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Effects of Fibroblasts and Endothelial Cells on Inactivation of Target Proteases by Protease Nexin-1, Heparin Cofactor II, and C1-Inhibitor

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Previous studies have shown that glycosaminoglycans in the extracellular matrix accelerate the inactivation of target proteases by certain protease inhibitors. It has been suggested that the ability of the matrix of certain cells to accelerate some inhibitors but not others might reflect the site of action of the inhibitors. Previous studies showed that fibroblasts accelerate the inactivation of thrombin by protease nexin-1, an inhibitor that appears to function at the surface of cells in extravascular tissues. The present experiments showed that endothelial cells also accelerate this reaction. The accelerative activity was accounted for by the extracellular matrix and was mostly due to heparan sulfate. Fibroblasts but not endothelial cells accelerated the inactivation of thrombin by heparin cofactor II, an abundant inhibitor in plasma. This is consistent with previous suggestions that heparin cofactor II inactivates thrombin when plasma is exposed to fibroblasts and smooth muscle cells. Neither fibroblasts nor endothelial cells accelerated the inactivation of C1s by plasma C1-inhibitor.

Key words: extracellular matrix, heparan sulfate, heparitinase, thrombin, C1s

Protease nexin-1 (PN-1) is a protease inhibitor that is synthesized and secreted by cultured human fibroblasts [1–3]. It inhibits certain proteases, including thrombin, urokinase, and plasmin, by forming a covalent complex with their catalytic site serine [1,2]. The complexes then bind back to the cells that secrete PN-1 and are rapidly internalized and degraded [4]. This provides a localized mechanism for both inactivating and clearing certain serine proteases.

Several results suggest that the site of action of PN-1 is in the extravascular compartment at or near the surface of cells. First, PN-1 is secreted by a number of cultured cells including fibroblasts, heart muscle cells, myotubes, kidney epithelial cells, and fibrosarcoma cells [5]. Second, it is present in only barely detectable levels

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in plasma [6]. Third, the surface of fibroblasts accelerates the inactivation of thrombin by PN-1 [7]. This activity resides in the extracellular matrix (ECM) [7] and is mostly due to heparan sulfate [8].

There is evidence that the ability of cell surfaces to accelerate the inactivation of proteases by protease inhibitors might reflect the site of action of the inhibitors. For example, the inactivation of thrombin by antithrombin III is accelerated by endothelial cells [9,10] but not by fibroblasts [7,11] or smooth muscle cells [11], consistent with the known functions of antithrombin III in the vascular compartment of the body. If the hypothesis about cell-surface specificity in the acceleration of these reactions were true, then endothelial cells might not accelerate the inactivation of thrombin by PN-1 since PN-1 appears not to be a physiological inhibitor of thrombin in plasma. We tested this in the present studies by examining the ability of cultured endothelial cells to accelerate the reaction between PN-1 and thrombin. We also examined the ability of both fibroblasts and endothelial cells to accelerate the reactions between two plasma protease inhibitors and their target proteases: heparin cofactor II and thrombin and C1-inhibitor and C1s.

MATERIALS AND METHODS

Cell Culture and Preparation of Cellular Materials

Bovine aortic endothelial cells (obtained from Dr. Corinne Gajdusek, University of Washington) were cultured in 100-mm tissue culture dishes in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (Gibco, Grand Island, NY). The cells were between passages 4 and 6. Human foreskin fibroblasts were prepared as previously described [12] and cultured in DMEM containing 5% calf serum. For use in experiments, bovine aortic endothelial cells were seeded at 1.5×10^4 cells per 35-mm tissue culture dish and grown to confluence; the serum was reduced to 0.4% fetal calf serum 24 hr prior to use. Human fibroblasts were seeded at 1.5×10^4 cells per 35-mm plate, grown to confluence, and serum starved in DMEM containing 1.0 mg per ml bovine serum albumin (BSA) 24 hr prior to use. Control plates without cells were treated in the same manner.

Fixed cells were prepared with 2% paraformaldehyde. Serum-reduced or serum-starved cultures of endothelial cells or fibroblasts were rinsed five times with phosphate-buffered saline (PBS) and fixed for 15 min at room temperature with 2% paraformaldehyde. The fixed cells were rinsed three times with PBS prior to use.

ECM was prepared from cells grown as described above except that the cell culture dishes were coated with 0.1% gelatin before seeding the cells; 50 μg/ml ascorbic acid was included in the growth medium to stimulate deposition of the matrix. To prepare ECM, the cells were rinsed once with PBS followed by three 10-min incubations at 4°C in 0.1% sodium deoxycholate, 1 mM phenylmethane sulfonylfluoride, 2 mM Tris, pH 8.0 [13]. Then, the ECM preparations were rinsed three times with 2 mM Tris, pH 8.0, for 5 min each at 4°C prior to use.

Purified plasma membranes from bovine aortic endothelial cells were prepared by the procedure of Thom et al [14].

Proteins and Radioiodination

PN-1 was purified by the method of Farrell et al [15]. Purified heparin cofactor II and C1-inhibitor were provided by Dr. David Farrell and Dr. William Van

Nostrand, respectively, from this laboratory. Purified human thrombin was provided by Dr. John W. Fenton II [16] (New York State Department of Health, Albany, NY). Purified C1 was provided by Dr. Robert Ziccardi (Scripps Clinic and Research Foundation, La Jolla, CA).

Thrombin and C1s were iodinated with $Na^{125}I$ (New England Nuclear, Boston, MA) by using chloroglycouril (Pierce Chemical Company, Rockford, IL) as previously described [17]. The specific activity was approximately 1.3–3.2 $\times$ 10^4 cpm per ng for thrombin and 4.0–10.0 $\times$ 10^3 cpm per ng for C1s.

Assay of Protease-Inhibitor Complexes

For experiments examining thrombin-inhibitor complex formation in the presence of fixed cells or ECM, 0.5 ml of buffer (0.15 M NaCl, 20 mM imidazole, pH 7.5) containing either 64 nM PN-1 or heparin cofactor II and 6.4 mM ^{125}I-thrombin were incubated at 37°C for the indicated times with either fixed fibroblasts or endothelial cells, ECM prepared from endothelial cells or control dishes without cells. The reaction was stopped with 0.5 ml electrophoresis sample buffer containing 1% sodium dodecyl sulfate (SDS), 10% glycerol, and bromphenol blue; 40 μl of each sample was then subjected to electrophoresis by using a 7.5% SDS polyacrylamide gel [18]. The amount of ^{125}I-thrombin in complex with either inhibitor was determined by locating the ^{125}I-thrombin-inhibitor complexes on gels, cutting them out, and measuring radioactivity as in previous studies [1,7]. Complex formation between C1-inhibitor and C1s was monitored in a similar fashion by using 12 nM ^{125}I-C1s and 60 nM C1-inhibitor in a reaction volume of 0.5 ml. The reaction was terminated by adding 0.5 ml of electrophoresis sample buffer; 40 μl of each sample was then electrophoresed by using a 7.5% polyacrylamide gel, and radioactivity in ^{125}I-C1s-C1-inhibitor complexes was measured as described above for ^{125}I-thrombin-inhibitor complexes. To determine the extent to which PN-1, heparin cofactor II, or C1-inhibitor was present on fixed cells or ECM, ^{125}I-C1s or ^{125}I-thrombin alone was incubated with either fixed cells or ECM. Radioactivity in ^{125}I-protease-inhibitor complexes was subtracted as background and was less than 2% of the radioactivity present in complexes when PN-1 or the other inhibitors were added.

Similar conditions were utilized for experiments examining the effect of purified membranes on ^{125}I-thrombin-PN-1 complex formation. Reaction mixtures of 20 μl contained 6.4 nM ^{125}-I-thrombin and 64 nM PN-1 and indicated concentrations of purified membranes. The mixtures were incubated at 37°C for 2 min; 20 μl of electrophoresis sample buffer was added, and radioactivity in ^{125}I-thrombin-PN-1 complexes was determined as described above.

Glycosidase Digestion of Membranes

In experiments involving glycosidase digestion of purified endothelial cell membranes, 2 μg of membranes were treated with either heparitinase or chrondroitinase ABC (Miles, Elkhart, IN) at concentrations ranging from 0 to 4 units per ml for 30 min at 37°C. Upon completion of the 30-min incubation, 6.4 nM ^{125}I-thrombin and 64 nM PN-1 were added to the reaction; incubation was continued for an additional 5 min at 37°C. The reaction was carried out in a volume of 20 μl. It was stopped by adding an equal volume of electrophoresis sample buffer. Radioactivity in ^{125}I-thrombin-PN-1 complexes was measured as described above.

RESULTS

Acceleration of Thrombin-PN-1 Complex Formation by Fixed Endothelial Cells

Recently, we demonstrated that thrombin-PN-1 complex formation is accelerated by the surface of human fibroblasts and that this acceleration is mostly due to heparan sulfate in the ECM [7,8]. Since PN-1 is probably not a physiological inhibitor of thrombin in plasma, we checked the ability of cultured endothelial cells to accelerate the inactivation of thrombin by PN-1. The endothelial cells were fixed with 2% paraformaldehyde to prevent secretion of PN-1 and endocytosis of thrombin-PN-1 complexes [7]. As shown in Figure 1, the inhibition of thrombin by PN-1 was accelerated two- to threefold by the presence of fixed endothelial cells. The observed increase was not due to residual PN-1 on the fixed cells since ^{125}I-thrombin incubated alone with fixed endothelial cells resulted in less than 2% of the radioactivity in ^{125}I-thrombin-PN-1 complexes that occurred when PN-1 was also included in the incubation.

Acceleration of Thrombin-PN-1 Complexes by Purified Membranes and ECM

To demonstrate that the accelerative activity of fixed bovine endothelial cells on thrombin-PN-1 complex formation was not due to an artifact of fixation we examined the effect of purified endothelial cell membranes on the rate of complex formation. Membranes prepared from unfixed endothelial cells were added in increasing concentrations to reaction mixtures of PN-1 and ^{125}I-thrombin and incubated at 37°C for 2 min. As shown in Figure 2, the acceleration showed a dose-dependence on added membranes; at the highest concentration they accelerated the reaction approximately 7.5-fold. This result indicated that the accelerative activity was membrane-associated.

To further localize the accelerative activity of endothelial cells, we examined the ability of fixed cells and of ECM prepared from parallel cultures of unfixed cells to accelerate the inactivation of ^{125}I-thrombin by PN-1. Figure 3 shows that both preparations accelerated the reaction about two- to threefold. As with the acceleration of thrombin-PN-1 complex formation by fibroblasts [7], this demonstrates that virtually all of the accelerative activity resided in the ECM.

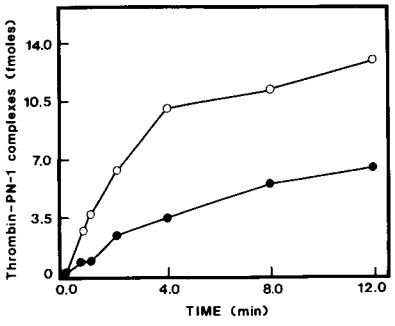

Fig. 1. Fixed bovine aortic endothelial cells accelerate the rate of ^{125}I-thrombin-PN-1 complex formation. Confluent cultures of endothelial cells were incubated for 24 hr in medium containing 0.4% serum, rinsed, and then fixed with 2% paraformaldehyde as described in Materials and Methods. The rate of formation of complexes between ^{125}I-thrombin and PN-1 was measured in the presence (○) and absence (●) of the fixed cells as described in Materials and Methods.

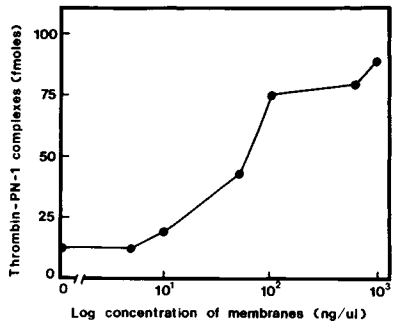

Fig. 2. Bovine aortic endothelial cell membranes accelerate the rate of ^{125}I-thrombin-PN-1 complex formation. Purified membranes from unfixed endothelial cells were added at the indicated concentrations to reaction mixtures containing ^{125}I-thrombin and PN-1; the rate of complex formation was determined as described in Materials and Methods.

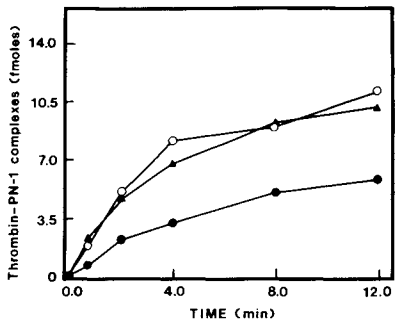

Fig. 3. Bovine aortic endothelial cell ECM accelerates the rate of ^{125}I-thrombin-PN-1 complex formation to the same extent as fixed endothelial cells. Confluent cultures of endothelial cells were incubated for 24 hr in medium containing 0.4% serum and either fixed with 2.0% paraformaldehyde or treated with sodium deoxycholate to prepare extracellular matrix (ECM) as described in Materials and Methods. The rate of formation of complexes between ^{125}I-thrombin and PN-1 was monitored in control dishes (●) and in the presence of fixed cells (○) or ECM (▲) as described in Materials and Methods.

Glycosidase Digestion of Endothelial Cell Membranes

Previous studies showed that the acceleration of thrombin–antithrombin III complex formation by endothelial cells [9,10] and of thrombin-PN-1 complex formation by fibroblasts [7,8] was mostly due to heparan sulfate. To determine whether heparan sulfate was also responsible for the accelerative activity of endothelial cells on thrombin-PN-1 complex formation, we incubated purified endothelial cell membranes with glycosidases which are specific for certain glycosaminoglycans and exmained the effect of this on the ability of the membranes to accelerate the formation of complexes. When endothelial cell membranes were digested with increasing concentrations of chondroitinase ABC, which specifically digests chrondroitin sulfates A, B, and C, there was little effect on complex formation (Fig. 4). In contrast, when endothelial cell membranes were treated with increasing concentrations of heparitinase, which specifically digests heparan sulfate, they lost about 85% of their ability to accelerate the inactivation of thrombin by PN-1 (Fig. 5). Thus, most of the accelerative activity was due to heparan sulfate.

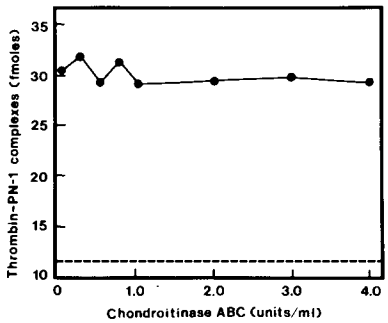

Fig. 4. Chondroitinase ABC does not inhibit the accelerative activity of endothelial cell membranes on ^{125}I-thrombin-PN-1 complex formation. Chondroitinase ABC was added at the indicated concentrations to purified membranes for 30 min at 37°C; the ability of the treated membranes to accelerate the rate of formation of complexes between ^{125}I-thrombin and PN-1 was then measured as described in Materials and Methods. As indicated by the dotted horizontal line, 12 fmol of thrombin-PN-1 complexes were formed in the absence of added membranes.

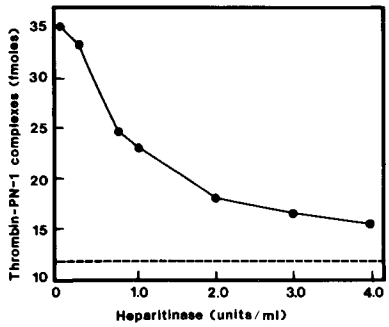

Fig. 5. Heparitinase inhibits the accelerative activity of endothelial cell membranes on ^{125}I-thrombin-PN-1 complex formation. Heparitinase was added at the indicated concentrations to purified membranes for 30 min at 37°C; the ability of the treated membranes to accelerate the rate of formation of complexes between ^{125}I-thrombin and PN-1 was then measured as described in Materials and Methods. As indicated by the dotted horizontal line, 12 fmol of thrombin-PN-1 complexes were formed in the absence of added membranes.

Ability of Endothelial Cells and Fibroblasts to Accelerate the Formation of Other Protease-Inhibitor Complexes

To further examine the role that endothelial cells and fibroblasts might play in accelerating the inactivation of proteases by protease inhibitors, we examined their effects on the reactions between heparin cofactor II and thrombin and between C1-inhibitor and C1s. Both of these reactions are accelerated by glycosaminoglycans [19,20]. The heparin cofactor II and thrombin reaction was chosen because heparin cofactor II, like PN-1, is an inhibitor of thrombin [19]. The C1-inhibitor and C1s reaction was chosen because C1-inhibitor recently was shown to have structural and functional similarities to PN-1 [21]. These reactions were measured as a function of time in the presence and absence of fixed endothelial cells or fixed fibroblasts. Panel A of Figure 6 shows that fibroblasts accelerated the heparin cofactor II and thrombin reaction about 1.5- to 2-fold; Panel B of Figure 6 shows that there was no detectable acceleration of the reaction by endothelial cells. Figure 7 shows that there was no

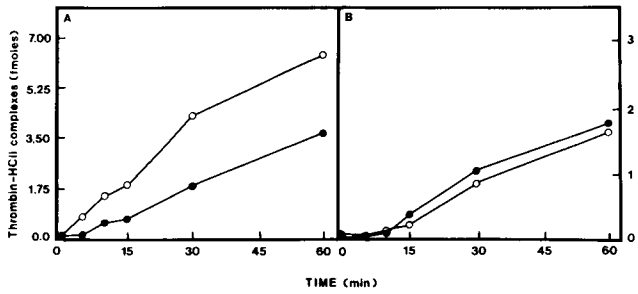

Fig. 6. Ability of fixed fibroblasts and endothelial cells to accelerate the rate of complex formation between ^{125}I-thrombin and heparin cofactor II. **A:** Confluent cultures of fibroblasts were incubated for 24 hr in serum-free medium and then fixed with 2.0% paraformaldehyde. **B:** Confluent cultures of endothelial cells were incubated for 24 hr in medium containing 0.4% serum, rinsed, and then fixed with 2.0% paraformaldehyde. The rate of formation of complexes between ^{125}I-thrombin and heparin cofactor II was then determined in the presence (○) and absence (●) of the fixed cells as described in Materials and Methods.

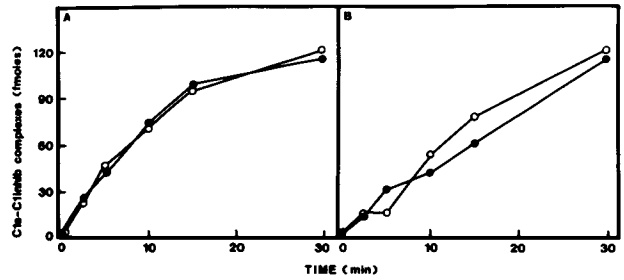

Fig. 7. Ability of fixed fibroblasts and endothelial cells to accelerate the rate of complex formation between ^{125}I-C1s and C1-inhibitor. **A:** Confluent cultures of fibroblasts were incubated for 24 hr in serum-free medium and then fixed with 2.0% paraformaldehyde. **B:** Confluent cultures of endothelial cells were incubated for 24 hr in medium containing 0.4% serum, rinsed, and then fixed with 2.0% paraformaldehyde. The rate of formation of complexes between ^{125}I-C1s and C1-inhibitor was then determined in the presence (○) and absence (●) of the fixed cells as described in Materials and Methods.

detectable acceleration of the reaction between C1-inhibitor and C1s by either endothelial cells or fibroblasts.

A summary of the ability of various cell types to accelerate the inactivation of thrombin by PN-1, antithrombin III, heparin cofactor II, and C1-inhibitor is presented in Table I.

DISCUSSION

It has been known for some time that the reactions between certain protease inhibitors and their target proteases are accelerated by glycosaminoglycans. In particular, the ability of heparin to accelerate the inactivation of thrombin by antithrombin III has been studied in much detail [22–24]. More recently, it has been realized that the surface of cells can also accelerate the reactions between certain protease inhibi-

TABLE I. Summary of the Ability of Cell Types to Accelerate the Inactivation of Thrombin by PN-1, Antithrombin III, Heparin Cofactor II, and C1-inhibitor

	Fibroblasts	Smooth muscle cells	Endothelial cells	Presumed polysaccharide involved in activation
PN-1	+ [7]	ND	+	Heparan sulfate [8]
Antithrombin III	− [7,11]	− [11]	+ [9,10]	Heparan sulfate [9,10]
Heparin cofactor II	+ [11,a]	+ [11]	− [11,a]	Dermatan sulfate [11]
C1-inhibitor	− a	ND	− a	−

+, acceleration observed; −, no acceleration observed; ND, not determined. The numbers in the table refer to the references from which this summary was prepared; "a" refers to this manuscript.

tors and proteases and that this activity is due to glycosaminoglycans. The first example identified was the ability of endothelial cells to accelerate the inactivation of thrombin by antithrombin III [25,26]. It has been shown that this is due to cell-surface heparan sulfate, a glycosaminoglycan that is closely related to heparin. Interestingly, it appears that fibroblasts do not accelerate the reaction between thrombin and antithrombin III even though their cell surfaces contain heparan sulfate [7,11]. While the present studies were in progress, it was reported that the inactivation of thrombin by heparin cofactor II is accelerated by fibroblasts and smooth muscle cells but not by endothelial cells [11]. The ability of fibroblasts to accelerate this reaction was dependent on cell-surface dermatan sulfate [11], consistent with earlier studies which showed that this glycosaminoglycan effectively accelerated the inactivation of thrombin by heparin cofactor II [27].

These results have suggested the possibility that the acceleration of reactions between certain protease inhibitors and their target proteases by the surfaces of cells may reflect the site of action of the inhibitors. This is particularly suggested in the ability of endothelial cells to accelerate the inactivation of thrombin by antithrombin III in view of the known functions of antithrombin III in plasma. Also, McGuire and Tollefsen suggested that the ability of fibroblasts and smooth muscle cells to accelerate the inactivation of thrombin by heparin cofactor II indicates that plasma heparin cofactor II may inhibit thrombin when plasma is exposed to these cells [11].

We recently showed that the surface of fibroblasts accelerates the inactivation of thrombin by PN-1 and that virtually all of this activity resides in the ECM [7]. About 80% of the activity was due to heparan sulfate [7,8]. These results are consistent with previous studies which indicated that the site of action of PN-1 is at or near the surface of cells in extravascular tissues [3,28]. This has been further supported by the finding that PN-1 is localized on the extracellular matrix of cultured fibroblasts [29]. Another point which argues for a role of PN-1 in the extravascular compartment is that PN-1 is present in only minute quantities in plasma compared to antithrombin III. The present results, however, indicate that endothelial cells accelerate the inactivation of thrombin by PN-1 and that they do this about as effectively as fibroblasts. As with fibroblasts, the activity is accounted for by the ECM and is mostly due to heparan sulfate. Although little PN-1 is present in plasma, it is noteworthy that Gronke et al recently found a molecule on the surface of platelets that appears identical to PN-1 except that it does not bind urokinase [30]. Thus, the endothelial surface might accelerate the inactivation of thrombin by platelet PN-1, a possibility that has not yet been tested.

It is clear that glycosaminoglycans account for most of the ability of cell surfaces to accelerate reactions between certain protease inhibitors and their target proteases. There are, however, key unanswered questions about the requirements for acceleration to occur. For example, heparan sulfate is present at the surface of both fibroblasts and endothelial cells, yet only the latter accelerate the inactivation of thrombin by antithrombin III. This could reflect differences in the structures of the heparan sulfates on these cells, the amounts present, or possibly differences in other cell-surface molecules.

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