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Fibrinolytic System of Cultured Endothelial Cells: Regulation by Plasminogen Activator Inhibitor

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Cultured bovine aortic endothelial cells have a relatively complex fibrinolytic system that is responsive to both the physiological state of the cell itself and to a variety of agents added to the culture medium. The fibrinolytic activity of these cells results from the production of both urokinase-type and tissue-type plasminogen activators and is regulated by an inhibitor capable of neutralizing their activities. The properties of these fibrinolytic components will be reviewed, and their respective roles in initiating and regulating the fibrinolytic activity of the cells will be summarized. A cDNA coding for the inhibitor has been isolated, and its sequence will be compared to that of other serine proteinase inhibitors.

Key words: fibrinolysis, endothelial cells, tissue-type plasminogen activator, urokinase-type plasminogen activator, serine protease inhibitor, fibrin autography, reverse fibrin autography, cell cultures, activated protein C, cDNA cloning, sequence analysis

Abnormal thrombus formation and dissolution are associated with several cardiovascular diseases including atherosclerosis and both thromboembolic and hemorrhagic conditions [1,2]. The walls of blood vessels undoubtedly contribute to the pathogenesis of these disorders. More specifically, vascular endothelial cells appear to have the potential to participate in the hemostatic process through the synthesis of molecules that may function either to promote thrombosis (eg, von Willebrand factor [3]; procoagulant activity [4]), or to maintain vessel patency (eg, plasminogen activator, PA [5]). The fibrinolytic system of endothelium thus assumes pivotal importance in maintaining this hemostatic balance.

We have been studying cultured bovine aortic endothelial cells (BAEs) as a model of endothelium [6,7]. The overall fibrinolytic activity of these cells changes

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with their growth state [8] and in response to the presence of a variety of factors [9–12]. Although it has been suggested that such changes reflect changes in PA, our unexpected finding [13,14] that these cells also synthesize an unusually stable PA inhibitor (PAI) makes accurate interpretation of such results difficult. For example, do the altered fibrinolytic states of these cells following various treatments or in certain human diseases reflect changes in PA, inhibitor, or both?

This review will emphasize the concept that the fibrinolytic "state" of endothelial cells, or of any cell or individual for that matter, is really a reflection of the balance between the activities of PAs and those of their natural inhibitors. Examples will be provided to demonstrate that agents that alter the fibrinolytic state may do so by changing PA, inhibitor, or both. In addition, we have recently succeeded in purifying the inhibitor and in isolating and characterizing a complimentary DNA (cDNA) that codes for the mature protein [15]. Some of the properties of the inhibitor will be summarized, and its sequence will be compared to that of antithrombin III and α_1-antitrypsin (α-1-protease inhibitor [α-1-PI]).

ENDOTHELIAL-CELL-MEDIATED FIBRINOLYSIS

Table I summarizes a large number of studies conducted over the years. It shows first of all that cultured BAEs are fibrinolytically active and in general secrete 2–8 IU of total PA activity per 10^6 cells, per 16 hours. It also demonstrates that a diverse number of agents are capable of altering this activity. For example, simply feeding the confluent monolayers with media containing fresh serum caused an 80% decrease in intracellular fibrinolytic activity as compared to unfed controls [8]. Similar decreases were observed when the cells were incubated in the presence of thrombin [10,11], dexamethasone [16], or calcium ionophore [11]. In contrast, activated protein C (APC), a vitamin K-dependent plasma protein [17], was shown to stimulate the fibrinolytic activity of these cells quite dramatically [12]. Bovine APC has been shown to have profibrinolytic activity in vivo [18].

Experiments were performed to determine whether the fibrinolytic activity summarized in Table I resulted from the presence of single or multiple forms of PA. Conditioned media (CM) and cell extracts were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by fibrin autography [19,20]. PAs are stable to SDS and can be assayed following electrophoresis. To do this, the SDS gel is placed on an indicator film containing agar, fibrin, and plasminogen. The fibrin makes the indicator film opaque. PAs in the sample gel diffuse into the indicator film and are thus revealed by the formation of a lysis zone

TABLE I. Effect of Various Treatments on the Cell-Associated Fibrinolytic Activity of BAEs

Treatment	Concentration	Fibrinolytic activity (% of control)
Untreated	—	100[a]
Serum	1%	22
Thrombin	0.025 units/ml	10
Dexamethasone	10^{-6} M	15
Calcium ionophore	1 μg/ml	10
Activated protein C	3.4 μg/ml	1,000

[a]Five units/10^6 cells in cell extracts; 2–8 units/16 hr/10^6 cells in conditioned medium.

as plasminogen in that region is converted to plasmin. When cell extracts or conditioned media from confluent BAEs were fractionated by SDS-PAGE and analyzed by fibrin autography [20], a complex lysis pattern developed with major zones at 52,000, 74,000, and approximately 116,000 daltons (Fig. 1). Thus, the fibrinolytic activity of these cells results from the presence of at least three distinct PA forms.

This general approach can be modified to detect fibrinolytic inhibitors [13,21]. In this case, lytic agents are incorporated directly into the fibrin preparation itself. The presence of these enzymes will eventually cause the whole fibrin indicator film to lyse. In the example shown in Figure 1, it is the interaction of exogenously added PA with plasminogen that generates plasmin throughout the film and in turn causes clearing. Inhibitors in the SDS gel diffuse into the indicator film and prevent this lysis. Thus, inhibitor activity in the SDS gel is revealed by the development of an opaque, lysis-resistant zone in the otherwise clear indicator film. This approach ("reverse fibrin autography" [RFA]) was used to analyze CM from BAEs, again following its fractionation by SDS-PAGE (Fig. 1). The gel was placed on a fibrin film containing plasminogen and urokinase and then photographed after the majority of the film had lysed. One area of the film appeared to be relatively resistant to this lysis. It migrated with an apparent M_r of 55,000. The lysis resistant zone in the indicator film resulted from the presence of a fibrinolytic inhibitor in the SDS gel since this region of the SDS-gel was excised, extracted into buffer, and shown to inhibit fibrinolysis [13,14]. Thus, the fibrinolytic system of endothelial cells is relatively complex, consisting of multiple forms of PA as well as an inhibitor. Some of the properties of these fibrinolytic components are summarized in the following sections.

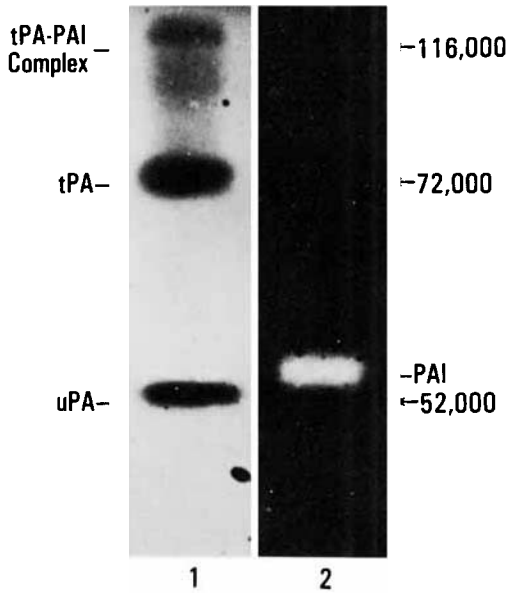

Fig. 1. Analysis of BAE CM by fibrin and reverse fibrin autography. BAE CM (100 μl) was fractionated by SDS-PAGE and analyzed for the presence of PAs by fibrin autography (**lane 1**) and for inhibitors by reverse fibrin autography (**lane 2**).

Plasminogen Activators

There are two kinds of PA, the so-called tissue-type (tPA; M_r 72,000) and the urokinase-type (uPA; M_r 54,000) molecules [2,7,22]. One of the major differences between these two PAs is that tPA binds to fibrin but urokinase does not. Thus, to begin to differentiate between the various PAs made by these cells, the ability of each to bind to fibrin was assessed [23]. Fibrinogen was added to CM and clotted by the addition of thrombin. The clots were removed by centrifugation, fractionated by SDS-PAGE, and analyzed by fibrin autography. Surprisingly, all of the high molecular weight forms (M_r 72,000–116,000) bound to the clot, suggesting that they are tPA-like. The low M_r form (M_r 52,000) did not bind but remained in the supernatant, which suggests that it is a uPA. These conclusions were substantiated by the observations that antibodies to human urokinase specifically inhibited the activity of the 52,000- non-fibrin-binding form but did not affect the activity of the other forms [20]; antibodies to tPA neutralized the activity of all the fibrin-binding forms but not that of the M_r 52,000 form.

The implication of these studies is that BAEs produce multiple forms of tPA. However, there is apparently only a single gene for tPA [24,25], and it codes for a protein of M_r 66,000. Thus, the M_r 116,000 form of tPA is much larger than it should be. To investigate this discrepancy, we analyzed BAE CM by Western blotting [26]. The M_r 116,000 form was recognized by antibodies to both tPA and to the inhibitor, indicating that it is a complex between the two molecules. Most of the tPA antigen in CM collected from endothelial cells is in complex with the inhibitor [26–28].

Inhibitor

The inhibitor was purified from BAE CM by a combination of concavalin A affinity chromatography and preparative SDS-PAGE and further characterized [14]. It was shown to be a single-chain glycoprotein of molecular weight 50,000 daltons with an isoelectric point of between 4.5 and 5. It was an unusually stable molecule in that full activity could be recovered from samples exposed to 1 M acetic acid, 5% β-mercaptoethanol or to denaturants such as SDS, guanidine, and urea [13]. These properties are in contrast to plasma protease inhibitors, which in general are rapidly inactivated by such treatments [29]. The inhibitor is actually synthesized by endothelial cells and represents between 2.5 and 12% of the total ^{3}H-leucine-labeled protein secreted by the cells in a 24-hour period [14]. Most importantly, the inhibitor is an antiactivator, and can neutralize the activity of both tPA and uPA but not that of plasmin. Inhibition is associated with the formation of enzyme-inhibitor complexes that are not dissociated by SDS [14].

This PA inhibitor (PAI) has a number of other interesting properties. For example, it seems to be unusually sensitive to inactivation by oxidants and is rapidly inactivated by low concentrations of chloramine T, n-chlorosuccinimide, and hydrogen peroxide [30]. In fact, the PAI is approximately one order of magnitude more sensitive to chloramine T than is α-1-PI, a protein proteinase inhibitor previously characterized by its extreme sensitivity to oxidants [31]. It is known that α-1-PI is unusual because it has a methionine residue at the P_1 position of its reactive center, and that the conversion of this methionine to methionine sulfoxide is responsible for the observed loss of activity [32]. This comparison raises the possibility that the PAI may also have a methionine at or near its reactive site.

Attempts were made to relate the PAI to other plasma and cellular protease inhibitors on the basis of their electrophoretic mobilities [33]. In these experiments, the purified PAI was fractionated by agarose zone electrophoresis and compared to whole plasma. While most plasma protease inhibitors migrate with α-mobility [29], the endothelial cell inhibitor migrates with β-mobility, whether analyzed by immunoblotting or activity. On the basis of this behavior, it was suggested that this class of PAIs be referred to as β-PAIs to distinguish them from other PAIs that migrate with α-mobility [33].

The β-PAI is also unusual in that it appears to be present in CM in an inactive form but can be "activated" by treatment with a variety of denaturants [34]. For example, less than 0.01 units/μl of PAI activity was detected in untreated CM, but medium treated with SDS (1.7 mM), guanidine HCL (4 M), urea (12 M), or KSCN (6 M) contained 0.9, 1.9, 0.8, and 0.5 units/μl, respectively. This effect was dose dependent with respect to the particular agent used, and the same concentration of reagent that induced PAI activity also stimulated the ability of the latent PAI to form complexes with exogenously added PAs. Activation did not appear to result from the removal of either a small dialyzable component from the medium or of a large M_r component that is bound to the latent PAI. Rather, it seemed to result from a conformational change in the molecule induced by each of the activating agents [34]. The identity of the biological equivalent of these denaturants remains to be elucidated. The recognized role of surfaces in coagulation (eg, phospholipids, platelets [35]) and fibrinolysis (eg, fibrin [36]; cells [37]) raises the possibility that cellular, lipoprotein, and protein surfaces may be involved in the activation of the latent PAI in vivo.

A number of additional insights about the β-PAI have been obtained from the sequence analysis of a cDNA coding for the PAI [15]. Preliminary observations indicated that human placenta was an excellent source of the β-PAI. Placental mRNA was therefore prepared and copied into DNA. The resulting cDNA was used to construct a λgt_{11} expression library that contained about 10^6 independent clones [38]. This cDNA library was then screened immunochemically, using affinity-purified IgG specific for the bovine β-PAI [14]. Several positive clones were identified by this approach and shown to contain inserts of two different lengths (ie, either 2 or 3 kb). Expression of the 2-kb clone in *Escherichia coli* resulted in the synthesis of a large molecular weight fusion protein, which was detected by Western blotting using the affinity-purified IgG. This fusion protein was not expressed in a strain lysogenized with the parental λgt_{11} vector (ie, lacking the human cDNA insert). Most importantly, the fusion protein was shown to have PAI activity when analyzed by RFA. The *E coli* extract also contained a second PAI zone, most likely derived from the fusion protein by limited proteolysis. It had an M_r of approximately 43,000. Northern blot analysis of RNA prepared from a human fibrosarcoma cell line (HT 1080) using the PAI cDNA as a probe, revealed two major species of RNA, approximately 3.0 and 2.2 kb in length. The 3-kb species appeared to be the predominant form of PAI mRNA in these cells. These two forms may represent the products of two alternative polyadenylation signals, or of alternative splicing events. Only the 3.0-kb form was detected when BAE mRNA was analyzed in this way.

Analysis of the sequence of the human β-PAI revealed a number of interesting features. For example, Figure 2 shows the amino acid sequence around the presumptive reactive center as deduced from the nucleotide sequence. The numbering is according to the original system employed by Hunt and Dayhoff [39] to describe α-

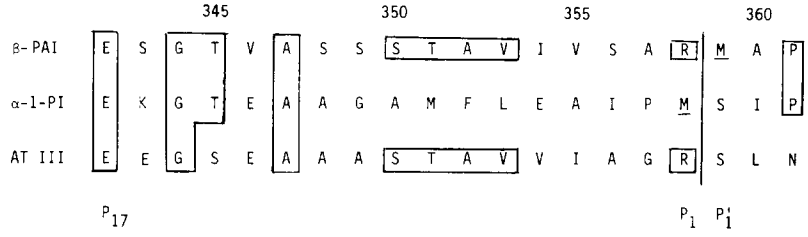

Fig. 2. Comparison of β-PAI, α-1-PI, and AT III. Alignment of the sequences of β-PAI, α-1-PI, and AT III around their reactive centers according to Hunt and Dayhoff [39]. The reactive site peptide bonds are indicated by the vertical line, and the terminology of the reactive site residues is adapted from Travis and Salvesen [31]. The reactive site methionines are underlined, and amino acids in α-1-PI and AT III homologous to those in β-PAI are boxed.

1-PI. In this figure, the Pearson Fast Protein Analysis program was used to align and compare the PAI with human α-1-PI and antithrombin III (AT III). The homology between the PAI and the other inhibitors in this region is indicated by the boxes. Although the P_1-P_1' amino acids [31,40] of the reactive site have not been isolated, there are a number of reasons to conclude that this computer alignment is the correct one. For example, the reactive site residue, P_1, generally corresponds to the specificity of the cognate enzyme [40]. The P_1 amino acid in this alignment is arginine, and PAs are known to have arg specificity [2,22]. In addition, the P'_1 amino acid is methionine, a readily oxidized amino acid [41]. Finding a methionine in the P_1' site is thus consistent with the demonstration that the β-PAI is extremely sensitive to oxidants. And finally, the presence of a glutamine in the P_{17} or "hinge" region appears to be a conserved feature of the strained loop region of this family of inhibitors [40].

Obviously, the discussion about the reactive site will remain somewhat speculative until the P_1 and P_1' residues are actually isolated and indentified. What is clear from these studies is that the β-PAI is most certainly a member of the serine protease inhibitor family (serpins [40]). It consists of 379 amino acids and has approximately 30% homology with both human α1-P-I and AT III, two other members of this family. This homology is maintained along the entire length of the molecule. It should be noted that no cysteine residues were present in the deduced protein sequence, an observation that may explain why this molecule is still fully active after reduction [13].

Regulation

The above discussion of the PAs and PAI produced by BAEs indicates that the fibrinolytic system of these cells is complex. It is based on the production of multiple PAs and an inhibitor that can neutralize them. It is thus apparent that the overall fibrinolytic activity of these cells is a balance between these opposing activities, and that agents that alter the fibrinolytic activity of these cells, may do so by changing either PA or inhibitor. The end result may be the same. For example, agents such as thrombin (Table I) that suppress the fibrinolytic activity of these cells, may do so by decreasing PA or increasing PAI. Conversely, agents such as activated protein C that enhance fibrinolytic activity, may do so by increasing PA or decreasing inhibitor. There are a number of published observations showing that altered fibrinolytic activity

reflects altered PA [2,22]. But what about the inhibitor? Are there any data to support the hypothesis that altered fibrinolytic activity may result from changes in PAI?

Bovine-activated protein C was shown by Comp and Esmon [18] to have a pronounced profibrinolytic effect in blood when infused into dogs. These authors suggested that this effect was due to the release of tPA from the endothelium. To further investigate this hypothesis, BAEs were cultured in the presence of bovine APC and were shown to have elevated fibrinolytic activity, both in CM and cell extracts [12]. Similar results were obtained when human APC and human endothelial cells [42] were used, even though human APC does not appear to be profibrinolytic in vivo [43]. The APC caused a dose-dependent increase in the activity of all PA forms but did not affect the activity of purified tPA, uPA, or tPA-PAI complexes (unpublished observation). However, preincubation of APC with PAI prior to the addition of tPA prevented complex formation, which suggests that APC interacts directly with the inhibitor itself. In direct experiments to test this possibility, we showed that complexes between purified bovine APC and β-PAI readily form and can still be detected after SDS-PAGE [44]. APC and PAI appear to be inhibited as a consequence of this interaction (unpublished observation). Thus it seems that the APC-mediated increase in the fibrinolytic activity of these cells is due primarily to a decrease in PAI activity and not to an increase in PA.

CONCLUSIONS

Cultured bovine endothelial cells have a relatively complex fibrinolytic system, which is responsive both to the physiological state of the cell itself and to a variety of agents added to the culture medium. The fibrinolytic activity of these cells results from the production of both urokinase-type and tissue-type PAs and an antiactivator capable of inhibiting their respective activities. The inhibitor is immunologically and biochemically related to the rapidly acting antiactivator detected in human platelets [45,46] and in the plasma of some patients [33], and may represent a novel class of fibrinolytic inhibitors (the antiactivators), which are likely to play a critical role in modulating the fibrinolytic potential of tissues, blood, and the vascular wall itself. It is a member of the serpin family of inhibitors [40].

Preliminary experiments indicate that there is at least 100 times more potential β-PAI than PA in BAE CM. And yet, the cells are fibrinolytically active under most conditions. At least part of the explanation for this apparent inconsistency is the finding that the majority of the inhibitor exists in a latent form that can be activated with denaturants such as SDS. The active inhibitor in CM (less than 10% of the potential inhibitor) readily forms complexes with cellular tPA but not with cellular uPA, presumably because the uPA is a proenzyme. Understanding the mechanism of the "activation" of the PAI by denaturants and the nature of the biological equivalent to SDS will be central to our understanding of this system, which as yet remains to be elucidated.

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