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Plasma Osteopontin in Acute Liver Failure

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Abstract

Background—Osteopontin (OPN) is a novel phosphoglycoprotein expressed in Kupffer cells that plays a pivotal role in activating natural killer cells, neutrophils and macrophages. Measuring plasma OPN levels in patients with acute liver failure (ALF) might provide insights into OPN function in the setting of massive hepatocyte injury.

Methods—OPN levels were measured using a Quantikine® ELISA assay on plasma from 105 consecutive ALF patients enrolled by the US Acute Liver Failure Study Group, as well as controls including 40 with rheumatoid arthritis (RA) and 35 healthy subjects both before, and 1 and 3 days after undergoing spine fusion (SF) surgery as a model for acute inflammation.

Results—Median plasma OPN levels across all etiologies of ALF patients were elevated 10- to 30-fold: overall median 1055 ng/mL; range: 33 – 19127), when compared to healthy controls (median in pre-SF patients: 41 ng/mL; range 2.6 – 86.4). RA and SF post op patients had elevated OPN levels (37 ng/mL and 198 ng/mL respectively), well below those of the ALF patients. Median OPN levels were highest in acetaminophen (3603 ng/mL) and ischemia-related ALF (4102 ng/mL) as opposed to viral hepatitis (706 ng/mL), drug-induced liver injury (353 ng/mL) or autoimmune hepatitis (436 ng/mL), correlating with the degree of hepatocellular damage, as reflected by aminotransferase values (R value: 0.47 for AST, $p < 0.001$).

Conclusions—OPN levels appeared to correlate with degree of liver necrosis in ALF. Very high levels were associated with hyperacute injury and good outcomes. Whether OPN exerts a protective effect in limiting disease progression in this setting remains uncertain.

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1. Introduction

Acute liver failure (ALF) results from severe hepatic injury of any kind, features coagulopathy (international normalized ratio: INR > 1.5) and varying degrees of hepatic encephalopathy (HE) [1]. On a pathophysiological level there is sterile inflammation, progression to multi-organ failure and functional immunoparesis. The progression of ALF seems to depend on the balance of pro- and anti-inflammatory responses in the liver, paralleling many features of sepsis and the systemic inflammatory response syndrome (SIRS) [2].

Many inflammatory and immune mediators are currently under investigation, including osteopontin (OPN) which was originally identified in bone [3]. OPN is composed of approximately 300 amino acids, with two distinct isoforms: a secreted and an intracellular form. At its center, OPN contains a classical binding motif, an arginine-glycine-aspartic acid (RGD) domain that is recognized by cell surface integrins. Near the RGD domain, OPN may be cleaved by proteases (thrombin and plasmin) [4]. Through RGD binding to cell surface receptors on target cells, secreted OPN can modulate cell adhesion and serve as a chemoattractant to other inflammatory mediators. OPN also acts as an autocrine and paracrine factor, playing an important role in induction and secretion of cytokines, macrophage and neutrophil migration, and subsequent activation [5, 6]. Apart from its role in bone remodeling and regulation of osteoclast activity, OPN appears to play a key role in triggering inflammation in autoimmune diseases (rheumatoid arthritis, multiple sclerosis), other chronic inflammatory states, acute inflammatory conditions (trauma) and malignancies [4, 7]. Additionally, there has been interest in use of OPN as a tumor marker in patients with hepatocellular carcinoma [8, 9]. While its exact functions remain unclear, intracellular OPN affects cell motility, cytoskeletal rearrangement, mitosis, signal transduction pathways downstream of innate immune receptors [10].

Elevations in serum and plasma OPN levels have been found in small studies of ALF patients [11, 12]. The correlation of OPN level with eventual outcomes in ALF patients has not been well defined. Our present goals were to compare OPN levels in a large series of ALF patients, examining how etiology, disease severity and prognosis are related to OPN levels while comparing OPN levels in ALF to those observed in healthy controls, in patients with RA (chronic inflammation) with varying levels of disease activity and in spinal fusion patients pre- and post-op as an example of an acute injury/trauma model.

2. Materials and Methods

2.1 Patients and specimens

The US Acute Liver Failure Study Group (ALFSG) was established in 1998 as a consortium of liver centers interested in better defining the causes and outcomes of acute liver failure. To date, more than 2,500 subjects have been enrolled prospectively at 23 tertiary centers within the US, all of which have liver transplantation programs. All enrolled subjects met standard criteria for acute liver failure: presence of coagulopathy (prothrombin time >15 seconds or INR > 1.5) and any degree of hepatic encephalopathy (HE), occurring within 26 weeks of the onset of first symptoms in a patient without previous underlying liver disease

[1, 13]. Since the subjects were encephalopathic by definition, written informed consent was obtained from their legal next of kin. Detailed demographic, clinical, laboratory and outcome data as well as daily sera for 7 days were collected prospectively. All centers were in compliance with their local institutional review board requirements. A Certificate of Confidentiality was obtained from the National Institutes for Mental Health for the entire study.

To obtain a representative selection of ALF patients of all etiologies we studied plasma samples from 105 patients enrolled consecutively into the US Acute Liver Failure Study between 2006 and 2008, obtained on study day 1 and stored at -80°C for 1–3 years prior to testing. Causes for ALF in this group included acetaminophen toxicity (APAP); ischemia (Shock); idiosyncratic drug-induced liver injury (DILI); autoimmune hepatitis (AIH); viral hepatitis (A or B); indeterminate (IND – cases in which a specific cause could not be determined); and other (a miscellaneous group including heat stroke, cancer, Budd Chiari syndrome). Among the 12 AIH patients studied, none had received immunosuppression (usually prednisone in moderate to high doses) for longer than two weeks at the time of admission to study.

To serve as a healthy control group and as an example of an acute inflammatory state, we obtained plasma samples from patients prior to posterior lumbar spinal fusion and on two occasions following the procedure. The 35 subjects studied prior to and after lumbar fusion (Rigshospitalet, Copenhagen, DK) all had symptomatic degenerative disc disease with chronic low back pain but were considered to be in good health otherwise, and without any autoimmune diseases. These patients would presumably mimic the findings observed in trauma patients or other similar acute inflammatory settings. Each patient's sample prior to surgery provided a control for subsequent plasma samples collected on day 1 and 3 after the operation. All patients underwent posterior lumbar un-instrumented fusion without decompression. Fusion was done from L4 to S1. Samples were obtained at 24 hours prior to surgery and at 24 and 72 hours after surgery and stored at -80°C for approximately up to 10 years prior to testing; no patient was receiving corticosteroids prior to surgery. As an example of a chronic inflammatory state (rheumatoid arthritis: RA), we obtained 40 RA plasma samples from the longitudinal Veterans Affairs Rheumatoid Arthritis Registry, a database recruiting RA patients at 10 VA hospital sites in the US (VARA) [14]. From VARA, 20 patients were chosen with high disease activity (Disease Activity Score-28 (DAS-28) average 6.90) and 20 were selected with low disease activity (average DAS-28 0.97). All RA subjects were receiving either NSAIDs or immune modulating therapy at the time of the blood draw. Blood samples were obtained and the plasma samples were stored at -80°C for 2 – 4 years prior to testing. In both high and low DAS score groups, 60% were receiving immunosuppressive therapy and 65% NSAIDs; all were receiving one or both forms of treatment. We did not see any difference in levels between those with only NSAIDs and those with immunosuppressive or combined treatment

2.2 ELISA

The plasma OPN levels were determined for all samples using the Quantikine® Osteopontin ELISA assay (R&D Systems Inc. Minneapolis, MN). The lower detection limit for plasma

samples with this assay was 0.011 ng/mL. We utilized plasma samples for these analyses since serum levels of OPN are recognized to be considerably lower than plasma due to cleavage of OPN by thrombin [15, 16].

2.3 Statistical methods

Statistical analyses were performed using IBM® SPSS® Statistics 17.0 (SPSS Inc, Chicago, IL USA). Non-parametric analysis was used for comparing continuous variables between groups (Kruskal-Wallis with post hoc testing (Dunn method) for multiple groups and Mann-Whitney for 2 groups). Logistic regression analysis was performed to identify independent factors associated with severe hepatic encephalopathy and spontaneous survival.

3. Results

3.1 Demographic and clinical features

Detailed clinical and demographic data for the 105 ALF patients are shown in Table 1. APAP overdose patients were around 10 years younger than patients with other etiologies as has been previously observed. APAP overdose patients and those with ischemic liver injury had significantly higher alanine aminotransferase (ALT) levels, lower bilirubin levels and higher creatinine levels compared to the remainder of ALF patients.

3.2 Plasma osteopontin levels in ALF patients compared to controls

Significantly higher plasma OPN levels were observed in the overall ALF patient group (Figure 1, median: 1055 ng/mL; Range: 33 – 19,127) when compared to healthy controls (SF pre-surgery group: median 41 ng/mL; range 3 – 86, $P<0.001$), or chronic inflammatory controls: (RA group median: 37 ng/mL; range 4 – 136, $P<0.001$) or the acute post-surgery group (median 198 ng/mL; range 58 – 459, $P<0.001$).

3.3 Factors associated with plasma osteopontin levels in ALF patients

Patients who developed ALF due to APAP or shock had significantly shorter duration between onset of symptoms and admission to study ($P<0.001$). Overall, for ALF patients, plasma OPN levels correlated moderately well with serum ALT and aspartate aminotransferase (AST) levels (Table 2, R values of 0.47 and 0.46 for AST and ALT levels, both $P<0.001$); OPN levels were inversely correlated with serum total bilirubin levels ($P<0.001$). No significant correlation was observed between plasma OPN levels and alkaline phosphatase (ALP) levels ($P=0.45$). There was a significant correlation noted between MELD scores and OPN levels. Plasma OPN levels did not correlate with white blood cell (WBC) counts in the peripheral blood ($P=0.65$), but were negatively correlated with blood platelet counts ($P<0.001$) and hemoglobin levels ($P=0.02$), the higher OPN levels being associated with lower platelet and Hgb levels.

3.4 Plasma osteopontin levels in different etiologies of ALF

As shown in Figure 1, the highest median OPN levels were observed in patients who developed ALF due to APAP overdose (3603 ng/mL, range 54 – 19,127) and shock (4103 ng/mL, range 517 – 16,386). Median values for those who developed ALF due to DILI (353 ng/mL, range 166.7 – 1524), AIH (436 ng/mL, range 186 – 1,650) were lower. The median

plasma OPN levels in those of indeterminate etiology (251 ng/mL, range 166 – 14,457) and those with viral hepatitis-related ALF (706 ng/mL range 33 – 1,650) were intermediate but still far below median APAP and ischemia OPN levels. P value for comparison of Shock and APAP OPN levels with those of all other groups was <0.007. All other pairings were not different from each other.

3.5 Plasma osteopontin levels and hepatic encephalopathy

By definition, all ALF patients in this study had some degree of HE. The number of individual patients with grade I, II, III and IV hepatic coma were 25, 25, 19 and 36, respectively (Table 3). Patients with grade I/II coma were grouped as mild HE, patients with grade III/IV coma were grouped as severe HE. Those subjects with severe HE had a two-fold higher median OPN level (1,395 ng/mL) when compared to those with mild HE (714 ng/mL, $p = 0.048$). By univariate analysis, lower platelet count ($P = 0.030$) and higher creatinine ($P = 0.001$) were found to correlate with severe HE, while bilirubin level, age and duration of symptoms did not. Logistic regression revealed that patient age [Odds ratio=1.04 (1.005–1.07), $P = 0.021$], bilirubin level [Odds ratio=0.94 (0.90–0.99), $P = 0.014$] and creatinine level [Odds ratio=1.36 (1.05 – 1.75), $P = 0.018$] were independent factors associated with presence of severe hepatic encephalopathy.

3.6 OPN levels and prognosis

As shown in Figure 2, the patients who survived without undergoing transplantation (SS, $n = 52$) tended to have higher median OPN levels (1470 ng/mL) compared to those who died and/or underwent transplantation (non-SS: 825 ng/mL, $n = 53$), but the difference did not quite reach statistical significance ($P = 0.067$). The spontaneous survivors also had shorter duration of illness compared to non-spontaneous survivors ($P = 0.002$; Table 4). However, when the patients who underwent liver transplant were separated from the group, there was no significant difference between OPN levels of subjects who survived without transplant and subjects who died ($n = 30$, p -value = 0.799). Of interest, the OPN levels for the transplant group were much lower than either the SS or death group ($p < 0.001$).

When OPN levels of SS patients were compared by etiology (Figure 3), there was a significant difference between the groups (p -value = 0.003): those who developed ALF due to Shock had higher OPN levels than those with AIH etiology (p -value = 0.034). However, there were no other differences observed between OPN levels among the other groups $p = 0.065$ or greater.

3.7 Results in control groups: serial plasma osteopontin levels in spinal fusion and rheumatoid arthritis patients

Levels of OPN in those about to undergo spinal fusion (SF pre-surgery group) had a OPN 3SD range of 0 – 115.1 ng/mL. These values did not differ from other normal range estimations with upper limits of normal of 103.14 ng/mL ($n = 30$), 123 ng/mL ($n = 10$) and 117.04 ng/mL ($n = 20$) [16 – 18].

The OPN levels among RA patients differed somewhat between those considered to have low levels of disease activity ($n = 20$, 31.2 ng/mL) when compared to those RA subjects

with higher disease activity scores ($n = 20$, 55.0 ng/mL, $p = 0.033$). However, the RA subjects' OPN values did not differ from those observed in the healthy control subjects ($n = 35$, $p = 0.890$; Figure 1). Those undergoing spinal fusion demonstrated considerable increases in levels after surgery, more pronounced on day 3 than day 1. Compared to baseline plasma OPN levels (median = 41 ng/mL), the plasma OPN levels in SF post-operative values increased significantly to roughly 3.4-fold (range 1~19) on the first day (median = 144 ng/mL) and 3.7-fold (range 2~40) on the third day after surgery (median = 198 ng/mL). The Day 1 and Day 3 post-surgery values were significantly different from the pre-surgery OPN values (p -values <0.001 ; Figure 1) and were significantly different from each other (p -value 0.026).

4. Discussion

The pathophysiology of acute liver failure remains complex. Understanding cytokine production and the cell signaling pathways might shed additional light on pathogenesis and strategies for modulating the intense reactions observed in these patients. This study demonstrated that ALF patients had plasma OPN levels 10 to 200 fold those observed in healthy controls, or even post-op spine surgery patients and patients with RA, the latter two representing acute and chronic inflammatory settings. The ALF patients who spontaneously survived tended to have higher plasma OPN levels compared to patients who died or received liver transplantation, likely reflecting the better overall survival of the APAP and shock groups that demonstrated the greatest increase in OPN and the highest aminotransferase levels. By design, we selected as comparison groups individuals who might have varying degrees of inflammatory activity, using patients prior to elective surgery and the same patients immediately following spinal surgery, as well as those with active or inactive RA. We were unable to control for prior use of immunosuppressive or other disease modifying treatment in the RA group this likely have affected the OPN results, if treatment ameliorates the changes in OPN levels that might be observed in the absence of any ongoing anti-inflammatory agents. Despite this, we did see a small but significant difference between the group deemed to have high disease activity from the low disease activity group.

OPN acting as a pro-inflammatory cytokine may play a pivotal role in inflammatory liver diseases associated with liver cell necrosis. In normal liver, expression of OPN is minimal in resting Kupffer cells. Once liver injury occurs, Kupffer cells or natural killer T lymphocytes are activated, express and secrete OPN and other cytokines [20]. OPN attracts neutrophils, lymphocytes and macrophages to hepatic injury sites where these mediator cells are subsequently activated, produce TH1 cytokines, and cause massive liver necrosis [21]. We noted an inverse correlation of OPN levels with serum bilirubin level suggesting that OPN is most associated with hyperacute injury (primarily necrosis), as opposed to those associated with adaptive immunity such as viral or autoimmune hepatitis that evolve more slowly (characterized by apoptosis more than necrosis). Since the highest levels of OPN were observed in patients with APAP or ischemic liver injury that have a very short disease interval and a good prognosis, the association of high OPN with promising outcomes suggests that this marker may be aligned with the degree of hepatocyte necrosis per se. Of interest, a correlation was observed between MELD on admission to study and OPN levels, perhaps reflecting the severe alterations in laboratory values (INR, Cr, Bili) that typify these

very sick patients; note that MELD is not generally used in the setting of ALF and was developed for assessment of patients with cirrhosis [22]. An alternative explanation would be that OPN is associated with hepatic regeneration or in some way exerts a protective effect, limiting further cell injury.

There are several lines of evidence supporting the idea that OPN has a hepato-protective function in the liver diseases. Transgenic mice that over-express OPN, after treatment with CCl₄, demonstrate less severe liver injury and inflammation [23]. In addition, OPN expression has been found in a mouse oval cell induced model to be associated with activation of hepatic stem cells [24]. Additional study of the dynamic changes of OPN levels in ALF patients might give us a clearer understanding of the role OPN plays and its possible predictive value in the ALF setting.

HE (and cerebral edema) in ALF patients has been thought to possibly reflect a central neuro-inflammatory response to system inflammation [25]. OPN over-expression has been observed in other central nervous system diseases, such as multiple sclerosis and Alzheimer's diseases [26, 27]. Over-expressed pro-inflammatory cytokines including OPN and other metabolites in ALF patients might pass an impaired blood-brain barrier, activate microglial cells and induce this inflammatory state in the brain. The highest levels of OPN were observed in 'hyperacute' patients, those with shock or acetaminophen injury, and this is indeed the patient group most susceptible to development of brain edema.

As a matricellular protein, OPN has been found to enhance platelet adhesion to matrix surface [28, 29]. This might explain the inverse correlation between plasma OPN levels and platelet counts. While platelet counts are observed to be lower in severe ALF, we have been unable to find a correlation between thrombopoietin levels and platelets in ALF patients [30, 31].

Limitations of the present study include the use of stored serum samples, and the aforementioned uncertainties about the use of anti-inflammatory or immunosuppressive medication in this setting and their effects on OPN values. The patients studied were otherwise well-characterized for etiology and for clinical disease state at the time of blood sampling.

In conclusion, this study plasma OPN levels were considerably higher in a large number of ALF patients (regardless of etiology) than were observed in healthy controls or in patients with other acute and chronic inflammatory conditions. Plasma OPN levels within the ALF group varied greatly by etiology, correlating in a general way with severity of liver injury as demonstrated by a variety of markers. Given the very wide range of values measured, OPN may exert a hepatoprotective effect under certain conditions or simply be a passive marker of liver necrosis in these settings.

Highlights

- Osteopontin (OPN) is a cytokine that is elevated in settings associated with inflammation.

- Plasma OPN levels in acute liver failure patients were increased from 10- to 500-fold over normal.
- OPN levels in acute or chronic inflammation (RA, post-op) were only minimally elevated.
- Highest OPN values occurred with severe hepatocyte necrosis (acetaminophen or ischemia).
- The significance of OPN release remains to be determined, it may play a cytoprotective role.

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Abbreviations

ALF	acute liver failure
ALP	alkaline phosphatase
ALT	alanine aminotransferase
APAP	acetaminophen
AST	aspartate aminotransferase
DAS-28	Disease Activity Score-28
HE	hepatic encephalopathy
INR	international normalized ratio
OPN	osteopontin
RA	rheumatoid arthritis

RGD	arginine-glycine-aspartic acid domain
SF	spinal fusion
SIRS	systemic inflammatory response syndrome
SS	spontaneous survival
USALFSG	US Acute Liver Failure Study Group
VARA	Veterans Affairs Rheumatoid Arthritis Registry
WBC	white blood cell

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Figure 1. Plasma OPN levels in ALF patients and controls

Plasma OPN levels are shown for 105 acute liver failure patients as overall group and as specific etiologies of ALF as well as 40 patients with rheumatoid arthritis (RA) and 35 patients undergoing posterior lumbar spinal fusion (SF). Each dot represents the OPN level. The horizontal line represents the median value in each group. ALF: acute liver failure, APAP: N-acetyl-p-aminophenol, the active compound of acetaminophen; DILI: drug-induced liver injury; IND: indeterminate; RA: rheumatoid arthritis; SP: spinal fusion, prior to surgery.

Figure 2. Correlation of OPN levels and outcomes in ALF patients

Patients who survived without transplantation (SS, n = 52) tended to have higher median admission plasma OPN levels compared to those who died (Death, n = 30) or received liver transplantation (Transplant, n = 23) (P=0.067).

Figure 3. Correlation of OPN values by etiology in spontaneous survivors

Median plasma OPN levels are shown for 52 ALF patients who spontaneously survived as the overall group and by specific etiologies: ALF: acute liver failure, Shock (n = 14), APAP: N-acetyl-p-aminophenol, the active compound of acetaminophen (n = 30); DILI: drug-induced liver injury (n = 12); IND: indeterminate (n = 12); AIH: autoimmune hepatitis (n = 12), Viral (n = 17), and Other (n = 8).

Table 2

Correlation between plasma OPN level and clinical parameters in ALF patients

Parameter	N	Correlation coefficient	P value
ALT	101	0.44	<0.001
AST	104	0.47	<0.001
Total Bilirubin	104	−0.44	<0.001
ALP	101	0.076	0.45
Albumin	88	0.27	0.011
INR	104	−0.07	0.48
Platelet	105	−0.48	<0.001
WBC	105	0.045	0.65
Hemoglobin	104	−0.23	0.02
Creatinine	104	0.61	<0.001
MELD	105	0.365	<0.001
Days from onset of symptoms	98	−0.48	<0.001

Table 3

Coma Grade: Median Values with Minimum/Maximum range

	Coma Grade			
	Mild		Severe	
	I	II	III	IV
n	25	25	19	36
OPN [ng/mL]	825.2 (166.4 – 5749.3)	504.0 (33.3 – 19126.9)	1185.7 (138.8 – 14457.4)	1469.6 (53.9 – 13347.9)
Age (years)	38 (22 – 62)	40 (17 – 65)	51 (18 – 84)	41.5 (19 – 78)
Duration illness (days from onset till study admission)	9 (1 – 95)	8 (1 – 60)	6 (1 – 44)	6 (1 – 63)
WBC ($4.0\text{--}11.0 \times 10^3/\text{uL}$)	8.8 (3.5 – 33.5)	8.8 (0.1 – 20.4)	8.8 (1.5 – 23.9)	11.2 (2.2 – 28.1)
Hgb (12–16 g/dL)	11.3 (7.8 – 15.8)	11.4 (7.9 – 15.5)	10.7 (6.9 – 16.2)	10.0 (7.8 – 16.2)
Platelets ($150\text{--}450 \times 10^3/\text{uL}$)	124 (48 – 277)	160 (16 – 262)	119 (46 – 266)	98 (7 – 367)
INR (0.9–1.3 %)	2.8 (1.3 – 11.0)	2.6 (1.4 – 8.0)	3.0 (1.2 – 6.6)	2.1 (0.9 – 9.2)
ALT (0–40 IU/L)	1258 (90 – 9326)	1373 (38 – 7851)	2333 (23 – 8113)	1230 (71 – 16090)
AST (0–40 IU/L)	827 (92 – 13872)	997 (119 – 5728)	1842 (45 – 15760)	930 (61 – 28800)
ALP (35–104 IU/L)	120 (61 – 369)	144 (35 – 720)	141 (60 – 1448)	115 (55 – 401)
Bilirubin (0.2–1.3 mg/dL)	14.1 (1.7 – 34.2)	13.0 (1.7 – 35.8)	8.2 (1.8 – 40.3)	7.7 (0.8 – 45.5)
Creatinine (0.5–1.0 mg/dL)	0.95 (0.5 – 8.3)	1.2 (0.4 – 6.1)	2.3 (0.7 – 7.7)	2.65 (0.6 – 6.1)

Table 4

Outcomes: Median Values with Minimum/Maximum Ranges

	SS	non-SS	Transplant	Died
n	52	53	23	30
OPN [ng/mL]	1470 (33.3 – 19126.9)	825 (138.8 – 14457.4)	324 (138.8 – 8559.7)	1399 (294.4 – 14457.4)
Duration illness (days from onset till study admission)	5 (1 – 95)	13 (1 – 50)	16 (3 – 50)	8 (1 – 49)