

**MECHANICAL AND LYTIC STABILITY OF THE  
FIBRIN MESH MODIFIED WITH VESSEL WALL  
COMPONENTS**

Ph.D. Thesis

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## INTRODUCTION

Thrombi that develop *in vivo* contain not only fibrin. During their formation cellular elements (platelets, red blood cells, leukocytes), cell-derived macromolecules and other blood proteins can be incorporated in the clot. These components can directly modify the structure of the fibrin mesh or influence the enzymatic activity of thrombin that mediates clot formation or plasmin that degrades fibrin.

During their formation clots are attached to the vessel wall. Earlier work from our research team has shown that upon thrombi generation proteases derived from neutrophil granulocyte are able to change the vascular structure.

Rearrangements of vessel wall extracellular matrix components can be observed within the areas of atherosclerotic plaques. For example, increased production of the collagen types I and III or the glycosaminoglycan chain attached decorin can be observed, but on the other hand degradation of these components by matrix metalloproteinases also occurs. On the surface of the ruptured atherosclerotic plaque or through the action of leukocyte proteases the environment of the forming thrombi contains large amounts of partially digested extracellular matrix molecules. Degraded macromolecular components can be also incorporated into the fibrin clot and they are able to influence the fibrin mesh mechanical and lytic stability.

## **OBJECTIVES**

Through the action of proteases, vessel wall derived proteins and proteoglycans incorporate in thrombi formed *in vivo*. Our goal was to identify *in vitro* the effects of some typical blood vessel wall derived extracellular matrix molecules on:

- fibrin structure
- fibrin lytic and mechanical stability
- plasminogen activation on fibrin surface.

## **METHODS**

### **TURBIDIMETRIC FIBRINOLYTIC ASSAYS**

In microtiter plates fibrinogen and the investigated vessel wall components (collagen fragments/decorin core protein/aorta decorin/dermatan sulfate/chondroitin sulfate) were mixed. Clotting and lysis were initiated with simultaneously added thrombin and plasmin. Turbidity change was detected by spectrophotometer with continuously monitored absorbance. Lysis time was defined as the time needed to reduce the turbidity of the clot to a half-maximal value.

## SCANNING ELECTRON MICROSCOPIC STUDIES

To analyze the surface of the modified fibrin structures, composite fibrin clots (fibrin mixed with extracellular matrix proteins of blood vessels) were prepared. After fixation and dehydration the samples were mounted on adhesive carbon discs, sputter coated with gold and images were taken using scanning electron microscope. The scanning electron microscopic images were analyzed to determine the diameter of the fibrin fibers, whereby the length of the line drawn perpendicular to the longitudinal axis of the fibers was given. On each image at least 300 fibrin filaments were measured. Empiric and theoretical distributions were defined and plotted as probability density function.

## CONFOCAL MICROSCOPIC IMAGING

Composite clots were prepared with the investigated components as described above. In this case the fibrin clots were prepared from fibrinogen 2% of which was conjugated with red fluorochrome. To analyse the lytic properties of the modified fibrin structures tPA coupled with Yellow Fluorescent Protein was loaded to the edge of the clots and the lytic front movement (YFP-tPA concentrated on the fibrin surface) was monitored using confocal microscope. Sequential images were taken from the fluid-fibrin interface to follow the progress of lysis.

## PLASMINOGEN ACTIVATION ASSAY

Fibrinogen mixed with vessel wall components was clotted with thrombin. tPA and a synthetic plasmin substrate Spectrozyme-PL were loaded on the surface of the clot. tPA activated the incorporated plasminogen which generated p-nitroaniline from the synthetic substrate. Accumulation of the yellow product was monitored with the continuously recorded absorbance. Measured values were plotted against the square of time which resulted in linear correlations. Slopes of the plots reflected the apparent activation rates of plasminogen.

## RHEOLOGICAL MEASUREMENTS

We investigated the viscoelastic properties of the modified clots with oscillation rheometer. Changes in the storage modulus and loss modulus were monitored. Determination of the flow limit of the fibrin gels was performed by increasing the applied shear stress gradually and measurements of the resulting strain were used for calculation of the viscosity. The critical shear stress determined by extrapolation of the fall in viscosity to 0 was used as indicator of the gel/fluid transition in the fibrin structure.

## STATISTICAL ANALYSIS

Theoretical distributions were fitted to the empirical data sets and compared using Kuiper test and Monte Carlo simulation procedures. The statistical evaluation of other experimental

measurements in our studies was performed with the Kolmogorov – Smirnov test ( $p = 0,05$ ).

## **RESULTS**

### **EFFECTS ON FIBRIN STRUCTURE**

To analyze the effect of extracellular matrix molecules on the fibrin structure SEM images were taken from the prepared fibrin clots and they were analyzed morphometrically. The most significant change occurred in the case of purified aorta glycosylated decorin: the diameter of the fibrin fibers increased compared to pure fibrin clot (from  $85 \pm 40$  nm to  $187 \pm 121$  nm). Presence of collagen fragments or decorin protein also increased significantly the diameter of the fibrin filaments. Collagen fragments tested in the concentration range between 5 - 100  $\mu\text{g} / \text{ml}$  caused a continuous increase in the fiber diameter and above this range caused a decrease the diameter of the fibrin filaments,.

Dermatan-sulfate (DS) and chondroitin-sulfate (CS) did not change significantly the fiber diameter compared to control.

### **LYTIC RESISTANCE OF THE MODIFIED CLOTS**

The lytic resistance of the modified clots was examined with plasmin. Fibrinogen was mixed with collagen fragments, decorin protein, glycosylated decorin, dermatan sulfate and chondroitin sulfate and then the coagulation and clot lysis

were monitored. These investigated substances are present locally in high quantities in the vascular wall, but when incorporated at low concentrations in the fibrin mesh, they had significant destabilizing effects, which was manifested in faster plasmin induced lysis. The most obvious change was in the case of composite clots modified with CS and DS, when lysis times decreased about two-fold.

The observed differences may result from direct effects of blood vessel molecules on the enzymatic activity of thrombin and plasmin. To answer this question we examined thrombin and plasmin enzyme activity on synthetic substrates in fibrin-free environment. In our test conditions the amidolytic activity of the enzymes did not change.

#### INVESTIGATION OF PLASMINOGEN ACTIVATION

Plasminogen incorporated in the fibrin mesh can be activated by tPA placed on the surface of the clot. Activation process can be followed when the synthetic substrate of plasmin, Spectrozyme PL is added in significant molar excess in the system. The apparent maximal plasminogen activation rate decreased to a statistically significant degree in fibrin modified by the presence of extracellular matrix components during the formation of the clot.

Because the investigated modifiers did not affect the plasminogen activation rate in fibrin-free environment, the background of the altered plasminogen activation is probably

the observed changes is the structure of the modified reactive interface layer that is known to profoundly affect both tPA and plasminogen binding to fibrin.

#### PENETRATION OF YFP-tPA INTO THE CLOTS

In all examined clots, YFP-tPA accumulated in a sharp interfacial layer, which moved as a result of fibrin lysis by the generated plasmin. The rate of migration of this front correlated with the rates of plasmin-mediated fibrin lysis rather than with the rate of overall plasmin generation. One of the modifiers, chondroitin sulfate, resulted in a broader homogenous tPA-rich band in fibrin, whereas in the presence of the other additives tPA co-localized with aggregates of partially degraded fibrin.

The most significant change was in the lysis of clots modified by glycosylated decorin, where the relative migration rate of the tPA front was two-fold faster compared to pure fibrin control clots.

#### LYSIS OF BLOOD PLASMA CLOTS MODIFIED WITH VESSEL WALL COMPONENTS

Blood vessel components had a similar effect on tPA-induced lysis of plasma clots as in the pure fibrin containing system: in all cases faster lysis could be observed as in the case of plasmin-mediated lysis of fibrin. The antithrombotic effect of dermatan-sulfate was confirmed because in this case fibrin



formation was not observed.

### CLOT MECHANICAL STABILITY

The mechanical stability of the composite fibrin clots was investigated measuring their rheological parameters. All modifiers had no significant effect on  $G'$  (storage modulus) and  $G''$  (loss modulus) values except aorta decorin and collagen fragments. Collagen fragments slightly decreased the  $G'$  value. Aorta decorin exerted the most pronounced effects on the rigidity of fibrin. The critical shear stress was significantly decreased (16 - 45%) by all extracellular matrix components, except the core protein of decorin.

### CONCLUSIONS

The mechanical stability of the *in vitro* generated fibrin clots is significantly lower when vessel wall components are present during clot formation. In parallel, plasmin-mediated fibrinolysis rates are significantly increased.

These results suggest that when thrombi formed *in vivo* incorporate vessel wall-derived proteolyzed macromolecular components generated by proteases, they can detach easier from its location on the blood vessel wall by blood mechanical forces and thrombolytic process, leading to more frequent embolic complications and ischemic damage.

## **PUBLICATIONS LIST**

### **ARTICLES PUBLISHED RELATED TO THE THESIS:**

Rottenberger Z, Komorowicz E, Szabó L, Bóta A, Varga Z, Machovich R, Longstaff C, Kolev K (2013) Lytic and mechanical stability of clots composed of fibrin and blood vessel wall components. *J Thromb Haemost*, 11: 529-538.

Rottenberger Z, Kolev K (2011) Matrix metalloproteinases at key junctions in the pathomechanism of stroke. *Cent Eur J Biol*, 6: 471-485.

### **ARTICLE BUBLISHED NOT RELATED TO THE THESIS:**

Szabó E, Lódi C, Korpos E, Batmunkh E, Rottenberger Z, Deák F, Kiss I, Tokés AM, Lotz G, László V, Kiss A, Schaff Z, Nagy P. (2007) Expression of matrilin-2 in oval cells during rat liver regeneration. *Matrix Biol*, 26: 554-560.