The alternative pathway and lipid modulators of fibrinolysis in thrombi

Ph. D. thesis

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Introduction

Thromboembolic diseases are the main cause of death in the developed world. This fact validates the development of the ideal thrombolytic agent (effective, eliminates quickly, few adverse effects, no bleeding complications, no re-occlusion). We can only achieve this if we know the thrombus structure on molecular level, identify the biochemical processes which occur in the compartments during the thrombolysis on enzymological level, and evaluate this data in the individual patient. Technical development which has occurred in the last few years creates an ability to examine the presence of the non – fibrin constituents of the thrombi and describe their role quantitatively in the fibrinolyitc process. These results suggest that an alternative, cell – dependent pathway of the fibrinolysis also exists.

Morphological studies have proved that the haemostatic processes occur in compartments. During the thrombus formation, platelets and leukocytes appear in these temporary and dynamically changing compartments, forming a heterogeneous environment, which has impact on the fibrinolysis.

Aims

Because of the special circumstances of the clotting process the forming thrombi differ in each patient. This “thrombi fingerprint” under the influence of various factors (medicine therapy, susceptibility, age, underlying conditions) develops into a special tissue with heterogeneous structure and characteristic.

1. During our work, we aimed to describe quantitatively the impact of the thrombi occluded leukocytes and platelets on the dissolution of the *in vivo* formed thrombi, and find a connection between the presence of leukocyte – elastase digested fibrin and the leukocyte, platelet and fibrin content of the thrombi.
2. In the thrombi, free fatty acids are released from the thrombi occluded phospholipids. We aimed to develop an in vitro model of the fibrinolysis, in which we can study the fatty acid effect on the different steps of plasminogen activation and on plasmin activity.

Methods

Patients

Twenty-eight patients subjected to thrombectomy were enrolled in the study. Twenty-five of them had obliterating thrombosis localized in large arteries. Three patients had venous thrombosis.

Molecular biochemical experiments

Turbidimetric fibrinolytic assay

The course of clot formation and dissolution was monitored by measuring the light absorbance of the fibrin samples at 340 nm. In certain experiments, different substances were added to fibrinogen prior to clotting.

Plasminogen activation assays

Plasminogen activation in the presence of fibrin was measured on the surface of clear fibrin clots. Plasminogen activator in buffer containing Spectrozyme-PL was layered on the clot surface. Changes of light absorbance at 405 nm (A405)

indicate the release of p-nitroaniline by the generated plasmin, which was continuously recorded.

Detection of phospholipid and free fatty acids in thrombi

Sections of thrombi were immersed in Nile-blue for phospholipid staining.

Thrombi were prepared without fixation, and immersed in ADIFAB (a fluorescent free fatty acid probe) on glass plates. Thereafter, the spatial pattern of fluorescence at 460 nm (excitation 390 nm) was measured.

Immunhistochemistry

Thrombus samples were incubated with mouse anti-human NE-digested FDP monoclonal antibody, mouse anti-human fibrin monoclonal antibody or mouse anti-human CD41 (platelet GPIIb/IIIa) antibody. Thereafter the slides were incubated with fluorophore-conjugated anti-mouse immunoglobulin antibody, then the sections were mounted with 4',6-diamidino-2-phenylindole (DAPI), which recognizes DNA, and images were taken with microscope.

Image processing

All the image pixels were allocated to the whole image of the thrombus section. Image processing was performed by self-devised Matlab scripts running in Matlab environment. Triple system of notation was applied: the NE-degraded FDPs were allocated to the red channel, the fibrin or platelets were green and nuclei were blue in the same thrombus section. All statistical analyses were performed on integrated intensity values.

Statistical analyses

Linear regression model was applied for the quantification of the association between the neutrophil cell count and the NE-digested FDP content of the same thrombus sample on one side, and between the neutrophil cell count in the thrombus and in the blood samples of the same patient on the other. Hierarchical, agglomerative clustering was performed to find similarity classes of patients using Ward's method and Euclidean distances. Discriminant function analysis was used for the verification of the identified clusters. For the comparison of cluster mean and variance values Hotelling's multivariate T^2 and F-statistics was implemented.

Results

1. Based on the fluorescent intensity values for the NE-digested FDPs and the nuclear DAPI staining of sections from surgically removed thrombi, the association between the elastase-digested fibrin and the leukocyte content of the thrombi can be quantified by Pearson correlation coefficient, which proves to be significant ($r = 0.71$, $p = 0.00002$). According to the coefficient of determination 51 percent of the variability of the elastase digested fibrin content can be attributed to the presence of leukocytes, the remnant 49 percent to other factors. Based on the similarity of the composition of the patients' thrombi, patients can be classified into different clusters depending on the used criteria. The first applied criterion is the amount of elastase-digested fibrin, leukocyte content and platelet GPIIb/IIIa antigen in the thrombus section. An agglomerative, hierarchical clustering approach, which tries different three-way combinations until clusters emerge, reveals two main clusters (ELP1 and ELP2) verified by discriminant function analysis. The statistics identifies the clusters as significantly different in their thrombus composition ($T^2=96.0$ $F(3,24)=29.5$, $p<0.00001$). Thrombi isolated from patients of cluster ELP2 had significantly higher elastase-digested fibrin

(p=0.0005) content than thrombi of patients belonging to cluster ELP1. The difference in platelet-related antigen content is nearly significant (p=0.06). The mean values for leukocyte content (estimated from nuclear staining) are also different (p=0.005), while variances do not differ significantly. Moreover, we found that leukocyte content of thrombi predicts elastase-digested fibrin significantly in cluster ELP2 (multiple correlation R^2= 0.58), but not in cluster ELP1 (R^2= 0.25). This association explains the greater variability of cluster ELP2, and that consequently it is less homogeneous than cluster ELP1.

2. The second applied criterion for classification is the amount of elastase-digested fibrin, leukocyte content and fibrin antigen in the thrombus section. Using the same hierarchical, agglomerative clustering approach as above, two main clusters (ELF1 and ELF2) emerge. The statistics identifies significant differences between the clusters based on their thrombus composition (T^2=36.4 F(3,24)=11.2, p<0.00009). The two clusters differ both in content of leukocytes (p=0.009) and elastase-digested fibrin (p=0.00000) according to univariate comparisons, while the amounts of fibrin are not significantly different. Thrombi in cluster ELF1 contain lower amounts of leukocytes and therefore have lower amounts of elastase-

digested fibrin. The variances of all constituents are significantly higher in cluster ELF2.

3. In order to evaluate the interrelations of fibrin and NE-FDP on one hand, and platelet and NE-FDP on the other, independently of the effect of leukocyte quantity, normalized values for NE-FDP, fibrin and platelet antigen were derived by dividing the integrated NE-FDP, fibrin and platelet signal of each thrombus image by their nuclear-stain signal. Based on the combination of normalized NE-FDP and normalized fibrin three clusters of patients emerge (REF1, REF2 and REF3). The thrombi in cluster REF1 contain small amounts of normalized NE-FDP and normalized fibrin. Cluster REF2 is characterized by higher amounts of normalized NE-FDP and even higher amounts of normalized fibrin. In cluster REF3 the normalized NE-FDP shows inverse ratio to the normalized fibrin. The elastase-related fibrinolysis is at a low level in cluster REF1 (thrombi contain much lower amount of digested than undigested fibrin). At the same level of normalized undigested fibrin there is a higher amount of normalized NE-FDP in the thrombi of cluster REF2, therefore the fibrinolysis is at a more advanced stage. The most progressed phase of elastase-related fibrinolysis is seen in cluster REF3, where the more the

normalized NE-FDP, the less the normalized undigested fibrin. Evaluation of the interrelations of normalized platelet antigen and normalized NE-FDP provides insights into the role of platelets in the cell-dependent fibrinolysis. Based on the combination of normalized platelet antigen and normalized NE-FDP content patients are classified in two clusters: REP1 and REP2. Thrombi of cluster REP1 are characterized by a relatively small amount of normalized NE-FDP and the normalized platelet antigen is also low. In cluster REP2 the normalized NE-FDP is in inverse ratio to the normalized platelet antigen. It is plausible to assume that the cell-dependent fibrinolytic process is scarcely progressed in cluster REP1, but is very intense in cluster REP2. Thus, the degree of cell-dependent fibrinolysis is inversely proportional to the content of platelet antigen.

4. The probe ADIFAB indicated highly variable amounts of free fatty acids in the thrombectomy samples. In five of eight thrombi examined by us, the amount of free fatty acids in thrombi spans a range from undetectable levels up to millimolar concentrations. The original fluorescence of ADIFAB, however, was not changed in three of the examined thrombi.

5. We modelled the overall course of thrombolysis induced by t-PA under the changing conditions of ongoing hydrolysis of phospholipids with fibrin–phospholipid clots containing phospholipase A_2. However, when the concentration of free fatty acid released by phospholipase A_2 increased to over 0.24 mmol/l (measured with ADIFAB), the dissolution process was significantly accelerated. Using an assay specific for plasminogen activation on a fibrin surface, significant acceleration of plasmin generation was measured in the presence of oleic acid with the fibrinbinding activators t-PA. The activation with reteplase (a recombinant variant of t-PA containing the kringle-2 and protease domains) was more sensitive to the effect of the oleic acid than that with t-PA; identical fatty acid concentrations caused greater stimulation of the reaction rate with reteplase. The template function of fibrin seems to be essential for the stimulatory effect of oleic acid, because no activation was seen in the absence of template, and only a transient plasmin activity could be detected in the presence of a soluble template, cyanogen bromide-digested human fibrinogen fragment. When fibrinogen clotting and plasminogen activation were initiated simultaneously, during the ascending phase of the turbidity curves plasminogen

activation occurred in a fluid fibrinogen environment, where plasmin is susceptible to the action of oleic acid. Thus, at increasing oleic acid concentrations, more fibrinogen was spared from plasmin digestion, resulting in higher values of absorbance. During the descending phase of the curves, when most of the fibrinogen had already been converted to fibrin, the fibrinolytic rate was hardly affected by oleic acid. The effect of oleic acid depended on the type of activator; although less efficient in the fluid phase, reteplase showed fibrinolytic efficiency similar to that of t-PA in the fibrin-dependent stage of the assay.

Conclusions

1. Massive presence of neutrophil elastase-digested fibrin (NE-FDP) can be detected in thrombi.

2. The co-localization of NE-FDP and leukocytes is confirmed by the significant Pearson correlation coefficient between the elastase-digested fibrin and the leukocyte content of the thrombi.

3. The leukocyte – dependent thrombi degradation rate is inversely related to the fibrin and platelet content of the thrombi.

4. We demonstrated the presence of free fatty acids in thrombi spanning a range from undetectable levels up to millimolar concentrations.

5. Oleic acid at relevant concentrations reversibly inhibits more than 90% of the amidolytic activity of plasmin on a synthetic substrate (Spectrozyme PL), but only partially inhibits its fibrinolytic activity.

6. Plasminogen activation by tissue-type plasminogen activator (t-PA) is completely blocked by oleic acid in the fluid phase.

7. Plasminogen activation by tissue-type plasminogen activator is accelerated by oleic acid on a fibrin matrix.

8. A recombinant derivative of t-PA (reteplase) develops higher fibrin specificity in the presence of oleic acid than with wild-type t-PA.

9. Fibrin partially protects plasmin and t-PA against inhibition by oleic acid.

Publications in the field of the thesis:

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3. Rábai Gy., Szilágyi N., Sótonyi P., Kovalszky I., Machovich R., Kolev K. Contribution of neutrophil elastase to the lysis of obliterating thrombi in the context of their platelet and fibrin content. *Submitted for publication*

Congress abstracts:

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2. Rábai Gy., Machovich R., Kolev K. Fibrinolízis lipidkörnyezetben: a szabad zsírsavak moduláló szerepe. Thrombosis és Haemostasis Társaság Magyar Belorvosi Archivum 2007.