Control of type IV collagenase activity by components of the urokinase–plasmin system: a regulatory mechanism with cell-bound reactants

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The urokinase-type plasminogen activator (uPA) and the matrix-degrading metalloproteinases MMP-2 and MMP-9 (type IV collagenases/gelatinases) have been implicated in a variety of invasive processes, including tumor invasion, metastasis and angiogenesis. MMP-2 and MMP-9 are secreted in the form of inactivezymogens that are activated extracellularly, a fundamental process for the control of their activity. The physiological mechanism(s) of gelatinase activation are still poorly understood; their comprehension may provide tools to control cell invasion. The data reported in this paper show multiple roles of the uPA–plasmin system in the control of gelatinase activity: (i) both gelatinases are associated with the cell surface; binding of uPA and plasminogen to the cell surface results in gelatinase activation without the action of other metallo- or acid proteinases; (ii) inhibition of uPA or plasminogen binding to the cell surface blocks gelatinase activation; (iii) in soluble phase plasmin degrades both gelatinases; and (iv) gelatinase activation and degradation occur in a dose- and time-dependent manner in the presence of physiological plasminogen and uPA concentrations. Thus, the uPA–plasmin system may represent a physiological mechanism for the control of gelatinase activity.

Keywords: activation/cell surface/gelatinase/plasmin/urokinase

Introduction

The extracellular matrix (ECM) degradation that occurs during a variety of tissue remodeling processes, including tumor invasion, angiogenesis and metastasis formation, requires the concerted action of a number of extracellular proteinases. Among these enzymes the urokinase-type plasminogen activator (uPA) and a variety of matrix-degrading metalloproteinases (MMPs) play important roles (Mignatti et al., 1986, 1989; Kleiner and Stetler-Stevenson, 1993; Mignatti and Rifkin, 1993). Two MMPs, MMP-2 or gelatinase A, and MMP-9 or gelatinase B, degrade a variety of ECM proteins, including type IV collagen, fibronectin, laminin and interstitial (type I) collagen; in addition, they efficiently degrade denatured collagens (i.e. gelatins) of all types (Matrisian, 1990; Kleiner and Stetler-Stevenson, 1993; Aimes and Quigley, 1995). These proteinases, collectively referred to as type IV collagenases or gelatinases, have been implicated in a variety of invasive processes, including tumor invasion and metastasis both in experimental models and in human malignancies (Garbisa et al., 1987, 1992; Bernhard et al., 1990, 1994; Levy et al., 1991; Librach et al., 1991; Juarez et al., 1993; Mignatti and Rifkin, 1993; Montgomery et al., 1993; Watanabe et al., 1993; Crawford and Matrisian, 1995; Himmelstein et al., 1995; Kohn and Liotta, 1995; Stetler-Stevenson, 1995).

Both uPA and MMPs are secreted in the form of inactivezymogens (prouPA, proMMPs) that are activated extracellularly by limited proteolysis. Trace amounts of plasmin can activate prouPA (Petersen et al., 1988), thus generating a self-maintained feed-back mechanism of prouPA and plasminogen activation. The relatively high concentration of plasminogen in virtually all tissues (Robbins and Summara, 1970; Isseroff and Rifkin, 1983) can be rapidly converted to plasmin by uPA. Plasmin degrades a variety of ECM components and activates several MMPs, including MMP-1 (collagenase) and MMP-3 (stromelysin-1) but appears to have no effect on MMP-9. ProuroPA and proMMPs, being tools to control cell invasion. The data reported in this paper show multiple roles of the uPA–plasmin system in the control of gelatinase activity: (i) both gelatinases are associated with the cell surface; binding of uPA and plasminogen to the cell surface results in gelatinase activation without the action of other metallo- or acid proteinases; (ii) inhibition of uPA or plasminogen binding to the cell surface blocks gelatinase activation; (iii) in soluble phase plasmin degrades both gelatinases; and (iv) gelatinase activation and degradation occur in a dose- and time-dependent manner in the presence of physiological plasminogen and uPA concentrations. Thus, the uPA–plasmin system may represent a physiological mechanism for the control of gelatinase activity.

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focal contact sites (Hebert and Baker, 1988; Pollanen et al. 1985). Binding of uPA to uPAR accelerates plasminogen activation on the cell surface and localizes the enzyme to cell membrane components involved in both binding and activation of proMMP-2 (Brown et al., 1990; Ward et al., 1991; Murphy et al., 1992b; Strongin et al., 1993). The transmembrane proteins MT-MMPs (membrane-type MMPs) activate proMMP-2 but have no effect on proMMP-9 (Sato et al., 1994; Cao et al., 1995; Takino et al., 1995). MT1-MMP also appears to serve as a high-affinity binding site for MMP-2/TIMP-2 complex (Strongin et al., 1995). Expression of MT1-MMP stimulates in vitro invasion by several cell types, and is associated with invasive tumor cells or with stromal components of a variety of human neoplasia (Sato et al., 1994; Nomura et al., 1995; Okada et al., 1995).

To characterize physiological mechanisms that control gelatinase activity in cell cultures we studied proMMP-9 and proMMP-2 activation by human fibrosarcoma HT1080 cells that express high levels of uPA and uPAR, and by clones of mouse L fibroblasts transfected with the human genes for different components of the uPA–plasmin system. These culture models offer several advantages relative to cell-free systems using pure reagents in soluble phase: (i) gelatinase activation can be studied in the presence of cell membrane and ECM components involved in the control of uPA and MMP activities; (ii) the level and cellular distribution of different components of the uPA–plasmin system can be modulated by co-cultivating the L cell transfectants in different relative proportions; and (iii) this co-culture model affords constant levels of the reactants to be maintained for many hours. The results show that physiological concentrations of plasmin can activate both progelatinases by a mechanism independent of metallo- or acid proteinase activities that requires interactions of the reactants with the cell surface. In contrast, in soluble phase plasmin degrades both MMP-9 and MMP-2. Thus, the uPA–plasmin system may represent a physiological mechanism for the control of gelatinase activity.

Results

Plasmin activates proMMP-9 and proMMP-2 in cell cultures without the action of other MMPs

To characterize the role of plasmin in type IV collagen degradation, HT1080 cells that produce high levels of uPA and uPAR (Mazzieri et al., 1994) were tested for their ability to degrade 3H-type IV collagen films in the presence or absence of plasminogen. As shown in Figure 1A and B, low type IV collagenase activity was measured in the absence of plasminogen. Addition of 2 &micro;g/ml of pure plasminogen to HT1080 cell cultures considerably increased substrate degradation both by the cell-conditioned medium and by cells grown on a 3H-type IV collagen layer. This effect was abolished by 1,10-phenanthroline or EDTA (not shown), which inhibit metallo-proteinases, and by aprotinin, a serine proteinase inhibitor, or α2-antiplasmin, the physiological inhibitor of plasmin (Figure 2A). In contrast, pepstatin A, an inhibitor of acid proteinases, was ineffective. Since in the absence of cells plasmin had no collagenolytic activity (see legend to Figure 1), and none of these inhibitors was cytotoxic (Mignatti et al., 1986, 1989; data not shown), these results showed that type IV collagen degradation by HT1080 cells requires the concerted action of both metallo- and serine proteinases.

Gelatin zymography of serum-free cell-conditioned medium concentrated by ultrafiltration in Centricon tubes (not shown) or by gelatin–Sepharose as described in Materials and Methods (Figure 1C) showed major bands with Mr >100 kDa, Mr 92, 84 and 72 kDa, and a minor band of 84 kDa. In contrast, cell extracts (Figure 1D) showed major 92 and 84 kDa bands, and a minor band of 79 kDa, but no 72 kDa band. By Western blotting the 92, 84 and 79 kDa bands, and the 72 kDa band were recognized associated with MMP-9 and MMP-2. As partial purification of MMP-2 or MMP-9 (Goldberg et al., 1989, 1992), stained weakly only with anti-MMP-9 antibody. Biotinylation experiments showed that 92 and 84 kDa MMP-9, as well as low amounts of 72 kDa MMP-2 are located at the cell surface (Figure 1F); in contrast, the 84 and 72 kDa bands are exclusively intracellular (Figure 1F). Thus, the gelatinases expressed by HT1080 cells are both secreted into the conditioned medium and associated with the cell surface. No bands with Mr 92 and 84 kDa were identified with any of the MMP-3 or MMP-7 (matrilysin) could be detected by gelatin or casein zymography of cell extracts or conditioned medium concentrated by ultrafiltration (data not shown), indicating that the type IV collagenase activity of HT1080 cells is associated with MMP-9 and MMP-2. As partial purification with gelatin–Sepharose afforded a better detection of the gelatinases by zymography, this method was used instead of ultrafiltration in all subsequent experiments.

Addition of plasminogen to HT1080 cell cultures had no effect on the gelatinases associated with the cell extracts (Figure 1D). In contrast, gelatin zymography of medium conditioned in the presence of plasminogen (Figure 1C) showed a considerable increase in the intensity of the 84 kDa band, and the generation of a 64–68 kDa band. These bands comigrated with those obtained by treatment of HT1080 cell-conditioned medium with trypsin (not shown) or with p-aminophenylmercuric acid (APMA), which activate proMMP-9 and proMMP-2 (Figure 2B)
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**Fig. 1.** Effect of plasminogen and proteinase inhibitors on type IV collagen degradation and gelatinase activation by HT1080 cells. Type IV collagen degradation. (A) Serum-free medium conditioned by HT1080 cells in the absence (–) or in the presence (+) of 2 μg/ml of pure human plasminogen with or without 100 μg/ml of aprotinin (Aprot), 10 μg/ml of pepstatin A (Peps) or 10 μg/ml of 1,10-phenanthroline (Phen) was assayed for type IV collagenase activity as described in Materials and methods. (B) HT1080 cells were grown in 3H-type IV collagen-coated wells with serum-free medium in the absence (–) or in the presence (+) of 2 μg/ml of pure human plasminogen with or without addition of 100 μg/ml of plasminogen, incubated as a control in parallel wells in the absence of cells, released 3–6% of the total radioactivity. Mean and variability of duplicate samples from a representative experiment are shown. This experiment was repeated three times with comparable results. Gelatin zymography of serum-free conditioned medium (C) or Triton X-100 extracts (D) of HT1080 cells grown for 16 h in the absence (–) or in the presence (+) of 2 μg/ml of pure human plasminogen with or without addition of 100 μg/ml of aprotinin (Aprot), 10 μg/ml of plasminogen, incubated as a control in parallel wells in the absence of cells, released 3–6% of the total radioactivity. Mean and variability of duplicate samples from a representative experiment are shown. This experiment was repeated three times with comparable results. (E) Immunological characterization of the gelatinases associated with HT1080 cells. Western blotting of serum-free conditioned medium or Triton X-100 extracts of HT1080 cells with antibody to human MMP-9 (lane 1) or MMP-2 (lane 2). The experimental procedures are described in Materials and methods. This experiment was repeated three times with comparable results. (F) Biotinylation of the gelatinases associated with HT1080 cells. (Lanes a–c) To evidence intracellular forms of the gelatinases, Triton X-100 extracts of HT1080 cells were biotinylated and the gelatinases purified with gelatin-Sepharose. 20 μl (a), 10 μl (b) or 5 μl (c) of the resin were loaded on a SDS/3–8% polyacrylamide gel, and biotinylated proteins were evidenced as described in Materials and methods. (Lane d) HT1080 cells were surface biotinylated and lysed, and the gelatinases purified with gelatin-Sepharose. 50 μl of the resin was loaded on the gel. (Lane e) gelatin zymogram of HT1080 cell extract, to show the correspondence of the lysis bands with the bands evidenced by Western blotting and by biotinylation. Arrowheads show the different Mr forms of MMP-9 and MMP-2. proMMP-9 (92 kDa) is present both on the cell surface and intracellularly. In contrast, 84 kDa MMP-9 is exclusively found on the cell surface, and 79 kDa intracellularly. proMMP-2 not evidenced by gelatin zymography is biotinylated both inside the cell and on the cell surface. This experiment was repeated three times with comparable results.
Fig. 3. Type IV collagenase (A) and plasmin (B) activities of extracts or conditioned medium of HT1080 cells grown in the presence (+) or absence (−) of plasminogen. Confluent HT1080 cells were grown for 16 h in serum-free medium in the absence or in the presence of 2 μg/ml of pure plasminogen. At the end of the incubation, Triton X-100 cell extracts and conditioned media were assayed for type IV collagenase and for plasmin activities as described in Materials and methods. This experiment was repeated three times with comparable results.

Fig. 2. Effect of plasminogen and α2-antiplasmin on type IV collagen degradation and gelatinase activation by HT1080 cells. (A) Serum-free medium conditioned by HT1080 cells in the absence or in the presence of 2 μg/ml of pure human plasminogen with or without 10 μg/ml of α2-antiplasmin was assayed for type IV collagenase activity as described in Materials and methods. Means and variability of duplicate samples from three experiments are shown. (B) Gelatin zymography of serum-free medium conditioned by HT1080 cells for 16 h in the absence (−) or in the presence of 2 μg/ml of pure human plasminogen with or without addition of 10 μg/ml of α2-antiplasmin. APMA: medium conditioned in the absence of plasminogen and α2-antiplasmin, incubated with p-aminophenylmercuric acetate 1 mM at 37°C for 1 h. The samples were concentrated by gelatin–Sepharose chromatography, and analyzed by gelatin zymography as described in Materials and methods. This experiment was repeated three times with comparable results.

by other MMPs, including MMP-1, MMP-3 or MT-MMPs (He et al., 1989; Goldberg et al., 1992; Sato et al., 1994), and does not result from autocatalysis (Shapiro et al., 1992; Desrivieres et al., 1993; Watanabe et al., 1993).

To test whether plasmin-mediated activation of proMMP-9 and proMMP-2 requires the action of other proteases, we characterized the effect of different proteinase inhibitors. In the presence of plasminogen, gelatinase activation was abolished by α2-antiplasmin (Figure 2) or by aprotinin but not by pepstatin A (Figure 1C), showing that the generation of active MMP-9 and MMP-2 in cell-conditioned medium requires plasmin or plasmin-activated proteinase(s). Inhibition of metalloproteinase activity by 1,10-phenanthroline did not block gelatinase activation (Figure 1C): therefore, in the presence of plasmin MMP-9 and MMP-2 activation is not mediated

(Brown et al., 1990; Nagase et al., 1992; Strongin et al., 1993; Itoh et al., 1995).

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Fig. 4. Zymographic analysis of the gelatinases associated with serum-free conditioned media (A) or Triton X-100 extracts (B) of L cells transfected with the human genes for different components of the uPA plasmin system. Three mg of cell extract protein, or volumes of conditioned medium (3–4 ml) normalized to the protein concentration of the corresponding cell extracts were analyzed by gelatin zymography as described in Materials and methods. This experiment was repeated three times with comparable results. The MMP-2 band comigrates with that of the proMMP-2 secreted by HT1080 cells (not shown). The nature of the band present in Lc PIT cell-conditioned medium with M₄ higher than that of the MMP-2 secreted by the other clones has not been characterized. This band did not appear consistently in all the zymograms.

by specific antibodies to human MMP-9 and MMP-2, respectively (data not shown). No bands with Mₛ consistent with those of MMP-3 or matrilysin could be detected by gelatin or casein zymography of L cell extracts or conditioned media concentrated by ultrafiltration. All the L cell clones showed no major differences in the expression of MMP-1, the type-1 and type-2 tissue inhibitors of metalloproteinases (TIMP-1 and TIMP-2), and uPA inhibitors other than PAI-1 (data not shown).

By the ³H-type IV collagen assay all the L cell clones showed low collagenolytic activity in the absence of plasminogen. Addition of 2 μg/ml of pure plasminogen increased the type IV collagenolytic activity of LᵤPA cells by ~7-fold but did not affect substrate degradation by the other cell clones (Figure 5A). LᵤPA cells secrete a high level of human uPA comparable with that of HT1080 cells, whereas the other clones express no uPA. In the absence of cells, an amount of plasmin comparable with that generated by LᵤPA cells did not degrade ³H-type IV collagen (Figure 5A).

Addition of plasminogen had no effect on the gelatinases associated with LᵤPA cell extracts (data not shown). In contrast, gelatin zymography of cell-conditioned medium showed that addition of plasminogen to serum-free LᵤPA cell cultures resulted in the generation of active MMP-9 (84 kDa) and MMP-2 (64–68 kDa). This effect was abolished by aprotinin but not by pepstatin A. The metalloproteinase inhibitor 1,10-phenanthroline did not block gelatinase activation (Figure 5B); thus, as is the case for HT1080 cells, proMMP-9 and proMMP-2 activation by L cells requires plasmin but not other MMPs, and does not result from autocatalysis.

**uPA binding to uPAR increases type IV collagenase activity**

To test the effect of uPA binding to uPAR on the type IV collagenase activity of L cells, increasing amounts of pure human uPA or LᵤPA cells were added to Lᵢ cells grown on ³H-type IV collagen films in the absence or presence of 2 μg/ml of pure plasminogen. Lᵢ cells secrete no uPA but bind human uPA efficiently (Roldan et al., 1990). Addition of pure uPA or of LᵤPA cells increased substrate...
repeated four times with comparable results. The number of LuP A cells was indicated on the abscissa as the percentage of 1,10-phenanthroline, EDTA or aprotinin inhibited uPA activity as described in Materials and methods. Each point represents mean and variability of duplicate samples from a representative experiment. This experiment was repeated three times with comparable results. Effect of PAI-1 on the uPA (C) and type IV collagenase (D) activities of co-cultures of LR with LuP A cells. Increasing amounts of LuP A cells were grown in 3H-type IV collagen-coated wells in serum-free medium supplemented with 2 μg/ml of plasminogen. The number of LuP A cells is indicated on the abscissa as the percentage of the number of LR cells. At the end of the incubation substrate degradation was measured as described in Materials and methods. Each point represents mean and variability of duplicate samples from a representative experiment. This experiment was repeated five times with comparable results. Gelatin zymography of medium conditioned by LR cells or by co-cultures of LR with 10% LuP A cells in the presence of 2 μg/ml of plasminogen. The samples were concentrated and analyzed as described in Materials and methods. Gelatin zymography of an amount of LuP A cells equivalent to that used for the co-culture showed no visible bands. This experiment was repeated three times with comparable results. (D) uPA activity of co-cultures of LR with LuP A cells. Increasing amounts of LuP A cells were co-cultivated with 3 × 10⁵ LR cells for 16 h in microculture wells in serum-free medium. The number of LuP A cells is indicated on the abscissa as the percentage of the number of LR cells. At the end of the incubation soluble (C) and cell-bound (●) uPA activity was measured as described in Materials and methods. Each point represents mean and variability of duplicate samples from a representative experiment. This experiment was repeated four times with comparable results.

degradation in a dose-dependent manner in the presence but not in the absence of plasminogen (Figure 6A and B). By gelatin zymography, medium conditioned by LR cells alone in the presence of plasminogen showed MMP-9 and MMP-2 in proenzyme form; in contrast, in medium conditioned by co-cultures of LR with 10% LuP A cells in the presence of plasminogen both the gelatinases were converted into their active forms (Figure 6C). Addition of 1,10-phenanthroline, EDTA or aprotinin inhibited type IV collagen degradation by co-cultures of LR + L_uPA cells. α2-antiplasmin (not shown) had an equivalent effect. In contrast, pepstatin A was ineffective (Figure 7A and B).

To confirm that the type IV collagenolytic activity of the co-cultures was mediated by the uPA secreted by L_uPA cells, increasing amounts of L_PA-1 cells were added to co-cultures containing 10% L_uPA cells. The PAI-1 secreted by L_PA-1 cells inhibited both cell-associated and soluble uPA activity in a dose-dependent manner (Figure 7C). Likewise, substrate degradation by co-cultures grown on 3H-type IV collagen films decreased as a function of the number of L_PA-1 cells. In contrast, the type IV collagenase
activity of the conditioned medium was not affected (Figure 7D).

Although L_{uPA} cells produce gelatinase levels higher than those of L_R cells (Figure 4), type IV collagen degradation by L_{uPA} cells was 6- to 7-fold lower than that of co-cultures with L_R cells (Figure 6B), suggesting that uPA binding to uPAR increased gelatinase activation. The collagenolytic activity of the co-cultures increased slowly in the presence of a proportion of L_{uPA} to L_R cells lower than 1%, and more rapidly in co-cultures containing 1–10% L_{uPA} cells (Figure 6B). The level of uPA activity also increased with the number of L_{uPA} cells. In co-cultures containing <1% L_{uPA} cells uPA was predominantly cell-associated. With higher proportions of L_{uPA} to L_R cells the amount of cell-bound uPA leveled off, and the level of uPA in the conditioned medium increased dramatically (Figure 6D). Saturation of the uPA-binding sites of L_R cells and subsequent release of excess uPA in soluble phase paralleled the rapid increase in 3H-type IV collagen degradation. These findings indicated that efficient type IV collagen degradation requires either uPAR-bound uPA or high levels of soluble uPA.

To discriminate between these two hypotheses, increasing amounts of L_{uPA} cells were co-cultivated on 3H-type IV collagen films with a constant number of either L_R or L_c cells. L_R cells do not bind human uPA (Roldan et al., 1990). In the presence of 2 μg/ml of plasminogen co-cultures of L_{uPA} with LR cells efficiently degraded the substrate. In contrast, co-cultures with L_c cells showed no collagenolytic activity (Figure 8A). Type IV collagen degradation by co-cultures of L_R cells with 10% L_{uPA} cells also increased as a function of plasminogen concentration, whereas co-cultures with L_c cells showed virtually no collagenolytic activity even with a high plasminogen concentration (Figure 8B). In a second set of experiments, increasing amounts of L_{ATF} cells were added to co-cultures of L_R cells with 10% L_{uPA} cells in the presence of 2 μg/ml of plasminogen. L_{ATF} cells secrete an N-terminal, non-catalytic peptide (ATF) of human uPA that contains the uPAR-binding sequence (Appella et al., 1987; Pedersen et al., 1991). As ATF competes with uPA for binding to uPAR, addition of L_{ATF} cells resulted in a dose-dependent decrease in the level of cell-bound uPA and in increased soluble uPA in the culture supernatant (Figure 8C). Type IV collagen degradation by the cells or conditioned medium also decreased with increasing L_{ATF} cell number in a dose-dependent manner (Figure 8D). This effect paralleled the decrease in cell-bound uPA. Thus, uPA binding to uPAR on L_R cell membrane strongly accelerates gelatinase activation.

**Gelatinase activation by plasmin requires interactions with the cell surface**

To understand whether gelatinase activation occurs in soluble phase or requires interaction with the cell surface, we studied activation of proMMP-9 and proMMP-2 in the absence of cells. 125I-labeled recombinant MMP-2 was incubated at 37°C for 1 h in the presence or absence of 4 μg/ml of plasminogen and increasing uPA concentration, and analyzed by SDS-PAGE and autoradiography. As shown in Figure 9A, addition of plasminogen and uPA resulted in dose-dependent MMP-2 degradation.

Because recombinant MMP-2 may be more sensitive to proteolytic degradation than the native enzyme, we characterized the effect of plasmin on MMP-2 secreted by L cells. Serum-free medium conditioned by L_R cells was incubated for 1 h in the absence or presence of plasminogen and increasing uPA concentration, and MMP-2 levels were measured by a substrate-capture ELISA (Wacher et al., 1990). In the presence of plasminogen the level of MMP-2 detected by this assay decreased with increasing uPA concentration in a dose-dependent manner (Figure 9B). Thus, plasmin generated MMP-2 degradation products that either did not bind to the gelatin used as a substrate or were not recognized by the antibody.

To characterize further the effect of plasmin on MMP-2 and MMP-9 in the absence of cells, HT1080 cell-condi-
tioned medium was incubated with pure uPA and plasminogen for 16 h—i.e. the time used to monitor gelatinase activation in cell cultures—and analyzed by gelatin zymography. As shown in Figure 9C, active MMP-2 and MMP-9 were not generated; in contrast, the intensity of the bands corresponding to the progelatinases decreased with increasing uPA concentration in a dose-dependent manner. Thus, in the absence of cells plasmin does not convert proMMP-9 and proMMP-2 into their active forms but rapidly generates degradation products that lack gelatin affinity and/or catalytic activity. These findings indicated that interactions with the cell surface are required for plasmin-mediated activation of proMMP-9 and proMMP-2.

To test this hypothesis, HT1080 cell-conditioned medium was incubated with LR cells in the presence or absence of 4 μg/ml of pure plasminogen. After 0–4 h of incubation, the conditioned medium was characterized by gelatin zymography. As shown in Figure 10A and B, incubation with LR cells resulted in time-dependent generation of active MMP-2 and MMP-9 in the presence but not in the absence of plasminogen. To understand whether plasmin activates the gelatinases directly or through an intermediate serine proteinase(s), serum-free medium conditioned by HT1080 cells in the presence of 1,10-phenanthroline (10 μg/ml) and pepstatin A (10 μg/ml) was incubated with confluent LR cells in the presence or in the absence of 4 μg/ml of plasmin. After 2 h incubation, α2-antiplasmin (10 μg/ml) or α2-antiplasmin plus aprotinin (100 μg/ml) was added and the incubation was continued for 4 h. Gelatin zymography of the conditioned medium (Figure 10C) showed that after 2 h incubation with plasmin active gelatinases were generated; the subsequent addition of α2-antiplasmin prevented the further generation of active forms. Surprisingly, inhibition of plasmin activity also resulted in the disappearance of the 84 and 64–68 kDa forms of MMP-9 and MMP-2 generated during the initial 2 h of incubation, indicating that these active forms of the gelatinases are rapidly processed to degradation products that lack either catalytic activity or gelatin affinity.
To understand whether plasmin binding to the cell surface is required for gelatinase activation, serum-free HT1080 cell-conditioned medium was incubated for 2 h at 37°C with L929 cells in the presence or absence of 4 µg/ml of plasminogen and 500 µg/ml of ε-aminocaproic acid (EACA), which prevents plasmin(ogen) binding to the cell surface. Gelatin zymography of the cell-conditioned medium showed that addition of EACA inhibited gelatinase activation (Figure 10D). Thus, gelatinase activation requires the generation of plasmin activity on the cell surface.

Discussion

The data reported show multiple roles of the uPA–plasmin system in the control of gelatinase activity: (i) in the presence of cells plasmin generates active forms of MMP-9 and MMP-2 without requiring the action of other metallo- or acid proteinases; (ii) this effect results in a 5- to 7-fold increase in type IV collagen degradation; (iii) binding of uPA and plasmin(ogen) on the cell surface, where the gelatinases are also located, accelerates gelatinase activation; (iv) in the absence of cells plasmin degrades soluble MMP-9 and MMP-2; and (v) progelatinase activation and degradation occur in a dose- and time-dependent manner in the presence of relatively low concentrations of plasminogen (1–4 µg/ml) and uPA (0.3–100 mU/ml). Thus, the uPA–plasmin system may represent a physiological mechanism for the control of gelatinase activity.

By gelatin zymography, addition of pure plasminogen to HT1080 or L929 cells results in the generation of bands with M, 84 and 64–68 kDa consistent with physiologically or chemically activated MMP-9 and MMP-2, respectively (Stetler-Stevenson et al., 1989; Brown et al., 1990; Okada et al., 1990, 1992; Keski-Oja et al., 1992; Nagase et al., 1992; Juarez et al., 1993; Montgomery et al., 1993; Strongin et al., 1993; Itoh et al., 1995). Under the same conditions in which the 84 and 64–68 kDa bands are generated, degradation of type IV collagen films also occurs. Several observations show that the collagenolytic activity we measured by the 3H-type IV collagen assay is associated with active MMP-9 or MMP-2: (i) by gelatin zymography, conditioned medium or extracts of HT1080 or L cells showed the presence of only MMP-9 and MMP-2; no bands with M, s consistent with those of matrixins or stromelysin, which also degrade type IV collagen, were detected; (ii) pure MMP-9 or MMP-2 efficiently solubilize type IV collagen films in a dose-dependent manner in the absence of other proteinases (Atkinson et al., 1992); and (iii) other MMPs, including MMP-3, are at least two orders of magnitude less efficient than MMP-9 or MMP-2 in degrading type IV collagen films by the same assay we used (Atkinson et al., 1992). Under our experimental conditions generation of plasmin resulted in partial activation of MMP-9 and MMP-2, as evidenced by gelatin zymography. However, this effect was associated with a 5- to 7-fold increase in type IV collagen degradation, indicating that the specific activity of the active forms of gelatinases generated by plasmin is considerably higher than that of the correspondingzymogens.

Plasmin generated on the cell surface can activate other MMPs, including MMP-1 and MMP-3 that in turn activate the gelatinases (He et al., 1989; Matrisian, 1990; Nagase et al., 1991; Goldberg et al., 1992; Kleiner and Stetler-Stevenson, 1993; Mignatti and Rifkin, 1993; Sato et al., 1994; Cao et al., 1995; Takino et al., 1995). The activation site of MT1-MMP appears to be compatible with a serine proteinase such as plasmin or uPA (Strongin et al., 1995). However, our finding that gelatinase activation by plasmin is not inhibited by metalloproteinase inhibitors rules out that in the presence of plasmin progelatinase activation is mediated by other MMPs or occurs by autolysis (Shapiro et al., 1992; Desrivieres et al., 1993; Watanabe et al., 1993).

Gelatinase activation was completely inhibited by α2-antiplasmin (Figure 2). This finding is consistent with previous reports that α2-antiplasmin or serum efficiently block or dramatically retard cell-mediated degradation of the ECM (Wong et al., 1992; Baricos et al., 1995) as well as tumor cell invasion in vitro (Mignatti et al., 1986). Thus, although ECM-bound plasmin is partly protected from α2-antiplasmin inhibition (Knudsen et al., 1986), in vitro experiments requiring relatively long incubation of the cells with PAI-1 blocked collagen degradation by cells grown on 3H-type IV collagen layers but appeared to have no effect on the collagenolytic activity present in their cell-conditioned medium (Figure 7D). This effect may result from the experimental conditions used to measure gelatinase activity in cell-conditioned medium. During incubation at 37°C for 16 h plasminogen might be activated through uPA-independent mechanisms. In addition, small amounts of gelatinase(s) may be activated during incubation with the cells; inhibition of plasmin formation by PAI-1 prevents plasmin-mediated gelatinase degradation in soluble phase.

Activation of proMMP-2 or proMMP-9 in vitro has been reported by other authors to be independent of plasmin (Brown et al., 1990; Okada et al., 1990, 1992; Reith and Rucklidge, 1992; Montgomery et al., 1993), or to require high concentrations of plasminogen (100–200 µg/ml) or uPA (Okada et al., 1990, 1992; Keski-Oja et al., 1992; Murphy et al., 1992a; Desrivieres et al., 1993; Montgomery et al., 1993). We found that plasminogen or uPA concentrations 2–3 orders of magnitude lower than those used by other authors (Keski-Oja et al., 1992) can efficiently activate proMMP-2 and proMMP-9. Because the concentration of plasminogen in all tissues is relatively high (Robbins and Summara, 1970; Isseroff and Rifkin, 1983), the concentrations we used (1–4 µg/ml) are likely physiological or below physiological. Whereas different cells may use different mechanisms for gelatinase activation, the discrepancies between our results and those reported by other authors can be explained by the experimental conditions we used. Our cell culture models afforded the characterization of gelatinase activation in the presence of cell membrane and ECM components that contribute to modulating a variety of extracellular proteolytic activities. These conditions cannot be reproduced in the test tube using pure reagents in soluble phase. In addition, the use of co-cultures afforded constant levels of reactants to be maintained throughout the experiments. In contrast, addition of pure reagents to cell-free systems may result in relatively rapid inactivation. Several observations show that the results we obtained with our L cell
co-cultures do not reflect nonspecific effects generated by our co-culture conditions: type IV collagen degradation occurred only in the presence of pure plasminogen, and was inhibited by the same proteinase inhibitors that blocked the gelatinase activity of single cell types; moreover, addition of a specific transfectant had parallel, dose-dependent effects on both PA and type IV collagenase activities.

Our data show that plasmin-mediated gelatinase activation occurs when the reactants—uPA, plasmin(ogen) and the gelatinases—can interact with the cell surface. Binding of uPA to uPAR accelerates plasminogen activation on the cell membrane (Ellis et al., 1991); high amounts of plasmin generated by uPAR-bound uPA can more rapidly activate the gelatinases, and contribute to further degrading type IV gelatin generated by MMP-9 or MMP-2 cleavage. Our \(^{3}H\)-type IV collagen film assays were done at physiological temperature (37°C). Under these conditions, the \(\frac{3}{4}\) and \(\frac{1}{2}\)-length fragments of type IV collagen generated by MMP-9 or MMP-2 denature spontaneously into randomly coiled peptides that can be cleaved by a variety of enzymes, including plasmin and the same type IV collagenases (gelatinases). However, the single cleavage of the collagen triple helix is the rate-limiting step in collagen degradation (Welgus et al., 1981; Atkinson et al., 1992). In the absence of cells, soluble plasmin in control medium had no collagenolytic activity (Figures 1 and 5A). In addition, although HT1080 cell extract and conditioned medium possessed comparable levels of plasmin activity, the type IV collagenase activity of the cell-conditioned medium was considerably higher than that of the cell extract, which contained lower amounts of gelatinases (Figure 3).

Therefore, the increase in collagen degradation in the presence of uPAR-bound uPA reflects efficient gelatinase activation, and is not the result of increased gelatin degradation by soluble or cell-associated plasmin. This effect may result from the juxtaposition of all the reactants on the cell surface. Our data show that both gelatinases are associated with the cell surface (Figure 1F), where uPA and plasmin(ogen) are also localized. Binding of uPA to uPAR accelerates gelatinase activation in a dose-dependent manner (Figure 8A); inhibition of uPA or plasmin binding to the cell surface blocks gelatinase activation (Figures 8D and 10D). Although L\(_{uPA}\) cells do not bind human uPA, they bind plasmin(ogen) on their cell surface or ECM as efficiently as other cell types (data not shown; Miles and Plow, 1985; Plow et al., 1986; Plow and Miles, 1990). The high level of uPA secreted by L\(_{uPA}\) cells may afford the generation of sufficient amounts of cell-bound plasmin to activate proMMP-9 and proMMP-2. Thus, gelatinase activation in cell cultures requires either high levels of soluble uPA and plasminogen or uPA binding to uPAR.

Our observation that progelatinase activation can occur in the presence of metalloproteinase inhibitors is consistent with previous findings (Lohi and Keski-Oja, 1995), and indicates that the proteolytic activity of MT-MMP(s) is not required for MMP-2 activation. However, MT-MMP(s) may serve as a cell membrane binding site for MMP-2 or MMP-2-TIMP-2 complex (Strongin et al., 1995). On the membrane of HT1080 cells MMP-2 and TIMP-2 form a trimolecular complex with an active form of MT1-MMP generated by proteolytic cleavage of its propeptide. The propeptide of MT1-MMP-1 appears to be an excellent substrate for serine proteinases, including plasmin or uPA (Strongin et al., 1995). In the light of these observations it can be speculated that plasmin plays a dual role in a proteolytic cascade which leads to proMMP-2 activation on the cell membrane. Plasmin may activate proMT1-MMP, that binds TIMP-2 and proMMP-2; bound proMMP-2 is then activated by plasmin.

In addition to activating the gelatinases, plasmin also provides a negative control for gelatinase activity by rapidly degrading both MMP-9 and MMP-2 in soluble phase. Thus, type IV collagenase activity in the presence of plasmin results from a balance between gelatinase activation and degradation; this balance being modulated by enzyme interactions with the cell surface. These conclusions prompt consideration of the mechanisms that control gelatinase activity in the extracellular microenvironment.

Conformational changes resulting from gelatinase–cell surface interactions may partially protect these enzymes from plasmin degradation, and render the gelatinases available only to limited cleavage. In contrast, in soluble phase multiple cleavage sites may be available for degradation by plasmin. Autolysis or other plasmin-independent mechanisms can also contribute to degradation of active gelatinases. Consistent with previous findings (Nagase et al., 1992), our results indicate that the 84 and the 64–68 kDa forms of MMP-9 and MMP-2 generated in the presence of plasmin are rapidly degraded after addition of \(\alpha_2\)-antiplasmin (Figure 10C).

Binding sites for MMP-2 have been described on the membrane of several cell types (Emonard et al., 1992; Monsky et al., 1993; Strongin et al., 1995). We found that both MMP-2 and MMP-9 are located on the surface of HT1080 cells (Figure 1E). MMP-9 was consistently associated with extracts of HT1080 or L cells, whereas MMP-2 could be detected by zymography or Western blotting only in conditioned medium. These results can be explained by several hypotheses, including an apparently higher intracellular concentration of MMP-9 resulting from a longer time for synthesis and/or post-translational modifications. Interestingly, gelatin zymography and Western blotting showed that in both HT1080 and L cells grown in the absence of plasminogen 84 kDa MMP-9 was more abundant in cell extracts than in conditioned medium where, on the contrary, 92 kDa MMP-9 was prevalent (Figure 1C and D; Figure 4). By pulse–chase experiments we found that 84 kDa MMP-9 remains associated with cell extracts, but not with the ECM, for several hours, whereas MMP-2 is rapidly secreted. Deglycosylation experiments showed that 84 kDa MMP-9 is not a glycosylation variant of proMMP-9. Inhibition of protein secretion with Brefeldin A prevents the generation of cell-associated 84 kDa MMP-9, indicating that this form of MMP-9 is generated extracellularly, and bound to the cell membrane (R.Mazzieri, S.Monea, L.Zanetta and P.Mignatti, manuscript in preparation). The generation of 84 kDa MMP-9 associated with HT1080 or L\(_{uPA}\) cell extracts appears to be independent of plasmin (Figures 1D and 4), whereas in cell-conditioned medium 84 kDa MMP-9 is upregulated by plasmin (Figures 1C, 5B and 6C). A further characterization of cell-associated 84 kDa gelatinase may be important to understand the mechanism of proMMP-9 activation.
Our results show that, like proMMP-2 (Brown et al., 1990; Ward et al., 1991; Murphy et al., 1992b; Strongin et al., 1993, 1995; Sato et al., 1994, Cao et al., 1995), proMMP-9 activation also requires interaction with the cell surface. A form of MMP-2 with Mr 84 kDa similar to that of the active form generated in the conditioned medium is associated with the cell surface. In contrast, no active forms of MMP-2 appear to be associated with the cells. Incubation of proMMP-2 with cells overexpressing MTI-1 also results in generation of soluble forms of the active enzyme; no active MMP-2 is found associated with the cell surface (Sato et al., 1994; Cao et al., 1995). These findings indicate that, unlike MMP-9, MMP-2 interacts with the cell membrane only transiently, and is immediately released after activation. Thus, as proposed by several authors (Docherty et al., 1992; Kleiner and Steetler-Stevenson, 1993; Strongin et al., 1993; Lohi and Keski-Oja, 1995), MMP-9 and MMP-2 may have different mechanisms of activation. Our data show that plasmin represents a common pathway for the activation of both gelatinases.

ECM degradation during tissue remodeling requires a protease cascade that involves plasminogen activators and several MMPs (Mignatti et al., 1986, 1989; Mignatti and Rifkin, 1991; Steeter-Stevenson et al., 1993). This protease cascade can be modulated by specific interactions of both serine proteinases and MMPs with the cell surface. The comprehension of the molecular mechanisms that mediate gelatinase-cell surface interactions can provide useful tools for the development of novel strategies aimed at inhibiting type IV collagenase activity and controlling a variety of tissue remodeling processes, including tumor invasion and angiogenesis.

Materials and methods

Materials

Human or bovine plasminogen and fibrinogen were purified as described (Unkeless et al., 1973). Human recombinant proMMP-2 (Fridman et al., 1993) and rabbit antiserum to human MMP-2 and MMP-9 were a generous gift from Dr W.G. Steetler-Stevenson (NCI, Bethesda). Pure human uPA (Ukidan; M, 54 kDa; 100 000 IU/mg) was purchased from Serono (Rome, Italy), biosynthetically [3H]proline-labeled type IV collagen from Amersham (Amersham, England), gelatin-2-Sepharose from Pharmacia Biotech AB (Uppsala, Sweden), gelatin from Merck (Darmstadt, Germany), aprotinin, t-aminocaproic acid, 1,10-phenanthroline, phenylalanine A, p-aminophenylmercuric acetate (APMA) and α2-antiplasmin from Sigma (St Louis, MO). Protein concentration was measured by the BCA protein assay reagent (Pierce Chemical Co., Rockford, IL) using bovine serum albumin (BSA; Sigma) as a standard. Human fibrinogen and recombinant MMP-2 were labeled with 125I (Amersham) and iodogen (Pierce Chemical Co.) as described (Mignatti et al., 1991).

Cells and culture medium

The following clones of mouse L cells were used for the experiments described: LpA cells: mouse L cells transfected with human prouPA cDNA and the neomycin resistance gene (Cajot et al., 1989), kindly provided by Dr J.F.Cajot (ISREC, Lausanne, Switzerland); LpA cells: mouse LB6 cells transfected with human uPAR cDNA and the neomycin resistance gene (LB6 clone 19; Roldan and Keski-Oja, 1990), kindly provided by Drs F. Blasi and N. Pedersen (University of Copenhagen, Denmark); LpA cells: mouse L cells transfected with human PAI-1 cDNA and the neomycin resistance gene (clone A7; Cajot et al., 1990), kindly provided by Drs J.F. Cajot and B Sordat (ISREC, Lausanne, Switzerland); LATF cells: mouse LB6 cells transfected with a cDNA encoding a non-catalytic, N-terminal peptide of human uPA that contains the uPAR-binding sequence, and with the neomycin resistance gene (clone A7F; Pedersen et al., 1991), kindly provided by Drs F. Blasi and N. Pedersen (University of Copenhagen, Denmark); Lc cells: control L cells transfected with the neomycin resistance gene alone, kindly provided by the above laboratories.

The cells were grown in Dulbecco’s minimum essential medium (DMEM; Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum (FCS; Biochrom KG, Berlin, Germany), 100 units/ml of penicillin, 100 μg/ml of streptomycin and 0.25 μg/ml of amphotericin B (Gibco BRL). Every 4–5 weeks, the cells were grown for 5–7 days in the presence of 200 μg/ml of genetin (Sigma, St Louis, MO). The transfected cell clones were characterized for PA inhibitors by measuring inhibition of pure uPA activity by dilutions of conditioned medium in [125I]-fibrin assay (see below), and for MMP-1, MMP-2, MMP-3, MMP-9, TIMP-1 and TIMP-2 expression by semiquantitative RT-PCR as described (Onisto et al., 1993). The results obtained by RT-PCR for MMP-2 and MMP-9 were consistent with gelatin zymography results.

Human HT1080 fibrosarcoma cells were originally obtained from the American Type Culture Collection and grown in our laboratories for several years in DMEM supplemented with 10% FCS and L-glutamine 2 mM.

Preparation of cell extracts and conditioned media

L cells or HT1080 cells were seeded into 16-mm tissue culture wells at a density of 3 × 105 cells/well, or into 10-cm culture dishes at a density of 2.6 × 106 cells/dish. Where indicated, a constant number of Lc or LpA cells (3 × 104 or 5 × 105 cells/well or 2.6 × 106 cells/dish) were co-cultivated with increasing amounts of LpA, LpA-L or LpA-L cells. The relative proportion of these cell types in the co-cultures is indicated as a percentage of LpA cell number. After 4–6 h incubation at 37°C in DMEM supplemented with 10% FCS, the cells were washed twice with phosphate-buffered saline (PBS) to remove residual FCS, and incubated for 16 h with 1 ml/well or 4 ml/dish of serum-free DMEM with or without the indicated concentrations of plasminogen and the proteinase inhibitors to be tested. The culture supernatants were harvested, and cellular debris removed by centrifugation at 300 g for 10 min at 22°C. The cells were washed twice with PBS, lysed for 10 min on ice with 100 μl/well or 1 ml/dish of Triton X-100 0.5% (v/v) in Tris-HCl 0.1 M, pH 8.1 under constant shaking, and scraped with a rubber policeman. The cell lysates were centrifuged at 800 g for 10 min at 4°C. Conditioned media and cell extracts were immediately processed for proteinase assays as described below.

Gelatin zymography

Cell extracts (1 ml) or conditioned media (4 ml) were incubated at 4°C for 1 h in an end-over-end mixer with 50 μl of gelatin-Sepharose equilibrated with Tris-HCl 50 mM, NaCl 150 mM, CaCl2 5 mM, 0.02% (v/v) Tween-20, EDTA 10 mM, pH 7.6 (Collier et al., 1988). After 4 washes with 1 ml of equilibration buffer containing NaCl 200 mM, the beads were resuspended in 30 ml of 4 × non-reducing Laemmli buffer, and loaded on SDS/3–8% polyacrylamide gels containing 1 mg/ml of gelatin. After electrophoresis, the gels were washed twice with 200 ml of 2.5% (v/v) Triton X-100 for 2 h at 22°C to remove SDS, and 3 times with H2O for 5 min to remove Triton X-100. The gels were incubated at 37°C for 24–48 h in Tris–HCl 50 mM, NaCl 0.2 M, CaCl2 20 mM, pH 7.4, stained overnight with Coomassie Brilliant Blue R-250 5% (w/v) in 45% (v/v) methanol, 10% (v/v) acetic acid and destained in the same solution without dye (Reaves and Dowd, 1980). The Mr of the bands was determined by reference to high-Mr (14.3–200 kDa) standards (Rainbow Markers; Amersham, UK).

Western blotting

Gelatinases purified from cells extract (1 ml) or conditioned medium (4 ml) by gelatin-Sepharose as described above were electrophoresed in a SDS/3–8% polyacrylamide gel and electrophoretically to a nitrocellulose membrane (Hybond-C Extra, Amersham). The membrane was prehydrized at 42°C for 1 h in Tris base 20 mM, NaCl 137 mM, HCl 1.0 M, 0.1% Tween-20, pH 7.6 (TBS-T) containing 5% blocking agent (Amersham), hybridized at 22°C for 1 h in TBS-T containing 1% BSA and the indicated rabbit antiserum diluted 1:200–1:300, and incubated at 22 °C for 45 min in TBS-T containing horseradish peroxidase (HRP)-labeled anti-rabbit IgG. Each step was followed by extensive washing in TBS-T (4 ml/cm2) at 22°C. After removing the TBS-T buffer, the membrane was incubated for 1 min at 22°C with 0.125 ml/cm2 of ECL detection solution (Amersham) and exposed to films (Hyperfilm MP, Amersham) for 10 s to 5 min.

Cell surface biotinylation

To label cell surface protein, subconfluent HT1080 cells in 50 cm2 tissue culture dishes were washed twice with cold PBS, and incubated at 4°C
for 30 min with 3–5 ml of biotinylation buffer (bicarbonate buffer, pH 8.6; Amersham). At the end of the incubation, the cells were washed twice with PBS and lysed with Triton X-100 as described above (preparation of cell extracts).

To biotinylate total cell proteins, Triton X-100 cell extract protein was diluted to 1 mg/ml in 50 mM biotinylation buffer containing 40 μl of biotinylation reagent per mg protein, and incubated at 22°C for 1 h. Cell extracts were chromatographed on a G-25 column to remove unreacted biotin.

The eluted protein was concentrated with 50 μl of gelatin-Sepharose and run in an SDS-polyacrylamide gel as described above for gelatin zymography. After blotting to a nitrocellulose membrane, blocking and washing with TBS-T as described above for Western blotting, the membrane was incubated at 22°C for 1 h with horseradish peroxidase-labeled streptavidin (Amersham) 1:1500 in TBS-T. Following extensive washing with the same buffer, biotinylated proteins were evidenced by addition of ECL detection solution (Amersham) as described above. Films (Hyperfilm, Amersham) were exposed for 10 s to 1 min.

**Assay for type IV collagenase activity**

One ml of serum-free conditioned medium, or 3×10⁵ cells or 150–250 μg of cell extract protein in 1 ml DMEM were added to 1-H⁴ type IV collagen-coated microculture wells (2 cm²; 3000–4000 d.p.m./well) (Garbisa et al., 1980). After 4–6 h incubation, when the cells had spread on the substrate, the medium was removed. The cells were washed twice with PBS to remove residual FCS, and incubated for 16 h with 1 ml of serum-free DMEM in the presence or absence of the indicated concentrations of plasminogen and the proteinase inhibitors to be tested. Serum-free medium with or without plasminogen and proteinase inhibitors was incubated in parallel wells as a control. At the end of the incubation, 800 μl aliquots of the supernatants were collected, and radioactive degradation products measured in a Camberra Packard liquid scintillation counter (Garbisa et al., 1980). Samples and controls were assayed in duplicate. Unless otherwise indicated, type IV collagen degradation is reported in the figures as percentage of the total substrate after subtraction of background radioactivity released by control medium with or without plasminogen and the proteinase inhibitors (3–5% of the total radioactivity).

**Assay for plasminogen activator (PA) or plasmin activity**

1–10 μg of cell extract protein or 10–50 μl of cell-conditioned medium was tested for PA activity by the 125I-fibrin assay (Gross et al., 1982) in the presence of 2 μg/ml of pure plasminogen. To measure receptor-bound uPA activity, confluent cells in 16-mm culture wells were washed twice with PBS and incubated with 100 μl/well of 50 mM glycine-HCl, 0.1 M NaCl, pH 3.1 at 22°C for 3 min with mild shaking (Stoppelli et al., 1986). After neutralization with 100 μl of 0.5 M HEPES, 0.1 M NaCl, pH 7.5, 50 μl aliquots of the acid wash were tested for uPA activity by the 125I-fibrin assay (Gross et al., 1982). Equivalent volumes of a mixture of glycine-HCl with the HEPES buffers were used as a control. To measure plasmin activity 150–250 μg of cell extract protein or 150–250 μl of cell-conditioned medium were tested by the 125I-fibrin assay without adding plasminogen to the assay buffer. Samples and controls were assayed in duplicate.

**Assay for PA inhibitor (PAI) activity**

Increasing volumes of media conditioned by the different L cell clones, diluted in 0.5 ml of 0.1 M Tris–HCl, pH 8.1 containing 250 μg/ml BSA, 4 μg/ml plasminogen and 32 μl/m (International Units)/ml of pure human uPA, were tested for their ability to inhibit uPA activity in a 125I-fibrin assay (Gross et al., 1982). Equivalent volumes of DMEM were used as controls. Samples and controls were assayed in duplicate.

**ELISA for MMP-2**

The level of MMP-2 in Lg cell-conditioned medium was quantitated by a modified substrate-capture immunonassay (Wacher et al., 1990). Ten μl of conditioned medium diluted with 90 μl of TSE (50 mM Tris–HCl, 20 mM NaCl, 10 mM EDTA, pH 7.6) were incubated overnight at 4°C in gelatin-coated, flat-bottom microtiter wells (Nuncson, Roskilde, Denmark). The captured MMP was detected by 3 h incubation at 4°C with a 1:500 dilution of rabbit antisem (4221) to affinity-purified human MMP-2, followed by incubation for 2 h at 4°C with peroxidase-conjugated goat anti-rabbit IgG antibody (1:5000). After addition of o-phenylenediamine (Bio-Rad) for 10 min at 22°C, OD₄₅₀ was measured with a Titertek spectrophotometer. The specificity of the antibody has been assessed as described (Wacher et al., 1990). Human recombinant MMP-2 was used as a standard and irrelevant IgG as a negative control.

The OD₄₅₀ readings of the samples were within the linear range of the assay. Samples and controls were assayed in triplicate.

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