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Inhibition of tumor-cell-mediated extracellular matrix destruction by a fibroblast proteinase inhibitor, protease nexin I

(plasminogen activator/urokinase/growth inhibition)

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ABSTRACT **Human fibrosarcoma (HT-1080) cells, in contrast to normal fibroblasts, rapidly hydrolyze the glycoprotein, collagen, and elastin extracellular matrix (ECM) synthesized by cultured rat aortic smooth muscle cells. This degradation occurs at a rapid rate in the presence of serum, indicating that the cellular proteases responsible are relatively insensitive to serum proteinase inhibitors. Here it is shown that protease nexin I (PNI), a fibroblast-secreted inhibitor of urokinase, plasmin, and certain other serine proteinases, effectively inhibited the HT-1080 cell-mediated degradation of this ECM. PNI at 2.0 nM significantly inhibited matrix destruction for 1–2 days and at 0.2 μM caused a virtually complete inhibition that persisted for the entire 10-day period of observation. Inhibition of ECM destruction was accompanied by a transient arrest of HT-1080 cell proliferation that took place during the first 3 days after PNI addition. PNI did not inhibit the growth of normal fibroblasts and also did not inhibit the growth of HT-1080 cells that were seeded onto plastic dishes rather than onto ECM. Like many types of malignant cells, HT-1080 cells release large amounts of urokinase. Antibody against this plasminogen activator partially protected ECM from HT-1080 cell-mediated hydrolysis, indicating that it may have been a target of PNI. One potential physiological function of PNI could be to help maintain the integrity of connective tissue matrices, protection that malignant cells could overcome by secreting proteinases in excessive amounts.**

Fibroblasts and other connective tissue cells release into culture medium several novel serine protease inhibitors, called protease nexins (PNs), which effect the direct inhibition and cell-receptor-mediated clearance of protease targets (1). PNI is a relatively fast-acting inhibitor of trypsin, thrombin, plasmin, and urokinase (2, 3). Recent evidence from several sources strongly suggests that urokinase-mediated plasminogen activation may play a role in the invasive behavior of malignant cells. With few exceptions, malignant cells release urokinase in abnormally great amounts (4). Immunochemical staining of tissue sections has shown that urokinase is present in particularly high concentration at the invasive edges of tumors (5, 6). Ossowski and Reich (7) recently reported that anti-urokinase antibody inhibited the metastasis of human epidermoid carcinoma cells seeded onto chicken embryo chorioallantoic membranes. In view of the ability of PNI to inhibit urokinase and plasmin, the present investigation was undertaken to determine the effect of this inhibitor on tumor-cell-mediated destruction of extracellular matrix (ECM). Jones and DeClerk (8) have shown that a number of human tumor cells, but not normal fibroblasts, destroy the ECM elaborated by rat aortic smooth muscle (R22) cells. Study of the ECM destruction mediated by HT-1080 fibrosarcoma cells, the most destructive of the cells examined, indicated that the matrix degradation (*i*) involves all of the major components of the ECM (collagen, elastin, and glycoproteins), (*ii*) is largely plasminogen-dependent, and (*iii*) is relatively insensitive to a number of natural and synthetic proteinase inhibitors. Here we report that HT-1080 cell-mediated degradation of R22 cell ECM is effectively inhibited by PNI, which also suppresses the growth of HT-1080 cells on the ECM.

MATERIALS AND METHODS

PNI was purified as described by Scott *et al.* (3). Urokinase (Winthrop Laboratories, New York) was a gift from G. Murano (National Institutes of Health; Bethesda, MD). Elastin and trypsin used in the ECM degradation experiments were purchased from Worthington (trypsin code TRTPCK; elastase code ESFF). The trypsin was preincubated with bovine elastin (Sigma) to absorb possible contaminating elastase activity. Collagenase was purchased from Sigma (type VII). Serum depleted of plasminogen was obtained by passage over a lysine-Sepharose column as described by Deutsch and Mertz (9). Serum was obtained from Dutchland (Denver, PA). L-[4,5-^{3}H]Proline (32 Ci/mmol; 1 Ci = 37 GBq) was purchased from Research Products International (Mt. Prospect, IL). Antibody against 54-kDa human urokinase was raised in rabbits as described by Eaton *et al.* (2). IgG was purified by ammonium sulfate precipitation, and chromatography on Affi-Gel Blue (Bio-Rad) was according to the instructions of the manufacturer. The anti-urokinase IgG at 18 μg/ml inhibited 1 Committee on Thrombolytic Agents (CTA) unit of urokinase per ml by 50%. Rabbit anti-tissue-type plasminogen activator antibody (IgG) was a gift from R. R. Schleef and D. A. Loskutoff (Scripps Clinic and Research Foundation, La Jolla, CA). This antibody at 10 μg/ml inhibited 1 CTA unit of tissue-type plasminogen activator per ml by 50%. Normal human fibroblasts were isolated from explants of neonatal foreskins. HT-1080 fibrosarcoma cells were purchased from the American Type Culture Collection. Rat aortic smooth muscle cells (R22) were a gift from R. Kao (University of California, San Francisco).

Cell Culture and Assay of Extracellular Matrix Digestion. All culture media were supplemented with penicillin at 100 units/ml and streptomycin at 0.1 mg/ml. For stock cultures, 1–2 × 10^5 fibroblasts or HT-1080 cells were seeded into 100-mm-diameter culture dishes (Corning) and grown to confluence in Dulbecco-modified Eagle's medium (DMEM) containing 5% fetal calf serum. Cells were detached by incubation at 37°C in phosphate-buffered saline (0.15 M NaCl/10 nM phosphate, pH 7.4) that contained 0.05% trypsin (KC Biological, Lenexa, KS) and 1 mM EDTA. Radiolabeled ECM was prepared essentially as described by Jones and

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Abbreviations: ECM, extracellular matrix; PN, protease nexin; CTA units, Committee on Thrombolytic Agents.

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DeClerk (8). R22 cells were cultured in 24-well dishes (Costar, Cambridge, MA) in Eagle's minimal essential medium that contained 10% fetal calf serum, 2% tryptose phosphate and 0.5–1.0 μCi of [^{3}H]proline per ml. ECM synthesis was stimulated by the daily addition of 0.14 mM ascorbic acid for 1 wk, after which the cells were extracted with 25 mM NH_4OH. The extracted matrices were sterilized under an ultraviolet lamp and stored at 5°C until use. Fibroblasts or HT-1080 cells were seeded onto the ECMs in 1 ml of DMEM containing 10% fetal calf serum. In cases where IgG was to be added, the serum was preincubated for 30 min at 60°C to inactivate complement. ECM hydrolysis was monitored by assaying radioactivity in 50-μl aliquots of medium. The distribution of [^{3}H]proline in matrix glycoprotein, collagen, and elastin was determined as described by Jones and DeClerk (8). Briefly, ECMs were rinsed three times with phosphate-buffered saline and incubated sequentially with trypsin, elastase, and collagenase (all at 10 μg/ml in 0.1 M Tris/10 mM $CaCl_2$, pH 7.6) for 3-hr intervals at 37°C. The ECMs were rinsed three times between enzyme additions. Hourly samples were taken to monitor the rate of hydrolysis. Less than 15% of the ECM protein remained after enzymatic hydrolysis as determined by an overnight incubation with 2 M NaOH at 25°C.

Enzyme-Linked Immunosorbant Assay for Urokinase. Cells (2×10^5) were seeded onto 35-mm-diameter culture dishes that contained 2 ml of medium and 5% calf serum. After growth to confluence, the cultures were rinsed twice and incubated in serum-free medium that contained 0.1% bovine serum albumin. At the indicated times, the cells were counted with a Coulter Counter, and the proteins in the fibroblast-conditioned medium were concentrated 10-fold by ultrafiltration using an Amicon YM10 membrane. Nine hundred microliters of these conditioned media or fresh medium containing 52-kDa urokinase (at 1 ng to 1 μg) was mixed with 50 μl of phosphate-buffered saline that contained 0.3 μg of purified anti-urokinase antibody and was incubated for 2 hr at 37°C. The amount of free, unreacted antibody was then measured as follows. Urokinase at 0.4 μg/ml in 0.01% bovine serum albumin in phosphate-buffered saline was incubated in assay wells (Corning assay well strips) for 4 hr at room temperature on a rocker platform. The wells were rinsed twice with phosphate-buffered saline containing 0.05% Tween 20 and once with phosphate-buffered saline containing 0.05% Tween 20 and 0.01% bovine serum albumin and were incubated for 1 hr at 25°C with phosphate-buffered saline containing 3% bovine serum albumin (RIA grade; Sigma). This solution was aspirated, and 0.2 ml of the antibody and medium or antibody and urokinase mixtures was added and incubated overnight at 5°C. The wells were rinsed three times as described above, and bound antibody was measured by incubation with protein A-peroxidase conjugate (Zymed Laboratories, Burlingame, CA) and 4-aminoantipyrene. The concentration of urokinase antigen in the conditioned medium was determined by reference to the urokinase standard curve.

RESULTS

As reported by Jones and DeClerk (8), HT-1080 cells but not normal fibroblasts rapidly digested R22 cell ECM (Fig. 1 *Left*). The radioactivity released from ECM seeded with normal fibroblasts did not exceed the radioactivity spontaneously released from ECM that had not been seeded with cells. The radioactivity released from ECM seeded with HT-1080 cells was more than 10-fold above this background. HT-1080 cell-mediated matrix degradation was most rapid between days 1 and 2 of culture.

Because the culture medium used in these experiments contained 10% serum and because proteinase inhibitors cumulatively account for about 10% of the total protein content of serum (10), the capacity of HT-1080 cells to destroy ECM was evidently relatively resistant to serum proteinase inhibitory activity. In fact, pretreatment of the

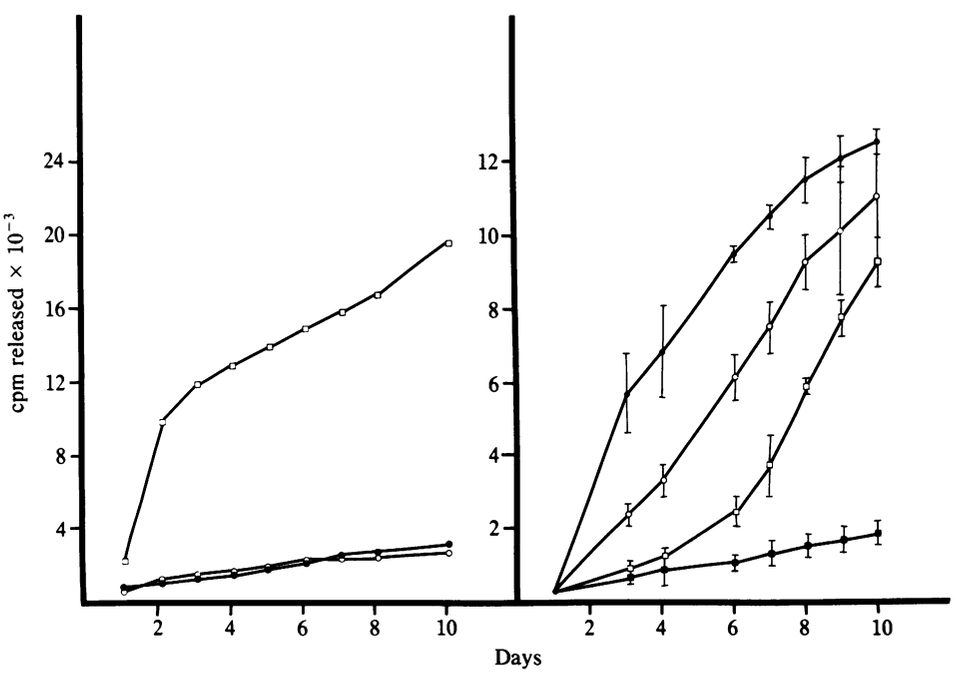

FIG. 1. Effect of PNI on HT-1080 cell-mediated extracellular matrix degradation. Cells were seeded onto [^{3}H]proline-labeled ECMs at 2.5×10^4 cells per cm^2. The radioactivity in aliquots of medium was measured after 24 hr (day 0) and on the succeeding days indicated. (*Left*) HT-1080 cells (□), normal fibroblasts (●), and control cultures (○) were incubated without cells. (*Right*) At day 0, HT-1080 cells were given either no supplement (●) or PNI, which was added daily at 0.1 μg/ml (○), 1.0 μg/ml (□), or 10 μg/ml (■). Medium was changed every 24 hr. The radioactivity at each time point represents the cumulative total for that day plus the previous days. The data shown are the mean values ± standard deviations calculated from triplicate data. Deviations were smaller than the symbols except where shown.

serum at pH 3, which inactivates several of the abundant plasma proteinase inhibitors (11), resulted in only a 30–40% increase in the rate at which HT-1080 cells destroyed the ECM (data not shown), a finding in agreement with earlier results of Jones and DeClerk (8).

Purified human fibroblast PNI added at 0.1 μg/ml (2.0 nM) reduced the rate that HT-1080 cells degraded ECM by about 50% during the first 2 days of incubation (Fig. 1 *Right*). After day 3, HT-1080 cells treated with PNI at this dose digested ECM as rapidly as untreated cells. Increasing the PNI dose to 1.0 μg/ml (20 nM) greatly suppressed ECM digestion for 5 days, but again the inhibition was subsequently overcome. At 10 μg/ml (0.2 μM), PNI effectively inhibited ECM destruction for the entire 10-day duration of the experiment.

About half of the radioactivity that the HT-1080 cells released from the ECM came from glycoproteins, with the remainder coming from collagen (≈30%) and elastin (≈20%), as judged by sequential removal of matrix radioactivity by trypsin, collagenase, and elastase, respectively. PNI protected all of these constituents (Table 1). Because PNI is specific for serine proteinases that cleave at lysine or arginine residues, its protection of elastin and collagen was probably not caused by direct inhibition of elastase or collagenase (see *Discussion*).

Inhibition of Cell Proliferation. Fig. 2 *Left* shows that PNI rapidly but transiently inhibited the growth of HT-1080 cells on ECM. Cell counts made 3–5 days after addition of PNI showed that cultures treated with PNI contained significantly fewer cells than did untreated cultures. Furthermore, cell counts made at earlier times revealed that PNI arrested the growth of HT-1080 cells within 24 hr after it was added (Fig. 2 *Left*).

Table 1. ECM components remaining after a 10-day exposure to HT-1080 cells

PNI, μg/ml	% control radioactivity remaining, mean ± SD		
	Trypsin^S	Elastase^S	Collagenase^S
0.0	14.6 ± 4.0	18.2 ± 8.9	28.7 ± 27.8
0.1	21.2 ± 0.1	26.9 ± 7.6	40.0 ± 3.7
1.0	34.3 ± 1.2	41.7 ± 1.1	53.7 ± 2.9
10.0	106.4 ± 19.3	112.1 ± 23.2	107.2 ± 19.9

On day 10 HT-1080 cells were removed from ECMs by incubation with 0.025 M NH_4OH for 15 min at 25°C, and the remaining radioactivity was measured as described. Control ECMs that had been incubated without HT-1080 cells were treated identically. The radioactivity removed after a 3-hr incubation with each protease is presented as a percentage of the radioactivity removed from control ECMs. Digests were done in triplicate wells except for 0.0 PNI which was done in duplicate. Trypsin^S, elastase^S, and collagenase^S, trypsin-, elastase-, and collagenase-sensitive.

Examination of the arrested cells by phase-contrast microscopy showed no evidence of increased cell death. In fact, cultures incubated with PNI contained fewer refractive, vacuolated, or detached HT-1080 cells than did untreated control cultures. Three to five days after PNI addition, the cells overcame the growth inhibition and began to proliferate as rapidly as did untreated cells (Fig. 2 *Left*). By day 10 cell numbers in control and treated cultures usually differed by <30% (data not shown).

The finding that PNI-treated cultures contained fewer HT-1080 cells than did control cultures probably accounts for part but not all of the ECM-sparing effect of PNI. Comparison

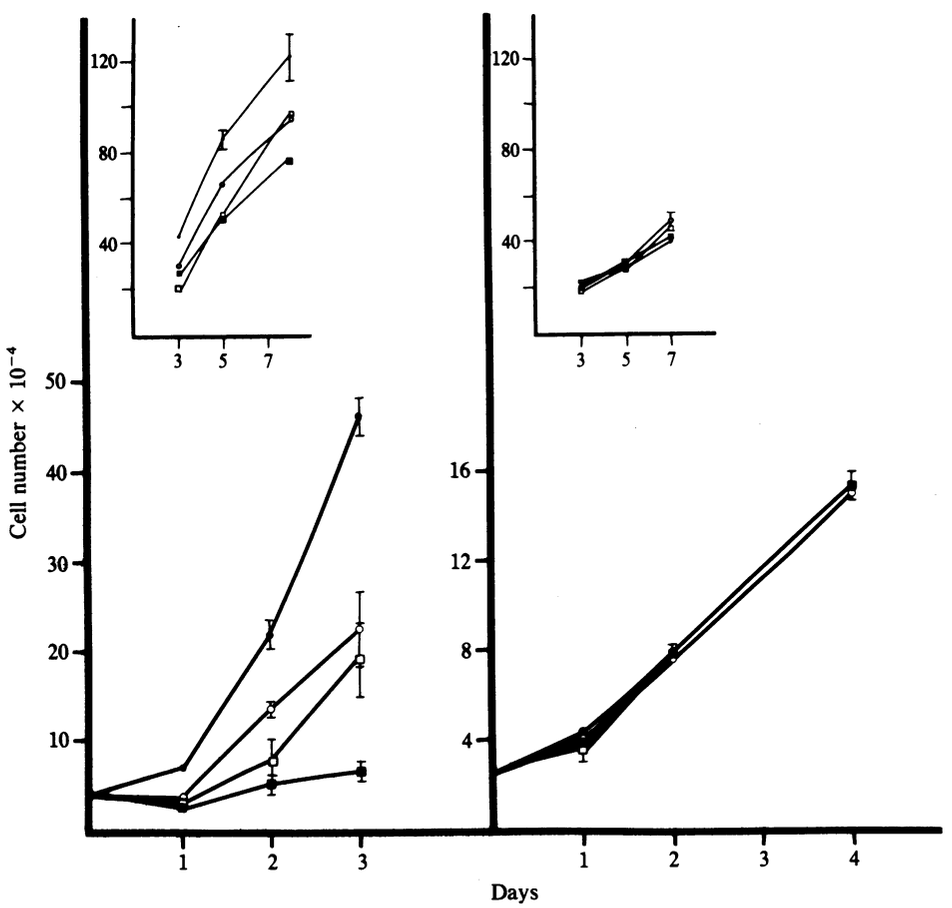

FIG. 2. Effect of PNI on the proliferation of cells on extracellular matrix. HT-1080 cells (*Left*) or normal fibroblasts (*Right*) seeded on ECM were incubated without PNI (●) or with PNI at 0.1 μg/ml (○), 1.0 μg/ml (□), or 10.0 μg/ml (■) as described in Fig. 1 *Right*. On the indicated days, the cells were detached from the wells and counted in an electronic particle counter (Coulter).

of Fig. 1 *Right* and Fig. 2 *Left* shows that PNI at 20 nM or 0.2 μM continued to cause pronounced inhibition of ECM destruction, even when the difference in cell numbers in treated and untreated cultures was less than 2-fold. Note also that untreated HT-1080 cells caused rapid ECM degradation between days 1 and 2, when there were still relatively few cells per culture.

PNI did not significantly influence the rate at which normal fibroblasts grew on the ECMs (Fig. 2 *Right*). Moreover, it did not inhibit the growth of HT-1080 cells that were seeded directly onto plastic culture dishes rather than onto ECM (Fig. 3). Thus, the antiproliferative action of PNI was restricted to HT-1080 cells seeded onto ECM.

Effect of Anti-Urokinase Antibody on Matrix Destruction. Enzyme-linked immunosorbant assays demonstrated that medium conditioned by HT-1080 cells contained great amounts of urokinase: about 40-fold more than medium conditioned by normal human fibroblasts (Table 2). Furthermore, HT-1080 cells released somewhat less PNI than did normal fibroblasts. Thus, whereas the ratio of secreted PNI to secreted urokinase was about 4 for the normal fibroblasts, it was about 0.07 for HT-1080 cells (Table 2). To test the possibility that this proteinase/proteinase inhibitor imbalance promoted the destruction of ECM by HT-1080 cells, anti-urokinase antibody (IgG) was incubated with HT-1080 cells seeded on ECM. This antibody caused a dramatic reduction in matrix degradation during the first 3 days of culture (Fig. 4 *Left*). Thereafter it was far less effective. The loss of effectiveness of the antibody cannot be interpreted as evidence for urokinase-independent matrix destruction after day 3 because a maximally effective dose of the antibody was not clearly reached. Note that the rate of matrix degradation from day 3 to day 6 (Fig. 4 *Left*) was clearly less when the antibody was added at 2 mg/ml than when it was added at 1 mg/ml. Antibody against tissue-type plasminogen activator did not detectably influence the rate of HT-1080 cell-mediated ECM destruction at any time interval (Fig. 4 *Right*).

Table 2. Amounts of urokinase antigen and PNI secreted by HT-1080 cells and normal fibroblasts

Cells	Incubation time, hr	Secretion, ng per 10^6 cells	
		PNI*	Urokinase†
Normal fibroblasts	24	—	5.5
	48	28.9	6.9
HT-1080 cells	24	19.3	139.0
	48	19.3	303.0

*Averages of duplicate measurements, determined by incubating ^{125}I-labeled thrombin with medium samples and measuring the amount of radioactivity that migrated at 77-kDa on NaDodSO$_4$ gels as described (12).

†The polyclonal anti-urokinase antibody used does not discriminate between urokinase and urokinase proenzyme. Recent experiments (D. Eaton, R. Gronke, and J.B.B., unpublished data) suggest that most or all of the urokinase released by HT-1080 cells is in the proenzyme form. Urokinase proenzyme is rapidly activated by plasmin at trace (subfibrinolytic) concentrations (13, 14).

DISCUSSION

The idea of using proteinase inhibitors to prevent the destruction and invasion of tissues by tumor cells is an old one (15). However, the nature of inhibitors that may be most effective is an open issue. The present results show that the fibroblast proteinase inhibitor PNI in low doses inhibited the digestion of vascular smooth muscle ECM by HT-1080 cells, an invasive line of human tumor cells. The potency of PNI is indicated by its action over a background of 10% serum. In cultures that

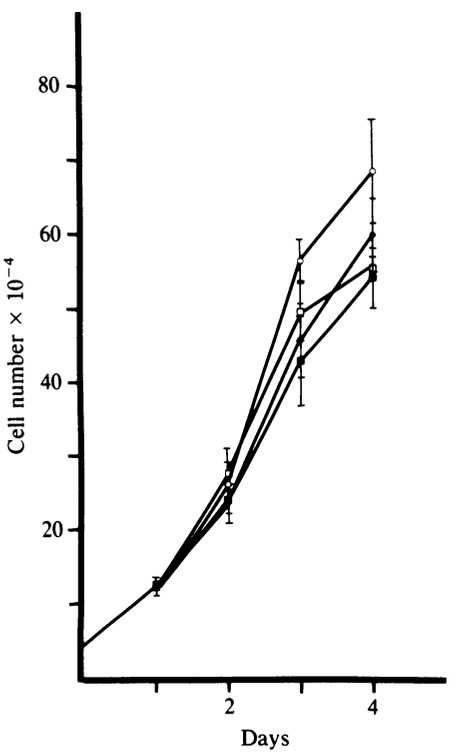

FIG. 3. Effect of PNI on the proliferation of HT-1080 cells seeded onto a plastic substratum. HT-1080 cells were seeded in plastic culture wells at 2.5 × 10^4 cells per cm^2. After 24 hr, the cells were rinsed and incubated with medium containing serum with no addition (●) or with PNI at 0.1 μg/ml (○), 1.0 μg/ml (□), or 10 μg/ml (■). The medium and PNI were replenished daily, and cells were counted on the days shown.

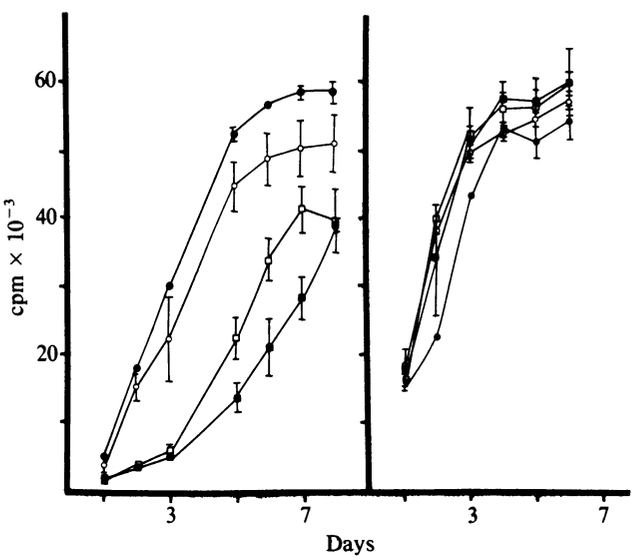

FIG. 4. Effects of anti-urokinase antibody (□, ■) or anti-tissue-type plasminogen activator antibody (○, ●) on the degradation of extracellular matrix by HT-1080 cells. Cells were seeded on ECM at 2 × 10^4 cells per cm^2 in medium containing 10% heat-inactivated fetal bovine serum. After 24 hr (on day 0) immune or nonimmune IgG was added, and every 48 hr the medium/IgG mixture was replaced with fresh medium/IgG. (*Left*) Anti-urokinase antibody was at 1 mg/ml (□) or 2 mg/ml (■); nonimmune IgG was at 1 mg/ml (○) or 2 mg/ml (●). The data shown are the mean values ± SD of triplicates. (*Right*) Anti-tissue-type plasminogen activator antibody was at 0.25 mg/ml (□) or 0.5 mg/ml (■); nonimmune IgG was at 0.25 mg/ml (○) or 0.5 mg/ml (●). The bars show the range of duplicate data. The range was smaller than the symbols except where shown.

contained serum proteinase inhibitors at a cumulative concentration of about 1 mg/ml, PNI significantly inhibited HT-1080 cell-mediated ECM degradation when added at 0.1 μg/ml (2 nM) and inhibited this degradation virtually completely at 10 μg/ml (0.2 μM). Jones and DeClerk (8) previously tested eight proteinase inhibitors for their effects on the HT-1080 cell-mediated degradation of the same (R22 cell) ECM used here. The most potent were the trypsin/plasmin inhibitors, soybean trypsin inhibitor and trasylol, each of which at 20 μM reduced by 50–60% the ECM degradation caused at 48 hr. In our studies PNI at 20 nM inhibited matrix destruction by ≈90% over this time period. Thus, the present findings suggest that PNI may have antiinvasive activity. Because it is secreted by fibroblasts and smooth muscle cells (Walter Laug, personal communication), it could function as a physiological inhibitor of ECM hydrolysis.

Several observations, taken together, indicate that HT-1080 cell urokinase may have been an important target of PNI in the above experiments. First, the HT-1080 cells secreted urokinase at a 40-fold faster rate than did normal fibroblasts. Second, antibody against urokinase but not against tissue-type plasminogen activator, significantly inhibited HT-1080 cell-mediated ECM destruction. Third, PNI is one of the fastest acting urokinase inhibitors that has been described to date (2). Fourth, serum is relatively deficient in urokinase inhibitory activity. Urokinase added to human serum at 80 ng/ml loses its activity with a half life of ≥2 hr (16), a finding that may help to explain the ability of HT-1080 cells to degrade ECM in the presence of serum. Implication of urokinase in the HT-1080 cell-mediated destruction of ECM is consistent with accumulating evidence that links urokinase to the invasive activity of tumor cells (5–7). Because PNI inhibits plasmin almost as rapidly as it inhibits urokinase (3), inhibition of plasmin might contribute to its protection of ECM. However, this may not be of major importance, as serum contains several effective plasmin inhibitors at micromolar or greater concentration (17, 18).

The evidence that plasminogen activator mediated HT-1080 cell degradation of ECM is consistent with the previously reported finding (8) that the rate at which HT-1080 cells destroy ECM is markedly reduced when plasminogen-depleted serum (prepared by passage through lysine-Sepharose) is substituted for whole serum in the culture medium. The basis for this effect could be complex, however. The relative capacity of lysine-Sepharose preadsorbed serum to support HT-1080 cell growth was not reported in the above study. In our hands HT-1080 cells multiply several fold more rapidly when the culture medium contains whole serum rather than lysine-Sepharose-preadsorbed serum (B.L.B. and J.B.B., unpublished data).

PNI protected ECM collagen and elastin, even though its protease specificity indicates that it is not an inhibitor of collagenase(s) or elastase(s) (3). Its collagen- and elastin-sparing effect may arise from a requirement that ECM glycoproteins (which PNI protects from plasmin-mediated hydrolysis) must be hydrolyzed before matrix collagen and elastin become accessible to collagenase(s) and elastase(s). That matrix glycoproteins protect matrix elastin and collagen from hydrolysis is suggested by the finding that HT-1080 cell-mediated hydrolysis of matrix collagen and elastin requires plasminogen unless matrix glycoproteins have been removed by trypsinization (8).

The inhibition of HT-1080 cell-mediated ECM degradation that was caused by anti-urokinase antibody or certain low doses (≤20 nM) of PNI lasted for only several days. Why inhibition was overcome in these cases is unclear, but there may have been multiple causes—e.g., accumulation of HT-1080 cell proteases bound to the ECM; eventual removal of matrix components that, although nonabundant, were important for matrix integrity; and eventual onset of plasminogen-independent degradation of matrix collagen and elastin. The possibility that cells began to release qualitatively different protease activities after several days on ECM also cannot be ruled out, but it is important to note that PNI at 0.2 μM was effective at all time points and that a maximally effective concentration of anti-urokinase antibody was not clearly reached.

In addition to inhibiting ECM destruction, PNI caused a pronounced, though transient, arrest of proliferation of HT-1080 cells seeded onto ECM. This effect only occurred under highly specific circumstances because PNI did not inhibit the growth of HT-1080 cells seeded onto plastic and did not inhibit the growth of fibroblasts. It is premature to speculate on the mechanism of this inhibition until a number of fundamental features are known—e.g., whether it requires the proteinase inhibitory activity of PNI, and whether it is also caused by anti-urokinase antibody. PNI could inhibit cell growth by inactivating a mitogenic proteinase, as has been shown previously with fibroblasts exposed to thrombin (19).

These effects of PNI on tumor cell interactions with ECM should prompt an examination of its effects in both *in vitro* and *in vivo* model systems for metastasis. Because entry into and departure from capillaries are critical steps in tumor colonization of distant sites (20), it will be of particular interest to determine whether PNI inhibits tumor cell destruction of endothelial cell basement membranes.

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