ORIGINAL ARTICLE

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Increased Serum Activity of Matrix Metalloproteinase-9 in Patients with Acute Variceal Bleeding

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Background/Aims: Matrix metalloproteinases (MMP)-2 and -9 can degrade essential components of vascular integrity. The aim of this study was to investigate the association between those MMPs and variceal bleeding (VB). **Methods:** Fifteen controls, 12 patients with acute ulcer bleeding (UB) group, 37 patients with varix (V group), and 35 patients with acute VB group were enrolled. Serum was obtained to measure MMP-2 and -9 activity by zymogram protease assays. **Results:** The activity levels of these compounds were compared with the controls' median value. The median MMP-9 activity was 1.0 in controls, 1.05 in the UB group, 0.43 in the V group, and 0.96 in the VB group. The level of MMP-9 activity was higher in the VB group than in the V group ($p<0.001$). In the VB group, there was a significant decrease in MMP-9 activity over time after bleeding ($p<0.001$). The median MMP-2 activity level was 1.0 in controls, 1.01 in the UB group, 1.50 in the V group, and 1.55 in the VB group. The level of MMP-2 activity was similar in the VB and V groups. **Conclusions:** The level of MMP-9 activity increased in association with VB. The role of MMP-9 in the pathogenesis of VB should be verified. **(Gut Liver 2012;6:249-255)**

Key Words: Hemorrhage; Esophageal and gastric varices; Matrix metalloproteinase 2; Matrix metalloproteinase 9

INTRODUCTION

Matrix metalloproteinase (MMP)-2 and -9 are members of the MMP family that can degrade several components of the extracellular matrix (ECM). MMP-2 is a gelatinase A or 72kDa type IV collagenase. MMP-9 is a gelatinase B or 92kDa type IV collagenase.[1] The substrates of MMP-2 and -9 are denatured interstitial collagens (gelatins), type V collagen, and type IV collagen.[1] These MMPs can cleave noncollagenous ECM including elastin, fibronectin, and laminin.[1]

The collagen, elastin, fibronectin, and laminin support the cellular components of blood vessels.[2] These ECMs are important for the maintenance of the structure of blood vessels. Abnormalities of the ECMs are associated with variety of vascular diseases.[3]

The degrading effect of the ECM associated with MMP-2 and -9 plays an important physiological role in vascular remodeling.[4-6] However, the over expression of MMP-2 and -9 is associated with pathologic changes in the vascular wall. The development of aortic artery aneurysms is associated with an increased production or activity of MMP-2 and -9.[7-9] In cases with aneurysm rupture, the MMP-2 and -9 are increased not only in the aortic wall but also in the blood.[7,10-13] The mechanisms involved in the development of aneurysm formation and rupture and the association of MMPs with this process may be attributed to the degradation of the ECM proteins and subsequent weakening of the aortic wall.[14] The blood and venous tissue levels of MMP-2 and -9 are increased in varicose veins.[15-17] The expression and activity of these MMPs are increased in thrombophlebitic varicose veins.[16] The MMP-2 and -9 may be associated with the mechanism of the development of varicose veins and thrombophlebitis formation.

Gastroesophageal variceal bleeding (VB) is the most serious life threatening complication of cirrhosis. Important predictors of VB include large varices, decompensated cirrhosis (Child B or C), and the endoscopic findings of red whale marks.[18] Although the risk factors for VB have been evaluated by numerous prior studies, there are few reports explaining the mechanisms involved in VB.

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It is well known that the formation of gastroesophageal varices is attributed to increased portal pressure. In addition, there may be variceal ECM changes that enlarge and weaken the vessel wall similar to the mechanism of the development of aortic aneurysms and varicose veins. We assume that there may be a marked increase of portal pressure before VB occurs. In addition, MMP-2 and -9 may also increase. Therefore, we hypothesized that increased those MMPs may degrade vascular matrix proteins and induce VB. The aim of this study was to evaluate whether the changes of MMP-2 and -9 is associated with acute VB.

MATERIALS AND METHODS

1. Patients

Thirty five cirrhotic patients with acute VB group, 12 patients with acute gastric or duodenal ulcer bleeding (UB group), 37 cirrhotic patients with varices (V group), and 15 healthy controls were enrolled from January 2004 to December 2005. All patients and controls were Korean and admitted to Gil Hospital, Incheon, Korea.

Patients with hematemesis or melena that showed fresh blood in the stomach and had bleeding stigmata on the varices or active VB at endoscopy were considered to have acute VB. All bleeding signs and symptoms such as hematemesis and melena occurred within 6 hours prior to admission. One patient with gastric VB and 34 patients with esophageal VB were enrolled in this study.

All cirrhotic patients in the V group had varix, but no current VB. They were admitted to the hospital for spontaneous bacterial peritonitis (n=5), hepatic encephalopathy (n=3), jaundice (n=3), ascites (n=2), abdominal pain due to hepatocellular carcinoma (HCC) (n=1), and 23 patients had no specific symptoms.

The types of esophageal varices were classified into three categories according to the report of Beppu *et al*.[19]

Acute UB was defined in patients with hematemesis and melena that showed fresh blood in the stomach and bleeding stigmata on the ulcer or active UB at endoscopy. All bleeding signs and symptoms such as hematemesis and melena occurred within 6 hours prior to admission. Eleven patients with gastric UB and one patient with duodenal UB were enrolled. None of the enrolled patients had chronic viral hepatitis or liver cirrhosis.

Fifteen controls were healthy and had no current or past history of liver diseases or any specific diseases. Their median age was 37 (29 to 62) years old and number of male was 9.

2. Blood samples

The serum from patients with acute variceal or UB was collected initially during routine blood examination when the patients were admitted to the emergency room. Subsequent serum sampling was obtained at 2 and 6 days after admission. However, the most patients with UB were usually discharged early, serum samples at 6 days were not obtained in those patients. The serum from the V group and controls was taken during visits to the hospital for the study period. However, the serum from the patients who have spontaneous bacterial peritonitis or hepatic encephalopathy, was obtained after all problems were resolved. All blood samples were stored at -70°C for determination of MMPs and tumor necrosis factor (TNF)-α.

3. Determination of the serum activity of MMP-2 and -9

The serum activity of MMP-2 and -9 were measured by zymogram protease assays. Ten percent sodium dodecyl sulfate (SDS) polyacrylamide gels (acrylamide/bis acrylamide, 29/1) containing 0.05% gelatin were used for electrophoresis. Serum samples (containing 30 μg protein) were prepared with standard SDS-gel-loading buffer (0.1% SDS). Electrophoresis was performed at 60 V for 6 hours and then the gel was washed twice with 100 mL distilled water containing 2.5% Triton X-100 on a shaker for 30 minutes at room temperature. The gel was then incubated in 100 mL developing buffer (50 mM Tris-HCl, 0.2 M NaCl, 5 mM $CaCl_2$, 1% Triton X-100) for 16 hours at 37°C, stained with Coomassie brilliant blue R-250, and destained with methanol-acetic acid-water (50:75:875, v:v:v).

4. Quantification of MMPs activity

Relative MMP activity was quantified by scanning the zymogram photograph on a gel documentation and analysis system (Kodak Image Station 4000MM; Eastman Kodak, Rochester, NY, USA). To normalize the possible difference between zymographs, two healthy control samples were used for each gel to serve as internal controls. The digitized data of each photograph were normalized by their own internal controls.

5. Determination of serum TNF-α level

The serum level of TNF-α was determined by an enzyme-linked immunosorbent assay (ELISA, Endogen Human TNF-α ELISA kit; Pierce Biotechnology Inc., Rockford, IL, USA).

6. Statistical analysis

Continuous variables were expressed as the median and range. The Mann-Whitney non-parametric test was used for comparing continuous variables between groups. Within group, the Wilcoxon signed-rank test or Friedman test were used for the comparison of each change of MMP-2, -9, and TNF-α. The χ^2-test or Fisher's exact test were used for comparing categorical variables between groups. A p-value less than 0.05 was considered statistically significant. The SPSS version 12.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis.

RESULTS

1. Comparison of clinical characteristics and hemodynamic values between the VB and UB groups

The median age and sex ratio were not different between the VB and UB groups. The levels of AST, ALT, and total bilirubin were higher and prothrombin time was more prolonged in the VB group than in the UB group. The level of albumin, hemoglobin, and platelet count were lower in the VB group compared to the UB group (Table 1).

Hemodynamic values such as systolic, diastolic, mean arterial blood pressure, and pulse rate were not different between the two groups. The amount of blood transfusion and intravenous fluid administration during the initial 24 hours were not different between the two groups (Table 2).

2. Comparison of clinical characteristics and hemodynamic values between the VB and varix groups

The median age was higher in the VB than in V groups. The sex ratio and causes of cirrhosis were not different between the two groups. Alcohol was the most common cause of cirrhosis in both two groups. The levels of AST, ALT, total bilirubin, platelet count, albumin, prothrombin time, and the distribution of Child-Pugh class were not different between the two groups. The hemoglobin level was lower in the VB group than in the V group. The size of the varix was not different between the two groups. The frequency of HCC was not different between the two groups. Except for one case of HCC, all HCC cases had

Table 1. Baseline Clinical and Laboratory Characteristics

Characteristic	Ulcer bleeding (n=12)	p-value	Varix bleeding (n=35)	p-value	Varix (n=37)
Age, yr	51 (37-88)	0.641	50 (30-85)	0.027	58 (34-72)
Sex, male/female	10/2	0.703	26/9	0.4	31/6
Causes				0.089	
Alcohol			23		18
HBV			7		17
HCV			4		1
Cryptogenic			1		1
AST, U/L	17 (11-28)	<0.001	64 (7-1143)	0.195	47 (19-256)
ALT, U/L	12 (5-234)	0.005	26 (7-690)	0.963	26 (7-156)
Total bilirubin, mg/dL	0.6 (0.4-0.9)	<0.001	2.2 (0.7-13.5)	0.297	2.9 (0.3-18.6)
Albumin, g/dL	3.5 (3.1-4.6)	<0.001	2.9 (1.9-4.1)	0.125	3.1 (1.8-4.5)
Hemoglobin, g/dL	10.0 (2.8-12.9)	0.033	8.3 (3.0-14.8)	<0.001	11.0 (7.9-17.5)
Platelet, $\times 10^3/\mu L$	262 (205-425)	<0.001	103 (35-241)	0.112	75 (23-242)
PT, sec	12.6 (12.1-14.3)	<0.001	16.9 (12.7-60)	0.083	15.2 (11.5-26.2)
Varix size, F1/F2/F3			6/18/11	0.468	8/22/7
Child-Pugh class, A/B/C			6/17/12	0.97	7/17/13
HCC			4	0.736	6

Data are presented as numbers of patients or the median (range).
HBV, hepatitis B virus; HCV, hepatitis C virus; AST, aspartate aminotransferase; ALT, alanine aminotransferase; PT, prothrombin time; HCC, hepatocellular carcinoma.

Table 2. Baseline Hemodynamic Values and Patient Management

	Ulcer bleeding (n=12)	p-value	Varix bleeding (n=35)	p-value	Varix (n=37)
BP, mm Hg					
Systolic BP	100 (70-140)	0.844	100 (50-150)	0.024	113 (90-148)
Diastolic BP	60 (40-100)	0.8	60 (0-90)	0.003	70 (50-90)
Mean arterial BP	77 (50-113)	0.624	77 (25-110)	0.018	83 (63-108)
Pulse rate, /min	84 (78-106)	0.292	87 (68-156)	<0.001	72 (52-92)
Managements					
IV fluid, mL	3,000 (2,180-5,100)	0.617	3,076 (1,730-4,880)		
Blood transfusion, P	3 (1-6)	0.444	3 (1-7)		

Data are presented as number of patients or the median (range).
BP, blood pressure; IV, intravenous; P, pack.

a stage less than III (according to the modified International Union against Cancer Tumor-Node-Metastasis stage) (Table 1).

The systolic, diastolic, and mean arterial pressures were lower in the VB group than in the V group. The heart rate was more rapid in the VB group than in the V group (Table 2). Beta blocker had been administrated to 11 patients in the VB group and 21 patients in the V group prior to enrollment.

3. Serum activity of MMP-9 in controls and patients at enrollment

The median serum activity of MMP-9 in the V group (0.43; range, 0.14 to 1.31) was the lowest value among all patient groups and controls. The MMP-9 activity in the UB group at bleeding (1.05; range, 0.83 to 1.31) was not different from controls (1.0; range, 0.7 to 1.4). The MMP-9 activity in the VB group at bleeding (0.96; range, 0.5 to 4.24) was higher than in the V group ($p<0.001$) (Figs 1 and 2A).

4. Change of serum activity of MMP-9 after bleeding

The median serum activity of MMP-9 activity in the UB group was significantly decreased from 1.05 to 0.89 (0.77 to 1.22) at bleeding and 2 days after bleeding, respectively ($p=0.015$) (Fig. 1).

The MMP-9 activity in the VB group decreased over time ($p<0.001$). The MMP-9 activity decreased from 0.96 at bleeding to 0.81 (0.32 to 2.39) after 2 days ($p<0.001$), and to 0.65 (0.15 to 1.33) after 6 days after bleeding ($p=0.01$) (Figs 1 and 2B).

5. Serum activity of MMP-2 in patients and controls at enrollment

The median serum activity of MMP-2 in controls (1.0; range, 0.85 to 1.16) was not different from the UB group (1.01; range, 0.59 to 2.2) at bleeding. The MMP-2 activity in the VB group (1.55; range, 0.38 to 7.01) at bleeding was not different from the V group (1.5; range, 0.97 to 1.85). Its activity in the UB group at bleeding was lower than in the VB group at bleeding ($p=0.002$) and the V group ($p=0.001$) (Figs 2A and 3).

6. Change of serum activity of MMP-2 after bleeding

The median serum activity of MMP-2 in the UB group was decreased from 1.01 at bleeding to 0.95 (0.45 to 2.68) at 2 days after bleeding. However, there was no significant change in its activity between the two time points. The MMP-2 activity in the VB group decreased from 1.55 at bleeding to 1.49 (0.37 to 4.42) at 2 days ($p>0.05$), and to 1.44 (0.27 to 6.42) at 6 days ($p>0.05$) after bleeding. There was no significant change in its activity between each two time points (Fig. 3).

7. Basal serum TNF-α level and change of serum TNF-α level after bleeding

The median serum level of TNF-α was less than 1 pg/mL in controls and the UB group at bleeding. The TNF-α level in the VB group (52.5; range, 7.8 to 182.7 pg/mL) at bleeding was higher than that in the V group (23.7; range, 0 to 195.4 pg/mL). The TNF-α level was very high in the V group and the VB group at bleeding compared to controls and the UB group ($p<0.001$) (Fig. 4).

The TNF-α level in the UB group was remained at a very low level at 2 days after bleeding. The TNF-α level in the VB group was decreased from 52.5 pg/mL at bleeding to 44.8 pg/mL (range, 12.8 to 302 pg/mL) at 2 days ($p>0.05$), and decreased to 39 pg/mL (range, 8.9 to 157.6 pg/mL) at 6 days ($p>0.05$) after bleeding. There was no significant change in the level of TNF-α between each two time points (Fig. 4).

DISCUSSION

The serum activity of MMP-9 might increase before VB and

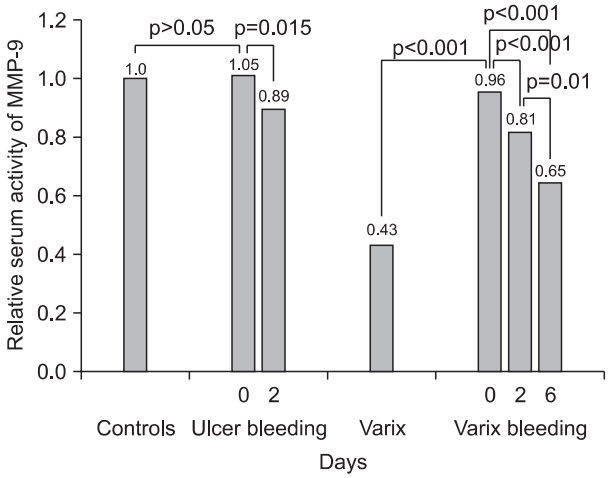

Fig. 1. Relative median serum activity of matrix metalloproteinase (MMP)-9. After the median serum activity of MMP-9 in controls is determined to be 1.0, each measurement of MMP-9 serum activity is compared with the controls' median value.
Day 0, at the time of bleeding; Days 2 and 6, days after bleeding.

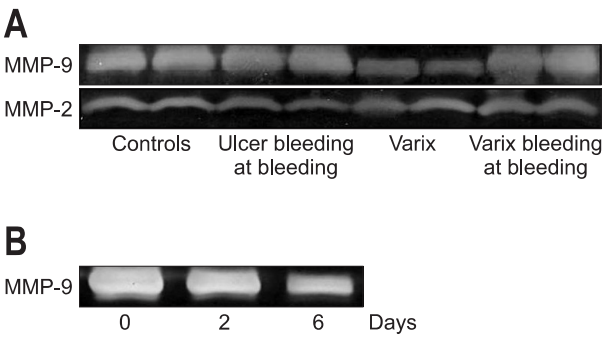

Fig. 2. The serum activity levels of matrix metalloproteinase (MMP)-2 and -9 are measured using zymogram protease assays. (A) The level of MMP-9 activity is lowest in the varix group. The level of MMP-2 activity is higher in the varix group and varix bleeding group at the time of bleeding compared with the controls and ulcer bleeding group at the time of bleeding. (B) The level of MMP-9 activity decrease over time.
Day 0, at the time of bleeding; Days 2 and 6, days after bleeding.

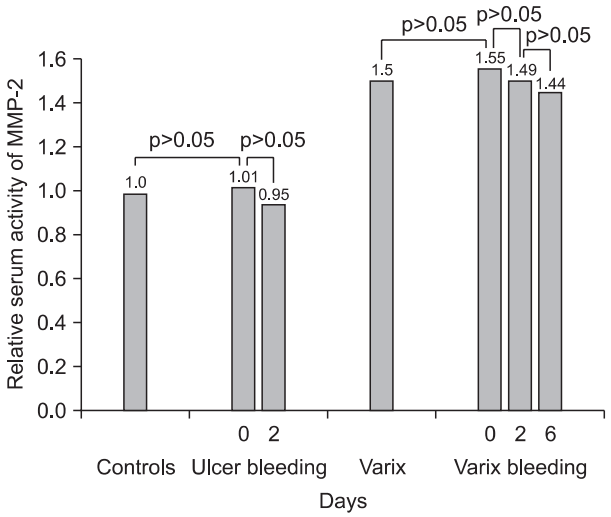

Fig. 3. Relative median serum activity level of matrix metalloproteinase (MMP)-2. The median serum activity of MMP-2 in the controls is considered to be 1.0; the serum activity of MMP-2 is expressed by a comparison with the median value observed among the controls.
Day 0, at the time of bleeding; Days 2 and 6, days after bleeding.

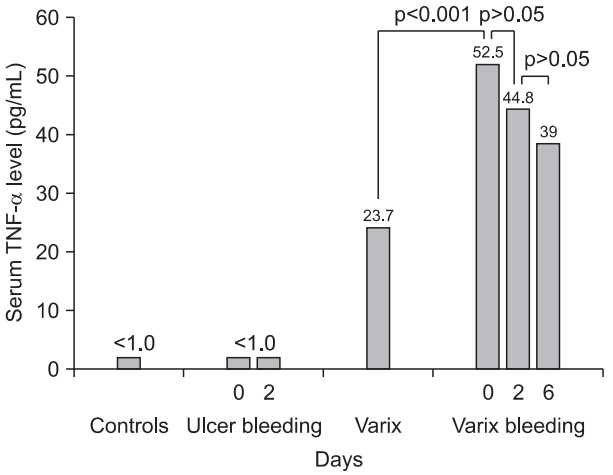

Fig. 4. The median level of serum tumor necrosis factor (TNF)-α.
Day 0, at the time of bleeding; Day 2 and 6, days after bleeding.

subsequently decrease to basal level over time. The MMP-9 activity in the VB group at bleeding was higher than in the V group that had varix without current bleeding. The MMP-9 activity in the VB group subsequently decreased to basal level that was still higher level than that in the V group. Therefore, the serum activity of MMP-9 might have increased before VB. Although it was not possible to measure the serum activity of MMP-9 just before and at the time of on set of bleeding, the following evidences might support the above possibility. First, all patients with VB in this study had bleeding related signs and symptoms within 6 hours before admission. Second, blood samples were collected immediately in the emergency department when the patients were admitted. Third, seventeen patients (50%) in the VB group had current active bleeding at endoscopy. Fourth, the sudden hemodynamic change due to acute blood loss occurred in both the VB group and UB group. However, the increased MMP-9 activity was observed only in the VB group. Fifth, one VB case had re-bleeding at 8 days after bleeding. The MMP-9 activity in this case decreased from 1.34 at bleeding to 0.76 after 2 days, but re-increased to 0.89 after 6 days after bleeding. Therefore, the MMP-9 activity might be increased before VB and the increased MMP-9 activity may be involved in the mechanisms associated with VB.

In this study, the basal MMP-9 activity in the V group was the lowest among all patient groups and controls. The MMP-9 activity is low in cirrhotic patients compared to normal controls and decreases over time as chronic liver disease progresses to liver cirrhosis.[20-22] The reason why the MMP-9 activity is low in cirrhotic patients compared to healthy controls is unclear. Further study is needed to evaluate the role of MMP-9 in the pathogenesis of liver cirrhosis.

MMP-9 plays an important role in aortic aneurysm formation[7-9] and rupture,[7,10-13] this has been demonstrated in experimental models of aortic aneurysm formation in mice.[23] MMP-9 is the most abundantly expressed MMP in aortic aneurysm tissue, it is produced mainly by the aneurysm-infiltrating macrophages.[7] It is also detected in the liver and is produced mainly by Kupffer cells.[1] It is increased in the histological layers of varicose veins and associated with thrombophlebitic varicose veins.[16] Therefore, increased MMP-9 is associated with inflammation and tissue macrophages. Some have suggested that infection and inflammation are associated with the development of VB. Bacterial infections have been documented in 35% to 66% of cirrhotic patients with VB.[24] The endotoxemia secondary to bacterial infection may be occurred and increase portal pressure by induction of endothelin, the most potent vasoconstrictor in portal vein.[25] These effects induce sudden increases of portal vein pressure in patients that already have large varices with high wall tension and portal pressure, and finally lead to VB.[25] In addition, we hypothesize that the sudden increased serum activity of MMP-9 might degrade the ECM that supports the vessel wall and finally lead to VB. Although we did not evaluate directly whether the endotoxemia could induce MMP-9, it is possible that the increased MMP-9 activity might be associated with the endotoxemia through induction of TNF-α in cirrhotic patients.[26-30]

TNF-α is a potent inflammatory cytokine that has a variety of functions and is induced by endotoxin.[26] TNF-α promotes MMP-9 induction through the activation of the NF-κB pathway.[27-30] Therefore we investigated whether change of the serum TNF-α level in VB paralleled the change of MMP-9 activity over time. The increasing and decreasing patterns of TNF-α level was similar to those of the MMP-9 activity in the VB group although the TNF-α level was not significantly decreased after bleeding event. Therefore, TNF-α appears to play an important

role in the mechanism associated with VB and may initiate an increase in MMP-9 activity. However, further study is needed to evaluate the factors involved in initiating increase of TNF-α and determines whether the endotoxin increases TNF-α and MMP-9 activity sequentially. And additional study is also needed to investigate whether the increase in MMP-9 activity is the simple reflection of the increase of TNF-α or really associated with destruction of vascular wall.

An important risk factor associated with VB is red marks or red spots on the varix. Histological studies of esophageal transaction rings from the area of bleeding show that the red spots on varices are dilated blood-filled channels that are not lined by endothelial cells and basement membrane.[31] The endothelial cell is an important cell that secretes many vasoactive substances and regulates vascular tone.[32] The basement membrane is essential for the survival and function of the endothelial cell.[33] Portions without endothelial cells and basement membrane in blood vessels may be vulnerable to changes in vascular pressure, and subsequently may be easily ruptured. Type IV collagen is the major ECM component of basement membrane.[34] MMP-9 has a strong proteolytic effect on type IV collagen.[1] Its increase might induce weakening of the basement membrane and detachment of endothelial cells. Therefore, increased MMP-9 activity may be associated with the mechanism of VB.

After bleeding, the MMP-9 activity in the UB group decreased slightly over time. The cause of the decrease in the MMP-9 activity is unclear. However, the hemodynamic supports with intravenous fluid administration might have had a dilution effect on the MMP-9 activity. Compared to the UB group, the MMP-9 activity in the VB group remarkably decreased over time. This decreasing pattern means that the MMP-9 activity returns to the basal level over time after bleeding.

The MMP-9 is associated with tumor invasiveness and metastasis in HCC by its proteolytic effect of surrounding ECM.[35] In this study, all of HCC cases were not advanced stage. The prevalence of HCC was not different between V and VB groups. Even if cases with HCC were excluded in analysis, the MMP-9 activity in VB group at bleeding was still increased than that in V group (data not shown).

In contrast to the MMP-9, the serum activity of MMP-2 in the VB group at bleeding was not different from the V group. In addition, there was no significant change of the MMP-2 activity after bleeding. Therefore, MMP-2 dose not appear to be associated with VB.

The MMP-2 activity in liver tissues and blood increases when chronic liver disease progresses to liver cirrhosis[20,21,36] because hepatic stellate cells that mainly produce the MMP-2 in the liver are activated.[1] In this study, the MMP-2 activity in all cirrhotic patients was higher than that in controls and the UB group.

The limitations of this study included the followings. First, this study indirectly evaluated the role of MMP-9 in the mechanism of VB. Further study that can elucidate the mechanism through *in vitro* or *in vivo* experiments is needed. Second, serum samplings were not taken before VB. Serial samplings before VB are needed to elucidate whether the MMP-9 activity is increased before onset of VB. Third, there is possibility that the increased MMP-9 activity is simple reflection of the associated conditions with VB such as endotoxemia or acutely decreased liver function. Comparison study between endotoxemia with and without VB, and between UB and VB in patients with liver cirrhosis is needed.

In conclusion, the increased serum activity of MMP-9 present at VB in cirrhotic patients may be associated with the mechanism of VB. The increased MMP-9 activity may be involved in the destruction of the variceal matrix and facilitate rupture of the varix. Further studies are needed to specifically determine the pathophysiology of this process.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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