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CLEAVAGE OF PROTHROMBIN BOUND IN IMMUNE COMPLEXES RESULTS IN HIGH THROMBIN ENZYMATIC ACTIVITY.

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Thrombin plays a pivotal role in blood clotting as well as in the regulation of vascular remodeling and oxidative stress. Recent evidence suggests that auto-antibodies directed against prothrombin, may play an important role in the pathogenesis of atherosclerosis. It is however not clear, if prothrombin bound in an immune complex retains its clotting and regulatory properties or acts solely by increasing vascular inflammation. In order to answer this question, we used a newly developed stain for the detection of thrombin activity of such complexes. Plasma and serum samples were subjected to rocket immunoelectrophoresis in an anti-prothrombin antiserum containing agarose gel. Gel plates, covered with a nitrocellulose membrane were soaked with chromogenic thrombin substrate. The product of thrombin activity was diazotized to red azo dye bound to nitrocellulose. Activity stain revealed barely discernible rockets in plasma, but heavily stained ones in serum. Pre-incubation with trypsin enhanced activity of immunoprecipitates deriving from plasma, but not from serum. Densitometric analysis showed, that the trypsin-enhanced activity in plasma derived immune complexes was twice as high as in serum derived immunoprecipitates. Thrombin active centre is not blocked by anti-prothrombin antiserum allowing to retain thrombin activity. Moreover, prothrombin in immunoprecipitate is readily cleaved by proteolytic enzymes. This cleavage could potentially be enhanced by antibody binding, although these results need to be confirmed using different antibodies.

Key words: *prothrombin, thrombin, activity staining, immune complexes, atherosclerosis*

INTRODUCTION

The importance of autoimmune syndromes in the pathogenesis of atherosclerosis has been implicated by several cell culture and animal models (1). Recent clinical studies confirmed (by means of intima-media thickness or coronary artery calcium score) that atherosclerotic plaque development is enhanced in patients with antiphospholipid syndrome, lupus erythematodes or other connective tissue disorders (2, 3). Moreover immune complex deposition within the vascular wall has been associated with endothelial dysfunction and atherosclerosis (1). It is however not clear whether the contribution of immune complexes to vascular inflammation and atherosclerosis is dependent solely on pro-inflammatory properties of immune complex or, maybe, molecules trapped by auto-antibodies may preserve their biological functions and properties. Prothrombin may be one of the examples of such molecules. Presence of auto-antibodies against prothrombin have been indicated in anti-phospholipid syndromes (4, 5), which in turn present with early atherosclerosis (6). Prothrombin in plasma may get cleaved to active thrombin by variety of stimuli that regulate hemostasis (7). Diversity and importance of thrombin interactions with its plasma inhibitors is also well established. For instance, thrombin formation during blood clotting has long been known to coincide with the appearance of proteolytic activity bound to alpha-2 macroglobulin (8). Much less information is available on the effect of the specific anti-prothrombin antibodies on the enzyme activity. These could prevent prothrombin cleavage or could lead to the loss of the enzyme activity. However, retention of its activity in some immune complexes may also be anticipated, which could explain some surprising associations of the presence of anti-prothrombin antibodies in the sera of patients with certain thrombotic events (9), and even the risk of myocardial infarction (10). Of a special interest, is the group of patients with lupus inhibitors in whom abnormal prothrombin crossed immunoelectrophoresis has been shown by Edson et al (11).

Accordingly, we aimed to develop a method, based on the activity stain reaction, for the detection of thrombin activity in thrombin and prothrombin-containing immune complexes.

MATERIALS AND METHODS

Plasma and serum samples were collected from healthy volunteers and diluted 1:3 in PBS. Rocket immunoelectrophoresis of plasma or serum samples was then performed in anti-prothrombin antiserum containing (1μL/cm^2, DAKO) 1% agarose gel in 0.05M Tris-HCl buffer, pH 8.6 to form immune complexes containing thrombin or prothrombin. In order to avoid activation of clotting enzymes in contact with glass, gel was placed on plastic gel-fix membrane, 50 mm x 75mm (Serva). The electrophoresis was conducted for 20 hours at 50V. Then, gel plates were carefully pressed and washed 3 times for 15 minutes in 0.9% NaCl and finally for 15 minutes in the incubation buffer (0.2M Tris-HCl + 0.05M $CaCl_2$, pH 7.8). Each wet gel plate was carefully covered with a strip of nitrocellulose membrane, soaked with chromogenic tripeptide thrombin substrate H-

D-Phe-Pip-Arg-pNA (S-2238), dissolved in the incubation buffer (0.2M Tris-HCl+0.05M $CaCl_2$, pH 7.8). A sandwich formed was placed between two glass or plexiglass plates, tightly bound with rubber rings, and incubated in a humid chamber for 1h at 37°C. Then, nitrocellulose membrane was separated from agarose gel and yellowish product of thrombin activity (p-nitroaniline), blotted onto nitrocellulose was diazotized with N-(1-naphthyl)ethylenediamine (12), to readily visible red azo dye. Simultaneously, immunoprecipitates in agarose gel were stained for proteins with Coomassie Blue R-250.

In order to analyze the possibility of in vitro activation of prothrombin bound within immune complexes by trypsin, agarose gel with prothrombin or thrombin containing rocket immunoprecipitates were pre-incubated with this enzyme. A method described above was followed, but additionally, agarose gels with rocket immunoprecipitates, pressed and washed were incubated in 0.1M buffer Tris-HCl, pH=8.0 with 10mM $CaCl_2$, containing 25 µg/ml or 5 µg/mL of trypsin, at 37°C for 20 min. Then, in order to remove trypsin, plates were washed 4 x 15 minutes in 0.9% NaCl and finally in the incubation buffer for 10 min., using a shaker. Staining for thrombin activity was then continued as described above.

Image obtained using photography of stained nitrocellulose membranes was digitally analyzed using Scion Image (Scion Corporation; NIH) software. Statistical analysis was performed using t-test and Mann-Whitney U test.

RESULTS

Thrombin activity of serum and plasma derived immune complexes

Immune complexes were generated from plasma and serum in the agarose gel, using specific anti-prothrombin antibodies. Activity stain for thrombin of serum and plasma, run by rocket immunoelectrphoresis in anti-prothrombin antiserum containing agarose gel, revealed barely discernible thrombin activity in plasma rocket immunoelectrophorograms, but a very high level of the activity in serum rocket immunoelectrophorograms (*Fig. 1*).

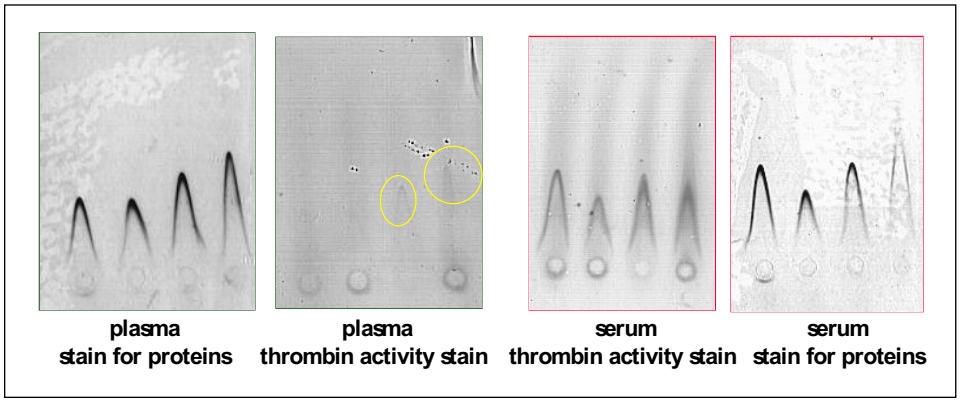

Fig.1. Activity stain for thrombin of 4 serum and plasma samples run by rocket immunoelectrphoresis in anti-prothrombin antiserum containing agarose gel.

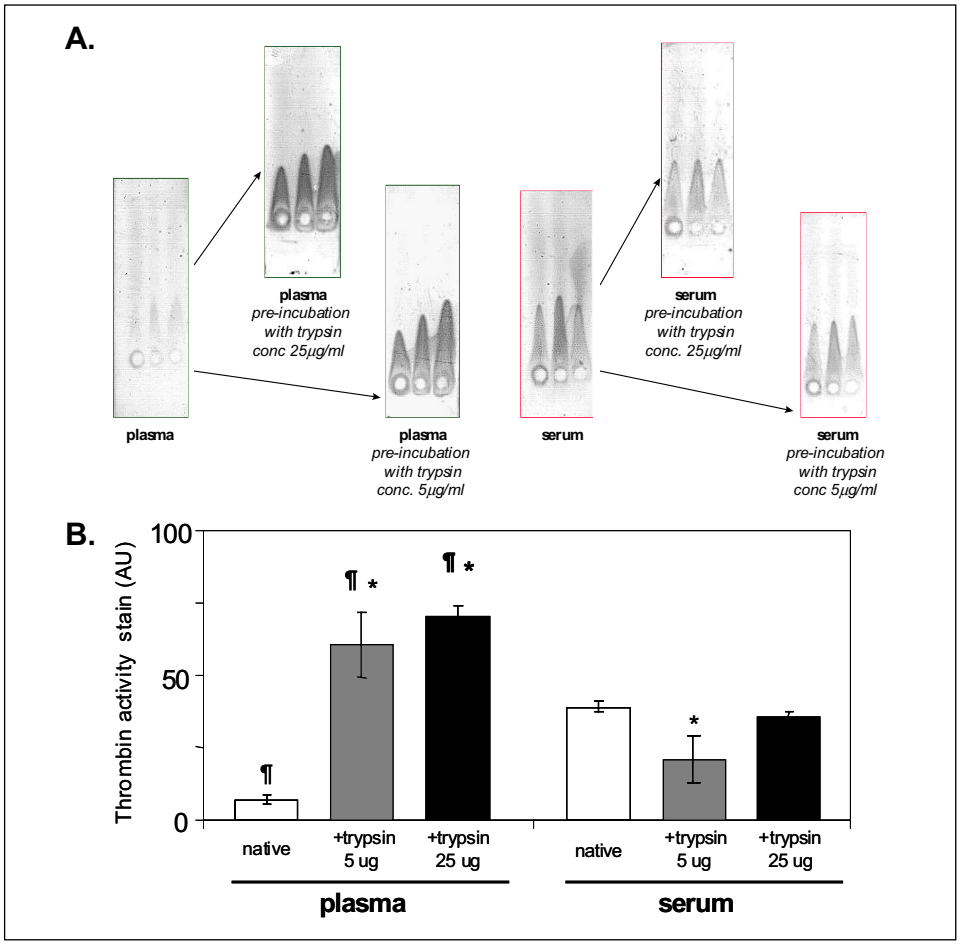

Fig. 2. Effect of the pre-incubation with trypsin on the activity stain for thrombin of immunoprecipitates derived from serum and plasma run by rocket immunoelectrphoresis in anti-prothrombin antiserum containing agarose gel. Panel A. Representative photographs of nitrocellulose membrane; Panel B. Effect of trypsin incubation on thrombin activity of immune complexes (mean±SEM; *-$p<0.05$ vs native; -$p<0.001$ vs native serum).

Activation of prothrombin trapped in immune complex

The effects of the pre-incubation with trypsin on the activity stain for thrombin of immunoprecipitates derived from serum and plasma run by rocket immunoelectrphoresis in anti-prothrombin antiserum containing agarose gel were investigated (*Fig. 2*). Results clearly indicate, that pre-incubation with trypsin enhances activity of immunoprecipitates deriving from plasma, but not from serum, which results from cleaving of prothrombin by trypsin. Plasma derived

immunoprecipitates showed significantly greater activity than serum, also in the experiment where paired plasma samples and serum samples from the same subjects were subjected to trypsin.

DISCUSSION

In the present study, we describe a method of the detection of thrombin complexed with polyclonal anti-prothrombin antiserum and retaining enzymatic activity. Anti-prothrombin antisera tested in our experiments were not directed towards the catalytic site of thrombin and the enzyme, trapped in immunoprecipitate, readily split the peptide substrate. Moreover our data may suggest that antibody bound prothrombin may, in some way, be more prone to cleavage, therefore allowing for more thrombin generation than other physiological stimuli. To our knowledge, this is the first demonstration of this phenomenon, and the potential mechanisms of this phenomenon are unclear. It would be interesting to perform similar experiments with an antiserum directed to the active form of thrombin as control, however to our knowledge, it is not available on the market. Several studies in the past have shown that antibody binding to enzyme molecules may change their properties. In particular, antibodies, directed toward active enzyme molecules may reduce, block (13), not change (14), or enhance (15) their activities.

Immune complexes, retaining enzymatic activity and forming immunoprecipitates may be stained using cytoenzymatic methods, provided that products formed are insoluble or may be insolubilized. The thrombin substrate, S-2238 is converted by the enzyme into a soluble, yellowish product, p-nitroaniline, freely diffusing to the surrounding medium.

Insoluble immunoprecipitates, containing thrombin may not be stained *in situ* for the enzyme activity because of an extensive diffusion of p-nitroaniline. Also a transfer of the intact complex (without dissociation into antigen and antibody components) is rather difficult, if possible. We offer a simple method (based on assay described by Ohlsson et al (12)), based on the close contact of insoluble (immunoprecipitated) thrombin with nitrocellulose membrane soaked with the substrate, what prevents diffusion of the reaction product and results in a sharp localization of the enzyme activity. Also, investigation of the activation processes of the proenzyme (prothrombin) in immune complexes, as well as the possibility of the detection of complexes of autoantibodies with thrombin or prothrombin as autoantigens, will make, we hope, this method useful.

Our findings might be especially important in the light of the new findings of the possible role of anti-enzyme antibodies, also anti-prothrombin antibodies, in human pathology. Their presence and increased level was shown to be involved in the pathogenesis of "lupus anticoagulant" as shown by Swadzba et al. (4) and Ieko et al. (16) and occur frequently in patients with systemic autoimmune

disease (17), and also in habitual abortions patients (9). Vaarla et al. showed, that antibodies to prothrombin imply risk of myocardial infarction which may be mediated by hypercoagulative mechanisms (10). Indeed the presence of these antibodies seems to increase the risk of thrombosis, while other antibodies, like antiplasminogen don't seem to have this effect (17). A recent study showed that injection of anti-prothrombin antibodies (similar to used in the present study), in an animal model, results in thrombosis (18). Our results show, that binding of prothrombin by antibodies does not lead to its activation by itself, however it appears to make it more prone to cleavage by proteolytic enzymes like thrombin. Activities detected in immune complexes within the rockets in the gel following trypsin treatment were significantly greater in plasma than when thrombin (rather than pro-thrombin) was bound directly (serum). Our results may explain why anti-prothrombin antibodies may increase thrombosis while not interfering with active site of the enzyme. Our results may suggest that binding of prothrombin by an antibody may increase its proteolytic cleavage capacity and therefore generate more thrombin activity. This mechanism may be particularly important as immune complexes from blood are very likely to be deposited in vascular wall and induce inflammation. Inflammatory cells, in turn, include granulocytes and macrophages which may release their granules containing numerous trypsin-like proteolytic enzymes (19). Generation of thrombin within an immune complex targeted already to the vascular wall may have other important implications for vascular biology. Thrombin has been shown to activate migration and proliferation of vascular smooth muscle cells, mainly by directly increasing vascular NAD(P)H oxidase activity (20). It has been shown that vascular NAD(P)H oxidase activity is important in the development of atherosclerosis and endothelial dysfunction (21, 22) and may be significantly related to risk factor profile (23). Thrombin bound to immune complexes may possibly activate this oxidase, increase superoxide production and contribute to the loss of nitric oxide bioavailability with it's numerous pathological consequences (24 ,25).

The participation of inflammatory cells in the activation of prothrombin bound in the immune complex may also be important in relation to the role of microbial antigens (*H. pylori* or *Ch. pneumoniae*) in endothelial dysfunction and atherosclerosis (26). Finally, we believe that the method we described here is not limited to visualization of thrombin in the immune complex. Activity stain with the use of nitrocellulose membrane, soaked with appropriate substrate seems to be easily adaptable also for the detection of enzymatically active immune complex forms of other p-nitroaniline liberating enzymes.

Concluding, we found that thrombin active centre is not blocked by anti-prothrombin antiserum allowing to retain thrombin activity. Moreover, the proenzyme (prothrombin) in immunoprecipitate is readily cleaved by proteolytic enzymes. This cleavage appears to be enhanced by antibody binding.

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