Urokinase Plasminogen Activator and Plasmin Efficiently Convert Hemofiltrate CC Chemokine 1 into Its Active [9–74] Processed Variant

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Urokinase Plasminogen Activator and Plasmin Efficiently Convert Hemofiltrate CC Chemokine 1 into Its Active [9–74] Processed Variant

Jalal Vakili,* Ludger Ständker,‡ Michel Detheux,§ Gilbert Vassart,*† Wolf-Georg Forssmann,‡ and Marc Parmentier*‡

We have previously isolated from human an N-terminally truncated form of the hemofiltrate CC chemokine 1 (HCC-1), and characterized HCC-1[9–74] as a strong agonist of CCR1, CCR5, and to a lower extent CCR3. In this study, we show that conditioned media from human tumor cell lines PC-3 and 143B contain proteolytic activities that convert HCC-1 into the [9–74] form. This activity was fully inhibited by inhibitors of urokinase-type plasminogen activator (uPA), including PA inhibitor-1, an anti-uPA mAb, and amiloride. Pure preparations of uPA processed HCC-1 with high efficiency, without further degrading HCC-1[9–74]. Plasmin could also generate HCC-1[9–74], but degraded the active product as well. The kinetics of HCC-1 cleavage by uPA and plasmin (Michaelis constant, $K_m$, of 0.76 ± 0.4 M for uPA, and 0.096 ± 0.05 M for plasmin; catalytic rate constant, $k_{cat}$; 3.36 ± 0.96 s$^{-1}$ for uPA and 6 ± 3.6 s$^{-1}$ for plasmin) are fully compatible with a role in vivo. The activation of an abundant inactive precursor into a broad-spectrum chemokine by uPA and plasmin directly links the production of uPA by numerous tumors to their ability to recruit mononuclear leukocytes, without the need for the transcriptional activation of chemokine genes. The Journal of Immunology, 2001, 167: 3406–3413.

Chemokines are a superfamily of 8- to 10-kDa secreted proteins predominantly involved in the trafficking of immune cells. They are structurally classified into two main groups (CC and CXC chemokines) according to the relative position of the first two conserved cysteines. Functionally, one can distinguish inducible chemokines, involved in leukocyte recruitment to inflammatory sites, and constitutive chemokines, mediating the homing of leukocytes to lymphoid organs (1). Chemokines act through G protein-coupled receptors, the expression of which is regulated according to the maturation and functional status of the leukocyte populations (2). Chemokine receptors have also been reported on a variety of cell types including endothelial cells (3), smooth muscle cells (4), and various tumors (5). CC chemokines are involved in numerous diseases (6). Their expression by tumoral smooth muscle cells (4), and various tumors (5). 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and also influences cellular adhesion through interactions with integrins (22, 23).

Hemofiltrate CC chemokine-1 (HCC-1) is a CC chemokine originally isolated from the hemofiltrate of patients with chronic renal failure. It is constitutively expressed by numerous tissues, leading to high plasma levels in normal subjects (~10 nM). These levels may increase 10-fold in pathological conditions involving an inflammatory process (24). Full-size HCC-1 was shown to be a fairly weak agonist of CCR1 (25), and inactive on CCR5 (26). We have recently isolated from human hemofiltrate a truncated variant of HCC-1 lacking the first eight amino acids. HCC-1[9–74] was characterized as a strong agonist of CCR1 (EC50: 2.8 nM), of CCR5 (EC50: 4.8 nM), and a weaker agonist of CCR3 (27). Accordingly, it promoted the chemotaxis of a large array of leukocyte populations, including monocytes, macrophages, T lymphocytes, and eosinophils, and was shown to be a potent inhibitor of HIV entry. Moreover, several human tumor cell lines were found to process full-size HCC-1 into a form that could activate CCR5. Thus HCC-1 appeared as an abundant and widely expressed chemokine precursor processed by limited proteolysis into a highly potent and broad-spectrum chemoattractant factor. In this report, we extend the analysis of the proteolytic activities released by a larger panel of cell lines, and provide strong evidence that the uPA-plasmin system is involved in the conversion of HCC-1 into the active [9–74] form.

Materials and Methods

Chemicals

Chemicals were obtained from Sigma (St. Louis, MO) and cell culture media from Life Technologies (Grand Island, NY) unless otherwise stated. Aprotinin and AEBSF (4-(2-aminoethyl)-benzylsulfonyl fluoride) were obtained from Roche Molecular Diagnostics (Indianapolis, IN). Neutralizing anti-uPA mAb 394 (28) and U-stop, a synthetic inhibitor of tPA, were purchased from American Diagnostica (Greenwich, CT). HCC-1 and HCC-[9–74] were prepared by F-moc solid-phase synthesis as previously described (29).

Aequorin-based functional assay

Intracellular Ca2+ release was measured as described (27) by a functional assay based on the luminescence of mitochondrial aequorin (29).

Cells and conditioned media

Cell lines (American Type Culture Collection, Manassas, VA) were incubated at 37°C in a 5% CO2 incubator with 95% humidity. Cells (107) were seeded and grown in flasks to 60% confluence, with HAM-F12 (or DMEM for 143B cells) supplemented with 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, and 1 mM sodium pyruvate. For the preparation of conditioned media, the flasks were washed once and incubated in serum-free media for 5 days. Conditioned media were recovered, clarified by centrifugation at 13,000 rpm, filtered through 20-µm pore membranes, and immediately stored at −80°C.

Reversed phase (RP) chromatography and peptide analysis

RP chromatography was performed as described previously (27). Briefly, supernatants from cultured cell lines were fractionated on an analytical RP-C18 column (0.46 × 25 cm, 5-µm beads; Vydac, Hesperia, CA) using a linear acetonitrile gradient. Fractions were tested for their ability to stimulate CCR5 in the aequorin assay. Active fractions were analyzed by mass spectrometry and Edman degradation sequencing as previously described (27).

Assay of HCC-1 converting activity in cell cultures and conditioned media

Initial screening of the proteolytic activation of HCC-1 by tumoral cell lines was performed by incubating, at 37°C for 48 h, 1 µM HCC-1 in the culture media of subconfluent monolayers. Time course experiments with the PC-3 cell line were conducted by incubating 50 nM HCC-1 in conditioned media at 37°C. Samples were collected, supplemented with 50 µM U-stop, and kept on ice until tested. For inhibition experiments, conditioned media were preincubated with inhibitors for 30 min at 37°C, after which 50 nM HCC-1 was added and incubated for another 4–6 h at 37°C before being assayed. Potential interference of inhibitors with the aequorin assay was evaluated by testing the ability of 1 nM HCC-[9–74] to stimulate the CCR5-aequorin cell line in the presence of inhibitors.

Enzymatic assays and kinetics experiments

Purified tcHMW-uPA, with a sp. act. of 90,000 IU/mg, and purified plasmin (activated by matrix-bound uPA), with a sp. act. of 7.5 IU/mg, were obtained from American Diagnostica. tPA was obtained from Calbiochem (La Jolla, CA). These preparations were estimated to be over 95% homogeneous as determined by SDS-PAGE analysis. Experiments with uPA or tPA were conducted in a 10 nM Tris-HCl buffer, pH 8.0, containing 38 mM NaCl and 0.01% Tween 20. Plasmin activity was assayed in 10 nM Tris-acetate, pH 8.3, 0.01% Tween 20. For experiments involving tPA, fibrin degradation products (tPA stimulator; Chromogenix, Molndal, Sweden) were added to the reaction. Time course experiments with uPA or plasmin were conducted in the conditions described above. Concentration-activity experiments were performed by incubating the enzymes for 2 h (uPA) or 30 min (plasmin) at 37°C with 50 nM HCC-1.

For the determination of uPA enzyme kinetics data, conditions of steady-state production of HCC-[9–74] were first determined by incubating various concentrations of HCC-1 with 5 nM tcHMW-uPA at 37°C. Samples were taken between 0 and 10 min, and the activity was quenched by transfer of the tubes in an ethanol-ice slurry and addition of 50 µl EIA lysis buffer. The enzymatic activity was assayed for the determination of the kinetic parameters of HCC-1 (30) by incubating with 100 nM to 5 µM HCC-1 at 37°C, and the reaction was quenched after 5 min. All reactions were immediately tested in a CCR5-aequorin assay. Results in relative light units (RLU) were transformed into HCC-[9–74] concentrations by using a standard curve obtained with synthetic HCC-[9–74] and performed during the same assay session. Nonlinear regression was applied to the Michaelis-Menten model for the calculation of kinetics rates. All analyses were performed with Prism software version 3.02 (GraphPad Software, San Diego, CA).

For plasmin, determination of the linear interval of HCC-[9–74] production and kinetics studies (using 20 nM purified enzyme and 10 µM aprotinin as a quenching agent) were performed according to the uPA method.

Determination of the amidolytic activities of uPA or plasmin was performed with a photometric assay at 405 nm, using substrate S2444 (pyro-Glu-Gly-Arg-pNA; Chromogenix) for uPA, Chromozyme PL (Tosylglycyl-prolyl-lysine-4-nitranilide acetate; Roche Molecular Diagnostics) for plasmin, as described by the manufacturer, and an acid stop method. Reference uPA (high m.w. urokinase, code 875984) and plasmin (3rd international standard for plasmin, code 97536) preparations were obtained from the National Institute for Biological Standards and Controls (NIBSC, South Mimms, U.K.).

Western blotting

Samples (1 ml) of conditioned media from each cell line were precipitated by 15 µl of Strep-tactin (Stratagene, La Jolla, CA) and resuspended in 100 µl of SDS-PAGE loading buffer. Fractions (7 µl) of these samples or purified uPA were electrophoresed under reducing conditions on 10% polyacrylamide gels, then electrotransferred onto 0.45-µm nitrocellulose filters (Schleicher & Schuell, Keene, NH). Membranes were saturated by the same assay session. Nonlinear regression was applied to the standard curve obtained with synthetic HCC-[9–74] units (RLU) were transformed into HCC-[9–74] before being assayed. Potential interference of inhibitors with the aequorin assay was evaluated by testing the ability of 1 nM HCC-[9–74] to stimulate the CCR5-aequorin cell line in the presence of inhibitors.

Results

We have shown previously that HCC-[9–74] could be produced in vitro by limited trypsin digestion of HCC-1, and that human tumor cell lines could release uncharacterized proteases able to activate HCC-1 (27). In our search for specific proteases involved in HCC-[9–74] production, we screened an extended range of human tumoral cell lines for proteolytic activation of HCC-1. Subconfluent monolayer cultures of prostate carcinoma (PC-3, DU145) (30), osteosarcoma (143B) (31), melanoma (MeWo, Mel-Z2) (32), and breast adenocarcinoma (MCF-7) (33) cells were incubated for 48 h with full-length HCC-1 in culture medium. The medium was then recovered and tested for its ability to activate
CCR5 in an aequorin-based bioassay (Fig. 1a). For all cell lines, the background stimulatory activity (activity of the medium in the absence of HCC-1) was minimal. Following incubation with HCC-1, the media of PC-3 cell cultures contained the highest level of CCR5 stimulatory activity. Cell lines DU145 and 143B exhibited lower levels of HCC-1 activation, whereas MeWo, Mel-Z2, and MCF-7 produced little or no detectable activity on CCR5.

Thus, in several cultures, HCC-1 was processed into a form that activated CCR5, suggesting that proteases released by tumor cells could generate the active truncated form. To avoid the potential interference of serum-derived proteins with the proteolytic activities generated by tumor cells, and for purification purposes, acellular serum-free conditioned media (CM) were prepared from the PC-3 cell line. The presence of HCC-1 converting activity in these media was confirmed, and these preparations were used for subsequent experiments. A time course of HCC-1 (50 nM) activation by PC-3-conditioned media is shown in Fig. 1b. An activity was detectable by 1 h, and increased for several hours before reaching a plateau that was maintained for up to 9 h.

With the aim of identifying the protease(s) responsible for the conversion of HCC-1, we tested a range of protease inhibitors on the HCC-1-processing activity of PC-3 CM, using the CCR5-aequorin assay. We first selected class-specific inhibitors (Fig. 2a) active on serine, cysteine, aspartate, or metalloproteases. The most active were all among serine protease inhibitors, particularly AEBSF, which decreased the activity to near background levels at a concentration of 4 mM. Other inhibitors of serine proteases, such as leupeptin, aprotinin, and soybean trypsin inhibitor, decreased HCC-1 conversion by only ~25%. Inhibitors of metalloproteases (EDTA, 1,10-phenanthroline), of cysteine proteases (pepstatin), and of aspartic proteases (N-(N-(1-3-trans-carboxyamidine-2-carbon-yl)-1-leucyl)-agmatine, E-64) had no activity in this assay. The concentrations of leupeptin (0.02 mM) and aprotinin (0.003 mM) used in this assay were expected to inhibit most serine proteases, with the exception of a few enzymes known for their relative resistance to these potent inhibitors. PC-3 cells have been described to produce high levels of uPA (34, 35), and this enzyme is relatively resistant to leupeptin (36) and aprotinin (37). This led us to hypothesize regarding the involvement of uPA in the proteolytic activation of HCC-1 by the PC-3 cell line. To test this hypothesis, the effect of a selective inhibitor of PA (PAI-1) and of two specific inhibitors of uPA, the neutralizing mAb 394 and amiloride (38), was tested. As shown in Fig. 2b (open columns), all three inhibitors reduced CCR5 activation to near baseline levels, demonstrating an almost complete inhibition of HCC-1-processing activity. This inhibition was shown to be dose dependent, and full inhibition was obtained with 8 μg/ml mAb 394 or 20 μg/ml PAI-1 (Fig. 2c). The effect of uPA inhibitors was also tested on the other cell lines able to process HCC-1 (Fig. 1a). CM was prepared from these cells, and their HCC-1-processing activity was confirmed. HCC-1 activation by 143B medium was completely inhibited by all three uPA inhibitors. For DU145 medium, only PAI-1 produced 100% inhibition, whereas mAb 394 and amiloride reduced HCC-1 activation by 50% (Fig. 2b).

The presence of active tHMW-uPA in CM from cell lines PC-3, 143B, and DU145 was confirmed by Western blotting using a polyclonal anti-uPA Ab (Fig. 2d). In all cases, a band of ~55 kDa, corresponding to pro-uPA (single-chain) was found, together with two bands of 30 and 20 kDa, corresponding to active tHMW-uPA (two-chain) under reducing conditions. The uPA amidolytic activity in the three CM was measured, using reference uPA preparations as controls, and the chromogenic substrate S2444. The activities were 22.5, 29.5, and 19 IU/ml for PC-3,
DU145, and 143B cells, respectively (data not shown). Using a standard curve generated with synthetic HCC-[19–74], the estimated yield of HCC-[19–74] after incubating 50 nM HCC-1 for 5 h in CM was 0.88, 0.84, and 0.13 nM/μl for PC-3, DU145, and 143B cells, respectively (data not shown). All conditioned media were inactive when tested for amidolytic activity on the plasmin-specific substrate Chromozyme PL (data not shown). Therefore, these results support uPA as the main protease produced by PC-3 and 143B cells, and responsible for the production of HCC-[19–74]. For the DU145 cell line, proteases other than uPA, possibly contributing to HCC-1 cleavage, could not be excluded.

To determine the precise nature of CCR5-stimulatory products generated during incubation of HCC-1 with PC-3 CM, 50 μg of full-size HCC-1 were incubated for 8 h at 37°C in PC-3 medium. This material was separated by RP-chromatography as previously described (27), and fractions were tested on a CCR5-aquorin cell line (data not shown). A single peak of activity was obtained. The matrix-assisted laser desorption ionization-mass spectrometry (MS) spectrum of the active fraction (Fig. 3a) revealed two major peaks of 7.8 and 8.7 kDa (Fig. 3b). Further analysis by electrospray mass spectrometry revealed molecular masses of 7796 ± 2.0 and 8672 ± 0.8 Da, corresponding, respectively, to HCC-[19–74] and full-length HCC-1. Spectral data were further confirmed by N-terminal sequencing of the active fraction (Fig. 3b). These results suggest that the [9–74] form was the only active fragment of HCC-1 generated by incubation with PC-3 CM. Similar results were obtained with purified uPA (data not shown).

The interaction of HCC-1 with uPA was further investigated by using purified tcHMW-uPA. As suggested by Fig. 1b, and analysis by mass spectrometry, HCC-1 processing by uPA yields CCR5-stimulatory activities that remain stable over time. This suggests that HCC-[19–74] is not further processed into inactive degradation products. To confirm this observation, tcHMW-uPA was incubated with either full-length HCC-1 or HCC-[19–74], and the biological activity on CCR5-expressing cells was followed for up to 6 h (Fig. 4a). When 50 nM HCC-1 was incubated with 10 IU/ml tcHMW-uPA, CCR5 activation was detectable by 40 min, reached a plateau after 2 h, and was maintained for over 6 h. The same amount of uPA had no significant effect on the CCR5-stimulatory activity of 0.5 nM HCC-[19–74]. Overall, these results indicate that the dynamics of HCC-1 cleavage by uPA, measured for up to 6 h, favors stable production HCC-[19–74] without further degradation. We then assessed the amount of tcHMW-uPA needed to convert 50 nM HCC-1 during a 2-h incubation at 37°C (Fig. 4b). The lowest concentration of tcHMW-uPA that produced detectable CCR5-stimulatory activity was 0.375 IU/ml (~0.075 nM). At the highest concentration of uPA tested (500 IU/ml, ~100 nM), the activity reached 80% of the response obtained with 50 nM HCC-[19–74] (Fig. 4b, filled column). Fig. 4c shows a dose-response curve established with synthetic HCC-[19–74] on the CCR5-aquorin cell line, characterized by an EC50 of 1.6 ± 0.3 nM (mean ± SEM), in agreement with our previous data. These results indicate that the major part of HCC-1 can indeed be processed into the active [9–74] form without further degradation or significant production of other inactive variants.

Next, the CCR5-aquorin assay was used to measure the kinetics parameters for uPA activation of HCC-1. Production of HCC-1[9–74] was measured by reporting luminescence values to standard curves established for HCC-[19–74] during the same assay sessions. Initial rates were assessed using 5 nM tcHMW-uPA, and the incubations were stopped after 5 min, well within the interval of linear HCC-[19–74] production (Inset, 5 nM uPA was incubated with 125 (Δ) or 250 nM (△) HCC-1, as described in Materials and Methods). HCC-[19–74] production and initial rates of proteolysis, (v, μM min⁻¹) were determined by reporting luminescence values in RLU to standard curves of HCC-[19–74], as shown in c, established during the same assay session. This figure is representative of two independent experiments. Each point represents the mean ± SEM of duplicate data points.

FIGURE 4. Urokinase processing of HCC-1. a, Purified tcHMW-uPA (10 U/ml) was incubated at 37°C with medium alone (○), with 50 nM full-size HCC-1 (■), or with 0.5 nM HCC-[19–74] (△), and the incubation medium was tested for CCR5 activation at the indicated time points. b, Various concentrations of tcHMW-uPA were incubated for 2 h at 37°C with 50 nM full-size HCC-1 (□). The response evoked by 50 nM synthetic HCC-[19–74] tested during the same assay session is shown (●). The results, in RLU, represent the means ± SEM of triplicate data points, and are representative of three independent experiments. c, A standard curve of HCC-[19–74] activity on a CCR5-aquorin cell line was established as previously described (26), and is representative of three independent experiments. The values, in RLU, represent the means ± SEM of duplicate data points. d, Kinetics of HCC-1 cleavage by urokinase. Purified tcHMW-uPA (5 nM) was incubated with various concentrations of HCC-1, and the reaction was stopped after 5 min, well within the interval of linear HCC-[19–74] production (inset, 5 nM uPA was incubated with 125 (Δ) or 250 nM (△) HCC-1, as described in Materials and Methods). HCC-[19–74] production and initial rates of proteolysis, (v, μM min⁻¹) were determined by reporting luminescence values in RLU to standard curves of HCC-[19–74], as shown in c, established during the same assay session. This figure is representative of two independent experiments. Each point represents the mean ± SEM of duplicate data points.
of their polypeptide and polysaccharide components (39). Considering the broad spectrum of plasmin substrates, we used purified plasmin activated by matrix-bound uPA to investigate its potential activity on HCC-1. Fig. 5a shows the time course of the conversion of 50 nM HCC-1 into its active form by 20 nM plasmin. The activity, detected as for uPA on a CCR5 and apoequorin-expressing cell line, was detected after 3 min of incubation at 37°C, and reached by 20 min a plateau that was maintained for up to 40 min. In parallel, the stability of HCC-[9–74] was assessed in the presence of plasmin. The activity of 2.5 nM HCC-[9–74], incubated with 20 nM purified plasmin, decreased gradually to reach 75% of the initial value by 40 min of incubation. This decrease is probably the reflection of a low rate of proteolytic inactivation of HCC-[9–74], although globally, the dynamics of HCC-1 processing favors the production of HCC-[9–74]. The formation of HCC-[9–74] from 50 nM HCC-1 by a range of plasmin concentrations in solution is shown in Fig. 5b. Following a 30-min incubation, a dose-dependent activity was detected between 2 and 25 nM plasmin, and near total conversion of HCC-1 was obtained for 50 and 100 nM. The aequorin assay was used, as described for uPA, to derive kinetics parameters for the activity of plasmin on HCC-1. The linear range for HCC-[9–74] production was determined (Fig. 5c, inset), and a reaction time of 5 min was selected to measure initial rates of HCC-1 cleavage by plasmin. The conversion of HCC-1 to HCC-[9–74] by plasmin also followed a Michaelis-Menten kinetics, with an apparent \( K_m \) estimated at 0.096 ± 0.05 µM (mean ± SEM), a \( k_{cat} \) estimated at 6 ± 3.6 s⁻¹ (mean ± SEM), and a \( k_{cat}/K_m \) of 66.6 µM⁻¹ s⁻¹. Kinetic parameters of HCC-1 cleavage by plasmin are compared with those reported for fibrinogen in Table I.

Finally, the possible processing of HCC-1 by the tPA was assessed. Up to 500 IU/ml of purified tPA left unchanged the stimulatory activity of 2.5 nM HCC-[9–74] and failed to stimulate CCR5 when incubated with 50 nM HCC-1 (data not shown).

**Discussion**

In the present work we have extended our previous study, and showed that urokinase (uPA) present in CM from several human tumoral cell lines was able to convert the precursor chemokine HCC-1 into the potent truncated [9–74] form. Expression of uPA is widespread in tumor cells, and uPA production had been reported for all cell lines initially tested for HCC-1 activation, with the exception of 143B (Fig. 1a). Our ability to detect the HCC-[9–74] in CM using a CCR5:aequorin bioassay not only depended on the level of uPA expression, but also on the presence of inhibitors of uPA in the medium, and on the extent of pro-urokinase activation. Specific inhibitors of uPA completely abolished HCC-[9–74] production in CM from the PC-3 and 143B cell lines, although in the DU145 cell line, complete inhibition was obtained with PAI-1 only, whereas the neutralizing mAb 394 and amiloride reduced the activity by 50%. This raised the possibility that in this cell line, tPA might contribute to HCC-1 activation. However, high concentrations of purified tPA did not result in HCC-1 processing (data not shown), apparently excluding tPA as an HCC-1-processing enzyme. Experiments using class-specific protease inhibitors further revealed that, in addition to uPA, other trypsin-like proteinases were likely involved in the HCC-1 conversion by cell line DU145 CM (data not shown). Western blot analysis of CM confirmed the presence of active tHMW-uPA for all three cell lines, as did amidolytic activity on a uPA-specific substrate. No plasmin activity was found in these conditioned media, demonstrating that the activity of uPA is not mediated by the activation of plasminogen.

uPA has only one well documented substrate, plasminogen, which is activated into plasmin through the cleavage of a single Arg⁶⁶⁶-Val⁶⁶⁷ bond. In vitro, uPA has also been shown to activate the hepatocyte growth factor (39). Plasmin, in contrast, apart from its principal substrates, fibrin and fibrinogen, processes a number of other targets including major components of the extracellular matrix (18, 40, 41), and of the complement and coagulation pathways (42–44). Both uPA and plasmin establish protein

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### Table I. Michaelis-Menten kinetic constants of HCC-1 processing by uPA and plasmin

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<td>( k_{cat} ) (s⁻¹)</td>
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<tr>
<td>Urokinase</td>
<td>0.76 ± 0.4</td>
<td>3.36 ± 0.96</td>
<td>4.4</td>
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<tr>
<td>Plasmin</td>
<td>0.096 ± 0.05</td>
<td>6 ± 3.6</td>
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*These constants are compared to those of reference proteolytic activities of plasmin on fibrinogen (72), and of uPA on Glu-plasminogen (73). For HCC-1, the results are the mean ± SEM of five and two independent experiments for uPA and plasmin, respectively.
interactions that anchor the enzymes in direct contact with their relevant substrates. uPA interacts with its high-affinity receptor, uPAR, or low-affinity glycosaminoglycan binding sites (45), which are critical for the reciprocal activation of pro-uPA and plasminogen (20).

Using the CCR5-aequorin assay and purified enzyme preparations, we characterized the properties of HCC-1 cleavage by uPA, and uncovered plasmin as an HCC-1-processing enzyme. The characteristics of HCC-1 truncation by purified uPA and plasmin have revealed some differences. Incubation of synthetic HCC-1[9–74] with uPA for up to 5 h did not result in a decrease of its activity, whereas incubation with plasmin reduced its activity by 25% after 40 min. Thus HCC-1[9–74] remains stable in the presence of uPA, whereas plasmin further catalyzes a slow inactivating proteolysis of the active chemokine. The plasmin concentrations required for full conversion of 50 nM HCC-1 were in the range of 2–50 nM, corresponding to 0.1–2.5% of the plasmatic concentration of plasminogen. These plasmin concentrations are similar to those used for the in vitro processing of other plasmin substrates (42). The uPA concentrations needed for HCC-1[9–74] formation (1–100 nM) are well above the plasmatic concentrations of this enzyme (150 pM). However, as previously noted, uPA works essentially in the bound state, and the plasmin concentration of uPA is not an adequate reflection of the effective concentration of active enzyme. uPA and plasmin proteolysis of HCC-1 follows Michaelis-Menten kinetics (Table I). The apparent Michaelis constant, \( K_{\text{m}} \), of uPA for HCC-1 (0.76 \( \mu \text{M} \)) is ~8-fold higher than that of plasmin (0.096 \( \mu \text{M} \)), whereas the catalytic efficiency, \( k_{\text{cat}}/K_{\text{m}} \), of plasmin for HCC-1 processing (66.6 \( \mu \text{M}^{-1}\text{s}^{-1} \)) is ~15-fold higher than that of uPA. The kinetics constants of uPA and plasmin for HCC-1 were compared with those previously reported for classical and in vivo relevant substrates of these enzymes (Table I). The catalytic efficiency of uPA for HCC-1 is 7-fold higher than that for Glu-plasminogen, whereas plasmin processes HCC-1 60-fold more efficiently than fibrinogen. The concentration of HCC-1 in plasma (10–100 nM) (24) is lower than that of plasminogen (2 \( \mu \text{M} \)) or fibrinogen (10 \( \mu \text{M} \)). Nevertheless, the results of our kinetics analysis are compatible with a physiological role of both uPA and plasmin in the cleavage of HCC-1. Therefore, we believe that these two enzymes are likely involved in the in vivo generation of HCC-1[9–74]. However, the relative contribution of the two enzymes, and the precise conditions in which the cleavage occurs, remain to be determined.

Additional factors need to be considered when evaluating the significance of our findings. HCC-1 was previously shown to activate CCR1 with a IC_{50} in excess of 100 nM. However, HCC-1[9–74] activates CCR1 and CCR5 in the 2–6 nM range. As a consequence, even if a small percentage of HCC-1[9–74] is generated from the estimated 10–80 nM HCC-1 present in plasma, this may nevertheless be significant. Moreover, chemokines bind to glycosaminoglycans linked to cell surface proteins or to the extracellular matrix (46, 47), and this interaction is thought to be critical for the long-term stability of chemokine gradients in tissues and the presentation of chemokines by endothelial cells (48, 49). Plasminogen has been reported to bind to the lysin-rich C-terminal domain of macrophage-inflammatory protein-2\(\alpha\) (growth-related oncogene-\(\beta\)) when bound to the cell surface, possibly enhancing local plasmin production (50). The interaction of HCC-1 with glycosaminoglycans has not been reported so far, but is likely to be a critical factor influencing the local availability of HCC-1, its potential presentation to uPA or plasmin, and the formation of an effective gradient of HCC-1[9–74].

The importance of the N-terminal processing of chemokines as a way of modulating their activity is being increasingly recognized. With the exception of HCC-[9–74] and neutrophil-activating peptide-II, chemokines are usually secreted as active proteins, which may undergo further processing, generally resulting in a reduction of their agonistic activity. Dipeptidylpeptidase IV (CD26), a widely distributed membrane-bound protease, has been shown to cleave off the first two amino acids of a number of chemokines. Cleavage of stromal cell-derived factor-1\(\alpha\) generates a full CXCR4 antagonist (51); that of RANTES reduces its activity (52). Gelatinase A generates also an antagonist by monocyte chemoattractant protein (MCP)-3 N-terminal processing (53). Proteolytic activation of precursors was previously described for neutrophil-activating peptide-II, a selective CXCR2 agonist generated by proteolysis of the basic platelet protein by cathepsin G and other chymotrypsin-like proteases (54). However, the influence of the extracellular processing of chemokines in relevant physiological or pathological situations remains for the great part to be elucidated.

MCP-1, a CC chemokine produced by various human tumor cell lines, has been the most extensively studied chemokine in relation to tumor growth (55, 56). Production of MCP-1 and other CC chemokines by tumors has been correlated with the level of mononuclear cell infiltration. The influence of chemokine production and mononuclear infiltration on tumor progression remains to be clarified, as opposite effects have been reported depending on the experimental design (57–59). The strongest evidence yet for the active contribution of inflammatory cells to the development of the tumoral process was provided recently. Coussens et al. (60) used an HPV16 E6/E7 transgene in MMP-9 knockout mice to demonstrate the pivotal contribution of leukocyte-derived MMP-9 in tumor invasiveness.

A role for PA-mediated pericellular proteolysis in tumor invasiveness and metastasis has been derived from a number of observations. Increased levels of uPA, uPAR, and, somewhat unexpectedly, PAI-1, have been shown in a variety of human tumors, and have been established as independent markers of poor prognosis (61–63). Studies of tumor development in plasmin- or urokinase-deficient mice have confirmed that both urokinase and plasmin contribute to tumor-associated pericellular proteolysis, but that other proteases could efficiently substitute for each deficiency (64, 65). The effect of uPA in tumor development is not limited to proteolysis, as signal transduction by uPAR is believed to facilitate cancer cell proliferation (66–68). Using a transplantation chamber model, it was shown that transplanted malignant keratinocytes failed to invade the underlying host stroma in PAI-1-deficient mice or to initiate neoangiogenesis, as opposed to in control mice, suggesting a protumorigenic role for PAI-1 (69). The molecular basis of this intriguing effect has been clarified recently. In agreement with previous in vitro studies (70), an optimal level of extracellular matrix degradation was found to be required for efficient tumor invasion and capillary formation (65). Therefore, this observation attributes the protumorigenic effect of PAI-1 to the counterbalancing of excessive plasmin generation.

The influence of PA, plasmin, or PAI-1 gene invalidation, or of tumor uPA inhibition (71) on the inflammatory infiltration of tumors in mice has, to the best of our knowledge, not been specifically addressed. Also, the mouse ortholog of HCC-1 has not been described so far, and other chemokines (such as IL-8) have been demonstrated to be absent in rodents. Therefore, in this context it would be difficult to speculate regarding the expected consequences of uPA or plasmin deficiency on the possible processing of HCC-1 in mice.

We speculate that HCC-[9–74], the product of the proteolytic activation of HCC-1 by uPA and/or plasmin, might be generated in all situations where the uPA/plasmin cascade is activated in the
process of tissue remodeling, including in wound healing, leukocyte migration, neoangiogenesis, and tumor development (16). This would represent a mechanism by which a constitutively expressed chemokine precursor, present in abundance in the circulation and extracellular matrix, could be converted to a potent chemokine and initiate further leukocyte recruitment. This mechanism would act independently of the usual transcriptional control of most inflammatory chemokines and other mediators. It could also represent an amplification loop for migrating cells expressing the uPAR at their leading edge.

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