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Hematopoietic stem cells and liver regeneration: Differentially acting hematopoietic stem cell mobilization agents reverse induced chronic liver injury

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ABSTRACT

Bone marrow (BM) could serve as a source of cells facilitating liver repopulation in case of hepatic damage. Currently available hematopoietic stem cell (HSC) mobilizing agents, were comparatively tested for healing potential in liver fibrosis. Carbon tetrachloride (CCl_4)-injured mice previously reconstituted with Green Fluorescent Protein BM were mobilized with Granulocyte-Colony Stimulating Factor (G-CSF), Plerixafor or G-CSF + Plerixafor. Hepatic fibrosis, stellate cell activation and oval stem cell frequency were measured by Gomori and by immunohistochemistry for a-Smooth Muscle Actin and Cytokeratin-19, respectively. Angiogenesis was evaluated by ELISA and immunohistochemistry. Quantitative real-time PCR was used to determine the mRNA levels of liver Peroxisome Proliferator-Activated Receptor gamma (PPAR-γ), Interleukin-6 (IL-6) and Tumor Necrosis-alpha (TNFα). BM-derived cells were tracked by double immunofluorescence. The spontaneous migration of mobilized HSCs towards injured liver and its cytokine secretion profile was determined in transwell culture systems. Either single-agent mobilization or the combination of agents significantly ameliorated hepatic damage by decreasing fibrosis and restoring the abnormal vascular network in the liver of mobilized mice compared to CCl_4-only mice. The degree of fibrosis reduction was similar among all mobilized mice despite that G-CSF + Plerixafor yielded significantly higher numbers of circulating HSCs over other agents. The liver homing potential of variously mobilized HSCs differed among the agents. An extended G-CSF treatment provided the highest anti-fibrotic effect over all tested modalities, induced by the proliferation of hepatic stem cells and decreased hepatic inflammation. Plerixafor-mobilized HSCs, despite their reduced liver homing potential, reversed fibrosis mainly by increasing hepatic PPAR-γ and VEGF expression. In all groups, BM-derived mature hepatocytes as well as liver-committed BM stem cells were detected only at low frequencies, further supporting the concept that alternative mechanisms rather than direct HSC effects regulate liver recovery. Overall, our data suggest that G-CSF, Plerixafor and G-CSF + Plerixafor act differentially during the wound healing process, ultimately providing a potent anti-fibrotic effect.

Introduction

Liver transplantation is the only effective treatment for end-stage liver cirrhosis, but it is limited by donor shortage, postoperative morbidity and mortality, immune rejection, high costs and long-term side effects. In order to fulfill the unmet medical needs in the field, alternative, cell-based therapies for the treatment of end-stage hepatic diseases are under investigation.

Mature hepatocytes have been traditionally recognized as the major contributors to liver repair [1,2]. However, recent evidence suggests that intrahepatic stem cell populations, the so-called "*hepatic progenitor/stem cells*" or *oval cells* (OCs) become activated, expand and actively contribute to the regenerative responses by giving rise to hepatocytes and biliary epithelial cells when hepatocyte proliferation is overwhelmed

Abbreviations: a-SMA, a-Smooth Muscle Actin; BM, bone marrow; CCl_4, carbon tetrachloride; CK19, Cytokeratin-19; FCM, flow cytometry; FVIII, Factor VIII; G-CSF, Granulocyte-Colony Stimulating Factor; GFP, Green Fluorescent Protein; HSCs, hematopoietic stem cells; IL-6, Interleukin-6; LK, Lin^-/c-Kit^+; OCs, oval cells; panCK, pan Cytokeratin; PPAR-γ, Peroxisome Proliferator-Activated Receptor gamma; SDF-1, stromal cell-derived factor-1; SGPT, serum glutamic–pyruvic transaminase; TNFα, Tumor Necrosis Factor alpha; VEGF, Vascular Endothelial Growth Factor.

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by severe liver injury [3,4]. A third source of liver-repopulating cells, bone marrow (BM), has been shown to contribute to the liver healing process after tissue injury [5,6] and efforts were made to exploit this novel source for therapeutic purposes. The BM is the largest reservoir of pluripotent stem cells in adults, traditionally considered to give rise to only hematopoietic cell lineages. This concept was challenged by reports demonstrating that BM-derived stem cells can generate a variety of adult cell types that express non-hematopoietic cell markers [7–11]. Although it had been proposed earlier that BM cells could generate hepatocytes under tissue stress [12,13], the contribution of BM to liver regeneration, under either physiological or pathological conditions, was extremely low to replace even the physiological hepatocyte turnover. Thus, the concept of bone marrow-derived liver regeneration has been strongly questioned [14–16] and the current belief is that the clinical benefit observed in injured tissues after hematopoietic stem cell (HSC) therapies is produced by the activation of endogenous progenitor cells through paracrine signaling between donor and host cells providing cytokines and growth factors [16–18].

BM-resident HSCs can be mobilized into the peripheral blood at a low magnitude under specific stimuli such as tissue injury [19,20] or at high amounts after pharmacological priming with cytostatic drugs, chemokines or hematopoietic cytokines [21,22]. G-CSF is a hematopoietic growth factor that mediates HSC mobilization to peripheral blood and represents the most widely used mobilizing agent [23]. Several reports have suggested that G-CSF-mobilized HSCs contribute to liver repair in acute and chronic liver injury models [24,25]. However, it remains arguable whether liver repopulation is mediated through a paracrine signaling process from the recruited to the liver, HSCs, stimulating tissue progenitor cells or is a direct hepatotrophic G-CSF-effect.

A novel agent, Plerixafor (AMD3100, Mozobil), is a bicyclam molecule that reversibly antagonizes the binding of stromal cell-derived factor-1 (SDF-1) to its receptor CXCR4. Plerixafor results in the rapid mobilization of HSCs into the circulation and acts synergistically when combined with G-CSF, yielding large numbers of hematopoietic progenitor cells [29,30]. Plerixafor-alone or its combination with G-CSF have only been limitedly studied as liver regenerating factors in injury models [31].

In the present study, we comparatively investigated all currently available HSC mobilizing agents (G-CSF, Plerixafor or G-CSF + Plerixafor) as liver repopulating factors. In addition, the contributing cell subpopulations and the functional mechanisms involved in the regeneration process of the chronic liver damage were also evaluated.

Materials and methods

Animals and chronic liver injury model

All procedures were approved by and performed in accordance with the Animal Care and Use Committee of the Regional Veterinary Health Authority. Seven week old C57Bl6 mice were lethally irradiated (900 cGy) and injected *via* tail vein injection with 1×10^7 BM cells isolated from age-matched Green Fluorescent Protein (GFP) donors. After an 8-week period, chronic liver injury was induced to the recipients, by injecting 1.5 ml/kg carbon tetrachloride (CCl_4: Fluka, 1:1 dilution in corn oil: Sigma) intraperitoneally (i.p.), twice a week, up to a total of 23 doses.

Mobilization

Mice were mobilized with 250 μg/kg/day recombinant human G-CSF (Tevagrastim, TevaGenerics GmbH), administered i.p. for either 6 or 12 days or with 5 mg/kg Plerixafor (Mozobil, Sanofi-Genzyme) i.p. for three days. When the combination of G-CSF plus Plerixafor was used, Plerixafor was injected on the last 3 days of G-CSF administration. Control mice received saline injections. Animals were sacrificed one day after the last mobilization dose and 5 days after the last CCl_4 dose.

Flow cytometry

Donor GFP chimerism in the blood of transplanted mice was determined by flow cytometry (FCM), before the initiation of CCl_4.

At sacrifice, whole blood obtained by cardiac puncture was stained with APC-Mouse Lineage Cocktail (anti-CD3, anti-CD11b, anti-B220, anti-GR-1, anti-Ter-119) and PE-anti-c-Kit mAb (BD Pharmingen). The absolute numbers of Lin−/c-Kit$^+$ (LK) cells per ml of blood, were calculated based on the LK cell frequency by FCM and the absolute cell counts. Results were obtained on a FACSCalibur device (Becton Dickinson, BD) and analyzed with the CellQuest Pro6 software.

Assessment of liver injury

Serum glutamic–pyruvic transaminase (SGPT; Reflotron Roche) was measured using the Reflotron PLUS autoanalyzer.

Liver tissue samples were fixed in 10% formalin and embedded in paraffin (Paraplast Plus, Tissue Embedding Medium, Leica). Liver sections (2.5 μm) were stained with hematoxylin and eosin for morphological evaluation and connective tissue staining (Gomori and Masson, Bio-Optica). All samples were evaluated blindly by a pathologist and the total fibrosis score was based on the severity of parenchymal extinction, fibrosis and "arterialization" and "capillarization" of portal veins and sinusoides.

The extent of liver fibrosis was determined using a 4-scale injury grading score, similar to the one used in non-alcoholic steatohepatitis/cirrhosis [32], as follows: Grade I: periportal and mild perisinusoidal fibrosis, Grade II: periportal fibrosis with bridging to neighboring portal tracks, Grade III: collagen fibers connecting portal tracks and moderate fibrosis around the central vein, and Grade IV: collagen fibers connecting portal tracks and portal tracts with central veins.

Immunohistochemistry and double immunofluorescence

Immunohistochemistry of paraffin-embedded liver sections (3 μm) was performed with anti-a-Smooth Muscle Actin antibody (a-SMA, 1:800; Sigma), Cytokeratin-19 (CK19, 1:100; Abcam), ki-67 (MIB1, Clone TEC-3, 1:20; DAKO) and Factor VIII (FVIII, 1:800; Abcam) antibodies. Bound antibodies were visualized by Dako Real Envision Detection System Peroxidase/DAB+, and slides were then counterstained with hematoxylin, mounted and studied by light microscopy. For quantitation of positive cells, a minimum of 20 high power fields of view (×400) were evaluated. Double immunofluorescence was performed with FITC-labeled anti-GFP antibody (1:200; Abcam) and pan Cytokeratin (panCK: CK 4, 5, 6, 8, 10, 13, 18, 1:200; Sigma) or Cytokeratin-19 (CK19, 1:100; Abcam), followed by incubation with TRITC-labeled IgG antibody (1:40; DAKO). The slides were mounted in fluorescence media with 4′,6-diamidino-2-phenylindole (DAPI/Antifade, Q-BIOgene) and 10 randomly selected fields were screened by fluorescence microscopy.

Real-time quantitative reverse transcription PCR

Total RNA from liver tissues was extracted using the QIAmp RNA kit (Qiagen) and reverse transcribed with RT^2 First Strand kit (Superarray Biosciences) to make complementary DNA.

Hepatic gene expression of Tumor Necrosis Factor alpha (TNFα), Interleukin-6 (IL-6) and Peroxisome Proliferator-Activated Receptor gamma (PPAR-γ) were assessed by real time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) using predesigned gene expression assays (Applied Biosystems) in accordance with the manufacturer's protocol. GAPDH was used as endogenous control for relative quantification. All reactions were performed in duplicate.