Vascular Medicine 2005; **10**: 1–6

Plasma levels and zymographic activities of matrix metalloproteinases 2 and 9 in type II diabetics with peripheral arterial disease

Salvatore Santo Signorelli[a], Grazia Malaponte[b], Massimo Libra[b], Luigi Di Pino[a], Gabriella Celotta[a], Valentina Bevelacqua[b], Marcello Petrina[b], Giuseppina S Nicotra[b], Manuela Indelicato[b], Patrick M Navolanic[b,c], Giuseppe Pennisi[a] and Maria Clorinda Mazzarino[b]

Abstract: Deregulation of matrix metalloproteinases (MMPs) is an important factor contributing to the development of vascular lesions. Plasma levels and zymographic activities of MMP-2 and MMP-9 were investigated in type II diabetics with ($n = 51$) or without ($n = 42$) peripheral artery disease (PAD) and in normal volunteers ($n = 23$). Plasma MMP-2 levels were higher in type II diabetics with ($p < 0.01$) or without ($p > 0.05$) PAD in comparison with normal volunteers. Similarly, type II diabetics with ($p < 0.0001$) or without ($p > 0.05$) PAD had higher plasma MMP-9 levels than normal volunteers. Plasma zymographic activities of both MMP-2 and MMP-9 were positively correlated with their plasma levels. Plasma MMP-2 zymographic activity was higher in type II diabetics with PAD than type II diabetics without PAD ($p > 0.05$). Plasma MMP-9 zymographic activity was higher in type II diabetics with ($p < 0.0001$) or without ($p < 0.0001$) PAD in comparision with normal volunteers. Together, these results indicate that increased plasma levels and zymographic activities of MMP-2 and MMP-9 may contribute to PAD in type II diabetics. In particular, plasma MMP-9 may be a useful marker for the development of vascular disease in type II diabetics.

Key words: MMP-2; MMP-9; type II diabetes; vascular disease

Introduction

Matrix metalloproteinases (MMPs) are a family of Zn^{2+}-dependent enzymes that catalyze proteolysis of many connective tissue proteins of the extracellular matrix (ECM) such as collagen, gelatin, fibronectin, laminin, elastin, and proteoglycans.[1,2] Other MMP substrates are not components of the ECM. MMPs have been implicated in the degradation of myelin basic protein,[3] and interleukin-1β,[4] as well as proteolytic processing of tumor necrosis factor-α.[5] MMPs are secreted as proenzymes by many cell types,[6] including leukocytes, macrophages, astrocytes, neurons, and microglia, and are widely distributed in tissues and biological fluids such as blood and urine.[7] They are involved in many physiological processes, including tissue remodeling during development and platelet aggregation.[8] MMPs also have roles in pathophysiological processes such as inflammation, tissue repair, myocardial injury, vascular diseases, tumor invasion, and metastasis.[9–11]

Mechanisms have been identified for regulation of both expression and activity of MMPs. Transcription of MMP genes is modulated by growth factors, cytokines, and free radicals.[12–14] However, MMP-2 differs from other MMPs in that it is constitutively expressed. Activity of MMPs is negatively regulated by tissue inhibitors of matrix metalloproteinases (TIMPs).[15] The in vivo balance between MMPs and TIMPs dictates the level of MMP activity.[16]

Deterioration of MMP regulation contributes to the development of arterial lesions, in part, by facilitating monocyte invasion.[17] Gelatin zymography studies have shown that MMPs, especially MMP-2 (72-kDa gelatinase A)[18,19] and MMP-9 (92-kDa gelatinase B),[20] are involved in remodeling processes associated with atherogenesis.[21,22] MMPs are synthesized in atheromatous plaques[23] and are present at elevated levels in rupture-prone shoulder regions of arterial blood vessels.[24] Increased MMP activity has also been correlated with cardiovascular pathologies.[25,26] Since vascular complications such as acute coronary artery syndrome and peripheral arterial disease (PAD) are significantly more common among diabetics,[27] it is

Departments of [a]Internal Medicine and [b]Biomedical Sciences, School of Medicine, University of Catania, Catania, Italy; [c]Department of Microbiology & Immunology, Brody School of Medicine at East Carolina University, Greenville, NC, USA

Address for correspondence: Salvatore Santo Signorelli, Via Firenze n, 123, 95020 Acicastello, (CT) Italy. Tel: +39 95 7594079; Fax: +39 95 312165; E-mail: ssignore@unict.it

© 2005 Edward Arnold (Publishers) Ltd 10.1191/1358863x05vm582oa

hypothesized that MMPs are preferentially activated in diabetics with a history of PAD. The aim of the present study was to determine whether plasma levels and zymographic activities of MMP-2 and MMP-9 differed between type II diabetics with or without PAD.

Materials and methods

Patients

Heparin plasma samples were provided by the Angiology Unit of the University of Catania between October 2002 and March 2003. Subjects enrolled in the present study included 42 type II diabetics without PAD (group 1), 51 type II diabetics with PAD (group 2), and 23 normal volunteers (group 3). Informed consent was obtained from all patients, and peripheral blood sample collection was approved by the Institutional Review Board. PAD was diagnosed for patients having either an ankle/brachial index (ABI) of less than 0.9 or an absence of one or more arteries of the lower legs as determined by the ecoduplex technique (Apogée CX 800 ATL-Philips electronic probe; 7–10 mHz). The clinical characteristics of enrolled subjects are summarized in Table 1. The mean ages of groups 1, 2, and 3 were 65 ± 15, 68 ± 11, and 69 ± 13 years, respectively. Individuals with arterial hypertension, chronic renal failure, or a history of ischemic coronary artery disease were excluded. Venous blood samples collected from each subject were deposited into lithium heparin-coated plastic tubes and kept at room temperature until centrifugation. Samples were centrifuged at 1500 RPM for 30 min within 60 min after collection. Plasma was then removed and stored at –80 °C until analysis.

MMP-2 and MMP-9 ELISA

ELISA was performed according to the manufacturer's instructions (Amersham Biosciences, UK). In brief, plasma samples were diluted with assay buffer to a total volume of 100 μl then incubated for 2 h at room temperature. Sulphuric acid was then added at a final concentration of 1 mM to stop the reaction. Absorbance of ELISA samples at 450 nm was measured by spectrophotometry (ThermoLabsystems, Finland). Protein levels are expressed in ng/ml.

The assay recognizes the precursor of MMP-2 but not the active form of MMP-2. It does not cross-react with MMP-1, -2, -7, -8, -9 and MT1-MMP. Regarding the specificity of the MMP-9 ELISA system, no detectable cross-reactivity was observed with proMMP-1, proMMP-2, proMMP-3, TIMP-1 and TIMP-2.

Gelatin zymography

MMP-2 and MMP-9 gelatinolytic activities were measured by zymography. Zymography was performed as described by Lorenzl et al and Gruber et al with slight modifications.[28,29] Protein concentrations of plasma samples were quantified with the Bio-Rad Protein Assay (Bio-Rad, USA). Aliquots containing 100 μg of protein were mixed with sample buffer then subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) without boiling or reduction. Gels contained 7.5% (w/v) polyacrylamide copolymerized with 0.2% (w/v) gelatin. After electrophoresis, gels were washed twice for 30 min in 2% (v/v) Triton X-100 then incubated at 37°C for 20 h in incubation buffer [50 mM Tris, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, and 0.01% (w/v) sodium azide (pH = 7.5)]. After incubation, gels were fixed in 20% trichloroacetic acid (Sigma, Germany) for 30 min then stained in 0.5% (w/v) Coomassie Brilliant Blue R-250 (Bio-Rad) for 90 min. Gels were then washed for 60 min in destaining solution (35% (v/v) ethanol and 10% (v/v) acetic acid). Gelatinolytic activity generated clear bands against the blue background of the gel. Gelatinases A and B (Chemicon International, USA) were used as positive controls to indicate sizes of MMP-2 and MMP-9 present in plasma samples, respectively. Identities of MMP-2 and MMP-9 were

Table 1 Clinical characteristics of the individuals studied.

Clinical characteristic	Diabetic patients without PAD	Diabetic patients with PAD	Normal volunteers
Age (years)	65 ± 15	68 ± 11	69 ± 13
Sex (M/F)	12/8	16/9	10/5
Fasting glucose (g/dl)	6.5 ± 1.1	6.3 ± 1.0	3.5 ± 1.3
Duration of diabetes (years)	5.3 ± 1.6	5.4 ± 1.9	Not applicable
Total cholesterol (mg/dl)	198.2 ± 7.2	201.3 ± 2.7	165.3 ± 4.6
Triglycerides (mg/dl)	113.5 ± 7.9	110.3 ± 7.2	98.5 ± 6.4
HbA_{1c} (%)	6.5 ± 1.1	6.3 ± 1.0	3.5 ± 1.3
BMI (kg/m^2)	<27	<27	<27
ABI	1.0–1.3	0.6–0.9	>1.0

The mean value of each characteristic is presented together with its corresponding standard deviation.
Both Student's *t*-test and ANOVA tests were performed to analyze the statistical significance of differences between groups versus normal volunteers for each clinical characteristic. Statistical significance was observed only for ABI ($p < 0.005$).

also confirmed by demonstrating that their zymographic activities were inhibited by addition of EDTA to the incubation buffer at a final concentration of 10 mM. EDTA inhibits MMP-2 and MMP-9 zymographic activities by chelating Zn^{2+}. Quantity One software (Bio-Rad) was used for densitometry analysis of MMP-2 and MMP-9 zymographic activities. Zymographic activities are expressed in pixels.

Statistical analysis

Mean values are presented together with their corresponding standard deviations. SPSS software was used for statistical analysis. The data are normally distributed and were expressed as mean ± standard error of the mean (SEM). Student's *t*-tests were performed to analyze the statistical significance of differences between each group. ANOVA was used to compare the parameters among PAD, non-PAD and control groups. If ANOVA analysis showed significant differences, an unpaired *t*-test using Bonferroni correction was performed in order to analyze which groups had significantly different values and to calculate *p*-values. A difference among groups was defined to be statistically significant if the corresponding *p*-value was less than 0.05. Correlations between variables were determined by Spearman rank-order correlation coefficients (*r*-values).

Results

MMP-2 and MMP-9 plasma levels

Mean plasma MMP-2 levels in type II diabetics with or without PAD and in normal volunteers are shown in Figure 1. Mean plasma MMP-2 levels were higher in type II diabetics with (1121 ± 456 ng/ml) or without (903 ± 440 ng/ml) PAD in comparison with normal volunteers (701 ± 362 ng/ml). The only statistically significant difference in mean plasma MMP-2 levels between the three groups was between type II diabetics with PAD and normal volunteers ($p < 0.01$).

Mean plasma MMP-9 levels were slightly higher in type II diabetics without PAD (39 ± 24 ng/ml) in comparison with normal volunteers (25 ± 17 ng/ml), but this difference was not statistically significant. In contrast, a statistically significant increase in mean plasma MMP-9 levels was present in type II diabetics with PAD (62 ± 30 ng/ml) in comparison with normal volunteers ($p < 0.0001$) (Figure 2). Furthermore, there was a statistically significant difference in mean plasma MMP-9 levels between type II diabetics with and without PAD ($p < 0.01$).

MMP-2 and MMP-9 zymographic activities

Representative SDS-PAGE zymograms indicating plasma MMP-2 and MMP-9 zymographic activities in normal volunteers (CT) and type II diabetics with (DIA + PAD) or without (DIA) PAD are shown in Figure 3A. Results of densitometry analysis of MMP-9 and MMP-2 zymographic activities are indicated in Figures 3B and 3C, respectively.

Statistically significant differences in mean plasma MMP-9 zymographic activities were observed in type II diabetics with (29 213 ± 13 797 pixels) or without (15 984 ± 8522 pixels) PAD in comparison to normal volunteers (6182 ± 4513 pixels) ($p < 0.0001$). The difference in mean plasma MMP-9 zymographic activities between type II diabetics with PAD and type II diabetics without PAD was also statistically significant ($p < 0.0001$). Mean plasma MMP-2 zymographic activities were higher in type II diabetics with (107 714 ± 72 799 pixels) or without (80 361 ± 28 078 pixels) PAD in comparison with normal volunteers (62 856 ± 27 527 pixels). The only statistically significant difference in mean plasma MMP-2 zymographic activities between the three

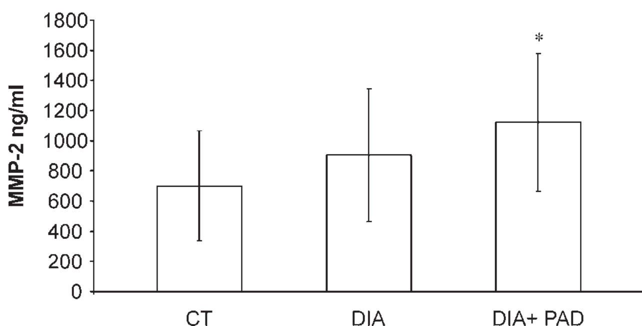

Figure 1 Plasma MMP-2 levels in normal volunteers (CT) and in type II diabetics with (DIA + PAD) or without (DIA) PAD. Plasma MMP-2 levels were measured by ELISA as described in 'Materials and methods'. Mean values are presented together with their corresponding standard deviations. A statistically significant increase in the mean MMP-2 level was observed in type II diabetics with PAD in comparison with normal volunteers (*).

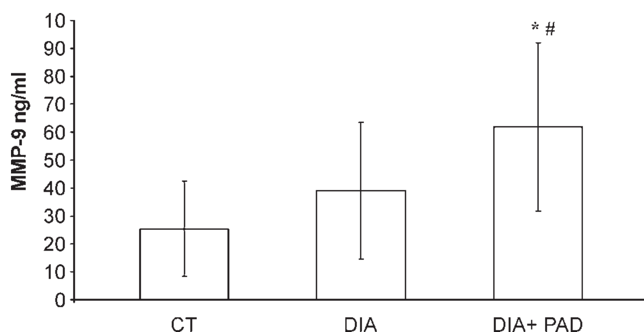

Figure 2 Plasma MMP-9 levels in normal volunteers (CT) and in type II diabetics with (DIA + PAD) or without (DIA) PAD. Plasma MMP-9 levels were measured by ELISA as described in 'Materials and methods'. Mean values are presented together with their corresponding standard deviations. A statistically significant increase in the mean MMP-9 level was observed in type II diabetics with PAD in comparison with both normal volunteers (*) and type II diabetics without PAD (#).

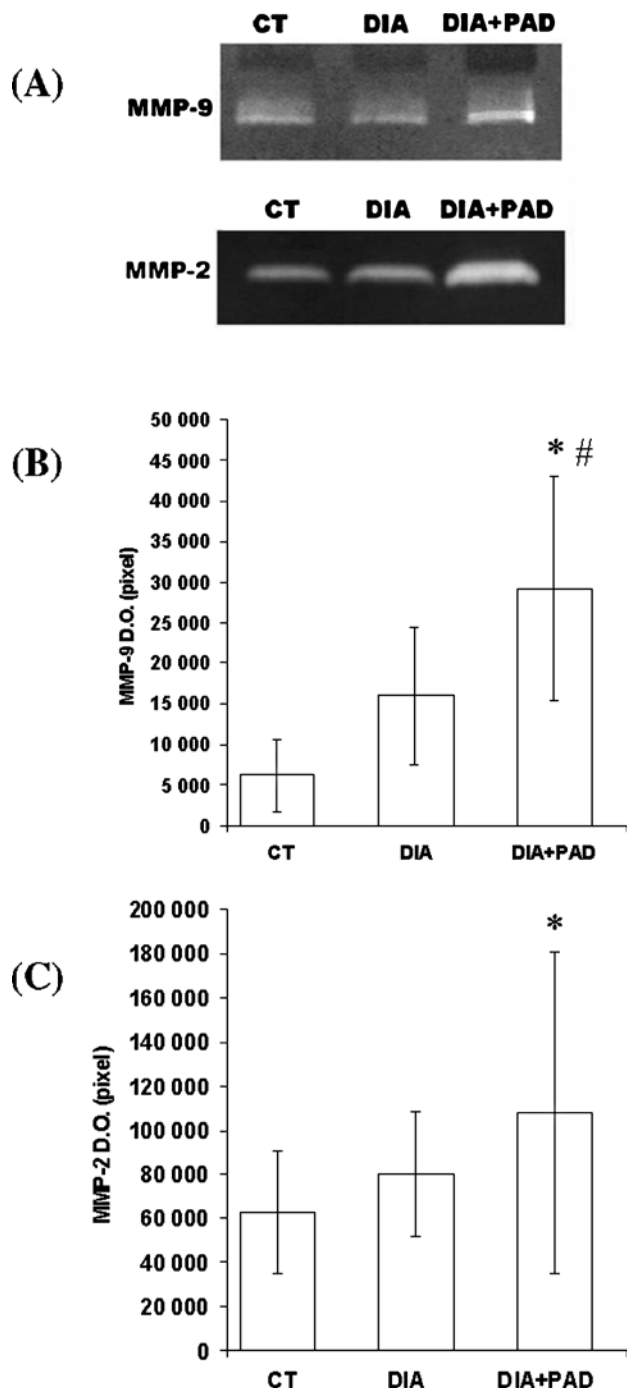

Figure 3 Plasma MMP-2 and MMP-9 zymographic activities in normal volunteers (CT) and in type II diabetics with (DIA + PAD) and without (DIA) PAD. (A) Representative analysis of MMP-2 and MMP-9 zymographic activities. MMP-9 zymographic activity was detected at 92 kDa (upper panel) and MMP-2 zymographic activity was detected at 72 kDa (lower panel). (B) Mean MMP-9 zymographic activities are presented together with their corresponding standard deviations. A statistically significant increase in mean MMP-9 zymographic activity was observed in type II diabetics with PAD in comparison with both normal volunteers (*) and type II diabetics without PAD (#). (C) Mean MMP-2 zymographic activities are presented together with their corresponding standard deviations. A statistically significant increase in mean MMP-2 zymographic activity was observed in type II diabetics with PAD in comparison with normal volunteers (*).

groups was between type II diabetics with PAD and normal volunteers ($p < 0.05$).

Relationships between plasma levels and activities of MMP-2 and MMP-9

Plasma levels and zymographic activities of MMP-2 and MMP-9 in type II diabetics with or without PAD were analyzed to determine whether correlative relationships were present (Table 2). MMP-2 plasma levels did not correlate with either MMP-9 plasma levels or zymographic activities in type II diabetics with or without PAD. MMP-2 zymographic activities also did not correlate with either MMP-9 plasma levels or zymographic activities in type II diabetics with or without PAD. However, zymographic activities of MMP-2 and MMP-9 did positively correlate with MMP-2 and MMP-9 plasma levels, respectively, among type II diabetics with and without PAD (Table 2).

Discussion

It is well known that type II diabetics have a high risk of developing cardiovascular diseases. These conditions include PAD, which is considered to be a common manifestation of atherosclerosis. We have previously observed increased levels of proinflammatory cytokines and soluble adhesion molecules associated with systemic inflammation in patients with PAD.[30] Furthermore, increased expression of non-traditional markers of subclinical inflammation, including C-reactive protein (CRP) and interleukin-6 (IL-6), has been associated with type II diabetes.[31,32]

Several studies have investigated the potential involvement of MMPs in atherosclerosis. For example, expression of MMP-2 was shown to be increased within atherosclerotic plaques.[33,34] Additionally, smooth muscle cells and inflammatory macrophages surrounding plaques prone to acute disruption exhibited increased levels and activities of MMP-9. These results suggest that MMP-2 and MMP-9 released during macrophage activation may be important causes of vascular damage.

Other studies have implied that expression and activity of MMPs may be increased among type II diabetics. Death et al showed that exposure to a high concentration of glucose induced increased expression and activity of MMP-1 and MMP-2 in endothelial cells and MMP-9 in macrophages.[11] Plasma levels of MMP-9 and other markers of inflammation were decreased in type II diabetics by treatment with rosiglitazone, which has been introduced recently for the management of diabetes.[35,36] These effects were evident both after long-term treatment as well as after only two weeks of treatment. The decrease in plasma MMP-9 levels strictly correlated with decreased

Table 2 Correlative analysis of MMP-2 and MMP-9 levels and zymographic activities in type II diabetics with (DIA + PAD) or without (DIA) PAD.

	MMP-2 activity		MMP-2 protein		MMP-9 activity		MMP-9 protein	
	DIA *r*-values	DIA + PAD *r*-values	DIA *r*-values	DIA + PAD *r*-values	DIA *r*-values	DIA + PAD *r*-values	DIA *r*-values	DIA + PAD *r*-values
MMP-2 activity	–	–	0.67 ($p < 0.001$)	0.80 ($p < 0.0001$)	0.13 ($p > 0.05$)	0.30 ($p > 0.05$)	0.20 ($p > 0.05$)	0.02 ($p > 0.05$)
MMP-2 protein	0.67 ($p < 0.001$)	0.80 ($p < 0.0001$)	–	–	0.33 ($p > 0.05$)	0.36 ($p > 0.05$)	0.12 ($p > 0.05$)	0.31 ($p > 0.05$)
MMP-9 activity	0.13 ($p > 0.05$)	0.30 ($p > 0.05$)	0.33 ($p > 0.05$)	0.36 ($p > 0.05$)	–	–	0.65 ($p < 0.001$)	0.76 ($p < 0.0001$)
MMP-9 protein	0.20 ($p > 0.05$)	0.02 ($p > 0.05$)	0.12 ($p > 0.05$)	0.31 ($p > 0.05$)	0.65 ($p < 0.001$)	0.76 ($p < 0.0001$)	–	–

Each *r*-value is presented together with its corresponding *p*-value.

plasma glucose levels and was independent of effects of rosiglitazone on metabolism.

In the present study, plasma levels and zymographic activities of MMP-2 and MMP-9 were studied in type II diabetics with or without PAD and in normal volunteers. Statistically significant differences in plasma MMP-2 levels and zymographic activities were only observed between type II diabetics with PAD in comparison with normal volunteers. However, statistically significant differences in plasma MMP-9 levels and zymographic activities were observed between type II diabetics with PAD in comparison with both type II diabetics without PAD and normal volunteers.

Portik-Dobos et al have reported that MMP-9 levels are lower within blood vessels isolated from type II diabetics than from normal individuals.[37] Our findings suggest that MMP-9 is released from blood vessels into the bloodstream in type II diabetics to a greater extent than in healthy individuals, which may contribute to increased chronic local inflammation of blood vessels among type II diabetics. Additionally, the endothelium in blood vessels from diabetics may activate MMP-producing cells in the circulation.

In conclusion, results of the present study suggest that plasma MMP-9 may be a useful marker for development of macrovascular complications, such as PAD, in type II diabetics. Additional studies are required to further characterize the effects of MMP-9 on the development and treatment of cardiovascular diseases in type II diabetics.

Acknowledgements

The authors wish to thank Luigina Mei for assistance during the preparation of this manuscript.

References

1 Van den Steen PE, Opdenakker G, Wormald MR, Dwek RA, Rudd PM. Matrix remodelling enzymes, the protease cascade and glycosylation. *Biochim Biophys Acta* 2001; **1528**: 61–63.

2 Borkakoti N. Structural studies of matrix metalloproteinases. *J Mol Med* 2000; **78**: 261–68.

3 Gijbels K, Proost P, Masure S, Carton H, Billisu A, Opdenakker G. Gelatinase B is present in the cerebrospinal fluid during experimental autoimmune encephalomyelitis and cleaves myelin basic protein. *J Neurosci Res* 1993; **36**: 432–40.

4 Ito A, Mukaiyama A, Itoh Y et al. Degradation of interleukin 1β by matrix metalloproteinases. *J Biol Chem* 1996; **271**: 14 657–60.

5 Gearing AJH, Beckett P, Christodoulou M et al. Processing of tumour necrosis factor-α precursor by metalloproteinases. *Nature* 1994; **370**: 555–57.

6 Bartholome EJ, Van Aelst I, Koyen E et al. Human monocyte-derived dendritic cells produce bioactive gelatinase B: inhibition by IFN-β. *J Interferon Cytokine Res* 2001; **21**: 495–501.

7 Lein M, Nowak L, Jung K et al. Analytical aspects regarding the measurement of metalloproteinases and their inhibitors in blood. *Clin Biochem* 1997; **30**: 491–96.

8 Sawicki G, Sanders EJ, Salas E, Wozniak M, Rodrigo J, Radomski MW. Localization and translocation of MMP-2 during aggregation of human platelets. *Thromb Haemost* 1998; **80**: 836–39.

9 Creemers EE, Cleutjens JP, Smits JF, Daemen MJ. Matrix metalloproteinase inhibition after myocardial infarction: a new approach to prevent heart failure? *Circ Res* 2001; **89**: 201–10.

10 Itoh Y, Nagase H. Matrix metalloproteinases in cancer. *Essays Biochem* 2002; **38**: 21–36.

11 Death AK, Fisher EJ, McGrath KC, Yue DK. High glucose alters matrix metalloproteinase expression in two key vascular cells: potential impact on atherosclerosis in diabetes. *Atherosclerosis* 2003; **168**: 263–69.

12 Uzui H, Harpf A, Liu M et al. Increased expression of membrane type 3-matrix metalloproteinase in human atherosclerotic plaque: role of activated macrophages and inflammatory cytokines. *Circulation* 2002; **106**: 3024–30.

13 Chen H, Li D, Saldeen T, Mehta JL. TGF-β_1 attenuates myocardial ischemia-reperfusion injury via inhibition of upregulation of MMP-1. *Am J Physiol Heart Circ Physiol* 2003; **284**: 1612–17.
14 Gurjar MV, DeLeon J, Sharma RV, Bhalla RC. Mechanism of inhibition of matrix metalloproteinase-9 induction by NO in vascular smooth muscle cells. *J Appl Physiol* 2001; **91**: 1380–86.
15 Brew K, Dinakarpandian D, Nagase H. Tissue inhibitors of metalloproteinases: evolution, structure and function. *Biochim Biophys Acta* 2000; **1477**: 267–83.
16 Visse R, Nagase H. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. *Circ Res* 2003; **92**: 827–39.
17 Dollery CM, McEwan JR, Henney AM. Matrix metalloproteinases and cardiovascular disease. *Circ Res* 1995; **77**: 863–68.
18 Gohlke U, Gomis-Ruth FX, Crabbe T, Murphy G, Docherty AJ, Bode W. The C-terminal (haemopexin-like) domain structure of human gelatinase A (MMP2): structural implications for its function. *FEBS Lett* 1996; **378**: 126–30.
19 Asanuma K, Magid R, Johnson C, Nerem RM, Galis ZS. Uniaxial strain upregulates matrix-degrading enzymes produced by human vascular smooth muscle cells. *Am J Physiol Heart Circ Physiol* 2003; **284**: H1778–84.
20 Opdenakker G, Van den Steen PE, Dubois B et al. Gelatinase B functions as regulator and effector in leukocyte biology. *J Leukoc Biol* 2001; **69**: 571–79.
21 Galis ZS, Khatri JJ. Matrix metalloproteinases in vascular remodeling and atherogenesis: the good, the bad, and the ugly. *Circ Res* 2002; **90**: 251–62.
22 Galis ZS, Johnson C, Godin D et al. Targeted disruption of the matrix metalloproteinase-9 gene impairs smooth muscle cell migration and geometrical arterial remodeling. *Circ Res* 2002; **91**: 852–59.
23 Galis ZS, Sukhova GK, Lark MW, Libby P. Increased expression of matrix metalloproteinases and matrix degrading activity in vulnerable regions of human atherosclerotic plaques. *J Clin Invest* 1994; **94**: 2493–503.
24 Shah PK, Galis ZS. Matrix metalloproteinase hypothesis of plaque rupture: players keep piling up but questions remain. *Circulation* 2001; **104**: 1878–80.
25 Gao CQ, Sawicki G, Suarez-Pinzon WL et al. Matrix metalloproteinase-2 mediates cytokine-induced myocardial contractile dysfunction. *Cardiovasc Res* 2003; **57**: 426–33.
26 Shah PK, Falk E, Badimon JJ et al. Human monocyte-derived macrophages induce collagen breakdown in fibrous caps of atherosclerotic plaques: potential role of matrix-degrading metalloproteinases and implications for plaque rupture. *Circulation* 1995; **92**: 1565–69.
27 Schneider DJ, Sobel BE. Determinants of coronary vascular disease in patients with type II diabetes mellitus and their therapeutic implications. *Clin Cardiol* 1997; **20**: 433–40.
28 Lorenzl S, Albers DS, Narr S, Chirichigno J, Beal MF. Expression of MMP-2, MMP-9, and MMP-1 and their endogenous counterregulators TIMP-1 and TIMP-2 in postmortem brain tissue of Parkinson's disease. *Exp Neurol* 2002; **178**: 13–20.
29 Gruber BL, Sorbi D, French DL et al. Markedly elevated serum MMP-9 (gelatinase B) levels in rheumatoid arthritis: a potentially useful laboratory marker. *Clin Immunol Immunopathol* 1996; **78**: 161–71.
30 Signorelli S, Mazzarino MC, Di Pino L et al. High circulating levels of cytokines (IL-6 and TNFalpha), adhesion molecules (VCAM-1 and ICAM-1) and selectins in patients with peripheral arterial disease at rest and after a treadmill test. *Vasc Med* 2003; **8**: 15–19.
31 Ridker PM, Hennekens CH, Buring JE, Rifai N. C-reactive protein and other markers of inflammation in the prediction of cardiovascular disease in women. *N Engl J Med* 2000; **342**: 836–43.
32 Pradhan AD, Manson JE, Rifai N, Buring JE, Ridker PM. C-reactive protein, interleukin 6, and risk of developing type 2 diabetes mellitus. *JAMA* 2001; **286**: 327–34.
33 Loftus IM, Naylor AR, Bell PR, Thompson MM. Plasma MMP-9 – a marker of carotid plaque instability. *Eur J Vasc Endovasc Surg* 2001; **21**: 17–21.
34 Loftus IM, Naylor AR, Goodall S, et al. Increased matrix metalloproteinase-9 activity in unstable carotid plaques. A potential role in acute plaque disruption. *Stroke* 2000; **31**: 40–47.
35 Haffner SM, Greenberg AS, Weston WM, Chen H, Williams K, Freed MI. Effect of rosiglitazone treatment on nontraditional markers of cardiovascular disease in patients with type 2 diabetes mellitus. *Circulation* 2002; **106**: 679–84.
36 Marx N, Froehlich J, Siam L et al. Antidiabetic PPARγ-activator rosiglitazone reduces MMP-9 serum levels in type 2 diabetic patients with coronary artery disease. *Arterioscler Thromb Vasc Biol* 2003; **23**: 283–88.
37 Portik-Dobos V, Anstadt MP, Hutchinson J, Bannan M, Ergul A. Evidence for a matrix metalloproteinase induction/activation system in arterial vasculature and decreased synthesis and activity in diabetes. *Diabetes* 2002; **51**: 3063–68.