# Blood plasma proteins and cellular components as modulators of fibrinolysis

Ph.D. thesis

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Budapest
2008

## Introduction

It is generally accepted that in the fibrinolytic process plasminogen activators (e.g. tissue type plasminogen activator, tPA) convert plasminogen to plasmin, which dissolves fibrin by hydrolytic cleavage of peptide bonds. These enzymatic reactions are well-known, but their course in vivo often differs from their in vitro kinetics. The complex composition of thrombi can modify, inhibit or enhance, the effectiveness of the fibrinolytic system. Thus, in recent years it has become clear that the models and studies of thrombolysis should take into consideration the composition of in vivo thrombi, the concentration of various molecules in the thrombus compartment and the flow conditions. We investigated the effects of different macromolecules originating from the cellular elements of thrombi (platelets, leukocyte) - structural proteins (myosin), phospholipids and enzymes (neutrophil leukocyte elastase: NE) - on the thrombolytic process. The immunoglobulin-G (IgG) antibodies present at high molar concentration in the blood plasma, and especially IgGs from patients with antiphospholipid syndrome were also studied. We investigated their effect on the thrombolytic process and their putative interaction with phospholipids.

## Methods

Normal (N-) and APS-IgGs, human plasminogen were isolated from normal and APS patients' blood plasma. For the in vivo experiments with NE and $\alpha_1$-protease inhibitor ($\alpha_1$-PI), plasmas were taken from patients with pulmonary thromboembolism.

The IgG fraction of the plasma samples was isolated by affinity chromatography on Protein A Sepharose. The Fab and Fc fragments of the IgG molecules were prepared by papain digestion. Phospholipid vesicles containing phosphatidyl-choline (PC) and phosphatidyl-serine (PS) used in our experiments were prepared with brief sonication following extrusion through a polycarbonate filter.

The coagulation and the solubilization of the fibrin gel were followed at 340 nm with turbidimetry (a method based on light scattering or on changes in the light scattering of the samples). To determine the penetration of tPA into the fibrin clot $Eu^{3+}$-labeled tPA (Eu-tPA) was applied. Plasminogen activation and the amidolytic activity of plasmin generated from plasminogen were measured on the chromogenic substrate spectrozyme-PL (SPPL, *H*-D-norleucil-hexahidrotirosil-lizin-*p*-nitroanilid). SPPL was applied on fibrin surface containing modulator molecules. The kinetics of the myosin-fibrinogen association and dissociation was measured by immobilized myosin and $^{125}I$- or $Eu^{3+}$-labeled fibrinogen. The interaction between the fibrin and the myosin was also examined by BIA (biomolecular interaction analysis). This method detects the surface plasmon resonance (SPR) signal of the molecules binding to the immobilized surface. The $\alpha_1$-proteinase-inhibitor ($\alpha_1$-PI) – NE complex concentration in the blood plasma was determined by ELISA.

## Results

*The effects of IgGs on the fibrinolytic process in phospholipid-free enviroment*

The prolongation of the lysis time of fibrin clots containing N-IgG is by at least 25%, up to 2-fold compared to the pure fibrin, whereas with the IgG fraction from the APS patients – at least 60% up to 6-fold. Likewise, the dissolution of plasma clot samples from APS patients is definitely slower than in the control normal plasma samples. The IgGs from APS patients retard the dissolution of clots prepared from normal plasma supplemented with APS IgGs. Our results show that IgG antibodies present at high molar range in the plasma enhance the fibrinolytic resistance of the clots, and APS-IgGs exert a stronger antifibrinolytic effect. These effects are specific: the variable Fab portion of the IgG molecules prolongs the lysis times, while the constant Fc fragments do not.

The IgGs do not inhibit the amidolytic activity of plasmin. Neither the APS- nor the N-IgGs affected the plasmin action on SPPL substrate. The N-IgGs did not influence, but the APS-IgGs obliterated the competition of the fibrin and the SPPL for the protease. This effect raises the possibility for interaction between the APS IgG and the fibrin or between the APS IgG and the fibrin bound plasmin that makes the fibrin less susceptible for the protease or changes the kinetic properties of the fibrin bound enzyme.

Under flow conditions both the N- and APS-IgGs stabilize the fibrin clot, the samples containing IgGs disassembly later. However the APS-IgGs enclosed in fibrin result in larger soluble fibrin degradation products. In conclusion both the N- and APS-IgGs stabilize the fibrin degradation products in the fibrin structure, but in a different degree.

*The effects of IgGs on the fibrinolytic process in phospholipid enviroment*

Platelet phospholipids render thrombi resistant to fibrinolytic effects. We investigated the impact of normal and APS-IgG antibodies on the elementary steps of fibrinolysis (tPA penetration into fibrin clots; plasminogen activation by tPA; dissolution of fibrin by plasmin) in phospholipid environment.

Phospholipids fill in the pores of the fibrin gel and thus impair the penetration of the activator or other exogenously applied proteins. Our experiments show that IgGs have the same impact; both the N- and APS-IgGs decreased the amount of tPA penetrating into the fibrin clots. This finding can be explained by the phenomenon that the presence of IgG antibodies results in thinner fibers and narrow pores in the fibrin structure. Phospholipids and IgGs applied together at near-physiological concentrations also decrease the penetration of tPA into the fibrin clot acting in synergy. However, some pathological IgGs do not affect the diffusion of tPA into the fibrin clot and do not modify the barrier effect of the phospholipids. The loss of function of certain APS-IgGs may contribute to the variability in the fibrinolytic activity in APS patients.

The tPA diffusion into the reactive surface layer of fibrin determines the rate of activation of plasminogen closed into the clot. The phospholipids also decrease the rate of plasmin formation by binding the components of the fibrinolytic system. The IgGs may enhance or obliterate this inhibition in parallel with their impact on the tPA penetration. However, the effect of some APS-IgGs on the plasminogen activation process cannot be attributed only to the impaired diffusion, but to the optimized formation of the ternary tPA-fibrin-plasminogen complex, which results in increased plasmin generation.

Phospholipids render the fibrin clots resistant to plasmin. According to our experiments, APS-IgGs modify this resistance, whereas N-IgGs do not. APS-IgGs initially enhance the antifibrinolytic effect of the phospholipids, but some of the APS-IgGs favor the plasmin action in the advanced stage of fibrin dissolution. When such an APS-IgG was applied, the initial fibrinolytic resistance of the clot decreased, and it was the first to be completely dissolved. The background of this duality can be the additivity of the phospholipids and IgG effects in the basal state of the fibrin matrix followed by the displacement of plasmin from the phospholipid surface due to some APS-IgGs in the loosened structure of the clot in the later stages of the process. This phenomenon may be relevant to the variations in fibrinolysis at different stages of thrombus ageing.

In conclusion, in the elementary steps of the fibrinolysis the APS-IgGs may have antifibrinolytic effects, while in the other phases of the process the same APS antibodies may enhance the fibrinolysis. The overall impact of the IgGs on the whole fibrinolytic process cannot be easily predicted, as it arises from these contradictory effects. The effects of the APS-IgGs examined do not show consistent deviation from the pattern of normal IgG effects in our experimental setting. This implies that a high degree of heterogeneity can be expected in the APS patients' population, if future studies evaluate the discrete steps of fibrinolysis in more patients.

*Myosin, a stabilizer of the fibrin structure*

Both IgGs and phospholipids impair the fibrinolytic process, while in combination they act in synergy. Arterial thrombi contain a significant amount of various macromolecules, e.g., it is estimated that the concentrations of platelet myosin and fibrin are in the micromolar range (0.5 – 5 μM). Fibrin and fibrinogen are not passively surrounded by other molecules; fibrinogen circulates in weak noncovalent association with a

number of plasma proteins which may interfere with the thrombotic and fibrinolytic processes.

The dissolution of fibrin clots slowed down in the presence of myosin compared to the myosin-free samples. Myosin, and only free myosin (not complexed with fibrin), contributes as a cofactor to tPA activation. At low concentrations, myosin binds to fibrin resulting in the retarded dissolution of the fibrin clot. At a high concentrations of myosin (> 1 μM), the inhibition of fibrinolysis is obliterated if the plasminogen is activated by tPA: at high molar range the myosin acts as a cofactor, compensating the antifibrinolytic effect of fibrin-bound myosin. The uPA dependent plasminogen activation does not require a cofactor, so linear inhibition of the fibrinolysis by increasing myosin concentration can be observed only if plasminogen is activated by uPA.

However, myosin is also the substrate of plasmin. The retarded dissolution of fibrin clots containing myosin can be attributed to the competition of the two alternative substrates. The mechanism of this retarded fibrinolysis can be that fibrin-bound myosin renders fibrin less susceptible for the plasmin.

An investigation into the interaction between immobilized myosin and $^{125}I$- or $Eu^{3+}$-labeled fibrinogen revealed that both the dissociation and the association phases are slow; equilibrium was achieved in approximately 2 hours. The binding is reversible, noncovalent. The association and dissociation rate constant values are 180.6 $M^{-1}s^{-1}$ and $3.07 \times 10^{-4}$ $s^{-1}$. According to our data, there is a difference of 4 orders of magnitude between the rate constant of the myosin-fibrinogen binding and that of the fibrin-monomer association. Thus, due to the low rate of the interaction, myosin cannot influence the fibrin polymerization, the formation and the structure of the fibrin clot.

Our findings were confirmed by BIA. The interaction of fibrin and myosin was characterized with BIA technology. When soluble fibrin at various concentrations was injected over a surface with immobilized myosin, the SPR response produced signals typical of weak ligand interaction. The equilibrium dissociation constant calculated for the interaction of this soluble fibrin species and myosin is 0.94 μM. The higher affinity of the self-aggregating fibrin monomers (Kd=0.156 μM) dominates, and apparently the fibrin architecture is not affected by myosin.

When soluble myosin was injected over the surface of immobilized fibrin, the slowly progressing increase of the SPR response showed fibrin-initiated myosin aggregation rather than simply myosin-fibrin association. In solution, myosin self-associates (aggregates), which is dependent on ionic strength and pH. At physiologic ionic strength and pH, myosin starts to self-associate. The fibrin network may serve as a matrix core that initiates the aggregation of myosin and promotes the forming of myosin dimers and larger aggregates.

GPRP-peptide prevents fibrin polymerization due to binding to fibrinogen at a site in the molecule that is responsible for the association of 2 fibrin monomers. Its application allowed us to exclude the involvement of this polymerization site in the fibrinogen-myosin interaction. Since both myosin and fibrin are substrates of FXIIIa, we checked the possibility for myosin being covalently crosslinked to fibrin by FXIIIa. According to our findings, neither is myosin covalently attached to fibrin, nor does it influence the time course of the fibrin crosslinking at biologically relevant FXIIIa concentrations.

*The contribution of $\alpha_1$-proteinase inhibitor levels to in vivo fibrinolytic potential*

The solubility of fibrin clots is affected not only by plasma or platelet proteins, but also by enzymatic effects, typical of the thrombus compartment. Accordingly, the interaction between the neutrophil leukocyte elastase (NE) and its inhibitor, $\alpha_1$-proteinase inhibitor ($\alpha_1$-PI), was studied.

In vivo the reaction between NE and $\alpha_1$-PI is a pseudo-first order reaction as is generally assumed. According to our observation, there is a weak, but significant correlation between $\alpha_1$-PI and $\alpha_1$-PI–NE complex concentration. This implies that the $\alpha_1$-PI–NE complex concentration does not only depend on the enzyme concentration, but also on the availability of the inhibitor. We have to take into consideration that the complex is mainly generated in the thrombus compartment, where PMN-leukocytes release a large amount of NE, whose concentration can be near equimolar with its inhibitor. This means that at sites where NE is released, the $\alpha_1$-PI + NE → $\alpha_1$-PI – NE reaction can follow a second-order kinetics.

The $\alpha_1$-PI present at high molar range in the circulation inhibits immediately the NE released and affected in the thrombus compartment, so no free NE can be observed in the plasma; its concentration can be estimated indirectly. According to our previous findings, we can assume a local significant elastase action or increased fibrinolytic potential, if NE-generation relates to lower $\alpha_1$-PI levels. The increased fibrinolytic potential mentioned above is caused by NE, which has a direct fibrinolytic effect, but also cleaves plasminogen. The residual derivative is miniplasminogen, which can be activated by plasminogen activators faster than plasminogen. Likewise, higher plasma $\alpha_1$-PI concentrations result in decreased NE action and impaired fibrinolytic potential in the thrombus compartment.

This assumption is supported by the correlation observed between

the lysis times and the concentration of the $\alpha_1$-PI–NE complex, if fibrinolysis is induced by tPA; but there is no correlation if it is induced by plasmin. Lower $\alpha_1$-PI level relates to lower complex concentration, which locally results in enhanced NE action. As a consequence of this NE effect, miniplasminogen is generated and activated by tPA at high molar range. The forming miniplasmin dissolves fibrin like plasmin.

In our experiments human plasmas were clotted and lysed, so their fibrin structures were stabilized with plasma FXIIIa. Miniplasmin is a more effective fibrinolytic enzyme on covalently crosslinked fibrin than plasmin, and resists more to plasmin inhibitors. The increased NE action and elevated fibrinolytic potential followed by increased miniplasminogen and, subsequently, by tPA-induced miniplasmin generation lead to shorter lysis times. Likewise, a high $\alpha_1$-PI–NE complex concentration relates to impaired NE action and, consequently, to impaired miniplasminogen generation, which results in slow fibrinolysis induced by tPA.

The same correlation between the lysis times and the concentration of the enzyme-inhibitor complex cannot be observed, if fibrinolysis is induced by plasmin. Thus, the correlations between the lysis times and the complex concentrations mentioned above can be attributed to the mechanisms in the zymogen activation phase of the tPA-induced fibrinolytic process.

The $\alpha_1$-PI affects the fibrinolytic properties of the fibrin clot indirectly by modifying its structure. A decrease in the thrombin activity results in thicker fibers and a coarse fibrin structure. The fibrin gel network like this is a better cofactor of tPA, but a weaker substrate of plasmin. One of the alternative targets of $\alpha_1$-PI is thrombin, decreasing the amount of the active enzyme. We demonstrated that elevated levels of $\alpha_1$-PI render the fibrin clot structure more resistant to the effects of fibrinolytic enzymes (tPA, plasmin) by decreasing thrombin action.

## Conclusions

1. The IgGs both from normal human plasma and from patients suffering in APS inhibit the course of the fibrinolysis, but APS-IgGs exert an even stronger antifibrinolytic effect. The antifibrinolytic effects are caused by the variable Fab portion of the IgG molecules.
2. The IgGs do not affect directly the amidolytic activity of plasmin; the APS-IgGs rather modify the fibrin-plasmin interaction.
3. The normal and APS-IgGs reduce the tPA diffusion into fibrin clots, and they act in synergy with phospholipids. However, some pathological APS-IgGs do not have this property.
4. The effects of the IgGs on the tPA penetration determine the rate of the plasminogen activation in the reactive surface layer of the fibrin clot.
5. The IgG antibodies from APS patients initially enhance the resistance of fibrin clots containing phospholipids to plasmin, but in the advanced stage of fibrin dissolution they may have divergent effects.
6. Myosin stabilizes fibrin clots, and renders them more resistant to fibrinolytic effects.
7. The fibrinogen-myosin dissociation and association are both slow (180.6 $M^{-1}s^{-1}$ and 3.07 $\times$ $10^{-4}$ $s^{-1}$). Myosin binds reversibly, noncovalently to fibrinogen ($K_d$=1,35-1,70 μM).
8. The low $\alpha_1$-PI level of the blood plasma relates to the plasma's elevated tPA-dependent fibrinolytic potential. This phenomenon may be connected with the better activatability of the miniplasminogen generated by PMN-elastase and the different structural properties of the fibrin gel related to the various inhibitor levels.

## Publications in the topic of the dissertation

1. Kolev, K., **Gombás, J.**, Váradi, B., Skopál, J., Mede, K., Pitlik, E., Nagy, Z., Machovich, R. Immunoglobulin G from patients with antiphospholipid syndrome impairs the fibrin dissolution with plasmin. *Thromb Haemost* 2002; 87: 502-508

   **IF4.357**

2. Kolev, K., Tenekedjiev, K., Ajtai, K., Kovalszky, I., **Gombás, J.,** Váradi, B., Machovich, R. Myosin: a non-covalent stabilizer of fibrin in the process of clot dissolution. *Blood* 2003; 101: 4380-4386

   **IF10.120**

3. **Gombás, J.**, Kolev, K., Tarján, E., Machovich, R. Impaired fibrinolytic potential related to elevated $\alpha_1$-proteinase inhibitor levels in patients with pulmonary thromboembolism. *Ann Hematol* 2004; 83: 759-763

   **IF1.292**

4. **Gombás, J.**, Tanka-Salamon, A., Skopál, J., Nagy, Z., Machovich, R., Kolev, K. Modulation of fibrinolysis by the combined action of phospholipids and immunoglobulins. *Blood Coagul Fibrinolysis* 2008; 19: 82-88

   **IF1.370**

## Other publications

1. Léránt, I., Kolev, K., **Gombás, J.**, Machovich, R. Modulation of plasminogen activation and plasmin activity by methylglyoxal modification of the zymogen. *Biochim Biophys Acta* 2000; 1480: 311-320

   **IF1.399**