Phorbol Ester and Mitogens Stimulate Human Fibroblast Secretions of Plasmin-activatable Plasminogen Activator and Protease Nexin, an Antiactivator/Antiplasmin

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ABSTRACT Tumor-promoting phorbol esters have been reported to greatly increase plasminogen activator (PA) activity produced in numerous cell types. Many of these studies have employed a widely used fibrinolysis assay for PA activity that involves large-scale dilution of cell lysates or conditioned medium (CM) into buffer containing plasminogen and the plasmin substrate ^{125}I-fibrin. This assay indicates that phorbol ester and the mitogens epidermal growth factor (EGF) and thrombin all stimulate secretion of PA activity in our human foreskin fibroblast cultures. However, these effects are not observed in a modified fibrinolysis assay employing undiluted conditioned culture medium unless the medium is first treated at pH 3, which inactivates the secreted protease inhibitor, protease nexin (PN). Moreover, a direct assay for plasminogen activator activity based on cleavage of ^{125}I-plasminogen indicates that conditioned culture medium contains little if any active plasminogen activator either before or after treatment of the cultures with phorbol ester or EGF. Phorbol ester and mitogens do stimulate secretion of (*a*) an inactive PA that can be activated by plasmin and (*b*) PN, which inhibits both the activated form of the PA and plasmin. Secretions of the inactive PA and PN are further correlated in that release of both is stimulated most by phorbol ester, somewhat less by EGF, and least by thrombin. Significantly, these effects are not accompanied by increases in total protein secretion. We propose that fibroblasts secrete PA in an inactive form in the presence of PN to confine PA activity to an as yet undefined location or event.

Numerous investigators have reported that treatment of cell cultures with tumor-promoting phorbol esters or growth factors greatly increases cellular production of plasminogen activator (PA)[1] activity (1–7). These findings are intriguing because elevated PA activity produced by malignant cells contributes to several aberrant properties of these cells, including altered morphology (8) and the abilities to hydrolyze fibrin clots (9) and grow in soft agar (10). Recently, a widely used PA assay that has been employed in a number of the phorbol ester/growth factor studies has been shown to artifactually activate latent PA in culture medium conditioned by several different cell types (11, 12). This assay involves dilution of cell-conditioned medium or cell lysates into ^{125}I-fibrin-coated wells containing Tris·Cl buffer and plasminogen. PA activity is measured by the extent of plasmin-mediated hydrolysis of ^{125}I-fibrin. Unfortunately, small amounts of plasmin, which can contaminate plasminogen preparations, activate inactive PA secreted by several types of cells (12–14). The absence of NaCl in the assay buffer also apparently destabilizes inactive secreted PA (11). Furthermore, dilution of conditioned medium or cell lysates reduces the concentrations of fibrinolytic inhibitors that may be present. These problems should prompt reexamination of PA activity in a number of systems.

The present manuscript describes the influence of phorbol ester and growth factors on fibrinolytic regulation by normal human foreskin fibroblasts (HF cells). We have recently shown that in unsupplemented serum-free culture these cells impose two kinds of negative control on secreted PA (14). First, although HF cells release PA, it is in an inactive form. This material is similar or identical to a proenzyme form of the PA urokinase that has recently been purified from human

[1] *Abbreviations used in this paper:* CM, conditioned medium; EGF, epidermal growth factor; HF, human foreskin fibroblasts; PA, plasminogen activator; and PN, protease nexin.

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tumor cell-conditioned medium (12). Both the urokinase proenzyme and HF cell PA are activated by plasmin, and are artifactually activated in the standard ^{125}I-fibrin well assay. Second, HF cells also secrete the protease inhibitor, protease nexin (PN) (15), which inhibits activated HF cell plasminogen activator. Below we present evidence that neither phorbol ester nor growth factors stimulate accumulation of active PA in HF cell culture medium. However, these agents stimulate cellular release of both the plasmin-activatable PA and PN.

MATERIALS AND METHODS

Proteins: Proteins were obtained from the following sources: bovine serum albumin (BSA) (Cohn fraction V), gelatin, and soybean trypsin inhibitor from Sigma Chemical Co. (St. Louis, MO), fibrinogen from Calbiochem-Behring (La Jolla, CA); pancreatic elastase, chymotrypsin, trypsin, and pancreatic trypsin inhibitor from Worthington Biochemical Co. (Freehold, NJ); urokinase from Collaborative Research (Lexington, MA); mouse epidermal growth factor (EGF) was a gift from Patricia Comens (University of Kansas); human α-thrombin originally purified in the laboratory of Roger Lundblad (University of North Carolina) was a gift from Louis Houston (University of Kansas); plasminogen was purified from outdated human plasma by the procedure of Deutsch and Mertz (16); plasmin was prepared as described by Danø and Reich (17) and was stored at 5°C in the presence of 50% glycerol. Human fibroblast PN (50,000 daltons) was purified from serum-free microcarrier culture conditioned medium by heparin-Sepharose chromatography. The purification and characterization of PN will be presented later.

Cell Culture: Human foreskin fibroblasts (HF cells) were grown in Dulbecco's modified Eagle's medium (DME) containing antibiotics and 5% calf serum (M. A. Bioproducts, Walkersville, MA) as previously described (18). Quiescent cultures were prepared for experiments by seeding 2×10^{-5} cells into 35-mm diameter culture dishes (Falcon Plastics, Oxnard, CA) in 2 ml of the above serum-containing medium and allowing the cells to grow to confluence over 4–5 d. The confluent cultures were rinsed, and incubated with DME containing 0.01% BSA ("serum-free medium"). Phorbol myristate acetate (Sigma Chemical Co.) and growth factors were diluted in DME and filter-sterilized before addition to cultures.

Experimental Procedures: Plasmin, plasminogen, and thrombin were radioiodinated by incubation of 10 μg of protein with 0.3 mCi of Na ^{125}I (Amersham, Arlington Heights, IL) in the presence of chloroglycoluril (Pierce, Rockford, IL) and separated from unreacted ^{125}I as previously described (15). Fibrinogen was iodinated as above, except that it was incubated in the iodination reaction mixture at 1 mg/ml. Radioiodinated plasmin and plasminogen were stored at −80°C in the presence of 50% glycerol.

Fibrinolysis assay of plasminogen activator (PA) activity in Tris-Cl buffer was carried out as described by Loskutoff and Edgington (19). Assay of PA activity by cleavage of ^{125}I-plasminogen was carried out by the method of Danø and Reich (17) using modifications as described in Fig. 3.

To measure the concentration of PN in conditioned culture medium, we assumed that PN and thrombin are present in equimolar amounts in thrombin-PN complexes (15), and determined the amount of ^{125}I-thrombin that was incorporated into SDS-stable 72-kdalton complexes when the labeled protease (0.2 μg/ml) was incubated with medium samples for 1 h at 37°C. ^{125}I-thrombin in the complexes was resolved by SDS PAGE and quantitated in a gamma scintillation counter. SDS PAGE was carried out in 7% polyacrylamide gels according to the method of Weber and Osborn (20). Collagen secretion was assayed as described by Peterkofsky and Diegelmann (21).

RESULTS

Consistent with observations made on a variety of other cell types, incubation of HF cells with phorbol ester dramatically increased cellular production of PA activity as measured by the standard fibrinolysis assay of PA (Fig. 1*A*). In the assay, 50 μl of serum-free cell-conditioned culture medium (CM) was incubated with plasminogen in 1 ml Tris·Cl buffer inside ^{125}I-fibrin-coated plastic wells. CM from cultures treated for 1 d with phorbol myristate acetate at 10 ng/ml caused nearly complete hydrolysis of ^{125}I-fibrin in the wells in 2 h, whereas only 5% of the ^{125}I-fibrin was hydrolyzed in wells containing CM from untreated control cultures. In both cases fibrinolysis was mediated by cell-released PA activity because CM incubated without plasminogen was inactive (data not shown).

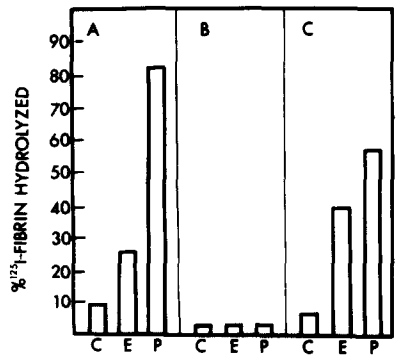

FIGURE 1 Fibrinolytic activity of HF cell-conditioned media incubated with plasminogen under various conditions. Quiescent cultures of HF cells were incubated for 24 h with no supplement (*C*), EGF at 10 ng/ml (*E*), or phorbol myristate acetate at 10 ng/ml (*P*), and the conditioned culture media were assayed for plasminogen activator activity by three different protocols. In all cases the media were incubated in ^{125}I-fibrin-coated wells containing plasminogen at 5 μg/ml. Plasminogen was added to each well in 50 μl of 0.1 M Tris-Cl buffer, pH 8.1. (*A*) the media were diluted 20-fold in 0.1 M Tris-Cl buffer (pH 8.1) containing 0.1% gelatin, and 1-ml volumes of these mixtures were added to the wells. (*B*) 1-ml volumes of undiluted media were added to the wells. (*C*) the media were preincubated at pH 3 for 2 h on ice. After raising the pH to 7.5, the assay was carried out as described for *B*.

Fig. 1*A* shows that, in the above assay, CM from cultures incubated for 1 d with EGF also exhibited greatly increased PA activity, although less than that caused by phorbol ester treatment. This stimulation is in accord with the finding that EGF stimulated secretion of PA by 3T3 cells (22). These increases in apparent PA activity in HF cultures did not reflect increases in cell mass or number in phorbol ester- or EGF-treated cultures. Those parameters increase significantly only after 2–3 d of continuous treatment (reference 18, and our unpublished data).

The effects of phorbol ester and EGF on PA activity were measured using a modification of the above assay. Plasminogen was added to undiluted CM, and this mixture was incubated in the ^{125}I-fibrin-coated wells. In this case, CM from control, EGF-, and phorbol ester-treated cultures all contained similar, barely detectable levels of PA activity (Fig. 1*B*). Although the observed fibrinolysis was significantly above background, this represented far less PA activity than was present in even control CM in the standard fibrinolysis assay. Note that the latter assay involved a 20-fold dilution of secreted PA and only a 2-h fibrinolysis incubation time (Fig. 1*A*), yet in all cases it detected greater fibrinolysis than occurred over 16 h in the assay employing undiluted CM (Fig. 1*B*). The decreased fibrinolytic activity observed in the assay employing undiluted CM was not surprising in view of the presence of NaCl in CM (11). However, this did not explain why there was not more PA activity in undiluted CM from phorbol ester- or EGF-treated cultures than in undiluted CM from untreated cultures. Because HF cells secrete the PA inhibitor, PN (14), the lack of difference between undiluted CM could be caused by elevated levels of PN in CM from phorbol ester or EGF-treated cultures.

Stimulation of PN Secretion by Phorbol Ester and Growth Factors

PN is inactivated by incubation at pH3 (14). To check whether the similarly low levels of fibrinolysis-stimulating

activity in CM from control, phorbol ester- and EGF-treated cultures could have resulted from differential cellular secretion of PN, we acid-treated CM and then measured PA activity in undiluted CM samples. As shown in Fig. 1 *C*, acid treatment increased fibrinolysis stimulated by undiluted CM from both control and treated cultures. Furthermore, acid-treated CM from cultures incubated with phorbol ester exhibited far more PA activity than acid-treated CM from untreated cultures. Acid-treated CM from cultures incubated with EGF stimulated an intermediate level of fibrinolytic activity. The relationship between these activities is approximately the same as when the CM samples were diluted 20-fold in the standard fibrinolysis assay (compare Fig. 1, *A* and *C*).

Because the above data suggest that phorbol ester and EGF may stimulate PN secretion, we measured PN levels in CM treated with these agents. Additionally, we checked CM from cultures incubated with thrombin, another mitogen for HF cells (23). The amount of PN in CM samples was measured by incubating ^{125}I-thrombin with CM for 1 h at 37°C, using SDS PAGE to resolve the SDS-stable ^{125}I-thrombin-PN complexes, and quantitating the complexes in a gamma counter. By this criterion, PN was present at 20–25 ng/ml in CM from control cultures, up to 35 ng/ml in CM from thrombin-treated cultures, up to 65 ng/ml in EGF-treated cultures, and up to 150 ng/ml in phorbol ester-treated cultures (Fig. 2, *A–C*).

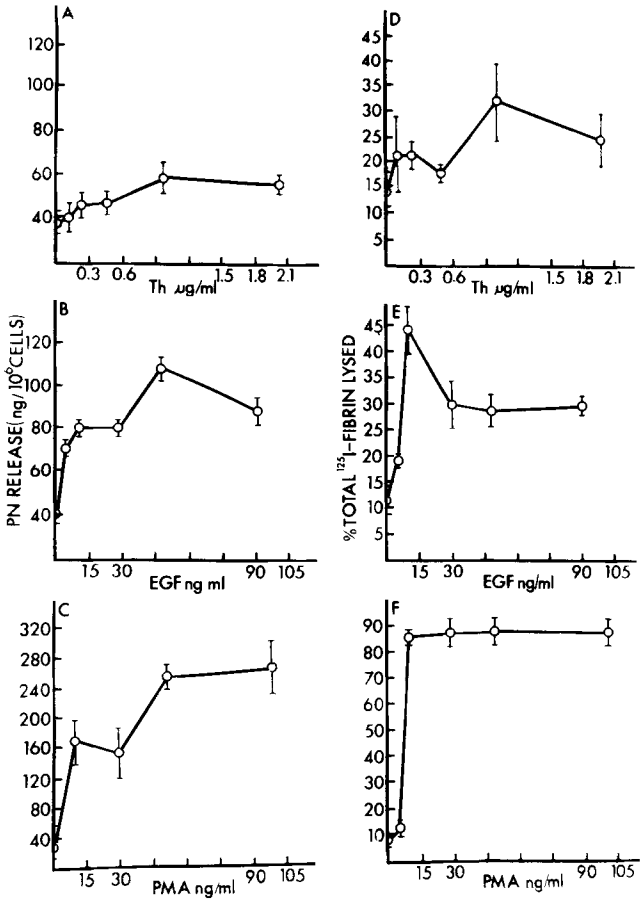

FIGURE 2 Effects of phorbol ester, EGF, and thrombin on the cellular release of PN and plasminogen activator. Quiescent cultures of HF cells were incubated with phorbol myristate acetate (*PMA*), EGF, or thrombin (*Th*) at the concentrations shown for 24 h. PN and plasminogen activator in the culture media were then monitored, except in the case of cultures treated with thrombin. The medium in the latter cultures was aspirated. The cultures were rinsed five times and incubated for 24 h with serum-free medium, which was then monitored for PN and plasminogen activator. In all cases, plasminogen activator activity was measured using the conventional fibrinolysis assay described in the legend for Fig. 1*A*. Cell number was measured with a Coulter Counter. The bars represent the range between duplicate determinations.

Samples of the CM assayed for PN were diluted into the standard fibrinolysis well assay for PA activity. As shown in Fig. 1, this assay indicated that CM from cultures treated with phorbol ester or EGF contained greatly increased levels of PA activity relative to control cultures (Fig. 2, *E* and *F*). CM from cultures incubated with thrombin also exhibited increased PA activity (Fig. 2*D*). Although the fibrinolysis assay is only semiquantitative, comparison of the effects shown in Fig. 2 indicates that the secretions of PA and PN were correlated in several ways. Each of the tested factors that stimulated PA release also stimulated PN release. For each factor, stimulation of both PA and PN occurred over approximately the same range of factor doses. Thrombin significantly stimulated PA and PN secretion at doses of 0.5 μg/ml or greater, whereas phorbol ester and EGF stimulated PA and PN secretion at doses >10 ng/ml and 1 ng/ml, respectively. (The implication of Fig. 2, *C* and *F*, that more phorbol ester was required for maximal PN release than for maximal PA release is misleading. Fig. 2*F* does not portray an accurate dose-response curve for stimulation of PA release because CM from phorbol ester-treated cultures caused nearly complete fibrinolysis at all but the lowest phorbol ester dose.) Furthermore, the rank order of potency of these factors for stimulating PN secretion (phorbol ester > EGF > thrombin) paralleled their order of potency for stimulating PA secretion.[2] Increased secretions of PA and PN were not reflections of a general increase in protein accumulation in CM of phorbol ester or growth factor-treated cultures. For example, total protein concentration in serum-free medium of EGF-treated and control cultures after 24 h was 12 ± 3 μg/ml and 14 ± 4 μg/ml, respectively, according to the Bradford protein assay (24). Additionally, the amount of collagen in 24-h CM from growth factor-treated and control cultures did not vary by >20% (data not presented).

Thrombin, EGF, and phorbol ester all perturb cell growth regulation (23, 25, 26), indicating that PN secretion is sensitive to the growth state of the cells. In addition to being a mitogen, thrombin is also a serine protease, suggesting that cells might regulate PN secretion in response to the presence of serine proteases. However, Table I shows that, unlike thrombin, neither urokinase, trypsin, chymotrypsin, nor elastase stimulated PN secretion. Both urokinase and trypsin bind PN (15, 27). Significantly, of all the proteases examined, only thrombin is mitogenic for HF cells (reference 23 and our unpublished data). Thus, PN secretion was not stimulated in response to the presence of extracellular protease activity per se, consistent with the possibility that the stimulatory effect of thrombin is accounted for by its mitogenic activity.

[2] In Fig. 2, after EGF or phorbol ester treatment the assays for PN and PA were carried out on the CM containing these factors, whereas after thrombin treatment the assays were carried out on thrombin-free medium conditioned by cultures that had been pretreated with thrombin. Assay of CM containing thrombin was not done because thrombin is fibrinolytic. The results shown in Fig. 2 were essentially unchanged when a parallel pretreatment protocol was used for all three agents (data not presented).

Phorbol Ester and EGF Stimulate Secretion of Inactive PA That Is Activated by Plasmin

Because the standard fibrinolysis assay can cause activation of inactive PAs (11, 12, 14), PA activity in CM was assayed directly by measuring conversion of ^{125}I-plasminogen to ^{125}I-plasmin in the presence of plasmin inhibitors. This assay did not detect PA activity in CM from untreated cultures, or CM from cultures treated with phorbol ester or EGF (Fig. 3). In view of our previous finding that CM from untreated HF cell cultures contains inactive PA that is activated by plasmin (14), we measured PA activity in CM from phorbol ester or EGF-treated cultures when these media were pretreated with plasmin. Fig. 3 shows that CM from unsupplemented HF cell cultures contained detectable plasmin-activatable PA, as previously described. This material was present at an increased concentration in CM from EGF-treated cultures, and at an even greater level in CM from phorbol ester-treated cultures. Thus, phorbol ester and EGF increased the secretion of the plasmin-activatable, inactive PA that was also released in untreated cultures. When CM not pretreated with plasmin was incubated with ^{125}I-plasminogen at 37°C for prolonged periods (24 h or longer), small but detectable amounts of ^{125}I-plasmin were occasionally generated and this activity was greatest in CM taken from cultures treated with phorbol ester or EGF (data not presented). It is uncertain whether these results were caused by very low levels of PA activity that perhaps actually exist in CM or were caused by slow spontaneous activation of inactive PA during the assay incubations.

TABLE I

Effect of Proteases on Cellular Release of PN

Treatment	PN ng/10^6 cells	% Control
None	49.5	100
Thrombin	76.0	153
Urokinase	32.1	65
Elastase	52.2	105
Chymotrypsin	31.2	63
Trypsin	47.7	96

Quiescent cultures of HF cells were incubated with the indicated proteases at 1 μg/ml, or, in the case of trypsin, 0.4 μg/ml. After 24 h, the culture medium was aspirated. The cultures were rinsed five times and incubated with serum-free medium for 24 h. The concentration of PN in this medium was measured. Cell number, determined with a Coulter Counter, differed by <15% in all cultures.

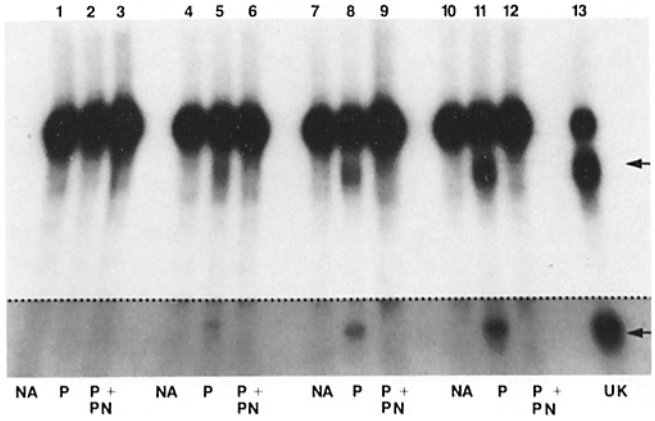

FIGURE 3 Effects of plasmin and PN on plasminogen activator secreted by HF cells treated with phorbol ester or EGF. Quiescent HF cells were incubated for 48 h with no supplement, EGF (10 ng/ml), or phorbol myristate acetate (10 ng/ml). The culture media were acid-treated as described for Fig. 1C, and concentrated tenfold by ultrafiltration through an Amicon PM10 membrane. The media were diluted tenfold with 0.1 M Tris-Cl buffer (pH 8.1) and again concentrated tenfold. Aliquots of these mixtures or the Tris-Cl buffer were incubated for 20 min at 37°C, either with no addition (*NA*) or with plasmin at 0.2 μg/ml (*P*). Then, bovine pancreatic trypsin inhibitor (0.125 mg/ml), soybean trypsin inhibitor (0.1 mg/ml), and ^{125}I-plasminogen (0.2 mg/ml) were added with or without *PN* (1.0 μg/ml). After 10 h at 37°C the mixtures were solubilized in SDS sample buffer and heated in a boiling water both for 5 min. SDS PAGE was carried out by the method of Laemmli (37) in a gel containing a 5–20% linear gradient of polyacrylamide, and an autoradiogram was prepared. Lanes *1–3*: buffer; lanes *4–6*: conditioned medium from untreated cultures; lanes *7–9*: conditioned medium from cultures treated with EGF; lanes *10–12*: conditioned medium from cultures treated with phorbol ester; lane *13*: buffer plus urokinase (0.75 CTA U/ml). The arrows denote the 70- and 26-kdalton chains of plasmin. To increase the visibility of the 26-kdalton bands, during printing the photograph was developed twice as long in this region (below the dashed line).

Inhibition of Plasmin by PN

As shown in Fig. 3, and previously (14), PN inhibits the plasmin-activated HF cell PA. If PN also inhibited plasmin it could inhibit fibrinolysis not only at the final proteolytic step but also at the level of activation of PA. The effect of purified PN on the fibrinolytic activity of plasmin is shown in Fig. 4. In the absence of added PN, plasmin at a concentration of 1×10^{-8} M, as indicated by active site titration (28), hydrolyzed most of the ^{125}I-fibrin present in the assay. Purified PN inhibited fibrinolysis by almost 90% at only 0.10 μg/ml (2×10^{-9} M).[3]

Fig. 4 *inset* shows that ^{125}I-plasmin (50 ng/ml; 5.6×10^{-10} M) added to HF cell CM was taken into SDS-stable complexes with a medium component (lane *1*). These complexes did not form when CM was pretreated at pH 3 (lane *2*). Incubation of ^{125}I-plasmin with purified PN resulted in the formation of ^{125}I-plasmin-PN complexes that were the same molecular weight as the SDS-stable complexes formed in CM (lane *3*). Urokinase inhibited formation of complexes between ^{125}I-plasmin and PN (lane *4*), suggesting that plasmin and urokinase compete for the same binding site on PN.

DISCUSSION

The results presented above show that HF cells incubated with agents that perturb cell growth regulation secrete increased quantities of plasmin-activatable PA plus PN, an inhibitor of both HF cell PA and plasmin. Previous work suggests that this inactive PA is urokinase proenzyme (14), and this has been supported by purification and preliminary characterization (Eaton and Baker, unpublished results). It

[3] The apparent inhibition of a molar excess of plasmin by PN is inconsistent with electrophoretic and other evidence that PN forms irreversible 1:1 complexes with serine proteases (14, 15). It is possible that the actual concentration of active plasmin present in the assay was less than predicted, either because of the extreme lability of plasmin (29) or because a fraction of plasmin that was reactive against the active site titrant was unreactive against fibrin. Moreover, the concentrations of PN were determined from a colorimetric assay (24) employing a serum albumin standard, and therefore rest on the assumption that PN and serum albumin bind similar amounts of dye per milligram protein.

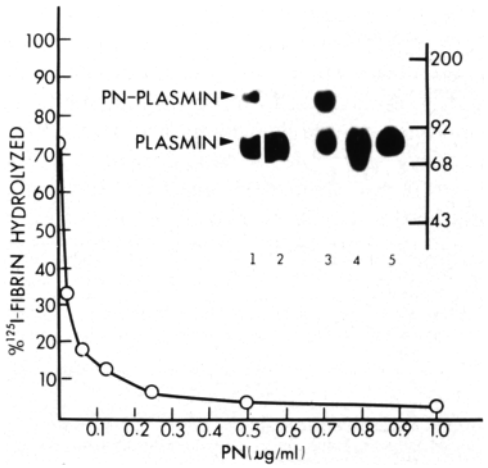

FIGURE 4 Inhibition of plasmin by protease nexin. Plasmin (1.1×10^{-8} M) was incubated for 30 min at 37°C alone or with PN at the indicated concentrations in 0.1 M Tris-Cl buffer (pH 8.1) containing 0.1% BSA. The reaction mixtures (1 ml) were then incubated in ^{125}I-fibrin-coated culture dishes for 4 h, and the radioactivity released into the buffer was measured in a gamma counter. The data show averages of duplicate determinations. *Inset:* ^{125}I-plasmin was incubated under the conditions described below and resolved by SDS PAGE (20) and autoradiography. Lane *1:* ^{125}I-plasmin (50 ng/ml) was incubated with 1 ml of medium conditioned by 48-h incubation with quiescent HF cells. Lane *2:* as in lane 1, except that the medium was pretreated at pH 3 as described in Fig. 1C. Lane *3:* the plasmin was incubated with 0.1 μg of purified PN in culture medium. Lane *4:* as in lane *3*, except that urokinase (1 μg/ml) was present in the incubation mixture. Lane *5:* ^{125}I-plasmin.

seems reasonable that secretion of HF cell PA as a proenzyme in the presence of PN (PA inhibitor) serves the same kind of regulatory function as does the coexistence of protease proenzymes with protease inhibitors in blood: to confine proteolytic activity to a particular location or event.

The precise function of urokinaselike PAs released by fibroblasts and many other normal cell types is unknown, but it is noteworthy that PA activity has been implicated in developmental processes that involve cell movement through tissues (30–32). The stimulation of HF cell PA proenzyme secretion by mitogens suggests that the PA plays some role in cell growth.

Whether the primary substrate of urokinase in tissues is plasmin or some other protein is unclear. Recently, Keski-Oja and Vaheri (33) showed that urokinase cleaves a 66-kdalton human fibroblast extracellular matrix protein, resulting in release of fibronectin from the matrix. Perhaps urokianse is protected from inhibition by PN when the protease is associated with a site on the extracellular matrix or cell surface. PN would then focus proteolytic activity at these locations by inhibiting both soluble active PA and plasmin generated by active PA. Growing cells would increase PN secretion in order to neutralize increased amounts of extracellular soluble PA.

The activation of HF cell PA by plasmin suggests that its physiologic mode of activation also involves proteolytic cleavage. As shown above, PN inhibits plasmin and so would limit this source of activation. Release of PA activity by SV 40 virus-transformed chick fibroblasts appears to involve the action of a chymotrypsin-like protease on the cell surface (34). It is possible that a PN-insensitive protease capable of activating HF cell PA is present on the surface of HF cells. On the other hand, our results have not provided any evidence that HF cell PA is activated in our cell culture system unless plasmin is present, so it is possible that the physiologic factors that activate this PA are not present in cell culture.

The present results raise questions about data from standard fibrinolysis assays that suggest that phorbol ester or mitogens increase cellular production of PA activity. In the case of HF cell CM, this apparent effect is not shown by other assays for PA activity. Whether HF cell CM from treated or untreated cultures contains any active PA is uncertain. The conventional fibrinolysis assay for PA involves dilution of CM into a buffer that facilitates activation of inactive PA (11, 12, 14) and as well decreases the concentration of PN. These effects provide a reasonable explanation for the indicated PA activity in HF cell CM and the apparently even greater PA activity in HF cell CM taken from cultures treated with phorbol ester or growth factors. Although dilution of CM dilutes the PA and PN equally, the PA (when activated) is a catalyst, so dilution only decreases its rate of reaction. PN, on the other hand, is a stoichiometric inhibitor (14) and its dilution decreases both the rate at which it reacts with proteases and the absolute amount of protease it can inhibit. In the fibrinolysis assay system this includes PA, plasmin generated during the fibrinolysis assay, and any plasmin present initially as a contaminant in the plasminogen added to the assay system. It has been proposed that initial plasmin contaminant activates urokinase proenzyme in the standard fibrinolysis assay for PA activity (12).

Several reports that phorbol ester or mitogen treatment stimulates cellular PA activity have been based solely on data from the standard fibrinolysis assay for PA activity (e.g., references 1 and 22). The present results suggest that these studies need to be reexamined. However, it is clear that phorbol ester treatment does increase production of PA activity by certain cell types (e.g., macrophages and Chinese hamster lung cells), at least when plaminogen is present (3, 6). In these cases, cells cultured on ^{125}I-fibrin clots in the presence of plasminogen exhibit greatly increased PA activity after phorbol ester treatment. This could reflect increased cell-associated PA activity and not increased PA activity in CM. It will be worthwhile to determine whether HF cells attached to ^{125}I-fibrin-coated substrate exhibit a dramatically increased capacity to promote plasmin-mediated fibrinolysis when these cells are treated with phorbol ester or growth factors. It is of interest that phorbol myristate acetate shifts the molecular weight and subcellular distribution of PA in 3T3 cells (35, 36).

The results presented here support the view that PN is a key negative regulator of fibrinolytic activity in the extracellular environment of human fibroblasts. PN is the major urokinase inhibitor secreted by HF cells, and therefore is the major inhibitor of activated HF cell PA (14). As shown above, PN also inhibits plasmin. Whether PN is the only significant plasmin inhibitor secreted by HF cells remains to be determined.

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