

Impaired function of bone marrow-derived endothelial progenitor cells in murine liver fibrosis

Katsuya Shirakura^{1,2}, Haruchika Masuda¹, Sang-Mo Kwon⁴, Syotaro Obi¹, Rie Ito¹, Tomoko Shizuno¹, Yusuke Kurihara¹, Tetsuya Mine², Takayuki Asahara^{1,3,*}

¹Department of Regenerative Medicine Science, Tokai University School of Medicine, Isehara, Japan;

²Internal Medicine (Gastroenterology), Tokai University School of Medicine, Isehara, Japan;

³Vascular Regeneration Research Group, Institute of Biomedical Research and Innovation, Kobe, Japan;

⁴Laboratory of Vascular Medicine and Stem Cell Biology, CHA University, Seoul, Korea.

Summary

Liver fibrosis (LF) caused by chronic liver damage has been considered as an irreversible disease. As alternative therapy for liver transplantation, there are high expectations for regenerative medicine of the liver. Bone marrow (BM)- or peripheral blood-derived stem cells, including endothelial progenitor cells (EPCs), have recently been used to treat liver cirrhosis. We investigated the biology of BM-derived EPC in a mouse model of LF. C57BL/6J mice were subcutaneously injected with carbon tetrachloride (CCl₄) every 3 days for 90 days. Sacrificed 2 days after final injection, whole blood (WB) was collected for isolation of mononuclear cells (MNCs) and biochemical examination. Assessments of EPC in the peripheral blood and BM were performed by flow cytometry and EPC colony-forming assay, respectively, using purified MNCs and BM c-KIT⁺, Sca-1⁺, and Lin⁻ (KSL) cells. Liver tissues underwent histological analysis with hematoxylin/eosin/Azan staining, and spleens were excised and weighed. CCl₄-treated mice exhibited histologically bridging fibrosis, pseudolobular formation, and splenomegaly, indicating successful induction of LF. The frequency of definitive EPC-colony-forming-units (CFU) as well as total EPC-CFU at the equivalent cell number of 500 BM-KSL cells decreased significantly ($p < 0.0001$) in LF mice compared with control mice; no significant changes in primitive EPC-CFU occurred in LF mice. The frequency of WB-MNCs of definitive EPC-CFU decreased significantly ($p < 0.01$) in LF mice compared with control mice. Together, these findings indicated the existence of impaired EPC function and differentiation in BM-derived EPCs in LF mice and might be related to clinical LF.

Keywords: Liver fibrosis, carbon tetrachloride, endothelial progenitor cells, differentiation

1. Introduction

Since the discovery of endothelial progenitor cells (EPC) in 1997 (1), many researchers have demonstrated the critical role of bone marrow (BM)-derived EPC in postnatal vasculogenesis through pivotal bioactivities, mobilization, homing, migration, differentiation, and proliferation in angiogenic tissues (2).

Based on this 'dogma', the number of circulating EPC is considered to reflect *in vivo* vasculogenic activity under pathophysiological conditions. For example, in a physiological feature, the menstrual cycle of human females is reportedly to lead to cyclic fluctuations in the number of circulating EPC (3).

On the other hand, pathological features, such as cardiovascular risk factors, including coronary heart disease or heart failure (4), diabetes mellitus (5), hypercholesterolemia (6), and smoking (7) have been reported to decrease the number of circulating EPC, indicating an impairment of EPC differentiation and/or mobilization. Recently, chronic inflammatory disorders, such as ulcerative colitis (8), rheumatoid arthritis (9),

*Address correspondence to:

Dr. Takayuki Asahara, Department of Regenerative Medicine Science, Tokai University School of Medicine Science, Isehara, Japan.

e-mail: asa777@is.icc.u-tokai.ac.jp

uremia (10), and Alzheimer's disease (11) also have been reported to decrease the number of BM-derived EPC, indicating the potential value of monitoring circulating EPC levels as a diagnostic biomarker of individual diseases.

However, in hepatological view, there are few scientific or clinical studies on EPC kinetics in chronic inflammatory liver injury. Using a murine liver fibrosis (LF) model induced by injection of carbon tetrachloride (CCl_4), we recently used an EPC colony forming assay of ancestral stem/progenitors, lineage negative, c-KIT⁺, Sca-1⁺, and Lin⁻ (KSL) cells to show that BM-EPC production was impaired in LF.

In the present study, we used our established model to further investigate BM-derived EPC bioactivity, particularly its potential for differentiation in LF.

2. Materials and Methods

2.1. Animals

Male C57BL/6J mice were purchased at 6 weeks of age from Crea Japan, Inc. (Tokyo, Japan). All animal experiments were conducted in accordance with the institutional guidelines of Tokai University School of Medicine (Isehara, Japan).

2.2. Preparation of the liver fibrosis model

To induce LF, mice were subcutaneously injected with CCl_4 (1 mL/kg body weight) mixed with olive oil every 3 days for 90 days. Control mice were injected with olive oil alone (12). All mice were sacrificed 2 days after the last injection. After liver tissues were obtained, hematoxylin and eosin (H-E) and Azan staining were performed. In addition, the animals' bodies were weighted, and their spleens also were separately weighed.

2.3. Isolation of whole blood mononuclear cells (WB-MNCs) and biochemical examinations

Blood samples were collected by intracardiac needle aspiration into heparinized containers. MNCs and plasma were isolated by density-gradient centrifugation (Histopaque 1083; Sigma-Aldrich, Missouri, USA), and erythrocytes were lysed with ammonium chloride. Nucleated cells were washed twice with phosphate-buffered saline/ethylenediaminetetraacetic acid (PBS-EDTA) to eliminate platelets. Biochemical examinations of blood were performed by the Central Clinical Laboratory of Tokai University Hospital (Isehara, Japan) and SRL (Atsugi, Japan). Blood was analyzed for aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transpeptidase (γ -GTP), alkaline phosphatase (ALP), total bilirubin, total protein, albumin, and hyaluronic acid (HA).

2.4. Isolation of BM-KSL cells

A BM stem cell population of KSL cells was isolated by cell sorter, FACS Aria (Becton Dickinson; BD, New Jersey, USA), according to the reported protocol (13), as described below. Whole BM cells were harvested from mouse tibias and femurs as described previously (14), then washed with PBS-EDTA, followed by ammonium chloride hemolysis to remove erythrocytes.

Isolated BM-MNCs were initially stained for 20 min at 4°C with a lineage-positive antibody cocktail containing CD45R/B220 (BD), TER119 (BD), CD3e (BD), CD11b (BD), Ly-6G, and Ly6C (Gr-1) (BD). After labeling the lineage positive (Lin⁺) antibodies with biotin-labeled magnetic beads, cells were subjected to a negative selection process with a magnetic cell sorting system (MACS). Lin-negative (Lin⁻) cells were counted and then incubated for 20 min at 4°C with rat-fluorescein isothiocyanate anti-mouse Ly-6A/E (Sca-1) (BD) and rat-PE CD117 (c-KIT) (BD), washed three times, and resuspended in 20% Iscove's modified Dulbecco medium (GIBCO, Invitrogen, California, USA). Fluorescein isothiocyanate-conjugated Sca-1 and phycoerythrin-conjugated c-KIT double-positive cells were then obtained by FACS.

The absolute number of BM stem cells was calculated by multiplying the proportion of KSL cells by the total number of Lin⁻ cells.

2.5. EPC colony forming assay

The vasculogenic potential of BM-KSL cells as a stem cell fraction of BM-derived EPCs (15) was assessed using an EPC colony-forming assay. A total of 500 BM stem cells per dish were seeded into a 35-mm hydrophilic tissue culture dishes (Falcon, BD), and EPC colony-forming-units (CFUs) were counted 7 days later. To characterize the EPC colony-derived cells, adherent colonies were stained with Alex 488-conjugated Griffonia simplicifolia isolectin B4 (GS-IB4) (Invitrogen) and 1,1'-Dioctadecyl-3,3',3'-tetramethyl indocarbocyanine perchlorate-labeled acetylated low-density lipoprotein (DiI-acLDL) (Biomedical Technologies, Wisconsin, USA) as previously described (5). In brief, after removing the methylcellulose by gently washing with ice-cold PBS twice, 1 mL of EBM-2 (Lonza, Basel, Switzerland) medium (5% fetal bovine serum) containing 10 μL of DiI-acLDL and 2 μL of Alex-488-conjugated GS-IB4 was added and further incubated for 3-5 h. After staining the colonies with 4',-6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, California, USA), cells were photographed through a fluorescence microscope (IX70; Olympus, Tokyo, Japan).

3. Results

3.1. Assessment of LF-mice model

In control mice, surfaces of the liver sections were smooth, and fatty changes, characterized by large droplets in hepatocytes, were observed (Figures 1A and 1B). On the other hand, in the LF group, surfaces of the liver sections were irregular, bridging fibrosis, pseudolobule formation, and micronodular formation (Figures 1C and 1D). Splens of CCl₄ injected mice were larger than those of the control group (Table 1). The ratio of spleen weight/body weight was significantly increased in CCl₄ mice, compared with control mice ($p < 0.005$). Serum AST, ALT, and ALP were significantly increased in CCl₄ mice compared with control mice ($p < 0.005$) (Table 2). However, there were no significant difference in mice serum γ -GTP, total bilirubin, total protein, albumin, and HA levels (data not shown). Finally, LF was clearly evident in all mice injected with CCl₄.

3.2. Impaired commitment and differentiation potentials of BM-KSL cells in LF mice

EPC colony forming activity of BM-KSL cells was performed by EPC colony-forming assay to

morphologically detect two types of EPC-CFUs, that is, primitive EPC-CFU and definitive EPC-CFU with small round and large spindle cells, respectively as previously described (16) (Figure 2). Both types of EPC-CFUs featured typical endothelial aspects of acLDL-DiI uptake and isolectin B4-FITC binding (Figures 2C and 2F).

In the EPC-colony forming assay of BM-KSL cells, the number of definitive EPC-CFU ($p < 0.0001$) and total EPC-CFU ($p < 0.05$) significantly decreased in LF mice, compared with controls, although primitive EPC-CFU did not differ between groups (Figure 3). These findings indicate that the differentiation potentials of BM-KSL cells into endothelial lineage are impaired in LF mice.

3.3. Attenuated EPC differentiation potential in circulation of LF mice

In the EPC-colony forming assay of WB-MNCs, the number of definitive EPC-CFU ($p < 0.005$) and total EPC-CFU ($p < 0.01$) significantly decreased in LF mice, compared with controls, although primitive EPC-colony forming cells did not differ between groups (Figure 4).

4. Discussion

In the current study, we successfully prepared an LF-

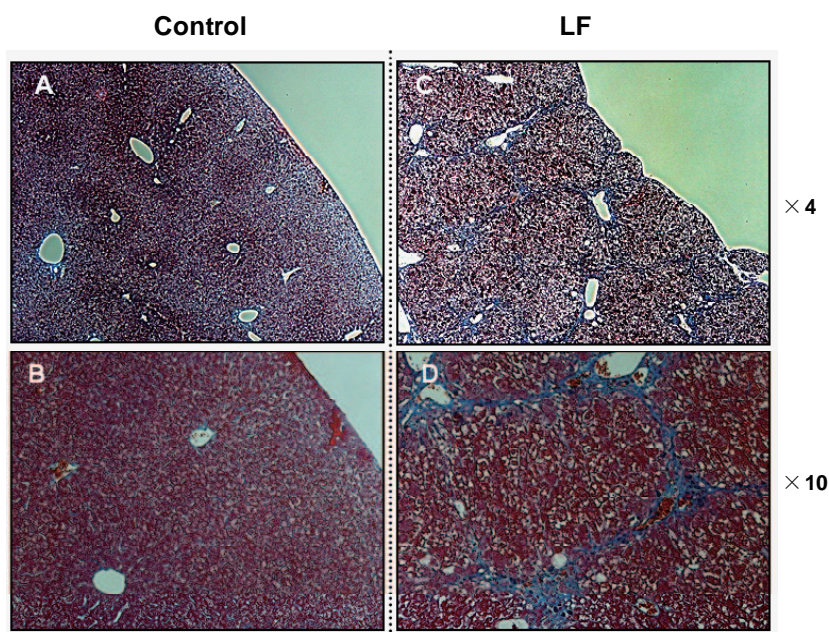


Figure 1. Liver fibrosis was observed in all mice injected with carbon tetrachloride (CCl₄). (A) and (B) Control mice (Azan stain: A, $\times 4$; B, $\times 10$). (C) and (D) CCl₄ mice (Azan stain: C, $\times 4$; D, $\times 10$)

Table 1. Spleen weight/body weight ratios in LF and control mice

Group	Spleen weight (mg) (Mean \pm S.E.M.)	Spleen weight/Body weight (Mean \pm S.E.M.)
Control	82.7 \pm 3.6	2.86 \pm 0.13
LF	93.5 \pm 3.5	3.58 \pm 0.13*

* $p < 0.005$ vs. control mice.

Table 2. The serum AST, ALT, and ALP levels in LF and control mice

Group	AST (IU/L)	ALT (IU/L)	ALP (IU/L)
Control	23.1 \pm 2.1	20.4 \pm 3.8	152.6 \pm 3.5
LF	233.8 \pm 2.4*	207.1 \pm 38.9*	201.6 \pm 5.7*

* $p < 0.005$ vs. control mice.

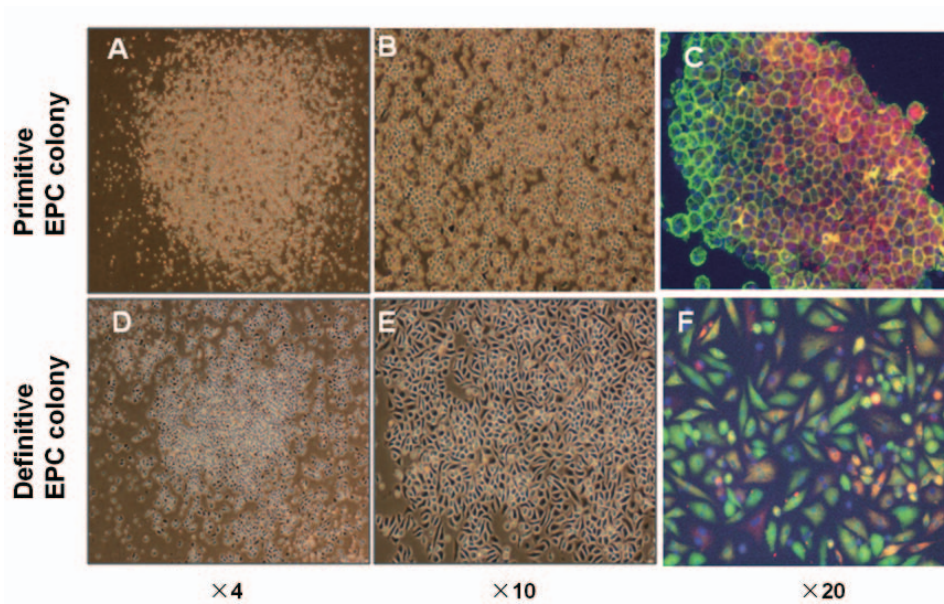


Figure 2. EPC colony-forming assay revealed two distinct colonies. (A) and (B) Primitive EPC colony (A, $\times 4$; B, $\times 10$). (C) and (F) Both colonies expressed acLDL (red), isolectin B4 (green), and DAPI (blue) cells (C and F, merge $\times 20$). (D) and (E) Definitive EPC colony (D, $\times 4$; E, $\times 10$).

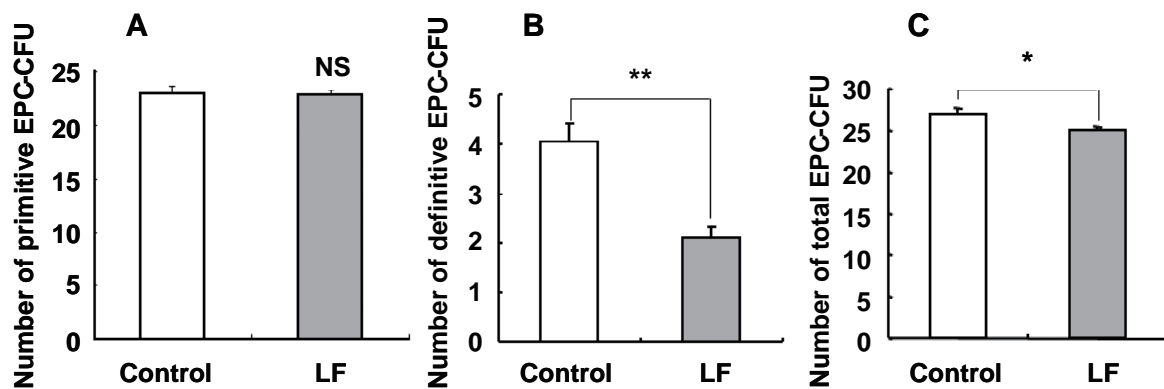


Figure 3. In BM-KSL cells, the number of definitive EPC colony-forming-units was significantly decreased in LF-mice. (A) Number of primitive EPC colony-forming-units. (B) Number of definitive EPC colony-forming-units. (C) Number of total EPC colony-forming-units. Control group ($n = 9$), LF group ($n = 13$). * $p < 0.05$, ** $p < 0.0001$ vs. control mice.

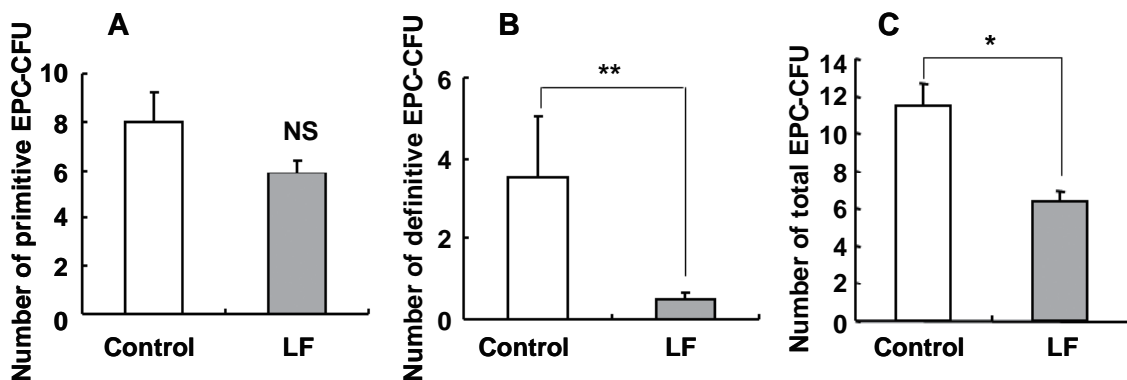


Figure 4. In WB-MNCs, the number of definitive EPC colony-forming-units was significantly decreased in LF-mice. (A) Number of primitive EPC colony-forming-units. (B) Number of definitive EPC colony-forming-units. (C) Number of total EPC colony-forming-units. Control group ($n = 4$), LF group ($n = 13$). * $p < 0.01$, ** $p < 0.005$ vs. control mice.

mice model *via* CCl₄ administration to evaluate EPC kinetics in LF using an EPC colony forming assay. Results showed that in BM and within the circulation of LF mice, the frequency of primitive colony forming EPC was not attenuated, but that of definitive colony forming EPC was significantly decreased, resulting in a decreased number of whole colonies forming EPC in LF-mice *versus* controls. These findings indicate that the function of EPC differentiation is downregulated in LF mice.

Chronic inflammatory LF induces vascular remodeling exerted by proangiogenic growth factors produced under hypoxic conditions *via* inadequate blood supply triggered by fibrotic tissue in humans (16). Based on the progression of LF, the replacement of functional hepatocytes with an excess amount of extracellular matrix is considered to decrease the production of proangiogenic growth factors or cytokines, such as vascular endothelial growth factor (17), cyclooxygenase-2-induced eicosanoids (18), hepatocyte growth factor (19), and thrombopoietin (20).

Various studies have demonstrated the pivotal roles of BM-derived EPCs, including their role in postnatal vasculogenesis, that is, mobilization, differentiation, and in foci recruitment, and shown that these cells are upregulated by proangiogenic growth factors, cytokines, and hormones (2). Previous reports show that EPC induce vasculogenesis and repair the injured tissues in several diseases (21,22). One of the mechanisms of this effect is that EPCs express several growth factors that exert paracrine effects for tissue regeneration, including hepatocyte growth factor, vascular endothelial growth factor, and transforming growth factor- α (23). Therefore, it is predicted that disturbed EPC differentiation might give rise to inadequate production for tissue regeneration, although the productivity of growth factors accompanying EPC differentiation need to be investigated.

Alternatively, it has recently been reported that the physiological homeostasis of EPC function is impaired in patients with diabetes (5), hypertension (24), hyperlipidemia (6), rheumatic disease (9), Alzheimer disease (11), aging, and smoking (7). In these conditions, oxidative stress is a key factor in inducing senescence or apoptosis of EPCs, resulting in impairments of physiological functions of EPC (25). Also, oxidative stress has been demonstrated to be a major inducer of pathological progression into LF in chronic liver disease as well (26). Therefore, in the present study, it is understandable that oxidative stress causing LF may impair EPC differentiation potential, as previously discussed in diabetes (16,27).

A therapeutic vascular improvement induced by using EPCs for chronic lower-limb ischemia including arteriosclerosis obliterans or Burger's disease, as well as myocardial infarction or angina pectoris, has been identified (28). In the field of liver regeneration therapy, autologous bone marrow cell infusion therapy, using

MNC (so-called non-selected EPCs), or selected EPCs (CD34⁺ or CD133⁺) has been used to treat liver cirrhosis patients in clinical studies, and favorable therapeutic efficacy has been reported (29,30). However, although accumulating evidence has indicated that EPC therapy may be a useful treatment for liver cirrhosis patients, there have been few reports on EPC kinetics during the development of LF.

Our study demonstrated that the homeostasis of EPC function was disrupted in LF, indicating a weakened regenerative capability. Given these results, it appears necessary to precisely and adequately investigate the efficacy of autologous bone marrow cell infusion using 'naïve EPCs' or to develop a novel strategy for improvement of EPC differentiation potential. Regarding this, G-CSF is reported to promote VEGF secretion from neutrophil for angiogenesis (31). Accordingly, the administration of G-CSF may be considered as one of strategies to repair its potential by induction of EPC differentiation as well as mobilization, because VEGF augments EPC differentiation and mobilization (32). The transplantation of EPCs after G-CSF administration may further enhance the recovery from LF. Alternatively, *ex vivo* culture system to improve the impaired EPC function, when successfully developed, might be another promising strategy.

In conclusion, LF exhibits impaired EPC commitment and differentiation potentials, providing information applicable not only for the pathological diagnosis, but also for the development of an effective EPC therapy.

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References

1. Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM. Isolation of putative progenitor endothelial cells for angiogenesis. *Science*. 1997; 275:964-967.
2. Hristov M, Erl W, Weber PC. Endothelial progenitor cells: Mobilization, differentiation, and homing. *Arterioscler Thromb Vasc Biol*. 2003; 23:1185-1189.

3. Masuda H, Kalka C, Takahashi T, *et al.* Estrogen-mediated endothelial progenitor cell biology and kinetics for physiological postnatal vasculogenesis. *Circ Res.* 2007; 101:598-606.
4. Kawamoto A, Asahara T. Role of progenitor endothelial cells in cardiovascular disease and upcoming therapies. *Catheter Cardiovasc Interv.* 2007; 70:477-484.
5. Fadini GP, Agostini C, Avogaro A. Endothelial progenitor cells and vascular biology in diabetes mellitus: Current knowledge and future perspectives. *Curr Diabetes Rev.* 2005; 1:41-58.
6. Chen JZ, Zhang FR, Tao QM, Wang XX, Zhu JH, Zhu JH. Number and activity of endothelial progenitor cells from peripheral blood in patients with hypercholesterolaemia. *Clin Sci (Lond).* 2004; 107:273-280.
7. Kondo T, Hayashi M, Takeshita K, Numaguchi Y, Kobayashi K, Iino S, Inden Y, Murohara T. Smoking cessation rapidly increases circulating progenitor cells in peripheral blood in chronic smokers. *Arterioscler Thromb Vasc Biol.* 2004; 24:1442-1447.
8. Masuda J, Mitsuyama K, Yamasaki H, Takedatsu H, Okamura T, Andoh A, Murohara T, Asahara T, Sata M. Depletion of endothelial progenitor cells in the peripheral blood of patients with ulcerative colitis. *Int J Mol Med.* 2007; 19:221-228.
9. Avouac J, Uzan G, Kahan A, Boileau C, Allanore Y. Endothelial progenitor cells and rheumatic disorders. *Joint Bone Spine.* 2008; 75:131-137.
10. de Groot KD, Bahlmann FH, Sowa J, Koenig J, Menne J, Haller H, Fliser D. Uremia causes endothelial progenitor cell deficiency. *Kidney Int.* 2004; 66:641-646.
11. Lee ST, Chu K, Jung KH, Park HK, Kim DH, Bahn JJ, Kim JH, Oh MJ, Lee SK, Kim M, Roh JK. Reduced circulating angiogenic cells in Alzheimer disease. *Neurology.* 2009; 72:1858-1863.
12. Inagaki Y, Higashi K, Kushida M, Hong YY, Nakao S, Higashiyama R, Moro T, Itoh J, Mikami T, Kimura T, Shiota G, Kuwabara I, Okazaki I. Hepatocyte Growth factor suppresses profibrogenic signal transduction *via* nuclear export of Smad3 with galectin-7. *Gastroenterology.* 2008; 134:1180-1190.
13. Kwon SM, Suzuki T, Kawamoto A, Ii M, Eguchi M, Akimaru H, Wada M, Matsumoto T, Masuda H, Nakagawa Y, Nishimura H, Kawai K, Takaki S, Asahara T. Pivotal role of Ink adaptor protein in endothelial progenitor cell biology for vascular regeneration. *Circ Res.* 2009; 104:969-977.
14. Asahara T, Masuda H, Takahashi T, Kalka C, Pastore C, Silver M, Kearne M, Magner M, Isner JM. Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. *Circ Res.* 1999; 85:221-228.
15. Tanaka R, Wada M, Kwon SM, Masuda H, Carr J, Ito R, Miyasaka M, Warren SM, Asahara T, Tepper OM. The effects of flap ischemia on normal and diabetic progenitor cell function. *Plast Reconstr Surg.* 2008; 121:1929-1942.
16. Medina J, Arroyo AG, Sanchez-Madrid F, Moreno-Otero R. Angiogenesis in chronic inflammatory liver disease. *Hepatology.* 2004; 39:1185-1195.
17. Asahara T, Takahashi T, Masuda H, Kalka C, Chen D, Iwaguro H, Inai Y, Silver M, Isner JM. VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. *EMBO J.* 1999; 18:3964-3972.
18. Kuwano T, Nakao S, Yamamoto H, Tuneyoshi M, Yamamoto T, Kuwano M, Ono M. Cyclooxygenase 2 is a key enzyme for inflammatory cytokine-induced angiogenesis. *FASEB J.* 2004; 18:300-310.
19. Asano Y, Iimuro Y, Son G, Hirano T, Fujimoto J. Hepatocyte growth factor promotes remodeling of murine liver fibrosis, accelerating recruitment of bone marrow-derived cells into the liver. *Hepatology.* 2007; 37:1080-1094.
20. Eguchi M, Masuda H, Kwon SM, Shirakura K, Shizuno T, Ito R, Kobori M, Asahara T. Lesion-targeted thrombopoietin potentiates vasculogenesis by enhancing motility and enlivenment of transplanted endothelial progenitor cells *via* activation of Akt/mTOR/p70S6kinase signaling pathway. *J Mol Cell Cardiol.* 2008; 45:661-669.
21. Asahara T. Stem cell biology for vascular regeneration. *Ernst Schering Res Found Workshop.* 2005; (54):111-129.
22. Khoo CP, Pozzilli P, Alison MR. Endothelial progenitor cells and their potential therapeutic applications. *Regen Med.* 2008; 3:863-876.
23. Ueno T, Nakamura T, Torimura T, Sata M. Angiogenic cell therapy for hepatic fibrosis. *Med Mol Morphol.* 2006; 39:16-21.
24. Pirro M, Schillaci G, Menecali C, Bagaglia F, Paltriccia R, Vaudo G, Mannarino MR, Mannarino E. Reduced number of circulating endothelial progenitors and HOXA9 expression in CD34⁺ cells of hypertensive patients. *J Hypertens.* 2007; 25:2093-2099.
25. Case J, Ingram DA, Haneline LS. Oxidative stress impairs endothelial progenitor cell function. *Antioxid Redox Signal.* 2008; 10:1895-1907.
26. Loguercio C, Federico A. Oxidative stress in viral and alcoholic hepatitis. *Free Radic Biol Med.* 2003; 34:1-10.
27. Tepper OM, Galiano RD, Capla JM, Kalka C, Gagne PJ, Jacobowitz GR, Levine JP, Gurtner GC. Human endothelial progenitor cells from type II diabetics exhibit impaired proliferation, adhesion, and incorporation into vascular structures. *Circulation.* 2002; 106:2781-2786.
28. Shantsila E, Watson T, Lip GY. Endothelial progenitor cells in cardiovascular disorders. *J Am Coll Cardiol.* 2007; 49:741-752.
29. Terai S, Ishikawa T, Omori K, Aoyama K, Marumoto Y, Urata Y, Yokoyama Y, Uchida K, Yamasaki T, Fujii Y, Okita K, Sakaida I. Improved liver function in patients with liver cirrhosis after autologous bone marrow cell infusion therapy. *Stem Cells.* 2006; 24:2292-2298.
30. Mohamadnejad M, Namiri M, Bagheri M, Hashemi SM, Ghanaati H, Zare Mehrjardi N, Kazemi Ashtiani S, Malekzadeh R, Baharvand H. Phase 1 human trial of autologous bone marrow-hematopoietic stem cell transplantation in patients with decompensated cirrhosis. *World J Gastroenterol.* 2007; 28:3359-3363.
31. Ohki Y, Heissig B, Sato Y, Akiyama H, Zhu Z, Hicklin DJ, Shimada K, Ogawa H, Daida H, Hattori K, Ohsaka A. Granulocyte colony-stimulating factor promotes neovascularization by releasing vascular endothelial growth factor from neutrophils. *FASEB J.* 2005; 19:2005-2007.
32. Dimmeler S, Aicher A, Vasa M, Mildner-Rihm C, Adler K, Tiemann M, Rütten H, Fichtlscherer S, Martin H, Zeiher AM. HMG-CoA reductase inhibitors (statins) increase endothelial progenitor cells *via* the PI 3-kinase/Akt pathway. *J Clin Invest.* 2001; 108:391-397.

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