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METALLOPROTEINASES AND ANTIBODIES AGAINST THEIR SUBSTRATES IN CHRONIC ISCHEMIC STROKE

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ABSTRACT
The aim of the present study was to investigate the serum levels of matrix metalloproteinases (MMP-9 and MMP-2), anti-α-elastin (AEAb) and anti-collagen type IV antibodies (AC4Ab) – class IgG and IgM, in circulation of patients with stroke in chronic phase, compared to healthy control subjects. The levels of anti-elastin and collagen type IV antibodies (IgG, IgM) were measured by home-made indirect ELISA and the levels of MMP-9 and MMP-2 quantity and activity by gelatin zymography. Thirty-one patients with stroke (mean age 63.4±11.9; age range 39-84 years; 18 women) were studied in chronic phase (3-12 months after the onset) with clinical evaluation, MRI, CT of the brain, Duplex/Doppler sonography of extracranial vessels. To obtain sera from healthy controls, 37 age-matched (58.4±14.8) subjects were enrolled. Analysis of variance (ANOVA), LSD test and two tailed correlation of Pierson were used for statistical analysis. The levels of AEAb and MMP-2 were not changed in the sera of investigated patients compared to healthy controls. The levels of AC4Ab (IgG) were significantly elevated in stroke patients compared to controls. We found significantly increased MMP-9 activity ($p=0.036$) compared with controls. Despite the unchanged AC4Ab (IgM) levels a correlation with MMP-9 serum level was observed ($r = 0.416$, $p<0.05$). Our results demonstrate that higher serum levels of MMP-9 in patients with stroke in chronic phase are associated with elevated AC4Ab (IgG) and correlate with AC4Ab (IgM).

Key words: anti-elastin antibodies, anti-collagen type IV antibodies, MMP-9, MMP-2, stroke

INTRODUCTION

Matrix metalloproteinases (MMPs) are a major group of enzymes that regulate cell-matrix composition by using zinc for their proteolytic activities (1). Increased matrix metalloproteinase-9 (MMP-9) and matrix metalloproteinase-2 (MMP-2) activity is associated with destruction of the elastic laminae of arteries, because of their ability to degrade almost all components of extracellular matrix and basal lamina such as elastin, fibrillins, laminin, or collagen type IV (2).

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Abnormal expression and increased activity of matrix metalloproteinase-9 (MMP-9) and matrix metalloproteinase-2 (MMP-2) play an important role in the pathogenesis of stroke. More recently, plasma MMP-9 levels have been identified as a novel predictor of cardiovascular risk in patients with coronary artery disease and stroke (3).

Elastin and collagen type IV are the major extracellular matrix proteins and essential mechanical component. Elastin, the main provider of tissue elasticity, is long-lived connective tissue protein as with a very slow turnover (4). Degradation of elastic fibers is mediated by elastolytic enzymes, liberated from different types of cells including granulocytes, monocytes, fibroblasts, cancer

cells and others. These enzymes could be classified as elastases from neutrophils and platelets, metaloproteinases and macrophage metalloelastase 12 (5). Although the small amount of elastin is degraded normally, in pathological conditions and aging increased degradation and fragmentation of elastic fibers is observed (6). AEAb are antibodies which are established as a part of normal homeostatic mechanism, with a role in clearance of the fragments of elastin degradation – elastin derived peptides (7). It is established that AEAb (G) correlate positively with products of elastin degradation (8). AEAb may play a role in remodeling of injured elastic structures by binding and clearance of EDP resulting from physiological or pathological turnover of elastin. Abnormal variations in elastin metabolism were established in a variety of autoimmune diseases (9). An increase of AEAb has been registered in patients with diabetes (10), atherosclerosis (11), autoimmune diseases – systemic lupus erythematodes (12), scleroderma (13), systemic diseases – polymialgia rheumatica (14), fibromyalgia syndrome (15).

AIM

Until now, elastin turnover as a result of activity of MMP-9 has not been an object of investigation in stroke. The aim of this study was to investigate elastin metabolism in association with MMP-9 in stroke patients in chronic phase. Our study suggests that chronic phase of ischemic stroke is characterized with change of the immune response to degraded elastin associated with high activity of MMP-9.

MATERIALS AND METHODS

Patients

Thirty-one patients with stroke (mean age 63.4±11.9; age range 39-84 years; 18 women) were studied in chronic phase (3-12 months after the onset) with clinical evaluation, MRI, CT of the brain, Duplex/Doppler sonography of extracranial vessels. To obtain sera from healthy controls, 37 age-matched (58.4±14.8) subjects were enrolled. At the time of the subject inclusion, a 10 ml blood sample was obtained by peripheral venipuncture, into a dry tube, for later serum MMP, anti-elastin IgG and IgM antibody. Serum was stored at –20°C until analysed using home made enzyme-linked immunosorbent asay (ELISA).

Gelatinolytic zymography

For the determination of gelatinolytic activity of MMP-2 and MMP-9 substrate-specific zymography was performed in SDS-PAGE gels (7%) containing 0.1% gelatin. Molecular weight standards were run with samples (20 μg). Following electrophoresis the gels were washed in renaturation buffer (2.5% Triton X-100, pH 7.5) for 1 h. Then the zymograms were incubated for 18 h at 37°C in incubation buffer (0.2 M NaCl, 5 mM CaCl, 50mMTris-HCl, pH 7.5). Enzymatic bands were visualized after staining for 3 hours with Coomassie brilliant blue G-250 and destained with methanol and acetic acid. Enzymatic activities were ensured by incubating identical zymograms with the addition of 20 mM EDTA. To measure gelatinase activity, gels were scanned and the intensity of the bands was read by ImageJ image analysis software.

Immunoblotting

MMP-9 and 2 expressions were detected by Western blot analysis. Twenty μg of total protein were loaded in SDS-PAGE (7%) and transferred to a nitrocellulose membrane. After blocking nonspecific bindings, the membrane was incubated for 2 h with rabbit anti-human MMP-2 and MMP-9 antibody (Sigma, Saint Louis, USA) at 1:500 in 0.1% Tween Tris Bufferd saline. Secondary peroxidase-conjugated antibody (BulBio, Sofia, Bulgaria), diluted 1:1000, was used for incubation of the membrane overnight at 4°C. Orto-phenylenediamine plus 0.1% H_2O_2 was used as colorimetric substrate. The membrane was scanned and the intensity of the bands was read by ImageJ image analysis software.

Statistical analysis

Differences in between the groups were analyzed for statistical significance ($P<0.05$) with one-way analysis of variance (ANOVA) and multiple comparison test – Post Hoc test, Least Significant Difference (LSD method), and for non parametric distributed data with Kruskal-Wallis method. Two tailed correlation between the parameters was tested by the method of Pierson. Statistical package SPSS, v. 15 was used for data analysis.

RESULTS

Antibodies directed to ECM components

The levels of AEAb (IgM) were significantly elevated in healthy controls compared to stroke patients (p=0,002), **(Table 1)**. The levels of AC4Ab (IgG) were significantly elevated in stroke patients compared to controls (p=0,003), **(Table 1)**.

Table 1. *Serum levels of anti-α-elastin (AEAb) and anti-collagen type IV (AC4Ab) antibodies. The mean difference is significant at the 0.05 level.*

Groups	AEAb (OD 492 nm)		AC4Ab (OD 492 nm)	
	IgG	IgM	IgG	IgM
Controls	0,190	0,468	0,151	0,254
Stroke	0,210	0, 287	0,241	0,281
P	0,216	**0,002***	**0,003***	0,504

MMP-2 and MMP-9

Gelatin zymography showed significantly increased levels of MMP-9 (p=0.036) compared to controls (**Figure 1 and 2**). MMP-2 serum levels were not changed. We established the higher levels of MMP-9 and anti-collagen type IV antibodies (IgG) in patients with stroke in chronic phase. Despite the unchanged AC4Ab (IgM) levels moderate correlation (r=0.416, p<0.05) was observed between MMP-9 and anti-collagen type IV antibodies (IgM).

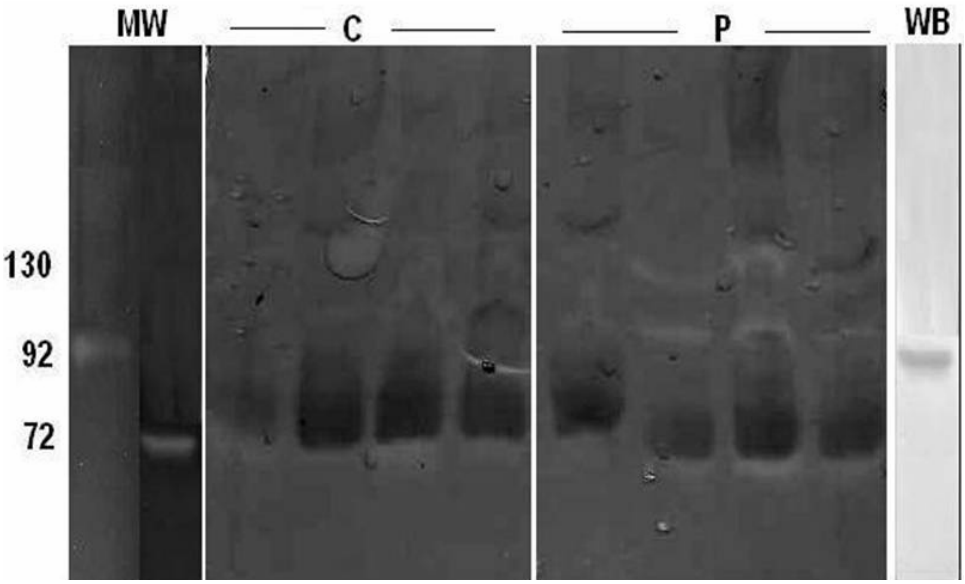

Figure 1. Gelatin zymography of sera from ischemic stroke patients and healthy subjects. (MW) Molecular weight standards of pro-MMP-9 (92kDa) and pro-MMP-2 (72kDa); (C) Sera of control healthy subjects; (P) Sera of ischemic stroke patients; (WB) Western blotting of pro-MMP-9.

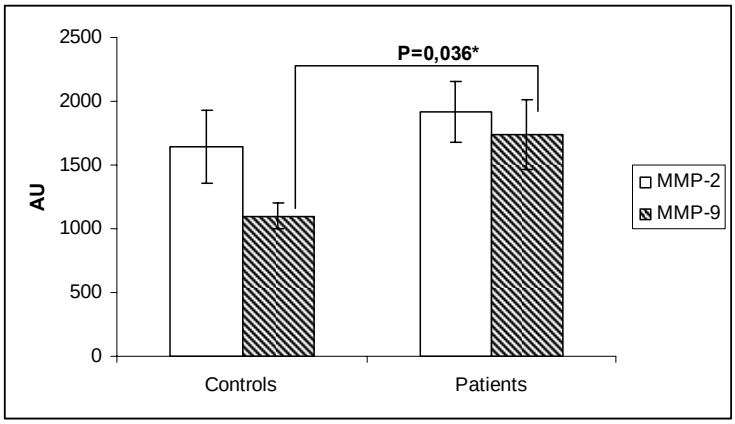

Figure 2. MMP-9, measured as arbitrary units (AU), *P = 0.036

DISCUSSION

MMP-2 and MMP-9 are involved in the degradation of matrix proteins (collagen type 4 and 5, elastin and tropoelastin). Their increase in the serum of patients with micro- and microangiopathy of the central and peripheral nervous system is an indicator of increased degradation of extracellular matrix proteins (16). The increased levels of anti-collagen type IV antibodies in the peripheral blood are related to MMP-9 provoked degradation of collagen type IV in basal lamina, that compromise blood brain barrier integrity. In some prospective studies found that patients with high initial levels of MMP-9 have an increased risk of stroke and death from cardiovascular disease in the following 5 years (17).

The results of these epidemiological studies are consistent with the results of clinical evaluation (high resolution MRI angiography, CT of the brain, Duplex/Doppler sonography of extracranial vessels), pathomorphological and immunological studies clearly show that lesion of the "fibrous cap" on the atherosclerotic plaque is the main mechanism for the transformation of stable in unstable (active, high risk) atherosclerotic plaque (18). Given that "fibrous cap" is composed primarily of substrates for matrix metalloproteinases 2 and 9, i.e elastin and collagen, arise the questions whether the change in levels of the decomposed products and humoral immune response against them correlate with an increased risk of acute vascular driven ischemia, causing stroke, heart attack, gangrene, and chronic nature of events (as a result of remodeling small cerebral vessels and vessels of the peripheral nerves).

CONCLUSIONS

The present study does not identify differences in the serum activity of MMP-2 - a fact which confirms the study of Montaner et al. (Montagner et al., 2001). The high serum activity of MMP-9 is also in agreement with the results of studies in patients with a recent stroke, but for the first time the chronic phase of ischemic stroke is associated with high serum anti-collagen type IV and anti-α-elastin antibody. The altered immune response against extracellular matrix proteins is probably due to increased degradation of the "fibrous cap" in atherosclerotic plaques. The parameters studied by us could be used as prognostic markers of vascular damage, but for their approval, further studies with a large number of patients need to be conducted.

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