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BASIC AND TRANSLATIONAL—LIVER

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

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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 SPCs 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 LSECs at 24 hours, followed by centrilobular hemorrhagic necrosis at 48 hours. Proliferation of BM SPCs, the 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 LSECs came from engrafted BM SPCs. Allogeneic resident SPCs, infused 24 hours after injection of dimethylnitrosamine, repopulated the sinusoid as LSECs and reduced liver injury. Expression of hepatic VEGF messenger RNA 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 46%, completely prevented formation of fenestration after engraftment as LSECs, 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 (LSECs) is necessary for both repopulation of the hepatic microcirculation[1,2] and for normal liver regeneration after partial hepatectomy.[2] Liver sinusoidal endothelial cell progenitor cells (SPCs) from the bone marrow provide hepatocyte growth factor that stimulates hepatocyte proliferation and liver regeneration.[2] In addition, BM SPCs repair monocrotaline-induced liver injury.[1] Thus, the BM SPC response is a repair response to disparate forms of liver injury.

Because recruitment of BM SPCs 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 SPCs 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. Increases of serum vascular endothelial growth factor (VEGF) increase the number of circulating endothelial progenitor cells,[3] and therapeutic approaches that increase tissue expression of VEGF promote engraftment of BM-derived endothelial cells, as was first shown in tumors.[4]

The current study uses the dimethylnitrosamine (DMN) model of hepatic necrosis to examine whether VEGF-A (referred to hereafter as VEGF) promotes BM SPC recruitment, and, if so, which steps of recruitment are regulated by VEGF, and whether VEGF-mediated recruitment of BM SPC modifies injury. The study shows that DMN causes extensive loss of LSECs before 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 SPCs in the bone marrow, mobilization of BM SPCs to the circulation, BM SPC

Abbreviations used in this paper: ASO, antisense oligonucleotides; BM, bone marrow; DMN, dimethylnitrosamine; FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; LSEC, liver sinusoidal endothelial cell; PCNA, proliferating cell nuclear antigen; PCR, polymerase chain reaction; PE, phycoerythrin; SPC, liver sinusoidal endothelial cell progenitor cell; TRITC, tetramethylrhodamine isothiocyanate; VEGF, vascular endothelial growth factor.

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engraftment in the liver, differentiation of BM SPCs to LSECs, and the effect this has on DMN injury. The effect of VEGF on BM SPC recruitment also was confirmed in the partial hepatectomy model.

Materials and Methods

Materials

All chemicals were obtained from Sigma-Aldrich Chemical Corporation (St Louis, MO) unless stated otherwise. Antibodies used were as follows: mouse anti-rat CD31 (Abcam, Inc, San Francisco, CA); tetramethylrhodamine isothiocyanate (TRITC)-conjugated anti–proliferating cell nuclear antigen (PCNA), fluorescein isothiocyanate (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 phycoerythrin (PE)-conjugated goat anti-mouse IgG (BD Biosciences, San Diego, CA). Microbeads used were as follows: CD133 Cell Isolation Kit and the anti-FITC MultiSort Kit (Miltenyi Biotec, Inc, Auburn, CA). Formaldehyde-treated serum albumin was a kind gift from Dr. Bård Smedsrød (University of Trømso, Norway).

Animal Studies

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

Acute liver injury was induced by intraperitoneal injection of DMN (25 mg/kg). Partial hepatectomy was performed under general anesthesia. Seventy percent of the liver was resected.

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

VEGF (cat no. PRG0114; Invitrogen, Grand Island, New York) supplementation was given through an Alzet pump (Alzet Corporation, Cupertino, CA) implanted in the peritoneum that infused 1 μL/h. VEGF infusion was started 24 hours before giving DMN and continued until rats were killed 24 hours after DMN. Hepatic vein VEGF levels were measured by a rat VEGF immunoassay kit (cat no. RRV00; R&D Systems, Minneapolis, MN).

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 NIH (publication 86–23, revised 1985).

LSEC Isolation

LSECs 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 more than 95% viability. Purity of these cells was 99%, as determined by uptake of formaldehyde-treated serum albumin, a function specific to LSECs,[7–9] peroxidase staining to exclude Kupffer cell contamination, and the presence of fenestrae organized in sieve plates.

SPC Isolation

BM and circulating SPCs were isolated by double-label immunomagnetic selection for CD133 and CD45 followed by a fluorescence-activated cell sorter (FACS) for CD31, or by CD133 immunomagnetic selection followed by FACS 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 μL beads for up to 10^7 cells) for 30 minutes 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 μL beads for up to 10^8 cells) for 30 minutes 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 minutes. The percentage of $PCNA^+$ $CD133^+CD45^+CD31^+$ cells was determined by flow cytometry using a FACSCalibur (BD Biosciences). Data were analyzed by Cell Quest Pro software (BD Biosciences).

Engraftment of BM SPCs 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 LSECs sufficient time to differentiate.

Resident SPCs are present in the same elutriation fraction as LSECs (ie, at 27.6 mL/min at 2500 rpm of the first elutriation step),[2] and all $CD133^+$ cells isolated from the LSEC fraction are resident LSEC label-retaining cells (ie, putative stem cells) or resident SPCs.[2] Thus, resident SPCs were obtained by isolating LSECs and selecting for $CD133^+$ cells by immunomagnetic separation with the autoMACS Pro as described earlier.

Immunostaining

Frozen sections of liver tissue were fixed with acetone and coverslips with LSECs 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, Thornwood, NY). For Figure 2, red was converted to magenta in Photoshop (Microsoft, Redmond WA) as per the instructions to authors.

Histology

Liver necrosis and congestion were identified in H&E-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

$CD133^+$ (1×10^6) resident SPCs were suspended in 1 mL phosphate-buffered saline and injected into the tail vein of experimental rats.

Real-Time Polymerase Chain Reaction

Total RNA of LSECs was prepared using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Complementary DNA was prepared using the RT[2]