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Hepatic Vascular Endothelial Growth Factor Regulates Recruitment of Rat Liver Sinusoidal Endothelial Cell Progenitor Cells

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Abstract

Background & Aims—After liver injury, bone marrow-derived liver sinusoidal endothelial cell progenitor cells (BM SPCs) repopulate the sinusoid as liver sinusoidal endothelial cells (LSECs). After partial hepatectomy, BM SPCs provide hepatocyte growth factor, promote hepatocyte proliferation, and are necessary for normal liver regeneration. We examined how hepatic vascular endothelial growth factor (VEGF) regulates recruitment of BM SPC and their effects on liver injury.

Methods—Rats were given injections of dimethylnitrosamine to induce liver injury, which was assessed by histology and transaminase assays. Recruitment of SPCs was analyzed by examining BM SPC proliferation, mobilization to the circulation, engraftment in liver, and development of fenestration (differentiation).

Results—Dimethylnitrosamine caused extensive denudation of LSEC at 24 hours, followed by centrilobular hemorrhagic necrosis at 48 hours. Proliferation of BM SPCs, number of SPCs in the bone marrow, and mobilization of BM SPCs to the circulation increased 2- to 4-fold by 24 hours after injection of dimethylnitrosamine; within 5 days, 40% of all LSEC came from engrafted BM SPC. Allogeneic resident SPCs, infused 24 hours after injection of dimethylnitrosamine, repopulated the sinusoid as LSEC and reduced liver injury. Expression of hepatic VEGF mRNA and protein increased 5-fold by 24 hours after dimethylnitrosamine injection. Knockdown of hepatic VEGF with antisense oligonucleotides completely prevented dimethylnitrosamine-induced proliferation of BM SPCs and their mobilization to the circulation, reduced their engraftment by

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46%, completely prevented formation of fenestration after engraftment as LSEC, and exacerbated dimethylnitrosamine injury.

Conclusions—BM SPC recruitment is a repair response to dimethylnitrosamine liver injury in rats. Hepatic VEGF regulates recruitment of BM SPCs to liver and reduces this form of liver injury.

Keywords

endothelial progenitor cells; toxic hepatitis; animal model; liver damage

Recruitment of bone marrow (BM) progenitors of liver sinusoidal endothelial cells (LSEC) is necessary for both repopulation of the hepatic microcirculation^{1, 2} and for normal liver regeneration after partial hepatectomy². Liver sinusoidal endothelial cell progenitor cells (SPC) from the bone marrow provide hepatocyte growth factor (HGF) that stimulates hepatocyte proliferation and liver regeneration². In addition, BM SPC repair monocrotaline-induced liver injury¹. Thus the BM SPC response is a repair response to disparate forms of liver injury.

Since recruitment of BM SPC to the liver ameliorates injury and promotes liver regeneration, understanding regulation of BM SPC recruitment could lead to therapeutic strategies to promote recruitment of BM SPC and to enhance engraftment of stem cell therapy.

The SPC, previously characterized as CD133⁺45⁺31⁺1⁺2⁺, differs from the CD45⁺ endothelial progenitor cell, the progenitor of vascular endothelial cells. Despite differences between the SPC and the endothelial progenitor cell, there are likely to be commonalities in their respective regulation. Elevations of serum VEGF increase the number of circulating endothelial progenitor cells³ and therapeutic approaches that increase tissue expression of VEGF promote engraftment of BM-derived endothelial cells, as was first demonstrated in tumors⁴.

The current study utilizes the dimethylnitrosamine (DMN) model of hepatic necrosis to examine whether VEGF-A (referred to hereafter as VEGF) promotes BM SPC recruitment; if so, which steps of recruitment are regulated by VEGF; and whether VEGF-mediated recruitment of BM SPC modifies injury. The study demonstrates that DMN causes extensive loss of LSEC prior to necrosis, elicits a marked BM SPC response at the time of LSEC loss, and that treatment with infused allogeneic SPC at this time attenuates the injury. Having established that SPC recruitment ameliorates DMN injury, we examined the effect of hepatic VEGF on proliferation of SPC in the bone marrow, mobilization of BM SPC to the circulation, BM SPC engraftment in the liver, differentiation of BM SPC to LSEC, and the effect this has on DMN injury. The effect of VEGF on BM SPC recruitment was also confirmed in the partial hepatectomy model.

METHODS

Materials

All chemicals were obtained from Sigma-Aldrich Chemical Corporation (St Louis, MO) unless stated otherwise. Antibodies: mouse anti-rat CD31 (Abcam Inc., San Francisco, CA); TRITC conjugated anti-PCNA, FITC conjugated donkey anti-mouse IgG, and TRITC conjugated donkey anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA); FITC conjugated mouse anti-rat CD45 and PE conjugated goat anti-mouse IgG (BD Biosciences, San Diego, CA). Microbeads: CD133 Cell Isolation Kit, anti-FITC MultiSort Kit (Miltenyi

Biotec Inc., Auburn, CA). Formaldehyde-treated serum albumin was a kind gift from Dr. Bård Smedsrød, University of Trømsø, Norway.

Animal studies

Lewis rats were obtained from Harlan Corp (Placentia, CA). Breeding pairs of Lewis (CAG-EGFP) Lewis rats were obtained from the NIH Rat Resource and Research Center at the University of Missouri.

Acute liver injury was induced by i.p. injection of dimethylnitrosamine (DMN), 25mg/kg. Partial hepatectomy was performed under general anesthesia. 70% of liver was resected.

VEGF-A was knocked down *in vivo* using anti-sense oligonucleotides (ASO). VEGF ASO and scrambled ASO control were a kind gift from ISIS Pharmaceuticals Inc (Carlsbad, CA). Hepatic VEGF knockdown was performed using i.p. injection of 20 mg/kg VEGF ASO twice weekly for 4 weeks.

VEGF (Invitrogen, Cat# PRG0114) supplementation was given through an Alzet pump (Alzet Corporation) implanted in the peritoneum that infused 1 μ l/hr. VEGF infusion was started 24 hours before giving DMN and continued until rats were sacrificed 24 hours after DMN. Hepatic vein VEGF levels were measured by rat VEGF immunoassay kit (R&D Systems, Cat #RRV00).

All protocols were reviewed and approved by the Animal Care and Use Committee at the University of Southern California to ensure ethical and humane treatment of the animals. This study followed the guidelines outlined in the NIH "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23 revised 1985).

LSEC isolation

LSEC were isolated by collagenase perfusion, iodixanol density gradient centrifugation, and centrifugal elutriation as previously described^{5, 6}. Yields averaged 84 million cells per normal rat liver with >95% viability. Purity of these cells is 99%, as determined by uptake of formaldehyde-treated serum albumin, a function specific to LSEC⁷⁻⁹, peroxidase staining to exclude Kupffer cell contamination, and the presence of fenestrae organized in sieve plates.

SPC isolation

Bone marrow (BM) and circulating SPC were isolated by double-label immunomagnetic selection for CD133 and CD45 followed by FACS sorting for CD31, or by CD133 immunomagnetic selection followed by FACS sorting for CD45 and CD31. For double-label immunomagnetic selection, BM and circulating mononuclear cells were incubated with anti-CD45 FITC antibody (1:10 dilution, 30 min at 4°C), followed by incubation with anti-FITC microbeads (20 μ l beads for up to 10⁷ cells) for 30 min at 4°C. After magnetic selection using the autoMACS Pro (Miltenyi Biotec), release reagent was used to clip off the magnetic bead. CD45⁺ cells were incubated with anti-CD133 microbeads (100 μ l beads for up to 10⁸ cells) for 30 min at 4°C.

To investigate BM SPC proliferation, CD133⁺CD45⁺ BM cells were isolated by immunomagnetic selection, permeabilized and incubated with TRITC conjugated anti-PCNA antibody (1:100 dilution) and PE conjugated anti-CD31 antibody (1:100 dilution) at 4°C for 30 min. The percentage PCNA⁺ CD133⁺CD45⁺CD31⁺ cells were determined by

flow cytometry using a FACSCalibur (BD Biosciences). Data were analyzed by Cell Quest Pro software.

Engraftment of BM SPC was determined on day 5 after DMN to allow resolution of DMN-induced congestion: congestion impairs perfusion of the liver needed for LSEC isolation. In the VEGF ASO pretreated group, engraftment and differentiation were determined together on day 14 to permit LSEC sufficient time to differentiate.

Resident SPC are present in the same elutriation fraction as LSEC, i.e. at 27.6 ml/min at 2500 rpm of the first elutriation step², and all CD133⁺ cells isolated from the LSEC fraction are resident LSEC label-retaining cells (i.e. putative stem cells) or resident SPC². Thus resident SPC were obtained by isolating LSEC and selecting for CD133⁺ cells by immunomagnetic separation with the autoMACS Pro as described above.

Immunostaining

Frozen sections of liver tissue were fixed with acetone and coverslips with LSEC were fixed with 4% paraformaldehyde. Liver sections or coverslips were incubated with FITC or PE conjugated anti-CD31 (1:100 dilution) overnight at 4°C. Images were taken using a confocal microscope (Zeiss LSM 510, Carl Zeiss Microimaging Inc.). For, red was converted to magenta in Photoshop as per the instructions to authors.

Histology

Liver necrosis and congestion were identified in hematoxylin-eosin stained liver sections.

Bone marrow transplantation

Cells were obtained from the BM of one tibia and femur from the Lew-Tg(CAG-EGFP)_{ys} Lewis donor rat. Recipients underwent 1000 cGy total body irradiation and were injected via tail vein with 50×10^6 BM cells. BM was allowed to engraft for 2–3 months before use.

Allogeneic, resident SPC infusion

1×10^6 CD133⁺ resident SPC were suspended in 1ml PBS and injected into the tail vein of experimental rats.

Real-time PCR

Total RNA of LSEC was prepared using RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. cDNA was prepared using RT² First Strand Kit from SABiosciences (Frederick, MD). Real-time PCR was performed using Real-Time RT²qPCR Primer Assays (SABiosciences) and the ABI PRISM 7900 real-time PCR system (Applied Biosystems, Foster City, CA), with β -actin as a reference control. All primers were purchased from SABiosciences.

Immunoblot

Frozen liver tissue (~40mg) was homogenized in triple lysis buffer (50 mM Tris-Cl pH 8.0, 150 mM sodium chloride, 0.02% w/v sodium azide, 0.1% w/v SDS, 1 % v/v NP-40, 0.5% w/v sodium deoxycholate plus protease inhibitors). Protein concentrations were quantified with DC Bio-Rad reagent (Bio-Rad Laboratories, Richmond, CA). Total protein (200 μ g) was separated by acrylamide gel and transferred to nitrocellulose. Immunoblotting was performed with a mouse monoclonal anti-VEGF-A antibody (Santa Cruz, cat no sc7269) and a secondary goat anti-mouse antibody conjugated with Li-Cor IRDye® 800CW (Li-Cor Biosciences, Lincoln, NE, cat no 926-3221). Bands were detected and both the 21kDa and 42kDa bands were quantified using the Odyssey Infrared Imaging System (Li-Cor

Biosciences). Membranes were stripped and re-blotted with β -actin antibody (Santa Cruz). Values were normalized to β -actin.

Scanning Electronic Microscopy (EM)

LSEC cultured on collagen-coated Thermanox coverslips (Thermo Fisher Scientific, Rochester, NY) were fixed with 2% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA) in 0.1 M cacodylate buffer, PH 7.4 for 30 minutes. After washing, LSEC were treated with 1% tannic acid (Electron Microscopy Sciences) in 0.15M cacodylate buffer for 1 hour, post-fixed with 1% osmium tetroxide (Ted Pella, Redding, CA) in 0.1M cacodylate buffer for 30 minutes, dehydrated with graded alcohols, dried with hexamethyldisilazane (Ted Pella), sputter-coated with 10-nm gold, and examined using a JSM-6390LV scanning electron microscope (JEOL, Tokyo, Japan).

Statistics

Values were compared by two-factor analysis of variance (ANOVA) with replication or by two-sided Student's t-test using Microsoft Excel (Microsoft) analysis toolpak. If ANOVA was significant, a posteriori comparison of individual points was performed by Least Significant Difference. $p < 0.05$ was considered significant.

RESULTS

DMN-induced loss of LSEC precedes hemorrhagic necrosis

Twelve hours after DMN injection, there was minimal sinusoidal congestion (Figure 1A). At 24 hours, there was mild centrilobular hemorrhage and congestion (Figure 1A) and immunostaining for CD31 demonstrated extensive loss of LSEC (Figure 1B). At 48 hours there was centrilobular hemorrhagic necrosis (Figure 1A), which progressed in severity by 72 hours (Supplemental figure 1). On DMN day 5, histological evidence of injury had largely resolved (Supplemental figure 1).

LSEC progenitor response to DMN induced liver injury

If BM SPC contribute to repopulation of the sinusoidal endothelium, then DMN toxicity should elicit a progenitor cell response. In these studies we defined the BM SPC response as the three phases that lead to BM SPC recruitment: proliferation of SPC in BM, mobilization of SPC from BM to the circulation and engraftment of BM SPC in the liver. 24 hours after DMN injection (i.e. after development of LSEC injury and prior to hepatocyte necrosis), the percentage of proliferating SPC in the BM increased from 20% in the control group to 56% in the DMN group (Figure 2A). The number of CD133⁺CD45⁺31⁺ SPC in the BM and circulation increased significantly 24 hours after DMN injection, with a 2.6-fold increase in BM and a 4.2-fold increase in circulating SPC (Figure 2B and 2C).

Engraftment was determined on day 5 (figure 2), after necrosis, congestion, and hemorrhage had largely resolved. Wild-type Lewis rats transplanted with bone marrow from Lew-Tg(CAG-EGFP)ys were used to determine BM SPC engraftment in the liver. On DMN day 5, $40.6 \pm 2.1\%$ of isolated LSEC were GFP⁺, i.e. bone marrow-derived (representative example of FACS analysis shown in Figure 2D). Immunofluorescent staining of the liver on day 5 after DMN demonstrated extensive GFP staining of CD31⁺ LSEC (Figure 2E), confirming engraftment of BM SPC as LSEC after DMN-induced liver damage.

Resident SPC infusion ameliorates DMN injury

1×10^6 allogeneic, resident SPC were infused on day 1 after administration of DMN (i.e. after LSEC loss, but prior to hepatocyte necrosis). Two days after allogeneic, resident SPC

infusion (DMN day 3), hemorrhage, congestion and necrosis were markedly improved compared to control (Figure 3A and 3B). ALT on DMN day 3 went from 124 ± 9 to 86 ± 4 IU/ml after SPC infusion ($n=3$, $p<0.005$), whereas AST went from 383 ± 39 to 225 ± 12 IU/ml after SPC infusion ($n=3$, $p<0.005$). Moreover, CD31 immunostaining showed significant restoration of the sinusoidal endothelium in livers that had received infusion of allogeneic, resident SPC (Figure 3C).

VEGF regulation of BM SPC proliferation, mobilization, and engraftment

24 hours after DMN administration (i.e. at the time of LSEC loss), gene and protein expression of VEGF were upregulated 5-fold (Figure 4A–C).

To examine the role of VEGF in the BM SPC response, antisense oligonucleotides (ASO) were used to knockdown liver expression of VEGF prior to treatment with DMN. After 8 doses of ASO, VEGF gene and protein expression were decreased by 60 and 75%, respectively (Figures 4D–F). In contrast, bone marrow VEGF did not decrease significantly after VEGF ASO (Supplemental figure 2). VEGF knockdown completely prevented the DMN-induced proliferation of BM SPC and mobilization of BM SPC to the circulation (Figure 5, Supplemental table 1), and decreased the number of SPC in the bone marrow by 50% (Figure 5). VEGF knockdown did not alter the percentage of LSEC that were resident SPC in normal liver (data not shown).

Engraftment: To investigate the effect of VEGF knockdown on BM SPC engraftment in injured liver, rats were transplanted with BM from Lew-Tg(CAG-EGFP)^{ys} Lewis rats, followed 2 months later by 4 weeks of treatment with the VEGF ASO. After knockdown of VEGF, rats were treated with DMN. Two weeks later LSEC were isolated and sorted by FACS for GFP. As shown in Figure 6A, on day 14 after DMN $69.7 \pm 5.2\%$ of total LSEC were bone marrow derived in rats who had received a scrambled control ASO, whereas VEGF ASO decreased engraftment of BM SPC by 46% to $37.6 \pm 4.1\%$ of total LSEC. Our previous studies have shown in bone marrow transplanted controls not subjected to injury that less than 1% of LSEC are of bone marrow origin².

One explanation for the decrease in engraftment by VEGF ASO is that VEGF regulates engraftment. An alternate explanation is that decreased mobilization of SPC by the VEGF ASO reduced the number of circulating SPC, so that the decrease in circulating SPC becomes the rate-limiting step leading to decreased engraftment. A second alternate explanation is that the markedly greater congestion after VEGF ASO (Figure 7) decreases the number of circulating SPC that reach the liver. To circumvent these two possibilities, engraftment of infused allogeneic, resident SPC was examined in a partial hepatectomy model, which lacks the severe congestion seen in the DMN model after VEGF ASO. Rats were treated for 4 weeks with VEGF ASO, followed by partial hepatectomy, and infusion of 1×10^6 allogeneic, resident SPC 24 after partial hepatectomy. Engraftment of infused allogeneic, resident SPC 2 weeks later was reduced from $8.2 \pm 1.1\%$ in the scrambled ASO control to $0 \pm 0\%$ in the VEGF ASO treated group (Supplemental figure 3). This confirms that VEGF regulates SPC engraftment.

VEGF regulation of BM SPC recruitment after partial hepatectomy

To confirm VEGF regulation of BM SPC recruitment in a second model, the effect of VEGF ASO was examined on day 3 after partial hepatectomy. Rats were treated with VEGF ASO or scrambled ASO for 4 weeks followed by partial hepatectomy and BM SPC proliferation and number of BM SPC, mobilization of BM SPC to the circulation, and engraftment were examined. The percentage PCNA⁺ BM SPC on day 3 after partial hepatectomy was $60 \pm 7\%$ in rats receiving scrambled ASO and decreased by 58% to $25 \pm 3\%$ in the VEGF ASO group

($n=3$, $p<0.05$); the number of BM SPC decreased by 78% from $36,928 \pm 7188$ per femur in rats receiving scrambled ASO to $7,985 \pm 1159$ per femur in rats that were treated with VEGF ASO ($n=3$, $p<0.05$); and circulating BM SPC decreased by 77% from $4,575 \pm 977/\text{ml}$ to $1,053 \pm 234/\text{ml}$ in rats treated with VEGF ASO. In wild-type Lewis rats transplanted with bone marrow from Lew-Tg(CAG-EGFP)_{ys}, followed after 2 months by 4 weeks of antisense treatment, and then partial hepatectomy, BM SPC engraftment (i.e. % GFP+ LSEC) was $36 \pm 3\%$ on day 3 after partial hepatectomy in rats that had been treated with a scrambled ASO and was reduced by 52% to 17 ± 2 in rats that received the VEGF ASO ($n=3$, $p<0.01$).

VEGF regulation of differentiation of engrafted BM SPC

SPC within the bone marrow lack the fenestrae organized in sieve plates characteristic for LSEC, whereas BM SPC that engraft after partial hepatectomy are well fenestrated within two weeks². To determine the effect of VEGF ASO-induced knockdown on differentiation of engrafted LSEC, GFP⁺ LSEC were isolated 2 weeks after DMN treatment in rats transplanted with GFP⁺ bone marrow. GFP⁺ LSEC from rats treated with scrambled ASO followed by DMN had fewer fenestrae per sieve plate and fewer sieve plates than normal LSEC, whereas there was a complete absence of fenestrae in LSEC from rats treated with VEGF ASO prior to DMN (Figure 6B).

VEGF knockdown exacerbates DMN induced liver injury

Knockdown of VEGF markedly exacerbated DMN-induced injury, with severe centrilobular hemorrhagic necrosis, greater elevation in ALT, and some increase in AST on day 1 compared to the scrambled ASO control (Figure 7).

Effect of VEGF supplementation on DMN liver injury

We examined whether the converse was true: would VEGF supplementation ameliorate DMN injury. Hepatic vein VEGF in normal rat was 8.6 pg/ml and was 56 pg/ml 24 hours after DMN. Continuous intraperitoneal infusion of VEGF by Alzet pump at a rate of 0.5 and 5 ng/hr, starting 24 hours before giving DMN, resulted in hepatic vein VEGF concentrations 24 hours after giving DMN of 3,621 and 4,649 pg/ml, respectively, but had no effect on the degree of DMN-induced liver injury at 24 hours (data not shown).

DISCUSSION

This study demonstrates that DMN-induced hemorrhagic necrosis elicits a BM SPC response. The injury is markedly ameliorated by infusion of SPC at the time of LSEC injury, demonstrating that recruitment of BM SPC attenuates DMN injury. Thus the DMN model is a good model to study regulation of BM SPC recruitment. Hepatic VEGF is increased at the time of DMN-induced loss of LSEC (and prior to hepatocyte necrosis), whereas prevention of the increase in hepatic VEGF by VEGF ASO impacts the three steps that lead to recruitment of SPC to the liver: it completely prevents increased proliferation of SPC in the bone marrow and mobilization of BM SPC to the circulation, and reduces engraftment of BM SPC in the liver. VEGF knockdown also completely prevents the development of fenestration in engrafted SPC. Thus VEGF plays an essential role in the regulation of BM SPC recruitment. In contradistinction to the amelioration of injury by SPC infusion, DMN injury was exacerbated when VEGF knockdown impaired SPC recruitment. Findings were also confirmed in a partial hepatectomy model: VEGF knockdown significantly decreases partial hepatectomy-induced BM SPC proliferation, mobilization of BM SPC to the circulation and engraftment.

Hepatic VEGF is increased in various models of toxic liver injury and after partial hepatectomy^{10–14}. Exogenous VEGF has been reported to enhance proliferation of LSEC and hepatocytes^{13, 15–17} and improve recovery from toxic liver injury^{17, 18}, whereas VEGF antibodies block these effects^{13,16}. Studies have hypothesized that VEGF works through endothelial cells to promote liver regeneration^{13, 15–17, 19}. The findings of the current study in conjunction with our earlier work² support an alternate mechanism by which VEGF ameliorates liver injury: hepatic VEGF recruits BM SPC, which repopulate the sinusoidal endothelium with actively proliferating cells and are the main source of increased LSEC HGF, and recruitment of BM SPC promotes hepatocyte proliferation and liver regeneration².

Infused allogeneic SPC engraft as LSEC and ameliorate DMN-induced hemorrhagic necrosis and monocrotaline-induced SOS¹, two forms of toxic liver injury in which LSEC are denuded prior to hepatocyte injury. These findings suggest that BM SPC recruitment might play an important role in other forms of liver injury in which LSEC are damaged, such as acetaminophen poisoning^{20–23} and ischemia-reperfusion injury^{24–27}. However the role of BM SPC recruitment extends beyond liver injury due to LSEC damage. There is extensive repopulation of LSEC by BM SPC after partial hepatectomy and BM SPC recruitment is necessary for normal liver regeneration², demonstrating that regulation of BM SPC recruitment is important for disparate types of liver injury.

DMN is a classic experimental toxin used to induce hepatic fibrosis. The current study demonstrates that DMN initially causes marked denudation of the LSEC lining, followed by repopulation by BM SPC. The engrafted BM SPC differentiate into LSEC, but remain poorly fenestrated for at least 2 weeks after a single dose of DMN. This contrasts with the partial hepatectomy model, in which engrafted BM SPC differentiate into LSEC with normal fenestration by 2 weeks². Thus DMN causes initial extensive loss of LSEC and then delayed normalization of the sinusoidal endothelium. Differentiated LSEC prevent HSC activation and promote reversion of activated HSC to quiescence^{28, 29}. This raises the possibility that DMN-induced fibrosis is due to prolonged changes to the sinusoidal endothelium induced by DMN, but this speculation will require experimental confirmation.

In summary, hepatic VEGF is an essential regulator of toxicity-induced proliferation of LSEC progenitors in the bone marrow, of mobilization of these bone marrow progenitors cells to the circulation, and of differentiation of the progenitors to liver sinusoidal endothelial cells, and contributes to engraftment of the progenitors in the liver.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ANOVA	analysis of variance
ASO	anti-sense oligonucleotides
BM	bone marrow

HGF	hepatocyte growth factor
LSEC	liver sinusoidal endothelial cell(s)
PCNA	proliferating cell nuclear antigen
SPC	liver sinusoidal endothelial cell progenitor cell(s)
VEGF	vascular endothelial growth factor

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Figure 1. Acute liver injury induced by DMN

(A) Hematoxylin-eosin stain of control liver and of liver 12–48 hours after DMN injection.

(B) CD31 (FITC, green) immunostaining of control liver and of liver 24 hours after DMN treatment.

Figure 2. Proliferation, mobilization and engraftment of BM SPC following DMN injection
(A) BM SPC proliferation. The percentage of PCNA⁺ SPC in the bone marrow in control rats and 1 day after DMN (n=3; **p<0.01); **(B) Number of SPC in BM.** The number of SPC in BM on day 1 after DMN injection (n=3; **p<0.01). **(C) Mobilization of BM SPC.** The number of circulating BM SPC (“PB SPC”) on day 1 after DMN injection (n=3; **p<0.01). Abbreviation: PB SPC, SPC in peripheral blood. **(D) Engraftment of BM SPC.** Representative example of FACS analysis demonstrating the percentage GFP⁺ LSEC 5 days after DMN injection in a wild type rat that had received a bone marrow transplantation from a Lew-Tg(CAG-EGFP)ys rat. Mean ± SEM of this group was 40.6 ± 2.1% GFP⁺ LSEC on day 5. **(E) Engraftment of BM SPC.** Engraftment in the sinusoid of bone marrow-derived GFP⁺ SPC on DMN day 5 is demonstrated by immunostaining for CD31 (magenta), GFP (green), and the overlay (white) demonstrating co-localization.

Figure 3. Effect of allogeneic, resident SPC infusion after DMN treatment

Rats received either an infusion of PBS (top row) or 1×10^6 allogeneic, resident SPC (bottom row) on day 1 after DMN and were sacrificed on day 3. Compared to the PBS control, in rats that received an infusion of resident SPC, (A) **whole liver** shows decreased hemorrhage, (B) **histology** shows decreased hemorrhage, congestion, and necrosis, and (C) **CD31 staining** (red) demonstrates a marked increase in engrafted LSEC.

Figure 4. VEGF gene and protein expression

Increased VEGF expression 1 day after DMN (A–C) and knockdown of VEGF expression by ASO (D–F). (A) Hepatic VEGF mRNA expression determined by real-time PCR in control and 1 day after DMN (n=3, *p<0.05). (B) Hepatic VEGF protein expression examined by immunoblot in control and 1 day after DMN. (C) Quantification of immunoblot in (B) (n=3, ***p<0.0001). (D) Hepatic VEGF mRNA expression determined by real-time PCR after control ASO and VEGF ASO knockdown (n=3, ***p<0.05). (E) Hepatic VEGF protein expression examined by immunoblot after control ASO and VEGF ASO knockdown. (F) Quantification of immunoblot in (E) (n=3, *p<0.05).

Figure 5. Effect of VEGF knockdown on BM SPC proliferation and mobilization

Rats received scrambled control ASO or VEGF ASO for 4 weeks, were treated with DMN and then sacrificed on day 1 after DMN. (A) **Proliferation of BM SPC.** The percentage of PCNA⁺ SPC in the bone marrow (n=3); (B) **Number of SPC in the bone marrow.** (n=4). (C) **Mobilization of BM SPC.** The number of BM SPC in the circulation (n=4). **p<0.01 compared to control.

Figure 6. Effect of VEGF knockdown on engraftment and differentiation of BM SPC

Wild type rats received a bone marrow transplantation from a Lew-Tg(CAG-EGFP)ys, were treated 2 months later with scrambled control ASO or VEGF ASO for 4 weeks, and were then injected with DMN. **(A) Engraftment of BM SPC.** Percentage of GFP⁺ LSEC was determined 14 days after DMN (n=3, ** p<0.01); **(B) Differentiation of engrafted SPC.** GFP⁺ LSEC were isolated 14 days after DMN and examined by scanning electron microscopy for the presence of fenestrae. Circles indicate sieve plates. Scale bar: 5 μ m.

Figure 7. Effect of VEGF ASO knockdown on DMN injury

Rats were treated with scrambled control ASO or VEGF ASO for 4 weeks and sacrificed on day 1 after DMN. (A) H&E staining. (B, C) Serum AST and ALT (n=3, *p<0.01, **p<0.001).