Title: Serum levels of soluble adhesion molecules as prognostic factors for acute liver failure.

Running title: Serum sPECAM-1 and sICAM-1 level in ALF.

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Abstract

Background/Aims: In patients with septic shock, the degree of liver dysfunction is correlated with serum levels of soluble intercellular adhesion molecule (sICAM)-1. We aimed to assess the usefulness of serum levels of soluble adhesion molecules as prognostic factors for acute liver failure (ALF).

Methods: Serum levels of soluble platelet endothelial cell adhesion molecule (sPECAM)-1, sICAM-3, soluble endothelial (sE)-selectin, sICAM-1, soluble platelet-selectin, and soluble vascular cell adhesion molecule-1 on admission were measured in 37 ALF patients and 34 healthy controls.

Results: Twenty-two ALF patients (59%) reached to fatal outcomes. Serum levels of sPECAM-1, sICAM-3, sE-selectin and sICAM-1 were higher in ALF patients than healthy controls. In 37 ALF patients, by the multivariate logistic regression analysis, ratio of direct to total bilirubin (per 0.1 increase; odds ratio 0.11, 95% CI 0.01-0.99), serum sPECAM-1 level (per 100 ng/ml increase; odds ratio 4.37, 95% CI 1.23-15.5) and serum sICAM-1 level (per 100 ng/ml increase; odds ratio 0.49, 95% CI 0.27-0.89) were associated with fatal outcomes. Using receiver operating characteristics curve, each area under the curve of serum sPECAM-1 and sICMA-1 levels as prognostic factors was 0.71 and 0.74, respectively.

Conclusion: Serum sPECAM-1 and sICAM-1 levels may be useful for predicting the prognosis of ALF.

Introduction

Acute liver failure (ALF) is the clinical manifestation of liver cell death of a critical degree with insufficient hepatocellular regeneration and characterized by hepatic encephalopathy and coagulopathy [1]. The survival rate without liver transplantation is over 60% in patients with acetaminophen-induced ALF and 20-30% in those with non-acetaminophen-related ALF [2]. Many of ALF patients rapidly progress to death from multiple organ failure (MOF). In order to rescue more ALF patients, it is important to accurately predict their prognosis.

Up to now, elevated serum levels of soluble adhesion molecules such as soluble intercellular adhesion molecule-1 (sICAM-1), soluble vascular cell adhesion molecule-1 (sVCAM-1), and soluble endothelial (sE)-selectin have been reported to be associated with the development of MOF [3,4]. Furthermore, in acute pancreatitis and severe burn, serum levels of sICAM-1 and sVCAM-1 have been shown to reflect the severity of the disease and be associated with the prognosis [5,6].

In patients with sepsis or septic shock, serum sICAM-1 levels have been reported to be correlated with serum bilirubin levels [7,8]. Furthermore, in patients with alcoholic liver cirrhosis, serum sICAM-1 levels have been shown to be correlated with prothrombin activities and serum bilirubin levels and be associated with the prognosis

[9]. On the other hand, in an endotoxic shock model, inhibition of adhesion molecule such as platelet (P)-selectin and ICAM-1 have been reported to reduce the degree of liver injury [10]. Thus, we speculated that serum levels of soluble adhesion molecules might be associated with the clinical outcomes of ALF patients.

This study aimed to investigate whether serum levels of soluble adhesion molecules were useful to predict the prognosis of ALF patients.

Methods

This study was approved by the Institutional Review Board at Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences.

Patients

The study-subjects consisted of 37 ALF patients and 34 healthy controls.

In this study, patients showing prothrombin activities of 40% or less of the standardized values due to severe liver damage within 8 weeks of the onset of disease symptoms were diagnosed as ALF [11]. Hepatic coma was graded on the standard scale of I to IV [12]. However, those who’s computed tomography showed the features of chronic liver disease (splenomegaly or varices, collaterals) were excluded.

Etiology of ALF

A diagnosis of hepatitis A and B was made based on the presence of IgM anti-hepatitis A virus antibody, and IgM anti-hepatitis B virus core antibody or hepatitis B surface antigen, respectively [13]. A diagnosis of autoimmune hepatitis was made according to the criteria revised by the International Autoimmune Hepatitis Group in 1999 [14]. A diagnosis of drug-induced liver injury was made based on the distinctive clinical course. A diagnosis of indeterminate liver failure was established when all of IgM anti-hepatitis A virus antibody, IgM anti-hepatitis B virus core antibody, hepatitis B surface antigen, hepatitis C virus-RNA, anti-nuclear antibody and anti-smooth muscle antibody were negative with no obvious cause such as drug, acute fatty liver of pregnancy, ischemic hepatitis, Wilson’s disease, malignant infiltration, cytomegalovirus infection, Epstein-Barr virus infection and herpes simplex virus infection.

Measurement of serum level of soluble adhesion molecule

Serum was collected when each patient admitted to our hospital, and stored at -80°C.

Serum levels of 6 soluble adhesion molecules were measured using the FlowCytomix Multiple Analyte Detection System with the Adhesion 6plex (eBioscience, San Diego, CA, USA), according to the manufacturer's protocol. This panel consisted of soluble platelet endothelial cell adhesion molecule (sPECAM)-1, sICAM-3, sE-selectin,

sICAM-1, sP-selectin and sVCAM-1. In brief, the Adhesion 6plex Standard diluted in assay buffer and samples were added to a 96 well filter plate. Antibody-coupled beads were added to all wells and incubated with phycoerythrin-conjugated second antibodies for 2 hours with continuous shaking. The beads were washed twice with assay buffer and re-suspended in assay buffer. The reaction mixture was analyzed using the MACSQuant Analyzer with MACSQuantify Software v2.2 (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Each serum level of soluble adhesion molecule was automatically calculated by FlowCytomix Pro 2.4 software (eBioscience, San Diego, CA, USA) using the appropriate standard curve.

Statistical Analysis

SPSS statistical program (release 11.0.1 J, SPSS, Chicago, IL, USA) was used for the statistical analysis.

Dichotomous variables were compared by the χ^2-test. Continuous variables were expressed as median (range). The Mann–Whitney U test was used to evaluate differences in the continuous variables between two groups, and the Kruskal-Wallis test was carried out among three groups. Spearman correlation coefficient was used to evaluate the consistency in the continuous variables between two groups. To identify prognostic factors for fatal outcome (liver transplantation or death), we developed the

univariate logistic regression model. The variables, which showed p <0.05 by the univariate analysis, were included into the multivariate logistic regression model. The prognostic accuracy of each factor which elicited by the logistic regression analyses was evaluated based on the area under the curve (AUC) using receiver operating characteristics (ROC) curve analysis. P-values <0.05 were considered significant.

Results

Clinical characteristics

Table 1 shows clinical characteristics and laboratory data on admission in 37 ALF patients. Of 37 ALF patients, 28 were diagnosed with ALF with hepatic coma (≥ grade II) and 9 with ALF without hepatic coma (grade 0 or I). All patients underwent culture (blood, tracheal aspirate, and urine) and computed tomography (head, chest, and abdomen) at the diagnosis of ALF; however, none clinically developed bacterial or fungal infection.

Overall, 15 survived without liver transplantation, 14 received living donor liver transplantation, and 8 died without liver transplantation. Thus, 22 patients reached to fatal outcomes (liver transplantation or death).

Serum level of soluble adhesion molecule

Table 2 shows serum levels of 6 soluble adhesion molecules on admission in 37 ALF patients and 34 healthy controls. Serum levels of sPECAM-1, sICAM-3, sE-selectin and sICAM-1 in 37 ALF patients were higher than those in 34 healthy controls. In 37 ALF patients, 22 patients reaching fatal outcome showed higher serum sPECAM-1 levels (P = 0.030) and lower serum sICAM-1 levels (P = 0.014) than 15 survivors without liver transplantation. On the other hand, there was no difference in serum levels of 6 soluble adhesion molecules between 28 ALF patients with hepatic coma and 9 ALF patients without hepatic coma (Table 3). Furthermore, in 15 survivors, there were no differences in serum levels of sPECAM-1 {577 (495-784) ng/ml versus 558 (194-898) ng/ml; P = 0.48} and sICAM-1 {1993 (1748-2246} ng/ml versus 1892 (1264-2737) ng/ml; P = 0.91} between 6 ALF patients with hepatic coma and 9 ALF patients without hepatic coma.

In 28 ALF patients with hepatic coma, 22 patients reaching fatal outcomes showed lower serum sICAM-1 levels {1423 (124-2839) ng/ml versus 1993 (1748-2246) ng/ml; P = 0.036} than 6 survivors; however there were no differences in serum levels of other 5 soluble adhesion molecules between the 2 groups.

Serum level of soluble adhesion molecule as prognostic factor for ALF

In the univariate logistic regression model, platelet count, ratio of direct to total

bilirubin (D/T ratio), serum sPECAM-1 level and serum sICAM-1 level on admission were associated with fatal outcomes in 37 ALF patients. However, the association of prothrombin activity on admission with the prognosis was equivocal (Table 4).

In the multivariate logistic regression analysis, D/T ration (odds ratio 0.11, 95% confidence interval 0.01-0.99), serum sPECAM-1 level (odds ratio 4.37, 95% confidence interval 1.23-15.5) and serum sICAM-1 level (odds ratio 0.49, 95% confidence interval 0.27-0.89) were associated with fatal outcomes in 37 ALF patients (Table 5).

Based on the ROC curves of serum levels of sPECAM-1 and sICAM-1 for estimating fatal outcomes in the 37 ALF patients, the AUC was 0.71 ($P = 0.007$) and 0.74 ($P = 0.005$), respectively. On the other hand, the AUC of platelet count, prothrombin activity, and D/T ratio was 0.77 ($P = 0.014$), 0.70 ($P = 0.050$), and 0.85 ($P < 0.0001$), respectively.

In 37 ALF patients, serum sPECAM-1 level was inversely correlated with prothrombin activity ($r = -0.52$, $P = 0.0017$). Serum sICAM-1 level was significantly correlated with platelet count ($r = 0.50$, $P = 0.0025$) and D/T ratio ($r = 0.53$, $P = 0.0015$).

When ALF patients showing serum sPECAM-1 level ≥650 ng/ml on admission were estimated to reach fatal outcomes, the sensitivity and specificity were 68% and

73%, respectively. On the other hand, when patients showing serum sICAM-1 level ≤1750 ng/ml on admission were estimated to reach fatal outcomes, the sensitivity and specificity were 68% and 73%, respectively.

Discussion

Recently, the prognosis of ALF patients has been improved due to the advances in supportive intensive care: however liver transplantation is the only effective intervention for those with fatal outcomes [15]. On the other hand, in Asian countries, the donation from deceased donors is severely limited because of various cultural and social reasons [16]. Approximately 50% of patients listed for emergency liver transplantation have died while awaiting a graft because of the lack of a timely suitable donor [17,18]. So, in order to rescue more patients in a setting of the shortage of liver grafts, prognostic factors useful to determine the suitable timing for liver transplantation are required.

In ALF, elevated serum levels of pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ have been reported to be associated with the disease pathogenesis and clinical outcomes [19-22]. These pro-inflammatory cytokines promote the secretion of soluble adhesion molecules such

as sICAM-1, sVCAM-1 and sE-selectin from the endothelial cells [23-25]. Thus, we speculated that some soluble adhesion molecules might be associated with the clinical outcomes of ALF.

In this study, serum levels of sPECAM-1 and sICAM-1 were significantly associated with the prognosis of ALF patients. Furthermore, the accuracy of serum levels of sPECAM-1 and sICAM-1 for predicting the prognosis of ALF seemed approximately equal to platelet count and prothrombin activity, which have been reported as important prognostic factors for ALF [26]. On the other hand, although ALF patients with hepatic coma has been reported to reach fatal outcomes more frequently than those without hepatic coma [11], there were no differences in serum levels of sPECAM-1 and sICAM-1 between these 2 groups in this study. So, we consider that serum levels of sPECAM-1 and sICAM-1 may be worth investigating as biomarkers for predicting the prognosis and determining the suitable timing for liver transplantation in ALF patients.

ICAM-1 is a member of the immunoglobulin-superfamily of cell adhesion molecules and expressed on both hematopoietic and non-hematopoietic cells [27]. ICAM-1 binds to its main leukocyte ligand, lymphocyte function associated molecule (LFA)-1, and plays an important roles in the trans-endothelial migration of leukocytes to

sites of inflammation and the activation of T cells [27,28]. On the other hand, sICAM-1, which consists of the five extracellular immunoglobulin-domains of the membrane-bound ICAM-1 molecule and lacks the transmembrane and cytoplasmic domains, inhibits ICAM-1 interaction with LFA-1 and attenuates inflammation [28-30]. Furthermore, sICAM-1 has been reported to promote angiogenesis [31]. Angiogenesis plays an important role in liver regeneration [32]. Thus, insufficient elevation of serum sICAM-1 levels in ALF patients may lead to the continuation of intra-hepatic inflammation and be associated with the failure of liver regeneration.

Generally, serum sICAM-1 levels have been reported to be higher in patients with inflammatory disorders, especially in those with poor prognosis [3,33,34]. However, this study indicated that, in ALF patients, lower serum sICAM-1 levels were associated with their fatal outcomes. Part of sICAM-1 has been reported to be secreted from hepatocytes stimulated with pro-inflammatory cytokines such as IFN-γ, IL-1β and TNF-α [35]. After hepatic resection, serum sICAM-1 levels have been shown to decrease [36]. In this study, serum sICAM-1 level was correlated with D/T ratio, which reflects hepatic bilirubin conjugation capacity, in ALF patients. Thus, serum sICAM-1 levels may reflect the grade of hepatic dysfunction in ALF patients. In order to confirm these findings, a further study with a larger sample size is required.

This study firstly showed the association of serum sPECAM-1 levels with the prognosis of ALF patients. PECAM-1 is a member of the immunoglobulin-superfamily of cell adhesion molecules and expressed on most cells of the hematopoietic lineage including platelets [37]. sPECAM-1 lacks the cytoplasmic and trans-membrane domains. In this study, serum sPECAM-1 level was inversely correlated with prothrombin activity. In ALF, the intra-hepatic and intra-vascular activation of coagulation, which decreases prothrombin activity and platelet count, results in micro-thrombus formation and local ischemia and contributes to the progression of the disease [38]. Serum sPECAM-1 levels have been reported to be associated with the development of ischemic stroke and acute coronary syndrome [39,40]. Thus, elevated serum sPECAM-1 levels in ALF patients are considered to reflect coagulation activation.

We consider that, in order to assess the usefulness of serum sPECAM-1 and sICAM-1 levels as biomarkers for predicting outcomes of ALF patients, the relation between the changes of these levels during the clinical course and the prognosis of ALF patients should be assessed, although, in this study, we could not for lack of the serum collection after the introduction of treatment in ALF patients. Hereafter, to clarify this point is necessary.

In conclusion, this study indicated that serum levels of sPECAM-1 and sICAM-1

on admission were associated with the prognosis of ALF patients. We consider that serum levels of sPECAM-1 and sICAM-1 may be worth investigating as biomarkers for predicting their outcomes and determining the suitable timing for liver transplantation. A further study with a large sample size is required.

Acknowledgments

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Table 1. Clinical characteristics and labolatory data on admission in 37 patients with acute liver failure.

	Survive	Fatal outcome	P value
Patients, n	15	22	
Age, yr	37 (16-71)	38 (27-73)	0.40
Gender, female (%)	8 (53)	14 (64)	0.39
Etiology, n (%)			
Viral hepatitis	8 (53)	9 (41)	0.55
Hepatitis A virus	3	0	
Hepatitis B virus	5	9	
Autoimmune hepatitis	4 (27)	5 (23)	
Drug-induced liver injury	2 (13)	3 (13)	
Indeterminate	1 (7)	5 (23)	
Hepatic coma, n (%)			0.0001
0 or I	9 (60)	0 (0)	
II	5 (33)	16 (73)	
III or IV	1 (7)	6 (27)	
Laboratory data			

White blood cell, /mm^3	7800 (4200-28000)	10165 (2300-25300)	0.27
Hemoglobin, g/dl	13.1 (7.3-18.4)	13.2 (8.4-16.4)	0.86
Platelet, $x10^4/mm^3$	14.6 (9.0-30.9)	9.1 (2.4-40.3)	0.007
Bilirubin, mg/dl	8.9 (3.9-26.0)	11.2 (2.3-32.8)	0.44
D/T ratio	0.67 (0.57-0.72)	0.52 (0.31-0.75)	0.0003
AST, IU/l	1036 (215-17340)	497 (41-18360)	0.18
ALT, IU/l	2504 (220-7990)	1177 (24-10470)	0.23
Creatinine, mg/dl	0.6 (0.4-2.6)	0.7 (0.4-4.8)	0.34
Prothrombin activity, %	32 (11-40)	23 (6-40)	0.034
Prognosis, n (%)			
Liver transplantation	0 (0)	14 (64)	
Death without liver transplantation	0 (0)	8 (36)	

ALT, alanine aminotransferase; AST, aspartate aminotransaminase; D/T ratio, ratio of direct to total bilirubin.

Table 2. Serum level of soluble adhesion molecule on admission.

	Acute liver failure patient				
	Overall	Survive	Fatal outcome	Healthy control	P-value
Patients, n	37	15	22	34	
sPECAM-1, ng/ml	664 (194-2049)	558 (194-898)	850 (313-2049)	338 (171-1072)	<0.0001
sICAM-3, ng/ml	286 (43-1170)	286 (43-674)	273 (51-1170)	88 (8-290)	<0.0001
sE-selectin, ng/ml	422 (140-1162)	440 (140-1098)	360 (169-1162)	108 (35-404)	<0.0001
sICAM-1, ng/ml	1783 (124-2839)	1892 (1264-2737)	1423 (124-2839)	666 (279-1604)	<0.0001
sP-selectin, ng/ml	185 (21-499)	139 (59-499)	192 (21-418)	201 (44-475)	0.33
sVCAM-1, ng/ml	2468 (719-3898)	2415 (719-3574)	2442 (898-3898)	2208 (1262-3574)	0.84

Each parameter was compared between 37 patients with acute liver failure and healthy controls. E-selectin, endothelial-selectin; ICAM-1, intercellular adhesion molecule-1; ICAM-3, intercellular adhesion molecule-3; PECAM-1, platelet endothelial cell adhesion molecule-1; P-selectin, platelet-selectin; s, soluble; VCAM-1, vascular cell adhesion molecule-1.

Table 3. Serum level of soluble adhesion molecule on admission in 37 patients with acute liver failure.

	Hepatic coma grade		
	0 or I	II or higher	P-value
Patients, n	9	28	
sPECAM-1, ng/ml	558 (194-898)	742 (313-2049)	0.061
sICAM-3, ng/ml	167 (43-674)	298 (51-1170)	0.13
sE-selectin, ng/ml	472 (140-1098)	360 (165-1162)	0.42
sICAM-1, ng/ml	1892 (1264-2737)	1676 (124-2839)	0.15
sP-selectin, ng/ml	154 (59-499)	182 (21-418)	0.96
sVCAM-1, ng/ml	3086 (719-3574)	2389 (898-3898)	0.70

E-selectin, endothelial-selectin; ICAM-1, intercellular adhesion molecule-1; ICAM-3, intercellular adhesion molecule-3; PECAM-1, platelet endothelial cell adhesion molecule-1; P-selectin, platelet-selectin; s, soluble; VCAM-1, vascular cell adhesion molecule-1.

Table 4. Prognostic factor for acute liver failure by univariate logistic regression model.

	OR	95% CI	P-value
Age, per 1 year increase	1.02	0.97-1.07	0.45
Gender, female	1.53	0.40-5.81	0.53
Etiology, viral hepatitis	0.61	0.16-2.28	0.46
Hepatic coma, III or IV	5.26	0.56-50.0	0.15
White blood cell, per 100/mm^3 increase	1.01	0.99-1.02	0.30
Hemoglobin, per 1 g/dl increase	0.93	0.71-1.23	0.63
Platelet, per 1 x 10^4/ mm^3 increase	0.90	0.82-0.99	0.030
Bilirubin, per 1mg/dl increase	1.04	0.96-1.13	0.36
D/T ratio, per 0.1 increase	0.14	0.03-0.59	0.007
AST, per 100 IU/l increase	1.00	0.99-1.01	0.99
ALT, per 100 IU/l increase	0.99	0.97-1.02	0.58
Creatinine, per 1 mg/dl increase	1.95	0.68-5.60	0.22
Prothrombin activity, per 1% increase	0.94	0.88-1.00	0.064
sPECAM-1, per 100 ng/ml increase	1.39	1.04-1.88	0.029
sICAM-3, per 100 ng/ml increase	1.13	0.89-1.43	0.32

sE-selectin, per 100 ng/ml increase	0.99	0.80-1.23	0.92
sICAM-1, per 100 ng/ml increase	0.84	0.72-0.97	0.016
sP-selectin, per 100 ng/ml increase	1.17	0.67-2.04	0.58
sVCAM-1, per 100 ng/ml increase	1.01	0.94-1.08	0.75

ALT, alanine aminotransferase; AST, aspartate aminotransaminase; CI, confidence interval; D/T ratio, ratio of direct to total bilirubin; OR, odds ratio; E-selectin, endothelial-selectin; ICAM-1, intercellular adhesion molecule-1; ICAM-3, intercellular adhesion molecule-3; PECAM-1, platelet endothelial cell adhesion molecule-1; P-selectin, platelet-selectin; s, soluble; VCAM-1, vascular cell adhesion molecule-1.

Table 5. Prognostic factor for acute liver failure by multivariate logistic regression model.

	OR	95% CI	P-value
Platelet, per 1 x 10^4/ mm^3 increase	1.26	0.98-1.63	0.074
D/T ratio, per 0.1 increase	0.11	0.01-0.99	0.049
sPECAM-1, per 100 ng/ml increase	4.37	1.23-15.5	0.022
sICAM-1, per 100 ng/ml increase	0.49	0.27-0.89	0.020

ALT, alanine aminotransferase; AST, aspartate aminotransaminase; CI, confidence interval; D/T ratio, ratio of direct to total bilirubin; ICAM-1, intercellular adhesion molecule-1; PECAM-1, platelet endothelial cell adhesion molecule-1; s, soluble.