Acta Med. Okayama, 2014
Vol. 68, No. 3, pp. 157–162

Acta Medica Okayama
http://escholarship.lib.okayama-u.ac.jp/amo/

Original Article

Usefulness of High Mobility Group Box 1 Protein as a Plasma Biomarker in Patient with Peripheral Artery Disease

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Atherosclerosis is often associated with chronic vascular inflammation. High-mobility group box 1 protein (HMGB1) plays various roles, not only as a transcriptional regulatory factor in the nucleus, but also as an inflammatory mediator. A previous study suggested that fibrinogen is an important factor associated with atherosclerosis progression. The present study was performed to examine the levels of plasma HMGB1 protein in atherosclerosis patients. We studied 24 patients with peripheral artery disease (PAD) with atherosclerosis, and 10 healthy controls. We found that the concentrations of HMGB1 were increased in the plasma of the patients with atherosclerosis, and there were significant correlations between the plasma HMGB1 and fibrinogen levels. Plasma HMGB1 may play a key role in the pathogenesis of clinical and experimental atherosclerosis.

Key words: HMGB1, fibrinogen, atherosclerosis, peripheral artery disease

Atherosclerosis is the one of the most common causes of cardiovascular disease. Although atherosclerosis is associated with hypertension, diabetes and hyperlipidemia, recent studies suggest an additional association with inflammation and the coagulation system.

High-mobility group box 1 protein (HMGB1) is a nuclear protein present in many cells. When inflammation occurs, HMGB1 is released into the extracellular space in both an active and a passive manner. It functions as a signal for inducing inflammation and as an activator for inducing the immune response [1, 2]. The action of extracellular HMGB1 appears to be dependent on its interactions with several cell surface receptors, *e.g.*, the receptor for advanced glycation end products (RAGE) and toll-like receptor 2/4 (TLR-2/4). Extracellular HMGB1 has been reported in a variety of clinical conditions associated with inflammation and reperfusion injury [3–6].

It's was suggested that atherosclerosis is characterized by a chronic inflammatory response to arterial wall injury [7]. RAGE is expressed in human atherosclerotic lesions [8]. Smooth muscle cells with atherosclerosis show increased expressions of HMGB1 and RAGE [9, 10]. RAGE expression is significantly up-regulated in macrophages associated with atherosclerotic lesions [8], and inhibition of RAGE signaling prevents the progression of atherosclerotic injury [11].

In the coagulation system, serum fibrinogen is one of the factors associated with blood coagulation and viscosity. Fibrinogen may therefore be closely related to atherosclerosis [8–10]. It was suggested that the mortality rate of individuals with cardiovascular disease is associated with high levels of serum fibrinogen [12]. Fibrinogen is essential for fibrin formation

Received September 20, 2013; accepted February 12, 2014.
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under the influence of thrombin and platelet aggregation [13]. In addition, fibrinogen synthesis is stimulated by cytokines from activated macrophages, and thus fibrinoge behaves as an acute-phase protein [14].

These observations suggest that fibrinogen, like HMGB1, might be associated with the coagulation and inflammation systems. In the present stud, we examined the expression of plasma HMGB1 in patients with atherosclerosis and we evaluated the correlation between plasma HMGB1 and the inflammatory or coagulation systems.

Materials and methods

The plasma levels of HMGB1 were measured in 10 healthy controls (age 66.3 ± 11.7 years, 7 males and 3 females) and 24 PAD patients (age 64.5 ± 17.4 years, 21 males and 3 females). All patients and volunteers provided informed consent, and the study was approved by the institutional review board at Okayama University Hospital (Okayama, Japan). First blood samples (0.5 mL) were collected from the subjects' peripheral vein and then centrifuged (3,000 rpm, 10 min) to obtain plasma samples. Second blood samples (0.5 mL) were collected using citric acid and centrifuged to obtain plasma samples. After centrifugation, these samples were stored at −80℃ until they were analyzed. The last blood samples (0.5 mL) were collected using EDTA 2Na for the blood cell counts.

The concentration of HMGB1 in plasma samples was determined using an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's protocol (Shino-Test, Sagamihara, Japan). The HMGB1 levels are expressed as nanograms per milliliter (ng/mL). We then analyzed the white blood cells count (WBC: $\times 10^4/mm^2$), C-reactive protein (CRP: mg/dL), platelet count (Plt: $\times 10^4/mm^2$), activated partial thromboplastin time (APTT: sec), and fibrinogen (Fib: mg/dL) using the standard methods established by the Department of the Central Clinical Laboratory, Okayama University Hospital.

The patients were divided into 2 groups according to the ankle brachial pressure index (ABI): the mild PAD group (ABI ≥ 0.6) and the severe PAD group (ABI < 0.6). All data are expressed as the mean ± SD. Group comparisons were performed using an analysis of variance (ANOVA) followed by the Mann-Whitney U-test. Correlation coefficients were determined using the Spearman rank test. In order to identify possible confounders of the correlation between HMGB1 and PAD both a univariate and a multivariate analysis with a multiple linear regression model were performed. A probability of < 0.05 was considered to be significant.

Results

HMGB1 expression in systemic circulation. The patients with PAD showed higher plasma levels of HMGB1 compared to the healthy controls (n = 10, median 4.63 ng/mL; 95% confidence interval [CI] 3.56–5.41). In addition, there were significant differences in the plasma HMGB1 levels between the mild (n = 12, median 6.16 (3.51–11.25; 95% CI) ng/mL; $p < 0.05$ vs. severe PAD group) and severe PAD patients (n = 12, median 16.18 ng/mL; 95% CI 3.18–29.00; $p < 0.01$ vs. control group) (Fig. 1).

Relationship between plasma HMGB1 and inflammatory or coagulation factors. The demographic data for the 24 subjects are presented in Table 1. The PAD grade also showed a significant correlation with the plasma level of fibrinogen (438 ± 99 mg/dL in the mild PAD group vs. 559 ± 151 mg/dL in the severe PAD group; $p < 0.05$). Table 2 shows the results of the multivariable analysis for factors correlated with plasma HMGB1 levels. Both the ABI ($p = 0.003$) and the plasma level of fibrinogen ($p < 0.001$) were significantly associated with the subjects' HMGB1 levels. However, CRP and other coagula-

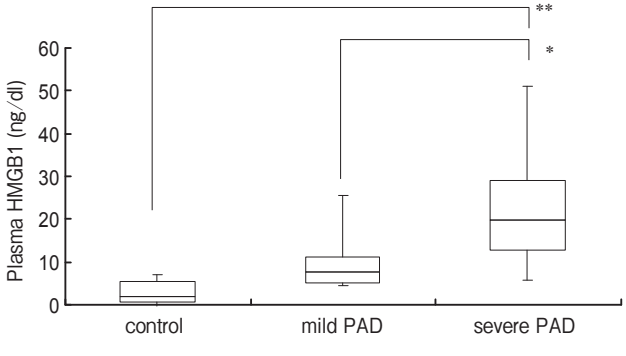

Fig. 1 The plasma levels of HMGB1 in atherosclerosis patients and relations between atherosclerosis progress and plasma levels of HMGB1. Data represent the median ± 95% confidence interval (CI). $^*p < .05$, $^{**}p < .01$ in comparison to the severe PAD group. PAD; peripheral artery disease.

Table 1 Comparison of baseline characteristics according- Fontaine in PAD Patients

PAD Patients Variables	mild PAD *(n=12)*	severe PAD *(n=12)*	*p value*
Male	9 (75.0)	12 (100.0)	
Age, years	63.9 ± 15.9	65.1 ± 18.0	0.869
Body weight, kg	63.3 ± 13.1	59.6 ± 12.2	0.504
Systoric BP, mmHg	145.4 ± 16.2	134.2 ± 12.4	0.109
Diastolic BP, mmHg	77.1 ± 8.0	77.6 ± 6.7	0.880
Ambulatory distance, m	174.3 ± 294.4	34.5 ± 30.2	0.116
ABI	0.72 ± 0.10	0.32 ± 0.15	<.001
Smoking	12 (100.0)	11	91.6
Medication for			
Hypertention	11 (91.6)	7 (83.3)	
Diabetes	7 (58.3)	3 (25.0)	
Hyperlipidemia	7 (58.3)	8 (32.0)	
Laboratory values			
WBC, $\times 10^4/mm^2$	6,536 ± 3,576	8,583 ± 3,623	0.209
Hb, mg/dl	12.1 ± 2.4	12.8 ± 2.4	0.509
Hct, %	36.6 ± 6.8	38.3 ± 6.6	0.556
Plt, $\times 10^4/mm^2$	22.2 ± 7.2	34.4 ± 19.7	0.078
APTT, sec	32.9 ± 4.8	31.8 ± 6.7	0.657
Fibrinogen, mg/dl	438 ± 99	559 ± 151	0.045
CRP, mg/dl	1.25 ± 2.13	1.38 ± 3.00	0.912
HMGB1, ng/ml	7.38 ± 6.09	19.08 ± 15.59	0.024

Data are expressed as mean ± SD or number (percentage).
PAD, peripheral artery disease; BP, blood pressure; ABI, ankle brachial pressure index; WBC, white blood cell number; Hb, hemoglobin; Hct; Plt, platelet number; PT, prothorombin time; APTT, active partial thromboplastin time; CRP, C-reactive protein.

Table 2 Correlation coefficients for HMGB1 in PAD patients

Variables	Correlation coefficients	*p value*
Age, years	−0.022	0.922
Body weight, kg	0.170	0.225
Systoric BP, mmHg	−0.288	0.231
Diastolic BP, mmHg	−0.301	0.210
Ambulatory distance, m	−0.194	0.353
ABI	−0.566	0.003
Smoking	0.124	0.555
Medication for		
Hypertention	−0.126	0.438
Diabetes	−0.231	0.307
Hyperlipidemia	−0.313	0.127
Laboratory values		
WBC, $\times 10^4/mm^2$	0.097	0.677
Hb, mg/dl	0.127	0.584
Hct, %	0.093	0.688
Plt, $\times 10^4/mm^2$	0.359	0.110
APTT, sec	0.211	0.371
Fibrinogen, mg/dl	0.754	<0.001
CRP, mg/dl	0.229	0.331

PAD, peripheral artery disease; WBC, white blood cell number; APTT, active partial thromboplast; CRP, C-reactive protein.

tion-related factors demonstrated no significant relationship with HMGB1.

Fig. 2 shows the relationships between the plasma HMGB1 and fibrinogen levels. The correlation coefficient was 0.455. Table 3 shows the results of the multivariable analysis for factors correlated with plasma HMGB1 levels. The only factor significantly associated with the HMGB1 level was fibrinogen ($p = .003$). The multiple stepwise regression analysis showed that fibrinogen was the only factor independently related to the HMGB1 levels ($R^2 = 0.353$) (Table 4).

Discussion

Atherosclerosis and extracellular HMGB1. Our results showed an association between atherosclerosis and increased plasma HMGB1 levels. Plasma HMGB1 levels have been reported to increase during inflammation, such as sepsis [1] and lung inflammation [3]. In the present study, the plasma HMGB1 levels in patients with PAD were much

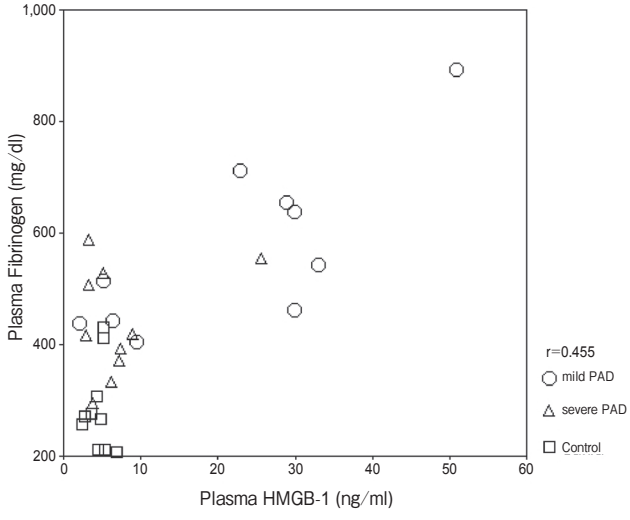

Fig. 2 The scatterplot shows that the relations between Plasma HMGB1 levels and fibrinogen for healthy control and peripheral artery disease. r, correlation coefficient.

Table 3 Multivariable analysis for correlates of HMGB1 levels

Characteristics	β	SE	p
ABI	−0.563	10.118	0.310
WBC	0.201	0.774	0.455
Hb	−0.133	4.412	0.892
Plt	−0.474	0.019	0.100
APTT	1.843	2.758	0.056
Fibrinogen	0.783	0.021	0.003
CRP	−1.400	3.006	0.065

Table 4 Multiple stepwise regression analysis for correlate of plasma HMGB1

Characteristics	β	SE	p
Fibrinogen	0.594	0.019	0.006

$R^2 = 0.353$

higher than those in healthy controls (Table 1). The source of circulating HMGB1 in atherosclerosis patients has not been determined, but 2 mechanisms are speculated to increase the general circulating HMGB1 levels. A local inflammatory secretory release of HMGB1 might be induced from immune responder cells such as monocytes, macrophages and endothelial cells with cytokine stimulation. Atherosclerotic lesions express high levels of HMGB1, thus leading to inflammation in atherosclerotic lesions [1, 2, 9]. However, the source of plasma HMGB1 may be not only immune responder cells but also vascular smooth muscle cells in atherosclerotic plaques [10].

In atherosclerotic plaques, endothelial cells and smooth muscle cells contain cytoplasmic HMGB1. HMGB1 is accumulated from the cytoplasm into secretory vesicles. However, atherosclerotic plaques contain extracellular HMGB1, which is released from necrotic cells [9]. While atherosclerotic inflammation continues, HMGB1 may be released into circulating blood.

It was suggested that HMGB1 is associated with the inflammatory and coagulation systems [15]. During the last stage of atherosclerosis, plaque rupture induces the formation of a thrombus. Endothelial cells lose their anti-coagulation properties and adhere to platelets. As shown in Fig. 1, the plasma levels of HMGB1 correlate with the progression of atherosclerosis. Normally, patients with severe PAD have severe local infection and gangrene of the foot. However, our present findings indicate that the infection-related factors WBC and CRP were not significantly related to the ABI degree, and thus the plasma level of HMGB1 may be independent from local infection or necrosis. Therefore, the extracellular HMGB1 secreted from atherosclerotic lesions and local HMGB1 may contribute platelets to the formation of thrombosis and the progression of atherosclerosis.

Atherosclerosis and fibrinogen. Atherosclerosis has been demonstrated to be associated with inflammatory processes in the vascular walls [15, 16]. The activity and progression of atherosclerosis is influenced by vascular inflammation [17]. Fibrinogen is an acute protein marker as well as a coagulation factor and has various other functions such as determining the blood viscosity and stimulating vascular smooth muscle cell migration and proliferation. Fibrinogen provides an indirect measure of cytokine-dependent inflammatory processes in the atherosclerotic wall. In addition to its role as a nonspecific marker of inflammation, fibrinogen is involved in atherogenesis and thrombogenesis by acting as a bridging molecule for many types of cell-to cell adhesion events that are critical for atherogenesis [18].

Fibrinogen plays a central role in the coagulation cascade and has a critical impact on the formation of fibrin clots following the rupture of an atherosclerotic

plaque [19]. The agents used to treat atherosclerosis, such as statins and angiotensin-converting enzyme inhibitors, which improve clinical outcomes in primary and secondary prevention [20], decrease the fibrinogen levels [21]. It was thus suggested that increased plasma fibrinogen is one of the risk factors for PAD [8–10]. In the present study, there was a significant correlation between HMGB1 and fibrinogen (Fig. 2).

In addition, the present subjects' fibrinogen levels were independently correlated with HMGB1 levels (Table 3). The multiple stepwise regression analysis showed that fibrinogen's R^2 (multiple regression coefficient) was 0.353, and the *p*-value for fibrinogen was 0.006 (Table 4). Our results may indicate a causal relationship between HMGB1 and fibrinogen, and thus, high levels of plasma HMGB1 and fibrinogen may induce atherosclerotic progression. A prior study suggested that HMGB1 is a potential biomarker for subclinical inflammation [1]. HMGB1 may be a good candidate as a marker for atherosclerosis.

Here we found that the concentration of HMGB1 was increased in the patients with atherosclerosis, and the level of plasma HMGB1 was correlated with that of fibrinogen. We therefore speculate that the plasma HMGB1 level is increased in atherosclerotic patients, and a significant correlation between the plasma HMGB1 and plasma fibrinogen levels will be examined and perhaps confirmed in future studies.

However, our study also has some technical limitations. First, we evaluated a very small number of patients. Comparisons of large numbers of PAD patients may better elucidate the relationships among plasma HMGB1, fibrinogen and PAD. Second, we used only the ABI for our atherosclerosis classification. The ABI values were obtained with an oscillometric automated device, and small differences in the device's measurements may have altered the final numerical ABI values. Using another measurement device such as a skin perfusion pressure (SPP) or transcutaneous partial pressure ($tcPO_2$) device may better clarify these relations. Third, we do not yet know the mechanisms underlying how HMGB1 is related to atherosclerosis. A longitudinal study is needed to clarify the causal relationships among HMGB1, fibrinogen levels, and atherosclerosis progression. We also do not know whether HMGB1 as a biomarker has a strong relationship with atherosclerosis progression. Further investigations of the role of HMGB1 may make it possible to identify a novel biomarker for atherosclerosis.

Acknowledgments. We thank Dr. Nobuya Ohara and Mrs. Rika Takamoto for their technical assistance.

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