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BONE MARROW PROGENITOR CELLS REPAIR RAT HEPATIC SINUSOIDAL ENDOTHELIAL CELLS AFTER LIVER INJURY

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Abstract

Background and Aims—Damage to hepatic sinusoidal endothelial cells (SEC) initiates sinusoidal obstruction syndrome (SOS), which is most commonly a consequence of myeloablative chemo-irradiation or ingestion of pyrrolizidine alkaloids such as monocrotaline (Mct). This study examines whether SEC are of bone marrow origin, whether bone marrow repair can be a determinant of severity of liver injury and whether treatment with progenitor cells is beneficial.

Methods—Mct-treated female rats received infusion of male whole bone marrow or CD133+ cells at the peak of sinusoidal injury. The y-chromosome was identified in isolated SEC by fluorescent in situ hybridization. Bone marrow suppression was induced by irradiation of both lower extremities with shielding of the abdomen.

Results—SEC in uninjured liver have both hematopoietic (CD45, CD33) and endothelial (CD31) markers. After Mct-induced SOS, infusion of bone marrow derived CD133+ progenitor cells replaces more than one-quarter of SEC. All CD133+ cells recovered from the SEC fraction after injury are CD45+. CD133+/45+ progenitors also repaired central vein endothelium. Mct suppresses CD133+/CD45+ progenitors in bone marrow by 50% and in the circulation by 97%. Irradiation-induced bone marrow suppression elicited SOS from a sub-toxic dose of Mct, whereas infusion of bone marrow during the necrotic phase of SOS nearly eradicates histological features of SOS.

Conclusions—SEC have both hematopoietic and endothelial markers. Bone marrow-derived CD133+/CD45+ progenitors replace SEC and central vein endothelial cells after injury. Toxicity to bone marrow progenitors impairs repair and contributes to the pathogenesis of SOS, whereas timely infusion of bone marrow has therapeutic benefit.

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INTRODUCTION

Sinusoidal endothelial cells (SEC) are unique endothelial cells (EC) that function both as vascular lining as well as scavenger cells that clear circulating waste molecules and antigens.

SEC are the target of injury in peliosis hepatis and may be injured in cold preservation injury, nodular regenerative hyperplasia and experimental acetaminophen injury¹. Injury to SEC is the key initiating step in sinusoidal obstruction syndrome (SOS): toxins that cause SOS are selectively toxic to SEC¹ and protection of SEC prevents SOS²⁻⁴.

The two major causes of SOS are pyrrolizidine alkaloid intoxication and myeloablative chemo-irradiation used for hematopoietic cell transplantation. The bone marrow origin of some EC⁵⁻⁸ and the occurrence of SOS after bone marrow ablation raise several questions. Are SEC derived from a bone marrow progenitor after liver injury and what cell is the progenitor? Do bone marrow-derived progenitors play a significant role in the repair of SEC damage and is bone marrow-dependent repair impaired in SOS?

If SEC progenitors are bone marrow derived after liver injury, it makes sense that myeloablative modalities prolong injury and exacerbate SOS. However another major cause of SOS is the ingestion of pyrrolizidine alkaloids, which are not general bone marrow suppressants but rather lead to leukocytosis⁹. In this study we have attempted to answer the questions above, as well as to examine the effect of Mct, a pyrrolizidine alkaloid, on bone marrow-derived SEC progenitors.

MATERIALS AND METHODS

Materials

Chemicals were obtained from Sigma (St Louis MO) unless otherwise stated. Antibodies: goat polyclonal CD31 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), goat anti-rat CD33 antibody (Research Diagnostics Inc., Flanders, NJ), mouse anti-rat CD45 antibody (BD Pharmingen, San Diego, CA, catalog no. 554875), rabbit anti-human CD133 antibody (Abgent, San Diego, CA), sheep anti-rabbit IgG (DynaL Biotech, Oslo, Norway), donkey anti-rabbit IgG, phycoerythrin conjugate and rabbit anti-goat IgG rhodamine conjugate (Santa Cruz Biotechnology), goat anti-mouse IgG, FITC conjugate (BD Pharmingen).

Monocrotaline Model of SOS

Male Sprague-Dawley rats weighing 240–280 gm were obtained from Bantin and Kingman Laboratories (Fremont, CA). Rats were given 160 mg/kg Mct in phosphate buffered saline (PBS) by oral gavage (i.g.) on day 0. The liver injury score was determined by a published scoring system⁹. All protocols dealing with animals were reviewed and approved by the USC Animal Care and Use Committee to ensure ethical and humane treatment of animals. Experiments were performed in adherence with the guidelines outlined in the NIH "Guide for the Care and Use of Laboratory Animals" (revised 1985) prepared by the National Academy of Sciences.

Irradiation

Irradiation exposures were done using the 6 MV X-ray beam of a Varian 2300 c/d linear accelerator. The size of the beam was 5 by 7.9 cm, covering only the rear leg from the toes to the pelvic joint with shielding of the abdomen. The dose prescription was 800 cGy to 2-cm depth, including bolus thickness and half the thickness of the leg. Rats received a sub-toxic dose of Mct, 140mg/kg i.g., 7 days after irradiation. Control groups received either irradiation or Mct.

Bone marrow isolation

Rats received a lethal dose of pentobarbital i.p. Cells were flushed from the marrow cavity of the tibia and femur with PBS and centrifuged at 1500 rpm for 10 min.

Bone marrow infusion

Bone marrow cells were collected from the tibia and femur of inbred male Fischer rats (Charles River, Raleigh, NC). On day 5 after Mct 50 million cells were injected into the tail vein of female or male Fischer rats using a 25-gauge needle; for therapeutic infusions, 200 million cells were injected.

For CD133+ cell infusion, bone marrow cells collected from male Fischer rats were incubated with CD133 antibody (1:50) for 2 hours at room temperature, washed with PBS, incubated for 30 min with 4 beads/cell of magnetic beads coated with sheep anti-rabbit IgG, and harvested using a magnetic particle concentrator (Dyna). 4 million CD133+ cells from a male rat plus 200 million bone marrow cells from a female rat (to provide cells to support engraftment) were injected into the tail vein of female Fischer rats on day 5 following Mct.

Isolation of CD133/CD45 positive cells from the bone marrow

Aliquots of 10 million bone marrow cells were incubated with CD133 antibody (1:50), CD45 antibody (1:100), or both, for 2 hours at room temperature with gentle shaking, washed three times in 1% BSA in PBS, and incubated with donkey anti-rabbit IgG, phycoerythrin conjugated (1:100), goat anti-mouse IgG, FITC conjugated (1:100), or both for 30 min at room temperature. The fraction of double positive cells was detected using FACS.

Isolation of CD133/CD45 positive cells from the circulation

Rats were sedated with pentobarbital, injected with 0.1 ml of 5000 IU/ml porcine intestine heparin, and 3–5 ml of blood was collected from the inferior vena cava. Blood was layered over an equal amount of Histopaque 1083 and centrifuged at 2400 rpm for 30 min at room temperature. Buffy coat was collected and washed in PBS. A 10 million cell aliquot was incubated with CD45 antibody (1:100) and CD133 antibody (1:50) for 2 hours at room temperature on a shaker. Cells were washed 1% bovine serum albumin in PBS and incubated with Dynabeads (Dyna Biotech) coated with sheep anti-rabbit IgG at a concentration of 4 beads/cell, and goat anti-mouse IgG, FITC conjugated (1:100) with rotation and mixing for 30 min at 4°C. CD133+ cells were isolated using a magnetic particle concentrator. CD133+ cells were counted on a hemocytometer and the CD45+ fraction was determined using FACS.

Cell isolation

SEC were isolated by collagenase perfusion, density gradient centrifugation, and elutriation, as previously described². Yields of rat SEC are on average 100 million SEC/10 gram liver with viability of >95% and purity of > 98%, as determined by positive staining for fluorescent acetylated-low density lipoprotein and a negative peroxidase stain to reveal contaminating Kupffer cells.

Kupffer cells were provided by the non-parenchymal liver cell sub-core at USC. Kupffer cells were isolated by in situ sequential digestion of the liver with pronase and collagenase, arabinogalactan gradient ultracentrifugation and plated on coverslips.

Scoring of light microscopy

Histologic sections of liver stained by hematoxylin and eosin and Sirius red were evaluated blindly by a pathologist (G. K), using a previously published scoring system that classifies SOS as mild, moderate or severe⁹.

Fluorescent in situ hybridization (FISH)

Freshly isolated SEC were applied to slides by cytospin at 800 rpm for 5 min. Slides were treated with 100ug/ml RNase type A (Sigma) in 2X SSC (Invitrogen, Carlsbad, CA) at 37°C for 2 hours and fixed in 100% methanol for 30 min. The y-chromosome-specific probe was generously provided by Dr. Barbara Hoebee, National Institute of Public Health and Environmental Protection, The Netherlands. The y-chromosome probe was dissolved in hybridization mixture and pre-hybridized at 37°C for 2 hours. 10ng of probe DNA was added; slides were covered with glass coverslips, and placed on a hotplate at 80°C for 10 min to denature DNA. Coverslips were sealed with rubber cement and incubated at 37°C in a humidified environment for 3 days for hybridization. Post-hybridization, slides were washed with 2X SSC buffer and 0.1X SSC buffer at 65°C and blocked with 1% blocking reagent (Boehringer Mannheim-Roche, Indianapolis, IN) at 37°C for 2 hours. y-Chromosome was detected with FITC labeled anti-digoxigenin (Boehringer Mannheim-Roche) and anti-fade mounting medium (Dako Corporation, Carpinteria, CA) containing 100ng/ml propidium iodide (Molecular Probes, Eugene, OR). Cells were considered positive if a red nucleus with a single green dot was seen using a Zeiss LSM 510 confocal microscope. The presence of y-chromosome was determined in 20 cells/field in 15 random fields. The y-chromosome is only visualized when it is in the plane of visualization. To correct for non-visualization of y-chromosome, SEC were isolated from a control male rat and the percentage of y-chromosome positive cells was determined.

Flow Cytometry for Ploidy

One million SEC were isolated, fixed in 80% ethanol for 30 min at 4°C, lysed with 0.5% Triton-X-100, washed, and incubated with 5 µg/ml propidium iodide plus 50 µg/ml RNase for 30 min at room temperature. Cells were analyzed by FACS analysis at 488 nm excitation through a long pass 600nm filter.

Immunohistochemistry

DAB staining. Slides were deparaffinized in xylene followed by a series of graded alcohols. Endogenous peroxide was blocked using 0.3% hydrogen peroxide in methanol for 15 min. Antigen retrieval was performed using 4.7ml of Antigen Unmasking solution (Vector Laboratories, Burlingame, CA) diluted in 500ml distilled water. Slides were heated to 90°C in the Antigen Unmasking solution and cooled for 45 min to room temperature. Non-specific binding was blocked using 5% bovine serum albumin in PBS for 20 min at room temperature. Slides were washed in PBS and incubated with either CD45 or CD133 antibody overnight at 4°C. Slides were washed and incubated with Superenhancer (Biogenex, San Ramon, CA) for 20 min, followed by a secondary horseradish peroxidase conjugated antibody (Biogenex) for 30 min. Detection was performed using liquid 2 step 3,3'-diaminobenzidine (DAB) chromogen (Biogenex) for 5–7 min. Slides were counterstained with Mayer's hematoxylin for 3–4 min, washed, and then dehydrated in a series of graded alcohols and xylene.

Fluorescence staining

Normal rat liver was snap-frozen in liquid nitrogen and embedded in OCT compound. Cryostat sections (10µm) were fixed with cold acetone for 10 min and blocked with 5% rabbit serum for 30 min. For CD31 staining, tissue sections were incubated with a goat polyclonal CD31 antibody (1:50) for 2 hours followed by a rabbit anti-goat IgG rhodamine conjugate (1:50) for 45 min. Controls were stained with normal goat IgG (Santa Cruz Biotechnology). For CD45 staining, sections were incubated with CD45 antibody (1:50) for 2 hours followed by a rabbit anti-mouse IgG FITC conjugate (1:100) for 45 min. Controls were stained with a mouse isotype control antibody, a purified mouse IgG₁, κ immunoglobulin (BD Pharmingen). For CD31 and CD45 co-staining, sections were incubated with both primary antibodies described as above

followed by both secondary antibodies described. Slides were mounted with Prolong Gold Antifade reagent (Molecular Probes).

Immunocytochemistry

Coverslips were washed, fixed in 4% paraformaldehyde and cold methanol, and incubated with goat polyclonal CD31 antibody (1:50), CD45 antibody (1:150), or goat anti-rat CD33 antibody for 2 hours at room temperature. Coverslips were washed in PBS and incubated with either goat anti-mouse IgG, FITC conjugated (1:100), or rabbit anti-goat IgG, FITC conjugated, respectively, for 45 min at room temperature. Controls were incubated with isotype control IgG at the same concentration as the primary antibody. Coverslips were mounted on slides with Prolong Gold Antifade reagent.

Statistics

All data, expressed as mean $\pm$ SEM, were from at least three separate experiments. Groups were compared by analysis of variance (ANOVA) with *a posteriori* contrast by least significant difference; or by Student t-test using the Microsoft Excel Analysis ToolPak (Microsoft, Redmond, WA). $p < 0.05$ was considered significant.

RESULTS

Myeloid and endothelial markers of SEC

Isolated SEC and tissue sections from uninjured liver were examined (Figure 1, online supporting figure 1a shows isotype-control antibody staining). Isolated SEC were positive for both CD45, the common leukocyte antigen, and CD33, a myeloblast antigen (note: not to be confused with CD133, which is a marker of “stemness”)

Liver sections stained for CD45 and CD31 (classic endothelial marker present in SEC cytoplasm) demonstrated a continuous staining pattern along the sinusoid consistent with the pattern expected for SEC (Figure 2; online supporting Figure 2a shows isotype-control antibody staining). CD45 co-localized with CD31, demonstrating that SEC are CD45 positive. Staining of isolated SEC and Kupffer cells for CD31 confirmed positive cytoplasmic staining for SEC but showed no staining for Kupffer cells, confirming that CD31 positive cells were SEC and not Kupffer cells (Figure 1).

Bone marrow origin of SEC

Female Fischer rats treated with Mct on day 0, received an infusion of 50 million *male* bone marrow cells on days 2 or 5. Rats were sacrificed on day 12. Fluorescent in situ hybridization (FISH) for y-chromosome was performed on SEC isolated from the liver. In rats ($n=4$) that received bone marrow infusion on day 5 following Mct (i.e. at the time of maximal sinusoidal denudation), $27.1\% \pm 3.0\%$ of SEC isolated on day 12 were y-chromosome positive (Figure 3). Given that infused cells competed with residual native circulating progenitors, this approach may underestimate the percentage of bone marrow-derived SEC. In contrast to infusion on day 5, in rats that received bone marrow infusion on day 2, i.e. preceding significant loss of SEC, only 3 to 4% of SEC were of bone marrow origin (data not shown).

SEC progenitor markers

CD133 is expressed on hematopoietic stem and progenitor cells^{10, 11}, endothelial progenitor cells^{8, 5}, neuroepithelial stem cells¹², and cancer stem cells¹³. To examine whether the SEC progenitor was CD133+, CD133+ cells were isolated by immunomagnetic separation from male bone marrow and the CD133 depleted fraction was reserved. Female rats received Mct 160 mg/kg i.g. and on day 5 received an infusion of either 4 million cells from the CD133+

fraction or the CD133 depleted fraction of male bone marrow plus 200 million cells from a female bone marrow to provide cells to support engraftment. On day 12, SEC were isolated and the percentage of y-chromosome positive SEC was examined (n=3). $24.3 \pm 3.9\%$ of SEC were y-chromosome positive in female rats that received CD133+ cells and $6.1 \pm 2.4\%$ of SEC were positive in rats that received the CD133 depleted fraction (Figure 4, panel A). Given that infused cells competed with residual native circulating progenitors and progenitors from the con-infused female bone, this underestimates the percentage of SEC derived from CD133+ cells. Immunomagnetic separation does not remove all target cells, so that the “CD133 depleted” fraction contains fewer CD133+ cells but is not without CD133+ cells. Of note, engraftment of CD133+ cells only occurred after procedural improvements that significantly shortened isolation time. The isolation time was much longer when CD133+ cells were separated into a CD45+ and CD45- fraction; y-chromosome could not be detected in SEC isolated after infusion of either CD133+/45+ or CD133+/45- cells, presumably due to lengthier isolation time. Limited ex vivo survival of progenitors has also been seen with mesenchymal progenitor cells (Bui, Lutzko et al, submitted).

To confirm that the SEC progenitor is both CD133+ and CD45+, SEC were isolated from liver on day 6 after Mct. CD133+ cells were isolated by immunomagnetic separation from the SEC elutriation fraction, plated, and stained for CD45. 100% of CD133+ cells present in the SEC fraction stained positive for CD45 (Figure 4).

Differentiation of infused progenitor cells

To determine whether infused progenitor cells differentiated into fenestrated cells, Mct-treated rats received an infusion of 50 million bone marrow cells on day 5. On day 12, SEC were isolated and 15 cells per preparation were examined by scanning electron microscopy (n=3). $79.3 \pm 0.8\%$ of cells were fenestrated, compared to 100% of control cells (data not shown). Given that on average 27.1% of cells were derived from the infused bone marrow cells (see above), this suggests that 24% of progenitors had developed fenestrae within one week (i.e. $79.3\% \text{ fenestrated cells} - 72.9\% \text{ resident cells} = 6.4\% \text{ fenestrated cells derived from the bone marrow}$; $6.4\% \text{ divided by } 27.1\% \text{ equals } 24\%$).

Origin of central vein endothelial cells

Central vein EC from uninjured liver were negative for CD133 and CD45 (figure 5, panels A and C). Portal vein EC, which are not injured in SOS, stained negative for CD133 and CD45 after Mct injury (Figure 5, panels E and F).

In the Mct model, both SEC and central vein EC are injured. Immunohistochemistry for CD133 and CD45 on day 4 after Mct shows CD133+/CD45+ cells in various stages of flattening onto the denuded central vein, restoring the endothelium (Figure 5, panels B and D). Thus the CD133+/CD45+ progenitor repairs injury to both SEC and central vein EC. As expected, both SEC and central vein EC become CD133-, but SEC remain CD45+ whereas central vein EC in uninjured liver is CD45 negative.

Fusion versus engraftment of a bone marrow-derived progenitor

Male Fisher rats received Mct 160 mg/kg i.g. on day 0 followed by an infusion of 50 million *male* bone marrow cells on day 5. Rats were sacrificed on day 12, SEC were isolated, and FISH was performed for y-chromosome. If there was fusion, y-chromosome positive cells would be expected to have 2 y-chromosomes and there would be an increase in the number of cells with tetraploid DNA content. A single y-chromosome was detected in 97% of SEC, as expected of SEC isolated from male rats, but none of the SEC had 2 y-chromosomes (online supporting Table 1). To further rule out fusion, DNA ploidy was examined by FACS (online supporting Table 1). In the Mct/bone marrow infused group, the proportion of SEC in G2 (cell cycle

containing 4N DNA) was 20.3%, versus 20.5% in the control group (n=3; p NS). These findings are not consistent with fusion.

Monocrotaline toxicity to the SEC progenitor

CD133+/CD45+ cells were quantified in the circulation and bone marrow on days 1 to 7 after treatment with Mct 160 mg/kg i.g. (n=3 for each time-point; Figure 6). Controls received PBS. The mean number of CD133+/CD45+ cells in the bone marrow of controls was 6.0 ± 0.8 million cells, which is $3.4 \pm 0.0\%$ of the number of cells isolated from the bone marrow. CD133+/45+ cells in the bone marrow decreased to a nadir of 3.0 ± 0.6 million cells by day 1, stayed in that range through day 5, and returned to the normal range by day 6.

The mean number of circulating CD133+/45+ cells isolated from controls was $144,575 \pm 35,780$ cells/ml of blood (figure 6) and this represented $74 \pm 3\%$ of CD133+ cells. This number decreased to $89,735 \pm 14,420$ cells/ml blood by day 1, decreased by 97% to $4,925 \pm 2,691$ cells/ml on day 2, and remained low through day 5; the number of circulating CD133+/45+ cells increased on day 6 and 7, but continued to be significantly lower than controls. CD133+ cells followed the same pattern in the circulation after Mct as the double-labeled cells (data not shown).

In summary, on day 1 following Mct the number of CD133+/CD45+ cells is decreased by 50% in the bone marrow and 37% in the circulation, and on day 2 is decreased by 97% in the circulation. By day 6, full recovery is seen in the bone marrow and cell numbers begin to increase in the circulation.

Bone marrow suppression contributes to SOS

Bone marrow in both lower extremities of rats was irradiated with shielding of the abdomen (n=4). Seven days after irradiation, when the peripheral leukocyte count was markedly suppressed, rats received a subtoxic dose of Mct (140 mg/kg i.g.). Control groups received either irradiation or Mct. Rats were sacrificed on day 4 after Mct and livers were examined for severity of toxicity. Liver histology was normal in the group that received lower extremity irradiation alone (data not show). All 4 rats that received only the subtoxic dose of Mct showed mild SOS, with mild central vein endothelial damage, mild subendothelial hemorrhage and moderate inflammation, confirming that this was only a mildly toxic dose of Mct (Figure 7). In 3 of 4 rats that received irradiation followed by Mct, severe SOS was observed, with severe central vein endothelial damage, severe subendothelial and sinusoidal hemorrhage, and severe inflammation, whereas mild SOS was observed in 1 of 4 rats. Thus bone marrow suppression elicited toxicity from a sub-toxic dose of Mct.

Treatment of SOS with infusion of bone marrow

To determine whether progenitor cell infusion is therapeutic, 200 million bone marrow cells were infused into rats on day 4 after Mct (n=3). Severity of SOS on day 6 was scored by histology (figure 8) and ALT, AST and liver weight were examined (online supporting table 2). Rats that did not receive bone marrow had severe early SOS with severe damage to central vein EC, severe subendothelial and sinusoidal hemorrhage, severe inflammation within the central vein, and mild to severe centrilobular necrosis. In contrast, rats that received the bone marrow infusion did not fit criteria for SOS (figure 8). Residual evidence of injury in rats that received bone marrow infusion, notably mild subendothelial hemorrhage in terminal hepatic venules, and some decrease in SEC, did not qualify as SOS by the scoring system. There was minimal centrilobular fibrosis in both groups (not shown). AST, ALT and liver weight were significantly lower in the group that received a bone marrow infusion (online supporting table 2).

DISCUSSION

The major findings from these studies are that isolated SEC and SEC in sections from uninjured liver have both leukocyte and endothelial cell markers, that in an injury model there are SEC of bone marrow origin, and that infusion of bone marrow cells during acute injury eliminates evidence of SOS. Bone marrow-derived SEC progenitors replace a significant portion of SEC when there is extensive injury to the sinusoidal lining. Bone marrow-derived cells do not fuse with liver cells, but replace the resident SEC following SEC injury.

SEC are derived from CD133+/CD45+ progenitor cells after SEC injury as demonstrated by the derivation of SEC from infused CD133+ cells after injury, by the presence of CD45 in SEC from uninjured liver, and the finding that all CD133+ cells in the SEC fraction are also CD45+. Similarly, after Mct CD133+ and CD45+ cells adhered to the denuded surface of the central vein and transitioned to flattened cells along the vein, providing direct visual evidence of the transition from progenitor cell to central vein EC. Thus bone marrow-derived CD133+/CD45+ cells are progenitors to both SEC and central vein EC in an injury model. As would be expected, both endothelial cell types lose the progenitor cell antigen CD133 with maturation, but in uninjured liver SEC are CD45+ and central vein EC are not. Using a very different approach, studies have shown that portal vein EC can be derived from myeloid lineage progenitors¹⁴, but that the portal vein EC from the myeloid lineage do not persist. The studies reported here could not examine long-term engraftment, as rats treated with a toxic dose Mct die of lung disease between days 15 and 18. However persistent expression of CD45 in SEC but not in central vein EC suggests long-term engraftment of cells of the myeloid lineage in the sinusoids, but that, analogous to the portal vein¹⁴, there is not long-term engraftment in the central vein. Although EC of both the portal and central vein are bone marrow-derived^{15, 16}, the progenitor for long-term engraftment still needs to be identified.

SEC are also injured during ischemia-reperfusion. Stolz et al concluded that only a very small percentage of SEC were derived from the bone marrow after ischemia-reperfusion injury¹⁷. However in this study SEC were isolated by selecting a CD45 negative fraction. 40% of CD45+ sinusoidal cells were of bone marrow origin and we believe a fraction of these CD45+ cells may be SEC of bone marrow origin.

CD45 is one of the most abundant leukocyte cell surface glycoproteins and is expressed exclusively by nucleated cells of hematopoietic origin^{18, 19}. SEC in uninjured rat liver are positive for both CD45 and CD33, consistent with a hematopoietic cell, and CD31, consistent with an endothelial cell. Current thinking is that during embryonic development the hemangioblast gives rise to two lines of cells: the CD45+, CD31- hematopoietic precursor on the one hand and the CD31+, CD45- endothelial precursors, the angioblast in the embryo and the endothelial progenitor cell in the adult. Our findings suggest that the SEC is derived from a CD45+CD31+ precursor, which is therefore neither the traditionally defined hematopoietic precursor nor the endothelial progenitor cell.

One of the two most common settings for SOS is after myeloablative chemotherapy, which raised the question of whether bone marrow ablation itself contributes to the development of SOS. The studies presented here provide three pieces of evidence that support the concept that impaired repair by bone marrow derived progenitors contributes to SOS after SEC injury. First, bone marrow irradiation elicits severe toxicity from an otherwise sub-toxic dose of Mct, providing direct evidence that impaired bone marrow repair is a determinant of injury in SOS. Second, infusion of bone marrow eliminates histological features of SOS. Third, Mct was toxic to the CD133+/CD45+ SEC progenitor with 50% suppression of the SEC progenitor in bone marrow and 97% suppression in the circulation. Thus Mct causes SOS by injury both to SEC²⁰ and to the SEC progenitor that is needed to repair the damaged sinusoidal lining. The

decrease in bone marrow and circulating CD133+/CD45+ cells precedes significant loss of SEC and central vein EC ⁹, so that the decline is not due to consumption. The upturn in the number of CD133+/CD45+ cells in the circulation on day 6 correlates well with the early evidence of restoration of SEC lining the sinusoid and of sinusoidal perfusion on day 6 ^{9, 21}.

If these findings can be reproduced in humans, there are several clinical implications. First, a potential treatment for SOS from myeloablative regimens would be to reserve some of the cells harvested for hematopoietic cell transplantation and infuse these if the patient develops SOS. Second, SEC are CD33+ and this explains why gemtuzumab-ozagamicin, calicheamicin linked to a humanized monoclonal antibody to CD33, can cause SOS. Third, patients who undergo a second hematopoietic cell transplant with a myeloablative preparative regimen or who require gemtuzumab-ozagamicin within months of their transplantation are at greater risk for SOS. If myeloablation causes prolonged suppression of SEC progenitor cells in some patients, then it may be possible to predict who is at risk for SOS by quantifying circulating SEC progenitor cells.

In summary, the SEC in uninjured rat liver has both hematopoietic cell (CD45 and CD33) and endothelial cell (CD31) markers. In a monocrotaline rat model, bone marrow-derived CD133+/CD45+ progenitor cells replace more than one-quarter of SEC. The CD133+/CD45+ progenitor cell also repairs injury to the central vein EC, but central vein EC from uninjured liver do not express CD45 suggesting that repair of central vein EC by myeloid lineage cells is transient. Irradiation-induced bone marrow suppression elicits SOS from a dose of Mct that is not toxic in the absence of bone marrow suppression, indicating that inadequate bone marrow repair contributes to SOS, whereas bone marrow infusion during the necrotic phase markedly eliminates injury. Mct is toxic to CD133+/CD45+ progenitors in the bone marrow and circulation, thereby impairing repair of Mct-induced injury to SEC and central vein EC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

EC	endothelial cells
FACS	fluorescence-activated cell sorting
FISH	fluorescent in situ hybridization
i.g	intragastric or by gavage
Mct	monocrotaline

SEC

sinusoidal endothelial cells

SOS

sinusoidal obstruction syndrome

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Figure 1. Immunocytochemistry of cells from uninjured liver

Staining of isolated SEC for CD45 (A), CD33 (B) and CD31 (C). D. CD31 staining in isolated Kupffer cells. See online supporting figure 1a for staining with isotype control antibodies. (Note: CD33 is a myeloblast antigen, as opposed to CD133, which is a marker of “stemness”)

Figure 2. Immunohistochemistry of uninjured rat liver stained for CD31, CD45 and co-localization
Top left, CD45. Top right, CD31. Bottom panel, co-localization of CD45 and CD31 shows as white. See online supporting figure 2a for staining with isotype control antibodies. (Note: red has been converted to magenta)

Figure 3. Bone marrow origin of SEC

SEC were isolated from a male rat (left panel) and from a female rat (middle panel) as positive and negative controls for y-chromosome (white dots indicated by arrows). When a female rat was treated with Mct 160 mg/kg i.g. and received male bone marrow, more than one-quarter of SEC isolated on day 12 (right panel) were positive for y-chromosome. (Note: red has been converted to magenta).

Figure 4. Characterization of SEC progenitor cells

Panel A. Infusion of CD133+ cells and recovery from SEC in the liver. CD133+ and CD133 depleted cells were isolated from male bone marrow and infused into Mct-treated female rats on day 5 (n=3). The percentage y-chromosome positive SEC was determined. Panels B-D. CD133+ cells isolated from SEC fraction isolated on day 6 after Mct treatment in a rat treated with bone marrow infusion on day 4. Panel B: CD45 staining of CD133+ cells. Panel C: differential interference contrast (DIC) shows all cells in the field. Panel D: merge of DIC and CD45 demonstrates that all CD133+ cells stain positive for CD45.

Figure 5. CD133+/CD45+ cells are central vein EC progenitors

Central vein of uninjured liver stains negative for CD133 (A) and CD45 (C), whereas on day 4 after Mct CD133+ cells (B) and CD45+ cells (D) within the central vein range from round cells in the lumen to cells that have flattened out and lined the vein. Inserts are higher power magnification demonstrating presence (B,D) or absence (A,C) of staining of EC. Portal vein EC on day 4 after Mct stain negative for CD133 (E) and CD45 (F).

Figure 6. Monocrotaline suppresses CD133+/CD45+ SEC progenitors in bone marrow and the circulation

CD133+/45+ cells isolated from bone marrow (top panel) and the circulation (bottom panel) were quantified in control rats (day 0) and on days 1–7 after Mct 160 mg/kg ig. ANOVA for bone marrow $p < 0.005$, for blood $p < 0.0001$. Comparison of time points to day 0 by least significant difference: * $p < 0.025$, ** $p < 0.01$, *** $p < 0.005$, § $p < 0.0001$.

Figure 7. Bone marrow suppression exacerbates toxicity of sub-toxic dose of monocrotaline
 Liver sections from rats 4 days after a sub-toxic dose of Mct. Top panels (Panel A is low power and B is high power magnification) are rats treated with sub-toxic dose of Mct (140 mg/kg ig) alone and show minimal evidence of SOS. Arrows in panel B shows normal venous EC. Bottom panels (panel C is low power and D is high power magnification) are from rats that received irradiation to long bones followed 1 week later by the sub-toxic dose of Mct; panel C demonstrates centrilobular hemorrhagic necrosis (arrows show edge of centrilobular necrosis) and panel D shows loss of venular EC. Note the resemblance of cells within the lumen (indicated by arrows) to the CD133⁺/45⁺ cells seen in figure 5.

Figure 8. Effect of bone marrow infusion

Panel A and B show SOS on day 6 after Mct: panel A shows widespread centrilobular hemorrhagic necrosis consistent with severe early SOS; panel B shows higher magnification view with hemorrhagic necrosis and loss of venular endothelium. Panel C and D do not show SOS in Mct-treated rats treated with bone marrow infusion during severe SOS. Panel C. Liver does not meet histological criteria for SOS on day 6 in Mct-treated rats that receive a bone marrow infusion on day 4; panel D shows higher magnification view without necrosis and with intact venular endothelium.