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The role of protein C and thrombomodulin in the regulation of blood coagulation

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In recent years, two distinct mechanisms have been described which allow for regulation of blood coagulation. The first involves inhibition of the coagulation proteases by plasma protease inhibitors. Based on clinical observations, antithrombin III appears to be the most mportant protease inhibitor. Specifically, antithrombin deficiency is associated with recurrent thrombotic episodes[1]. An alternative regulatory pathway involving protein C has become the focus of recent studies. Protein C is a vitamin K dependent plasma zymogen[2)3)]. Once activated to form the serine protease, activated protein C, this enzyme functions as an anticoagulant[4~8)]. Protein C anticoagulant activity is mediated through the selective inactivation of factors Va[6)8)9)] and VIIIa[8)10)]. Like antithrombin III deficiency, protein C deficiency is associated with recurrent thrombosis, even when the levels are reduced by only 50% of normal[11~14)]. In this paper, I would like to review the role of the vascular endothelium ininitiating protein C activation and the mechanism of assembly of the anticoagulant complex.

One major obstacle in establishing a physiologic function for protein C hinged on the observation that little[15)16)] or no[17)] protein C is activated when blood clots in vitro. Furthermore, although activated protein C is a potent anticoagulant, removal of protein C from plasma does not influence standard coagulation assays[14)] (**Teble 1**). These observations clearly do not support the concept that protein C plays a major role in the regulation of coagulation.

Table 1 The influence of activated protein C on plasma clotting initiated by factor Xa

Reaction	Clottining time
Plasma	31 sec
Plasma+0.1 μg/ml APC	63 sec
Plasma depleted of PC	30 sec

Human plasma clotting was initiated by the addition of factor Xa phospholipid and Ca^{2+}. Protein C was inhibited by addition of excess monospecific goat antihuman protein C antibodies prior to the assay.

Thus, although thrombin can activate protein C[5~7)], the rate of activation in blood appears insufficient for protein to alter the hemostatic balance. Based on these observations, we felt that either protein C played

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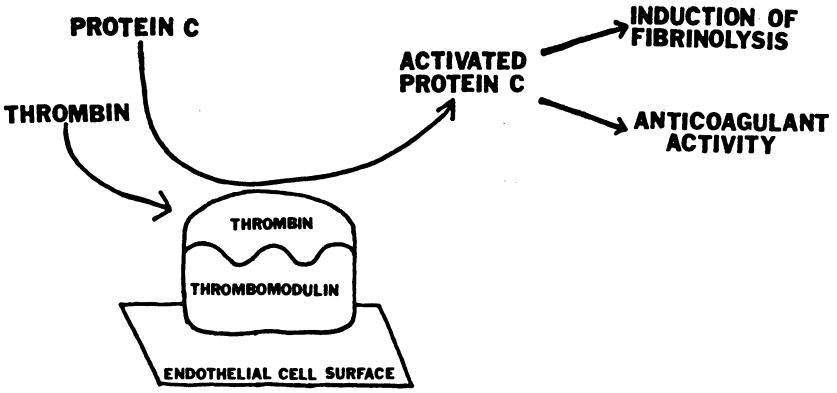

Fig. 1: A Model for Protein C Activation and Function (reproduced from the Journal of Clinical Investigation, 1982, 70 : 127-134 by copyright permission of the American Society for Clinical Investigation).

no role in regulating coagulation or that an alternative activation mechanism must exist. While several possible activators were considered, we felt the simplest and most directly testable hypothesis was that thrombin could bind to an endothelial cell surface receptor and that this interaction would alter the macromolecular substrate specificity of thrombin. A schematic representation of this hypothesis is presented in **Fig 1**.

This model was useful since it was readily testable. Specifically, the model predicted that the rate of protein C activation would be increased when thrombin binds to a specific receptor, thrombomodulin, on the surface of the vascular endothelium. The test of this model is to incubate thrombin and protein C over endothelial cells to determine if the cells accelerate the activation process. However, available in vitro systems suffer from a relatively low vascular surface area exposed to the fluid phase. In vivo, the surface area, and hence the receptor density, can be quite high. Busch et al.[18] calculated that the endothelial cell surface area exposed to blood increased approximately 1000 fold when blood moves from the macro to the microcirculation. Since high surface to volume ratios are not readily achieved in culture, a perfusion system was sought to investigate the involvement of the endothelial cell surface. One useful model is the Langendorf heart preparation[19]. In this system, the heart is perfused in reverse through the aorta. Since this closes the aortic valve, the perfusate is forced through the coronary microcirculation. Thrombin and protein C can be perfused and the effluent measured for activated protein C. The results of this experiment are indicated in **Table 2**. Protein C was activated only when thrombin and protein C were coperfused through the heart. From this experiment, we estimated that interaction of thrombin and protein C

Table 2 The effect of protein C perfusion through the myocardial microcirculation.

Perfusion mixture	Clotting time (sec)
Buffer	23±1.0
Thrombin	22±1.0
Protein C	23±0.8
Protein C+Thrombin	60±2.8
Protein C+Thrombin Control	23±0.6

Perfusion was performed through the rabbit myocardium as described previously[20]. The concentration of reagents were thrombin (0.1 US. unit/m*l*; protein C, 15 μg/m*l*.

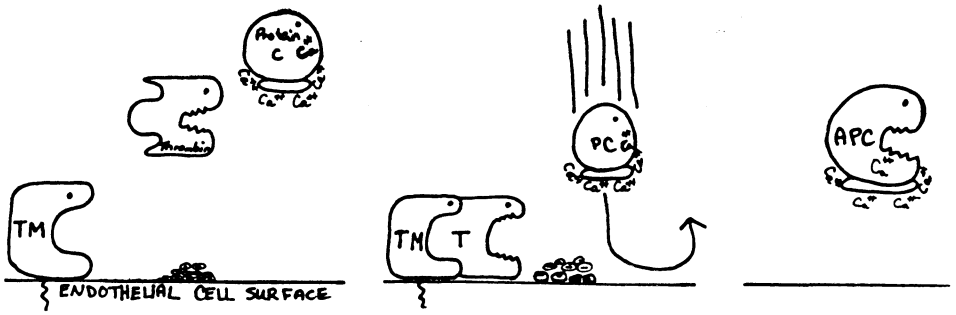

Fig. 2: Model of the Protein C Activation Complex on the Endothelial Cell. In addition to thombomodulin, the cell contributes an additional substrate binding site represented by negative patches of undefined chemical structure. APC, activated protein C; TM, thrombomodulin. (Modified from an original figure appearing in Pathobiology of the Endothelial Cell, "Activation of Protein C by a Complex between Thrombin and Endothelial Cell Surface Protein" pp. 121-136 (1982), H.L. Nossel, H.J. Vogel eds.), Academic Press, New York.

with the coronary microcirculaton enhanced the rate of protein C activation approximately 20,000 fold[20], DIP thrombin blocked the activatiom of protein C by the vascular endothelium. This observation was consistent with, but did not prove, the model depicted in **Fig. 2**. Furthermore, the interaction appeared to be independent of the active site on thrombin.

Further characterization of the system required establishment of an in vitro system to monitor mediated protein C activation. Fortunately, thrombomodulin accelerates protein C activation sufficiently that even though $\simeq$ 95% of the added thrombin is free in solution[21][22] the rate of protein C activation is nevertheless at least 30 fold faster than the thrombin alone control[20]. Activation was dependent on protein C, thrombin and the cell surface. In addition, the activation was inhibited by DIP-thrombin[20][23]. This system could be analyzed to determine 1) the affinity of thrombin for thrombomodulin 2) the affinity of the complex for protein C and 3) the mechanism by which DIP-thrombin inhibits protein C activation. The results indicate 1) a very high affinity interaction of thrombin with thrombomodulin (Kd$\simeq$0.5 nM) 2) that the complex reduces the Km for protein C approximately 100 fold relative to thrombin in soluton[23~25] and 3) DIP-thrombin inhibits protein C activation by competing with thrombin for binding to thrombomodulin.

The model suggests that thrombomodulin activity is associated with a protein. Evidence for this model was obtained by isolating the receptor. Briefly, thrombomodulin activity was solubilized with Triton X-100 from rabbit or rat lung and purified on DIP-thrombin agarose[26]. The purified receptor appeared to be homogeneous protein (Mr=74,000) when analyzed on acrylamide gel electrophoresis. Thrombomodulin activity is stable for at least two years stored at 4°C in buffers containing 0.05% Lubrol pX. This latter observation makes throbomodulin useful in the design of a functional assay.

With the aid of purified thrombomodulin, some of the details of the model depicted in Fig. 1 could be investigated. Kinetic atudies indicated that thrombin formed a 1:1 compley with thrombomodulin. Secondly, protein C activation by the complex Ca^{2+}. This latter observation is of interest since Ca^{2+} potently inhibits the activation of protein C by thro-

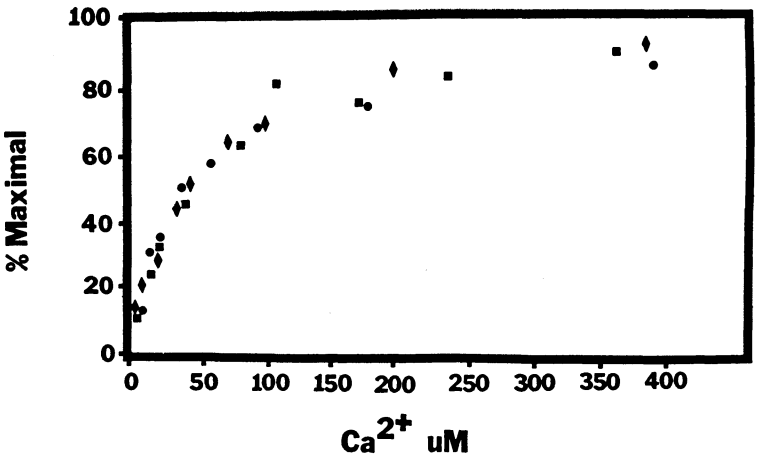

Fig. 3: Direct comparison of the Ca^{2+} dependence of binding, fluorescence, and activation rate obtained with Gla-domainless protein C. Data are expressed as a percentage of the maximal values obtained for Ca^{2+} binding (■) by equilibrium dialysis (moles of Ca^{2+}/mol of protein; for the decrease in intensity (●) of intrinsic fluorescence emission, and for the rate of activation (◆) by thrombin-thrombomodulin. [Reproduced from The Journal of Biological Chemistry, Vol. 258, No. 9, p. 5559, Structural Changes Required for Activation of Protein C are Induced by Ca^{2+} Binding to a High Affnity Site does not Contain γ-Carboxyglutamic Acid. Johnson, A.E., Esmon, N.L., Laue, T.M., Esmon, C.T. (1983)].

mbin in the absence of Ca^{2+} [24)25)].

These observations raised the question of how Ca^{2+} was involved in proten C activation. One possibility is that Ca^{2+} is required for formation of thrombin-thrombomodulin complex. The second possibility is that Ca^{2+} binds to protein C and alters the substrate conformation such that protein C fits better into the active site of the complex.

To investigate the involvement of Ca^{2+} in this system, we utilized the observation that thrombomodulin alters the macromolecular substrate specificity of thrombin such that thrombin no longer clots fibrinogen[27)], activates factor V[27)], or triggers platelet activation[28)]. The mechanism involves complex formation and is transient since active thrombin can be displaced from the complex. Since thrombomodulin the macromolecular substrate specificity, it was possible to determine if Ca^{2+} was involved in this change in specificity. Whether Ca^{2+} is present or not, thrombomodulin inhibits thrombins' ability to clot fibrinogen. Thus, neither complex formaton nor the change in macromolecular specificity require Ca^{2+}.

If complex formation does not require Ca^{2+}, then the most probable role for Ca^{2+} in the activation process involves a Ca^{2+} dependent change in protein C. Ca^{2+} binding to protein C has been demonstrated[24)]. This Ca^{2+} binding is predicted since protein C is a vitamin K dependent protein. We hypothesized that the Ca^{2+} dependent activation of protein C was mediated through binding Ca^{2+} to the Gla residues. A direct test of this hypothesis was suggested by recent studies of Morita and Jackson[29)]. They demonstrated a hinge region in factor X near the Gla domain that was especially susceptible to proteolysis with chy-

motrypsin. Since protein C and factor X are sequence homologous in this region[30], it was not surprising that chymotrypsin could be used to selectively remove the Gla domain from protein C as illustrated below.

Chymotrypsin
Protein C ——————→ Gla Domainless Protein C (Lacking residues 1-41 of the light chain) + Gla Containing Peptide

If the Ca^{2+} effects were mediated through the Ca^{2+} dependent interaction with the Gla peptide, then we predicted either 1) that removal of the Gla peptide would result in inhibition of activation by the thrombin thrombomodulin complex or 2) that the Ca^{2+} dependence would be eliminated. To our surprise, neither of these predictions was true. Gla domainless protein C and protein C were equivalent substrates. Furthermore, the reaction was still Ca^{2+} dependent.

Therefore, either the metal dependence is not mediated through the substrate or there is a Ca^{2+} binding site on protein C independent of the Gla residues. This site does exist and as shown in **Fig. 3**, the metal dependence of the activation correlated well with the metal dependence of a conformational change and the interaction of Ca^{2+} with a single high affinity site (as measured by equilibrium dialysis). Thus, from these data alone, vitamin K depenent modification does not appear to be required for protein C activation.

These experiments are misleading in the sense that protein C activation normally proceeds over the surface of the vascular endothelium. From analogies to the "prothrombinase system", the platelet appears to provide a substrate binding site facilitates prothrombin activtion[31] raising the possibility that the endothelial cell may provide a comparable surface for protein C activation. We used the Gla domainless protein C to invesigate this possibility. Since the two substrates are activated identically in soluton, we felt that, if the cell surface activated protein C preferentially, then this would indicate the existence of a substrate binding site on the endothelial cell surface. Direct comparisons of protein C and GD-protein C activation rates over the vascular endothelium revealed that the cell surface activation complex discriminates between these two substrates, preferring protein C$\simeq$50 fold over GD-protein C[25]. These differences are primarily a Km effect and the differential rates of activation are largely eliminated at very high substrate concentration. These observations are consistent with the cartoon model shown in **Fig. 2**. The chemical nature of the substrate binding site remains unknown. Since negatively charges surfaces, such as acidic phospholipids are known to

Table 3 Inhibition of endothelial cell mediated protein C activation by antithrombin III

Antithrombin III concentration	% Inhibition
100 μg/ml	92
30 μg/ml	58
10 μg/ml	25
3 μg/ml	3
0 μg/ml	20

Thrombin (50 μl of 0.2 unit/ml) was layered over rabbit endothelial cells grown in a 96 well tissue culture dish. After 5 min. 50 μl of protein C (2 μM) plus the appropriate concentration of antithrombin III was added. The cells were incubated with shaking at 37°C for 1 hour. At this time, supernatants were removed, excess antithrombin III added (200 μg/ml) and activated protein C production measured[13]. Values were related to those obtained in the absence of antithrombin III during the cell incubation period. The sample indicated as 0 antithrombin III represents an assay in which thrombin was allowed to bind to the endothelial cells, then free thrombin was washed off and protein C added. The small decrease in activity represents the maximum inhibition which would result from inhibition of free thrombin.

bind vitamin K dependent proteins, this surface is depicted as a patch of negative charges.

These observations raise the question of whether simple insertion of thrombomodulin into the membrane would result in this substrate discrimination. To approach this question, we examined the ability of several endothelial cell lines to discriminate between these substrates. We took advantage of the observation that endothelial cells in continuous cultures often alter their properties. In all of the cell studies, thrombomodulin remained surface bound. However, the ability to discriminate between substrates was not constant. Indeed, the relative discrimination varied from 1.2 to greater than 50. Thus, simple insertion of thrombomodulin into the membrane is insufficient to allow for discrimination. It appears that under some circumstances the cells either fail to synthesize the surface component or fail to couple it to the receptor.

Two potent anticoagulant effects appear to be mediated by thrombomodulin; 1) the activation of protein C and 2) the neutralization of thrombin procoagulant functions. Since the complex alters the specificity of tbrombin toward macromolecular substrates, it was not clear that protease inhibitors would inactivate thrombin when bound to thrombomodulin. However, the rate of thrombin inactivation is not altered when complexed with purified thrombomodulin in solution and complex can be inhibited by antithrombin III on the cell surface also (**Table 3**). Thus, antithrombin III can regulate protein C activation by inhibiting thrombin bound to thrombomodulin.

A functional assay for protein C

As indicated previously, thrombin does not readily activate protein C in vitro. However, thrombomodulin can facilitate protein C activation by increasing the rate of protein C activation and also preventing alternative substrates such as fibrinogen from competing with protein C. When the thrombin-thrombomodulin complex is added to recalcified plasma, protein C is activated and the activated protein C prevents plasma clotting. Direct clotting of fibrinogen is inhibited by thrombomodulin. Unfortunately, the activated protein C still cannot be monitored accurately.

Background hydrolytic activity is too high and variable to allow quantitation directly from the plasma. We have circumvented this problem by adsorbing the activated protein C onto antiprotein C antibodies. Coupled to agarose, these antibodies neutralize the anticoagulant activity of activated protein C but do not block the amidase activity[14]. Therefore, the hydrolytic activity can be quantitated with insolubilized APC free of the nonspecific hydrolytic activities present in the plasma.

The assay is more rapid than immunochemical techniques. Furthermore, it appears to be able to activate all forms of protein C present in the plasma of patients on oral anticoagulants. This suggests that a tentative diagnosis of abnormal protein C function could be made on patients without stopping oral anticoagulant therapy.

It is important to note that this assay does not detect abnormalities in proteolytic activity toward factor Va or VIIIa, the presence of normal lavels of carboxylation, the ability to interact with protein S (see below), with platelet membranes or to stimulate fibrinolysis. Thus, the assay may idicate normal function in patients deficient in one or more of the aforementioned functions.

Expression of anticoagulant activity

In vitro experiments suggest that activated protein C functions through the selective inactivation of factors Va and VIIIa and that rapid inactivation of these factors only occurs

in the presence of negatively charged phospolipids[6)8)]. These surfaces are thrombogenic and usually thought to be restricted to intracellular membrane surfaces. Thus, for protein C to play a role as a systemic anticoagulant, a cellular suface much exist to support factor Va inactivation. Platelets provide a surface which catalyzes prothrombin activation[31)]. Factor Va has been reported to bind to platelets preceding platelet activatiom[32)33)]. From these studies, it appears likely that a surface for factor Va binding may always be present on the platelet membrane. We felt that platelets could also provide a surface for activated protein C dependent factor Va inactivation. To test hypothesis, activated protein C was incubated with gel filtered platelets and the rate of factor Va inactivation measured. Prior to activation, the platelets did not accelerate factor Va inactivation. However, following activation the platelets did support factor Va inactivation. These studies indicate that platelets can provide a cellular surface for factor Va inactivation but that cellular activation was required[34)]. A requirement for platelet activation appeared to us to be incompatible with a role for platelets as a systemic regulator of activated factor Va.

Recent studies by Walker suggested that activated protein C requires another vitamin K dependent protein, protein S, to express plama ansicoagulant activity[35)]. In in vitro systems, protein S appears to facilitate activated protein C binding to synthetic phospholipid membranes[36)], much like factor Va enhances factor Xa binding to these membranes. Based on the analogy with the platelet prothrombinase system, we tested the possibility that protein S might form a receptor for activated protein C on the surface of unstimulated platelets. When protein S was incubated with activated protein C and factor Va, platelet dependent factor Va inactivation was observed. No requirement for platelet activation was observed. Thus, in the presence of protein S, the platelet can accelerate factor Va inactivation before the cells are activated[34)]. This observation is consistent with the concept that activated protein C may function as a systemic anticoagulant.

Summary

The protein C anticoagulant pathway represents a newly described system for investigating the regulation of blood coagulation. A role for protein C in clinial thrombosis has been suggested by recent findings that patients with 50% normal levels of protein C are at rick of thrombosis[11)14)] and that protein C levels decrease markedly during intravascular coagulation[37)38)]. The data presented here suggest that deficiencics in protein S, thrombomodulin, or the platelet receptor for activated protein C might also result in a thrombotic tendency. The interplay among the cellular, vascular and humoral components in this system suggest that the protein C anticoagulant pathway may be perturbed by a variety of disease processes. Certainly, the system provides a new approach to investigate the molecular basis of thrombotic disease.

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