The fibrin - neutrofil extracellular trap - von Willebrand factor axis in arterial thrombosis

Ph. D. thesis booklet

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INTRODUCTION

Heart attack and stroke are the leading causes of death on earth. Factors that increase the risk of thrombosis: atherosclerosis, hypertension, dyslipidemia, diabetes, tumors and smoking also affect the composition of the thrombus, making it more difficult to lyse.

Von Willebrand factor (vWF), which is released constitutively and in response to a stimulus from endothelial cells, is involved in platelet adhesion, binding to collagen and endothelial cells, as well as aggregation, i.e. binding to other platelets, white blood cells, fibrin, DNA and other molecules.

The formation of thrombi is accompanied by phenomena characteristic of inflammation, including neutrophil granulocyte infiltration. The neutrophil granulocyte that first appears at the site of inflammation either phagocytizes microbes and cell debris, or ejects its unfragmented DNA content in the company of cytoplasmic, nuclear and granular proteins during the formation of an extracellular trap (NET). NETosis is provoked by microbes, cytokines, antibodies, reactive oxygen derivatives, vWF, C-reactive protein (CRP), agents released from platelets. During NETosis, neutrophil elastase cleaves proteins, myeloperoxidase oxidizes, and peptidyl-arginine deiminase (PAD) citrullinates proteins. These enzymes, together with DNA and citrullinated histone, are NET markers and can also be detected in eroding or rupturing atherosclerotic plaques. Atherosclerosis risk factors maintain chronic inflammation, activate endothelial cells,

macrophages, neutrophil granulocytes, and result in elevated blood acute-phase protein, namely vWF-, CRP-, fibrinogen levels.

The PAD4 isoenzyme is required to initiate NETosis. Both PAD4 and PAD2 are released in the process and citrullinate a series of extracellular proteins: fibrin and fibrinogen, collagen, ADAMTS13, vimentin, filaggrin, etc. During citrullination, the arginine side chain of proteins is deiminated, ammonia is released and citrulline remains in the protein. Due to the loss of charge, the protein becomes structurally less dense, new epitopes appear on its surface, it may lose its function, and the cleavage site of various enzymes disappears. As a result, the body may recognize the citrullinated protein or its fragment as foreign, and an antibody may be produced.

OBJECTIVES

Quantitative immunohistochemical characterization of the interaction of von Willebrand factor, extracellular DNA and fibrin in thrombi of different localization (coronary, cerebral and peripheral).

Investigation of correlations between the composition of arterial thrombi (fibrin, vWF, DNA) and clinical and laboratory characteristics of patients.

Characterization of the fibrin structure of thrombi as a function of the thrombus composition (vWF, DNA, cellular elements).

The effect of citrullination of fibrinogen on the structure of the resulting fibrin.

METHODS

Patient characteristics, preparation and statistical evaluation of scanning electron micrographs of thrombus samples

Arterial thrombus samples from patients with coronary artery disease (18 CAD), peripheral artery disease (19 PAD) and cerebral ischemic stroke (17 AIS) were dissected immediately after removal from the patients, fixed with glutaraldehyde, and then dehydrated with a mixture of ethanol and acetone, carbon dioxide critical point dried using a drying method. The samples were coated with gold on a carbon disc, and 10 images per thrombus were taken with a scanning electron microscope (SEM). Using Photoshop, the images were divided into 864 squares of equal size and each region was classified: fibrin, platelets, other blood cells or a combination of these were visible. We calculated how many % of the total image area each component occupies.

Preparation and statistical evaluation of scanning electron micrographs of fibrin clot

The fibrin clot produced in vitro was prepared in the same way as the thrombus sample for SEM images. The fiber diameters were determined on the fibrin fibers intersecting the guide lines fitted to

the image. Theoretical distributions were fitted to the empirical data series of the fiber diameter and their deviations were evaluated using the Kuiper test.

Preparation of immunofluorescence recordings

Immunohistochemical von Willebrand factor (vWF), DNA and fibrin were detected from thrombi on cryosections. Extracellular DNA was visualized with TOTO-3 nucleic acid dye, vWF and fibrin with antibody, and fluorescent dye bound to the secondary antibody produced against it using a confocal laser scanning microscope. Image J software was used to quantify the area of the different fluorescent signals. Regions of interest were selected, their area in pixels was calculated, and vWF, DNA, and fibrin intensity thresholds were set to identify covered areas. To compensate for the slight spatial overlap between the two fluorescent signals due to the cross-absorption of the secondary antibodies, the ratio of the two percent coverage values was calculated and subsequently used as the fibrin/vWF and fibrin/DNA ratios, and then the fibrin/DNA (FN50) and fibrin/vWF (FW50) the median value of the ratio distribution. We used Kuiper statistical test for equality of distribution and Bootstrap one-tail statistical test for equality of median.

Citrullination of fibrinogen

Fibrinogen was citrullinated by adding PAD4 enzyme in the presence of FXIIIa inhibitor at 37 ºC. The reaction was stopped with

the pan-PAD inhibitor chloramidine. The samples prepared in this way were used for SEM and turbidimetric analysis.

Turbidimetric measurement of fibrin coagulation

Citrullinated and non-citrullinated fibrin were mixed in different proportions and coagulated with the addition of thrombin in a spectrophotometer in a microtiter plate. The absorbance was measured at 340 nm.

RESULTS

Ratio of DNA and von Willebrand factor content of arterial thrombus

Among the CAD and PAD patients, the values of FN50 and FW50 (the median of the ratio of the area occupied by fibrin and DNA, and fibrin and von Willebrand factor on confocal microscopy) changed significantly in those with dyslipidemia, but not in those with normal plasma lipid profile. In arterial thrombosis, in which the cause is atherosclerosis due to an abnormal lipid profile of the blood, the relationship described between the local activation of vWF and NETosis plays a greater role: vWF can provoke NETosis, and NET components stimulate the exocytosis of Weibel-Palade bodies, vWF cause its release, and both vWF and NET components are able to activate platelets, endothelial cells, and neutrophil granulocytes, thereby initiating blood coagulation and preventing fibrinolysis.

The evolution of the fibrin fiber diameter of arterial thrombi as a function of the vWF and DNA content of the thrombus

When both the DNA and vWF content of the thrombus is increased relative to the fibrin, the diameter of the fibrin fiber is smaller. It is known that a solid clot composed of thinner fibers, interwoven with DNA and vWF, is difficult to lyse. Acetylsalicylic acid causes the formation of a thicker fibrin fiber in men compared to untreated men, and all CAD patients, and approx. half of the others also were treated with the COX inhibitor. Among our patients, the fibrin fiber of smokers is thinner than that of non-smokers, and most of our PAD patients were smokers. Smokers' platelets, endothelial cells, etc. is in a more activated state, their plasma is more prone to clotting, and a thinner fibrin fiber is formed. In our patients, the thickness of the fibrin fiber was influenced by several factors.

Correlation of blood or thrombus white blood cell content and thrombus vWF content

The higher the number of white blood cells in the blood of hypertensive coronary and cerebral thrombosis patients, the higher the proportion of vWF compared to fibrin in their thrombus. The endothelial cells of hypertensive patients are in a dysfunctional, activated state, a lot of vWF can be released, in the case of atherosclerotic plaques, the high shear force can bring vWF into an active conformation, which promotes its binding to blood proteins and blood cells, and blood coagulation can start. The correlation

between white blood cells seen in the SEM images and the FW50 value of the thrombus is not as strong, but similar. We assume that leukocytes had a greater role at the beginning of thrombus formation between the thrombogenic surface and blood flowing under hyperdynamic conditions than in wide peripheral arteries, and their number decreased during the aging of the thrombus. Neutrophil granulocytes also respond to stimulation with NETosis, and the released proteases destabilize the plaque.

Von Willebrand factor and DNA content of the thrombus as a function of blood fibrinogen and C-reactive protein levels

If we look at the combined effect of blood fibrinogen and thrombus FN50 on thrombus FW50 in all patients, the measurement points are located on an asymmetric valley-shaped surface, higher Fg levels occur together with high thrombus DNA/fibrin and vWF/fibrin ratio. Not in all patients, but in atherosclerotic patients, blood C-reactive protein (CRP) and the FN50 value of the thrombus correlate similarly with the FW50 value: a high CRP level is associated with a high DNA/fibrin and vWF/fibrin ratio. CRP is not only an inflammatory marker, the synthesis of which is increased by IL-6, but also an inflammatory mediator, its activated forms attract and activate leukocytes, provoking NETosis. The synthesis of fibrinogen is also increased in inflammatory conditions. It is not only the main network-forming clot protein, but also increases blood viscosity. PAD patients had elevated levels of several inflammatory markers, several comorbidities and smoking contributed to the formation of

the thrombus, in contrast to the other two groups, in which only one inflammatory marker was elevated and even one risk factor could be enough, although there were typically several.

Structure of a clot formed from citrullinated fibrinogen

We were able to demonstrate the presence of neutrophil extracellular traps in thrombi from patients. During NETosis, in addition to DNA, a large number of cytoplasmic and nuclear proteins are released, several of which have an effect on the kinetics of clot formation and the properties of the resulting clot. PAD2 and PAD4 citrullinate fibrinogen, thus changing the structure of the clot and thinning the fibrin fibers. In our previous study, the lysis time of citrullinated fibrin (cFn) measured with the addition of plasmin was prolonged compared to non-citrullinated fibrin, which may be due to the fact that it is more difficult for plasmin to penetrate the dense network, and in vivo the fibrin network intertwined with NET and containing cFn is more difficult to digest. During our previous measurements (not in my own measurements), the citrullination of fibrin(ogen) by PAD enzymes changes the rheological properties of the clot, reduces its mechanical stability and elasticity, a lower shear force can cause the gel-sol transition in the rheometer. tPA layered on the surface of cFn penetrates more slowly, fibrinolysis is slower. Clots containing citrullinated fibrin are mechanically less stable, emboli can form more easily from such clots, but at the same time they become resistant to lysis and pharmacological treatment.

Kinetics of citrullinated fibrin clot formation

The preliminary citrullination of fibrinogen by PAD4 enzyme reduces the positive charge of the protein, changes the protein structure, as a result the resulting fibrin network consists of thinner fibers, its absorbance is lower in the turbidimetric test than that of non-citrullinated fibrinogen. During the turbidimetric test, the more citrullinated fibrinogen is present in the mixture, the later the coagulation starts after the addition of thrombin. The enzymes PAD2 and PAD4 are also able to deiminate the two arginines next to which thrombin would cleave fibrinopeptides A and B, i.e., due to citrullination, the cleavage site of thrombin in the Aα and Bβ chains of fibrinogen is lost, and cFg also prevents polymerization.

CONCLUSIONS

High DNA content of thrombi in patients with dyslipidemia was combined with high vWF content, which suggests that the atherosclerotic etiology of thrombi plays a role in this connection.

When presumably NET-derived DNA co-localized with elevated vWF content in clots, fibrin fibers were thinner.

The vWF content of the thrombi formed in the smaller diameter cerebral arteries and coronary arteries (but not in the peripheral arteries) of hypertensive patients was correlated with the white blood cell count in the blood, which suggests that the hydrodynamic

environment plays a role in the interaction between circulating leukocytes and the forming fibrin clots.

The correlation between thrombus vWF and systemic CRP was specific for thrombi of atherosclerotic etiology.

The fibrin network formed from citrullinated fibrinogen has thinner fibers and smaller pores than the one without citrullinated fibrin. This may explain the earlier observation that the dense fibrin network is less permeable, tPA and plasmin have a harder time getting inside the network, and therefore its lysis takes significantly longer.

Citrullinated fibrinogen begins to clot more slowly when thrombin is added, because thrombin loses its cleavage sites, since the arginines there are also transformed into citrulline.

LIST OF OWN PUBLICATIONS

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