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Osteopontin Deletion Drives Hematopoietic Stem Cell Mobilization to the Liver and Increases Hepatic Iron Contributing to Alcoholic Liver Disease

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The aim of this study was to investigate the role of osteopontin (OPN) in hematopoietic stem cell (HPSC) mobilization to the liver and its contribution to alcoholic liver disease (ALD). We analyzed young (14-16 weeks) and old (>1.5 years) wild-type (WT) littermates and global Opn knockout (Opn−/−) mice for HPSC mobilization to the liver. In addition, WT and Opn−/− mice were chronically fed the Lieber–DeCarli diet for 7 weeks. Bone marrow (BM), blood, spleen, and liver were analyzed by flow cytometry for HPSC progenitors and polymorphonuclear neutrophils (PMNs). Chemokines, growth factors, and cytokines were measured in serum and liver. Prussian blue staining for iron deposits and naphthol AS-D chloroacetate esterase staining for PMNs were performed on liver sections. Hematopoietic progenitors were lower in liver and BM of young compared to old Opn−/− mice. Granulocyte colony-stimulating factor and macrophage colony-stimulating factor were increased in Opn−/− mice, suggesting potential migration of HPSCs from the BM to the liver. Furthermore, ethanol-fed Opn−/− mice showed significant hepatic PMN infiltration and hemosiderin compared to WT mice. As a result, ethanol feeding caused greater liver injury in Opn−/− compared to WT mice. Conclusion: Opn deletion promotes HPSC mobilization, PMN infiltration, and iron deposits in the liver and thereby enhances the severity of ALD. The age-associated contribution of OPN to HPSC mobilization to the liver, the prevalence of PMNs, and accumulation of hepatic iron, which potentiates oxidant stress, reveal novel signaling mechanisms that could be targeted for therapeutic benefit in patients with ALD. (Hepatology Communications 2018;2:84-98)

Introduction

Many clinical studies have been conducted to investigate the safety and efficacy of bone marrow (BM)-derived cells for treating liver disease.1 Pluripotent hematopoietic stem cells (HPSCs) can participate in the repopulation of normal tissue renewal and severe liver injury to improve its function.2 Therefore, we focused on the long-term implication of BM-derived HPSC mobilization to the liver due to Opn deletion and its potential contribution to alcoholic liver disease (ALD).

Abbreviations: ALD, alcoholic liver disease; BM, bone marrow; CD, cluster of differentiation; CLP, common lymphoid progenitor; CXCL, chemokines (C-X-C motif) ligand; CXCR, CXC chemokine receptor; EPO, erythropoietin; GCSF, granulocyte-colony stimulating factor; HIF, hypoxia inducible factor; HPSC, hematopoietic stem cell; IL, interleukin; Kitl, kit-ligand; MCSF, macrophage-colony stimulating factor; MDA, malondialdehyde; MF, macrophage; MIP1α, macrophage inflammatory protein 1α; Ms, monocytes; mRNA, messenger RNA; OPN, osteopontin; Opn−/−, global osteopontin knockout; PMN, polymorphonuclear neutrophil; RANTES, regulated on activation normal T cell expressed and secreted; SDF, stromal cell-derived factor; TIBC, total iron-binding capacity; TNFα, tumor necrosis factor alpha; VEGF, vascular endothelial growth factor; WT, wild-type.

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Potential conflict of interest: Nothing to report.
To mobilize BM-derived pluripotent HPSCs, granulocyte-colony stimulating factor (GCSF)\(^{(1)}\) has demonstrated good tolerance in patients with decompensated ALD and increases the proliferative activity of both hepatic progenitor cells and mature hepatocytes in the short term\(^{(3)}\). However, larger studies are necessary to define the role of BM cell therapy in patients with chronic ALD and for those with the most severe forms of the disease.

Osteopontin (OPN) is a glycoprotein involved in cell adhesion, inflammation, angiogenesis, and tumor metastasis. It is a key constituent of the HPSC niche that drives HPSC localization and is a physiological negative regulator of HPSC proliferation\(^{(4)}\). OPN binds several integrins\(^{(5)}\) and the cluster of differentiation (CD)44 receptor. Our laboratory has demonstrated that OPN participates in the pathogenesis of liver fibrosis through integrin \(\alpha_v\beta_3\) signaling\(^{(7-9)}\). Moreover, OPN has been implicated in the development of autoimmune\(^{(10)}\) and allergic airway diseases\(^{(11)}\) up-regulation of interferon-\(\gamma\)\(^{(12)}\), interleukin (IL)-18 and IL-27 expression\(^{(13,14)}\), and inhibition of HPSC proliferation\(^{(15)}\). Yet, the contribution of OPN to the mechanisms behind hematopoiesis, HPSC lodgment, and control and retention within the BM along with mobilization to the liver are currently unknown.

Because the presence of polymorphonuclear neutrophils (PMNs) correlates with ALD severity in humans and \(Opn\) deletion has been associated with increased inflammation\(^{(16)}\) and alcoholic neutrophilic hepatitis\(^{(17,18)}\), we hypothesized that OPN stimulates BM-derived HPSC mobilization to the liver, creating a proinflammatory environment conducive to liver injury and ALD.

Besides the contribution of BM-derived macrophages (MFs)\(^{(19)}\) and PMNs\(^{(20)}\) to inflammation and steatosis, iron overload also promotes alcohol-induced inflammation\(^{(21)}\). Hepatic iron overload, a common adverse event in patients undergoing HPSC transplantation\(^{(22)}\), catalyzes the Fenton reaction and increases oxidative stress along with lipid peroxidation and as a result accelerates the progression of ALD. Yet, the role of OPN in regulating iron homeostasis in the liver remains undefined. Thus, the aim of this study was to investigate the contribution of OPN to HPSC mobilization to the liver and hepatic iron overload in ALD.

### Materials and Methods

**MICE**

Global osteopontin knockout (\(Opn^{-/-}\)) mice (C57BL/6) and their wild-type (WT) littermates were purchased from the Jackson Laboratories (Bar Harbor, ME). Male and female young (14-16 weeks) and old (>1.5 years) \(Opn^{-/-}\) mice and age-matched WT littermates were used in this study. All animals received humane care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health.

**GENERAL METHODOLOGY**

Serum and liver growth factors, chemokines, and proinflammatory cytokines (GCSF, macrophage-colony stimulating factor [MCSF], chemokine (C-X-C motif) ligand 1 [CXCL1], macrophage inflammatory protein 1 alpha [MIP1\(\alpha\)], regulated on activation normal T cell expressed and secreted [RANTES], IL-6, MIP2, tumor necrosis factor alpha [TNF\(\alpha\)], IL-1\(\beta\), and vascular endothelial growth factor [VEGF]) were analyzed using the Milliplex Map Kit (EMD Millipore Corporation, Billerica, MA). Hemosiderin staining was performed with the Iron Staining Kit (Thermo Fisher Scientific, Waltham, MA). Total iron-binding capacity (TIBC) was calculated from the quantitative

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determination of iron and unsaturated iron-binding capacity with the iron/TIBC Reagent Set (Pointe Scientific, Canton, MI). Serum ferritin was measured by enzyme-linked immunosorbent assay using a kit from Life Technologies (Carlsbad, CA). Lipid peroxidation was determined according to Yagi(23) Details on general methodology, hematoxylin and eosin, naphtol AS-D chloroacetate esterase, and immunohistochemistry have been described in our publications.8,9,16,18

STATISTICAL ANALYSIS

Data are expressed as mean ± SD. Statistical comparisons among groups were performed by a two-factor analysis of variance. All experiments were carried out in triplicate at least 4 times, and a representative image or blot is shown in all figures.

Results

Opn−/− DISPLAY INCREASED HEPATIC CD34+ AND CD127+ HEMATOPOIETIC PROGENITORS COMPARED TO WT MICE

Hematoxylin and eosin staining revealed small aggregates of cells with intense basophilic nuclei and cell clusters of different sizes in the liver of old Opn−/− male (Fig. 1A, bottom right panel and zoomed views) and old Opn−/− female mice (Fig. 1B, bottom right panel and zoomed views), which displayed hepatosplenomegaly compared to their age-matched WT littermates (Fig. 1C). In addition, old Opn−/− mice showed HPSC lodgment in the spleen, suggesting global HPSC mobilization (Supporting Fig. S1A). The pathology scores showed increased inflammation in the livers from old Opn−/− compared to WT mice (Fig. 1D). Quantitative polymerase chain reaction analysis of liver lysates for the stem cell factor kit-ligand (Kitl), which plays an essential role in hematopoiesis and stem cell maintenance,24 showed messenger RNA (mRNA) up-regulation in old Opn−/− compared to WT mice (Fig. 1E). Because CD34+ HPSCs interact with OPN through β1-integrins,4 flow cytometry was performed to identify the hepatic HPSC population involved. Increased hepatic CD34+ and CD127+ hematopoietic progenitors were found in old (Fig. 1F) and young (Fig. 1G) Opn−/− mice, suggesting that global Opn deletion likely promotes extracellular matrix breakdown, allowing the release of HPSCs from the BM to the circulation.

MYELOID PROGENITOR CELLS, CD34+ CELLS, AND CD127+ CELLS ARE LESS COMMON IN THE BM OF YOUNG COMPARED TO OLD Opn−/− MICE

Young and old Opn−/− mice and age-matched WT littermates were sacrificed, and hematopoietic progenitors in the BM, spleen, and liver were analyzed by flow cytometry. There were fewer common myeloid progenitors, such as granulocyte macrophage progenitors, macrophage dendritic cell progenitors, and common dendritic cell progenitors, in the BM of young compared to old Opn−/− mice (Fig. 2A,B). Short-term CD34+ CD135+ HPSCs (Fig. 2C) and common lymphoid progenitors (CLPs) CD127+ CD135+ (Fig. 2D) were also lower in number in the BM but not in the spleen of young Opn−/− compared to WT mice (Supporting Fig. S1B-E). These cell populations were significantly increased in old Opn−/− mice (Fig. 2E,F), suggesting a possible role for OPN in restricting HPSC mobilization. Next, we analyzed whether these cell populations were mobilized from the BM to other organs. However, no differences were observed in the lung, heart, and kidney (data not shown). Based on the characterization of these cells in the liver and the BM, next we examined the potential molecular mechanism involved in HPSC mobilization to the liver and its implications for ALD.

Opn−/− EXHIBIT INCREASED HEPATIC HEMATOPOIETIC GROWTH FACTORS AND CHEMOKINES

Because the ability of cells to home, proliferate, and mature in extramedullary organs involves local and systemic chemokine production, we measured growth factors, chemokines, and proinflammatory cytokines in serum and liver from WT and Opn−/− mice. Hepatic hematopoietic growth factors, such as GCSF and M-CSF (Fig. 3A), CXCL1, MIP1α, RANTES, and IL-6 (Fig. 3B) along with MIP2, TNFα, and IL-1β (Fig. 3C), were significantly up-regulated in Opn−/− compared to WT mice. As VEGF is required for the production of fully committed hematopoietic...
progenitors, we measured its production and found that serum but not hepatic VEGF (not shown) was increased in young $Opn^{−/−}$ compared to WT mice. Several mechanisms and soluble factors are involved in the mobilization of HPSCs from the BM to other organs: the CXC chemokine receptor (CXCR)4/stromal cell-derived factor 1 axis, soluble...
KITL, VEGF, and induction of hypoxic factors. Because HPSCs express CXCR4 and are chemotactored to and retained within the BM by SDF1α, the CXCR4/SDF1α signaling axis was analyzed in liver lysates of Opn−/− and WT mice; yet, there was no major difference in their mRNA levels (Supporting Fig. S2).

Taken together, these results suggest that proinflammatory and growth stimulatory factors likely regulate the differentiation of HPSCs into mature inflammatory cells, such as PMNs and MFs in the liver. Similarly, hepatic chemokine-dependent signaling but not the CXCR4/SDF1α axis could be responsible for the trafficking of HPSCs to the liver in Opn−/− mice. Consequently, a preceding proinflammatory environment in Opn−/− mice could possibly sensitize the liver to the hepatotoxic effects of alcohol.

**ALCOHOL FEEDING INCREASES LIVER PMN INFILTRATION IN Opn−/− COMPARED TO WT MICE**

To directly address the contribution of HPSCs found in the liver of Opn−/− mice in the setting of ALD, blood and liver of young and old WT and Opn−/− mice were analyzed by flow cytometry for PMNs and monocytes (Mo). Under basal conditions, young Opn−/− but not old mice (not shown) showed more circulating PMNs but less Mo, lymphocyte antigen 6 complex locus C Mo, and
eosinophils than WT mice (Fig. 4A-D). Hepatic PMNs but not Mo were significantly increased in young and had a trend toward an increase in old $Opn^{-/-}$ compared to aged-matched WT mice (Fig. 4E). Because peripheral neutrophilia and liver PMN infiltration are frequently found in patients with ALD, naphthol AS-D chloroacetate esterase immunostaining, markers of neutrophil activation, such as Neutrophil cytosolic factor (NCF) 1/2/4, heme-binding membrane glycoprotein (Gp91phox) and neutrophil cytochrome b light chain (P22Phox), together with $Il-8$ (a key mediator of PMN recruitment and degranulation) and its receptor $Cxr1$ mRNA were analyzed in total liver. Alcohol-fed $Opn^{-/-}$ showed more liver PMNs (Fig. 4F) and markers of neutrophil activation (Fig. 4G) but not increased $Il-8$ or $Cxr1$ mRNA compared to WT mice (Supporting Fig. S3A). Moreover, no changes were observed in $Cd11b$ mRNA in $Opn^{-/-}$ compared to WT mice (Supporting Fig. S3B). However, we recently...
demonstrated increased hepatic basal and alcohol-induced F4/80+ cells in Opn−/− mice.\(^{(18)}\)

**ALCOHOL FEEDING IMPAIRS ERYTHROPOIESIS AND INCREASES HEPATIC HEMOSIDERIN AND LIPID PEROXIDATION IN Opn−/− COMPARED TO WT MICE**

Hypoxia-inducible factor 1α (HIF1α) prevents hematopoietic cell damage due to overproduction of reactive oxygen species,\(^{(34)}\) suggesting that the hypoxic niche helps maintain the long lifespan of HPSCs. Because HPSCs exhibit hypoxic profiles,\(^{(35,36)}\) we performed quantitative polymerase chain reaction analysis for Hif1α; yet, no significant changes were observed (Supporting Fig. S4). HIF1α binds to and transactivates erythropoietin (Epo), which encodes for erythropoietin, a key hormone regulator of red blood cell production and master regulator of erythroid development and iron uptake by erythrocytes.\(^{(37)}\) We analyzed the mRNA of Epo and Hamp, the latter a central player in iron homeostasis,\(^{(38)}\) in livers of young and
old Opn^{−/−} and WT mice. Opn^{−/−} mice showed increased hepatic Epo yet decreased Hamp mRNA compared to WT mice. Hamp RNA was significantly reduced in young but not old Opn^{−/−} mice (Fig. 5A), suggesting abnormal erythropoiesis and impaired iron homeostasis in Opn^{−/−} compared to WT mice.  

Next, we looked at hepatic iron content due to its role in promoting lipid peroxidation, apoptosis, and liver injury. Serum TIBC decreased whereas hepatic iron, ferritin, and malondialdehyde (MDA), a lipid peroxidation product, increased in young compared to old Opn^{−/−} mice (Fig. 5B). Liver and BM were stained with Perls’ Prussian blue. At baseline, old Opn^{−/−} mice had more hemosiderin deposits in the liver (Fig. 5C) compared to young Opn^{−/−} mice (data not shown) and had far more in the BM (Fig. 5D), minimal amount in the lung, and deposits were totally absent in the heart and kidney (Supporting Fig. S5A-C). Furthermore, ethanol-fed Opn^{−/−} presented more hepatic hemosiderin compared to WT mice (Fig. 6A). Low serum TIBC and increased hepatic iron, ferritin, and MDA levels were found in ethanol-fed Opn^{−/−} compared to WT mice (Fig. 6B). Hepatic Epo mRNA was similar, whereas Hamp mRNA decreased in
alcohol-fed $Opn^{-/-}$ compared to WT mice (Fig. 6C). Thus, ethanol caused greater liver injury and lipid peroxidation in $Opn^{-/-}$ compared to WT mice. Collectively, these results suggest that $Opn$ deletion significantly increases HPSC mobilization to the liver, hepatic inflammation, iron deposits, and lipid peroxidation, all of which could contribute to the pathogenesis of ALD.

**Discussion**

The liver has the unique feature of inducing mobilization and engraftment of HPSCs\(^{(41)}\) in addition to supporting hematopoiesis following transplantation.\(^{(42)}\) Here, we highlight three mechanisms by which $Opn$ deletion aggravates ALD: HPSC mobilization, hepatic PMN extravasation, and iron overload.

Among other molecules that sustain HPSC lodgment, OPN appears to have a key role. It is at present an unrecognized component of endosteally located stem cell niches with an important physiologic role in the regulation of HPSC localization and proliferation. HPSCs bind circulating OPN, which results in a decrease in their differentiation and proliferation.\(^{(43,44)}\) We have previously shown that serum OPN levels increase in ALD.\(^{(18)}\) The increased
cellularity observed in $Opn^{−/−}$ mice along with HPSC mobilization and hepatosplenomegaly suggest that OPN plays a prominent role in HPSC niche interactions.

Nilsson et al.\(^{(4)}\) have shown that $Opn^{−/−}$ mice display BM hypercellularity and increased lineage-negative, stem cell antigen-1-positive, and c-kit receptor-positive cells compared to WT mice. They examined the ability of CD34$^+$ HPSCs to specifically bind OPN through integrins and the $Opn$ role on HPSC apoptosis, proliferation, and differentiation. These results, also validated by others,\(^{(45)}\) indicate a
biologically relevant role for Opn deletion in the multifaceted HPSCs in the BM, promoting expansion and mobilization of BM progenitors to peripheral blood due to the fact that stromal OPN anchors HPSCs to the BM niche through β1 integrin and the CD44 receptor.\(^{(46)}\)

It has been suggested that adhesion of human hematopoietic CD34\(^+\) cells to human liver is CD44 dependent and modulated by CXCR3/4.\(^{(41)}\) Hepatosplenomegaly and BM cellularity changes were observed in Opn\(^{-/-}\) deficient mice, indicating that absence of extrahepatic Opn may stimulate HPSC migration from the BM to other organs as mobilization of HPSCs occurred in the liver and other anatomic sites, such as spleen, kidney, and lung, in Opn\(^{-/-}\) mice. Yet, whether these events are mediated by CD44 or CXCR3/4 remains elusive.

The increase in GCSF, MCSF, CXCL1, MIP1\(\alpha\), RANTES, and IL-6 proteins but not in Cxcr4 and Sdf1\(\alpha\) mRNA levels, a pathway with a major role on HPSC mobilization and retention, could suggest that OPN fine-tunes engraftment of HPSCs through hepatic production of regulatory factors involved in proliferation and differentiation of HPSCs. This may affect HPSC homeostasis in the liver and tilt the balance toward increased inflammatory cells that could enhance ethanol-induced liver injury. In our study, Opn\(^{-/-}\) had neutrophilia, increased neutrophil activation, elevated cytokines (i.e., TNF\(\alpha\), IL-1\(\beta\), and MIP2), and more hepatic PMN, albeit similar
expression of IL-8 and Cxcr1 mRNA levels compared to WT mice, suggestive of production or additional infiltration of PMNs in the liver that could support oxidative stress-induced hepatic damage as observed by the MDA values in Opn−/− mice. The elevated TNFα and IL-1β levels could increase E-selectin, known to enhance PMN sequestration and transmigration into the liver. 

Moreover, we show that Opn deletion increases hepatic MFs and PMNs, which are further enhanced by alcohol consumption. This, in turn, can potentiate OPN-mediated alcohol-induced liver injury because MFs and PMNs are critical for the progression of ALD and our data suggest neutrophil activation. Overall, these findings suggest that Opn deletion can exacerbate alcohol-induced liver damage by inducing HPSC mobilization to the liver, PMN infiltration, iron deposition, and lipid peroxidation.

We further provide evidence that short-term HPSCs and CLPs accumulate in the liver of young and old Opn−/− mice under steady-state conditions. This is of singular interest as older mice are generally more susceptible to alcohol-induced liver injury and fibrosis, reinforcing how aging fuels HPSC egression from the BM in Opn−/− mice. As evidenced by the controls, both cohorts of young and old Opn−/− mice had increased intrahepatic PMNs, HPSCs, and CLPs, suggesting a potential mechanism driven by OPN to enhance alcohol-induced liver injury in Opn−/− mice. Future mechanistic studies linking OPN-dependent PMN infiltration with ALD are needed.

Chronic iron overload worsens alcohol-induced liver injury and vascular reactivity by increasing oxidative stress through the Fenton reaction, thereby promoting lipid peroxidation and accelerating the development of ALD. Hepcidin, a small polypeptide produced by hepatocytes, plays a central role in regulating iron uptake by promoting internalization and degradation of ferroportin, the only known cellular iron exporter. The increased hepatic expression of Epo resulting from the presence of proinflammatory cytokines could suppress Hamp expression and erythroid iron intake, leading to accumulation of hemichromes in the liver. This will support the likelihood of impaired iron homeostasis, uptake, and deposition in the liver of Opn−/− mice, hence increasing alcohol-induced liver injury in young but not in old Opn−/− mice. This may also result from greater HPSC turnover and lower circulating iron in old Opn−/− mice, both of which have a tendency to increase with aging.
Because OPN-dependent release of HPSCs from the BM promotes excessive homing to the liver, as observed by hepatic CD34+ and CD127+ HPSCs, our results suggest that Opn−/− mice develop a proinflammatory liver milieu that may predispose them to greater alcohol-induced liver injury. Although OPN is increased in alcoholic and nonalcoholic steatohepatitis (15,56) the ability of OPN to regulate the proinflammatory response may be indicative of its relevance when designing new therapies. Indeed, we have demonstrated that oral administration of OPN (i.e., from bovine milk) is efficient in treating early ALD in mice.16,18

Despite some experimental and clinical work having established association among iron overload, oxidative stress, and ALD, additional studies are needed to better understand the cell-specific mechanisms driven by OPN. One possibility is that OPN is key for maintaining gut integrity and permeability, as iron is absorbed in the gut (37) lipopolysaccharide translocation from the gut to the portal blood activates Kupffer cells to produce TNFα and OPN binds lipopolysaccharides blocking Kupffer cell activation and TNFα production,18 in that way contributing to lower alcohol-mediated injury. These studies are under way in our laboratory to reinforce the role of cell-specific OPN production in the pathogenesis of ALD. Overall, the present work demonstrates that global Opn deletion promotes HPSC mobilization, PMN infiltration, and iron deposits in the liver, thereby contributing to enhance the severity of ALD (Fig. 7).

**FIG. 7.** Opn deletion promotes HPSC mobilization to the liver and increases hepatic iron, neutrophil, and macrophage infiltration, thereby contributing to ALD. Besides OPN, aging, inflammatory cell infiltration, iron overload, and the accompanying lipid peroxidation are major events responsible for increased susceptibility to ALD. OPN as a negative regulator of HPSC proliferation, lodgment, and retention can regulate the response to ALD as continuous mobilization of BM-derived HPSCs to the liver could lead to a proinflammatory environment.

**REFERENCES**


Author names in bold designate shared co-first authorship.

Supporting Information