Cultured Rat Aortic Vascular Smooth Muscle Cells Digest Naturally Produced Extracellular Matrix

Involvement of Plasminogen-Dependent and Plasminogen-Independent Pathways

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Vascular smooth muscle (VSM) cell migration and proliferation play a major role in the development of atherosclerotic lesions, graft occlusion, and restenosis after angioplasty. Cell migration implies the digestion of the surrounding extracellular matrix. Cell-associated proteolysis has been extensively studied in neoplastic and inflammatory cells, but very little is known about the proteolytic properties of VSM. We have evaluated the ability of rat cultured VSM cells to solubilize [H]amino acid–labeled extracellular matrices produced by bovine VSM. When plated at a density of 30,000 cells per well in 24 multiwell plates, VSM cells were able to solubilize 63.3±7.0% of the extracellular matrix after 10 days in culture. Extracellular matrix digestion occurred also when the cells were cultured in plasminogen-depleted serum but was higher in the presence of 10 µg/ml purified plasminogen (net percent digestion after the subtraction of the appropriate control, 8.6±3.0% versus 21.2±3.5% after 3 days in culture, p<0.005, respectively). The involvement of other enzymes in addition to plasmin is confirmed by the ability of VSM cells to degrade extracellular matrices from which the plasmin-sensitive component was removed with plasmin pretreatment. Rat VSM cells were able to solubilize 52.3±2.0% of this residual extracellular matrix–associated radioactivity after 6 days in culture versus 26.1±1.5% in the control dishes (p<0.01, n=5). Cell contact was required for extracellular matrix degradation: cell-conditioned medium did not have any effect on extracellular matrix digestion. Similarly, no extracellular matrix digestion was observed when the cells were cultured on porous membranes suspended at 1 mm above the extracellular matrices. These experiments demonstrate that VSM cells can potently digest naturally produced extracellular matrices and that both plasminogen-dependent and plasminogen-independent mechanisms requiring cell contact are involved. A better knowledge of the fibrinolytic/proteolytic properties of VSM cells might contribute to the design of new strategies to prevent VSM cell migration from the intima to the media in the vascular wall. (Circulation Research 1992;71:385–392)

KEY WORDS • extracellular matrix degradation • vascular smooth muscle • plasminogen • proteolysis

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several extracellular matrix glycoproteins. It can also activate procollagenase and cooperate with elastase in the digestion of elastin. It is now established that plasminogen activation plays a central role in the extracellular matrix digestion by neoplastic and inflammatory cells, and its inhibition can prevent cell migration and metastasis in some in vitro models.

Recently, we have been able to demonstrate that VSM cells are able to synthesize in vitro and in vivo the enzymes necessary to modulate plasminogen activation. They express the mRNA for tissue-type plasminogen activator (t-PA) and its inhibitor, plasminogen activator inhibitor type 1 (PAI-1). PAI-1 was present in the cell-conditioned medium both in an active free form and in complex with t-PA. In this article we demonstrate that VSM cells are able to digest naturally produced extracellular matrices, solubilizing both plasmin-sensitive and plasmin-insensitive extracellular matrix proteins.

Materials and Methods

Cell Culture

Rat aortic VSM cells were obtained by enzymatic dissociation according to the method of Gunther and Gimbrone. These cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% neonatal calf serum. Passages 6–20 were used for the experiments.

Bovine smooth muscle cells were a kind gift of Dr. David Crossman, Clinical Research Centre, Harrow, Middlesex, UK. They were obtained from calf aorta with a tissue explant technique and cultured in the same medium supplemented with 10% fetal calf serum. Passages 10–25 were used.

M14 and BLM are two highly metastatic cell lines obtained from human melanoma metastasis. They were kindly provided by Dr. G.N.P. van Muijen, Department of Pathology, University Hospital, Nijmegen, The Netherlands. Both cell lines have been characterized for extracellular matrix digestion. They were cultured in DMEM with 10% fetal calf serum in a manner essentially identical to that used for VSM cells.

Plasminogen Depletion

Human serum was plasminogen-depleted by lysine-Sepharose affinity chromatography by following the method of Deutsch and Mertz. Aliquots of 40 ml human serum were eluted three times in a C 16/20 Pharmacia column containing 5 g lysine-Sepharose (bed volume, 8.3 ml) at a rate of 30 ml/hr. Lysine-bound plasminogen was displaced after each elution with 0.2 M e-aminocaproic acid. The eluate was continuously monitored in a UV spectrophotometer at a wavelength of 280 nm, and the plasminogen peak was no longer detectable after the second elution. The effectiveness of the procedure was confirmed by assessing plasminogen activity in the final eluate with a streptokinase activation chromogenic assay kit (Kabi, Uppsala, Sweden).

Production of Labeled Extracellular Matrices

Labeled extracellular matrices were prepared according to the methods of Jones et al. and Chapman et al. Briefly, bovine smooth muscle cells were plated in 24 multiwell cluster plates and grown for 7–9 days until they reached confluence. The cultures were then grown for 4 more days in DMEM containing 10% fetal calf serum supplemented with 1 μCi/mI [3H]amino acids with the medium changed every other day. The cells were then lysed with 0.5% Triton X-100 in phosphate buffer (pH 7.4) for approximately 10 minutes. The cytoskeletal elements and the nuclei were removed with a 5-minute incubation in 25 mM NH4OH. These steps were carefully monitored by phase-contrast microscopy. The dishes were washed twice with sterile distilled water, rinsed with 75% ethanol–25% water, dried at room temperature for 8–10 minutes, and stored at 20°C. Before being used for the experiments, the [3H]extracellular matrix–coated dishes were incubated in serum-free medium for 6 hours and then washed twice.

Composition of Extracellular Matrix

The composition of the extracellular matrix was estimated by enzymatic digestion as described by Jones et al. Digestion experiments were performed with the following enzymes: trypsin (type III, Sigma Chemical Co., St. Louis, Mo.), 20 μg/ml; elastase (type I from porcine pancreas, Sigma), 5 μg/ml; collagenase (type I from Clostridium difficilis, Sigma), 10 μg/ml; and plasmin (10 WHO units/mg protein, Sigma), 10 μg/ml. Elastase and collagenase were used in the presence of 10 μg/ml soybean trypsin inhibitor (Sigma). All enzymes were dissolved in Hank's balanced salt solution. Preliminary experiments determined that treatment for 2 hours at 37°C with the specified enzyme concentrations was sufficient to completely remove the enzyme-sensitive components from the extracellular matrices.

Thymidine Incorporation and Plating Efficiency

Rat aortic VSM cells were plated at a density of 10,000 cells per dish on extracellular matrix–coated 24 multiwell dishes in the presence or in the absence of 10 μg/ml plasminogen. After 24 hours, 1 μCi [3H]thymidine was added to the dishes. After another 24 hours, the dishes were washed twice with 0.5 ml of 0.9% saline, once with 0.5 ml of 10% trichloroacetic acid, and then again twice with 0.9% saline. The cells were dissolved in 0.4N NaOH and acidified with the proper amount of 1N HCl, and the radioactivity was counted in a liquid scintillation counter (model 1803 LS, Beckman Instruments, Inc., Fullerton, Calif.).

Cells prelabeled with [3H]thymidine were plated on dishes coated with nonradioactive extracellular matrix at a density of 20,000 cells per well. After 24 hours, the cultures were washed, dissolved, and counted as for thymidine incorporation.

Extracellular Matrix Digestion

Extracellular matrix digestion experiments were performed by plating the specified number of cells on dishes coated with radiolabeled extracellular matrices. Control wells were treated identically to the experimental ones, but no cells were plated. The medium was changed at the specified intervals, and the radioactivity that had been released into the supernatant was counted. At the end of the experiments, the extracellular matrix remaining in each well was dissolved with 0.4N NaOH, acidified with the appropriate amount of 1N HCl, and counted. Extracellular matrix digestion
was expressed as a percentage of the total amount of radioactivity initially present in each dish. The latter was defined as the radioactivity recovered in the supernatant plus the amount of radioactivity recovered in the dish at the end of the experiment.

Plasminogen reconstitution experiments were performed by adding 10 μg/ml purified plasminogen (P5661, purified by lysine-Sepharose affinity chromatography from human plasma and containing less than 0.0001 unit plasmin per unit plasminogen, Sigma) to extracellular matrices prepared from bovine smooth muscle cultures grown in DMEM supplemented with plasminogen-depleted human serum.

The ability of VSM cells to digest extracellular matrix components that are sensitive to proteinases other than plasmin was assessed by plating VSM cells on matrices from which the plasmin-sensitive component had been removed with plasmin digestion. These experiments were performed both in plasminogen-free conditions and in the presence of 10 μg/ml purified plasminogen. Plasmin contamination of plasmin-pretreated extracellular matrices was assessed by monitoring the cleavage of the chromogenic peptide S2251 (Kabi) at a wavelength of 405 nm. An aliquot of S2251 to a final concentration of 0.15 mg/ml was added directly to the extracellular matrix–coated dishes, which were treated and washed as previously described but in the absence of cells. The sensitivity of the method was assessed by adding a known amount of plasmin to extracellular matrix–coated dishes. An amount of plasmin ≥100 pg/ml was reliably detectable with this method.

A crude cell homogenate was prepared by scraping the cells from confluent 35-mm dishes, resuspending them in 0.5 ml of 10% neonatal calf serum medium, and homogenizing them in a glass homogenizer.

The relevance of cell contact in extracellular matrix digestion by VSM was evaluated by growing VSM cells on porous membranes (Transwell, 3-μm pore diameter, Costar Corp., Cambridge, Mass.) suspended at 1 mm above the labeled extracellular matrices. The radioactivity released in the supernatant was counted as previously described. Medium conditioned for 24 hours by rat VSM cell cultures was also used on extracellular matrix digestion experiments.

Statistical Analysis

Data are expressed as mean±SEM. Student’s t test for paired data was used for statistical analysis.

Results

Composition of Extracellular Matrix

Trypsin, elastase, and collagenase respectively solubilized 62.0±8.1%, 43.0±4.4%, and 21.0±3.7% (mean±SEM, n = 4) of the total extracellular matrix–associated radioactivity. The amount of extracellular matrix–associated radioactivity sensitive to plasmin was 74.4±1.0% of the total (mean±SEM, n = 4, Figure 1).

Extracellular Matrix Digestion

Rat aortic VSM cells can digest naturally produced extracellular matrix (Figure 2). When plated at a density of 30,000 cells per well, the cultures became confluent after approximately 3–4 days. As characteristic of

smooth muscle cells, these cultures continued growing to form a “hill and valley” pattern.

When 30,000 rat aortic VSM cells were plated on dishes coated with labeled matrix, the amount of radioactivity released in the supernatant was 64.3±5.8% (mean±SEM, n = 5) of total after 10 days in culture. Some extracellular matrix–associated radioactivity was nonspecifically released in the supernatant of the control dishes where only medium was added: 27.4±6.7% of total after 10 days in culture (mean±SEM, n = 5).

FIGURE 1. Bar graph showing the composition of extracellular matrices as determined by enzymatic digestion. [3H]Amino acid–labeled extracellular matrices produced by bovine aortic vascular smooth muscle cells were treated with one of the following enzymes for 2 hours at 37°C: plasmin (10 WHO units/ml protein), 10 μg/ml; trypsin (type III), 20 μg/ml; elastase (type I from porcine pancreas), 5 μg/ml; and collagenase (type I from Clostridium difficilis), 10 μg/ml. Total radioactivity was determined for each experiment as the released amount plus the amount recovered by dissolving the extracellular matrices with 0.4N NaOH at the end of the enzymatic digestions. Data are the mean±SEM of triplicates determined in four separate experiments.

FIGURE 2. Graph showing cumulative digestion of extracellular matrices by vascular smooth muscle (VSM) cells. Cells (30,000 per well) were plated on 24 multwell dishes coated with labeled extracellular matrices. The cells were grown in Dulbecco’s modified Eagle medium containing 10% neonatal calf serum. In the control dishes, only the medium was added. The medium was changed at various intervals, and the amount of radioactivity present in the supernatant was counted. The total radioactivity of each well was determined by adding the radioactivity recovered in the medium to the radioactivity recovered by dissolving the extracellular matrices with 0.4N NaOH at the end of the experiment. The data represent triplicate determinations (mean±SEM) in five separate experiments. The extracellular matrix digestion in the presence of cells was significantly higher than in the control wells at all time points (p<0.01, n = 5).
Matrix Plasminogen Dependency of Malignant Vascular Cells

The amount of extracellular matrix digestion in the presence of cells was significantly higher than the control value at all time points measured (p<0.01, n=5, Figure 2).

The digestion of extracellular matrices by VSM cells was also compared with that by two human melanoma cell lines (BLM and M14), selected for their metastatic and proteolytic properties. In parallel experiments, after 5 days in culture, rat aortic VSM cells had digested 54.0±0.5% (mean±SEM, n=3) of the total extracellular matrix-associated radioactivity. BLM and M14 cells had digested 86.0±1.0% and 82.3±1.0%, respectively (mean±SEM, n=3). A typical extracellular matrix digestion time course experiment for all three cell lines is shown in Figure 3.

In our experimental system, cell contact is an absolute requirement for extracellular matrix digestion. This is proved by the demonstration that 1) the medium conditioned by confluent rat aortic VSM cells did not increase the release of extracellular matrix-associated radioactivity into the supernatant (Figure 4) and 2) no difference in the release of radioactivity from the extracellular matrix was observed between the control wells and the Transwells containing the porous membranes with the VSM cultures (Figure 4).

To exclude the possibility that cell death with the release of neutral lysosomal proteinases could account for some extracellular matrix digestion, experiments were performed using a crude cell homogenate. A small increase in the amount of the release of extracellular matrix-associated counts was observed, but it was not statistically significant (Figure 4).

Plasminogen Dependency of Extracellular Matrix Digestion

To assess the role of plasminogen activation in extracellular matrix digestion by VSM cells, experiments were performed in the presence of plasminogen-depleted serum. To avoid the possible presence of plasminogen bound to extracellular matrix proteins, the extracellular matrices used in these experiments were produced by bovine smooth muscle cell cultures grown in the plasminogen-depleted serum. In these experiments, cells were plated at 60,000 cells per well, and the digestion was monitored daily for 3 days. These experiments demonstrate that some extracellular matrix digestion can occur in plasminogen-free conditions (Figure 5A). The amount of extracellular matrix-associated radioactivity released in the supernatant (percentage of total) is significantly higher than the control value at days 2 and 3: day 2, 23.3±3.5% versus 16.2±2.0%, respectively; day 3, 27.3±3.2% versus 18.7±2.4%, respectively (mean±SEM, p<0.01 for both, n=5).

Reconstitution experiments were also performed by adding a known amount of purified plasminogen to plasminogen-depleted cultures. Purified plasminogen preparations, however, can contain small amounts of plasmin that can release radioactivity from the extracellular matrices. Alternatively, plasminogen could be activated to plasmin by some plasminogen activator present in the extracellular matrix. In fact, the amount of radioactivity released from the extracellular matrices in the absence of cells was higher in the control wells in which plasminogen was added compared with the wells without plasminogen: day 1, 18.5±2.4% versus 11.5±1.2%, respectively, p<0.01; day 2, 23.4±3.2% versus 16.2±2.0%, respectively, p<0.01; day 3, 26.1±3.5% versus 18.7±2.4%, respectively, p<0.005 (mean±SEM, n=5). To take this into account, data were expressed as the net cell-associated extracellular matrix digestion (percentage of total), which was defined as the amount of radioactivity released in the presence of cells with or without plasminogen minus the amount of radioactivity present in the appropriate control condition, i.e., with or without plasminogen (Figure 5B).
**Figure 5.** Panel A: Graph showing digestion of extracellular matrices by vascular smooth muscle (VSM) cells in the absence of plasminogen. Cells (60,000) were plated in Dulbecco's modified Eagle medium supplemented with 10% human plasminogen–depleted serum. To exclude the presence of plasminogen bound to the extracellular matrix, these experiments were performed with extracellular matrix produced by cultures grown in medium supplemented with plasminogen-free human serum (mean±SEM, n=5). Panel B: Graph comparing net extracellular matrix digestion by VSM cells in the absence and in the presence of plasminogen. Net extracellular matrix digestion was defined as the amount of radioactivity released in the presence of cells minus the amount of radioactivity present in the appropriate control condition, i.e., in the presence or in the absence of plasminogen. Plasminogen-free extracellular matrices prepared as described in panel A were used. The medium with or without plasminogen was changed daily for 3 days. The net amount of cell-associated extracellular matrix digestion is higher in the presence of plasminogen at all time points (mean±SEM, n=5).

The addition of 10 μg/ml purified plasminogen to the cultures significantly increased the net cell-associated extracellular matrix digestion (percentage of total) at all time points (Figure 5B): day 1, 11.7±2.8% versus 2.7±0.9%, respectively, p<0.03; day 2, 15.38±3.1% versus 5.5±1.1%, respectively, p<0.02; day 3, 21.2±3.5% versus 8.6±3.0%, respectively, p<0.005 (mean±SEM, n=5). These differences are not due to an effect of plasminogen on plating efficiency or the growth rate. Plating efficiency was equally high both in the absence and in the presence of plasminogen: 95.3±0.4% and 93.1±1.6%, respectively (mean±SEM, n=4, p=NS). Thymidine incorporation was also not affected by plasminogen. The amount of [3H]thymidine incorporated in 24 hours in the absence and in the presence of plasminogen was 261,000±42,000 and 280,000±23,000 dpm per well, respectively (mean±SEM, n=5).

**Figure 6.** Graph showing digestion of plasmin-insensitive extracellular matrix components by vascular smooth muscle (VSM) cells and human malignant melanoma cell lines (BLM and M14). The labeled extracellular matrices were subjected to a pretreatment with 10 μg/ml purified plasmin to completely remove any plasmin-sensitive component from the matrices. All three cell types were plated at an initial density of 60,000 cells per well, and the extracellular matrix digestion was assessed as previously described in Figure 2. The residual plasmin-insensitive radioactivity was considered to be 100%. The extracellular matrix digestion in the presence of all three cell types was significantly higher than in the control wells at all time points (p<0.01, mean±SEM, n=5).

**Digestion of Plasmin-Pretreated Matrices**

Rat aortic VSM cells were able to digest extracellular matrices from which the plasmin-sensitive components had been previously removed (Figure 6). In this series of experiments, 22.6±1.0% of the initially extracellular matrix–associated radioactivity was not removed by plasmin treatment. After 6 days in culture, rat aortic VSM cells were able to digest 52.3±3.6% of this residual extracellular matrix–associated radioactivity. The amount of extracellular matrix radioactivity nonspecifically released in the supernatant was 26.1±2.0%. The difference between the extracellular matrix digestion in the VSM-containing dishes and the control dishes was highly significant (p<0.01, n=5) at all time points measured. Both tumor cell lines tested were able to digest a significant amount of plasmin-insensitive extracellular matrix (Figure 6). After 6 days in culture, the extracellular matrix digestion associated with the M14 cells was 53.0±1.2%, an amount very similar to that digested by the rat aortic VSM cells. The BLM cells were somewhat more potent, with a digestion of 67.9±3.1% at 6 days (Figure 6).

Plasmin can indirectly affect the digestion of substrates that are not directly sensitive to it, for instance, by cleaving and activating a different protease. For this reason, the digestion of plasmin-pretreated extracellular matrix was assessed in the presence and in the absence of plasminogen. Plasmin contamination of plasmin-pretreated extracellular matrix was assessed and found to be as low as 160±16 pg per well (n=3). Serum completely inhibited this residual plasmin activity: when plasmin contamination was assessed in the regular medium used in the extracellular matrix digestion experiments and containing 10% fetal calf serum, no activity was seen. In the absence of plasminogen, the
digestion of plasmin-pretreated extracellular matrices was significantly higher in the presence of cells at all time points measured: day 1, 36.0±1.4% versus 26.8±0.9%, respectively, p≤0.01; day 2, 48.5±2.2% versus 34.3±1.3%, respectively, p≤0.001; day 3, 57.0±2.1% versus 39.0±3.6%, respectively, p≤0.001; day 6, 72.8±1.8% versus 48.0±2.4%, respectively, p≤0.001 (mean±SEM, n=4) (Figure 7). The addition of plasminogen to the cultures did not further increase the amount of extracellular matrix digestion (Figure 7).

Discussion

This study demonstrates that cultured rat aortic VSM cells can potently digest both plasmin-sensitive and plasmin-insensitive components of naturally produced extracellular matrices.

A plasminogen-dependent component in extracellular matrix digestion by VSM cells is clearly demonstrated by the fact that the addition of plasminogen to plasminogen-depleted cultures significantly enhances extracellular matrix digestion. Several cell types are able to convert plasminogen to its active form plasmin by producing one or both of the two specific plasminogen activators, t-PA and urokinase-like plasminogen activator (u-PA). Both these activators can interact with their specific inhibitors, PAI-1 and plasminogen activator inhibitor type 2. Recently, we have shown that rat aortic VSM cells produce large amounts of PAI-1 both in vivo and in culture conditions identical to those of the extracellular matrix digestion experiments performed in this study. High levels of plasminogen activator inhibitor activity were present in the cell-conditioned medium, but no t-PA activity was detected. t-PA–PAI-1 complexes were detected by immunoblotting and fibrin autography. In the present series of experiments, the demonstration that VSM cell-associated extracellular matrix degradation is at least in part plasminogen dependent also implies that these cells are able to promote plasminogen activation in an environment in which the balance is overwhelmingly in favor of plasminogen activator inhibitor activity. Generally, plasminogen activation is not a reaction that occurs unchecked in the circulation; rather, it requires all the components to be bound or “anchored” to a substratum or to a cofactor. In the fibrinolytic cascade, the action of the inhibitors is partially escaped by the binding of plasmin to fibrin and by the enhancement of t-PA activity by its binding to fibrin. Similarly, in cell-associated proteolysis, the enzymatic activity is localized at the interface between the cells and the substrates. In many cell types, cell contact is either necessary or at least substantially enhances the degradation of the extracellular matrix substrates.

In the case of VSM cells, contact is an absolute requirement to digest the extracellular matrix components: VSM cell-conditioned medium was not effective in digesting extracellular matrix, and growing the VSM cells on porous membranes in proximity to (1 mm), but not in direct contact with, the extracellular matrix was not sufficient to achieve any extracellular matrix digestion. Specific binding of plasminogen to a variety of cells has been demonstrated, including monocytes/macrophages and endothelial cells. Receptor-bound plasminogen can be converted to plasmin by physiological plasminogen activators such as t-PA and u-PA.

This mechanism would provide a means of restricting plasmin proteolysis to a localized environment. Alternatively, plasmin generated from plasminogen in the soluble phase can bind to the same receptor with the same affinity as its zymogen. Recently, a specific receptor to which u-PA binds with high affinity has been purified and characterized. Receptor-bound u-PA is effective in activating plasminogen and, in particular, receptor-bound plasminogen. For this reason, it is believed to play a role in localizing proteolysis to the cell surface. Another function of the u-PA receptor has been postulated, namely the shielding of the u-PA molecule from PAI-1. According to recent evidence, however, it seems that this protection is, at most, partial. Specific high-affinity binding of active t-PA to the cell surface has also been described in several cell types, including endothelial and smooth muscle cells. The putative receptor for t-PA has not yet been characterized to the same extent as the u-PA receptor, but it appears to confer a high degree of protection from PAI-1. On the basis of our data, it is not possible to establish by which mechanism VSM cells activate plasminogen in our system. Because digestion requires contact, the activation of plasminogen by a plasminogen activator bound to the cell surface seems likely. The demonstration by fibrin autography of t-PA synthesis by these cells and the lack of evidence by this same technique for u-PA production make t-PA a more likely candidate.

VSM cells can solubilize a significant amount of extracellular matrix also in plasminogen-free conditions. All precautions were taken to exclude the presence of plasminogen from the experimental system. Because this enzyme binds to fibrin or extracellular matrix proteins, the bovine VSM cell cultures from which the labeled extracellular matrices were prepared were also grown in plasminogen-depleted serum. However, the presence of catalytic amounts of plasminogen
in the plasminogen-depleted serum or in the extracellular matrix or the production of small amounts of plasminogen by the rat VSM cells themselves cannot be excluded. For these reasons, extracellular matrices were predigested with purified plasmin to completely remove the plasmin-sensitive component and subsequently seeded with rat VSM cells. Also, in these conditions a significant amount of extracellular matrix digestion was observed, suggesting that proteases capable of digesting substrates other than those sensitive to plasmin are involved (Figure 6). Plasmin can indirectly be important in the proteolysis of substrates that are not directly sensitive to it. For instance, it can modulate collagen proteolysis by activating stromelysin and type IV procollagenase.21 Plasmin can also cooperate with elastases in the digestion of elastin.22,41 For these reasons, the role of plasminogen activation in the digestion of plasmin-sensitive substrates was also investigated. A significant amount of extracellular matrix degradation took place also in plasminogen-free conditions, supporting the existence of a truly plasmin-independent mechanism for extracellular matrix digestion in VSM cells (Figure 7). The extremely small amount of residual plasmin contamination in plasmin-predigested extracellular matrices is not likely to play a significant role, because it is inhibited by the fetal calf serum present in the culture medium. It cannot be excluded, however, that cell surface-bound plasmin might have a different specificity, not being inhibitable by fetal calf serum, and therefore contribute to the extracellular matrix digestion in these experimental conditions. However, this hypothesis seems unlikely because of the extremely small amount of plasmin residual in the system compared with the relatively high amount of extracellular matrix digestion observed in these conditions. When used in a cell-free system, this amount of plasmin is not able to cause any extracellular matrix digestion (data not shown). This pathway also requires cell contact, and mechanisms similar to the plasmin-dependent pathway to localize proteolysis can be hypothesized. The amount of extracellular matrix digestion by one cell type in a particular experimental system is difficult to compare with other cell types in different experimental conditions. The release of radioactivity from labeled extracellular matrices is usually monitored over days rather than hours, and during this time the number of cells is continuously changing. Most investigators have not normalized the amount of radioactivity released in the supernatant for the total amount of radioactivity present in the extracellular matrices. The use of naturally produced matrices versus purified substrates also complicates the comparison of data. For these reasons, we compared the VSM cells with two highly metastatic human melanoma cell lines, which have been proven to be very potent in digesting extracellular matrix produced by bovine VSM cells.29 Both neoplasic cell lines showed a large amount of extracellular matrix digestion (Figure 3). VSM cells were also very potent, and after 5 days in culture they digested an amount of matrix equal to approximately 50% of the amount digested by the BLM or M14 cells. Two factors have to be taken into account when comparing these two melanoma cell lines with nontransformed cells: 1) Despite being plated at the same initial density, the growth rate of the transformed cells is likely to be much faster than that of the VSM cells. 2) The two cell lines tested were the most potent among a series of malignant melanoma cells evaluated for their ability to digest labeled matrices.29 The study of the mechanisms underlying extracellular matrix digestion by human melanoma cell lines are beyond the purposes of the present article. However, it seems that all the cell lines tested can digest both plasmin-sensitive and plasmin-insensitive substrates (Figure 6). VSM cells are not the only nontransformed cell line able to digest extracellular matrices in culture. Macrophages46 and endothelial cells53 have been found to be able to digest both purified substrates such as elastin and naturally produced extracellular matrices. Conversely, not all cells seem to be able to digest extracellular matrix. For instance, human skin fibroblast54 and even tumor cells29 have been shown not to digest extracellular matrix substrates in an appreciable amount in experimental conditions similar to those described in this study. It cannot be excluded that even these cells could be able to do so in vivo when cooperating with other cell types. Ours represents the first study to demonstrate this capability by VSM cells and to investigate the mechanisms by which this occurs. More studies are necessary to further elucidate the mechanism(s) of plasminogen activation and the enzymes involved in the digestion of the plasmin-insensitive substrates and to investigate how extracellular matrix digestion can be inhibited. We believe that the interest of this study is in indicating a new potential way to prevent VSM cell migration. For example, this approach might be promising in the case of restenosis after angioplasty, where interference with physiological processes such as the healing of surgical wounds would not be a concern.

References
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doi: 10.1161/01.RES.71.2.385

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