Anticoagulant mechanisms are modulated by vascular endothelial cells

Mecanismele anticoagulante sunt modulate de către celulele endoteliului vascular

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Abstract

The function of the main anticoagulant mechanisms (antithrombin and protein C system) depends not only on the available amounts of these proteins, but also on the modulation of their activity by components located on vascular endothelial cells' membrane. Antithrombin is activated by the heparansulphate expressed on the endothelial cells, and impaired interaction between heparansulphate and the antithrombin molecule may occur in relation to an immune aggression (such as in heparin-induced thrombocytopenia) and/or to the enzymatic cleavage exerted by heparanase on heparansulphate. Heparanase has a prothrombotic effect through the downregulation of antithrombin anticoagulation and also through the induction of tissue factor and the dissociation of the tissue factor pathway inhibitor (TFPI) from the endothelial cells membrane. The protein C activation complex is represented by thrombin bound to endothelial thrombomodulin, which would act on protein C bound to a specific endothelial receptor, EPCR. Proinflammatory cytokines would exert deleterious effects on thrombomodulin and on EPCR, thereby impairing PC activation. The protein C bound to EPCR was reported to exert anti-inflammatory, cytoprotective and anti-apoptotic effects. Actually, by signaling through the same protease activated receptor (PAR-1), thrombin would elicit a proinflammatory response, while in the presence of either activated protein C (APC) or protein C bound to its specific endothelial receptor (EPCR), the signal transduced by this thrombin prototypic receptor would produce anti-inflammatory effects. The assessment of the circulating soluble forms of EPCR and thrombomodulin, as well as TFPI (dissociated from the cellular membrane by heparanase) could provide markers of endothelial damage.

Keywords: antithrombin, heparanase, protein C, EPCR, thrombomodulin, PAR-1, TFPI, endothelial damage

Rezumat

Funcţia principalelor mecanisme anticoagulante (antitrombina şi sistemul proteinei C) depinde nu doar de cantitatea în care sunt disponibile aceste proteine, ci şi de modularea activităţii lor de către componente localizate pe membranele celulelor vasculare endoteliale. Antitrombina este activată de către heparansulfatul exprimat pe celulele endoteliale, iar perturbarea interacţiunii dintre antitrombină şi heparansulfat se poate produce în contextul unei agresiuni imune (cum este cazul trombocitopeniei induse de heparină) sau prin clivarea enzi-

matică exercitată de heparanază asupra heparansulfatului. Efectul protrombotic al heparanazei se manifestă atât prin inhibarea acţiunii anticoagulante a antitrombinei, cât şi prin stimularea expresiei factorului tisular şi prin desprinderea inhibitorului căii mediate de factorul tisular (TFPI) de membrana celulelor endoteliale. Complexul de activare al proteinei C este reprezentat de trombina legată de trombomodulina endotelială, care acţionează asupra proteinei C fixate pe un receptor specific, EPCR. Citokinele proinflamatorii, prin favorizarea degradării trombomodulinei şi desprinderea EPCR de membrană diminuă efectul anticoagulant al sistemului proteinei C. Proteina C legată de EPCR are şi efecte antiinflamatoare, citoprotectoare şi antiapoptotice. De fapt, semnalizând prin intermediul aceluiaşi receptor activat de proteaze (PAR-1) trombina va produce efecte proinflamatorii, pe când în prezenţa proteinei C activate (APC) sau a proteinei C (PC) zimogen legate de receptorul endotelial specific (EPCR), semnalul transmis prin acest receptor prototipic trombinei va dezvolta efecte antiinflamatorii. Evaluarea nivelului circulant al formelor solubile ale EPCR şi trombomodulinei, precum şi cel al TFPI (desprins de pe membrana celulară de către heparanază) ar putea furniza indicatori ai agresiunii endoteliale.

Cuvinte cheie: *antitrombină, heparanază, proteina C, EPCR, trombomodulină, PAR-1, TFPI, agresiune endotelială*

Intravascular coagulation is prevented by anticoagulant mechanisms, with the main ones being antithrombin and the protein C system. It should be noted that, while antithrombin is a serine protease inhibitor (serpin) that binds thrombin and activated factor X, thereby producing inactive complexes, activated protein C is a protease, proteolitically degrading the activated coagulation cofactors Va and VIIIa. (1, 2).

The importance of the above mentioned anticoagulant mechanisms is illustrated by the recurrent thrombotic events occurring in subjects afflicted by inherited deficiencies in protein C (3,4) or in antithrombin (5,6,1).

Several apparently discordant situations have also been reported. Actually, increased plasma levels of protein C (7,8) and protein S (9) as well as to a lesser extent of antithrombin (10) were found in hyperlipidemic overweight patients with or without cardiovascular disease, who are known to be at risk for thrombotic complications.

Experimental acute inflammation produced by intramuscular injections of turpentine oil in rabbits, leading to the development of an acute phase reaction was found to be accompanied often by an increase in antithrombin level (154% of the control) and a decrease of protein C activity to 86%, while procoagulant fibrinogen level and coagulation factor VIII activity increased to much higher values, of 212% and 202% respectively (11).

It is noteworthy that addition of cytokines released from cultured macrophage to hepatocytes in culture had been previously reported to produce an increased synthesis of antithrombin to 150% of the control, while some typical acute phase reactants such as fibrinogen and $\alpha 1$ protease inhibitor ($\alpha 1$PI) increased to 188% and 200% of the control, respectively (12).

A more severe decrease of total plasma protein C was recently reported in mice injected intraperitoneally with an Escherichia coli lippolysaccharide (E. coli LPS). 6 hours after such an injection, plasma protein C activity decreased to 36% of the initial values and was accompanied by a dramatic decrease of plasma fibrinogen level to 16% (13). Such findings are highly suggestive for a disseminated intravascular coagulation and a subsequent consumption coagulopathy. One may therefore doubt the relevance of these last mentioned experimental findings for changes affecting haemostatic mechanisms developing in most inflammatory conditions occurring in human patients and associated with increased plasma fibrinogen levels.

Evidence had been provided that beside the increased ratio between the circulating procoagulant factors and the anticoagulant proteins, the rate of their activation may be pathogenically relevant. Essentially a faster activation of clotting factors and a delayed activation of the anticoagulants ones may lead to a prothrombotic state.

Antithrombin, heparin, heparan sulphate and heparanase

Antithrombin, newly synthesized and secreted by the hepatocytes, would bind and inhibit factor Xa and thrombin progressively and rather slowly. Binding of heparin to an antithrombin's side rich in basic aminoacids, such as arginine and lysine (Heparin Binding Site HBS) would however produce conformational changes in this protease inhibitor leading to an increased affinity for the activated clotting factors and amplifying as well as accelerating their inactivation. Mutations in the heparin binding site of antithrombin, resulting in impaired binding of heparin and delayed antithrombin activity, are associated with a thrombotic tendency, the so-called antithrombin deficiency type II HBS (14, 15).

It should be specified that pharmaceutical heparin is a product of mast cells that is isolated from porcine intestinal tissue. Although it is a potent activator of antithrombin, endogenous heparin plays a minor role in the control of coagulation in physiological conditions, being released by mast cells in small amounts into tissues in relation to inflammatory conditions. Actually the physiologic activator of antithrombin is heparansulphate, a component of the proteoglycans widely expressed on the surface of the endothelial cells, on the basal membrane under the endothelial cells layer and also within the extracellular matrix. The structure of proteoglycans includes a protein core and several heparansulphate lateral chains bound to the core protein through serine residues. The heparin sulphate is a highly sulphated polysaccharide consisting of the repeating of a disaccharide unit of $1 \rightarrow 4$ linked glucosamine and glucuronic/iduronic acid that may contain sulpho groups. Under physiological pH, the sulpho groups exhibit considerable negative charges that influence binding properties to more than one hundred proteins, including antithrombin. Removal of certain sulpho- or carboxyl groups was found to compromise the binding affinity to antithrombin, suggesting that these groups are critical to heparansulphate's anticoagulant role (16).

Disruption of the interaction between heparansulphate and the antithrombin molecule may occur in relation to immune aggression and/or can be enzymatically exerted by heparanase.

Heparin-induced thrombocytopenia (HIT type II) is illustrative for an immunologically mediated reaction characterized by severe thrombocytopenia, platelet activation and thrombosis. To be noted that two types of HIT are reported: HIT type I, and HIT type II. Apparently, HIT type I occurring in some heparin treated patients is due to a platelet pro-aggregating effect of heparin itself, producing a rather minor decrease in platelet count to about 30% of the basal value, which is transient and recovers within 2-5 days after the beginning of heparin therapy, while HIT type II is due to a true immunologic reaction. The presently accepted pathogenic model for this devastating disorder presents a three steps process: a) administration of heparin induces a release of platelet factor 4 (PF4) from platelets α granules; b) PF4 may bind to heparin in plasma and also to heparansulphate at the surface of the endothelial cells, and the complexes would become immunogenic, leading to progressively increasing levels of antibodies (mainly IgG); c) the antiheparin/PF4 antibodies can interact with platelet FcγRIIa receptors, leading to platelet activation, thrombin generation and increased expression of tissue factor (17).

Most importantly HIT IgG has also been demonstrated to activate endothelium in vitro by binding to endothelial cell surface (18). Such a process would impair local antithrombin activation and this downregulation of an anticoagulant mechanism, associated with the above-mentioned prothrombotic changes, may explain the development of thrombocytopenia/thrombosis or disseminated intravascular coagulation in patients sensitive to heparin.

Antithrombin activation would also be impaired by the cleaving and removal of some sulpho- and carboxyl rich fragments from the heparansulphate lateral chains of the proteoglycans on the endothelial cell surface. Such a process can be performed by heparanase. This endo-β-D-glucuronidase was primarily detected in placenta, keratinocytes and in activated cells of the immune system, while little or no expression of heparanase was found in connective tissue cells and in most epithelia. Later on, this enzyme's expression could be demonstrated in many other cells such as mast cells, neutrophiles, macrophages, and in T and B lymphocytes. Upregulated expression of heparanase was noted in essentially all human tumors, as well as in relation to inflammation and wound healing (19,20). Increased expression of heparanase was also emphasized in renal glomerular and tubular cells of patients with diabetic nephropathy (21). Noteworthy an upregulation of heparanase expression could be experimentally reproduced in high glucose or H_2O_2 treated endothelial cells in culture (22). It was also demonstrated that heparins are potent inhibitors of heparanase, while heparin trapped into heparanase would lose its anticoagulant effect (19, 20).

The prothrombotic effect of heparanase is not limited to the downregulation of antithrombin anticoagulation. Actually heparanase was reported to induce tissue factor and to produce a dissociation of tissue factor pathway inhibitor (TFPI) from the endothelial cells membrane, thereby generating a local prothrombotic condition (18).

Also by cleaving heparansulphate from the basal membrane under the endothelial cell layer, as well as the heparansulphate within the extracellular matrix, heparanase would favor the dissemination of malignant cells and would exert a prometastatic and a proangiogenic activity (20). Presenting details on these pathological aspects would however exceed the purpose of the present review.

Protein C, thrombomodulin, endothelial protein C receptor

Protein C system is a potent anticoagulant mechanism, resulting in proteolytic degradation of coagulation factors Va and VIIIa. The efficacy of this system depends not only on the level of PC and its cofactor PS, but also on the expression and function of thrombomodulin (TM) and endothelial protein C receptor (EPCR) located on the surface of endothelial cells membrane (23, 24).

Thrombomodulin functions as a cell surface receptor and an essential cofactor for active thrombin, which in turn activates protein C and thrombin-activatable fibrinolysis inhibitor (TAFI). When bound to thrombomodulin, thrombin loses its ability to cleave fibrinogen, factor XIII and factor V and activates PC by a limited proteolysis process (23).

Thrombomodulin is known to be released from endothelial cells by neutrophil elastase. Increased serum levels of soluble thrombomodulin fragments, due to protease mediated cleavage, have been associated with smoking, cardiac surgery, atherosclerosis, liver cirrhosis, diabetes mellitus, cerebral and myocardial infarction, and multiple sclerosis. An increase of plasma soluble TM level is reported to be associated with severity and worse outcome of coronary artery disease (25, 26)

EPCR was found to bind both PC and APC through their Gla domain (□-carboxyglutamic) and thereby facilitates PC activation and APC activity (25, 26). A soluble form of EPCR (sEPCR) is detectable in plasma at a concentration of approximately 100 ng/ml (27). Experimental data showed that sEPCR has no effect on protein C activation by the thrombin-TM complex, nor on APC inactivation by α1 antitrypsin or protein C inhibitor, but it is blocking the anticoagulant activity of APC (28).

It should be noted that there is an antagonism between the proinflammatory cytokines and the protein C system. Excess

proinflammatory cytokines would downregulate the hepatic synthesis of PC zymogen and would exert deleterious effects on thrombomodulin (TM) and on EPCR thereby impairing PC activation. Mechanisms involved in the abnormal behavior of TM and EPCR in vascular inflammation may differ. IL1 and TNF☐ would reduce TM expression and promote its internalization and intracellular degradation (29), while EPCR would be released from endothelial surface because a cytokine activated metalloproteinase (MP) would split the domain anchoring this receptor to endothelia (30).

The metalloproteinase responsible for the shedding EPCR from the surface of endothelial cells was recently identified to be the tumor necrosis factor converting enzyme (TACE), a member of the ADAM (a disintegrin and metalloproteinase) family. Enzymatic activity of this TACE/ADAM was reported to be involved in the shedding of many transmembrane proteins including several cellular receptors and this process plays an important role in the release of cytokines, growth factors and adhesion molecules. Among these enzyme's substrates one should mention pro TNF-α, L-selectin adhesion molecule, transforming growth factor α (TGF α), TNF-α receptors, interleukin-1 receptor, heparin-binding epidermal growth factor (EGF), vascular adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1), and growth hormone receptor (31,32).

High plasma levels of soluble EPCR may thereby occur in sepsis, diabetic angiopathy, systemic lupus erythematosus (SLE) (33), Behcet's disease (34) and various cases of vasculitis.

Besides its anticoagulant activity, the protein C system was reported to exert anti-inflammatory, cytoprotective and anti-apoptotic effects, acting through both EPCR and additional specific receptors detected on human mononuclear phagocytes (M∅ PCR) (35).

APC bound to EPCR in cytokines stimulated cells activates the prototypical thrombin protease – activated receptor (PAR-1), thereby inducing the downregulation of the transcripts for proapoptotic proteins such as p53 and thrombospondin-1, while thrombin-PAR-1 signaling produced the opposite effects (p53 unchanged and thrombospondin-1 upregulated) (36). The occupancy of EPCR by protein C switches the PAR-1 dependent signaling specificity of thrombin towards an anti-inflammatory response, through the inhibition of nuclear factor kB (NF - kB) (37). These data may have important significance in understanding the protective effects of protein C system and the involvement of NF-kB and of vascular PAR-1 in inflammatory disorders associated with thrombotic tendency.

It was actually demonstrated that an endothelial selective blockade of NF-kB pathway in transgenic mice overexpressing a mutant I-kB (an inhibitor of NF-kB) exerted a protective effect against the procoagulant proinflammatory conditions induced by intraperitoneal injections of E. coli lipopolysaccharide, while wild-type mice died with disseminated intravascular coagulation and multiple organ failure/injury (13).

Also, in agreement with the above-mentioned, studies performed on endothelial cells and peripheral blood mononuclear cells in culture provided evidence that the synthetic compound dehydroxymethylepoxyquinomicin (DHMEQ), a specific inhibitor of NF-kB, prevented the release of cytokines and the development of a thrombophilic diathesis induced by antiphospholipid antibodies (38).

Clinical relevance of endothelial – mediated anticoagulant mechanisms

Consistent evidence has been recently provided by data in the literature that vascular endothelial cells ensure the efficacy of anticoagulant mechanisms while injured or functionally disordered endothelium would favor the development of a prothrombotic condition.

Therefore, the investigation of plasma levels of the soluble, circulating anticoagulant proteins does not necessarily provide an accurate assessment of the haemostatic balance. Reliable markers informing about endothelial cells integrity or functionality are required.

C reactive protein (CRP), as well as plasma levels of proinflammatory cytokines, may point to an acute phase reaction and an inflammatory process which may or may not afflict vascular endothelia. On the other hand, the increased plasma levels of endothelial-derived von Willebrand factor is not necessarily caused by endothelial lesion and a leakage of this factor from such cells. Actually von Willebrand factor may be actively secreted as a response of endothelial cells to various stimuli, which may be proinflammatory cytokines, but also adrenergic ones (39 - 41).

Apparently an increase in the plasma level of endothelial protein C receptor (EPCR) shed from the surface of endothelial cells could be more appropriate as a marker of endothelial lesions and very high plasma levels of of this marker had been indeed detected in patients with sepsis or with systemic lupus erythematosus (33).

It was also demonstrated that, independently of its enzymatic activity, overexpressed heparanase in endothelial and tumor cells induces tissue factor pathway inhibitor (TFPI) and leads to an extracellular accumulation of this Kunitz-type protease inhibitor by dissociating it from the cell membrane surface (19, 20). In relation to these experimental findings, increased levels of TFPI have been noted in plasma of cancer patients (42). One may therefore presume that assessment of plasma TFPI may provide another marker of endothelial dysfunction. We have no knowledge so far about any clinical and laboratory study pertaining to the behavior of plasma TFPI in patients with cardiovascular disease.

Abbreviation list:

ADAM - a disintegrin and metalloproteinase,
APC - activated protein C,
CRP - C reactive protein,
DHMEQ –dehydroxymethylpoxyquinomicin,
EGF - epidermal growth factor,
EPCR - endothelial protein C receptor,
HBS - heparin binding site,
HIT - heparin induced thrombocytopenia,
ICAM-1 - intracellular adhesion molecule-1,
IL1 - interleukin 1,
MP - metalloproteinase,
NF-kB – nuclear factor kB,
PAR-1 - protease–activated receptor-1,
PC - protein C,
PF4 - platelet factor 4,
SLE - systemic lupus erythematosus,
TACE - tumor necrosis factor converting enzyme,
TAFI- thrombin activatable fibrinolysis inhibitor,
TFPI - tissue factor pathway inhibitor,
TGF α - transforming growth factor α,
TNF α - tumor necrosis factor α,
TM-thrombomodulin,
VCAM-1 - vascular adhesion molecule-1

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