The molecular basis of thrombolytic resistance

Ph.D. thesis

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Introduction

Blood vessel wall's injury launches the multistep process of blood coagulation, which leads to the formation of the thrombi. The structure of thrombi is formed by connected fibrin molecules following fibrinogen molecule's cleavage by thrombin. The fibrin network occludes molecular (immunoglobulins and other plasma proteins) and cellular (platelets, leukocytes) components. The dissolution of fibrin is carried out by plasmin, which has the ability to cleave fibrin molecules at specific sites, forming water soluble fibrin degradation products. Plasmin is formed from plasminogen by the action of plasminogen activators, which can cleave the peptide bond between the amino acids 561 and 562 of plasminogen, forming the two chain molecule of the plasmin. There are endogenous (tissue type plasminogen activator, urokinase), and exogenous (streptokinase) plasminogen activators. In ischemic heart disease the aim of the therapy is the recanalisation of the blood vessel, which is based on the application of plasminogen activators, but in a significant part of the patients this does not lead to the dissolution of thrombi. This suggests that fibrinolysis is not equivalent to thrombolyis, and prompts research aimed to clarify the role of the thrombi's non - fibrin components in the process of fibrinolysis. In my experiments I examined the fibrinolysis modulating effect of phospholipids and myosin as both are important components of thrombi.

The fibrinolysis

The term of fibrinolysis means the process when plasmin cleaves fibrin at specific locations, resulting smaller, water soluble products. Fibrinolysis takes place at the solid – liquid interface on the outer surface of thrombi, therefore the determination of the enzyme concentration involved in the process is difficult. The thrombi contain inactive plasminogen, and in order to start the fibrinolysis the plasminogen activators need to enter into the clot. The size of tPA would enable free diffusion in the pores of the fibrin network, but this is prevented by the great affinity of the enzyme to bind to fibrin. During fibrinolysis the tPA approaches the fibrin from the bloodstream, it binds on the surface of fibrin and its concentration increases several-fold in a thin, interface layer and the fibrinolysis starts in this reactive layer. In the presence of fibrin the tPA activates plasminogen, the generated plasmin digests the fibrin, and the digestion results in the exposure of new binding sites on fibrin, which leads to

the accumulation of plasminogen molecules on the surface of the clot. The plasmin cleaves the fibers in transversal direction, but the cleaved parts can form thicker fibrin bundles before they final splitting. Under these circumstances the fibrinolysis takes place in the outer thin layer of the fibrin, and the dissolution follows a layer-by-layer process. The speed of the fibrinolysis is influenced by the structure of the fibrin network, the size of the pores and by the other fibrin – occluded components, which can interact with the fibrinolytic enzymes. Other important factor is the flow rate of blood, what affects the diffusion of the fibrinolyic enzymes and the removal of the degradation products from the surface of the clot.

The structure of thrombi and the platelet membrane

When arterial thrombi are formed, the platelet content of 10 mL whole blood is compacted in a volume of 400 μL, whereas the fibrin content of the same thrombi corresponds to the fibrinogen concentration in blood plasma, and the concentration of the plasminogen and tPA is smaller in the thrombi than in the blood. The platelets occluded in the thrombi die six hours after the activation, but their cell membrane and proteins remain in the thrombi. That means, that the phospholipid content of the thrombi is high, its concentration is even higher than the fibrin concentration in the clot. The platelet membrane contains 83 % zwitterionic phospholipid (phosphatidylcholine (PC), ethanolamine) the rest 17 % has one negative charge (phosphatidylserine (PS), phosphatidylinositol). The other important feature of a phosphoplipid membrane is its phase state, this can be an ordered, gel - like structure, or at higher temperature a fluid - crystalline phase. The melting temperature is determined by the ratio of the saturated and unsaturated fatty acid content in the membrane. Higher unsaturated fatty acid content usually causes greater fluidity and lower melting temperature. 40 % of the fatty acids in the platelet membrane are unsaturated, this means that the platelet membrane at body temperature is in fluid –crystalline phase, but the presence of proteins in the membrane or activation of the platelet can lead to the formation of gel phase structures.

Aims

In order to reach effective thrombolysis, the molecular and cellular content of thrombi should be considered, because components occluded in the thrombi can affect their structure and the fibrinolytic process. Therefore our purpose was to develop an experimental model, which

resembles the real thrombi, and consider the effects of the shear force caused by the bloodstream.

Because it is impossible to build up an artificial thrombi, which contain all of the components at *in vivo* concentrations, in our experiments we focused on the effects of the phospholipid and myosin content of the thrombi, because these compounds are present at high concentration in thrombi, and published data suggest interaction among them and the fibrinolytic proteins.

Materials and methods

Plasma proteins (plasminogen, immunoglobulin G) were isolated from human blood plasma. Phospholipid vesicles were made from synthetic phospholipids with sonication and extrusion through a 50 nm pore - diameter membrane. Platelet membrane was prepared by sonication, and the lipids were gained by chloroform extraction. For the demonstration of the phospholipid content of thrombi we used frozen section of arterial thrombi, treated by Nile-blue staining. The process of the fibrinolysis was followed by turbdimetry. Synthetic substrate was used to measure the plasminogen activation on fibrin surface. The depth of the reactive layer was measured by confocal microscopy using FITC – labelled tPA. For the determination of the protein quantity penetrated into the clot, we used Eu-labelled proteins, and measured time-resolved fluorescence. We characterized the interaction between the phospholipids and the fibrinolytic proteins by SPR (surface plasmon resonance) and isothermal titration in microcalorimeter.

Results

Nile-blue staining of thrombi from human femoral artery shows massive presence of phospholipids, with concentration in the range of mg/ml. The source of this phospholipid content is presumably the thrombi occluded platelets. Therefore we tried to model the platelet membrane effect on the dissolution of fibrin. Platelet homogenate mixed into fibrin inhibits the rate of fibrinolysis significantly (the lysis time, a quantitative parameter used to characterize the speed of fibrinolysis, increases up to three-fold). This is partly caused by the plasminogen activator inhibitor - 1 content of platelets, but phospholipids isolated from the platelet membrane also inhibit fibrinolysis, although the increase of the lysis time is only two-fold. This inhibition can be reproduced by mixing synthetic phospholipid vesicles into fibrin.

The increase in the lysis time also depends on the membrane structure. Above the melting temperature, where the phospholipids are in fluid-crystalline phase, only the platelet membrane homogenate inhibits the fibrinolysis, the synthetic vesicles have no such effect. Phospholipids containing short chain fatty acids, unable to form a membrane structure do not affect the process of fibrinolysis. The inhibition of fibrinolysis caused by the gel-phase synthetic phospholipid vesicles is equivalent to the inhibiting effect of 440 nM α_2 – plasmin inhibitor mixed into the fibrin. The observed antifibrinolytic effect of phospholipids can be explained partly with the inhibition of plasminogen activation on the fibrin surface. The degree of inhibition correlates with the quantity of the anionic phospholipid fraction in the vesicle, and membrane structure is also required to achieve the inhibition. The phospholipids can also inhibit the diffusion of the fibrinolytic proteins into the thrombi. After incubation of fibrinolytic proteins on the surface of the fibrin clot, the size of the reactive layer occupied by the proteins reduces significantly when negatively charged phospholipid vesicles are mixed into fibrin. The presence of PCPS 1:1 and PCPS 3:1 vesicles in the fibrin reduces the depth of the reactive layer from 74 ± 9 μm to 36 ± 9 and 39 ± 4 μm, respectively. The diffusion of tPA is inhibited significantly only by the PCPS 1:1 vesicles, resulting a 30 % decrease in the depth of the reactive layer. The phospholipid content of thrombi influences the enzyme concentration in the reactive layer. Mixing PCPS 1:1 and PCPS 3:1 vesicles into the clot reduces the clot - penetrated tPA concentration to 25 %, whereas this reduction is only 50 % for plasmin. The zwitterionic PC vesicles have no effect on the penetrated enzyme concentration. Our results suggested the possibility of interaction between the fibrinolytic proteins and the phospholipids and prompted evaluation using SPR and microcalorimetry: the strength of the chemical binding correlates with the anionic fraction of the phospholipid vesicles, and the monolayer form binds stronger to plasmin and tPA than the vesicular form. The interaction is the strongest between the monolayer PCPS 1:1 and the plasmin, Kd value of the binding is in the nanomolar range according to the SPR method. The parameters of the interaction are in the same range when we use microcalorimetry, although the numeric Kd values are slightly higher. The examination of the monolayer form on the SPR chip resulted in measurable interaction between the mixture PCPS 1:1 and plasmin. Similar effects can be observed between tPA and phospholipids, but because of the weaker interactions only the calorimetry provides useful information.

Immunohistochemical evaluation of arterial thrombi indicates massive presence of myosin in its structure. Myosin is uniformly distributed in thrombi forming a mesh-like structure around

the fibrin fibers. The fibrin – mixed myosin inhibits the tPA-induced fibrinolysis in a concentration dependent way; the maximal degree of inhibition is achieved when the myosin concentration is between 0.6 – 1 μM, in this range the lysis time increases by up to 60%, but further increase in the myosin concentration reduces the degree of inhibition.

Under flow conditions the myosin content of fibrin results in longer lysis time, which suggests inhibition of fibrinolysis, but the disintegration of the clot starts at a lower degree of digestion, which can be caused by impaired fibrin – polymerisation.

Summary of the results

1. Arterial thrombi contain significant amount of phospholipids (5 g/L) and myosin.
2. Phospholipids from platelet membrane and synthetic phospholipids slow down the tPA – induced fibrinolysis, and their effect is additive to that of α_2-plasmin inhibitor.
3. Phospholipids inhibit the plasminogen activation on fibrin surface.
4. Phospholipids inhibit the penetration of tPA, plasminogen and plasmin in the fibrin clot, presumably due to their pore filling effect.
5. Phospholipids bind the fibrinolytic agents (especially plasmin).
6. Myosin inhibits fibrinolysis in a concentration dependent way, but the presence of myosin also destabilizes the structure of the clot.

Conclusions:

1. The concentration of phospholipids in thrombi is high enough to inhibit the tPA induced fibrinolysis.
2. This effect is definitely independent of the platelet proteins and contributes to the known fibrin stabilising effect of platelets based on their plasminogen activator inhibitor 1 (PAI - 1), factor XIIIa and α_2 – plasmin inhibitor content.
3. The described phospholipid effects are related to certain structural requirements: anionic head charge and low fluidity gel-phase membrane state are necessary for the maximal inhibition of fibrinolyis. Because membrane fluidity depends on the content of unsaturated fatty acid in the phospholipids, this phenomeon may be an additional

factor contributing to the known beneficial effect of the unsatuturated fatty acids in the progress of atherothrombosis.

4. The efficiency of the thrombolytic agents should be examined with methods, which approach the complex composition of *in vivo* thrombi, and model adequately the composite environment of fibrinolysis.

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