Urokinase upregulates matrix metalloproteinase-9 expression in THP-1 monocytes via gene transcription and protein synthesis

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Urokinase-type plasminogen activator (uPA) is suggested to exert its proliferatory, migratory and invasive action through binding with its membrane receptor, promoting pericellular proteolysis and mediating cell signal transduction. One of the possible actions of urokinase can be related to the regulation of activity and/or the expression of proteolytic enzymes participating in extracellular matrix degradation. In the present study, the role of uPA in regulating matrix metalloproteinase (MMP) expression and release by the monocyte cell line THP-1 was investigated. Recombinant uPA induced the release of MMP9/gelatinase B, as detected by zymography and Western blotting, and this release was abolished by actinomycin D and cycloheximide (inhibitors of DNA transcription and protein synthesis) and partially suppressed by monensin (an inhibitor of secretion). Proteolytically inactive urokinase with substitution of His^{204} for Gln was able to reproduce about 70% of the effect induced by the wild-type recombinant uPA. The reverse transcription-PCR and Northern blot data indicated that the action of r-uPA on THP-1 cells resulted in formation of MMP9 mRNA, which depended on time, within 6–48 h, of the cell incubation with r-uPA. These results suggest that urokinase upregulates MMP9 expression in monocytes via MMP9 gene transcription and protein biosynthesis.

Key words: mRNA transcription, protein secretion.

INTRODUCTION

Matrix metalloproteinases (MMPs) are a family of Zn-dependent proteolytic enzymes that specifically degrade protein components of the extracellular matrix. On the basis of their substrate specificity and structural features, MMPs can be subdivided into the following groups: collagenases, gelatinases, elastases, stromelysins and membrane-type matrix metalloproteinases (although at present some MMPs cannot be classified into these groups [1]). The group of gelatinases/type IV collagenases includes two enzymes, gelatinase A (MMP2, 72-kDa) and gelatinase B (MMP9, 92-kDa), which have similar specificities to type IV collagen, a component of the vascular basal membrane. MMP9 represents a significant part of the MMP activity released by leukocyte cells (neutrophils, lymphocytes, monocytes and macrophages) after induction by growth factors and cytokines [2–4].

Monocytes have been implicated in a number of vascular pathologies involving inflammation and vascular remodelling [5]. In atherosclerotic lesions, monocytes can secrete a variety of MMPs [6,7], which promote plaque instability and lead to myocardial infarction or stroke. Urokinase-type plasminogen activator (uPA), together with its membrane receptor, a glycosylphosphatidylinositol-anchored protein [8], has also been implicated in the pathophysiology of unstable atherosclerotic lesions, where its effects are thought to be mostly due to plasmin generation. Such mechanisms can contribute to gelatinase B activation [9]. Correlations between the expression of uPA and gelatinase B under many physiological and pathological conditions suggest that uPA might also regulate gelatinase B expression [10–12]. If this is true, such a mechanism would have additional implications for cell migration, cell invasion and the development of unstable atherosclerotic lesions.

In the present study we investigated whether uPA stimulates monocytes to produce and release MMPs. We demonstrate that uPA induces mRNA formation, protein synthesis and secretion of latent MMP9 by THP-1 monocytes.

EXPERIMENTAL

Materials

RPMI-1640 medium and fetal calf serum (FCS) were purchased from Life Technologies (Rockville, MD, U.S.A.), and actinomycin D, cycloheximide and monensin were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Mouse IgG1 anti-(rat/human MMP-9) monoclonal antibody (clone IIa5), which recognizes both latent and activated forms of MMP9 and show no cross-reaction with other MMPs, was from Research Diagnostics (Flanders, NJ, U.S.A.). The goat anti-(mouse IgG) conjugated with horseradish peroxidase, and chemiluminescent Super Signal Substrate were from Pierce (Rockford, IL, U.S.A.).

Random hexanucleotide primers, deoxynucleoside phosphates, Moloney-monkey-leukemia virus (MMLV) reverse transcriptase (RT), and Wizard™ PCR Preps DNA Purification System were from Promega (Madison, WI, U.S.A.). TaqStart antibody was from BD Biosciences ClonTech (Palo Alto, CA, U.S.A.), Taq polymerase was from Biomaster (Moscow, Russia), and the PCR primer pairs for glyceraldehyde-3-phosphate dehydrogenase (G3PDH) and MMP9 were provided by AZT Pharmaceutical Company (Moscow, Russia). Nylon membrane (Hybond™-N+) was purchased from Amersham (Little Chalfont, Bucks., U.K.) and tetramethylamonium chloride was from Fluka (Buchs, Switzerland). Recombinant uPA with wild-type structure (r-uPA) and proteolytically inactive urokinase, in which the active-

Abbreviations used: FCS, fetal calf serum; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; MMP, matrix metalloproteinase; RT, reverse-transcriptase; uPA, urokinase-type plasminogen activator; r-uPA, recombinant wild-type uPA; $r\text{-}uPA^{H/Q}$, proteolytically inactive uPA in which the active centre His^{204} residue is replaced by Gln; uPAR, urokinase plasminogen activator receptor.

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centre His^{204} residue is replaced by Gln (r-$uPA^{H/Q}$), were expressed in *Escherichia coli* and purified as described previously [13,14].

Cell culture and stimulation

THP-1 monocytic cells were cultured in a CO_2 incubator at 37 °C in RPMI 1640 medium supplemented with penicillin (100 units/ml), streptomycin (100 units/ml) and 10 % (v/v) FCS. Prior to experimentation with uPA and/or other substances, the concentration of cells was adjusted to 5×10^5/ml by suspension in RPMI 1640 supplemented with 0.5 % (v/v) FCS. Inhibitors of gene transcription and protein synthesis, actinomycin D and cycloheximide, as well as monensin, the Golgi-disturbing agent, were added to cell suspensions 1 h before a 48 h incubation with urokinase. To determine the dependency of MMP9 activity, protein and mRNA production on the time of incubation with r-uPA, the cells were exposed for 0, 6, 12, 24 and 48 h to 20 nM r-uPA, the concentration that induces submaximal MMP9 secretion by THP-1 cells as detected by zymography [15]. Following the cell incubation with uPA, the conditioned media was collected and immediately frozen until zymography for gelatinase activity and Western blotting with specific MMP9 antibody (see below) was performed. The cells treated with uPA, as well as control cells, were used for total RNA isolation and cDNA amplification.

MMP9 detection by Western blotting

Conditioned media were collected from THP-1 cells treated with either a saline control or r-uPA. The conditioned media were heated for 5 min at 100 °C in the sample buffer [0.0625 M Tris/HCl (pH 6.8), 2 % (w/v) SDS, 10 % glycerol (v/v), 5 % (v/v) β-mercaptoethanol and 0.001 % (w/v) Bromophenol Blue] then subjected to electrophoresis on a 7.5 % polyacrylamide/SDS gel, followed by transfer on to PVDF membranes. After overnight incubation (4 °C) in 5 % (w/v) skimmed milk in Tris-buffered saline, the membrane was incubated with mouse anti-(rat/human MMP-9) monoclonal antibody (clone IIa5; diluted at 1:500) for 1 h at 23 °C. MMP9 was detected by enhanced chemiluminescence with goat anti-[mouse IgG(H+L)]-horseradish peroxidase.

Gelatin zymography

Conditioned media were mixed with sample buffer (see above) without 2-mercaptoethanol and electrophoresed in a 7.5 % polyacrylamide/SDS gel containing 0.2 % (w/v) gelatin. The gels were washed in 2.5 % (v/v) Triton X-100 and incubated for 18 h in a buffer consisting of 50 mM Tris/HCl (pH 8.0), 50 mM NaCl, 0.05 % (v/v) Brij 35, and 10 mM $CaCl_2$. The gels were then stained with 0.25 % (w/v) Coomassie Brilliant Blue G-250 in 40 % (v/v) methanol and 10 % (v/v) acetic acid, followed by washing in a mixture of 40 % (v/v) methanol and 10 % (v/v) acetic acid. Proteolytic bands were detected by examining unstained regions on the gelatin-stained background. Zymograms were subjected to densitometric analysis using PCBAS software, and regions with gelatinolytic activity were expressed in arbitrary units.

RNA extraction and cDNA synthesis

Total RNA was extracted from human THP-1 cell line by a modified guanidine thiocyanate method [16]. Total RNA (1.5 μg) was reverse-transcribed at 37 °C for 1 h using a mixture (20 μl) containing 490 pmol of random hexanucleotide primers, 1 mM each of the deoxynucleoside triphosphates (dNTPs), 1 × RT buffer and 200 units of MMLV RT.

PCR

G3PDH and MMP9 were amplified using the following primers pairs: G3PDH reverse primer, 5′-TCCACCACCCTGTTGCTGTA-3′, and forward primer, 5′-ACCACAGTCCATGCCATCAC-3′ (coordinates of the primer's 5′-end are 1037 and 586 bp respectively; GenBank accession number NM_002046; PCR product size 452 bp); MMP9 reverse primer, 5′-GGAATGATCTAAGCCCAGCG-3′, and forward primer, 5′-GTGCGTCTTCCCCTTCACTTTCCT-3′ (coordinates of the primer's 5′-end 1256 and 1057 bp respectively; GenBank accession number XM_009491; PCR product size 199 bp). PCR amplification was carried out with cDNA, obtained from 30 ng of total RNA, in a 25 μl reaction mixture containing 10 mM Tris/HCl (pH 8.5), 50 mM KCl, 2 mM $MgCl_2$, 200 μM dNTPs, primers (0.5 μM each), 2.5 units of Taq polymerase and 1 μg of TaqStart antibody, using an Omn-E DNA Thermal Cycler (Hybaid Interactive, Teddington, Middx., U.K.). Tetramethylammonium chloride (60 mM) was added to the reaction mixture to increase the PCR yield and specificity [17]. The thermal profile was as follows: 94 °C for 5 min, followed by 33–39 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 40 s. Aliquots (5 μl) of each PCR mixture were run on a 1.5 % (w/v) agarose gel in Tris/borate/EDTA electrophoresis buffer with ethidium bromide (1 μg/ml). The fluorescence-stained bands corresponding to the PCR products were quantified using the AlphaImager™ System (Alpha Innotech Corporation, San Leonardo, CA, U.S.A.).

Quantitative PCR

The PCR products (amplicons) obtained during the THP-1 cDNA amplification with primer pairs specific for G3PDH and MMP9 genes were purified using Wizard™ PCR Preps DNA Purification System, and DNA concentration was determined spectrophotometrically at 260 nm. The DNA was then used as an internal standard in the PCR as described previously [18]. The same amount of target cDNA was co-amplified with serially diluted internal DNA standard, in concentrations varying from zero to values higher than the concentration of the target cDNA. PCR amplification was carried out using 35 cycles, the number chosen in preliminary experiments, to ensure that the product was detectable on gels, but its production did not reach the plateau. Aliquots of the products were subjected to agarose gel electrophoresis and bands corresponding to the expected PCR product size were processed as described above. The initial amount of cDNA amplified fragment flanked with the gene-specific pair of primers was determined as follows:

PCR amplification is described by

$$A = m \times (1 + b)^n \quad (1)$$

where A is the amount of the product synthesized after n cycles of amplification, m is the initial amount of target DNA and b is the amplification factor ($0 < b < 1$). In the presence of the internal standard

$$A_I = (m + M_s) \times (1 + b)^n \quad (2)$$

where M_s is the amount of added internal standard. For the exponential phase of the reaction

$$A_i/A_o = (m + M_s)/m \quad (3)$$

where A_o is the amount of product synthesized without added internal standard, or

$$(A_i - A_o)/A_o = M_s/m \quad (4)$$

From eqn 4 it follows that if the amount of the internal standard (M_s) is equal to the initial amount of the target DNA (m), then $(A_i - A_o)/A_o$ is equal to 1. So the initial amount of target DNA can be determined by identifying the intersection point of $(A_i - A_o)/A_o$ plotted against M_s and the horizontal line $(A_i - A_o)/A_o = 1$ (Figures 5A and 5B). In order to express the amount (m) as the number of molecules (q), the following equation can be used:

$$Q = m \times N_A / Mw_s \quad (5)$$

where N_A is Avogadro's number and Mw_s is the molecular mass of the internal standard.

Northern blot hybridization

Total RNA extracted from THP-1 cells (as described above) was denatured with 1 M glyoxal and 50 % (v/v) DMSO, separated on a 1 % (w/v) agarose gel and stained with ethidium bromide, to assess RNA quality, and transferred to a nylon membrane (Hybond™-N+) under alkaline conditions. Hybridization was performed at 42 °C for 16 h with an α-^{32}P-radiolabelled MMP9 probe obtained in a standard random oligonucleotide-primed reaction with Klenow fragment. The membrane was placed in a PhosphorImager SI exposure cassette for 14 days, and scanned using the PhosphorImager scanning instrument (Molecular Dynamics, Little Chalfont, Bucks., U.K.) and quantified using the ImageQuant® program supplied. The membrane was re-probed with the α-^{32}P-radiolabelled G3PDH probe to normalize data on the MMP9 mRNA.

RESULTS

Urokinase stimulates the release of MMP9/gelatinase B in THP-1 monocyte cells

We have recently shown that r-uPA stimulates monocyte THP-1 and U937 cells to release a $\approx$ 90 kDa protein with gelatinase activity, which was not inhibited by aprotinin, leupeptin and PMSF, but was abolished by EDTA, confirming its identity as a 92 kDa matrix metalloproteinase MMP9 (gelatinase B) [15]. In the present study, we definitively demonstrate by Western blotting that the previously detected 92 kDa protein with MMP activity is MMP9 (Figures 1A and 1B). Importantly, no proteins of less than 92 kDa with gelatinase activity were detected, in particular, the 85 kDa active form of MMP9. Thus uPA induces only latent MMP9 secretion. The effect of uPA on the secretion of latent MMP9 was time-dependent, detectable after 12 h and progressively increased during the 48 h period (Figure 1).

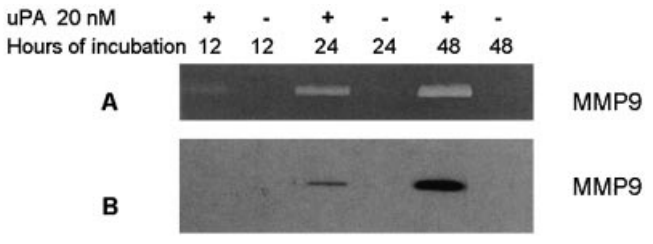

Figure 1 MMP9 production by THP-1 cells induced by urokinase

(**A**) A typical zymogram of conditioned media from THP-1 cells, incubated in the presence or absence of 20 nM urokinase (uPA) for the indicated time intervals. (**B**) A typical Western blot showing MMP9 protein detection in conditioned media from THP-1 cells incubated in the presence or absence of 20 nM urokinase.

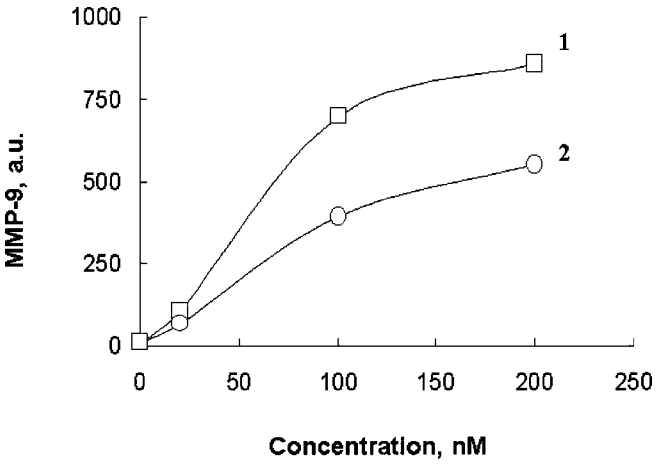

Figure 2 Dependence of MMP9 production by THP-1 cells on the concentration of r-uPA (1) and r-uPA$^{H/Q}$ (2)

THP-1 cells were incubated for 48 h with 0–200 nM of either r-uPA or r-uPA$^{H/Q}$, after which MMP9 activity in conditioned media was detected by gelatin zymography. a.u., arbitrary units.

Extracellular proteolysis and urokinase-induced gelatinase B secretion

According to our previous data, the ability of urokinase to induce MMP9 secretion by monocytes was partially dependent on its catalytic activity, since the effect of r-uPA was 50 % inhibited by plasminogen activator inhibitor-1 [15]. To verify this suggestion further, we performed incubation of THP-1 cells with catalytically inactive urokinase, in which the active centre His^{204} residue is replaced by Gln (r-uPA$^{H/Q}$). The data presented in Figure 2 indicate that catalytically inactive r-uPA$^{H/Q}$ induces about 30 % less MMP9 secreton in the concentration range 20–200 nM as compared with the effect of wild-type r-uPA when used at the same concentrations.

The effect of urokinase on MMP9 secretion is dependent on the protein synthesis

In order to determine whether transcriptional/translational mechanisms are also involved in the elevated MMP9 secretion stimulated by urokinase, we examined the ability of several inhibitors to affect MMP9 secretion (Figure 3). Pre-incubation (1 h) with cycloheximide greatly attenuated, in a dose-dependent manner, uPA-stimulated MMP9 secretion; cycloheximide (0.1 μg/ml) attenuated secretion by 50 $\pm$ 5 %, and higher concentrations (0.5 μg/ml) completely inhibited secretion. Actinomycin D (0.1 μg/ml), an inhibitor of gene transcription, also inhibited secretion by 97 $\pm$ 3 %. Monensin (1 μM), the agent that interferes with transfer across Golgi compartments [19] and inhibits MMP secretion in peritoneal macrophages [20], also partially suppressed MMP9 secretion.

Urokinase-stimulated MMP9 gene transcription in THP-1 cells

Urokinase-stimulated MMP9 secretion in THP-1 cells is inhibited by the agents affecting gene transcription and protein synthesis, therefore we next examined the effect of urokinase on MMP9 mRNA using both qualitative and quantitative RT-PCR. Expression levels were examined at 0–48 h after urokinase addition, relative to G3PDH, a housekeeping gene (see below).

In the absence of r-uPA, no MMP9 mRNA could be detected in the THP-1 cells after 35 cycles, whereas G3PDH mRNA could

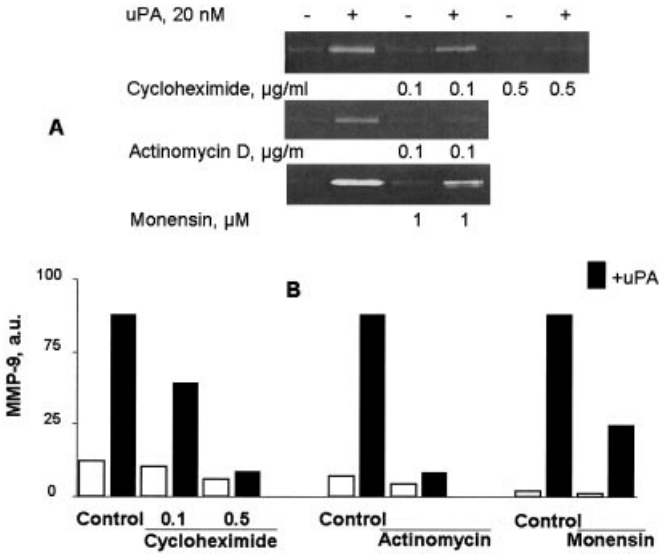

Figure 3 Inhibitory effects of cycloheximide, actinomycin D and monensin on urokinase-induced MMP9 secretion by THP-1 cells

(**A**) Cells were treated with cycloheximide (0.1 and 0.5 μg/ml), actinomycin D (0.1 μg/ml) or monensin (1 μM) for 1 h and then incubated for 48 h with either 20 nM r-uPA (+) or saline (−). (**B**) Conditioned media were harvested and subjected to zymography and densitometric analysis; a.u., arbitrary units.

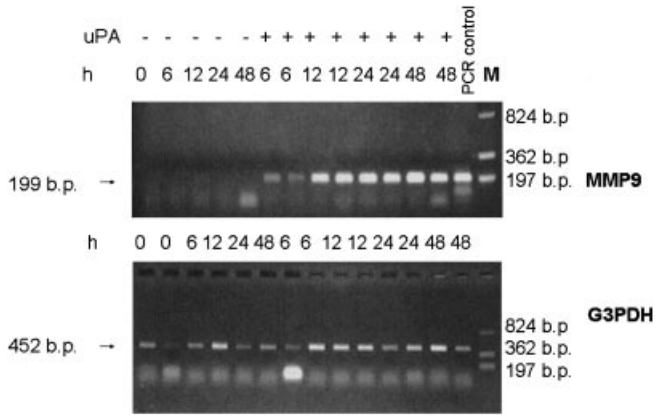

Figure 4 PCR analysis of human MMP9 and G3PDH mRNA from THP-1 cells treated with exogenous uPA

Electrophoretograms of MMP9 (upper panel) and G3PDH (lower panel) PCR-products were obtained using corresponding gene-specific primer pairs in 35 amplification cycles of cDNA synthesized from 30 ng of THP-1 total RNA. Size markers, positive PCR control, and incubation time of THP-1 cells with exogenous uPA are indicated.

be easily detected. In the presence of r-uPA, low levels of MMP9 mRNA were apparent at 6 h, increasing markedly by 48 h (Figure 4), whilst levels of G3PDH remained essentially similar.

To accurately quantitate the MMP9 mRNA level, a quantitative RT-PCR procedure, using an internal DNA standard identical with the amplified DNA fragment of either MMP9 or G3PDH cDNA, was used (see the Experimental section). Eight independent series of PCR amplifications of cells treated with uPA for 6, 12, 24 and 48 h were examined (Figure 5), using primer pairs in the presence of serially diluted internal MMP9 or G3PDH DNA standards. The amounts of MMP9 and G3PDH

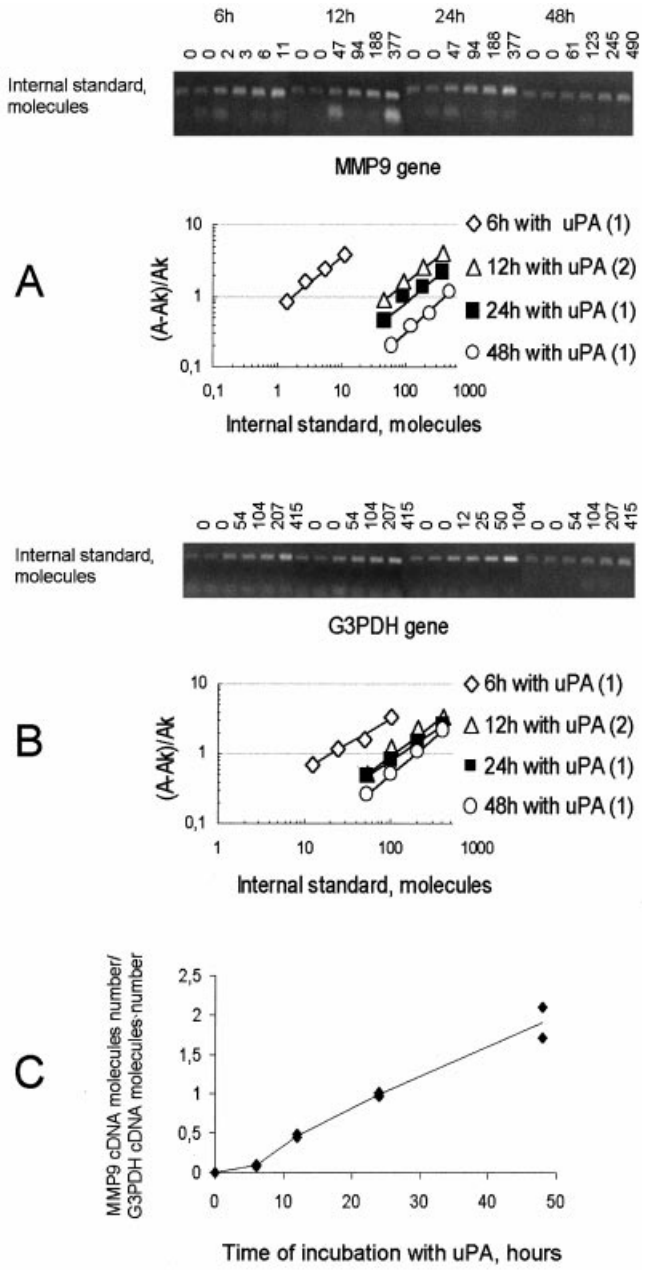

Figure 5 Quantitative RT-PCR analysis of human MMP9 and G3PDH mRNA from THP-1 cells

(**A**, upper panel) A typical electrophoretogram showing the PCR products of MMP9 cDNA and the co-amplification of the MMP9 internal DNA standard. The same amount of cDNA (synthesized from 30 ng of total RNA from exogenous uPA-treated THP-1 cells) was PCR amplified with the MMP9 primer pair and varying amounts of the MMP9 internal DNA standard (as indicated). Each experiment was performed in duplicate. Since the nucleotide sequence of the internal DNA standard is identical with the amplified region of MMP9 cDNA, the size of PCR product obtained on both templates is the same, and equal to 199 bp. (**B**, upper panel) Electrophoretogram showing the PCR products of G3PDH cDNA and the co-amplification of the G3PDH internal DNA standard. The same cDNA samples used for quantification of MMP9 gene were PCR amplified with the G3PDH primer pair and varying amounts of the G3PDH internal DNA standard (as indicated). The size of PCR products obtained from both templates is the same and equal to 452 bp. (**A**, lower panel) and (**B**, lower panel) show the mathematical processing of data from the electrophoretograms described above. The data are plotted on a logarithmic scale as a ratio of $(A_i - A_0)/A_0$ against the amount of internal DNA standard (see the Experimental section). The abscissa of the intersection point of the experimental curve with the ordinate equal to 1 indicates the initial amount of cDNA fragment flanked with either MMP9 or G3PDH PCR primer pair. (**C**) Normalization of the quantity of MMP cDNA product obtained in the experiments with internal DNA standard to the amount of G3PDH cDNA.

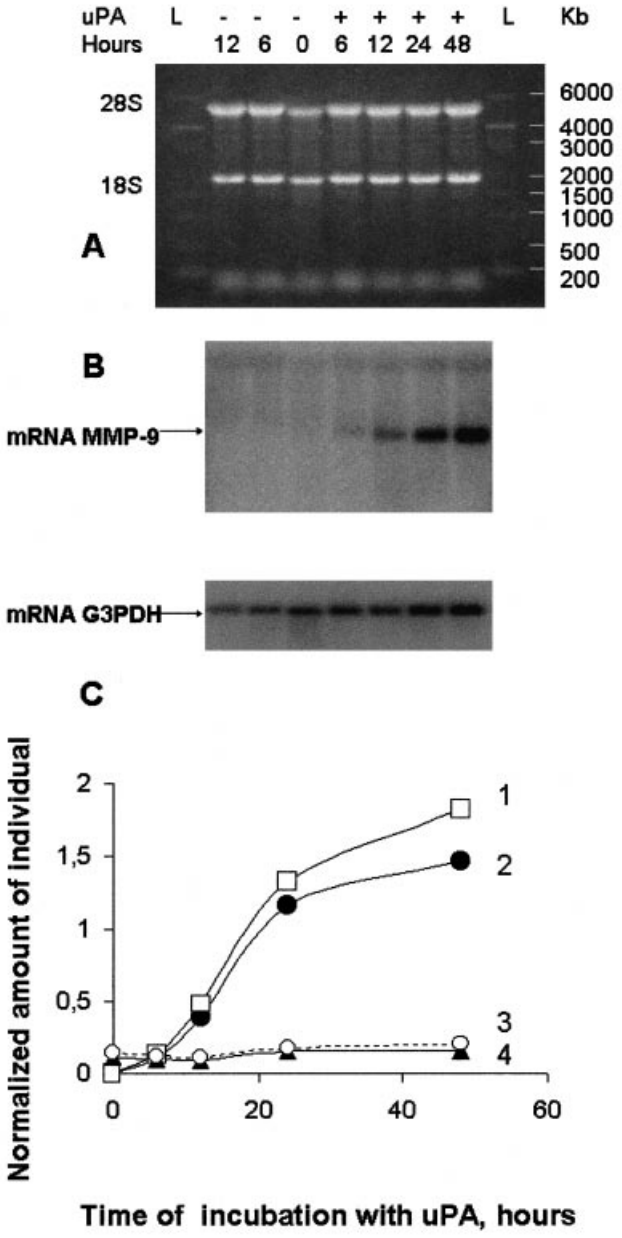

Figure 6 Northern blot hybridization assay of MMP9 and G3PDH mRNAs from urokinase-treated THP-1 cells

(**A**) Electrophoretogram of total RNA preparations isolated from THP-1 cells treated with r-uPA after staining with ethidium bromide. Molecular-mass standards are given on the right. (**B**) Northern blot hybridization of MMP9 (upper panel) and G3PDH (lower panel) mRNA after electrophoretic separation of RNA samples obtained from THP-1 cells incubated with r-uPA during the time periods indicated. (**C**) Normalization of MMP9 mRNA hybridization data to 18 S (curve 1) and 28 S (curve 2) RNA quantity. Curves 3 and 4 show the normalization of G3PDH mRNA to 18 S and 28 S RNA respectively.

PCR products generated in the absence of internal DNA standard (A_o) were compared with that formed in the presence of standard DNA (A_i), and the data were plotted on a logarithmic scale as a function of the internal DNA-standard concentration, as described in the Experimental section (Figures 5A and 5B).

From these curves, the initial amounts of MMP9 and G3PDH cDNA were calculated. To account for the possible difference in the efficiency of MMP9 mRNA RT in different samples, the data on the MMP9 cDNA molecule number were normalized to those of G3PDH cDNA molecule number (Figure 5C). These data indicate that the level of MMP9 mRNA converted into cDNA increases with the increment of the treatment duration of THP-1 cells with r-uPA.

In addition to the RT-PCR method, the MMP9 mRNA in THP-1 cells was detected by Northern blot hybridization. Total RNA from THP-1 cells incubated with r-uPA was resolved by electrophoresis under denaturing conditions (Figure 6A), transferred to nylon membrane and hybridized with a radiolabelled MMP9 probe. Following MMP9 mRNA detection by scanning the radioautograph (Figure 6B, upper panel), the blot was re-probed with radiolabelled G3PDH cDNA fragment and scanned to quantify G3PDH mRNA (Figure 6B, lower panel). These data show that the MMP9 mRNA in THP-1 cells is upregulated in a time-dependent fashion, whereas G3PDH mRNA is not significantly changed after treatment with r-uPA.

To evaluate the possibility that the G3PDH gene acts as a housekeeping gene, detecting changes in MMP9 mRNA levels due to the action of uPA on THP-1 cells, MMP9 mRNA data obtained by mRNA hybridization (Figure 6B) were normalized to the signal values obtained after scanning either 28 S or 18 S rRNAs in the ethidium bromide stained gel (Figure 6A). The results of such MMP9 mRNA normalization were plotted as a function of the time incubation with r-uPA (Figure 6C, curves 1 and 2).

The time-dependence observed is similar to that for MMP9 mRNA normalized to G3PDH mRNA (Figure 5C), and shows that the MMP9 mRNA level is approximately 15-fold greater by 48 h than by 6 h. On the contrary, normalization of G3PDH mRNA to that of 28 S and 18 S rRNA (Figure 6C, curves 3 and 4) shows that only 1.2–2-fold induction of G3PDH mRNA is observed during 24–48 h THP-1 cell incubation with r-uPA. Therefore these results demonstrate that the expression level of the G3PDH gene is almost constant, irrespective of the incubation time with r-uPA, and that it can be used as a control to normalize MMP9 mRNA data obtained by RT-PCR and Northern blot hybridization.

DISCUSSION

The present study shows that urokinase specifically induces MMP9 expression by THP-1 monocyte cells via transcription/translation processes. The detailed characterization of the effect of urokinase on mRNA levels by RT-PCR and Northern blot methods indicates that this effect is due to the accumulation of MMP9 mRNA in THP-1 monocytes. It appears to be a slow time-dependent increase. Moreover, the sensitivity of the effect to the inhibitory action of monensin, the anti-Golgi compound, suggests that this induction is also mediated via protein secretion from intracellular granules. At the same time, this effect differs principally from the secretion of macrophage elastase (MMP-12), which is induced by the serine proteinases plasmin and thrombin in peritoneal macrophages via protease-activated receptor-1. The latter does not include gene transcription and protein synthesis, but is due to post-translational secretion of pre-formed MMP-12 protein [20].

MMPs are usually expressed and secreted by cells as inactive enzymes, and further proteolytic processing is necessary to convert them into active forms. The activation of MMP9 is mediated via such proteolytic mechanisms and can involve plasmin and stromelysin [9,10,21]. Urokinase could participate in this proteolytic cascade via conversion of plasminogen into plasmin [22,23]. However, no other activated form of MMP9 was

found in our study, suggesting that proteolytic activation of gelatinase B is not involved in its expression by urokinase in THP-1 monocytes. We have also shown previously [15] that plasmin is not involved in this process, because the MMP9 secretion is not sensitive to the plasmin inhibitors, α-antiplasmin and aprotinin. In addition, the proteolytic activity of urokinase is not crucial for its secretory activity, since the recombinant enzyme r-uPA$^{H/Q}$ is also able, although with less efficiency, to induce the same effect. This action seems to be specific for urokinase, since tissue-type plasminogen activator is unable to induce significant MMP9 production by THP-1 cells [15].

Although uPA is best known for its ability to convert plasminogen into plasmin, it can exert a variety of additional effects on cells through interactions with its specific structural domains, resulting in degradation of extracellular matrix, cell adhesion and chemotaxis. For example, its ability to induce chemotaxis in smooth muscle cells has been attributed to the binding of the uPA-kringle domain to the surface of cells and the association of uPA with its receptor, urokinase plasminogen activator receptor (uPAR) [24]. Its growth factor-like domain has been implicated in vascular smooth muscle cell migration, in a manner dependent on fibroblast growth factor-2 and platelet-derived growth factor-BB [13]. In addition, uPAR is a multi-functional receptor, promoting pericellular proteolysis and matrix attachment and affecting proteinase expression during macrophage differentiation [25].

Here we demonstrate that the ability of uPA to increase MMP9 release is critically dependent on elevations in mRNA and protein synthesis. At present we can only speculate as to how these increases in MMP9 production and secretion occur. It may depend on the ability of uPA to interact with uPARs. In previous studies, some components of intracellular signal transduction (Src-kinases, extracellular signal-regulated protein kinase-2, p38 mitogen-activated protein kinase, casein kinase 2, nucleolin), as well as some nuclear factors identified in cells treated with urokinase, were found to be coupled with uPAR [26–29]. For example, overexpression of uPAR in Hep3 human carcinoma cells and its interaction with $\alpha 5\beta 1$ integrin has been shown to increase extracellular signal-related protein kinase activity whilst suppressing p38^{MAPK} [30]. Such intracellular signal transduction pathways could theoretically mediate the expression of MMP9 in monocytes induced by urokinase.

The interaction of uPA with uPAR not only localizes its proteolytic activity but also induces a variety of cellular responses important in tissue repair and remodelling, such as cell proliferation [31,32], cell adhesion and migration [24]. The secretion of latent MMP9 could be involved in these processes. The present study provides an explanation as to why uPA and MMP9 co-expression has been observed in many studies [10–12,33]. *In vivo* urokinase could well be responsible for the continual MMP9 upregulation via mechanisms which depend on activating gene transcription and protein synthesis. Future studies will clarify the precise mechanisms by which uPA induces these important effects on MMP9 secretion by monocytes.

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