Perspectives

Natural Anticoagulant Mechanisms

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Introduction

The coagulation cascade is composed of a series of linked proteolytic reactions. At each stage of the mechanism, a parent zymogen is converted to a corresponding serine protease, which is responsible for a subsequent zymogen-serine protease transition. In most instances, protein cofactors are present that can be activated by serine proteases and then possess the ability to bind the above reactants to specific cell surfaces. This process usually leads to a dramatic acceleration as well as a partial localization of the reactions. The end result of these transformations is the generation of thrombin, which is able to act upon fibrinogen and platelets to produce the hemostatic plug.

Given the above information, there has been a tendency to consider the coagulation cascade as a multi-stage amplifying system in which the development of an initial pathologic stimulus would lead to an explosive thrombotic outcome. During the past decade, considerable effort has been devoted to investigating several natural anticoagulant mechanisms that are able to exert a damping effect upon the coagulation cascade. These studies have significantly altered our understanding of the functioning of the hemostatic mechanism within the vascular tree. In this review, we shall discuss recent advances in our knowledge of the biochemistry and pathophysiology of three major anticoagulant mechanisms that are directed at regulating the three different types of protein transformations that are known to occur within the coagulation system. The latter events include generation of serine proteases, production of activated cofactors, and polymerization of fibrin. No attempt has been made to consider anticoagulant mechanisms that are able to modulate the behavior of platelets since this area of investigation is sufficiently complex to constitute the basis for a separate Perspectives article.

This review is dedicated to the memory of Dr. Hymie L. Nossel who made major contributions to our knowledge of how these natural anticoagulant mechanisms function in humans.

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Heparin-antithrombin mechanism

Antithrombin is a protein of 58,000 mol wt, which is present within human plasma at an average concentration of 150 μg/ml (1). The complete primary structure of this protease inhibitor has been reported by Petersen et al. (2). Antithrombin neutralizes the activity of thrombin as well as other serine proteases of the intrinsic coagulation cascade by formation of a 1:1 stoichiometric complex between enzyme and inhibitor via a reactive site (arginine)-active center (serine) interaction (3–6). In the absence of heparin, complex formation occurs at a relatively slow rate. When heparin is present, the mucopolysaccharide binds to lysyl residues on antithrombin and dramatically accelerates the rate of complex formation (3). It is possible that this phenomenon is due to a conformational alteration of the protease inhibitor that renders the reactive site arginine more accessible to the active serine center of thrombin (3). Bjork and co-workers (7) have examined the structure of the antithrombin molecule and demonstrated that the arginine reactive site of the protease inhibitor is located near the COOH-terminal of this protein at Arg_{385}-Ser_{386}. The heparin molecule possesses multiple functional domains, which are responsible for accelerating the various interactions between coagulation serine proteases and antithrombin (8, 9). Furthermore, the structure of the antithrombin binding site on the muccopolysaccharide has been demonstrated to consist of a unique sequence of sulfated and nonsulfated monosaccharide units (10–12).

In 1973, Damus et al. (4) suggested that the nonthrombogenic properties of blood vessels may be due, in part, to the presence of heparinlike species on the vascular endothelium. Heparan sulfate proteoglycans, heparinlike substances with increased amounts of glucuronic acid as well as *N*-acetyl glucosamine and decreased amounts of N and O sulfate groups, have been detected on the luminal surface of the vascular endothelium. Buonassisi and Root (13) have reported that radiolabeled heparinlike molecules were liberated from the surface of culture bovine aortic endothelial cells with *Flavobacterium* heparinase. Simionescu et al. (14) have demonstrated that in situ perfusion of capillary

endothelium with the above enzyme specifically removed cationized-binding sites from the fenestral diaphragms of these cellular elements. Marcum et al. (15, 16) have provided evidence that the above proteoglycans contained the appropriate monosaccharide sequences required for accelerating the action of antithrombin. On the one hand, heparinlike species with anticoagulant activity can be obtained from retinal microvascular tissue, which is completely free of mast cells. These products appear to be proteoglycans that are tightly bound to endothelial cells and function as anticoagulants in a manner virtually identical to commercial heparin. On the other hand, a small portion of heparan sulfates from calf cerebral microvasculature (~0.3%) possesses the molecular characteristics of heparan sulfate but can function to accelerate the protease inhibitor. Therefore, certain heparan sulfate subclasses contain the critical monosaccharide sequences required for catalyzing the action of antithrombin.

Thus, heparinlike proteoglycans active in anticoagulation are seemingly present on the luminal surface of the vessel wall and are available for interactions with antithrombin as well as other blood components. Several groups have used animal models to test this hypothesis. Lollar and Owen (17) have shown that injection of ^{125}I-thrombin into the circulatory system of rabbits results in almost immediate complexing of labeled enzyme with antithrombin. However, Busch and Owen (18) have reported that diisopropylfluorophosphate-treated thrombin is able to suppress the rapid neutralization of the enzyme by antithrombin and, on this basis, have claimed that thrombin receptors on the endothelium must be responsible for enhancing enzyme-inhibitor interactions. Marcum et al. (19) have used a rat hindlimb preparation to demonstrate that perfusion of thrombin and antithrombin through the vascular tree results in a 10–20-fold acceleration of enzyme-inhibitor complex formation when compared with either the calculated rate of reaction of thrombin and antithrombin in solution or to an appropriate sham control. The perfusion stream did not contain soluble heparinlike species that might catalyze enzyme-inhibitor interactions. However, the acceleratory phenomenon could be abrogated when antithrombin chemically modified at the Trp_{49} residue was used. The altered protease inhibitor interacts with thrombin at a normal rate in the absence of heparin but enzyme-inhibitor interactions are not catalyzed in the presence of the mucopolysaccharide. The acceleratory phenomenon was also eliminated when the heparin-degrading enzyme, *Flavobacterium* heparinase, was added to native antithrombin during perfusion. The above evidence indicates that a heparinlike proteoglycan active in anticoagulation (most likely a heparan-sulfate with an appropriate sequence for anticoagulant activity) is tightly bound to the luminal surface of the vascular tree of the rat and functions to accelerate the action of antithrombin in a manner virtually identical to that of commercially available heparin.

Several independent lines of clinical evidence suggest that the endogenous heparin-antithrombin mechanism described above is able to suppress the action of serine proteases of the coagulation system within the vascular tree of humans. Khoory et al. (20) have reported the isolation of heparan-sulfate proteoglycans with anticoagulant activity from the plasma of patients with multiple myeloma. Bauer et al. (21) have used specific radioimmunoassays to quantitate the levels of fibrinopeptide A (FPA)[1] and thrombin-antithrombin complex in patients with disseminated intravascular coagulation, deep vein thrombosis, and pulmonary emboli. The concentrations of these markers of thrombin action on fibrinogen and thrombin neutralization by antithrombin, corrected for their differences in metabolic behavior within the vascular system, were compared with the levels of FPA and thrombin-antithrombin complex obtained after addition of thrombin to whole blood under in vitro conditions. The data indicate that the vascular surface is responsible for accelerating the neutralization of thrombin by antithrombin vis a vis the action of the enzyme on fibrinogen by 20–50-fold. Numerous investigators have reported kindred in whom a reduced level of antithrombin of ~50% is associated with profound venous thromboembolic disease (22–24). Koide and co-workers (25, 26) have described a Japanese family with a congenital disorder characterized by multiple episodes of venous thrombosis in association with an inherited alteration of the antithrombin molecule in which Arg_{47} is replaced by Cys_{47}. The mutant antithrombin exhibits a functional defect similar to the chemically modified protease inhibitor used in the animal perfusion studies described above. The altered protein is able to neutralize various enzymes of the hemostatic mechanism in a normal manner under in vitro conditions but these interactions can not be catalyzed by exogenous addition of mucopolysaccharide. In conclusion, heparinlike proteoglycans within the vascular system of humans may accelerate interactions between hemostatic serine proteases and antithrombin. Furthermore, malfunction of the endogenous heparin-antithrombin mechanism appears to render the protease inhibitor less able to suppress coagulation system activity and lead to the development of thrombotic phenomena.

Protein C-thrombomodulin mechanism

Protein C is a vitamin K-dependent glycoprotein of 62,000 mol wt, which circulates in human plasma at a concentration of ~4 μg/ml (27). Stenflo and Fernlund (28) have determined the complete primary structure of the bovine form of this protein. This information reveals that protein C consists of a heavy chain of 41,000 D and a light chain of 21,000 D, which are joined by a single disulfide bridge. To perform an anticoagulant function, protein C must be converted to a component with serine protease activity, designated protein Ca. This process involves the cleavage of a single Arg^{12}-Leu^{13} bond at the amino-terminal end of the heavy chain of the zymogen with release of an activation peptide of ~1,400 mol wt (27). Both protein C and protein Ca possess gamma carboxyglutamic acid (Gla) residues on their light chains, which are required for the binding of either protein to calcium ions and cell membranes. Protein Ca is slowly neutralized by a specific plasma protease inhibitor of 57,000

1. *Abbreviations used in this paper:* FPA, fibrinopeptide A; FPB, fibrinopeptide B; PA, plasminogen activator.

mol wt but is not inactivated by antithrombin in the presence or absence of heparin (29).

Thrombin is the only physiologically relevant serine protease than can convert protein C to protein Ca (27). The rate of this reaction is quite slow when blood is allowed to clot under in vitro conditions. This observation raised a serious question about the biologic role of protein C within the human body. However, Esmon and Owen (30) were able to demonstrate that perfusion of protein C and thrombin through the Langendorff heart preparation resulted in a 20,000-fold increase in the rate of conversion of zymogen to serine protease. Given that this process can be saturated with either excess protein C or thrombin, it seemed likely that a receptor was present on the endothelium that could dramatically accelerate the reaction. These investigators were able to substantiate this hypothesis further by showing that cultured human umbilical vein cells possessed a high affinity receptor for thrombin (dissociation constant ~ 0.5 nM), which could greatly enhance the conversion of protein C to protein Ca (31). It is of interest that Salem et al. (32) have shown that the light chain of human Factor Va (thrombin-activated form of Factor V) is also able to accelerate the activation of protein C by thrombin. However, their recent experimental observations indicate that this interaction is much less efficient than thrombomodulin in enhancing production of protein Ca. The physiological significance of this interaction remains to be established.

The studies outlined above prompted Esmon and co-workers (33) to isolate the putative receptor—a protein of ~74,000 mol wt—from rabbit lungs. The addition of thrombin to this receptor, termed thrombomodulin, leads to the formation of a 1:1 stoichiometric complex of enzyme and cofactor that is able to activate protein C rapidly in the presence of calcium ions. It should be noted that thrombin attached to thrombomodulin can be neutralized by antithrombin at a rate equivalent to that of free enzyme. However, the thrombin-thrombomodulin complex exhibits a greatly diminished ability to clot fibrinogen, activate Factor V, or trigger platelet activation (34). Thus, this vascular endothelial cell receptor has the ability to accelerate the rate of thrombin-dependent protein C conversion, to allow neutralization of bound thrombin by antithrombin, as well as to directly inhibit the procoagulant activities of the enzyme.

Once evolved, protein Ca functions as a potent inhibitor of Factor V-Va and Factor VIII-VIIIa, which are important cofactors of the coagulation cascade (35, 36). Its first site of action is located at the surface of the platelet where Factor Va bound to specific sites on these cellular elements acts as a receptor for Factor Xa. This multimolecular prothrombinase complex rapidly converts prothrombin to thrombin. Protein Ca functions as a naturally occurring anticoagulant by specifically cutting Factor V or Factor Va (35). Factor Va seems to be particularly sensitive to destruction by protein Ca especially under in vivo conditions where the levels of this enzyme are exceedingly low. Thus, protein Ca possesses the requisite specificity to prevent assembly of the prothrombinase complex, and thereby suppress the production of thrombin (37). This inhibitory effect of protein Ca appears to be modulated by a variety of additional interactions. On the one hand, a slow rate of cleavage of Factor Va will allow Factor Xa to bind to the uneffected cofactor and thereby protect this protein against any subsequent action of protein Ca (38). Furthermore, Factor Xa, sequestered within the prothrombinase complex, is inaccessible to neutralization by antithrombin (39, 40). Hence, this limb of the protein C system is likely to play a particularly pivotal role in backstopping the endogenous heparin mechanism. On the other hand, various plasma proteins appear to be involved in the protein Ca-dependent destruction of Factor Va on the platelet surface. For example, protein S is able to enhance the binding of protein Ca to phospholipid-containing membranes and to accelerate the cleavage of Factor Va by this serine protease (41). The complement component C4b binding protein is known to complex with protein S and may be involved in regulating the function of the latter protein (42). Thus, it seems likely that a variety of interactions are responsible for determining the rate of translocation of protein Ca from its site of production on the endothelium to the surface of the platelet. Of course, one might expect that small amounts of protein Ca remain bound to the endothelial cell surface via gamma carboxyglutamic acid residues on this serine protease. In this manner, protein Ca could also regulate the Factor Va-dependent thrombin generation that is known to occur on the endothelial cell surface in a fashion analogous to that described for the platelet membrane.

The second site of action of protein Ca occurs at a locale where Factor VIIIa regulates the interaction between Factor IXa and Factor X. At the present time, little is known concerning the biochemical details of the protein Ca-dependent cleavage of this cofactor or of the biologic surface where these events take place (36). However, this inhibitory process would limit the generation of Factor Xa and thereby prevent production of thrombin.

Several independent lines of evidence obtained from animal studies and clinical observations indicate that the protein C-thrombomodulin mechanism functions under in vivo conditions to suppress thrombotic phenomena. Comp et al. (43) have infused low levels of thrombin into dogs and demonstrated that protein Ca is generated before any observable changes in the levels of Factor V, fibrinogen, or platelets. Bauer et al. (44) have devised a specific radioimmunoassay for quantitating the concentrations of protein C activation peptide in humans and have shown that dramatically elevated levels of this marker of protein C activation occur in clinical states associated with increased generation of thrombin such as disseminated intravascular coagulation and deep vein thrombosis. Griffin et al. (45), Bertina and co-workers (46), and Horellou et al. (47) have described several families with congenital reductions of ~50% in the antigenic levels of protein C who exhibit repeated thrombotic episodes. It is of interest to note that other kindred have been reported in which individuals who are heterozygous for Protein C deficiency have minimal symptoms whereas those who are homozygous for this trait die in infancy with massive venous

thrombosis and purpura fulminans (48). These data suggest that other factors such as the density of thrombomodulin on the endothelium, the levels of protein S within the blood, and the amounts of Factor Va present on the platelet surface are likely to modulate the effects of Protein C deficiency.

Plasminogen-plasminogen activator mechanism

Plasminogen is a plasma protein of 93,000 mol wt that circulates within human blood at a concentration of $\sim$150 μg/ml (49). During activation of the fibrinolytic mechanism, this zymogen is converted to the serine protease, plasmin. The latter transformation is characterized by the scission of a single peptide bond, Arg_{560}-Val_{561}, within plasminogen to form the two-chain disulfide-linked serine protease (49). Once generated, plasmin is able to cleave several Arg-X or Lys-X bonds within fibrinogen/fibrin in a sequential manner. The proteolytic activity of plasmin is limited mainly by α-2 plasmin inhibitor, which is present within human plasma at a concentration of $\sim$60 μg/ml and is able to rapidly complex with the latter enzyme (50–52). The activation of plasminogen is initiated by the proteolytic action of urokinase or tissue type plasminogen activator (PA), (53, 54). Urokinase exhibits a molecular weight of $\sim$54,000, transforms plasminogen to plasmin in the absence of a cofactor, and may be responsible for the continuous fluid phase generation of the latter enzyme (53). Tissue type PA exhibits a molecular weight of $\sim$70,000, possesses a high affinity for fibrin that it uses as a cofactor during the conversion of plasminogen to plasmin, and appears to be involved in the generation of the latter enzyme on fibrin polymers as well as within the interstices of the clot structure (54). Both types of PA are immunologically distinct and appear to be synthesized by a wide variety of cellular elements including microvascular and macrovascular endothelial cells (55–57). Thrombin has been reported to bind to endothelial cells and to inhibit the synthesis of urokinase as well as stimulate the production of tissue type PA (58). The latter effect may be mediated, in part, via the generation of protein Ca (59). A specific plasma inhibitor of urokinase and tissue type PA has recently been isolated from platelets and endothelial cells (57).

Several investigators have reported families with congenital abnormalities of the fibrinolytic mechanism who exhibit multiple episodes of venous thromboembolic disease. These have included functional defects in the plasminogen molecule (60, 61), reductions in the release of PA (62, 63), as well as alterations in the structure of fibrinogen (64). It has been tacitly assumed that thrombotic phenomena observed in these patients are due to their reduced ability to lyse small fibrin clots and prevent proximal extention. More recently, it has been suggested that plasmin may serve as a natural anticoagulant within the hemostatic mechanism and that the defects outlined above may also occur at an earlier stage in the coagulation system.

It is widely appreciated that thrombin is able to cleave a set of Arg_{16}-Gly_{17} bonds within the A-α-chains of fibrinogen with release of FPA and concomitant conversion of this macromolecule to fibrin I monomer. Subsequently, thrombin can split a second set of Arg_{14}-Gly_{15} bonds within the B-β-chains of fibrin I monomer with liberation of fibrinopeptide B (FPB) and concomitant generation of fibrin II monomer, which is capable of rapidly polymerizing to form a thrombus. Plasmin is also known to proteolyze a set of Arg_{42}-Ala_{43} bonds within the B-β-chains of fibrin I releasing B-β-1–42 and, thereby converting fibrin I monomer to fragment X, which is further degraded to form soluble cleavage products (65).

Nossel and co-workers (66) utilized radioimmunoassays for FPA, FPB, and B-β-1–42 (measured as thrombin-increasable FPB) to investigate the pathophysiology of intravascular coagulation and venous thrombosis. These investigators have examined patients receiving hypertonic saline to terminate pregnancy and have shown that immediately after intrauterine infusion, fibrin I monomer was generated by thrombin-mediated proteolysis of fibrinogen. Thereafter, fibrin I monomer was either cleaved by thrombin to liberate FPB or proteolyzed by plasmin to release B-β-1–42. These data led Nossel to hypothesize that the relative rates at which thrombin and plasmin split the B-β-chain of fibrin I monomer could determine the occurrence of thrombosis (67). Owen et al. (68) have applied these techniques to the study of naturally occurring venous thrombosis, as documented by ^{125}I-fibrinogen leg scanning in patients undergoing craniotomy. The results obtained indicated that individuals who developed thrombi, when compared with those who do not suffer from this complication, exhibited levels of FPA that were considerably greater than the concentrations of B-β-1–42 during the 4 days preceding the onset of this disorder. These observations lend credence to the hypothesis that a sustained imbalance between the procoagulant effects of thrombin and the anticoagulant actions of plasmin upon fibrin I monomer may lead to the development of thrombotic disorders in humans. The precise molecular defects which are responsible for these phenomena are currently unknown but most likely include abnormalities in the regulatory mechanisms that govern the release of plasminogen activators or their inhibitors from cellular sites.

Conclusion

This review has summarized our current knowledge of the biochemistry and pathophysiology of three natural anticoagulant mechanisms that function via a complex interplay between coagulation proteins, platelets, and endothelial cells. It is readily apparent that congenital abnormalities in the proteins of these regulatory processes lead to the development of thrombotic disease in humans. Indeed, it seems likely that careful dissection of the various soluble components of these mechanisms will result in a growing list of specific molecular abnormalities that can be associated with these disorders. However, most of the kindred described above exhibit profound venous thromboembolic disease, while only occasional families of this type have multiple episodes of arterial thrombosis. It is not surprising that the initial manifestations of these diffuse systemic hypercoagulable states occur within the deep veins of the lower extremity since blood flow is relatively slow in this segment of the cir-

culation. The reasons for the apparent absence of a dramatic increase in arterial thrombosis are less apparent. On the one hand, the traditional view is that the development of arterial thrombotic disease requires excessive activation of coagulation proteins in conjunction with gross defects in platelet or endothelial cell function. On the other hand, a more speculative possibility is that localized abnormalities in one or more of the endothelial cell components of these anticoagulant mechanisms might result in the emergence of these disorders. Detailed studies of these anticoagulant mechanisms will be needed to resolve this critical issue.

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