ROLE OF CELLULAR ELEMENTS IN THROMBUS FORMATION AND THROMBOLYSIS

PHD THESES

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INTRODUCTION

The complex structure of thrombi contains not only a fibrin meshwork, but also different other components. In the study of thrombus formation and thrombolysis these various constituents should not be ignored, because they profoundly influence haemostatic processes. Our research group has previously published abundant data about the role of thrombus components such as myosin, free fatty acids and phospholipids. The research reported in this PhD thesis focuses on the modulating effects of cellular elements (platelets, leukocytes, erythrocytes) accumulating in thrombi.

Platelets play a fundamental role in haemostasis as they participate in the first response to vessel wall injury. Their interaction with exposed collagen fibres initiates platelet adhesion and aggregation, thus the formation of the platelet-plug that stops the bleeding.

In small arteries and arterioles high shear forces develop. Under these conditions adhesion of platelets becomes VWF (von Willebrand factor)-dependent and it is localized to the adventitia and not to the media layer of the vessel wall.

Leukocytes are in a resting, low-adhesive state while circulating in blood. Stimulatory signals promote the migration of these cells through the endothelial layer. Neutrophil granulocytes represent 50-70% of leukocytes in

thrombi, thus we investigated their role in haemostasis with particular attention.

Erythrocytes are not only passive bystanders in haemostasis as during thrombus formation significant amount of red blood cells (RBC) are entrapped within the fibrin network where they influence fibrin structure and consequently the lysis of thrombi.

Research questions

1. How do enzymes originating from neutrophil granulocytes influence the thrombogenicity of vessel wall through the modulation of VWF-dependent platelet adhesion?

2. Do haemostatic enzymes and enzymes secreted by neutrophil granulocytes degrade VWF? How is the cleavage influenced by flow and the presence of platelets?

3. How do red blood cells influence thrombolysis?

METHODS

FLOW-CHAMBER

Artery cross-sections were perfused with citrate-anticoagulated whole blood at 3350 s^{-1} shear rate. Before perfusion the sections were enzymatically treated with plasmin, thrombin, neutrophil elastase, matrix metalloproteinases or incubated with activated neutrophil granulocytes. After the perfusion they were fixed and the adhered platelets were visualized by indirect immunofluorescence microscopy.

PREPARATION OF NEUTROPHIL GRANULOCYTES AND RED BLOOD CELLS

Neutrophil granulocytes were isolated from the buffy coat fraction of human blood with help of Percoll-gradient. Cells were activated with fMLP (formyl-Met-Leu-Phe), and the amount of secreted enzymes was determined by an ELISA assay. Red blood cells were prepared from citrate-anticoagulated whole blood. These cells were fluorescently labelled for measurements with confocal microscopy.

MICROSCOPIC TECHNICS

Changes in the vessel wall structure were visualized by atomic force microscopy (AFM) and scanning electron microscopy (SEM). AFM has the potential to achieve

submolecular resolution under physiological conditions whereas for SEM imaging fixation of samples is required. Cupromeronic Blue, a cationic dye enables SEM to detect proteoglycans in different vessel wall layers. SEM is also applicable to study *ex vivo* thrombus samples and artificial fibrin surfaces. Confocal microscopy was used to detect platelets adhered to the vessel wall or to follow the process of fibrinolysis in RBC-containing clots. This later study required fluorescent labeling of fibrinogen, tPA (tissue-type plasminogen activator) and RBCs.

STUDY OF VWF DEGRADATION

Under static and flow conditions we applied purified enzymes to study the degradation of VWF. Perfusion of the sections was realized in a flow-chamber. Lyophilized platelets were used for modeling the presence of platelets under static conditions and to avoid platelet activation. The study was performed in an aggregometer by measuring the ristocetin-induced platelet agglutination (RIPA). VWF-cleavage products were detected by Western Blot analysis in parallel experiments.

STUDY OF FIBRINOLYSIS IN RED BLOOD CELL CONTAINING CLOT

The *Ball sedimentation assay* was used to follow fibrin dissolution where a steel ball was placed on the surface of

the clot and the elapsed time was measured during which the ball reached the bottom of the tube (lysis time). Lysis and clotting were initiated simultaneously with thrombin and tPA. We followed plasminogen activation with the *plasminogen activation assay*. Activity of the generated plasmin was determined with its chromogenic substrate (Spectrozyme-PL).

MORPHOMETRIC ANALYSIS OF FIBRIN STRUCTURE AND STATISTICAL PROCEDURES

The SEM images of thrombi and fibrin were analyzed to determine the diameter of fibrin fibers. The distribution of the data on fiber diameter was analyzed, the theoretical distribution was identified. The best fitted distributions for different samples were compared using Kuiper test and p-values were calculated with Monte Carlo simulation procedures. In other experimental measurements we used the Kolmogorov-Smirnov test.

The quantification of platelets adhered to the vessel wall was performed with the Scion Image software selecting the region of interest, calculating its surface area in pixels and setting a threshold intensity value for automatic identification of the area covered by adhered platelets. The area covered by platelets is reported in percentage of the whole area. To estimate the statistical significance of differences, Student’s two-sample t-test was used.

RESULTS

CHANGES IN THE ARTERIAL WALL THROMBOGENICITY DUE TO HAEMOSTATIC AND NEUTROPHIL GRANULOCYTE-DERIVED ENZYMES

When citrated whole blood is perfused over cross-sections of human iliac artery at a surface shear rate of 3350 s^{-1}, platelets adhere mainly to the adventitia layer of the vessel wall and not to the media. Short (30 min) pretreatment of vessels with haemostatic enzymes, neutrophil granulocyte-derived enzymes at physiologically relevant concentrations or fMLP-activated neutrophil granulocytes promotes platelet adhesion to the media layer. The combined administration of MMP and serine protease inhibitors completely abolishes the effect of neutrophils on platelet adhesion to the media. The AFM images of native cryosections show a compact structure of the media, in which collagen fibres are covered by a homogenous amorphous layer, while in the adventitia the striped morphology of collagen fibers are clearly visualized. The striped collagen structure in the media layer is seen only after enzymatic or cellular pretreatment. In native SEM images the rough collagen bundle-arrangement in the adventitia stands in contrast with the bundle-free smooth surface of the media. The Cupromeronic Blue staining is weak in the adventitia indicating that collagen bundles are

hardly covered by proteoglycans, whereas the layer of the media shows a strong Cupromeronic Blue signal because of proteoglycan bunches. The specificity of Cupromeronic Blue staining for proteoglycans in the applied artery specimens was confirmed with chondroitinase ABC treatment, which is known to degrade proteoglycans and accordingly removes proteoglycan-related SEM morphological signs. Pretreatment of the vessel wall with enzymes or neutrophil granulocytes removed the proteoglycan meshwork from the surface of the media layer revealing collagen fibres. These data support the concept that neutrophil granulocyte-related proteolysis reverses the thromboresistance of the native media layer of arteries and renders deeper vessel wall layers platelet adhesive at sites exposed to blood.

MODULATION OF THE VON WILLEBRAND FACTOR-DEPENDENT PLATELET ADHESION THROUGH ALTERNATIVE PROTEOLYTIC PATHWAYS

VWF is an adhesive glycoprotein with a multimeric structure and it can be found in the blood circulation and subendothelial layer. At high shear rate (above 1000 s^{-1}) the adhesion of platelets to collagen fibres in the vessel wall is completely VWF-dependent. Regulation of VWF multimer size is important to avoid high-adhesive states. The most

important enzyme described to play a role in this process was the ADAMTS-13, a metalloproteinase that cleaves VWF multimers and thus decreases their sizes.

Haemostatic enzymes (plasmin and neutrophil elastase) are able to cleave VWF at fibrinolytically active concentrations while the presence of platelets inhibits the cleavage entirely by plasmin and partially by neutrophil elastase under static conditions. Flow and the presence of platelets promotes the degradation of VWF by plasmin, neutrophil elastase and thrombin but not by MMP-8 and -9. Under these conditions the enzymes mentioned above detach platelets from thrombogenic surfaces. This alternative pathway of VWF degradation is most probably localized to the area of thrombi where the enzymes are protected from plasma inhibitors.

LYTIC RESISTANCE OF FIBRIN CONTAINING RED BLOOD CELLS

Arterial thrombi contain variable amounts of red blood cells which interact with fibrinogen through an eptifibatide-sensitive integrin-type receptor and modify the structure of fibrin. The local amount of RBC in thrombi does not necessarily correlate with the systemic RBC count because some factors such as flow conditions and variable geometry influence the distribution of cellular elements in the lumen of blood vessels. Increasing the number of RBCs in

thrombi causes fibrinolytic resistance and decreasing fiber-diameter in both *clot-incorporated* (tPA is uniformly dispersed in the thrombus) and *surface-applied* (tPA is applied to the surface of pre-formed RBC-fibrin clots) *models*. This effect can be inhibited by eptifibatide that hinders RBC-fibrinogen binding. In *model 1*, where the total volume was constant while the RBC-volume and thus the extracellular fibrinogen concentration increased, the plasminogen activation increased too, because of the better cofactor function of the thinner fibers. In *model 2* with increasing total volume and RBC-number, but constant extracellular fibrinogen concentration, the plasminogen activation decreased. This was probably due to the poor availability of plasminogen related to the volume occupation of RBCs. Independently of plasmin generation the lysis time increased in both models which shows that the rate of the proteolytic phase in fibrinolysis is more important than the phase of plasminogen-activation.

According to our results different RBC numbers in thrombi could also explain why patients show different susceptibility towards thrombolytic therapies.

CONCLUSIONS

According to our results cellular elements in thrombi profoundly influence thrombus formation and thrombolysis. Platelets, neutrophil granulocytes and red blood cells modulate both anticoagulatory and procoagulatory processes. Thrombolytic therapies can only be improved by taking these fine regulatory mechanisms into consideration. Thrombolytic drugs should be tested in systems that are comparable with *in vivo* situations, simple fibrinolytic assays are not sufficiently informative.

PUBLICATIONS OF THE CANDIDATE

PUBLICATIONS RELATED TO THE PHD THESIS

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MANUSCRIPTS IN PREPARATION RELATED TO THE PHD THESIS

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