Title: Serum levels of PDGF-BB and VEGF as prognostic factors for patients with fulminant hepatic failure.

Short running title: Serum PDGF-BB and VEGF levels in FHF

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Abstract

Background: In animal models for acute liver injury, the administration of some angiogenic factors such as vascular endothelial growth factor (VEGF) and granulocyte-colony stimulating factor (G-CSF) are shown to reduce liver injury and improve liver proliferative capacity.

Aim: To assess the role of angiogenic factors in fulminant hepatic failure (FHF).

Methods: Serum levels of nine angiogenic factors {angiopoietin-2, follistatin, G-CSF, hepatocyte growth factor (HGF), interleukin-8, leptin, platelet-derived growth factor (PDGF)-BB, platelet endothelial cell adhesion molecule-1 and VEGF} were measured using the Bio-Plex Protein Array System in 30 patients, 17 of whom were diagnosed with FHF and 13 with acute hepatitis (AH), and 20 controls.

Results: Serum levels of PDGF-BB and VEGF were lower in FHF patients than AH patients and controls (PDGF-BB; 2050 ± 1572 pg/ml vs. 4521 ± 2419 pg/ml vs. 8506 ± 5500 pg/ml, VEGF; 39 ± 38 pg/ml vs. 144 ± 122 pg/ml vs. 205 ± 121 pg/ml). By using univariate logistic regression models, serum levels of PDGF-BB and VEGF were associated with poor outcomes. Serum PDGF-BB levels were strongly correlated with serum VEGF levels (r = 0.70). Furthermore, serum PDGF-BB levels were significantly correlated with platelet counts (r = 0.79), PT activity (r = 0.37) and D.Bil/T.Bil ratio (r =

0.50), while serum VEGF levels were significantly correlated with platelet counts (r = 0.68) and PT activity (r = 0.38).

Conclusions: Serum levels of PDGF-BB and VEGF are worth investigating as biomarkers for predicting outcomes of FHF patients.

Key Words: fulminant hepatic failure, platelet-derived growth factor–BB, vascular endothelial growth factor, hepatocyte growth factor, prognostic factor.

Introduction

Fulminant hepatic failure (FHF) is caused by liver cell death of a critical degree and characterized by hepatic encephalopathy and coagulopathy. The spontaneous survival rate was reported 60-70% in acetaminophen-related FHF and 20-40% in non-acetaminophen-related FHF.[1-3] While, in FHF patients with fatal outcomes, post liver transplantation survival ranges between 60% and 80%,[4] and liver transplantation is the most reliable treatment for FHF.

The condition of FHF changes dramatically. If liver transplantation is performed too late, there is increased risk of multiple organ failure and infection. A recent report showed that, in a setting of donor organ shortage, a fourth of the patients listed for emergency liver transplantation were unable to undergo surgery.[5] Thus, in addition to liver transplantation, new therapeutic modalities for promoting liver regeneration are desired.

In a rat model for acute severe liver injury, the administration of vascular endothelial growth factor (VEGF) is reported to promote hepatocyte proliferation and reduce the mortality.[6] Furthermore, in a rat model for FHF, granulocyte-colony stimulating factor (G-CSF) is shown to reduce liver injury and improve liver proliferative capacity.[7] Thus, the administration of these angiogenic factors may be

effective treatment for FHF.

Clinically, G-CSF administration is shown to induce proliferation of hepatic progenitors in patients with alcoholic steatohepatitis, which leads to liver regeneration.[8] However, the role of these angiogenic factors in FHF patients has been yet to be fully implemented. This study aimed to investigate the associations of serum levels of various angiogenic factors including VEGF and G-CSF with clinical characteristics and prognosis of FHF patients.

Methods

Patients

The study-subjects consisted of 30 patients, 17 of whom were diagnosed with FHF and 13 with acute hepatitis (AH), and 20 healthy controls.

Patients were diagnosed as FHF when hepatic encephalopathy of coma grade greater II developed within 8 weeks after the onset of disease symptoms with a prothrombin time less than 40% of the standardized values (Japanese diagnostic criteria).[2] However, those who's computed tomography showed the features of chronic liver disease (splenomegaly or varices, collaterals) were excluded.

Etiology of FHF

A diagnosis of fulminant hepatitis A, B and C was made based on the presence of immunoglobulin M antibody to hepatitis A virus, immunoglobulin M antibody to hepatitis B core antigen or hepatitis B surface antigen, and hepatitis C virus RNA identifiable by nested reverse transcription-polymerase chain reaction, respectively.[9] A diagnosis of autoimmune hepatitis was made according to the criteria revised by the International Autoimmune Hepatitis Group in 1999.[10] A diagnosis of Epstein-Barr virus infection was made based on measurement of Epstein-Barr virus load in whole blood by quantitative polymerase chain reaction amplification assays.[11] A diagnosis of drug-induced liver injury, acute fatty liver of pregnancy and ischemic hepatitis was made based on their distinctive clinical courses. A diagnosis of indeterminate FHF 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 angiogenic markers concentration

Serum was collected when patients were admitted to our hospital and before

treatment, and stored at -80°C.

Serum levels of angiogenic factors were measured using the Bio-Plex Protein Array System with the Bio-Plex Pro Human Angiogenesis 9-Plex Panel (Bio-Rad Laboratories, Hercules, CA, USA). This panel consisted of angiopoietin-2, follistatin, G-CSF, hepatocyte growth factor (HGF), interleukin 8 (IL-8), leptin, platelet-derived growth factor (PDGF)-BB, platelet endothelial cell adhesion molecule (PECAM)-1 and VEGF. In brief, the Bio-Plex Pro Angiogenesis Standard and samples diluted in Serum Diluent were added to a 96 well filter plate and incubated with the antibody-coupled beads for 1 h with continuous shaking. The beads were washed three times with wash buffer to remove unbound protein and incubated with biotinylated detection antibodies for 30 min with continuous shaking. Following three washes, premixed streptavidin-phycoerythrin was added to each well and incubated for 30 minutes. After incubation, the beads were washed and re-suspended in assay buffer. The reaction mixture was quantified using the Bio-Plex protein array reader. Each angiogenic marker level was automatically calculated by Bio-Plex Manager software 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 Fisher's exact test. Continuous variables were expressed as mean ± standard deviation. 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. The Spearman correlation coefficient was used to evaluate the consistency in the continuous variables between two groups. To identify the association of serum angiogenic factors with poor outcomes, we developed the univariate Cox proportional hazard models. The prognostic accuracy of each factor was evaluated based on the area under the curve (AUC) using receiver operating characteristic curve analysis. P-values <0.05 were considered significant.

Results

Characteristics on admission

Of 17 FHF patients, 5 survived spontaneously, 6 patients received living donor liver transplantation, and the remaining 6 patients died without liver transplantation. All 17 AH patients survived spontaneously. Table 1 shows clinical characteristics and laboratory data on admission of FHF patients and AH patients. Direct bilirubin/total bilirubin (D.Bil/T.Bil) ratio and prothrombin (PT) activity were lower in FHF patients

than AH patients. Age, gender, etiology and serum levels of T.Bil and transaminase were similar between FHF patients and AH patients.

Serum levels of angiogenic factors

Table 2 shows serum levels of 9 angiogenic factors on admission in FHF patinets, AH patients and controls. There were significant differences in serum levels of HGF, IL-8, PDGF-BB and VEGF among the 3 groups. Serum levels of PDGF-BB and VEGF were lower in FHF patients than AH patients and controls (FHF patients vs. AH patients: P = 0.002 and 0.004, respectively; FHF patients vs. controls: P = <0.0001 and 0.0005, respectively). Serum IL-8 levels were lower in FHF patients than AH patients (P = 0.002); however there were no differences in serum IL-8 levels between FHF patients and controls (P = 0.19). Serum HGF levels were higher in FHF patients than controls (P = 0.0004), while differences in serum HGF levels between FHF patients and AH patients were borderline (P = 0.069). On the other hand, there were no differences in serum levels of angiopoietin-2, follistatin, G-CSF, leptin and PECAM-1 among the 3 groups.

Association of serum levels of angiogenic factors with prognosis

In the 30 patients with FHF or AH, between 12 patients with good outcomes and 18 patients with poor outcomes (including death and liver transplantation), there were

significant differences in serum levels of HGF (3037 ± 2313 pg/ml vs. 13014 ± 24635: P = 0.004), PDGF-BB (4099 ± 2413 pg/ml vs. 1653 ± 1072 pg/ml: P = 0.002) and VEGF (120 ± 114 pg/ml vs. 32 ± 25 pg/ml: P = 0.010) (Figure 1). By univariate logistic regression models, serum levels of PDGF-BB and VEGF were associated with poor outcomes in the 30 patients with FHF or AH (Table 3). The association of serum HGF levels with poor outcomes was borderline. There were no associations between serum levels of the other angiogenic factors and the prognosis.

Table 4 shows the AUC of platelet count, D.Bil/T.Bil ratio, PT activity, and serum levels of HGF, PDGF-BB and VEGF as a prognostic factor. The AUC of PDGF-BB was equal to those of T.Bil/D.Bil ratio and PT activity.

In the 30 patients with FHF or AH, serum PDGF-BB levels were significantly correlated with serum VEGF levels (r = 0.70, P <0.0001) (Table 5). Furthermore, serum PDGF-BB levels were correlated with platelet counts (r = 0.79, P <0.0001), PT activities (r = 0.37, P-value = 0.044) and D.Bil/T.Bil ratio (r = 0.50, P = 0.006). On the other hand, serum VEGF levels were correlated with platelet counts (r = 0.68, P <0.0001) and PT activities (r = 0.38, P = 0.040).

Discussion

In a setting of donor organ shortage, it is important to accurately identify FHF patients with poor outcomes in order to rescue more patients with liver transplantation. However, biomarkers for predicting accurate prognosis fall short. Previously, serum copy number of transforming growth factor-alpha mRNA and serum HGF levels were reported as biomarkers for predicting the prognosis of FHF patients.[12,13] However, the specificity (65.5%) of serum copy number of transforming growth factor-alpha mRNA was not sufficient in the original report.[12] Furthermore, in this study, the association of serum HGF levels with the prognosis of patients with FHF or AH was borderline. So, another biomarker is required. This study suggests that serum levels of PDGF-BB and VEGF may be useful as biomarkers for predicting a poor prognosis of FHF patients. This report is the first concerning serum levels of PDGF-BB and VEGF in FHF patients although sample size of this study was limited. We consider that serum levels of PDGF-BB and VEGF are worth investigating as biomarkers for predicting outcomes of FHF patients. In order to confirm these findings, a further study with larger sample size is required.

In this study, serum levels of PDGF-BB and VEGF were well correlated with platelet counts. Platelet release angiogenic factors such as PDGF-BB and VEGF.[14,15] In FHF patients, platelet counts and serum thrombopoietin levels are decreased.[16]

Thrombopoietin, produced primarily in the liver but also in the bone marrow and kidney, binds to the thrombopoietin receptor expressed on the surface of stem cells, megakaryocyte progenitor cells, megakaryocytes, and platelets.[17] Thrombopoietin regulates the development and maturation of megakaryocytes and subsequent release of platelets. Additionally, thrombopoietin enhances platelet activation and function. Thus, we speculate that thrombocytopenia due to the decrease of serum thrombopoietin levels may lead to the decrease of serum PDGF-BB and VEGF levels. Furthermore, in this study, serum levels of PDGF-BB and VEGF were correlated with PT activities. So, serum levels of PDGF-BB and VEGF may reflect the extent of liver failure.

In this study, the decrease of serum PDGF-BB and VEGF levels were associated with poor outcomes of FHF patients. Hepatocytes proliferation and liver regeneration are stimulated by VEGF.[6,18] Furthermore, PDGF were reported to increase the expression of pleiotrophin, a potent mitogen for hepatocytes, in sinusoidal hepatic stellate cells.[19] Thus, we speculate that the decrease of serum PDGF-BB and VEGF levels may delay liver regeneration, and this may result in the increased risk of multiple organ failure and infection and the poor outcomes in FHF patients. On the other hand, in human, clinical trials using recombinant VEGF or VEGF gene transfer were already reported.[20,21] Treatment using PDGF-BB and VEGF may provide a new therapeutic

strategy for acute liver failure.

Recently, a clinical trial of recombinant human HGF, which stimulates the proliferation of mature hepatocytes and hepatic progenitor cells, in patients with FHF was reported from Japan.[22] In the study, 4 patients received the administration of recombinant HGF, of whom, 2 died due to progression of liver failure and 2 were rescued. However, from the first, serum HGF levels are higher in FHF patients than AH patients or healthy controls. On the other hand, this study indicates that serum levels of PDGF-BB and VEGF are lower in FHF patients. Thus, treatment using PDGF-BB and VEGF may be more reasonable.

We consider that, in order to assess the usefulness of serum PDGF-BB and VEGF levels as biomarkers for predicting outcomes of FHF patients, the relation between the changes of serum PDGF-BB and VEGF levels during the clinical course and the prognosis of FHF patients should be assessed, although, in this study, we could not for lack of the serum collection after the introduction of treatment in AH patients and FHF patients. On the other hand, in this study, 13 of 17 FHF patients (76%) received plasma exchange combined with continuous hemodiafiltration. Serum IL-8 levels were reported to be decreased by plasma exchange combined with continuous hemodiafiltration.[23] However, the effect of plasma exchange combined with continuous hemodiafiltration on

serum PDGF-BB and VEGF levels has been yet to be fully implemented. Hereafter, to clarify these points is necessary.

In conclusion, lower serum levels of PDGF-BB and VEGF were associated with poor prognosis of FHF patients in this study. Thus, we consider that serum levels of PDGF-BB and VEGF are worth investigating as biomarkers for predicting outcomes of FHF patients.

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Table 1. Clinical characteristics and labolatory data on admission.

	FHF	AH	P-value
Patients, n	17	13	
Age, yr	37 ± 14	41 ± 17	0.34
Gender, female (%)	12 (71%)	6 (46%)	0.26
Etiology, n (%)			
Viral hepatitis	5 (29%)	7 (54%)	0.26
HAV	1 (5%)	4 (31%)	
HBV	4 (24%)	3 (23%)	
AIH	3 (18%)	3 (23%)	
Drug-induced	4 (24%)	1 (8%)	
Indeterminate	5 (29%)	2 (15%)	
Period from initial symptoms to the diagnosis of fulminant hepatic failure, day			
	14 ± 12	—	—
Hepatic coma, n (%)			
II	14 (82%)	—	—
III or IV	3 (18%)	—	—
Laboratory data			

WBC, /mm^3	10829 ± 6698	7616 ± 2250	0.33
Hemoglobin, g/dl	13.2 ± 1.7	14.2 ± 2.2	0.18
Platelet, $x10^4/mm^3$	12.0 ± 6.9	18.0 ± 8.3	0.054
T.Bil, mg/dl	14.3 ± 8.8	11.1 ± 8.15	0.27
D.Bil/T.Bil ratio	0.59 ± 0.14	0.68 ± 0.03	0.027
AST, IU/l	2876 ± 3888	2967 ± 4696	0.98
ALT, IU/l	2817 ± 2563	2728 ± 2573	0.93
Cr, mg/dl	1.0 ± 1.1	0.9 ± 0.5	0.45
PT activity, %	23 ± 10	42 ± 12	0.0005

AH, acute hepatitis; AIH, autoimmune hepatitis; ALT, alanine aminotransferase; AST, aspartate aminotransaminase; Cr, creatinine; D.Bil, direct birilubin; FHF, fulminant hepatic failure; HAV, hepatitis A virus; HBV, hepatitis B virus; PT, prothrombin; T.Bil, total bilirubin; WBC, white blood cell.

Table 2. Serum levels of 9 angiogenic factors on admission.

	FHF	AH	Control	P-value
Patients, n	17	13	20	
Angiopoietin-2, pg/ml	1078 ± 689	1440 ± 982	998 ± 462	0.51
Follistatin, pg/ml	728 ± 752	1235 ± 1763	697 ± 207	0.11
G-CSF, pg/ml	129 ± 57	260 ± 222	347 ± 335	0.22
HGF, pg/ml	9958 ± 21015	3197 ± 2588	1981 ± 791	0.002
IL-8, pg/ml	161 ± 68	710 ± 1167	263 ± 259	0.015
Leptin, pg/ml	3441 ± 2774	3935 ± 2537	4530 ± 1728	0.15
PDGF-BB, pg/ml	2050 ± 1572	4521 ± 2419	8506 ± 5500	<0.0001
PECAM-1, pg/ml	8340 ± 3347	6842 ± 3366	7573 ± 4328	0.47
VEGF, pg/ml	39 ± 38	144 ± 122	205 ± 121	0.0005

AH, acute hepatitis; FHF, fulminant hepatic failure; G-CSF, granulocyte colony-stimulating factor; HGF, hepatocyte growth factor; IL, interleukin; PDGF, platelet-derived growth factor; PECAM, platelet/endothelial cell adhesion molecule; VEGF, vascular endothelial growth factor.

Table 3. Associations of serum levels of 9 angiogenic factors with poor outcomes in 30 patients with FHF or AH by univariate logistic regression models.

	Odds ratio	95% CI	P-value
Angiopoietin-2, per 1 pg/ml increase	1.00	0.99-1.00	0.78
Follistatin, per 1 pg/ml increase	1.00	0.99-1.00	0.58
G-CSF, per 1 pg/ml increase	0.99	0.98-1.00	0.15
HGF, per 1 pg/ml increase	1.00	1.00-1.00	0.058
IL-8, per 1 pg/ml increase	0.99	0.99-1.00	0.12
Leptin, per 1 pg/ml increase	1.00	1.00-1.00	0.48
PDGF-BB, per 1 pg/ml increase	0.99	0.99-1.00	0.012
PECAM-1, per 1 pg/ml increase	1.00	1.00-1.00	0.17
VEGF, per 1 pg/ml increase	0.97	0.95-0.99	0.045

AH, acute hepatitis; CI, confidence interval; FHF, fulminant hepatic failure; G-CSF, granulocyte colony-stimulating factor; HGF, hepatocyte growth factor; IL, interleukin; PDGF, platelet-derived growth factor; PECAM, platelet/endothelial cell adhesion molecule; VEGF, vascular endothelial growth factor.

Table 4. Prognostic accuracy by the AUC using receiver operating characteristic curve in 30 patients with FHF or AH.

	AUC	95% CI	P-value
Platelet	0.76	0.57-0.94	0.020
D.Bil/T.Bil ratio	0.83	0.66-0.99	0.003
PT	0.80	0.64-0.97	0.006
HGF	0.81	0.66-0.97	0.005
PDGF-BB	0.83	0.69-0.98	0.003
VEGF	0.77	0.60-0.94	0.015

AUC, area under the curve; CI, confidence interval; D.Bil, direct birilubin; HGF, hepatocyte growth factor; PDGF, platelet-derived growth factor; PT, prothrombin; T.Bil, total bilirubin; VEGF, vascular endothelial growth factor.

Table 5. Associations between serum levels of angiogenic factors (HGF, PDGF-BB and VEGF) and laboratory data in 30 patients with FHF or AH.

	HGF	PDGF-BB	VEGF
HGF	—	-0.31	-0.29
PDGF-BB	—	—	0.70**
Platelet	-0.26	0.79**	0.68**
T.Bil	0.20	0.09	0.22
D.Bil/T.Bil ratio	-0.34	0.50**	0.35
AST	-0.11	-0.15	-0.39*
ALT	-0.08	-0.13	-0.26
Cr	0.31	-0.10	-0.21
PT activity	-0.36	0.37*	0.38*

Values shown the correlation coefficient (r) between the variables.

*, P <0.05; P <0.01; Cr, creatinine; D.Bil, direct birilubin; HGF, hepatocyte growth factor; PDGF, platelet-derived growth factor; PT, prothrombin; T.Bil, total bilirubin; VEGF, vascular endothelial growth factor.

Figure Legends

Figure 1. Serum HGF, PDGF-BB and VEGF levels of 30 patients with FHF or AH.

A) Serum HGF levels were lower in patients with good outcomes than those with poor outcomes (including death and liver transplantation) (P = 0.004). B, C) Serum PDGF-BB and VEGF levels were higher in patients with good outcomes than those with poor outcomes (P = 0.002 and 0.010, respectively).