Published in final edited form as:

Dig Dis Sci. 2012 February ; 57(2): 516–523. doi:10.1007/s10620-011-1905-6.

Elevated Levels of Endothelin-1 in Hepatic Venous Blood Are Associated with Intrapulmonary Vasodilatation in Humans

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Abstract

Background—Hepatopulmonary syndrome is a pulmonary vascular complication of cirrhosis in which intrapulmonary vasodilatation (IPV) results in hypoxemia. Endothelin-1 (ET-1), produced by proliferating cholangiocytes, has been identified as a mediator of IPV in an animal model of HPS, but the pathophysiology of IPV in humans has not been defined.

Aim—The purpose of this study was to assess whether cirrhosis with IPV, which often leads to HPS, is associated with increased hepatic venous ET-1 blood levels.

Methods—We performed a prospective cohort pilot study of 40 patients with liver disease undergoing transjugular liver biopsy from November 1, 2008 to September 1, 2009. Patients were categorized according to absence (–) or presence (+) of IPV as determined by bubble-contrasted echocardiography. Hepatic venous blood was assayed for ET-1 by ELISA. The percent volume of cholangiocytes in the liver biopsy specimen was determined by morphometric analysis, as a measure of bile duct proliferation.

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Conflict of interest None.

Results—Nine subjects were excluded, due to absence of cirrhosis (6) and patent foramen ovale (3). Of the remaining 31 subjects, IPV was present in 18 (58%). Median hepatic venous ET-1 was higher with IPV+ than IPV− at levels of 9.1 pg/mL (range 7.5–11.7) versus 2.1 pg/mL (1.3–5.6), respectively ($P = 0.004$). ET-1 levels correlated positively with cholangiocyte percent volume ($r = 0.72$, $P < 0.001$) but not with measures of liver dys-function (bilirubin, INR, MELD score, or hepatic venous pressure gradient).

Conclusion—In human cirrhosis, increased hepatic venous ET-1 is associated with IPV and increased hepatic cholangiocyte volume.

Keywords

Cirrhosis; Hepatopulmonary syndrome; Endothelin-1; Bile duct proliferation

Background

Hepatopulmonary syndrome (HPS) is a syndrome of hypoxemia due to intrapulmonary vasodilatation (IPV) in patients with advanced liver disease. Intrapulmonary vasodilatation results in ventilation-perfusion (V/Q) mismatch and is accurately diagnosed by bubble-contrasted transthoracic echocardiography (BC-TTE) [1]. In referrals for liver transplantation, IPV is common, reported in over 40% of patients [1]; while HPS is less prevalent (15–20%) [1]. Hypoxemia in HPS is progressive, resulting in (1) poor overall survival [2–6], (2) exclusion from listing for liver transplantation (LT) [2–5], and (3) increased postoperative complications after LT [2–5, 7]. There is also no effective therapy for HPS besides LT.

Understanding of the pathogenesis of IPV and HPS is limited, and derives almost entirely from the rat common bile duct ligation (CBDL) model that recreates the pathophysiological features of human HPS. In the CBDL model, proliferating cholangiocytes produce and secrete endothelin-1 (ET-1) that binds to an upregulated ET_B receptor in the pulmonary vasculature causing IPV by increasing production of the vasodilator nitric oxide [8–13]. Other animal models of portal hypertension that do not result in bile duct proliferation (BDP) and subsequent biliary cirrhosis, do not cause IPV and HPS. However, IPV and HPS in humans are not exclusive to cholestatic liver diseases, but occur in cirrhosis of any cause. Also, unlike in the animal model, bile duct proliferation in humans is not confined to cholestatic liver diseases [14, 15]. Thus, in humans with cirrhosis (as in the CBDL rat) it is plausible that cholangiocyte-derived ET-1 is the primary mediator of IPV and HPS. Finally, since many patients with significant HPS-induced hypoxemia do not otherwise require LT for liver disease *per se*, treatments are needed that could allow LT to be postponed until dictated by the severity of liver disease. Although not all cases of IPV progress to HPS [16], IPV nonetheless, is a necessary, if not sufficient, condition for HPS to occur [17]. Other factors yet to be determined may operate for IPV to progress to HPS.

Establishing an association between ET-1 and IPV in humans could provide clinicians with a mechanism of disease and a predictive marker for the development of HPS and, thereby, could suggest a therapeutic target based on pathogenesis. To this end, we performed a pilot

study to determine if IPV in patients with cirrhosis is associated with elevated ET-1 levels in hepatic venous blood and with cholangiocyte proliferation in the liver.

Methods

The study was a prospective, cross-sectional evaluation of 40 patients undergoing transjugular (TJ) liver biopsy during evaluation for suspected cirrhosis, at the Medical University of South Carolina from November 1, 2008 to September 1, 2009.

Hepatic Venous ET-1

At the time of the TJ liver biopsy, HV blood was sampled, and the severity of portal hypertension was determined by measuring the hepatic venous pressure gradient (HVPg), as described previously [18, 19]. Plasma ET-1 in HV blood was quantified with an ELISA (R&D Systems). ET-1 data were excluded if the blood was hemolyzed (4 subjects), since hemolysis interferes with the assay.

IPV Detection

All subjects had a BC-TTE within 2 months of the TJ liver biopsy. IPV was defined as the late appearance of micro-bubbles in the left heart (≥ 3 cardiac cycles after injection), while the earlier appearance of the microbubbles (<3 cardiac cycles) was defined as intracardiac shunting. Both the right atrial and ventricular systolic pressures were assessed to evaluate for portopulmonary hypertension. Hypoxemia was assessed in all patients with pulse oxymetry while breathing ambient air. To avoid unnecessary risk of arterial puncture in patients with normal oxygenation in whom HPS is unlikely [20], arterial blood gases were only measured in those subjects with an oxygen saturation of <97% [20]. The alveolar-arterial oxygen tension difference (PA-aO₂) was calculated according to the formula:

$$PA-aO_2 = [(FiO_2 * (760 - 47)) - (PaCO_2 / 0.8)] - PaO_2.$$

Subject Stratification

Subjects with a positive BC-TTE were classified as having IPV; those with a PA-aO₂ >15 mmHg (>20 mmHg if age >64 years) were diagnosed with HPS, according to accepted criteria [17]. Subjects who had a positive BC-TTE (IPV+) but did not meet criteria for HPS because of normal age-adjusted PA-aO₂ or ambient air O₂ saturation ≥ 97% were classified as having “subclinical IPV.”

Additional data collected were: etiology of liver disease, laboratory evidence of cholestasis (i.e., elevated serum total bilirubin and alkaline phosphatase levels), severity of liver disease as judged by the Child-Turcotte-Pugh (CTP) and Model for End-Stage Liver Disease (MELD) scores, and complications of portal hypertension (namely, ascites, varices, and encephalopathy).

Liver Immunohistochemistry

Liver tissue specimens were sectioned at 5 µm and stained with haematoxylin–eosin and Masson's Trichrome; the presence of cirrhosis was determined by a pathologist who was masked to the rest of the patient data. For immunohistochemistry, sections were incubated with the primary antibody anti-cytokeratin 7 (CK7, Dako, Carpinteria, CA), a marker for biliary epithelium, at a dilution of 1:1,200, washed, incubated with goat anti-mouse IgG, developed with diaminobenzidine (DAB), and counterstained with haematoxylin. The specimens were examined with a Zeiss Axiovert 200 M inverted microscope using a 10× objective lens, and the images of the entire specimen were captured with a Zeiss AxioCam camera and Axiovision software.

Morphometric Analysis

Morphometric analysis of liver sections to assess for BDP was done using Image J software (NIH). We quantified the cholangiocyte percent volume from each specimen as a measure of BDP [21]. Images of all the liver sections for each subject were combined into a stack, and the anti-CK7 immunostained cells were visually isolated from the haematoxylin counterstained ones using the Color Deconvolution plugin [22] (see Fig. 1). A threshold was applied to each image that isolates the DAB stained profiles by selecting objects with greater pixel intensities than the background. The anti-CK7 stained cells from each image in the stack were quantified using the Analyze Particles plugin that restricts the measurement to the threshold tissue, and the values from each image of the stack were added to give a sum of anti-CK7 pixels for each subject ($\sum_{\text{CK7 Pixels}}$). The total amount of liver tissue present in each subject's total biopsy specimen was determined by quantifying the number of pixels of each specimen in the stack and summing the resulting values ($\sum_{\text{Total Pixels}}$). A measure of cholangiocyte volume was then determined by calculating the percent volume:

$$\sum_{\text{CK7 Pixels}} / \sum_{\text{Total Pixels}} \times 100\%$$

The unit of measure is volume rather than area since the liver tissue sections are of uniform thickness.

Data Analysis

Data were summarized as medians and interquartile ranges (IQR) or n (%) as appropriate. We sought candidate predictors of IPV, using univariate analyses with Fisher's exact test for dichotomous data and Wilcoxon rank-sum tests for continuous data. Severity of portal hypertension in mm Hg was assessed both as a continuous measure (HVPg) and as a dichotomous variable; a cutoff of 10 mm Hg was used to define clinically significant portal hypertension (CSPH) as has been previously determined [23–26]. Statistical significance was defined as a 2-tailed P value less than 0.05. Data analyses were performed using SAS (v 9.2 NC). The protocol for the study was approved by the Medical University of South Carolina Institutional Review Board.

Results

Study Population Demographics

Forty patients who had a TJ liver biopsy were enrolled into the study; nine were excluded (6 had advanced fibrosis but not cirrhosis and 3 had a patent foramen ovale), leaving 31 subjects. The median age was 55.0 (IQR 50.0–59.0) years with 55% men and 45% women; 90% were Caucasian. The median MELD score was 11.0 (6.5–16.5), and the majority (71%) had at least one complication of portal hypertension (Table 1). All subjects had portal hypertension (i.e. HVPG > 6 mmHg) with a median HVPG of 14.0 mmHg (10.0–18.0) and most had CSPH (26/31, 84%). None had echocardiographic evidence of portopulmonary hypertension by estimation of right atrial and ventricular systolic pressures. IPV was present in 18/31 (58%) subjects, of whom 11/31 (35%) had subclinical IPV while 7/31 (23%) had HPS. The subjects with HPS had a median PaO₂ of 78 mmHg (72–88 mmHg); three would be classified as mild and four as moderate, according to the current staging system for HPS [17].

Predictors of IPV

There was no difference in age, gender, race, blood pressure heart rate, or etiology of liver disease between IPV+ and IPV– subjects (Table 2). IPV+ subjects had worse liver dysfunction than those without IPV as determined by serum bilirubin, INR, and MELD score. Esophageal varices were more common in IPV– subjects than in the IPV+ group, but otherwise there was no difference in the prevalence of complications of portal hypertension (Table 2). Although CSPH was nearly universal in IPV+ subjects (17/18, 94%), there was not a statistically significant difference in portal hypertension between the groups when assessed either as a continuous (HVPG) or dichotomous (CSPH) variable (Table 2). However, ET-1 levels were 4.5-fold higher in the IPV+ subjects compared to IPV– ones (median 9.1, range 7.5–11.7 pg/mL vs. 2.11, 1.3–5.6 pg/mL, respectively, $P = 0.004$, Table 2 and Fig. 2a). Aside from IPV status, ET-1 weakly correlated inversely with the systolic blood pressure ($\rho = -0.41$, $P = 0.03$) but not with measures of cardiac function (right ventricular systolic pressure or left ventricular ejection fraction), MELD or CTP scores, degree of portal hypertension or the complications of portal hypertension (data not shown).

Next, we analyzed the data, taking into account whether or not those subjects with IPV also met criteria for HPS ($n = 7$). Subjects with HPS, compared to those without, had higher ET-1 levels (11.8, 10.4–14.6 pg/mL vs. 7.3, 2.1–9.2 pg/mL, $P = 0.04$). There was also a trend to worse liver dysfunction in the HPS group as determined by the serum bilirubin (1.45, 0.9–2.6 vs. 3.15, 2.2–5.5, $P = 0.57$), and MELD score (10.0, 6.0–13.0 vs. 16.5, 11.0–25.0, $P = 0.07$), but neither differences were statistically significant. There was also no difference in HVPG between the two IPV(+) cohorts, nor were there differences in any other clinical or laboratory variable tested between the subclinical IPV and HPS groups (data not shown).

Cholangiocyte Volume and ET-1 Levels

Of the 31 patients in the analysis with cirrhosis, 21 had sufficient liver tissue to permit immunohistochemical staining. Biliary profiles in the biopsy specimens were visualized in

the portal tracts and along fibrous bands; the cause of the liver disease did not influence BDP presence. There was also no difference in cholangiocyte percent volume among the causes of liver disease or according to its severity, and only one subject in the study had a primary biliary cause of cirrhosis. Cholangiocyte percent volume in IPV+ subjects (1.6, range 1.1–6.7%) was twice as big as in those without IPV (0.8, 0.5–1.3%, Fig. 2b). The cholangiocyte percent volume correlated strongly with HV ET-1 levels ($\rho = 0.72$, $P < 0.001$, Fig. 3), but not with degree of portal hypertension (HVPG) or severity of liver disease.

Discussion

Our prospective pilot study of patients with cirrhosis is the first to apply the findings of an animal model of HPS to human disease. The cardinal findings of the study are that the presence of IPV correlates with HV ET-1 and in turn both correlate with BDP. Whereas currently these findings are correlative, it is possible to hypothesize a mechanism.

Contrary to the animal model, BDP occurs in all forms of cirrhosis and is not restricted to cholestatic liver disease (Fig. 4), which is consistent with prior research findings [14, 15]. ET-1 levels did not differ by the cause of liver disease in our subjects, but rather by the degree of BDP. ET-1 also was not affected by the severity of liver dysfunction, portal hypertension, or complications of liver disease. The association between ET-1 and BDP suggests that cholangiocytes are potentially the source of increased circulating ET-1, as seen in the animal model. Other studies have documented elevated levels of ET-1 in plasma [27] and liver tissue [28, 29] of cirrhotic patients compared to normal controls and have suggested the cirrhotic liver to be a source [29]. However, direct evidence of cholangiocyte production of ET-1 that is seen in the CBDL model, has yet to be shown in humans.

It was not possible in our study to determine if HPS is simply an exaggeration of IPV (the main pathophysiological process under investigation here), or whether additional factors are present, since there were too few HPS patient to permit statistical comparison with subclinical IPV. Nonetheless, all patients with HPS had IPV, and levels of HV ET-1 were higher in HPS patients when compared to those without HPS, suggesting a role for ET-1 in the pathogenesis of HPS. In the CBDL model, the cause of HPS is multifactorial and includes increased circulating tumor necrosis factor- α [30, 31], carbon monoxide production by monocytes [32], and angiogenesis [33, 34], which should be sought now in human disease. Additionally, increased pulmonary uptake of ET-1 by the upregulated ET_B receptor is an important feature of HPS in the CBDL model that was not investigated in the present study. It is possible that elevated levels of ET-1 that reach the pulmonary vasculature are but one factor causing IPV and HPS, and that increased uptake of ET-1 in the lungs by upregulated ET_B receptors is additionally important. Finally, the relationship between severity of portal hyper-tension and ET-1 in the pathogenesis of IPV also requires further investigation. In our study, HVPG was higher in IPV+ compared to IPV– subjects, but the difference was not statistically significant, probably due to the small sample size and the high prevalence of CSPH.

In summary, HV ET-1 levels correlate positively with the presence of IPV and HPS in patients with cirrhosis. Bile duct proliferation that can occur in all forms of liver disease

correlates with HV ET-1 levels. Our results are the first to validate the rat CBDL model of HPS for human cirrhosis, and they support the concept that increased secretion by the liver of ET-1 (as a result of BDP) is a possible mediator of IPV and might provide a target for therapy.

Acknowledgments

This project was funded by the South Carolina Clinical & Translational Research Institute, Medical University of South Carolina's CTSA, NIH/NCRR Grant Number UL1RR029882 and the American College of Gastroenterology Junior Faculty Development Award. Imaging facilities were supported, in part, by Cancer Center Support Grant P30 CA138313 to the Hollings Cancer Center.

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Abbreviations

BDP	Bile duct proliferation
BC-TTE	Bubble-contrasted transthoracic echocardiography
CTP	Child-Turcotte-Pugh
CSPH	Clinically significant portal hypertension
CBDL	Common bile duct ligation
CK7	Cytokeratin 7
DAB	Diaminobenzidine
ET-1	Endothelin-1
HV	Hepatic venous
HVPG	Hepatic venous pressure gradient
HPS	Hepatopulmonary syndrome
IPV	Intrapulmonary vasodilatation
LT	Liver transplantation
MELD	Model of end stage liver disease

Fig. 1. Color deconvolution of liver biopsy specimens to show threshold CK7 cells visually isolated from the haematoxylin counterstained cells. **a** CK7 immunostained liver specimen at $\times 10$ magnification. **b** CK7 stained biliary profiles isolated from haematoxylin stained ones after color deconvolution. **c** Haematoxylin stained cells. **d** Threshold image of CK7 profiles

Fig. 2.
a Endothelin-1 levels ($P = 0.004$). **b** Cholangiocyte percent volume ($P = 0.03$), stratified according to IPV status. Data are represented as medians (*horizontal central lines*), IQR (*boxes*), minimum/maximum values (*whiskers*), and outliers (*X*)

Fig. 3. Relationship between cholangiocyte percent volume in liver biopsy and hepatic venous ET-1 ($r = 0.72, P < 0.001$)

Fig. 4.

Degrees of bile duct proliferation on cytokeratin 7 stained liver tissue. CK7 stained liver tissue at $\times 10$ (**a, c, e**) and $\times 20$ (**b, d, f**). Case 1 was a 68-year-old male with non-alcoholic steatohepatitis; minimal BDP seen in the portal tract; HV ET-1 2.3 ng/mL; IPV– (**a, b**). Case 2 was a 52-year-old female with primary biliary cirrhosis; moderate BDP with proliferating cholangiocytes extending into the liver lobule; HV ET-1 7.4 ng/mL; IPV+ (**c, d**). Case 3 was a 51-year-old female with hepatitis C cirrhosis; massive BDP with extensive lobular cholangiocyte proliferation; HV ET-1 14.6 ng/mL; IPV+ (**e, f**)

Table 1
Study population demographics

Characteristic	Study population, <i>n</i> = 31
Age	55.0 (50.0–59.0)
Gender	
Male	17 (55%)
Female	14 (45%)
Race	
Caucasian	28 (90%)
African American	2
Asian	1
Etiology of liver disease	
NASH	11
Hepatitis C	9
Alcohol	6
Other	5
MELD score	11.0 (6.5–16.5)
Esophageal varices	13 (42%)
Encephalopathy	7 (22%)
Ascites	18 (58%)

Data expressed as median (IQR) or *n* (%)

NASH non-alcoholic steatohepatitis, *MELD* model of end stage liver disease

Table 2
Univariate analyses of factors associated with IPV status

Characteristic	IPV- <i>n</i> = 13	IPV+ <i>n</i> = 18	<i>P</i> value
Age (years)	57.0 (range, 51.0–62.0)	54.0 (50.0–56.0)	0.42
Gender			0.28
Male	7	10	
Female	6	8	
Race			0.31
Caucasian	12	16	
Other	1	2	
Etiology of liver disease			
NASH	6	5	
Hepatitis C	3	6	
Alcohol	3	3	
Other	1	4	
Systolic blood pressure (mmHg)	125.0 (109.0–150.0)	118.0 (103.0–141.5)	0.52
White blood cell count (K/CUMM)	6.2 (3.6–7.2)	5.2 (4.2–6.7)	0.80
Platelet count (K/CUMM)	114.0 (71.0–125.0)	92.0 (71.0–112.0)	0.38
AST (IU/L)	56.5 (34.0–104.5)	64.5 (46.0–83.0)	0.60
ALT (IU/L)	39.0 (25.5–65.0)	37.0 (27.5–52.0)	0.35
Alkaline phosphatase (IU/L)	118.0 (112.0–137.5)	119.5 (84.5–166.5)	0.98
Bilirubin (mg/dL)	1.25 (0.8–1.6)	2.7 (1.5–4.5)	0.01
INR	1.2 (1.1–1.3)	1.4 (1.2–1.8)	0.03
Creatinine (mg/dL)	1.0 (0.8–1.3)	0.9 (0.7–1.3)	0.51
MELD	8.0 (5.0–11.5)	13.0 (10.0–18.5)	0.05
Child-Turcotte-Pugh Score	7.0 (6.5–8.0)	8.0 (7.0–11.5)	0.18
HVPG (mmHg)	12.0 (7.0–18.0)	15.5 (11.0–18.0)	0.35
Hepatic venous Endothelin-1 (pg/mL)	2.1 (1.3–5.6)	9.1 (7.5–11.7)	0.004
Cholangiocyte percent volume	0.008 (0.005–0.013)	0.016 (0.011–0.067)	0.03
CSPH			0.14
Yes	9	17	
No	4	1	
Esophageal varices			0.03
Yes	9	5	
No	4	13	
Encephalopathy			0.25
Yes	2	5	
No	11	12	
Ascites			0.28
Yes	7	10	
No	6	8	

Dichotomous data expressed as *n* and analyzed with Fishers exact test; Continuous data expressed as median (IQR) and analyzed with Wilcoxon rank sum

CSPH clinically significant portal hypertension, *HVPG* hepatic venous pressure gradient, *MELD* model of end stage liver disease, *NASH* nonalcoholic steatohepatitis

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