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Soluble latent membrane-type 1 matrix metalloprotease secreted by human mesangial cells is activated by urokinase

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Soluble latent membrane-type 1 matrix metalloproteinase secreted by human mesangial cells is activated by urokinase.
Background. Matrix metalloprotease 2 (MMP2) is secreted in a latent inactive form (pro-MMP2) that is activated on the cell surface by a membrane-type 1 MMP (MT1-MMP) in the presence of the tissue inhibitor of MMP (TIMP2). In spite of evidence for the synthesis of MT1-MMP shown by immunoblotting, immunocytochemistry and RT-PCR, and of TIMP2, MMP2 was found exclusively in a latent form in human mesangial cells (HMC) serum-free culture medium.

Methods and Results. On purified membranes of HMC, MT1-MMP was found in a 63 kD latent form and as a faint band of 55 kD. The 55 kD band was also present in the ultracentrifuged conditioned medium and likely represented MT1-MMP cleaved from its transmembrane domain, since Northern blot analysis showed only one transcription product. The addition of urokinase plasminogen activator (uPA, 100 nM) to HMC membranes induced the activation of pro-MMP2 via the activation of latent membrane-associated MT1-MMP as reflected by the cleavage of the 63 and 55 kD forms. In addition, when the conditioned medium was successively incubated with uPA and α_2-macroglobulin and analyzed by immunoblotting, MT1-MMP decreased, indicating that the soluble MT1-MMP was in a latent form and was activated by uPA.

Conclusion. Our results provide the first evidence, to our knowledge, of the existence of a soluble latent form of MT1-MMP secreted by primary human cells in culture, confirming that MT1-MMP is an ectoenzyme, and show that uPA can regulate MT1-MMP activity in a soluble phase.

The integrity of the extracellular matrix is a critical parameter in glomerular function. Proteinuria, due to an increase of the permeability of the glomerular basement membrane on the one hand, and mesangial expansion leading to glomerulosclerosis and renal failure on the other hand, are two major hallmarks of glomerular disease. In both cases, a dysregulation of the activity of proteolytic enzymes, particularly matrix metalloproteases (MMPs), and of the plasminogen activator system may be implicated [reviewed in 1, 2]. Several studies have demonstrated matrix degrading enzymes in human and rat glomeruli and, in particular, the existence of MMP2 (gelatinase A, 72 kD) and of MMP9 (gelatinase B, 92 kD) [3–6]. MMP2 and MMP9 have attracted much attention because they degrade type IV collagen, which is a major component of physiological basement membranes and extracellular matrices. MMP2 and MMP9 are secreted in a latent inactive form and their mechanisms of activation are still unclear. MMP2 activation was reported to be dependent on a newly discovered membrane-type MMP, MT1-MMP, in contrast to previous reports suggesting a role of plasmin [7, 8] and of urokinase [9]. Because mesangial cells play a central role in glomerular fibrosis, their proteolytic potential has been the focus of numerous studies. Under basal conditions, mesangial cells in culture secrete only MMP2 and no urokinase [10, 11], findings that are consistent with immunohistochemical studies of kidney tissue showing the expression of MMP2 [12] but not of urokinase by mesangial cells [13, 14]. In addition to its collagen-degrading activity, it has been recently suggested that active MMP2 may play a role in the proliferation and in the inflammatory phenotype of rat mesangial cells [15]. However, MMP2 is produced in a latent form by human mesangial cells in spite of the recent demonstration of MT1-MMP [16], in contrast to rat mesangial cells where MT1-MMP is absent [15]. Several lines of evidence indicate that MT1-MMP was also secreted in a latent inactive form [17, 18], but the mechanism of activation of latent MT1-MMP has not yet been elucidated. Since in pathological conditions such as inflammation uPA can be generated in large quantities in the mesangium by infiltrating neutrophiles and monocytes/macrophages, we questionned whether uPA could play a role in the activation of pro-MT1-MMP, and therefore in MMP2 activation, thus supporting the recent data of Turck et al [15]. Our study shows that uPA was able to induce the activation of pro-MMP2 secreted by human mesangial cells in the

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presence of cells as well as in a cell-free system, and thus in the absence of the membrane-associated form of MT1-MMP. Urokinase activation of MMP2 in the absence of cells was mediated by the activation of a latent soluble form of MT1-MMP, confirming that MT1-MMP is an ectoenzyme.

METHODS

Reagents

RPMI 1640, fetal calf serum (FCS), trypsin, agarose and the enzyme for reverse transcription were from GIBCO BRL (Gaithersburg, MD, USA); L-glutamine, collagenase type I and human thrombin were from Sigma Chemical Co (St Louis, MO, USA); human high molecular weight urokinase was from Hoechst-Behring (Puteaux, France) and recombinant tPA was from Boehringer Mannheim (Mannheim, Germany); human α_2-macroglobulin was from Calbiochem (La Jolla, CA, USA). The molecular weight standards for the SDS-PAGE gels were from Bio-Rad (Hercules, CA, USA); human recombinant pro-MMP2, monoclonal antibodies to human MMP2 [Ab-3] and to MT1-MMP [Ab-1 clone 114-1F2 (Sato et al [17]) were obtained from Oncogene Science (Genzyme, Cambridge, MA, USA). Monoclonal antibodies to the active site of human uPA (MuK4) were from Biopool (Umeå, Sweden) and to the uPA receptor (uPAR) were from American Diagnostica Inc. (Greenwich, CT, USA). The ECL kit, the goat anti-mouse IgG and (α-^{32}P) dCTP were purchased from Amersham Life Science (Buckinghamshire, UK). All other reagents were of analytical grade.

Cell culture

Human glomerular mesangial cells (HMC) were isolated as described previously [10], cultured in RPMI 1640 containing 2 mM L-glutamine and 10% heat inactivated FCS, and characterized as reported [10]. The cells were used between passes two and four. The human fibrosarcoma HT 1080 cell line was obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) and the cells were grown in DMEM containing 5% inactivated FCS and 2 mM L-glutamine. Conditioned medium of subconfluent mesangial cells was obtained after washing the cells and incubating them in serum-free RPMI 1640 in the presence or in the absence of plasminogen activators. At the end of the incubation period, the conditioned medium was withdrawn, centrifuged 15 minutes at 14,000 g or for some experiments ultracentrifuged two hours at 35,000 g and stored at −30°C until analysis. Conditioned medium was concentrated 25 times with Amicon centriprep columns (Witten, Germany).

Membrane preparation

Plasma membranes were prepared from HT 1080 and from mesangial cells. Briefly, the cells were washed in cold Krebs-Heinseleit buffer (118 mM NaCl, 5 mM KCl, 1.1 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, pH 7.4), resuspended in homogenization buffer (5 mM Tris-HCl pH 7.4, containing 0.25 M sucrose, 1 mM EGTA, 500 units/ml of Trasylol, and 1 mM PMSF) and homogenized at 4°C in a 50-ml Potter homogenizer. Three ml of the homogenate was loaded onto 2 ml of 1.45 M sucrose in 20 mM Tris-HCl, pH 7.4 and centrifuged at 35,000 g for 30 minutes in a Beckman SW 28 rotor. The membranes at the interface were collected, pelleted for 20 minutes at 40,000 g and washed three times in 10 mM HEPES pH 7.4, containing 0.25 M sucrose, 5 mM $MgCl_2$, 0.2 mM $CaCl_2$, 250 units/ml of Trasylol and 0.5 mM PMSF. The membranes were resuspended in the same buffer and stored at −80°C.

Gelatin zymography and reverse zymography

Gelatinolytic activity of the conditioned media was visualized by SDS-polyacrylamide gels (7.5% PAGE, unless otherwise stated) containing 1 mg/ml gelatin. After electrophoresis, the gels were soaked in Triton X100 2.5% for one hour and incubated in Tris-HCl 50 mM pH 7.5, $CaCl_2$ 5 mM, $ZnCl_2$ 1 μM overnight at 37°C and stained with Coomassie Blue dye. Reverse gelatin zymography was performed by incubating the gels in the same buffer containing 1 volume of HT 1080 serum-free conditioned medium for 24 hours at 37°C.

Immunoblot analysis

After electrophoresis on SDS-PAGE, the proteins were transferred onto PVDF membrane (Millipore) with a Trans-Blot semi-dry transfer cell (Bio-Rad). After blocking one hour at 37°C in 2% bovine serum albumin in Tris 50 mM pH 7.5, NaCl 100 mM (TBS) containing 0.1% Tween 20, the membrane was incubated with antibodies to MMP2 (1 μg/ml) or to MT1-MMP (10 μg/ml) for 18 hours at 4°C. After extensive washing in TBS 0.1% Tween 20, the goat anti-mouse IgG was added and the incubation prolonged for one hour at room temperature. The blots were revealed by chemoluminescence with the ECL kit.

Immunogold silver staining (IGSS)

Mesangial cells grown on cover slips were fixed in 1.25% glutaraldehyde in phosphate buffer saline (PBS, 0.13 M NaCl, 2.6 mM KCl, 1.4 mM KH_2PO_4, 4 mM Na_2HPO_4) for 10 minutes and rinsed in PBS. The cells were incubated with anti-human MT1-MMP (10 μg/ml) in PBS containing 1% bovine serum albumin (PBS-BSA), overnight at 4°C. After washing in PBS-BSA, the cells were incubated with gold-labeled (1 nm) anti-mouse IgG for three hours at room temperature. The slides were washed in distilled water before amplification with a silver enhancement reagent at room temperature, and counterstained with Giemsa. Photographs were taken with combined brightfield and epipolarization microscopy.

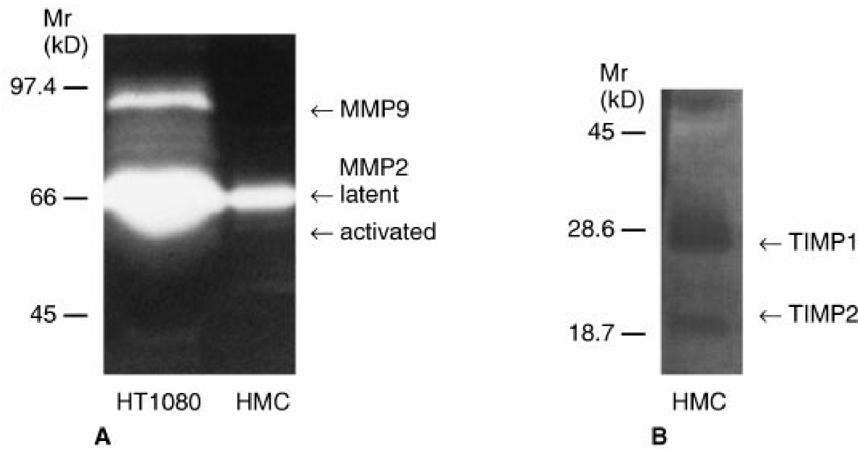

Fig. 1. Human mesangial cells secrete inactive latent form of MMP2 and TIMP2. (*A*) The gelatinolytic activity of the serum-free conditioned medium of human mesangial cells and of HT 1080 cells were analyzed in a 7.5% SDS-PAGE gel containing 1 mg/ml gelatin and incubated as described in the text. The gelatinolytic activity was visualized by Coomassie blue staining. (*B*) Visualization of TIMPs activities by reverse gelatin zymography analysis in a 14% SDS-PAGE gel. The molecular weight markers are on the left.

Northern blot analysis

Total RNA was isolated using the guanidium isothiocyanate method [19]. Twenty micrograms of total RNA were analyzed by Northern blot using a ^{32}P-labeled 550 bp human MT-MMP cDNA obtained by RT-PCR (see below). The membranes were hybridized overnight at 42°C, washed in 0.2 × SSC 0.1%SDS twice for 15 minutes at room temperature then twice at 42°C and finally at 65°C.

Amplification of MT1-MMP mRNA from HMC by RT-PCR

The RNA messenger from HMC and HT 1080 was reverse transcribed using random hexamers. Twenty-five nanograms of cDNA were used as a template in a PCR reaction. The sense primer 5′-CCCTATGCCTACATC-CGTGA-3′, corresponding to nucleotides 598 to 617 of human MT1-MMP and the antisense primer 5′-TCCATC-CATCACTTGGTTAT-3′, complementary to nucleotides 1129-1148 of human MT1-MMP were used as described [20]. PCR was performed by standard techniques using *Taq* polymerase, repeating 35 cycles of a 60-second denaturation step at 94°C, a 60-second annealing step at 60°C and a 60-second extension step at 72°C. The reaction amplified a 550-bp product which was analyzed on a 1.5% agarose gel. The mesangial cell PCR products of 10 reactions were combined, eluted and used as a probe for Northern analysis after sequencing.

Pro-MMP2 purification

Latent MMP2 was purified from HMC serum-free conditioned media. After SDS-PAGE electrophoresis, the gel was cut at position 68 kD band and pro-MMP2 was electro-eluted. The recovery of a latent form of MMP2 was further assessed by gelatin zymography and immunoblotting.

RESULTS

Mesangial cells secrete pro-MMP2, a membrane-associated pro-MT1-MMP, a membrane furin-processed MT1-MMP and a soluble pro-MT1-MMP

Human mesangial cells in culture secrete only MMP2 in a latent form, identified by a single lysis band with an apparent molecular weight of 68 kD on gelatin zymograms in culture media (Fig. 1A). Even in serum-free conditioned media of 48 hours, no lysis band of lower molecular weight indicative of MMP2 activation was visible (not shown). It is well established [4, 21], and we also confirmed by reverse gelatin zymography (Fig. 1B), that human mesangial cells in culture secrete TIMP2. The results of immunoblot analysis of a HMC membrane preparation (Fig. 2A), of RT-PCR (Fig. 2B) and of immunogold silver staining (Fig. 2D) demonstrated that HMC also synthesize MT1-MMP. In addition, Northern blot analysis showed only one transcript at the expected size of 4.5 kb (Fig. 2C). Immunoblot analyses of HMC membrane preparation showed two major bands that were always present, one at 63 kD that was specific for membrane-associated pro-MT1-MMP [22, 23], one at 45 kD [24], and a minor band at 55 kD. The 55 kD band occasionally observed associated with the membranes (Fig. 3, lane 1) could represent the furin-matured MT1-MMP [23] but could also correspond to latent MT1-MMP truncated at the transmembrane domain [24, 25] and being secreted in the conditioned medium. To test this hypothesis, we performed immunoblot analyses of the serum-free conditioned medium of HMC with a monoclonal antibody to human MT1-MMP. The results confirmed the existence of a soluble 55 kD protein in the concentrated ultracentrifuged serum-free conditioned medium (Fig. 3, lane 2). This 55 kD MT1-MMP could not be ascribed to alternative splicing since only one transcript was detected by Northern blot.

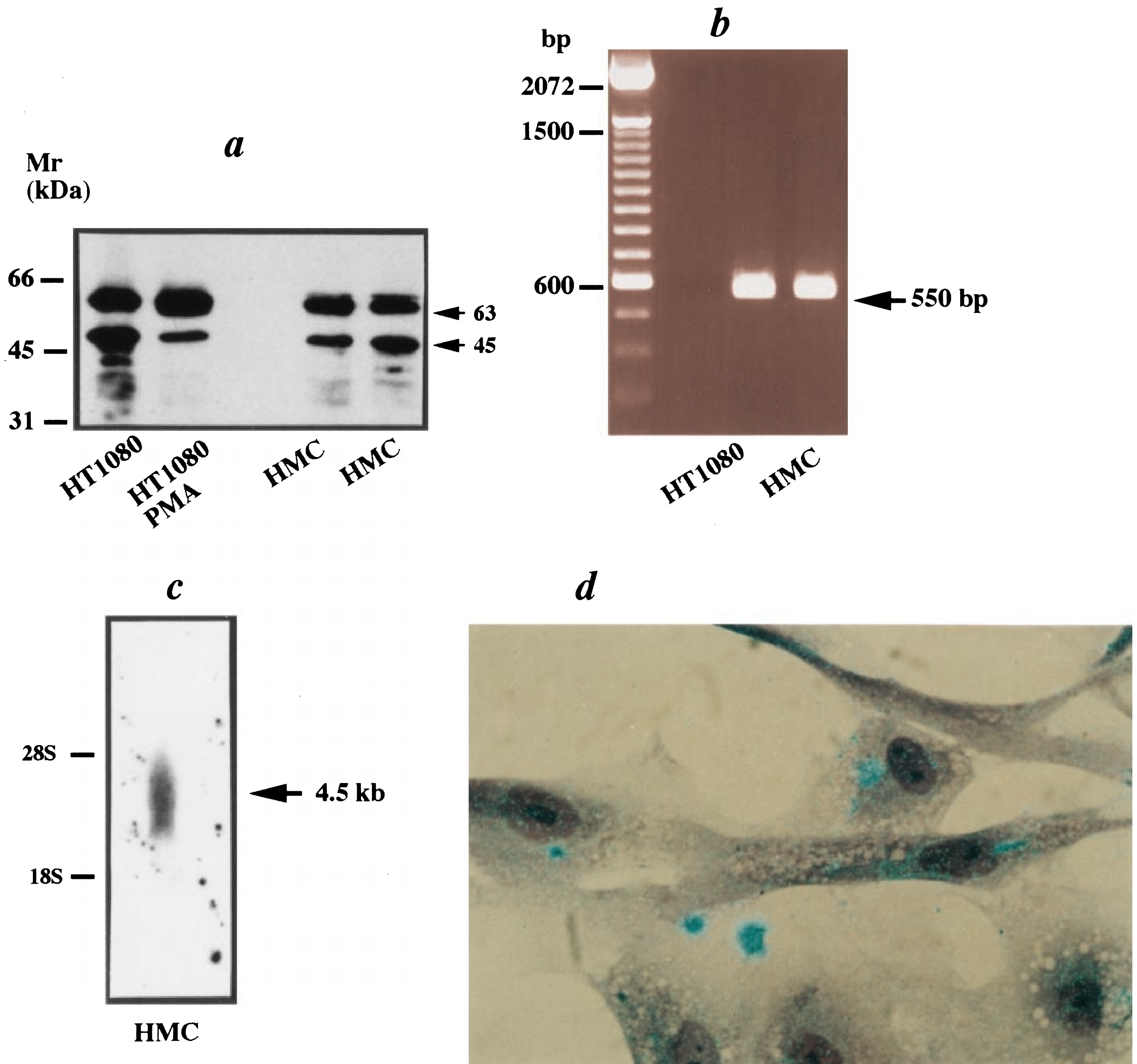

Fig. 2. Evidence for the synthesis of MT1-MMP by human mesangial cells. (*A*) Immunoblotting of human mesangial cells (HMC) membrane preparations from two different origins and from HT 1080 cells stimulated or not by PMA (5 nM) as a control (SDS-10% PAGE). The results showed two major bands at 63 and 45 kD in both cells types. (*B*) RT-PCR as described in the text. (*C*) Northern blot analysis of MT1-MMP mRNA in HMC. (*D*) Immunogold silver staining of HMC. MT1-MMP was detected in a granular pattern as bright blue staining on the surface of mesangial cells.

Activation of pro-MMP2 by addition of uPA to mesangial cells

Next we investigated the role of tPA and uPA on the generation of active MMP2. The uPA, but not tPA, could induce the activation of pro-MMP2, as shown by the appearance of a gelatinolytic band at 62 kD (Fig. 4). The activation of latent MMP2 by uPA was time-dependent (Fig. 5A). In the presence of 100 nM uPA, the activated forms of MMP2 were detectable after two hours of incubation at 37°C and after 24 hours, 30% of pro-MMP2 was activated. The activation of pro-MMP2 by uPA was also dose-dependent (Fig. 5B). The activation of MMP2 could be detected by zymography with 100 nM uPA, and this effect reached a plateau for 500 nM uPA, a concentration at which 50% of pro-MMP was cleaved. No further activation of MMP2 was observed even after prolonged incubation with uPA (not shown). Since mesangial cells secrete plasminogen activator inhibitor type 1 (PAI-1) and also possess the uPA receptor that mediates the internalization and degradation of uPA/PAI-1 complexes [26], we wanted to

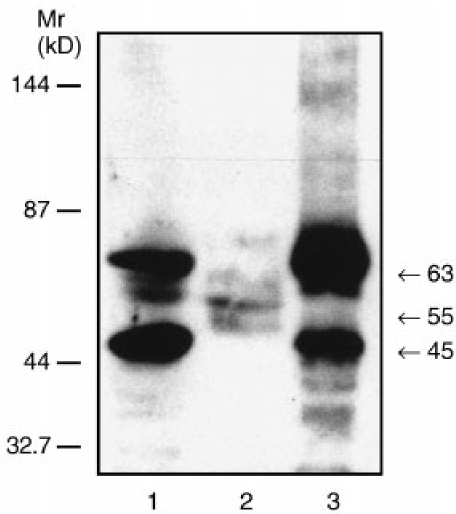

Fig. 3. Evidence for a soluble MT1-MMP in the conditioned medium of mesangial cells. Immunoblot analysis after electrophoresis by SDS-7.5% PAGE. Lane 1, HMC membranes showing the existence of the major 63 and 45 kD bands and a faint band of 55 kD; Lane 2, the ultracentrifuged conditioned medium of HMC concentrated 25 times by Amicon; Lane 3, HT 1080 cells membranes as control.

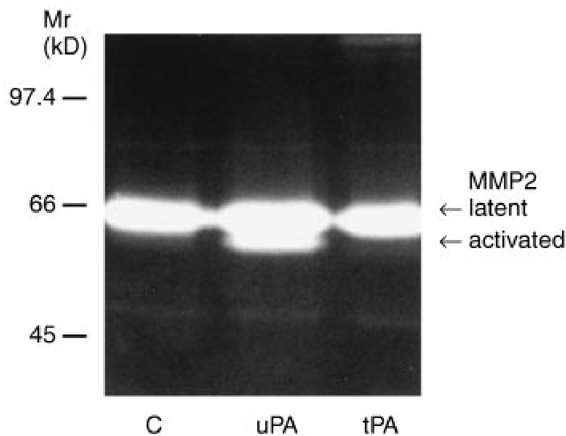

Fig. 4. Addition of uPA to mesangial cells induces the appearance of activated MMP2. uPA (100 nM) was added to serum-starved HMC and incubated for 24 hours, and the conditioned media were analyzed by gelatin zymography. As compared to control, the addition of uPA, but not of tPA, could induce the appearance of activated form of MMP2.

study the role of uPA binding to its specific receptor on the activation of pro-MMP2. The results showed no modifications of pro-MMP2 activation in the presence of antibodies blocking uPA-uPA receptor interactions, indicating that uPA binding to its receptor was not necessary. In contrast, addition of antibodies to the active site of uPA totally prevented the appearance of active forms of MMP2, indicating that the active site of uPA was necessary to pro-MMP2 activation (not shown). Next, we questionned whether the absence of total activation of pro-MMP2 could be due to the inhibition of active exogenous uPA by PAI-1 secreted by HMC, during the incubation period. The fibrin zymography confirmed the persistence of active uPA after 24 hours of incubation with HMC (not shown). Therefore the lack of total conversion of pro-MMP2 into active MMP2 was not due to active uPA exhaustion.

uPA activation of pro-MMP2 in the conditioned medium is mediated by activation of latent soluble MT1-MMP

Surprisingly, the activation of pro-MMP2 from HMC by uPA could be observed in the absence of cells. The incubation of uPA with ultracentrifuged serum-free conditioned media induced MMP2 activation to the same extent as that in the presence of cells (Fig. 6). To further study the effect of uPA on pro-MMP2, we incubated recombinant proenzyme-MMP2 with uPA at 37°C up to 24 hours. By gelatin zymography, no activation of pro-MMP2 could be observed (Fig. 7, lane 4), confirming the absence of direct cleavage of pro-MMP2 by uPA [27, 28]. In contrast, addition of ultracentrifuged serum-free conditioned medium to uPA and pro-MMP2 induced pro-MMP2 activation (Fig. 7, lane 5). One could argue that the higher concentration of pro-MMP2 in lane 5 could account for MMP2 auto-activation, as reflected by the lower lysis band, but the pro-MMP2 concentration was similar to that in lane 1 where no activation was observed. These results suggest that uPA had activated the latent soluble MT1-MMP, which in turn had activated pro-MMP2.

To confirm this hypothesis and distinguish between activated and latent proteinases we took advantage of the property of α_2-macroglobulin to form complexes with proteolytically active, but not with latent or inactive proteases. This test is based on the well documented finding that α_2-macroglobulin cannot bind to inactive proteinases [29]. Therefore, we incubated the conditioned medium with or without uPA (100 nM) for 24 hours at 37°C, followed by the addition of excess α_2-macroglobulin (100 μg/ml) and the incubation was prolonged for one hour. The conditioned media were analyzed by immunoblotting. The results showed in the control conditioned medium incubated with α_2-macroglobulin one strong band of 55 kD corresponding to soluble MT1-MMP, whereas in the conditioned medium preincubated with uPA and then with α_2-macroglobulin, the 55 kD band was hardly visible, suggesting that soluble MT1-MMP had been activated and trapped by α_2-macroglobulin (Fig. 8A). No complexes of MT1-MMP/α_2-macroglobulin could be visualized because it did not enter the gel under non reducing conditions. To strenghthen these results, the conditioned media were also analyzed for the molecular forms of MMP2 by immunoblotting (Fig. 8B). The results showed one single band of 66 kD representing latent MMP2 in the control conditioned medium incubated with α_2-macroglobulin. In contrast, in the presence of uPA and α_2-macroglobulin, two bands of 66 and 62 kD were visible, corresponding to active and activated and cleaved forms of MMP2. The extremely low level of staining of these two bands confirms that MMP2 has been activated

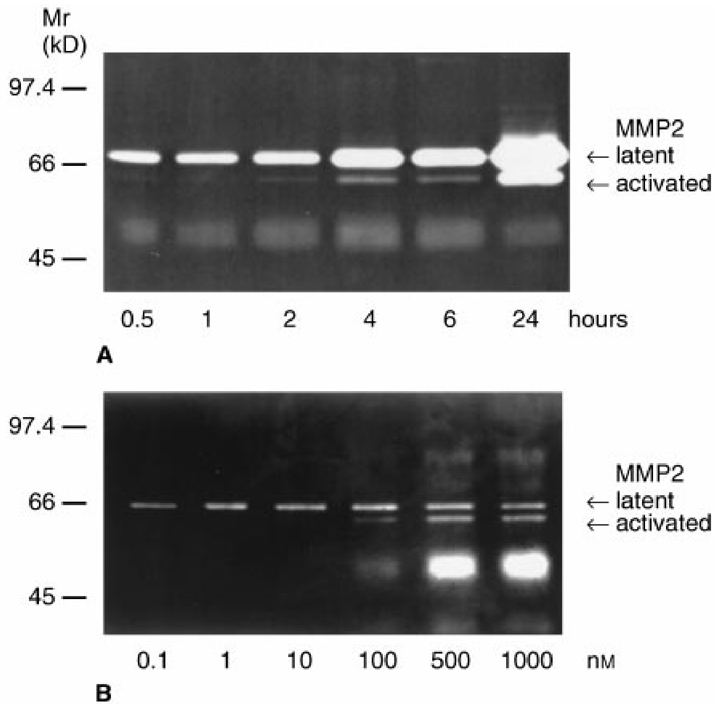

Fig. 5. Time course and dose-dependent generation of active MMP2 in the presence of uPA. (*A*) Serum-deprived HMC were incubated with uPA (100 nM), the conditioned media withdrawn at intervals and analyzed by gelatin zymography in a SDS-7.5% PAGE. A very faint band of activated MMP2 can be seen after two hours of incubation of HMC with uPA. The 54 kD lysis band was due to uPA since it was visible with uPA alone (not shown). (*B*) Serum-deprived HMC were incubated with uPA (0.1, 1, 10, 100, 500, 1000 nM). The conditioned media were withdrawn after six hours of incubation at 37°C and analyzed by gelatin zymography, as described before.

and trapped by α_2-macroglobulin (Fig. 8B). All together, these results indicated that soluble MT1-MMP was in latent form and was activated by uPA and that pro-MMP2 activation by uPA in the conditioned medium was mediated by the activation of soluble MT1-MMP.

Effect of uPA on membrane associated MT1-MMP

To study the effects of uPA on latent membrane-associated MT1-MMP, mesangial cell membranes were incubated with uPA 100 nM at 37°C for 15 minutes up to 24 hours, and the molecular form of MT1-MMP analyzed by immunoblot (Fig. 9). In the presence of uPA, the 63 kD and 55 kD bands of membrane-associated MT1-MMP progressively disappeared, and the intensity of the 45 kD band remained unchanged even after 24 hours of incubation with uPA. These results suggested that incubation with uPA provoked MT1-MMP activation followed by further degradation either due to uPA or to MT1-MMP autoproteolysis into lower molecular weight degradation products [18, 30].

DISCUSSION

Pro-MMP2 activation can be achieved *in vitro* by multiple ways. Autocatalysis may be observed in soluble phase for high concentrations of pro-MMP2 [15, 27, 31], and it has been recently demonstrated that pro-MMP2 activation could depend on a membrane-associated MMP, MT1-MMP [17, 24]. MT1-MMP would serve to concentrate MMP2 on the cell surface where pro-MMP2 may undergo activation by autocatalysis [32] after limited proteolysis by active MT1-MMP [18, 33, 34], or would be activated via the formation of a trimolecular complex with MT1-MMP and TIMP2 [33, 34]. The relationship between MT1-MMP structure and function has been extensively studied with a particular interest for the transmembrane domain. Contradictory results concerning the capacity of the transmembrane domain-deleted mutants to activate pro-MMP2 have been reported [17, 18, 23, 24], although several lines of evidence showed that this domain is not necessary for activation of pro-MMP2 [18, 23]. Even though the discrepancies may be explained by different experimental conditions, it is likely that pro-MMP2 activation is not an unequivocal process.

As for the cleavage of MT1-MMP from the cell surface, the mechanism of its activation is still unclear. Will et al have shown that trypsin, a serine protease, was effective *in vitro* for the activation of a soluble catalytic domain of MT1-MMP [18], and Strongin et al have shown the existence of a functional activated 60 kD form of MT1-MMP in HT 1080 fibroblastic cell line [34]. The latter authors suggested that serine proteases such as plasmin or plasminogen activators might be good candidates for the activation of latent MT1-MMP. Since HT 1080 are known to secrete large amounts of urokinase and some tPA [35, 36], it is likely that one of these plasminogen activators was responsible for the activation of pro-MT1-MMP. An interaction between uPA and the metalloprotease proteolytic pathways has been suggested for a long time [9, 37]. Although the synthesis of uPA by human mesangial cells in culture remains controversial, some authors claiming that there was no synthesis of uPA [10, 11] whereas others have

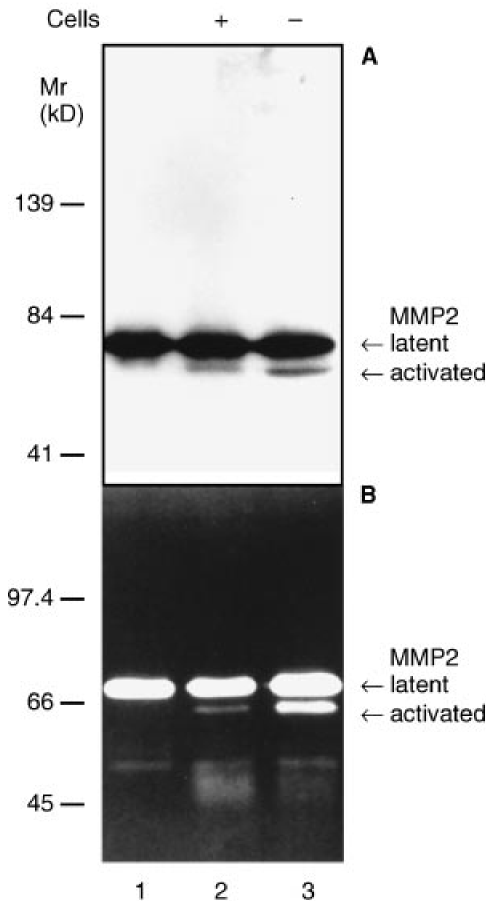

Fig. 6. Active MMP2 generation by uPA in a cell-free system. The molecular forms of MMP2 in the conditioned medium of HMC in the presence of uPA added to the cells or to the ultracentrifuged conditioned medium were analyzed (*A*) by immunoblotting and (*B*) by zymography. Lane 1, conditioned medium of serum-starved HMC; Lane 2, conditioned medium of HMC incubated with uPA (100 nM) for 24 hours; Lane 3, ultracentrifuged conditioned medium incubated with uPA (100 nM) for 24 hours. The molecular weight markers are on the left.

demonstrated the contrary [7], in our experimental conditions, human mesangial cells in culture do not synthesize uPA. They synthesize a little tPA that is complexed to plasminogen activator inhibitor-1 in the conditioned medium [10]. Therefore, mesangial cells constitute a good model to study the effect of uPA on pro-MMP2 and MT1-MMP activation. The fact that uPA but not tPA nor plasmin (personal observation) could activate MT1-MMP is of major importance given the recent data of a close correlation between active MMP2 and the inflammatory phenotype of rat mesangial cells *in vitro* [15]. Turck et al have shown that rat mesangial cells transfected with MMP2 antisense RNA had a reduced proliferation rate and that reconstitution with active MMP2 restored the proliferating rate [15]. These data are further supported by the *in vivo* data of increased MMP2 mRNA and activity, associated with increased glomerular cellularity in anti-Thy 1.1 glomerulonephritis in rat [12] and in sclerotic murine glomeruli from mice transgenic for the bovine growth hormone [38]. Human mesangial cells with an "activated" phenotype can be encountered *in vivo* in acute glomerulonephritis associated with mesangial cell proliferation and expression of smooth-muscle alpha actin, and with uPA production in large quantities by infiltrating cells, neutrophils and monocytes/macrophages. In these pathological conditions, uPA may play a crucial role in activating pro-MMP2 via activation of MT1-MMP. Our results support the recent data of Mazzieri et al [39], who found that urokinase plays a role in the activation of pro-MMP2, but we could not demonstrate the role of uPA/uPA receptor interaction on MMP2 activation, nor could we reproduce the activation of pro-MMP2 by plasmin.

The existence of a soluble form of MT1-MMP has been suspected by Okada et al, who found in human carcinomas that MT1-MMP transcripts were detected in stromal cells but not in cancer cells, whereas the protein was detected in cancer cells [40]. Therefore, they suggested that MT1-MMP may be an ectoenzyme generated by cleavage at the cell surface. Imai et al have described the secretion, in the culture medium of a human breast carcinoma cell line stimulated with Concanavalin A, of a 56 kD propeptide-deleted MT1-MMP complexed to TIMP2 [25]. MT1-MMP would have been processed at the cell surface, then complexed to TIMP2, and the complex released in the conditioned medium after pro-MMP2 activation. This may not be the case for human mesangial cells since we were unable to detect any active MMP2 in basal conditions. Furthermore, Cao et al have observed in the culture medium of COS cells transfected with a MT1-MMP deletion mutant lacking the transmembrane domain, the presence of a 54 kD protein recognized by two different antibodies to MT1-MMP [24]. Taken together with the reports of Pei and Weiss [23] and of Cao et al [24], our data strongly suggest that MT1-MMP exists in at least three forms: membrane-associated, furin-processed, and soluble MT1-MMP. The latter is a latent form and needs a cooperating factor(s) to be activated. The role of uPA on soluble MT1-MMP activation may have important physiological implication, since recently Sawicki et al have described a new pathway of platelet aggregation involving MMP2 [41]. They showed that the platelet releasate contained latent MMP2. However, platelet aggregation was induced by active but not latent MMP2, and the mechanism of MMP2 activation has not been elucidated. Since active uPA is associated to platelet membranes [42], it is possible that membrane-bound and soluble MT1-MMP activation by uPA may occur at the platelet surface or in a close vicinity, thus allowing the activation of pro-MMP2 released from the platelets. These issues are currently under investigation.

In conclusion, we have shown the existence of a soluble latent form of MT1-MMP secreted by primary human mesangial cells in culture, thus confirming that MT1-MMP is an ectoenzyme. We have also shown that uPA can

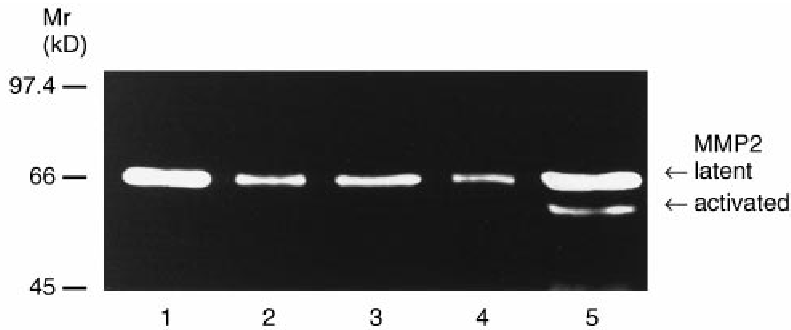

Fig. 7. The conditioned medium of HMC is necessary for uPA activation of pro-MMP2. Human recombinant pro-MMP2 was incubated with uPA alone or with conditioned medium. Lane 1, conditioned medium; Lane 2, recombinant pro-MMP2; Lane 3, conditioned medium added to recombinant pro-MMP2; Lane 4, pro-MMP2 incubated with uPA (100 nM) for 24 hours at 37°C; Lane 5, as in lane 4 plus conditioned medium. The molecular weight markers are on the left.

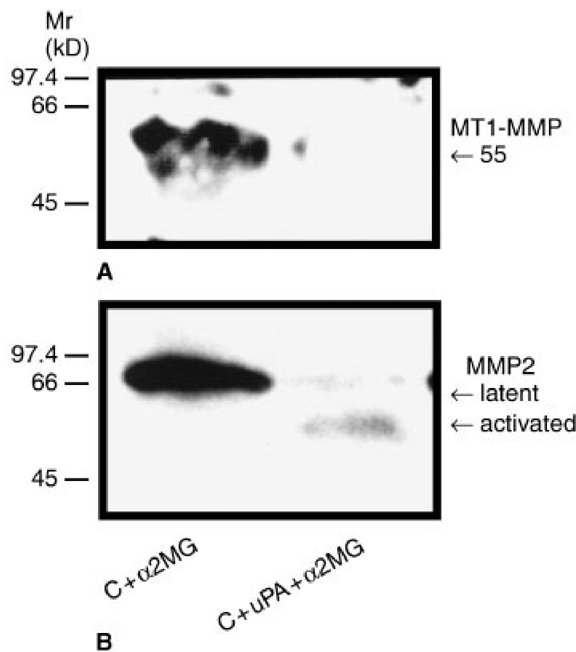

Fig. 8. Demonstration of the latency of the soluble MT1-MMP and activation by uPA. Concentrated conditioned medium was incubated for 24 hours at 37°C without or with uPA (100 nM), excess α_2-macroglobulin (100 μg/ml) was then added and the incubation prolonged for one hour. (*A*) MT1-MMP and (*B*) MMP2 were analyzed by immunoblotting.

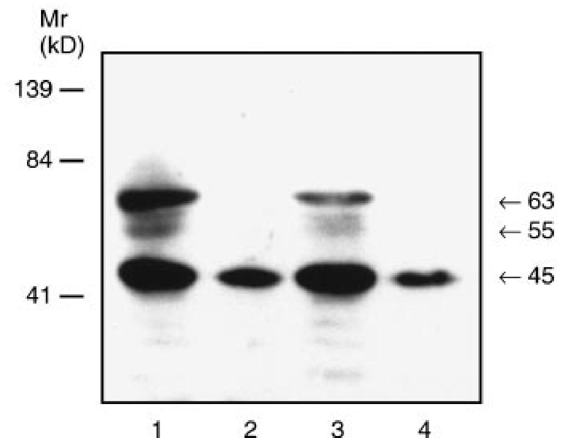

Fig. 9. uPA cleaves the membrane associated MT1-MMP. Purified membranes of human mesangial cells (10 μg) were incubated with uPA (100 nM) for 1 or 24 hours and the molecular forms of membrane-associated MT1-MMP analyzed by immunoblotting. Membranes alone incubated at 37°C, one hour (lane 1) and 24 hours (lane 3). Membranes incubated with uPA (100 nM), one hour (lane 2) and 24 hours (lane 4).

activate latent membrane-associated and soluble MT1-MMP.

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APPENDIX

Abbreviations used in this article are: FCS, fetal calf serum; HMC, human mesangial cells; IGSS, immunogold silver staining; MMP2, matrix metalloprotease 2; MT1-MMP, membrane-type 1 matrix metalloprotease; PAI-1, plasminogen activator inhibitor type-1; PBS-BSA, phosphate buffered saline-bovine serum albumin; RT-PCR, reverse transcribed-polymerase chain reaction; TIMP, tissue inhibitor of matrix metalloprotease; tPA, tissue plasminogen activator; uPA, urokinase plasminogen activator; uPAR, urokinase plasminogen activator receptor.

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