Plasma Levels of Matrix Metalloproteinase-8 in Patients With Carotid Atherosclerosis

Tamara Djurić,^{1*} Maja Živković,¹ Aleksandra Stanković,¹ Ana Kolaković,¹ Djole Jekić,² Vesna Selaković,³ and Dragan Alavantić¹

¹*Vinča Institute of Nuclear Sciences, Laboratory for Radiobiology and Molecular Genetics, Belgrade, Serbia*

²*Military Medical Academy, Department of Neurology, Belgrade, Serbia*

³*Institute for Medical Research, Military Medical Academy, Belgrade, Serbia*

Matrix metalloproteinases (MMPs) are involved in the remodeling of the extracellular matrix (ECM) in the arterial wall during atherogenesis. Collagens are the most abundant proteins in the ECM. MMP-8 is expressed by cells associated with the development of the atherosclerotic plaque. It cleaves collagen type I three times more potently than two other interstitial collagenases MMP-1 and MMP-13. The aim of this study was to investigate whether plasma MMP-8 values are associated with occurrence of carotid plaque (CP) and possible correlations with clinical and biochemical parameters in carotid atherosclerosis (CA) patients. Total plasma MMP-8 levels were quantified by ELISA in 63 patients with ultrasonographic evidence of CP presence

and 12 controls. Plasma MMP-8 values were significantly higher in patients with CA compared with controls (median 23.36 ng/ml vs. 13.02 ng/ml, $P < 0.001$) but they did not differ significantly according to gender, smoking and hypertensive status, associated diseases, and use of statins. Statistically significant positive correlations were observed between MMP-8 plasma values and C reactive protein ($r = 0.41$, $P = 0.001$), urea ($r = 0.50$, $P < 0.001$), aspartate transaminase ($r = 0.48$, $P = 0.001$), and creatinine levels ($r = 0.38$, $P = 0.006$). These results suggest association of MMP-8 plasma levels with occurrence of CP and correlation with certain biochemical markers. *J. Clin. Lab. Anal.* 24:246–251, 2010. © 2010 Wiley-Liss, Inc.

Key words: atherosclerosis; carotid plaque; collagenase; correlation; MMP-8

INTRODUCTION

Atherosclerosis is still the leading cause of death in both men and women in Western world. Atherosclerotic plaque in humans develops over many decades and involves accumulation of inflammatory cells, lipids, smooth muscle cells, and extracellular matrix (ECM) proteins in the intima of the vascular wall (1). Matrix metalloproteinases (MMPs) are the family of peptidase enzymes responsible for the degradation of ECM, clotting factors, lipoproteins, latent growth factors, and chemotactic and cell adhesion molecules (2). Matrix metalloproteinase-8 (MMP-8) or collagenase-2 is a member of the MMP family. Its role in atherogenesis has been neglected till recently because the neutrophils, which are thought to be the only cells that produce it, are rare in the atherosclerotic plaque. In 2001 it was shown that the cells associated with the development of the atherosclerotic plaque, particularly endothelial cells,

vascular smooth muscle cells, and macrophages express MMP-8 in vitro and in situ (3). Several MMPs are capable to degrade the collagens, major proteins of plaque ECM, but MMP-8 cleave collagen type I three times more potently than two other interstitial collagenases MMP-1 and MMP-13 (4). Imbalance between collagen synthesis and degradation determines remodeling of the arterial wall, plaque growth, or subsequently vulnerability of the plaques (1).

Grant sponsor: Serbian Government Research; Grant number: M145023.

*Correspondence to: Tamara Djurić, Institute of Nuclear Sciences “Vinča”, Laboratory for Radiobiology and Molecular Genetics, P.O. Box 522, 11001 Belgrade, Serbia. E-mail: tamariska@vinca.rs

Received 15 December 2009; Accepted 24 May 2010

DOI 10.1002/jcla.20393

Published online in Wiley InterScience (www.interscience.wiley.com).

The changes of MMPs at the cellular level could be reflected in blood stream, so the measurement of these proteases in the blood had been recommended as noninvasive tool in diagnosis and monitoring of certain diseases (5). Up to date most of the rather scarce data regarding MMP-8 in cardiovascular disease investigated its tissue activity or serum levels (6–10). It was previously suggested that blood sample processing influences the correct determination of circulating MMPs and that MMP-8 behave different in serum and plasma (11). Those who investigated plasma MMP-8 levels mainly focused on coronary heart disease (CHD) (12,13).

As circulating levels of MMP-8 have been associated with established risk factors for development of carotid atherosclerosis (CA), such as obesity and metabolic disease (14,15), as well as with CHD and as there is increased intraplaque activity of MMP-8 (8), we hypothesized that its plasma levels may be associated with occurrence of plaque in CA. The aim of our study was to elucidate whether plasma MMP-8 levels are higher in patients with CA than controls and to investigate correlation of plasma MMP-8 levels with clinical and biochemical parameters in CA patients.

MATERIAL AND METHODS

Study Population

Study group consisted of 75 participants. All of them were Caucasians with European descent, from Serbia. The 63 patients were recruited among subjects consecutively admitted to Military Medical Academy, Department of Neurology, Belgrade, Serbia during the period from year 2005 to 2006 with evidence of carotid plaque (CP) presence either on CCA or ICA. Exclusion criteria for all patients were history of previous carotid endarterectomy (possible restenosis), tumors, chronic inflammatory diseases or autoimmune disease, and renal failure.

A total of 15 individuals from the staff working in the Military Medical Academy, Department of Neurology, Belgrade, underwent clinical, ultrasound, and ECG examination. Twelve of them without evidence of CA, cerebrovascular or cardiovascular disease, any chronic inflammatory disease or diabetes mellitus (DM) were recruited as controls.

For all individuals enrolled in the study complete biochemical data were obtained, a complete medical history was collected and included smoking and drinking habits, presence of diabetes, coronary artery disease, peripheral arterial occlusive disease, and drug treatment. Subjects already diagnosed with DM, fasting glucose level of ≥ 7.0 mmol/l or taking insulin or oral hypoglycemic drugs were characterized to have DM; those with

previous myocardial infarction or with stabile angina pectoris evaluated by selective coronarography that confirmed or revealed coronary artery disease were characterized to have coronary heart disease. Peripheral artery disease was diagnosed as ankle brachial index lower than 0.9. Hypertension was defined as a systolic blood pressure (SBP) ≥ 140 mmHg, a diastolic blood pressure (DBP) ≥ 90 mmHg, or current treatment with an antihypertensive drug. Ultrasound assessment of CA was performed by high-resolution B-mode ultrasound (Toshiba, PowerVision 6000, 7.5 MHz, Riverside, CA). The scanning protocol entails the longitudinal scanning of the near and far wall of both carotid arteries at the distal CCA, carotid bifurcation, and proximal ICA. The IMT was defined as the distance from the leading edge of the lumen–intima interface to the leading edge of the media–adventitia interface. CP was defined as an echogenic thickening of intimal reflection that encroaches on the arterial lumen with minimal IMT 1.2 mm (16).

The study was approved by the research ethics committee of the Military Medical Academy, Belgrade, Serbia and each participant gave their written informed consent to participate in the study.

Measurement of Plasma MMP-8 Level and Biochemical Analysis

For the measurement of plasma MMP-8 concentration tubes coated with lithium heparin were used to prepare corresponding plasma samples. The supernatants were carefully removed and stored at -80°C until analysis. Concentrations of total MMP-8 in plasma samples were determined using the Quantikine Human MMP-8 (total) Immunoassay (R&D Systems Europe, Abingdon, UK) ELISA kit on BioTek Synergy HT Microplate Reader (BioTek Instruments, Inc., Bad Friedrichshall, Germany) at 450 nm with correction at 570 nm. The assay detects human active and pro-MMP-8 concentrations in plasma. MMP-8 levels of all samples were measured in duplicate and the results were then averaged. Our reference range for plasma MMP-8 in healthy controls was 12.48 ± 1.53 ng/ml ($n = 12$, 66.7% women, range 9.7–14 ng/ml). There was no cross-reactivity or interference with either the MMP-1, MMP-2, MMP-3, MMP-9, and MMP-13, or TIMP-1 and TIMP-2 according to manufacturer. The MMP-8 assay detection limit is 0.06 ng/ml. Analyses were performed according to manufacturer's instructions. Both, inter- and intra-assay coefficients of variation were $< 6.0\%$.

All other standard biochemical analyses were routinely performed at the hospital laboratory.

Statistical Analysis

Statistical analysis was performed using Statistica Version 5, software package (StatSoft Inc., 1997). Dichotomous variables were compared using chi-square test. Means of normally distributed continuous variables were compared by unpaired *t*-test and means of skewed continuous variables with nonparametric Mann–Whitney *U* test or Kruskal Wallis Analysis of variance (ANOVA). Values of continuous variables are expressed as mean $\pm$ standard deviation (SD). Correlations between MMP-8 plasma values and biochemical parameters in patients with CA were calculated using Spearman's rank correlation coefficients. In all tests, differences with two-tailed α -probability ($P \leq 0.05$) were considered significant.

RESULTS

Clinical and biochemical data of patients with CA and controls are summarized in Table 1. Patients were older, had higher SBP, DBP, total cholesterol (TC),

triglycerides (TG), apolipoprotein B, C reactive protein (CRP), D dimer, urea and creatinine (CRE) levels compared with controls. Also, there were more hypertensives in patients group. The percentage of patients using statins were 9.6%, and as additional diseases 32.1% of them had coronary heart disease, 30.2% DM and only one patient had peripheral arterial occlusive disease. There was no statistically significant difference according to body mass index, gender, percentage of hyperlipidemic subjects, family history of atherosclerosis, smoking status, statin use, high-density lipoprotein cholesterol (HDL) and low-density lipoprotein cholesterol (LDL), lipoprotein (a) (Lp(a)), apolipoprotein A1, aspartate transaminase (AST), alanine transaminase (ALT), von Willebrand factor (vWf), lactate dehydrogenase (LDH), and creatine kinase (CK) levels.

Plasma MMP-8 values were significantly higher in patients with CA compared with controls (median 23.36 ng/ml vs. 13.02 ng/ml, respectively, $P < 0.001$) (Fig. 1). Patients with CA more often had a MMP-8 level > 13.02 ng/ml than controls (93 vs. 25%, respectively, $P < 0.01$). Plasma MMP-8 levels did not differ

TABLE 1. Clinical and Biochemical Characteristics of Patients With Carotid Atherosclerosis (CA) and Controls

	Controls <i>n</i> = 12	CA patients <i>n</i> = 63	<i>P</i>
Age (years)	50.4 $\pm$ 5.3	69.1 $\pm$ 13.7	<0.01
Gender (male), <i>n</i> (%)	4 (33.3)	38 (60.3)	0.11
SBP (mmHg)	129 $\pm$ 14	150 $\pm$ 25	<0.01*
DBP (mmHg)	76.5 $\pm$ 5	90 $\pm$ 15	<0.01*
BMI (kg/m ²)	26.4 $\pm$ 3.3	25.5 $\pm$ 2.9	0.44
Hypertension, <i>n</i> (%)	2 (16.7)	53 (84.6)	<0.01
Hyperlipidemia <i>n</i> (%)	3 (25.0)	16 (24.5)	0.99
TC (mmol/l)	5.94 $\pm$ 0.95	6.23 $\pm$ 2.69	<0.05*
HDL (mmol/l)	1.29 $\pm$ 0.20	1.19 $\pm$ 0.26	0.29
LDL (mmol/l)	3.84 $\pm$ 0.69	4.42 $\pm$ 1.09	0.1
TG (mmol/l)	1.12 $\pm$ 0.41	2.28 $\pm$ 4.96	<0.05*
Family history of atherosclerosis, <i>n</i> (%)	2 (16.7)	6 (9.4)	0.60
Coronary heart disease, <i>n</i> (%)		20 (32.1)	
Diabetes mellitus, <i>n</i> (%)		19 (30.2)	
Peripheral arterial occlusive disease		1 (0.63)	
Current smoker, <i>n</i> (%)	6 (50.0)	26 (41.5)	0.75
Statins, <i>n</i> (%)	0 (0.0)	6 (9.6)	0.57
Lp(a) (mg/dl)	18.68 $\pm$ 19.0	22.6 $\pm$ 26.2	0.42*
Apolipoprotein A1 (g/l)	1.302 $\pm$ 0.274	1.208 $\pm$ 0.306	0.33
Apolipoprotein B (g/l)	0.888 $\pm$ 0.166	1.058 $\pm$ 0.278	<0.05*
ALT (U/l)	25.5 $\pm$ 6.8	35.3 $\pm$ 15.2	0.08
AST (U/l)	25.6 $\pm$ 9.6	27.1 $\pm$ 11.7	0.71
CRP (mg/l)	2.65 $\pm$ 1.92	17.16 $\pm$ 24.73	<0.05*
DDIM (μ g/l)	196.6 $\pm$ 171.9	266.5 $\pm$ 188.8	<0.05*
vWF (IU/ml)	1.09 $\pm$ 0.38	1.94 $\pm$ 1.32	0.12
Urea (mmol/l)	4.19 $\pm$ 1.19	7.12 $\pm$ 3.23	<0.05
LDH (U/l)	312.9 $\pm$ 52.0	345.0 $\pm$ 106.6	<0.05*
CK (U/l)	97.6 $\pm$ 48.4	169.5 $\pm$ 241.2	0.49*
CRE (μ mol/l)	61.9 $\pm$ 11.5	86.4 $\pm$ 31.7	<0.01*

For continuous variables values are mean $\pm$ SD; *P*—for categorical variables Fisher's exact two-tailed test was used; Students' *t*-test was used to compare values of normally distributed continuous variables between controls and patients with carotid atherosclerosis; *Mann–Whitney *U* test was used to compare values of skew distributed continuous variables between controls and patients with carotid atherosclerosis.

significantly according to gender, smoking and hypertensive status, associated diseases, and use of statins (data not shown). Statistically significant positive correlations were observed between MMP-8 plasma values and CRP ($r = 0.41$, $P = 0.001$), urea ($r = 0.50$,

$P < 0.001$), AST ($r = 0.48$, $P = 0.001$), and creatinine levels ($r = 0.38$, $P = 0.006$) in patients with CA (Fig. 2). The rest of investigated biochemical parameters did not show correlation with MMP-8 plasma levels.

DISCUSSION

In this study, we were focused to investigate whether plasma MMP-8 could be a possible marker for occurrence of carotid atherosclerotic plaque. In regard to notion that plasma levels are true and more precise measure of circulating MMPs in peripheral blood than serum (11), we measured plasma MMP-8 levels in control subjects and patients with CA to whom presence of carotid atherosclerotic plaque was ultrasonographically confirmed. We found that patients had significantly higher MMP-8 plasma values than controls and that significantly higher proportion of them had MMP-8 plasma values above the median value measured in our control population.

Until recently the role of MMP-8 in atherosclerosis was neglected compared with other MMPs, so there are only few studies so far that explored it. Those who investigated plasma MMP-8 levels were mainly focused on CHD (12,13) with same results as in our study on CA. The higher serum levels of MMP-8 also predicted cardiovascular death in men with subclinical

Fig. 1. Kruskal-Wallis ANOVA test was performed to compare plasma MMP-8 values between two groups. Values are presented as median and range. CA—patients with carotid atherosclerosis.

Fig. 2. (A) positive correlation with C reactive protein (CRP) ($P = 0.001$), (B) positive correlation with urea ($P < 0.001$); positive correlation with aspartate transaminase (AST) ($P = 0.001$); (D) positive correlation with creatinine (CRE) ($P = 0.006$). Exact Spearman's correlation coefficients are presented on the top left of each scattergraph. Each scattergraph contains a linear regression line of the best fit between the variables with border lines showing 95% confidence interval.

atherosclerosis and were associated with acute myocardial infarction and death from CHD (9). Also, it was suggested that the plasma MMP-8 values could be a biomarker for unstable angina pectoris (13). We investigated MMP-8 in plasma, which is a noninvasive approach, as potential marker for diagnosis and monitoring of CA. Most of the previous studies in CA investigated MMP-8 on tissue level. It was shown that in atherosclerotic plaque MMP-8 colocalize with cleaved type I collagen (3). Up to date increased MMP-8 activity was found in ruptured infarct tissue (6), unstable plaques (7,8) and was associated with CP progression (7). All of this data, along with results of our study, suggest that MMP-8 has very important role in atherosclerosis and vulnerability of the plaques.

Recent data showed that MMP-8 is associated with the well-known risk factors for the development of CA. Plasma MMP-8 was found to be higher in obese children and adolescents (14). Significantly higher serum MMP-8 levels were found in patients with metabolic syndrome who were without DM or cardiovascular disease compared with nonmetabolic ones (15). Association of MMP-8 plasma levels with CA, which we found in this study, was independent of these risk factors although almost one-third of patients had DM. Still, there was no association of plasma MMP-8 values with DM, which is in concordance with the result from another study conducted on patients with CA (17). In our study population, other additional diseases as well as age, gender, smoking and hypertensive status, and use of the statins did not influence MMP-8 plasma values either.

Further, we investigated possible correlations of plasma MMP-8 with high palette of biochemical parameters. Among patients, plasma MMP-8 significantly correlated with CRP, urea, AST, and creatinine values, markers of inflammation, myocardial infarction, and renal function. Two recent studies have shown significant correlation between high-sensitivity CRP and MMP-8 in patients with CAD, and correlation of creatinine and MMP-7 levels, but not MMP-8 in patients with CA (10,13). Unfortunately, possible explanation of the observed interactions is still unknown, so we can only speculate on potential explanation. It was recently shown that CRP is not only a biomarker, but also a mediator of tissue remodeling. The mechanisms by which CRP promotes cardiac fibrosis and inflammation could be the activation of the transforming growth factor- β /Smad and NF- κ B signaling pathways (18). It was previously shown that MMP-8 was upregulated in the human monocyte-derived macrophages by LPS, TNF- α , and IL-1 β , possibly through the NF- κ B pathway (3). Thus, positive correlation of MMP-8 and CRP, found in this study, may be the consequence of systemic inflammation, but

also the role of both molecules in vascular tissue remodeling. Besides CRP, the mean values of the other parameters, which were in significant correlation with MMP-8, were in the range of physiological reference values. Further studies are needed to either confirm or to elucidate functional relationship between biochemical markers and MMP-8. Future research should include the bigger sample size, which we considered as one of the limitation of our study. This could enable the deeper insight into correlation of plasma MMP-8 with the severity of atherosclerosis, which could have important clinical value.

In conclusion, our results suggest the association of MMP-8 plasma values with occurrence of CP and correlation with certain biochemical parameters. As it is of great interest to define, the useful biochemical markers that show significant correlation with disease presence or its complications these results represent promising direction. MMP-8 is undoubtedly one of the important players in the complex and multifactorial atherosclerotic disease, but much more studies are needed before MMP-8 could be established as significant and clinically useful marker for development/complications of atherosclerosis.

REFERENCES

1. Newby AC. Dual role of matrix metalloproteinases (matrixins) in intimal thickening and atherosclerotic plaque rupture. *Physiol Rev* 2005;85:1–31.
2. Nagase H, Woessner Jr JF. Matrix metalloproteinases. *J Biol Chem* 1999;274:21491–21494.
3. Herman MP, Sukhova GK, Libby P, et al. Expression of neutrophil collagenase (matrix metalloproteinase-8) in human atheroma: A novel collagenolytic pathway suggested by transcriptional profiling. *Circulation* 2001;104:1899–1904.
4. Hasty KA, Jeffrey JJ, Hibbs MS, Welgus HG. The collagen substrate specificity of human neutrophil collagenase. *J Biol Chem* 1987;262:10048–10052.
5. Zucker S, Hymowitz M, Conner C, et al. Measurement of matrix metalloproteinases and tissue inhibitors of metalloproteinases in blood and tissues. Clinical and experimental applications. *Ann N Y Acad Sci* 1999;878:212–227.
6. van den Borne SW, Cleutjens JP, Hanemaaijer R, et al. Increased matrix metalloproteinase-8 and -9 activity in patients with infarct rupture after myocardial infarction. *Cardiovasc Pathol* 2009; 18:37–43.
7. Turu MM, Krupinski J, Catena E, et al. Intraplaque MMP-8 levels are increased in asymptomatic patients with carotid plaque progression on ultrasound. *Atherosclerosis* 2006;187:161–169.
8. Molloy KJ, Thompson MM, Jones JL, et al. Unstable carotid plaques exhibit raised matrix metalloproteinase-8 activity. *Circulation* 2004;110:337–343.
9. Tuomainen AM, Nyssönen K, Laukkanen JA, et al. Serum matrix metalloproteinase-8 concentrations are associated with cardiovascular outcome in men. *Arterioscler Thromb Vasc Biol* 2007;27:2722–2728.
10. Heider P, Pelisek J, Poppert H, Eckstein HH. Evaluation of serum matrix metalloproteinases as biomarkers for detection of

- neurological symptoms in carotid artery disease. *Vasc Endovascular Surg* 2009. DOI: 10.1177/1538574409334826.
11. Jung K. Matrix metalloproteinase-8 and tissue inhibitor of metalloproteinase-1 in serum do not reflect the analytes circulating in blood. *Arterioscler Thromb Vasc Biol* 2008;28:e15–e16.
 12. Kato R, Momiyama Y, Ohmori R, Taniguchi H, Nakamura H, Ohsuzu F. Plasma matrix metalloproteinase-8 concentrations are associated with the presence and severity of coronary artery disease. *Circ J* 2005;69:1035–1040.
 13. Momiyama Y, Ohmori R, Tanaka N, et al. High plasma levels of matrix metalloproteinase-8 in patients with unstable angina. *Atherosclerosis* 2009. DOI: 10.1016/j.atherosclerosis.2009.07.037.
 14. Belo VA, Souza-Costa DC, Lana CM, et al. Assessment of matrix metalloproteinase (MMP)-2, MMP-8, MMP-9, and their inhibitors, the tissue inhibitors of metalloproteinase (TIMP)-1 and TIMP-2 in obese children and adolescents. *Clin Biochem* 2009; 42:984–990.
 15. Aquilante CL, Beitelshees AL, Zineh I. Correlates of serum matrix metalloproteinase-8 (MMP-8) concentrations in nondiabetic subjects without cardiovascular disease. *Clin Chim Acta* 2007;379:48–52.
 16. Wyman RA, Fraizer MC, Keevil JG, et al. Ultrasound-detected carotid plaque as a screening tool for advanced subclinical atherosclerosis. *Am Heart J* 2005;150:1081–1085.
 17. Krupinski J, Turu MM, Font MA, et al. Increased tissue factor, MMP-8, and D-dimer expression in diabetic patients with unstable advanced carotid atherosclerosis. *Vasc Health Risk Manag* 2007; 3:405–412.
 18. Zhang R, Zhang YY, Huang XR, et al. C-reactive protein promotes cardiac fibrosis and inflammation in angiotensin II-induced hypertensive cardiac disease. *Hypertension* 2010;55:953–960.