocytes via in vivo cell fusion. Hepatology 2006;43:108–116.
[26] Hirose J, Kouro T, Igarashi H, Yokota T, Sakaguchi N, Kincade PW. A developing picture of lymphopoiesis in bone marrow. Immunol Rev 2002;189:28–40.
[27] Baba S, Fujii H, Hirose T, Yasuchika K, Azuma H, Hoppo T, et al. Commitment of bone marrow cells to hepatic stellate cells in mouse. J Hepatol 2004;40:255–260.
[28] Yang X, Lu P, Ishida Y, Kuziel WA, Fujii C, Mukaida N. Attenuated liver tumor formation in the absence of CCR2 with a concomitant reduction in the accumulation of hepatic stellate cells, macrophages and neovascularization. Int J Cancer 2005.
[29] Moore BB, Kolodsick JE, Thannickal VJ, Cooke K, Moore TA, Hogaboam C, et al. CCR2-mediated recruitment of fibrocytes to the alveolar space after fibrotic injury. Am J Pathol 2005;166:675–684.
[30] Schmidt M, Sun G, Stacey MA, Mori L, Mattoli S. Identification of circulating fibrocytes as precursors of bronchial myofibroblasts in asthma. J Immunol 2003;171:380–389.
[31] Metz CN. Fibrocytes: a unique cell population implicated in wound healing. Cell Mol Life Sci 2003;60:1342–1350.
[32] Quan TE, Cowper S, Wu SP, Bockenstedt LK, Bucala R. Circulating fibrocytes: collagen-secreting cells of the peripheral blood. Int J Biochem Cell Biol 2004;36:598–606.
[33] Mori L, Bellini A, Stacey MA, Schmidt M, Mattoli S. Fibrocytes contribute to the myofibroblast population in wounded skin and originate from the bone marrow. Exp Cell Res 2005;304:81–90.
[34] Bucala R, Spiegel LA, Chesney J, Hogan M, Cerami A. Circulating fibrocytes define a new leukocyte subpopulation that mediates tissue repair. Mol Med 1994;1:71–81.
[35] Cowper SE, Bucala R. Nephrogenic fibrosing dermopathy: suspect identified, motive unclear. Am J Dermatopathol 2003;25:358.

[36] Lee VY, Schroedl C, Brunelle JK, Buccellato LJ, Akinci OI, Kaneto H, et al. Bleomycin induces alveolar epithelial cell death through JNK-dependent activation of the mitochondrial death pathway. Am J Physiol Lung Cell Mol Physiol 2005;289:L521–L528.
[37] Chesney J, Bucala R. Peripheral blood fibrocytes: novel fibroblast-like cells that present antigen and mediate tissue repair. Biochem Soc Trans 1997;25:520–524.
[38] Brittan M, Hunt T, Jeffery R, Poulsom R, Forbes SJ, Hodivala-Dilke K, et al. Bone marrow derivation of pericryptal myofibroblasts in the mouse and human small intestine and colon. Gut 2002;50:752–757.
[39] Barth PJ, Schenck zu Schweinsberg T, Ramaswamy A, Moll R. CD34+ fibrocytes, alpha-smooth muscle antigen-positive myofibroblasts, and CD117 expression in the stroma of invasive squamous cell carcinomas of the oral cavity, pharynx, and larynx. Virchows Arch 2004;444:231–234.
[40] Hartlapp I, Abe R, Saeed RW, Peng T, Voelter W, Bucala R, et al. Fibrocytes induce an angiogenic phenotype in cultured endothelial cells and promote angiogenesis in vivo. FASEB J 2001;15:2215–2224.
[41] Chesney J, Metz C, Stavitsky AB, Bacher M, Bucala R. Regulated production of type I collagen and inflammatory cytokines by peripheral blood fibrocytes. J Immunol 1998;160:419–425.
[42] Balmelli C, Ruggli N, McCullough K, Summerfield A. Fibrocytes are potent stimulators of anti-virus cytotoxic T cells. J Leukoc Biol 2005;77:923–933.
[43] Nagy N, Biro E, Takacs A, Polos M, Magyar A, Olah I. Peripheral blood fibrocytes contribute to the formation of the avian spleen. Dev Dyn 2005;232:55–66.