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Original Article

Increased MMP-2 in healthy postmenopausal women

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Abstract

Background: Matrix metalloproteases 2 (MMP-2) and 9 (MMP-9) are involved in the atherosclerosis process. The objective of the study was to evaluate MMP-2 and MMP-9 activities and other circulating inflammatory factors in healthy postmenopausal women (PMW) as a model of subclinical atherosclerosis.

Methods: Twenty-three PMW and 13 premenopausal women (PreMW) were selected following established criteria. The main measurements in plasma samples were: lipid–lipoprotein profile, high-sensitivity C-reactive protein (hs-CRP) (immunoturbidimetry), soluble vascular cellular adhesion molecules (sVCAM-1) enzyme-linked immunosorbent assay and MMP activity by zymography.

Results: The relative areas of MMP-2 were increased in PMW: 1.1 (0.1) versus 0.6 (0.05), $P < 0.02$. MMP-9 was only detected in three PMW and one PreMW. MMP-2 correlated with HDL-cholesterol ($r = -0.51$), triglycerides ($r = 0.67$), apolipoprotein B ($r = 0.47$), hs-CRP ($r = 0.42$), homeostasis model assessment ($r = 0.53$) and waist circumference ($r = 0.40$), at least $P < 0.02$. sVCAM-1 showed no difference between groups: 28.7 (5.5) versus 35.5 (20) ng/mL, but correlated with MMP-2 and hs-CRP ($r = 0.46$ and $r = 0.48$ respectively, $P < 0.05$).

Conclusions: In postmenopause, the increase in MMP-2 reflects the systemic specific inflammatory process that accompanies atherogenesis.

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Introduction

Atherosclerosis comprises a slow process characterized by several steps such as lipid deposits, oxidation and platelet aggregation involving structural elements of the arterial wall, inflammatory cells, cytokine secretion and vascular remodelling.[1–3]

It is known that women after menopause increase their atherogenic risk, even when they are apparently healthy. In a previous publication we studied some circulating factors evidencing subclinical endothelial injury in healthy postmenopausal women (PMW).[4] It would be an interesting advance to evaluate non-traditional risk factors that can reflect the inflammatory status and the vascular process in situations of subclinical atherosclerosis, such as postmenopause.

The synthesis and degradation of components of the vascular extracellular matrix (ECM) are usually kept under strict control. Matrix metalloproteases (MMPs) are a family of more than 20 zinc-dependent endopeptidases that collectively degrade most of the protein and proteoglycan core protein components of the extracellular matrix.[5] They contribute to maintaining homeostasis of the vascular structure; however, the disregulation or activation of MMP expression is a feature of numerous pathological conditions, such as tumour metastasis, vascular and cardiac dysfunction and rheumatic conditions. Thus, as MMPs play a significant role in vascular remodelling, they have been suspected to be partly responsible in 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 they have been suggested to be causally involved in the plaque rupture.[6]

Adherence of circulating leukocytes to the endothelium and their subsequent transmigration into the arterial intima is an early step in the formation of atherosclerosis. The recruitment of leukocytes into tissues is dependent on a diverse family of cellular adhesion molecules that are expressed on the surface of vascular endothelial cells, such as vascular cellular adhesion molecules (VCAM).[7] There are no previous references evaluating in circulation both MMPs and VCAM in healthy PMW as indicators of the atherogenic process.

Our aim was to evaluate the activities of MMP-2 and MMP-9, and VCAM-1 concentration in circulation, both factors involved in the endothelial injury and inflammation, in healthy (PMW) in comparison with premenopausal women (PreMW) controls, and then to associate the levels of these factors with other metabolic and inflammation biomarkers.

Materials and methods

A total of 36 healthy women were studied; 23 of them were PMW, clinically evaluated and consecutively recruited at the Climacteric Section of the Gynecology Division at Durand Hospital, Buenos Aires. The age of PMW ranged from 43 to 60 y (average 53 y), with at least one year of natural menopause and not more than 10 y of amenorrhea. In all the cases, serum levels of follicle-stimulating hormone above 40 IU/L confirmed the postmenopausal status. The control group included 13 women of reproductive age (23–44 y, average 33 y) with normal physical examinations and laboratory tests. They were consecutively recruited from patients who attended the same Centre for their routine health check.

None of the women were included if presenting history or underlying symptoms of diabetes or cardiovascular disease, as well as if they were receiving hormonal, hypolipidemic or any other drug known to modify lipid metabolism. Women were also excluded if having a history of hypothyroidism, neoplasia or renal disorder. Women whose weight had varied more than 5% in the last six months were also excluded. Four PMW and five PreMW consumed 2–6 cigarettes/ day, while the remaining subjects had been non-smokers for the last 10 y. In no case did alcohol consumption surpass 10 g/day. PMW and PreMW were not under regular training. Both groups of women had similar diets with the following distribution: 20% proteins, less than 30% fat and at least 50% carbohydrates. Calory intake varied according to individual body weight.

The weight and height of each patient were measured and body mass index (BMI) calculated as weight (kg)/height^2 (m^2) to evaluate the obesity degree. Waist circumference was measured midway between the lateral lower rib margin and the superior anterior iliac crest on a standing position and always by the same investigator.

Written informed consent was obtained from each subject before admission to the study, which was approved by the Ethic Committee of the School of Pharmacy and Biochemistry, University of Buenos Aires.

After a 12-h overnight fast, blood samples were drawn and collected into EDTA-containing tubes (2.7 mmol/L) and into dry tubes. In the PreMW group, blood samples were drawn at the follicular phase of the menstrual cycle (day 3–7). Samples were centrifuged at 1500*g* and 4°C for 5 min. Plasma was stored at −70°C for MMP-2 and 9 determinations. Serum was kept at 4°C for evaluation of fasting glucose, lipid and lipoprotein profile, high-sensitivity C-reactive protein (hs-CRP) and soluble VCAM (sVCAM-1) within 48 h.

Glucose, cholesterol and triglycerides (TG) were determined by enzymatic colorimetric methods with standards and controls in each run; in the case of TG, the glycerol blank was taken into account. All measurements were performed in a Hitachi 917 autoanalyser (Roche Diagnostics, Mannheim, Germany). Intra- and inter-assay coefficients of variation (CV) were <3% in all runs.

HDL- and LDL-cholesterol were determined by selective precipitation methods.[8,9] HDL was isolated in the supernatant obtained following the precipitation of apolipoprotein (apo) B-containing lipoproteins with 0.44 mmol/L phosphotungstic acid in the presence of magnesium ions. Intra and inter-assay CV for HDL-cholesterol were 3.2% and 3.8%, respectively. For LDL-cholesterol, a selective precipitation with 10 g/L polyvinylsulphate in polyetilenglycol (molecular weight 600; 2.5% w/v; pH 6.7) was assessed and LDL-cholesterol level was determined as the difference between total cholesterol and the cholesterol contained in the supernatant. The intra- and inter-assay CV were 4.7% and 5.0%, respectively.

Apo B was determined by immunoturbidimetry (Roche Diagnostics) in the Hitachi 917 analyser. The detection limit was 20 mg/dL and the intra- and inter-assay CV were 2.5% and 3.3%. hs-CRP was measured by a high-sensitivity immunoturbidimetric method (Tina-quant CRP, Roche Diagnostics) in the Hitachi 917 analyser. The detection limit was 0.01 mg/dL and the intra- and inter-assay CV were 1.9% and 3.0%, respectively. All measurements were under good quality control.

MMP activity was measured in plasma by zymography or a quantifiable gel electrophoresis.[10] Briefly, sodium dodecyl sulphate (SDS)-polyacrylamide gels (7.5%) were copolymerized with gelatin 0.1% (G-8150; Sigma, St Louis, MO, USA). Plasma (1 μL) was loaded in each well in non-reducing conditions, and gels were run for three hours in 25 mmol/L Tris, 192 mmol/L glycine, 0.1% SDS at 4°C, pH 8.3, in a Mini Protean-3 (Bio-Rad Laboratories, Hercules, CA, USA). After running, the gels were rinsed with 2.5% Triton X-100 for 30 min and then incubated 18 h in 0.15 mol/L NaCl, 10 mmol/L $CaCl_2$, Tris HCl, pH 7.4 at 37°C. After staining with Coomassie blue R-250 (B-0149; Sigma) and destaining with acetic acid–methanol–water (1:3:6), enzyme activity was detected as colourless bands against the blue-stained background. MMP-9, 84 kDa (active form), and 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. The CV were 4.8% (intra-assay) and 8.6% (inter-assay). Band intensities were quantified using Scion Image J software (Scion Corporation, Frederick, MD, USA), and relative activity was expressed as a ratio to the internal standard.

Levels of sVCAM-1 were determined by monoclonal antibody-based enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN, USA). Sample levels were calculated by analysing standards with known concentrations of recombinant adhesion molecules coincident with samples and plotting of signal versus concentration. Results were expressed as ng/dL.

Statistical analysis

Results were expressed as mean (SD) for normally distributed data and as median and range for skewed data. Differences between groups were tested using the unpaired Student's *t*-test for normally distributed data and the Mann − Whitney *U* test for skewed data. Correlations between variables were assessed using the Pearson or

Table 1 Anthropometric and biochemical characteristics in postmenopausal women (PMW) and premenopausal women (PreMW)

	PMW (n = 23) (43–60 y)	PreMW (n = 13) (23–44 y)
Waist circumference (cm)	86.6 (10.1)*	73.9 (5.8)
BMI (kg/m^2)	26.0 (3.5)*	21.4 (2.1)
HOMA	2.9 (2.3)***	1.7 (0.7)
Total cholesterol (mmol/L)	6.26 (1.24)*	4.86 (0.81)
Triglycerides (mmol/L)	1.56 (0.73)*	0.78 (0.41)
HDL-cholesterol (mmol/L)	1.35 (0.31)**	1.56 (0.34)
LDL-cholesterol (mmol/L)	4.37 (1.25)*	2.83 (0.81)
Apo B (mg/dL)	121 (30)***	102 (19)
hs-CRP (mg/L)	3.3*** (0.46–12.36)	1.1 (0.1–7.7)

BMI, body mass index; HOMA, homeostasis model assessment; Apo B, apolipoprotein B; hs-CRP, high-sensitivity C-reactive protein.
Results are expressed as mean (SD); except for hs-CRP, median (range)
*$P < 0.001$, **$P < 0.02$, ***$P < 0.05$

Spearman tests according to parameter distributions, and results were adjusted by age. Differences were considered significant at P less than 5% in the bilateral situation.

Results

Characteristics of the total sample of women are listed in Table 1. As expected, PMW presented higher values of waist circumference, BMI, TG, total cholesterol, LDL-cholesterol, Apo B, homeostasis model assessment index (HOMA) and hs-CRP in comparison to controls (at least $P < 0.05$). Besides, the group of PMW showed a significant decrease in HDL-cholesterol ($P < 0.02$).

Zymographic analysis revealed the expression of one major gelatinolytic band in all the plasma samples, corresponding to the pro-MMP-2 form (Figure 1a). The activity of this MMP was significantly increased in PMW than in controls ($P < 0.02$), even after adjustment by age (F: 6.51; $P < 0.03$). Gelatinolytic bands are shown in Figure 1b. MMP-9 was only detected in three PMW and in one preMW, and thus a reliable statistical analysis was not possible.

Table 2 shows significant correlations, in PMW and controls, between MMP-2 and lipid parameters, hs-CRP, waist circumference and HOMA index.

Regarding sVCAM-1, no differences were observed between patients, 28.7 (5.5) ng/mL and controls, 35.5 (20) ng/mL, $P = 0.32$. However, a positive correlation was observed between sVCAM-1 and MMP-2 ($r = 0.46$, $P < 0.05$) and between sVCAM-1 and hs-CRP ($r = 0.48$, $P < 0.05$) (Figure 2). These correlations remained significant even after age adjustment, F: 4.16, $P < 0.05$ and F: 11.8, $P < 0.004$, respectively.

Discussion

In this study, we evaluated some non-traditional factors in PMW that could reflect endothelial inflammation and a subclinical atherogenic process. In comparison to PreMW, asymptomatic PMW showed an increase in MMP-2 activity, which correlates with an atherogenic lipoprotein profile, hs-CRP and soluble VCAM levels.

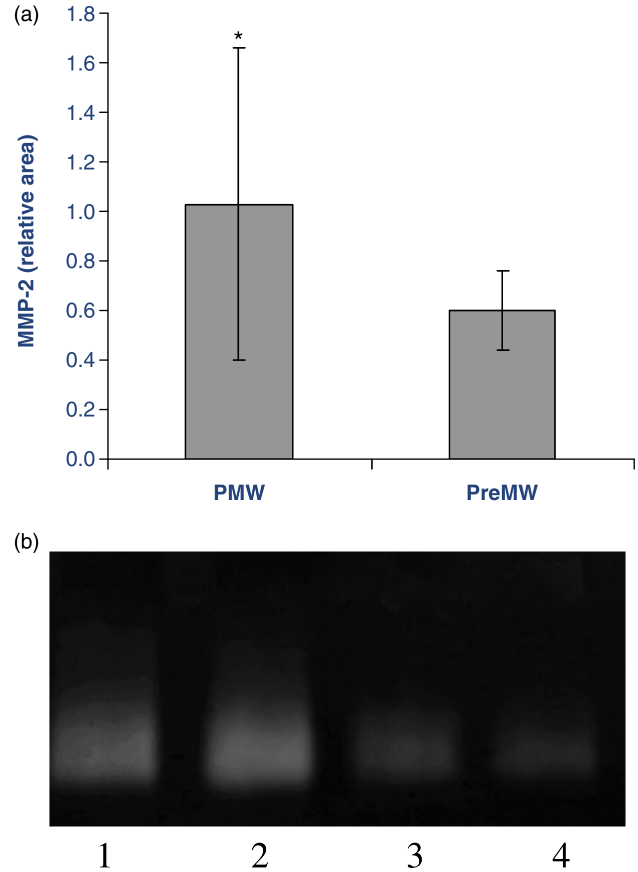

Figure 1 (a) Matrix metalloprotease (MMP) activities in postmenopausal women (PMW) and premenopausal women (PreMW). *$P < 0.02$. (b) proMMP-2 gelatinolytic bands. 1 and 2 correspond to two PMW; 3 and 4 correspond to two PreMW

Synthesis of MMPs in the vasculature is favoured by different stimuli such as local inflammation and oxidative stress;[11] both present in hypoestrogenism states.[4,12] There are few reports about MMP activities in apparently healthy PMW, considered an atherosclerosis preclinical step.

In the present study it has been observed that circulating MMP-2 activity was significantly increased in PMW, while practically, MMP-9 was not detected. The increased MMP-2 activity could be associated with the first steps of the atherogenic process mainly related to the vascular smooth muscle cell migration and intimal thickening. The high MMP-2 activity might be responsible for a greater matrix degradation of type IV collagen within the basement

Table 2 Significant correlations between MMP-2 and lipid parameters, insulin resistance and inflammatory markers

	r	$P <$
Triglycerides	0.67	0.001
LDL-cholesterol	0.47	0.005
Apo B	0.47	0.006
HDL-cholesterol	−0.51	0.002
hs-CRP	0.42	0.01
Waist circumference	0.40	0.02
HOMA	0.53	0.02

MMP, matrix metalloproteases; Apo B, apolipoprotein B; hs-CRP, high-sensitivity C-reactive protein; HOMA, homeostasis model assessment

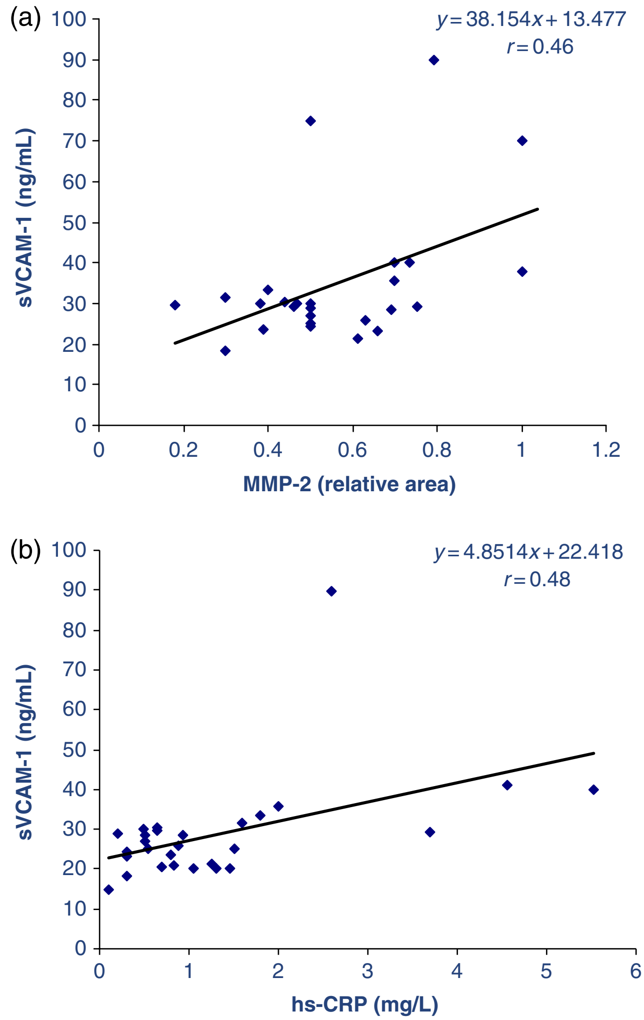

Figure 2 Correlations between (a) sVCAM-1 and MMP-2 and (b) sVCAM-1 and hs-CRP. sVCAM-1, soluble vascular cellular adhesion molecules; MMP, matrix metalloproteases; hs-CRP, high-sensitivity C-reactive protein

membrane, and also might activate several growth factors and cytokines, underlying the atherosclerotic process in the arterial vessel wall.[13]

The most prominent form of MMP-2 detected in this study was pro-MMP-2, given that this form is stable in circulation and could be reflecting the increase of its synthesis and activation in the subendothelium.

The lack of MMP-9 detection can be attributed to the fact that this MMP is mainly associated to the plaque rupture in advanced lesions.[14] In accordance with this concept, we found an increase in MMP-9 in patients with diagnosed coronary artery disease.[15]

In accordance to what it is known, PMW presented higher BMI and waist circumference. It must be taken into account that adipose tissue can also contribute to the release of MMP-2 into circulation, based on data reported by other authors showing that the expression of MMP-2 was induced in the adipose tissue.[16]

High adhesion molecule levels were demonstrated in different metabolic alterations such as impaired glucose tolerance and hyperinsulinemia, indicating endothelial dysfunction.[17,18] Herein, no differences were observed in sVCAM-1 between healthy PMW and controls. In a recent publication of a similar design, Schoppen *et al.*[19] also found no differences in sVCAM-1 levels in PMW. However, in the present study we have observed, in the whole population, a positive correlation between sVCAM-1 with MMP-2 and hs-CRP, which could reflect the mechanisms involved in the vascular remodelling and inflammation process contributing to the atherosclerotic plaque formation.

It is worth noting that MMP-2 could be considered as an emerging early marker of atherosclerosis. However, a limitation of this study is the fact that apart from MMP-2 and 9, there are other MMP and their tissue inhibitors (TIMPS) that could have an additive effect on the atherosclerotic plaque, and so it would be very interesting to extend the study to include other MMPs and TIMPS. Besides, the low number of patients in the control group only allows us to infer that there would be an increase in MMP-2 activity in postmenopause; increasing the number size of the groups would surely improve the results obtained.

Finally, our findings of increased MMP-2 and the significant associations with sVCAM-1 may contribute to the understanding of the early atherogenic process in menopause.

DECLARATIONS

Competing interests: None.

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Ethical approval: The Ethic Committee of the School of Pharmacy and Biochemistry, University of Buenos Aires approved this study (Res. CD 0156/06).

Guarantor: LS.

Contributorship: GB and LS researched literature, conceived the study and obtained ethical approval. MLM and VM participated in the development of the MMP method, recruited the samples, carried out most of the biochemical determinations and contributed to the analysis of the results. EMR participated in the development of MMP technique and quantification of the results. FB contributed to measuring one of the parameters, and analysis and interpretation of its results. MLM and LS wrote the first draft of the manuscript. All authors reviewed and edited the manuscript and approved the final version of the manuscript. MLM and VM contributed equally to the work.

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