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Metalloproteases 2 and 9, Lp-PLA$_2$ and Lipoprotein Profile in Coronary Patients

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Background and Aims. Many studies suggest that the different steps of the atherosclerotic process may be mediated by metalloproteases (MMPs). MMP-9 and MMP-2, which are highly expressed in the vulnerable regions of the atherosclerotic plaques, have been suggested to be causally involved in plaque rupture. In another manner linked with LDL, lipoprotein-associated phospholipase A_2 (Lp-PLA$_2$) hydrolyzes phospholipids generating proinflammatory and proatherogenic products. Our aim was to evaluate plasma activity of MMP-2 and 9, as well as Lp-PLA$_2$, in subjects with coronary artery stenosis in comparison with controls and to correlate these activities with lipoprotein profile and general biomarkers of inflammation.

Methods. Forty two subjects who had undergone coronary angiography were divided into two groups: patients with coronary vessels with at least 45% stenosis (CAD [coronary artery disease], $n = 24$) and patients without angiographically detectable coronary artery disease (controls, $n = 18$). Plasma activity of MMP-2 and MMP-9 was measured and correlated with markers of systemic inflammation (hs-CRP), subendothelial inflammation (Lp-PLA$_2$) and lipoprotein profile.

Results. Plasma activity of both MMPs was consistently higher in patients than in controls ($p < 0.01$). Pro-MMP-2 ($r = 0.34$, $p < 0.01$) and MMP-9 ($r = 0.51$, $p < 0.02$) activities correlated with apoprotein B. Pro-MMP-2 correlated with hs-CRP ($r = 0.47$, $p < 0.01$) and inversely with HDL cholesterol ($r = -0.35$, $p < 0.02$). No differences were observed in Lp-PLA$_2$ between patients and controls (15.2 ± 4.0 vs. 15.4 ± 4.5 μmol/mL/h, p = NS, respectively), and no correlation was observed with MMPs.

Conclusions. MMP activity was higher in CAD than in controls. The correlation observed between pro-MMP-2 and high-sensitive C-reactive protein (hs-CRP) may be due to specific systemic inflammatory processes. No correlation was observed between Lp-PLA$_2$ and MMPs.

Key Words: Metalloproteases, Plaque vulnerability, Lp-PLA$_2$, lipoproteins.

Introduction

Atherosclerosis is a multifactorial illness whose development and progression has been extensively studied (1–3). Many previous studies have suggested that the different steps of the atherosclerotic process may be mediated by metalloproteases. Matrix metalloproteases (MMPs) are a family of >20 zinc-dependent endopeptidases that collectively degrade most of the protein and proteoglycan-core-protein components of the extracellular matrix (ECM) (4). Increased expression and activity of these enzymes have been identified in various pathological processes such as general inflammation, tumor metastasis, myocardial injury and vascular remodelling (5). Thus, as

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MMPs play a significant role in vascular remodelling, they have been suspected to be partly responsible for the pathogenesis of cardiovascular disease. MMP-9 and MMP-2 are highly expressed in the vulnerable regions of the atherosclerotic plaques and, for this reason, have been suggested to be causally involved in plaque rupture (6). However, the hypothesis of the causal role of different MMPs in plaque rupture is controversial, given that they have been associated both with plaque instability (7) as well as with stability (8,9). Nevertheless, elevated plasma levels of different MMPs have been reported in patients with acute coronary syndrome and are associated with severe coronary stenosis and cardiovascular mortality.

Because high plasma concentration of low-density lipoprotein (LDL) is one of the principal risk factors for atherosclerosis, accumulation of this lipoprotein in the subendothelium has been extensively considered the primary event in atherosclerotic plaque formation. However, a substantial number of subjects with normal lipoprotein concentrations still develop atherosclerosis, thus supporting the presence of additional factors involved in this disease.

In association with LDL, the lipoprotein-associated phospholipase A_2 (Lp-PLA$_2$) has been described (10), an enzyme highly expressed in the damaged vessels that belongs to the A_2 phospholipase family that hydrolyzes phospholipids generating potent proinflammatory and proatherogenic products (11). Different studies also suggest that higher Lp-PLA$_2$ activity contributes to processes identified as pivotal to plaque vulnerability, including monocyte migration, proinflammatory effects of oxidized LDL and macrophage death (11,12). Recently, it has been demonstrated that Lp-PLA$_2$ induces pro-MMP-2 activation modulating the expression of tissue inhibitors of metalloproteases (TIMPs) by intracellular levels of cAMP (13). However, to our knowledge, no studies have evaluated the association between Lp-PLA$_2$ and MMPs activities in circulation.

It has been clearly established that vulnerable plaques are the underlying cause of most clinical coronary events. In some cases, a deep plaque injury cannot be identified despite a careful diagnostic search. Thus, it is important to identify predictors of instability in order to contribute to an early diagnosis and therapy and also to provide data to elucidate the mechanism underlying the process of instability.

Our aim was to evaluate plasma activity of MMP-2 and MMP-9, as well as Lp-PLA$_2$, in subjects with arterial stenosis in comparison with controls and to associate the activity of these enzymes with lipoprotein profile and biomarkers of general inflammation.

Materials and Methods

Studied Population

A total number of 42 Caucasian subjects were recruited at the Universidad Adventista del Plata, Paraná, Entre Rios, Argentina. These patients had undergone coronary angiography because of chest pain related to coronary heart disease, ischemic heart disease, myocardial infarction, and electrocardiography changes, as well as indication of valvular surgery. Patients presenting $>45\%$ stenosis were defined as cases (CAD [coronary artery disease], $n = 24$) warranting in all cases the presence of clinically significant lesions. Controls were selected among patients who had indication of coronary angiography previous to valvular surgery and who presented $<30\%$ stenosis ($n = 18$), without clinical significance (14). Patients presenting 30−45% stenosis were excluded. Each angiogram was performed with a Toshiba DS-TB (Toshiba, Tokyo, Japan) and subsequently reviewed independently by two experienced observers who were blinded to the clinical details. Patients taking cholesterol-lowering drugs were excluded, given that statins interfere in MMP synthesis (15,16). We evaluated the participants with regard to cigarette smoking, blood pressure and the presence of diabetes according to standard criteria. At the time of diagnosis, among those patients with hypertension, ten CAD and five controls were receiving antihypertensive treatment (beta blockers, calcium channel blockers or angiotensin receptor blockers). In addition, most of the diabetic patients were treated with oral hypoglycemic agents.

Written informed consent was obtained from each subject before admission, and the study protocol was approved by the Ethic Committee of the Faculty of Pharmacy and Biochemistry, University of Buenos Aires (Buenos Aires, Argentina).

Sample Collection

After an overnight fast, blood samples were obtained from peripheral vein puncture following a 15-min rest. Samples were collected in dry and chilled EDTA tubes for separating serum and plasma, respectively, within 1 h of the extraction. Serum samples were separated by centrifugation at 3,000 rpm during a period of 15 min at 4°C. For lipid and lipoprotein determinations, serum was kept at 4°C until its processing within 48 h. On the other hand, a serum aliquot was stored at −70°C for measurement of apoprotein B (apoB), Lp-PLA$_2$ and high-sensitive C-reactive protein (hs-CRP). For MMPs, a plasma aliquot was obtained because in serum samples MMPs are released from blood cells. In this case plasma was also kept at −70°C until processing.

Assay Procedure

Cholesterol and triglycerides (TG) were determined in a Hitachi 917 autoanalyzer by enzymatic methods (Roche Diagnostics, Mannheim, Germany). After selective precipitation methods, high-density lipoprotein (HDL) and LDL-cholesterol were determined (17,18). Serum lipid measurements were under good quality control with interassay coefficients of variation (CV) routinely $<3\%$. ApoB and hs-CRP were

determined by immunoturbidimetry (Roche Diagnostics) in a Hitachi 917 analyzer with CV routinely <3%.

Zymography for Gelatinases

Metalloprotease activity was detected by zymography (19). SDS-polyacrylamide gels (7.5%) were copolymerized with gelatin 0.1% (G-8150, Sigma). Plasma (1 μL) was loaded in each well, and gels were run for 3 h in Tris-glycine buffer (pH: 8.3) at 4°C in a Mini Protean-3 (Bio-Rad Laboratories, Hercules, CA). After running, gels were rinsed with 2.5% Triton X-100 and then incubated for 18 h in buffer (NaCl, $CaCl_2$, Tris HCl, pH: 7.4,) at 37°C. After staining with Coomassie blue R-250 (B-0149, Sigma, St. Louis, MO) and destained with acetic acid-methanol-water (1:3:6), enzyme activity was detected as colorless bands against the blue-stained background. Because the denaturizing condition activates the latent forms of the enzyme, both latent and active enzymes are detected by this method. MMP-9, 84 kDa (active form), MMP-2, 72 kDa (pro-form) and 67 kDa (active form) were identified by molecular weight. Conditioned media from the promyelocyte U-937 cell line was used as activity standard. Coefficients of variation were 4.8% (intra-assay) and 8.6% (inter-assay). Band intensities were quantified using Sion-Image J, software (Scion Corporation, Frederick, MD), by an operator who ignored which group each sample belonged. Relative activity was expressed as a ratio to the internal standard.

Lp-PLA_2 Activity

Lp-PLA_2 activity was measured following the radiometric assay described (20) with few modifications as previously published (21). Separation of the released radiolabeled acetate from the lipid substrate was carried out by phase-phase partitioning and measurement of the radioactivity in the aqueous phase. Briefly, incubation mixture contained 50 μL of 1/50 diluted serum and 10 μmol/L-1 hexadecyl-2-[^{3}H]acetyl-glycero-3-phosphocholine (specific activity = 25 μCi.μmol/L) in a total volume of 0.5 mL of PBS buffer (pH = 7.4). Tritiated substrate 1-hexadecyl-2-[^{3}H]acetyl-glycero-3-phosphocholine (13.5 Ci.mmol/L) (New England Nucleotides, Boston, MA), and nontritiated substrate (Cayman Chemical, Ann Arbor, MI) were mixed, the solvents were evaporated under nitrogen stream, redissolved in PBS, and sonicated. Incubation was carried out at 37°C for 5 min and the enzymatic reaction was stopped in an ice bath and with the addition of 1.5 mL of chloroform. Then, 0.5 mL of saturated sodium bicarbonate solution was added; after centrifugation, the aqueous phase was washed twice with 1.5 mL of chloroform. Radioactivity of the aqueous phase was measured by liquid scintillation using a Packard autoanalyzer. Radioactivity of the substrate buffer was also measured. Results were expressed as μmol/mL/h. Measurements were all carried out within the same assay. Within-run precision (CV) for Lp-PLA_2 activity was 5.1%.

Statistical Analysis

Results are expressed as mean ± standard deviation or median (range) according to data distribution. Clinical and biochemical data were analyzed using the Student's t-test, Mann-Whitney U test, and χ^2 test as appropriate. Univariate correlations between MMP activity and other selected variables were analyzed using the Pearson correlation coefficients or Spearman correlation according to data distribution. A p value <0.05 was considered significant. All analyses were performed using SPSS v.11.5 for Windows.

Results

Table 1 shows no differences in the prevalence of clinical risk factors such as diabetes, hypertension or smoking between groups. It can also be appreciated that CAD patients presented higher values of total cholesterol and apoB with no differences in triglycerides and HDL cholesterol. LDL cholesterol showed a tendency to higher values in CAD patients than in controls.

In CAD patients, hs-CRP values were higher than controls. In each group, a patient was detected who presented a high hs-CRP value in accordance with acute infection. Regarding MMPs activities, zymographic analysis revealed the expression of two major gelatinolytic bands in all plasma samples corresponding to pro-MMP-2 form and MMP-9 (Figure 1), where reliable quantification was possible. The gelatinolytic bands disappeared in parallel zymograms in which the development buffer contained EDTA, confirming the gelatinolytic activity to be caused by metalloproteases inhibited by EDTA.

Table 1. General features, lipid and lipoprotein profile Lp-PLA_2, and hs-CRP of patients (CAD) and controls

	CAD (n = 24)	Controls (n = 18)	p =
Age (years)	63 ± 9	68 ± 11	0.189
Males (n, %)	18, 75	11, 64.5	0.340
Diabetes (n, %)	5, 23	4, 23	1.000
Smoking (n, %)	9, 36	5, 26	0.120
Hypertension (n, %)	18, 73	11, 64	0.470
Antihypertensive treatment (n, %)	10, 40	5, 28	
Hypoglycemic treatment (n, %)	4, 17	4, 22	
TC (mmol/L)	5.69 ± 1.30	4.94 ± 0.88	0.037
TG (mmol/L)	2.12 ± 1.01	2.12 ± 0.99	0.992
HDL cholesterol (mmol/L)	1.01 ± 0.44	0.99 ± 0.26	0.889
LDL cholesterol (mmol/L)	3.90 ± 1.43	3.22 ± 0.68	0.075
ApoB (mg/dL)	121 ± 30	102 ± 19	0.023
hs-CRP (mg/L)	13 (0.1–56)	4.8 (0.1–39)	0.030
Lp-PLA_2 (μmol/mL/h)	15.2 ± 4.0	15.4 ± 4.5	0.875

Results are expressed as mean ± standard deviation except hs-CRP: median (range).

TC, total cholesterol; TG, triglycerides, HDL, high-density lipoprotein; LDL, low-density lipoprotein; hs-CRP, high-sensitive C-reactive protein; ApoB, apoprotein B.

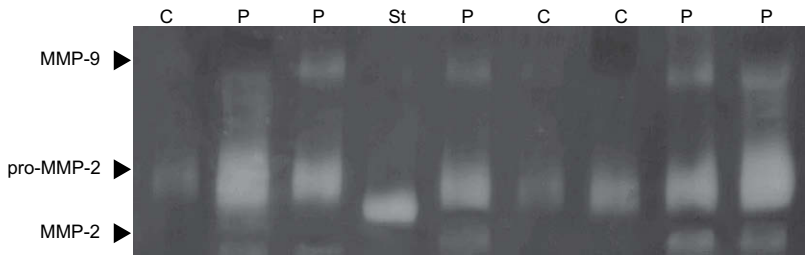

Figure 1. SDS-PAGE zymographic analysis of plasma from different cardiovascular patients and controls. Molecular weights are as indicated on the left. Pro-MMP-2 (MW: 72 kDa) and MMP-9 (MW: 84 kDa) were quantified as described in Materials and Methods. C, controls; P, patients; St, standard of Pro-MMP-2.

The activity of these MMPs, expressed as relative area, was consistently increased and statistically different in patients than in controls—relative areas: pro-MMP-2 0.99 ± 0.40 vs. 0.49 ± 0.28, $p < 0.01$; MMP-9: 1.25 ± 0.43 vs. 0.36 ± 0.19, $p < 0.04$, respectively (Figure 2).

As seen in Table 2, in CAD patients and controls, MMP-9 and pro-MMP-2 levels correlated significant and positively with apoB. Moreover, pro-MMP-2 correlated positively with hs-CRP but negatively with HDL cholesterol. For all studied subjects, there was an overall significant correlation between MMP-9 and pro-MMP-2 ($r = 0.72$, $p < 0.01$).

Regarding circulating Lp-PLA$_2$, no differences were observed between CAD patients and controls (15.2 ± 4.0 vs. 15.4 ± 4.5 μmol/mL/h, respectively, p = NS). Even though a positive correlation was observed between Lp-PLA$_2$ and LDL cholesterol ($r = 0.41$, $p < 0.03$), no correlation was found between Lp-PLA$_2$ and MMPs, hs-CRP or HDL cholesterol, considering both subgroups.

Discussion

In this study, using a sensitive zymographic assay, we found overactivity of MMP-9 and pro-MMP-2 in the circulation of coronary patients in comparison with controls. Additionally, our results evidenced that the increase in both MMP-9 and pro-MMP-2 correlated with the increment in apoB. Pro-MMP-2 was directly associated with hs-CRP and inversely with HDL cholesterol. In addition, no differences were observed in Lp-PLA$_2$ activity between CAD patients and controls, and no association was found between plasma activities of Lp-PLA$_2$ and MMPs.

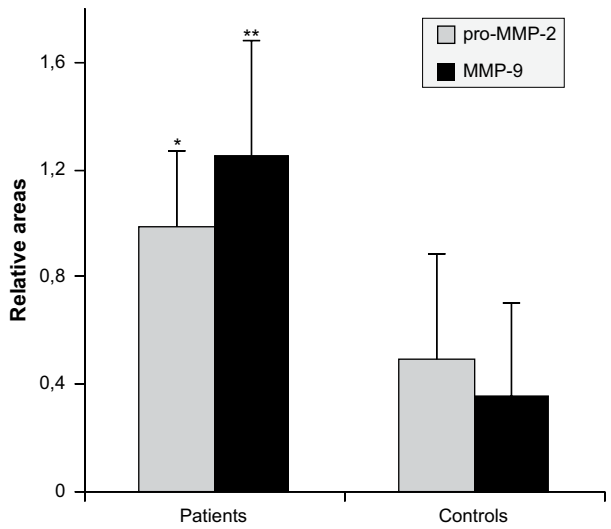

Figure 2. Box plot showing levels of pro-MMP-2 and MMP-9 activities in plasma of patients ($n = 24$) and controls ($n = 18$). *$p < 0.01$, **$p < 0.04$.

Atherosclerotic plaque rupture is the major cause of acute ischemic events. MMPs are capable of degrading the major components of the vascular extracellular matrix, contributing actively to increase plaque vulnerability. However, given the high number of different MMPs and the possibility to measure them in different biological compartments such as plasma or arterial plaques, controversies exist about their specific roles (8,22).

Johnson et al. (9), using apoE/MMP-9 double knockout mice, described that MMP-9 would play a protective role, limiting plaque growth and promoting a stable plaque phenotype. de Nooijer et al. (23), using apoE-deficient mice at different stages of plaque progression, demonstrating that in intermediate lesions, MMP-9 promotes outward remodelling, not accompanied by more adverse events. However, in advanced lesions, it promotes vulnerable plaque morphology and high incidence of ischemia. However, it is not clear if MMP circulating levels are altered in the above-described models.

Recently, during a follow-up period of 4 years, Blankenberg et al. (24) showed that plasma concentration of MMP-9 at baseline was significantly higher among patients who subsequently experienced a fatal cardiovascular event, compared with controls, even after adjustment for most potential clinical and therapeutic variables. Other authors found a decrease in MMP-2 and an increase in MMP-9 concentration in young coronary patients (25), which would increase the controversies. In our study, the significant association found between both MMPs indicates that the same mechanism may be involved in promoting MMP-9 and MMP-2 release to circulation.

Although the most prominent form of MMP-2 detected in this study was the latent enzyme pro-MMP-2, this fact should not be disregarded, given that these forms are stable in circulation and may be reflecting the increase of its synthesis and activation in the subendothelium (26).

Table 2. Correlations between MMPs and ApoB, hs-CRP and HDL cholesterol

	MMP-9 r ($p <$)	pro-MMP-2 r ($p <$)
ApoB	0.51 (0.01)	0.34 (0.02)
hs-CRP	0.21 (NS)	0.47 (0.01)
HDL cholesterol	−0.22 (NS)	−0.35 (0.02)

NS, nonsignificant.

In accordance with other authors, the positive correlation obtained between MMPs and apoB was significant (24,27). It may be related to previous *in vitro* findings where well-known injuring factors associated with the development of atherosclerotic plaque induce MMPs secretion from different cells (28), ApoB being a marker of injuring factors.

Pro-MMP-2 showed an inverse significant correlation with HDL. This would reinforce the antiatherogenic role of HDL and its protective function in the subendothelial space. On the other hand, MMP-9 did not show a correlation with HDL, although other authors reported the association, studying a higher number of patients (24).

The relationship observed between hs-CRP and pro-MMP-2 may reflect the specific systemic inflammatory process accompanying atherosclerosis and contributing to the MMP release. The possible activation of this pro form in situ, in a pro-inflammatory condition, would shift matrix remodelling towards a proteolytic state. Previous studies have described a correlation between MMP-9 and hs-CRP, but in acute coronary syndrome patients (26).

No differences were observed in Lp-PLA_2 between patients and controls. Currently, the role of Lp-PLA_2 as a pro-inflammatory or anti-inflammatory factor is controversial. Some authors suggest that Lp-PLA_2 may be additive to hs-CRP in its ability to predict coronary heart disease risk (29). We did not find any association between Lp-PLA_2 and hs-CRP or with other MMPs. As the mechanism of Lp-PLA_2 elevation in plasma has not yet been completely described, the lack of associations with MMPs and hs-CRP in this study may suggest that these parameters participate in the atherogenic process by different pathways.

In the present study we selected patients by means of an angiography procedure, which is still considered a gold standard method. In order to minimize possible overlapping between groups and also to reduce heterogeneity in each group, we excluded patients with 30–44% coronary stenosis. Despite the low number of studied subjects, we consider their careful selection as a favorable point. Another limitation of this study is the fact that we evaluated only two MMPs by zymographic assay, and it is known that several MMPs may have variable and additive effects on the atherosclerotic plaque. Finally, we have not studied metalloprotease inhibitors that could modulate MMP activity.

In conclusion, in coronary artery patients, particularly in those who are asymptomatic, measurement of soluble MMP activities with sensitive and standardized assays may improve risk assessment, early diagnosis and probably the prognosis of cardiovascular disease, also providing important information on its biology. Additional prospective observational and interventional studies are required to clarify the role of these molecules.

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