A Central Role for CD68(+) Macrophages in Hepatopulmonary Syndrome

Reversal by Macrophage Depletion

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Rationale: The etiology of hepatopulmonary syndrome (HPS), a common complication of cirrhosis, is unknown. Inflammation and macrophage accumulation occur in HPS; however, their importance is unclear. Common bile duct ligation (CBDL) creates an accepted model of HPS, allowing us to investigate the cause of HPS.

Objectives: We hypothesized that macrophages are central to HPS and investigated the therapeutic potential of macrophage depletion.

Methods: Hemodynamics, alveolar–arterial gradient, vascular reactivity, and histology were assessed in CBDL versus sham rats (n = 21 per group). The effects of plasma on smooth muscle cell proliferation and endothelial tube formation were measured. Macrophage depletion was used to prevent (gadolinium) or regress (clodronate) HPS. CD68(+) macrophages and capillary density were measured in the lungs of patients with cirrhosis versus control patients (n = 10 per group).

Measurements and Main Results: CBDL increased cardiac output and alveolar–arterial gradient by causing capillary dilatation and arteriovenous malformations. Activated CD68(+) macrophages (nuclear factor- κ B+) accumulated in HPS pulmonary arteries, drawn by elevated levels of plasma endotoxin and lung monocyte chemoattractant protein-1. These macrophages expressed inducible nitric oxide synthase, vascular endothelial growth factor, and platelet-derived growth factor. HPS plasma increased endothelial tube formation and pulmonary artery smooth muscle cell proliferation. Macrophage depletion prevented and reversed the histological and hemodynamic features of HPS. CBDL lungs demonstrated increased medial thickness and obstruction of small pulmonary arteries. Nitric oxide synthase inhibition unmasked exaggerated pulmonary vasoconstrictor responses in HPS. Patients with cirrhosis had increased pulmonary intravascular macrophage accumulation and capillary density.

Conclusions: HPS results from intravascular accumulation of CD68(+) macrophages. An occult proliferative vasculopathy may explain the occasional transition to portopulmonary hypertension. Macrophage depletion may have therapeutic potential in HPS.

Keywords: liver transplantation; arteriovenous malformations; clodronate; cirrhosis; portopulmonary hypertension

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Hepatopulmonary syndrome (HPS), characterized by hypoxemia and intrapulmonary shunting due to capillary dilation and arteriovenous malformations, is prevalent in patients with liver disease and has no medical treatment. Prior studies recognized the accumulation of intravascular phagocytic cells in the pulmonary arteries of rats with HPS; however, their importance and identity was uncertain.

What This Study Adds to the Field

We show that intravascular accumulation of activated macrophages in small pulmonary arteries is central to the pathogenesis of HPS. CD68(+) macrophages generate vasodilatory, angiogenic, and proliferative growth factors, including inducible nitric oxide synthase, vascular endothelial growth factor, and platelet-derived growth factors, respectively. *In vivo* depletion of pulmonary intravascular macrophages prevents and reverses HPS. We also unexpectedly discovered a proliferative, pulmonary vasculopathy characterized by muscularized, partially obstructed, small pulmonary arteries and an exaggerated vasoconstrictor diathesis that is masked by nitric oxide. This may explain the occasional transition from HPS to portopulmonary hypertension after liver transplantation. This study suggests that HPS results from intravascular accumulation of CD68(+) macrophages, and macrophage depletion may have therapeutic potential.

(Received in original form August 19, 2010; accepted in final form November 21, 2010)

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Supported by National Institutes of Health grants RO1-HL071115 and 1RC1HL099462-01 (S.L.A.), the American Heart Association, and the Roche Foundation for Anemia Research.

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Crit Care Med Vol 183, pp 1080–1091, 2011

Originally Published in Press as DOI: 10.1164/rccm.201008-1303OC on December 10, 2010
Internet address: www.atsjournals.org

Hepatopulmonary syndrome (HPS), characterized by hypoxemia and intrapulmonary shunting, occurs in 5 to 32% of patients with liver disease (1). HPS significantly increases mortality and worsens functional status and quality of life in patients with cirrhosis (2). The major pathological abnormalities in HPS include alveolar capillary dilatation and pulmonary arteriovenous malformations (3). Clinically, patients with HPS present with hypoxemia and signs of a hyperdynamic circulatory state that include low systemic vascular resistance, low pulmonary vascular resistance (PVR), and high cardiac output (CO) (1). The pathogenesis of HPS is unclear, and currently there are no effective medical therapies. Orthotopic liver transplantation is the only available treatment (1). It is noteworthy that some patients with cirrhosis with HPS can have coexisting portopulmonary hypertension or develop it after liver transplantation (4). The basis for transition from HPS to portopulmonary hypertension, a condition with low CO and high PVR, is unknown.

Common bile duct ligation (CBDL) in rats is an accepted experimental model of HPS (5). Biliary cirrhosis induced by

TABLE 1. COMPARISON OF LIVER FUNCTION TESTS AND HEMODYNAMICS IN STUDY GROUPS

Characteristics	Sham	CBDL	CBDL + GdCl3	CBDL + Clodronate
Total bilirubin, mg/dl	0.1 ± 0.01	8.1 ± 0.7*	4.8 ± 2*	8.9 ± 1.8*
Alkaline phosphatase, IU/L	125 ± 13	372 ± 41*	406 ± 209*	757 ± 126*
Aspartate aminotransferase, IU/L	77 ± 5	428 ± 61*	528 ± 94*	8,204 ± 11,913*
Alanine aminotransferase, IU/L	40 ± 3	62 ± 7	89 ± 17*	1,489 ± 2,369*
Hemodynamics				
Mean pulmonary artery pressure, mm Hg	17.5 ± 0.9	16.3 ± 0.6	17.5 ± 1.5	15.9 ± 6.2
Left ventricular end-diastolic pressure	4.3 ± 0.5	5.5 ± 0.7	6.2 ± 1.3	5.7 ± 5

Definition of abbreviations: CBDL = common bile duct ligation; GdCl3 = gadolinium chloride.

* $P < 0.05$ compared to sham group.

CBDL recapitulates the features of human HPS, including pulmonary precapillary and postcapillary vessel dilatation, pleural and pulmonary arteriovenous malformations, and increased alveolar–arterial (A–a) oxygen gradient from intrapulmonary shunting (6). In this HPS model, mononuclear cells with phagocytic activity accumulate in the pulmonary arteries (7). However, the importance and identity of these pulmonary intravascular phagocytic cells is incompletely understood. Prior studies have provided indirect evidence that certain features of HPS may result from increased nitric oxide (NO) and vascular endothelial growth factor (VEGF) secreted by the pulmonary intravascular macrophages (8, 9). Macrophage accumulation is known to promote neoangiogenesis and vascular remodeling in other conditions, such as atherosclerosis, cancer, and other pulmonary vascular disease, by secretion of growth factors (10–13).

Thus, we hypothesized that pulmonary intravascular macrophage accumulation may be central to the pathogenesis of HPS, serving as a source of vasodilatory, angiogenic, and proliferative growth factors, such as inducible nitric oxide synthase (iNOS), VEGF, and platelet-derived growth factor (PDGF), respectively. Conversely, depletion of intravascular macrophages should prevent or regress HPS. Here we show that *in vivo* depletion of pulmonary vascular macrophages, using two complementary techniques, is sufficient to normalize oxygenation and prevent or reverse the hemodynamic and histological features of HPS. In performing quantitative histology we also made the unexpected observation of a proliferative pulmonary vasculopathy. Some of the results of these studies have been previously reported in the form of an abstract (14).

METHODS

Animal Protocol

The University of Chicago Animal Care Committee approved all protocols. Male Sprague-Dawley rats weighing 200 to 300 g were purchased from Charles River Laboratories (Wilmington, MA). CBDL versus sham surgery ($n = 21$ in each group) was performed on Day 1, as described previously (15). Briefly, all animals underwent laparotomy under 2% isoflurane inhalational anesthesia. In the CBDL group, the common bile duct was isolated and double ligated with a hemoclip. The abdominal incision was then closed with sutures and animals were allowed to recover. In the sham group, the abdomen was closed after placing a hemoclip in the peritoneum, without disturbing the common bile duct. All animals were studied on Day 28.

Depletion of Macrophages

Two methods that deplete intravascular macrophages were used: serial intravenous injections of either gadolinium chloride (GdCl3) or liposomal clodronate (16). We used complementary methods to confirm that any beneficial effects observed reflects macrophage depletion rather than off-target effects of the drug. GdCl3 precipitates at neutral pH and circulating macrophages ingest the aggregates,

inducing apoptosis (17). Clodronate is a bisphosphonate that, when injected in liposomes, is ingested by circulating macrophages. The intracellular release and accumulation of clodronate also induces apoptosis (18).

GdCl3 was administered beginning 1 day before CBDL to prevent HPS. The CBDL+GdCl3 rats ($n = 6$) were injected intravenously with GdCl3 (10 mg/kg; Sigma Chemical Co, St Louis, MO) biweekly for 4 weeks. In additional rats, clodronate was administered on Day 14 post CBDL to reverse HPS. Rats were injected intravenously with either liposomal clodronate or liposomal vehicle biweekly for 2 weeks (15 mg/kg; Encapsula NanoScience, Nashville, TN; $n = 6$ per group). GdCl3 and clodronate doses were based on the literature (16).

Inhibition of iNOS- and VEGF/PDGF-dependent Pathways

To investigate the effects of inhibiting the individual pathways that are activated in the macrophages (iNOS, VEGF, and PDGF), we treated two additional groups of CBDL rats ($n = 6$ each). They received either a selective inhibitor of iNOS (L-N_G-[1-iminoethyl]-lysine, L-NIL) or the tyrosine kinase inhibitor, imatinib mesylate. L-NIL was given in the drinking water (8 mg/L) and imatinib was given by daily intraperitoneal injections (50 mg/kg) as described previously (19, 20). In these regression protocols, L-NIL and imatinib treatment started on Day 14 after CBDL surgery and were continued for 2 weeks.

Human Lung Sections

We studied human lung sections from 10 patients who died from cirrhosis versus 10 age- and sex-matched patients who died from other causes. These samples were obtained from autopsy specimens archived at The University of Chicago. Microvascular density was quantified by measuring the pixel density of CD31 expression in at least 10 high-power fields per lung section from each patient using a rabbit polyclonal anti-human antibody against human CD31 (Novus Biologicals, Littleton, CO). CD68 macrophage counts were performed on lung sections using a human monoclonal antibody to CD68 (Novus Biologicals). The total number of CD68(+) cells per high-power field and the number of pulmonary intravascular macrophages were quantified.

Statistics

Sample size for all experiments was six or more animals per group unless specified. Normality was confirmed with a Kolmogorov-Smirnov or D'Agostino and Pearson omnibus test. Values are listed as mean ± SEM. Intergroup differences between groups were assessed by an unpaired Student *t* test or analysis of variance with *post hoc* analysis using a Bonferroni correction for comparison among multiple groups. For data that were not normally distributed, we used a Mann-Whitney test or Kruskal-Wallis test. A *P* less than 0.05 is considered statistically significant. All analyses were performed using Prism statistical software version 5 (Graph Pad, La Jolla, CA).

Detailed methods for cardiac ultrasound, arterial blood gas measurements, cardiac catheterization, isolated perfused lung, quantitative lung histology, cell culture, proliferation assay, and quantitative polymerase chain reaction have been previously reported (21–23) and can be found in the online supplement. The methods for matrigel assay, micro-computed tomography (CT) angiography, and the endotoxin assay can also be found in the online supplement.

RESULTS

CBDL Recapitulates Human HPS

CBDL rats developed jaundice, ascites, elevated plasma transaminases and alkaline phosphatase levels, and biliary cirrhosis

(Table 1). The operative mortality rate was less than 10%. The diagnosis of cirrhosis was confirmed by the macroscopic appearance of the liver in all CBDL rats at autopsy and by Masson trichrome staining, which revealed increased collagen (Figure 1A). There was a progressive increase in CO after CBDL. The

Figure 3. Vasoconstrictor diathesis in hepatopulmonary syndrome (HPS) is revealed by nitric oxide synthase (NOS) inhibition. (A) There is enhanced pulmonary vasoconstriction in response to angiotensin II in common bile duct ligation (CBDL) versus sham animals. (B) Hypoxic pulmonary vasoconstriction is augmented in HPS lungs after inhibition of NOS by N^G-nitro-arginine methyl ester (L-NAME) ($n = 3$ per group). (C) Representative and quantitative data showing increased proliferative activity in CBDL versus sham rats. Images obtained using double-label immunofluorescent stains for proliferating cell nuclear antigen (red) and smooth muscle actin (green) ($\times 40$; $n = 4$ per group). hpf = high-power field; PCNA = proliferating cell nuclear antigen.

increase in CO was statistically significant by Week 3 and paralleled the increase in CBD diameter, as measured by ultrasound (Figure 1B). As previously described, CBDL animals developed HPS characterized by alveolar capillary dilatation (Figure 1C), an increased A–a oxygen gradient (data presented below), and increased lung microvessel density, as measured using von Willebrand factor immunostaining (Figure 1C).

CBDL Induces an Occult Obstructive Pulmonary Vasculopathy

In addition to the expected capillary dilatation and arteriovenous malformations, quantitative histology unexpectedly re-

vealed a pulmonary vasculopathy (Figure 2A), characterized by an increase in the percent medial thickness and loss of lumen. This was present in some, but not all, resistance pulmonary arteries (PA). Immunostaining for smooth muscle actin and von Willebrand factor revealed that CBDL lungs had increased percent medial thickness of resistance PAs (25–150 μ m) compared with the sham lungs (Figures 2B and 2C). To confirm this using a three-dimensional imaging technique, we performed micro-CT pulmonary angiography on barium-perfused lungs. This confirmed both the unexpected obstructive arteriopathy, with a dropout of resistance PA (~ 100 μ m diameter), and

Figure 4. Common bile duct ligation (CBDL) induces accumulation of CD68(+) macrophages in the lungs. (A) Note greater accumulation of CD68 (red) and nuclear factor (NF)- κ B (green) positive intravascular macrophages in CBDL versus sham animals ($\times 40$). (B) Representative immunofluorescent image demonstrating nuclear translocation of NF- κ B (green) in CBDL. Note colocalization with the nuclear marker, 4',6-diamidino-2-phenylindole (DAPI, blue), which indicates macrophage activation. (C) Plasma endotoxin level is increased in CBDL ($n = 8$ per group). (D) Monocyte chemoattractant protein (MCP)-1 mRNA is increased in CBDL versus sham lungs ($n = 7$ per group). PA = pulmonary arteries.

Figure 5. Increased inducible nitric oxide synthase (iNOS), vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF) in pulmonary intravascular macrophages in hepatopulmonary syndrome (HPS). (A–C) Representative and quantitative data showing greater accumulation of CD68(+) (red) macrophages and increased expression of (A) iNOS (green), (B) VEGF (green), and (C) PDGF (green) in common bile duct ligation (CBDL) versus sham animals ($\times 40$) ($n = 3$ per group; 10 high-power fields [hpf] per slide). Note iNOS, VEGF, and PDGF are largely expressed within the macrophages. (D) Representative immunoblots and mean expression shows greater expression of phosphorylated extracellular signal-regulated kinase (p-ERK1) in CBDL lungs. This is reduced in the gadolinium chloride (GdCl₃)-treated CBDL group ($n = 5$ per group).

the expected increase in alveolar capillary density in CBDL versus sham rats (Figure 2D).

Acute Inhibition of NOS Reveals Enhanced Vasoconstrictor Responses in Isolated CBDL Lungs

To evaluate the physiological relevance of this proliferative vasculopathy, we assessed the response of the lung circulation to vasoconstrictors using the isolated, perfused lung model, as previously described (24). In this model, flow is held constant so that changes in pressure reflect changes in PVR. Both the sham and CBDL lungs had a similar pressor response to hypoxia at baseline, but the vasoconstrictive response to angiotensin II was higher in the CBDL lungs than the sham lungs (Figure 3A). After N^G-nitro-arginine methyl ester (L-NAME), the increase in hypoxic pulmonary vasoconstriction was significantly higher in the CBDL versus sham lungs (Figure 3B), suggesting an enhanced vasoconstrictor capacity, consistent with the observed proliferative vasculopathy shown in Figure 2.

Increased Cell Proliferation in CBDL Lungs

To understand the role of cell proliferation in the observed capillary and PA remodeling, we quantified cell proliferation in

lung sections. CBDL lungs had significantly increased expression of proliferating cell nuclear antigen (PCNA) staining versus sham animals. Nuclear PCNA staining was increased threefold ($P < 0.05$) in alveolar microvessels and muscularized resistance PAs (Figure 3C).

Accumulation of Activated CD68(+) Macrophages in the PAs of CBDL Rats

The muscular and nonmuscular PA in CBDL lungs showed accumulation of large mononuclear macrophages that stained strongly positive for the tissue macrophage-specific marker CD68 (Figure 4A). Although the majority of CD68(+) macrophages were located within the pulmonary vasculature, they were also seen in the alveolar interseptal region. Some of the resistance pulmonary arteries were totally occluded by the accumulation of CD68(+) macrophages (Figure 2A). In contrast, sham lungs had low levels of CD68(+) tissue macrophages and had no significant intravascular macrophage accumulation. To further delineate if the accumulated CD68(+) macrophages were in an activated state, we performed immunostaining for the p65 subunit of the transcription factor nuclear factor- κ B (NF- κ B), which translocates to the nucleus during inflammatory

Figure 6. Hepatopulmonary syndrome (HPS) plasma increases endothelial tube formation and pulmonary artery smooth muscle cells (PASMC) proliferation. (A) Representative and mean data from vascular endothelial growth factor (VEGF) dot blot assay showing increased plasma VEGF level in common bile duct ligation (CBDL) and sham animals ($n = 6$ per group). (B) Representative images and mean data from matrigel assay showing a VEGF-dependent increase in endothelial tube formation in response to CBDL versus sham plasma ($n = 3$ per group). (C) Representative and mean data from platelet-derived growth factor (PDGF) dot blot assay showing increased plasma PDGF level in CBDL and sham animals ($n = 9$ per group). (D) The *in vitro* BrdU assay shows increased PDGF-dependent PASMC proliferation in response to CBDL versus sham plasma ($n = 3$ per group). hpf = high-power field.

activation. Nearly every CD68(+) macrophage in the CBDL lungs was positive for nuclear NF- κ B (Figures 4A and 4B). Conversely, the percentage of CD68(+) macrophages with nuclear immunostaining of NF- κ B was very low in the sham lungs, suggesting that HPS not only leads to an accumulation of macrophages but that these macrophages also exhibit inflammatory activation.

To explore the mechanism for the accumulation of CD68(+) macrophages in the pulmonary vasculature, we measured the circulating plasma endotoxin levels and the lung monocyte chemoattractant protein (MCP-1) expression, known stimuli for increased transmigration of circulating myeloid cells into tissues (25). Circulating plasma endotoxin levels were significantly higher in the CBDL versus sham rats (Figure 4C). Additionally, mRNA expression of the chemokine MCP-1 was significantly higher in the CBDL lungs compared with the sham lungs (Figure 4D). There was no difference in the expression of vascular cell adhesion molecule in the CBDL versus sham lungs (data not shown).

Increased iNOS, VEGF, and PDGF Expression in the Macrophages in CBDL Lungs

To determine the signaling pathway through which accumulation of CD68(+) macrophages may contribute to the described histological and hemodynamic changes in HPS, we assessed the lung expression of iNOS, VEGF, and PDGF by immunostaining. Compared with sham, CBDL lungs had significantly increased iNOS, VEGF, and PDGF immunostaining. The number of CD68(+) macrophages that coexpressed iNOS, VEGF, or PDGF was significantly higher in the CBDL versus sham rats (Figures 5A–5C).

Activation of Extracellular Signal-regulated Kinase Signaling Pathway

The extracellular signal-regulated kinase (ERK) signaling pathway mediates VEGF- and PDGF-induced vascular remodeling

(26, 27). To evaluate the activation of ERK signaling in CBDL lungs, we measured expression of the active form of ERK-1, using an anti-phospho-ERK antibody. Phosphorylation of ERK1 was markedly increased in CBDL versus sham lungs (Figure 5D).

HPS Plasma Increases Endothelial Tube Formation and Pulmonary Artery Smooth Muscle Cells Proliferation

The plasma VEGF levels were significantly higher in CBDL versus sham rats (Figure 6A). Consistent with this, the plasma from the CBDL rats increased endothelial tube formation in a two-dimensional matrigel tube formation assay in a VEGF-dependent manner. The tyrosine kinase inhibitor imatinib, which blocks proangiogenic signaling, significantly reduced the endothelial tube formation by CBDL plasma compared with sham plasma (Figure 6B).

Plasma PDGF levels were also higher in CBDL animals than in sham animals (Figure 6C). Consistent with this, the CBDL plasma caused increased proliferation of pulmonary artery smooth muscle cells (PASMC) *in vitro* (Figure 6D). However, there was significant biological variability with only 60% of CBDL plasma specimens causing greater proliferation than sham plasma (see Figure E1 in the online supplement). The observed increase in PASMC proliferation was largely due to PDGF and was inhibited by imatinib and a PDGF-inactivating antibody (Upstate Biotechnology, Lake Placid, NY; Figure 6D and Figure E2). These data suggest that circulating growth factors contribute to the observed angiogenesis as well as the newly discovered vasculopathy.

Depletion of CD68(+) Macrophages Prevents and Reverses HPS

To evaluate the role of CD68(+) macrophages in the pathogenesis of HPS, we depleted intravascular macrophages using chronic *in vivo* intravenous GdCl₃ and liposomal clodronate

Figure 7. Depletion of macrophages prevents and reverses hepatopulmonary syndrome (HPS). (A–D) Representative and mean data showing that macrophage depletion strategies reduce accumulation of CD68 (red) and nuclear factor (NF)-κB (green) intravascular macrophages in common bile duct ligation (CBDL) and normalize capillary density (CD31, red), cell proliferation (proliferating cell nuclear antigen [PCNA], green), and percent medial thickness of small pulmonary arteries (PAs) (smooth muscle actin, green; $\times 10$; $n = 4$ in each group). For CD68, CD31, and PCNA staining, 10 high-power fields (hpf) per animal at $\times 40$ were studied. Scale bar = 20 μm . For % medial thickness, 120 blood vessels less than 150 μm were studied. Scale bar = 200 μm . (D) Comparison of alveolar–arterial (A–a) O_2 gradient and hemodynamics in the four study groups (sham, $n = 8$; CBDL, $n = 8$; CBDL + gadolinium chloride [GdCl]₃, $n = 6$; and CBDL+clodronate, $n = 3$).

therapy. Accumulation of CD68(+) macrophages in the pulmonary vasculature was significantly reduced by both GdCl₃ and clodronate when compared with untreated CBDL animals (Figure 7A). Activation of NF-κB was also significantly suppressed with GdCl₃ and clodronate treatment (Figure 7A). Depletion of CD68(+) macrophages with either GdCl₃ or clodronate reversed the capillary dilatation and microvascular angiogenesis seen in the HPS animals (Figure 7B). In addition, both GdCl₃ and clodronate therapy reduced cell proliferation, as measured by PCNA staining (Figure 7C). Depletion of macrophages with GdCl₃ also prevented the increased muscularization of the resistance PA (Figure 7D).

As expected, CBDL animals had significantly higher A–a O_2 gradients than sham animals. Macrophage depletion normalized oxygenation (Figure 7E). Likewise, the hyperdynamic circulatory state in HPS, characterized by high CO, was normalized by GdCl₃ and clodronate treatment (Figure 7E). Furthermore, there was a trend toward normalization of PVR in GdCl₃-treated animals, but there was no change in PVR with clodronate treatment (Figure 7E). There was no observed difference

in mean pulmonary artery pressure and left ventricular end-diastolic pressure between any of the groups (Table 1).

Moreover, ERK1 activation was prevented by GdCl₃ treatment (Figure 5D), providing evidence that depletion of macrophages inactivates VEGF- and PDGF-signaling pathways in HPS. The angiogenic potential of HPS serum tended to be lower in the plasma from the GdCl₃- and clodronate-treated CBDL animals (Figure E3). Taken together, these findings suggest that the CD68(+) macrophages are the predominant cause for all features of the hepatopulmonary syndrome, including hemodynamic changes, excessive angiogenesis, and obstructive vascular remodeling.

Inhibition of iNOS Alone Does Not Reverse HPS

To determine the effects of inhibiting the iNOS/VEGF- and PDGF-driven pathways separately, we treated CBDL rats with either L-NIL or imatinib. L-NIL treatment successfully inhibited iNOS as evidenced by a compensatory increase in expression of lung iNOS mRNA expression (Figure E4). Although L-NIL treatment normalized the A–a gradient, there was no difference in CO, mean pulmonary artery pressure, or pulmonary vascular

TABLE 2. CLINICAL CHARACTERISTICS OF PATIENTS AND CONTROL SUBJECTS

Patient	Age (yr)	Sex	Diagnosis	A-a O ₂ Gradient	Capillary Density (Fold Control)*
Cirrhosis					
1	46	Female	Autoimmune hepatitis	>15 mm Hg	0.4
2	59	Male	Hepatitis C and alcoholic cirrhosis	NA	N/A
3	65	Male	Alcoholic cirrhosis	>15 mm Hg	N/A
4	61	Female	Cryptogenic cirrhosis	NA	N/A
5	36	Female	Hepatitis	>15 mm Hg	N/A
6	27	Male	Primary sclerosing cholangitis	>15 mm Hg	1.2
7	54	Male	Hemochromatosis	>15 mm Hg	2.0
8	64	Male	Hepatitis C	>15 mm Hg	1.0
9	64	Female	Nonalcoholic steatohepatitis	>15 mm Hg	2.6
10	67	Male	Alcoholic cirrhosis	>15 mm Hg	2.0
Control					
1	49	Female	Metastatic cervical cancer	>15 mm Hg	1.0
2	60	Male	Congestive heart failure	N/A	0.9
3	65	Male	Intracranial hemorrhage	N/A	N/A
4	62	Female	Myocardial infarction	N/A	N/A
5	36	Male	HIV	>15 mm Hg	N/A
6	30	Male	Thrombotic thrombocytopenic purpura	N/A	1.9
7	54	Male	Colon cancer	N/A	0.5
8	61	Male	Dementia	N/A	1.2
9	60	Female	Aortic dissection	N/A	0.43
10	65	Male	Congestive heart failure	>15 mm Hg	N/A

Definition of abbreviation: N/A = not available.

* Calculated as the ratio of capillary density for individual subjects (patients with cirrhosis or control patients) relative to the calculated average capillary density of all six control patients.

resistance between the untreated CBDL and L-NIL-treated CBDL rats (Figure E5). All imatinib-treated CBDL rats died suddenly within 3 to 5 days after starting the treatment.

Increased CD68(+) Macrophages in Patients with Cirrhosis

To determine whether CD68(+) macrophages also accumulate in the lungs of patients with cirrhosis, we performed immunohistochemical staining in paraffin sections from 10 patients with cirrhosis and 10 age- and sex-matched control patients. Table 2 describes the clinical characteristics of these patients. It is unknown whether the patients with cirrhosis had a clinical diagnosis of HPS, but each of them died of cirrhosis and the majority had an increased A-a gradient. Conversely, control subjects had no known liver or lung disease. Histologically, the patients with cirrhosis also exhibited abnormal dilated capillaries and increased capillary density based on CD31 staining (Figure 8A). Quantitative analysis of CD68(+) macrophages revealed that patients with cirrhosis had significantly increased pulmonary intravascular macrophages (Figure 8B). In addition, several patients with cirrhosis had marked plugging of small PAs with macrophages, similar to that what we had observed in CBDL rats (Figure 8C).

DISCUSSION

The purpose of the current study was to examine the hypothesis that pulmonary intravascular macrophages are central to the pathogenesis of HPS. The data support the hypothesis and show that macrophage depletion is sufficient to prevent or regress virtually all features of HPS. There are six new findings in this study. First, in CBDL there was accumulation of CD68(+) macrophages in the pulmonary vasculature. These macrophages are recruited to the lung by the observed endotoxemia and increased lung MCP-1. The macrophages are activated, as evident from the nuclear translocation of the proinflammatory transcription factor, NF- κ B (Figure 4). Second, the activated CD68(+) macrophages, often plugging small arteries, shower the vasculature with a variety of growth factors and cytokines that account for the features of HPS, specifically iNOS, VEGF,

and PDGF (Figure 5). The activity of these growth factors is confirmed by the prominent activation of the ERK signaling pathway in the lung (Figure 5D). Third, bioassays demonstrate that macrophage-derived VEGF and PDGF production spills over into the plasma, which has angioproliferative effects in bioassays, increasing both endothelial tube formation and PASMC proliferation (Figure 6). A fourth, and entirely unanticipated, finding is that CBDL induces an occult proliferative pulmonary vasculopathy that is masked by vasodilation and neoangiogenesis (Figure 2). Evidence of this muscularization of small PAs and vascular obstruction was first suggested in isolated perfused lungs by the observation that the NOS inhibitor, L-NAME, unmasks an enhanced vasoconstrictor diathesis (Figure 3). Subsequently, histology showed the muscularization of many small PAs. Micro-CT angiography corroborated this finding, demonstrating dropout of small PAs. We speculate that this may explain the occasional shift from HPS to portopulmonary hypertension that can occur in HPS, either spontaneously or post liver transplantation (when vasodilatory NOS and angiogenic VEGF effects have abated and no longer counterbalance the muscularized, obstructed arterial phenotype) (Figure 2). Perhaps the most exciting and therapeutically relevant finding is the observation that depletion of macrophages, using either GdCl₃ or clodronate, prevents and regresses, respectively, most of the features of HPS (Figure 7). These findings in rats are relevant to humans with cirrhosis. Patients with cirrhosis also had increased CD68(+) pulmonary intravascular macrophage accumulation. Taken together, these findings provide strong evidence that intravascular accumulation of CD68(+) macrophages plays a central role in the etiology of HPS (Figure 9).

Pulmonary intravascular macrophages are normally present in many species, including sheep, cattle, and horses (28); however they are usually absent in rats and humans, except in certain pathological conditions (29). Accumulation of CD68(+) macrophages has been reported to occur in the pulmonary vasculature in CBDL rats (7). A proposed mechanism for accumulation of these macrophages in HPS is the observed translocation of gram-negative bacteria from the gut and in-

Figure 8. (A) Increased capillary density based on CD31 staining in patients who died with cirrhosis versus age- and sex-matched control patients ($\times 20$). (B) Increased CD68(+) macrophages within the pulmonary vasculature in patients who died with cirrhosis versus age- and sex-matched control patients ($\times 40$). (C) Representative images showing plugging of small pulmonary arteries with pulmonary intravascular macrophages in a patient with cirrhosis versus age- and sex-matched control patient ($\times 40$). PIMS = pulmonary intravascular macrophages.

creased circulating tumor necrosis factor- α (TNF- α) that occur in HPS (30–32). Consistent with this, reduction of bacterial translocation using norfloxacin decreased pulmonary intravascular macrophage accumulation and reduced HPS (31). Moreover, pentoxifylline, a TNF- α antagonist, was effective in preventing and regressing HPS. Consistent with these reports, we noted increased plasma endotoxin levels (Figure 4C) and activation of NF- κ B within the pulmonary vascular macrophages (Figure 4A). Endotoxin interacts with Toll-like receptor 4 in the macrophages and activates the NF- κ B signaling pathway, ultimately leading to increased secretion of various cytokines and growth factors (33). Our study adds further to the literature by demonstrating increased expression of MCP-1 in the CBDL lungs. MCP-1 is a potent chemokine that attracts macrophages to the site of inflammation. Polymorphisms in the MCP-1 gene are known to predispose patients with cirrhosis to develop HPS (34). Together, these findings suggest that MCP-1 may play a role in recruiting the macrophages to the pulmonary vasculature in experimental HPS.

The role of CD68(+) pulmonary intravascular macrophages in the pathogenesis of HPS had not been directly assessed; however, previous reports indirectly implicated a role for pulmonary intravascular macrophage accumulation in the pathogenesis of HPS. Nunes and colleagues demonstrated that

increased lung NO in CBDL reflected increased iNOS expression in the macrophages. Administration of L-NAME, a non-specific NOS blocker, prevented HPS (8). Zhang and colleagues reported that inhibition of heme oxygenase reduced an excessive carbon monoxide production by the macrophages and partially reversed HPS (35). This group also showed that increased pulmonary angiogenesis in experimental HPS reflects activation of VEGF-signaling pathways in intravascular macrophages (9). These important studies targeted individual cytokines and growth factors; however, none of these prior studies directly targeted the pulmonary intravascular macrophages themselves, nor did they prove that the mediator originated solely from the macrophage. The current study builds on these previous reports by directly targeting the macrophages. Our results show that activated macrophages express vasodilating (iNOS), angiogenic (VEGF), and proliferative (PDGF) factors, and depletion of these activated macrophages by two different and complementary techniques prevent or reverse most of the hemodynamic and histological manifestation of experimental HPS, including A—a oxygen gradient, CO, capillary density, and PCNA(+) cells in the lungs. Macrophage depletion also inhibited the activation of ERK, which is central to the signaling of angiogenesis. On binding of their ligands, VEGF and PDGF receptors phosphorylate and activate ERK, thereby stimulating vascular cell pro-

Figure 9. Schematic illustrating the proposed central role of macrophages in the pathogenesis of hepatopulmonary syndrome. A-a = alveolar-arterial; CBDL = common bile duct ligation; iNOS = inducible nitric oxide synthase; NO = nitric oxide; PASMC = pulmonary artery smooth muscle cells; PDGF = platelet-derived growth factor; PVR = pulmonary vascular resistance; VEGF = vascular endothelial growth factor.

liferation. Depletion of macrophages reduced activation of ERK in CBDL lungs. Taken together, these observations persuasively prove a central role for pulmonary intravascular macrophages in the pathogenesis of HPS (Figure 9).

Currently, liver transplantation is the only available treatment for patients with HPS, and no effective medical therapies exist. The presence of HPS predicts increased mortality in patients with cirrhosis who undergo liver transplantation (36). The clinical implication of our work is that interventions that directly target the macrophages may have benefit in HPS. This is a complementary approach to the inhibition of individual pathways that are activated in the macrophages (i.e., NF- κ B, TNF- α , NOS, and heme oxygenase), as suggested by others (9, 30–32). However, targeting the macrophages, a major cellular source of multiple cytokines and growth factors, instead of targeting a single cytokine, may be a more comprehensive and beneficial approach. This is supported by the observation that inhibition of iNOS only normalizes hypoxemia but has no effect on the pulmonary hemodynamics (Figure E5). Targeting VEGF and PDGF separately using tyrosine kinase inhibitors at a therapeutic dose may be not possible in the presence of underlying liver disease. All CBDL rats treated with imatinib died suddenly within 3 to 5 days. We believe that this is probably due to an off-target effect of imatinib causing worsening hepatic failure (37–39). Future studies could also assess the therapeutic efficacy of directly inhibiting ERK activation, thereby attacking the common downstream signaling pathway involved in vascular remodeling in HPS.

The limited human data in this paper suggest that patients with cirrhosis, similar to the CBDL animals, also have increased pulmonary intravascular macrophage accumulation. Some of them had total plugging of small PAs with these macrophages, similar to what we observed in the CBDL rats. This observation is consistent with prior studies that have suggested accumulation of macrophages in the pulmonary vasculature in patients with liver disease (40, 41). During routine imaging of the liver with Tc-sulfur colloid, patients with liver disease showed increased uptake of colloid in the lungs due to enhanced phagocytic activity, which was attributed to the presence of

reticuloendothelial cells. None of the patients with cirrhosis in our study had a known clinical diagnosis of HPS; however, the majority of these patients had increased A-a oxygen gradient, abnormal capillary dilatation histologically, and increased capillary density, suggesting intrapulmonary shunting. Future studies are needed to confirm our finding specifically in patients with an established antemortem, clinical diagnosis of HPS.

The existence of an occult proliferative vasculopathy has not been described before in this experimental model of HPS. The proliferative vasculopathy we observed in this model is neither diffuse nor severe as that noted in monocrotaline or Sugen 5416/hypoxia-induced pulmonary arterial hypertension. Moreover, any potential hemodynamic effect is masked by the predominate effect of vasodilator excess and arteriovenous malformations, which lower PVR. However, we were able to confirm this arterial vasculopathy using quantitative micro-CT pulmonary angiography, which is a very sensitive way to detect vascular dropout. This technique has not previously been applied to the study of HPS. Moreover, we noted that once the vasodilator NOS was inhibited in isolated perfused lung experiments, the pulmonary vasculature of CBDL rats had greater hypoxic pulmonary vasoconstriction. This is also consistent with the presence of a subgroup of muscularized arteries that can severely constrict once the vasodilator forces are withdrawn. Carter and colleagues and Imamura and colleagues studied the effect of chronic administration of L-NAME in drinking water on hypoxic pressure response in CBDL animals (42, 43). In contrast to our study, in their observation, compared with sham animals, CBDL animals had decreased hypoxic pulmonary vasoconstriction, and this normalized with chronic L-NAME therapy. We suspect that the difference between our observation and the prior studies is due to the acute versus chronic administration of L-NAME. Unlike these prior studies, we investigated the effect of acute L-NAME administration in isolated perfused lung. Nonetheless, the latent enhancement of vasoconstrictor capability of the HPS lung is consistent with the observed obstructive vasculopathy (which we document using histology, immunofluorescence for PCNA, and micro-CT angiography).

The presence of the occult proliferative vasculopathy that we describe here may explain the sporadic transition from HPS to portopulmonary hypertension that can occur in some patients with HPS after liver transplantation. In contrast to HPS, patients with portopulmonary hypertension have increased PVR, low CO, and right-sided heart failure (44). The histopathological abnormalities of the intrapulmonary vessels in portopulmonary hypertension include intimal thickening, medial smooth muscle hypertrophy, and adventitial fibrosis, with or without *in situ* thrombosis (44). Despite the extreme difference in the clinical and pathological characteristics, HPS has been reported to coexist or transition to portopulmonary hypertension, especially after orthotopic liver transplantation (4). The basis for this transition is largely unexplained. The occult proliferative pulmonary vasculopathy noted in this study may explain this pulmonary hypertensive diathesis. The enhanced hypoxic response after acute administration of L-NAME in the isolated perfused CBDL lungs suggests that the phenotypic manifestation of this obstructive, proliferative vasculopathy is masked by lung NO overproduction. The mechanism that unmasks it after liver transplantation is unclear and needs further evaluation in future studies, although we suspect normalization of liver function may interrupt the pathway by preventing endotoxemia, a major driver of iNOS up-regulation.

It appears that the muscularization of small PAs relates, in part, to increased macrophage-derived PDGF. PDGF is a potent mitogen that induces PASMC proliferation, and it has been implicated in the pathogenesis of experimental and human PAH (20, 45). In the current study, the increased PDGF expression in the pulmonary intravascular macrophages was associated with increased plasma PDGF level. Moreover, the CBDL plasma caused increased proliferation of PASMC *in vitro* in a PDGF-dependent manner. Depletion of macrophages, the source of PDGF, with GdCl₃ prevented the increase in percent medial thickness of the resistance pulmonary arteries.

Limitations

We measured treadmill-walking time in HPS as a measure of functional capacity. Although this was depressed (sham vs. CBDL: 915 ± 6 and 574 ± 49 s, *P* < 0.05), therapy did not increase walking time (CBDL + GdCl₃: 552 ± 65 s) or survival. We interpret this as reflecting the overriding morbidity caused by the ongoing and untreated cirrhosis. Although macrophage depletion in a rodent model is feasible and effective, there are practical considerations for translation of this strategy to humans. Clodronate appears to be mildly hepatotoxic in some CBDL rats (Table 1), although this did not lessen its therapeutic efficacy. Interestingly, prior studies in rodents show clodronate is protective from liver injury (whether induced by pancreatitis or acetaminophen) (46, 47). However, there are human data indicating clodronate can increase AST levels without clinically significant liver injury (48). Clinical pharmacology studies would be needed to evaluate the potential toxicity of macrophage-depletion strategies before use in humans with cirrhosis. Nonetheless, the observed increase in liver function tests does prove the beneficial effects of clodronate are not due to prevention of liver disease; rather, they reflect macrophage depletion.

Conclusions

In conclusion, we have demonstrated that the macrophages play a critical role in the pathogenesis of experimental HPS by secreting vasodilating (iNOS), angiogenic (VEGF), and proliferating (PDGF) growth factors. We further describe a novel occult proliferative vasculopathy affecting the resistance PAs in CBDL rats, which may explain the transition of some patients with HPS to portopulmonary hypertension after liver transplantation.

Author Disclosure: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Acknowledgment: The authors thank Dr. Aliya Husain in the Department of Pathology at the University of Chicago Medical Center for providing us with the human slides and Dr. Kenneth E. Weir for his expert review of the manuscript.

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