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The Mast Cell as Site of Tissue-Type Plasminogen Activator Expression and Fibrinolysis

Christian Sillaber,†* Mehrdad Baghestanian,‡* Dorian Bevec,§ Martin Willheim,¶ Hermine Agis,* Stylianos Kapiotis,‖ Wolfgang Fürereder,* Hans C. Bankl,** Hans P. Kiener,† Wolfgang Speiser,‖ Bernd R. Binder,‡ Klaus Lechner,* and Peter Valent†*

Recent data suggest that mast cells (MC) and their products (heparin, proteases) are involved in the regulation of coagulation and fibrinolysis. The key enzyme of fibrinolysis, plasmin, derives from its inactive progenitor, plasminogen, through catalytic action of plasminogen activators (PAs). In most cell systems, however, PAs are neutralized by plasminogen activator inhibitors (PAIs). We report that human tissue MC as well as the MC line HMC-1 constitutively produce, express, and release tissue-type plasminogen activator (tPA) without producing inhibitory PAIs. As assessed by Northern blotting, highly enriched lung MC (>98% pure) as well as HMC-1 expressed tPA mRNA, but did not express mRNA for PAI-1, PAI-2, or PAI-3. The tPA protein was detectable in MC-conditioned medium by Western blotting and immunosassay, and the MC agonist stem cell factor (c-Kit ligand) was found to promote the release of tPA from MC. In addition, MC-conditioned medium induced fibrin-independent plasmin generation as well as clot lysis in vitro. These observations raise the possibility that MC play an important role in endogenous fibrinolysis. *The Journal of Immunology, 1999, 162: 1032–1041.

The enzymatic system of fibrinolysis has been implicated in a number of physiologic and pathophysiologic processes, including ovulation, embryogenesis, cell migration, clot lysis, tumor spread, angiogenesis, and wound healing (1–5). The key enzyme of fibrinolysis, plasmin, derives from the proenzyme plasminogen. This inactive progenitor is converted into the active enzyme by two plasminogen activators (PAs),* tissue-type plasminogen activator (tPA) and urinary-type plasminogen activator (uPA; urokinase) (1–5).

Recent data suggest that both tPA and uPA play a role in endogenous fibrinolysis. Thus, the functional disruption of the tPA gene in experimental animals (knockout mice) leads to impaired clot lysis (6). Mice with a combined deficiency (tPA and uPA genes) suffer from extensive fibrin deposition with impaired organ function, occurrence of vascular thrombosis, loss of fertility, and reduced survival (6). The emerging concept is that endogenous tPA and uPA are involved in the degradation of locally generated fibrin. In line with this concept, recombinant tPA (rtPA) acts fibrinolytically in vivo and has successfully been used as a thrombolytic agent in patients (7).

The plasmin-generating activity of tPA (and uPA) is under control of physiologic inhibitors (PA inhibitors (PAIs)). PAI-1, PAI-2, and PAI-3 are capable of neutralizing PAs by ligand binding and complex formation (5, 8, 9). Thus, in contrast to free, uncomplexed PA, the PA-PAI complexes are ineffective molecules with respect to plasminogen activation. Under various pathologic (inflammatory) conditions, the levels of circulating PAIs may increase (10).

The cellular basis of endogenous fibrinolysis has been a matter of numerous speculations and investigations. Endothelial cells (EC) apparently are a major source of tPA (11–14). However, these cells also produce significant amounts of PAI-1 in vitro (15, 16). Activated macrophages and smooth muscle cells are another source of PAIs and PAIs (17–20). However, MC are a source of PAIs and PAIs under a variety of circumstances, the production of PAIs is sufficient to antagonize and overcome tPA. By contrast, some tumor cells express tPA or uPA in excess over PAIs, and thereby can trigger fibrinolysis (21–23). A physiologic cell that would serve as a potent source of pro- fibrinolytic activity has not been identified yet.

The mast cell (MC) as multifunctional immune cells involved in the regulation of diverse (patho)physiologic processes (24–26). These cells are found in most organs and are located in strategic apposition to vascular cells and blood vessels as well as in loose connective tissues. MC and their products have been implicated in the regulation of vasodilation, EC activation, capillary leak and edema formation, angiogenesis, or leukocyte migration (27–30). Most of these activities (of MC) are due to production and release of distinct biologically active mediators, such as histamine, proteoglycans (heparin), PGs (PGD2), proteases (tryptase, chymase), or cytokines (TNF-α) (28, 30–34).

In the present study the human MC is identified as a source of active tPA. Thus, primary human lung MC and HMC-1 cells (human MC line) expressed and released enzymatically active tPA, but did not express detectable PAIs. Moreover, MC-conditioned
medium induced clot lysis in vitro. These observations suggest that MC contribute to the process of endogenous fibrinolysis.

Materials and Methods

Reagents, Abs, and buffers

Recombinant human (rh) stem cell factor (SCF) was purchased from Peprotech (Rocky Hill, NJ); collagenase type II was obtained from Sebaby (Sunderland, Austria); collagenase type IA was purchased from Sigma (St. Louis, MO); RPMI 1640 medium, genetamicin, amphotericin B, and FCS were obtained from Sera Lab (Crawley Down, U.K.); and Iovcée’s modified Dulbecco’s medium (IMDM), glutamine, penicillin, and streptomycin were purchased from Life Technologies (Gaithersburg, MD). EC basal medium and EC growth factor were obtained from Biogenesis (Poole, U.K.). An alkaline phosphatase-conjugated mAb against tryptase MPW5UK (G3) was purchased from Chemicon (Temecula, CA). The mAbs MPW3VPA (anti-IPA), MPW5UK (anti-IPA), and SPAH12 (anti-IPA-2) were purchased by Technoclone (Vienna, Austria). The anti-c-Kit mAb YB5.B8 (IgG1) (35) was provided by L. K. Ashman (University of Adelaide, Adelaide, Australia). One liter of Ca2+/Mg2+-free Tyrode’s buffer contained 0.2 g of KCl, 0.05 g of NaH2PO4, 0.8 g of NaCl, and 1 g of glucose. All oligonucleotide probes and primer pairs (for Northern blotting and RT-PCR) were obtained from MWG Biotech (Ebersberg, Germany).

Purification of primary lung MC

Lung tissue was obtained from 11 patients suffering from bronchiogenic carcinoma. Informed consent was given in each case. MC were isolated from surgical specimens according to published techniques (36, 37). In brief, tissue was chopped into small fragments, washed in Mg2+/Ca2+-free Tyrode’s buffer, and then incubated in collagenase type II (2 mg/ml) at 37°C for 1–3 h. Dispersed cells were recovered by filtration through nylon cloth, washed, and examined for the percentage of MC (Giems or toluidine blue staining). Cell suspensions were further enriched for MC by countercurrent flow centrifugation (elutriation) as previously described (37). In brief, cells were loaded at a flow rate of 12 ml/min into a Beckman elutriator equipped with a JE-6B rotor (Beckman Instruments, Palo Alto, CA). Fractions were recovered at increasing flow rates (14, 18, 20, and 30 ml/min). A selection was performed based on the content of MC. In one donor, two fractions contained >90% MC (total number of cells, 6 × 107). These MC were cultured overnight (37°C, 5% CO2), washed, and then exposed to rhSCF (100 ng/ml; 3 × 105 cells) or control medium (3 × 105) at 37°C in 5% CO2 for 2 h. Cell-free supernatants were recovered by aspirating the MC. MC were washed in RNA-free NaCl (0.9%) and subjected to RNA isolation (some cells were used for cytospin preparations). In the other donors (n = 10), the elutriated MC were further enriched by cell sorting using anti-c-Kit mAb YB5.B8 (37). MC were sorted as c-Kit+ cells, and were >98% pure as assessed by Giemsa staining. Six of the ten MC preparations (each >98% pure) were pooled (total cell number in pool, 7 × 109) for Northern blotting. In two donors pure MC were used for RT-PCR analyses, and in two donors MC were used forIPA measurements (by ELISA). Supernatants of MC were used forIPA measurements and clot lysis assay.

Purification and preparation of other cells

Apart from MC, several other cells were tested for expression of tPA and PAI-1. HUVEC were isolated using collage type IA and were purified by ELISA. Supernatants of MC were used for tPA measurements and clot lysis in vitro. Two healthy volunteers by Ficol gradient centrifugation, and bone marrow (bM) MNC were obtained from two patients with idiopathic thrombocytopenic purpura. Polymorphonuclear leukocytes were prepared from one healthy donor, and one patient suffering from hypereosinophilic syndrome using dextran (after isolation, eosinophil purity was 85% in the hypereosinophilic syndrome patient). Informed consent was obtained in all patients. Cell lysates were prepared by exposing cells (constant volume and cell number) to distilled water and freeze-thawing. Cell-free supernatants (supernatants = conditioned media) were prepared from cultured cells (2 or 24 h at 37°C) after centrifugation.

Cell lines

The human MC line HMC-1 (40) was provided by J. H. Butterfield (Mayo Clinic, Rochester, MN). HMC-1 cells were cultured in IMDM supplemented with 10% FCS and antibiotics at 37°C and 5% CO2. The human MC line HMC-1 was provided by J. H. Butterfield (Mayo Clinic, Rochester, MN). HMC-1 cells were cultured in IMDM supplemented with 10% FCS and antibiotics at 37°C and 5% CO2. The human MC line HMC-1 was provided by J. H. Butterfield (Mayo Clinic, Rochester, MN). Human skin-derived microvascular EC were obtained from Biogenesis (Poole, U.K.). Lung fibroblasts were cultured from a primary cell line obtained from one healthy donor infected with HIV-1 (B cell, Molt-4 (T cell), and A431 (epithelial). Cell lines were maintained in medium (RPMI 1640 or IMDM) with 10% FCS (37°C, 5% CO2). Conditioned medium (supernatant) was prepared by culturing cells for 2 or 24 h.

Stimulation of MC with rhSCF

Purified lung MC (91% pure) were incubated in RPMI 1640 medium and 10% FCS in the presence or the absence of rhSCF (100 ng/ml) for 2 h. HMC-1 were exposed to rhSCF (100 ng/ml) or control medium (IMDM) for 0.5–12 h. All incubations were performed at 37°C.

Northern blot analysis

Total RNA was extracted from lung MC (>98% pure (pool from six donors; total cell number, 7 × 109) and 91% pure (one donor; control medium vs rhSCF (2 h); cell number, each 3 × 1010)) and HMC-1, by the guanidinium isothiocyanate/cesium chloride extraction technique (42). Northern blot analysis was conducted as previously described (43). In brief, 1 μg of RNA were size fractionated on 1.2% agarose gels, transferred to nylon membranes (Hybond N, Amersham, Aylesbury, U.K.) with 20× SSC (1× SSC consists of 150 mM NaCl and 15 mM sodium citrate, pH 7.0) overnight, and cross-linked to membranes by UV irradiation (UV Stratalinker 1800, Stratagene, San Diego, CA). RNA was prehybridized at 65°C for 4 h in 5× SSC, 7% SDS, 10× Denhardt’s solution (1× Denhardt’s solution consists of 0.02% BSA, 0.02% polyvinyl pyrolidone, and 0.02% Ficoll), 10× dextran sulfate, 20 mM sodium phosphate (pH 7.0), sonicated salmon sperm DNA (100 μg/ml), and poly(A)+ (100 μg/ml). Hybridization was performed with 32P-labeled synthetic oligonucleotide probes (Table I). Blots were washed once in 3× SSC, 5% SDS, 10× Denhardt’s solution, and 20 mM sodium phosphate, pH 7.0, for 30 min at 65°C, and once in 1× SSC and 1% SDS for 30 min at 65°C. Bound radioactivity was visualized by exposure to XAR-5 films at −70°C using intensifying screens (Eastman Kodak, Rochester, NY).

RT-PCR

Total RNA was isolated from a known number (30,000) of pure (>98%) MC (lung, n = 2) using a modified guanidinium isothiocyanate-acid phenol extraction procedure (RNAzol B method, Biotechex, Houston, TX). After washing, cell pellets were resuspended in 0.8 ml of RNAzol B. Then, chloroform (80 μl) was added. The mixtures were kept at 4°C for 5 min. After centrifugation at 12,000 × g (4°C, 15 min), the upper aqueous phase was collected. Six micrometers of carrier RNA (yeast transfer RNA, stored as a 4 mg/ml solution in RNase-free water) were added before precipitation in isopropanol (~20°C, 12 h). Precipitated RNA was centrifuged at 12,000 × g (4°C) for 15 min, and pellets were washed thrice with 75% ethanol, dissolved in RNase-free water, and stored in liquid nitrogen. cDNA synthesis was performed using a single-stranded CDNA synthesis kit (First Strand cDNA Synthesis Kit, Pharmacia Biotech, Brussels, Belgium) according to the manufacturer’s instructions. Total RNA (dissolved in 20 μl of RNase-free water) was heated to 65°C for 10 min, quick-chilled on ice, and incubated with 11 μl of First-Strand Reaction Mix (closed, FPLCpur (Pharmacia Biotech) murine reverse transcriptase, RNAguard, RNase/DNase-free BSA, and 1.8 mM each of dATP, dCTP, dGTP, and dTTP in aqueous buffer), 1 μl DTT solution (200 mM aqueous solution), and 1 μl dNTP primer (random hexadeoxynucleotides at 0.2 μg/ml aqueous solution) at 37°C for 1 h. The reaction was terminated by heating to 90°C (5 min). Samples were chilled on ice immediately and stored at −20°C. Aliquots of the cDNA products, i.e., 3 μl for the constitutively expressed β-actin gene and 15 μl for the tPA gene, were used for RT-PCR
in a final volume of 100 μl containing PCR buffer (Perkin-Elmer/Cetus, Emeryville, CA), 2.5 U of Taq DNA polymerase (Perkin-Elmer/Cetus), and 0.25 μM of both upstream and downstream primers specific for tPA (5′ primer, 18-mer, gene position 1345–1362, 5′-CAGGGAGACGGCTGTC-3′; 3′ primer, 18-mer, gene position 1711–1728, 5′-GTCGGGTTGGTCTCTGCA-3′) or β-actin (5′ primer, 20-mer, gene position 969–988, 5′-AAGCCGGCTCTGCGGGCGAC-3′; 3′ primer, 21-mer, gene position 1327–1347, 5′-CTCGGAGACCCACACCGCTC-3′) and nucleotides (200 μM each of dATP, dGTP, dTTP, and dCTP). Samples were subjected to RT-PCR to amplify the 444-bp DNA fragment (tPA) and the 245-bp DNA fragment (β-actin) by running 35 cycles (94, 59, and 72°C, each for 1 min) after initial denaturation at 95°C (2 min). PCR products were subjected to electrophoresis and visualized by ethidium bromide.

**Detection and quantitation of tPA and PAI-1 protein**

Expression of the tPA protein and PAI-1 protein in cell lysates and/or supernatants was quantified by ELISA. The tPA ELISA (Chromogenix, Molndal, Sweden) showed a detection limit of 0.5 ng/ml and no cross-reactivity with tPA, PAI-1, and uPA, according to published techniques (15, 44). In brief, 100 μl of supernatants were applied on 10-cm resolving gels containing 10% acrylamide and 2-cm stacking gels with 4% acrylamide, separated proteins were transferred to a nitrocellulose membrane using a Bio-Rad Trans Blot with 0.025 M Tris, 0.192 M glycine, and 20% (v/v) methanol as transfer buffer at a constant voltage (30 volt) overnight. After transfer, the nitrocellulose strips were blocked with 1% rabbit serum or 1% horse serum (according to the species above) and then subjected to electrophoresis for 16 h. After electrophoresis, separated proteins were transferred to a nitrocellulose membrane using a Bio-Rad Trans Blot (Bio-Rad, Richmond, CA) with 0.025 M Tris, 0.192 M glycine, and 20% (v/v) methanol as transfer buffer at a constant voltage (30 volt) overnight. After transfer, the nitrocellulose strips were treated with 2% milk powder in TBS for 2 h. Thereafter, membranes were washed three times in 0.05% Tween-20 in TBS. Bound mAbs were incubated for 2 h with a peroxidase-labeled rabbit anti-mouse Ab (Amersham, Aylesbury, U.K.) dissolved in milk powder and TBS (Ab titer, 1/300). After incubation, membranes were washed, and Ab reactivity was made visible by coupled color reaction as previously described (15).

**Purification of tPA from MC supernatants and analysis of tPA activity**

The enzymatic activities of rtPA, purified cellular tPA (HMC-1), and crude HMC-1 supernatant, were analyzed and compared using a plasminogen-activation assay. HMC-1 supernatants were obtained by conditioning cells in serum-free medium at 37°C for 24 h. The tPA protein was purified from supernatants using an immunoaffinity column (3VPA-Sepharose) as described previously (47). The tPA activity was determined by measuring the generation of plasmin from native Glu-plasminogen in the absence or the presence of CB11 fragments of human fibrinogen or heparin. The plasmin generated was quantified by synthetic paraumbilical substrate S-2251 as previously described (47). Values for specific tPA activities were given in nanomolar concentrations of generated plasmin per nanogram of tPA (assessed by ELISA). The tPA activity was also quantified by a bioimmuno-assay (BIA; Chromogenix) using plasmin substrate S-2403 (48). The tPA BIA showed a detection limit of 0.017 U/ml (rtPA).

**Clot lysis assay**

Supernatants of various cells (HMC-1, n = 4; pure lung MC, n = 3; HUVEC, n = 1; lung fibroblasts, n = 1; blood MNC, n = 1; smooth muscle cells, n = 1), rtPA, or control medium were analyzed for clot lysis activity. The clot lysis assay was performed as previously reported (49). In brief, the clot was prepared by mixing plasminogen (0.1 μmol), fibrinogen (1 mg), CaCl₂ (4 mmol), and thrombin (0.5 U) in 300 μl. Cell supernatants (106 cells, 37°C, 2 h), rtPA (200 ng/ml), or control medium were added to the clot reaction mixture to a final volume of 1000 μl. Mixtures were placed in 24-well plates (Costar, Cambridge, MA) at 37°C. In selected experiments, PAI-1 (100 U/ml) or a blocking anti-tPA Ab (20 μg/ml; American Diagnostics, Greenwich, CT) were added together with MC or HMC-1 supernatants, or rtPA. After 2, 12, and 24 h (37°C), the wells were inspected by microscope. Clot lysis was defined as a complete dissolution of the fibrin meshwork within 24 h.

**Double immunohistochemistry**

Tissue was obtained from one explanted heart (heart transplant recipient suffering from dilated cardiomyopathy) at surgery, from lobular lung resection (patient suffering from bronchiogenic carcinoma), and from juvenile foreskin (circumcision, n = 1). Informed consent was obtained in each case. In addition, tissue was obtained from one heart at autopsy according to the guidelines of the local ethical committee. Tissue was fixed in neutral buffered formalin, embedded in paraffin, and cut into 2-μm sections.

Simultaneous double immunohistochemistry was performed essentially as previously described (32, 50) using Abs against tPA (1/200), PAI-1 (1/20), or PAI-2 (1/20) and an alkaline phosphatase conjugated monoclonal mouse Ab (clone G3) against human tryptase (1/500; Chemicon). Sections were treated with TBS and 0.1% protease type XIV (Sigma) for 10 min. After washing the slides in TBS at pH 7.6, nonspecific binding was blocked with 1% rabbit serum or 1% horse serum (according to the species of the second-step Ab; Vector, Burlingame, CA). First-step Abs were diluted in TBS and 1% rabbit (or 1% horse) serum, and applied for 60 min. Then, sections were washed and incubated with either a biotinylated horse anti-mouse Ab or a biotinylated rabbit anti-goat Ab (Vector) for 30 min. Subsequently, FITC-streptavidin complexes (Vector) were applied for 30 min. After washing, slides were photographed. Thereafter, anti-trypaste Ab was applied, and the reaction was visualized by fast red. Slides were counterstained in Gill’s hematoxylin. In control experiments, slides were similarly treated, with the primary Ab omitted or using isotype-matched control Abs. All Ab reactions were confirmed for single cells by double staining experiments in serial sections. The staining reaction of MC with anti-tPA Ab was also confirmed for isolated MC. For this purpose, purified lung MC (>90% pure) and HMC-1 were spun on cytosin slides, fixed in acetone or methanol, and stained with anti-tPA or anti-trypaste. Streptavidin-conjugated peroxidase and amino-ethylcarbazole were applied for tPA staining, and streptavidin-conjugated alkaline phosphatase and fast red were used for tryptase staining of isolated MC.

**Results**

**Expression of PA and PAI mRNA in MC**

As assessed by Northern blotting, purified unstimulated lung MC as well as HMC-1 expressed substantial amounts of tPA mRNA (Fig. 1). Transcripts for tPA were detectable in 91% pure MC (one
donor) as well as in >98% pure MC (MC pool, six donors) by Northern blotting. Expression of c-kit and glyceraldehyde-3-phosphate dehydrogenase mRNA in MC (positive control) was also demonstrable. By contrast, neither MC nor HMC-1 expressed detectable transcripts for uPA or PAIs. Exposure of lung MC (91% pure) to recombinant SCF (100 ng/ml, 2 h) was followed by a decrease in the expression of tPA mRNA (Fig. 1C). The same effect of rhSCF was seen in HMC-1 cells. Like primary MC, HMC-1 expressed tPA- and c-Kit mRNA. Exposure of HMC-1 to rhSCF (100 ng/ml) reduced expression of tPA mRNA compared to control medium (control). SCF caused down-regulation of tPA mRNA expression. The lower graph shows the tPA protein concentration (ELISA) in the supernatants of these MC.

Expression and release of the tPA protein

Expression of the tPA protein was analyzed by an ELISA quantifying both complexed (with PAI) and uncomplexed tPA. Pure lung
A second experiment. Similar results were obtained in the presence of tPA activity (BIA). Results represent the mean ± SD of triplicate determinations from one experiment. HMC-1 cells were grown in IMDM with 10% FCS at 37°C.

**FIGURE 3.** Time-dependent accumulation of tPA activity in HMC-1 cells in 1 ml contained 4.8 ± 0.12 ng tPA/ml. A time-dependent increase in tPA protein and activity in conditioned medium of unstimulated HMC-1 was found. Fig. 3 shows the time-dependent increase in tPA activity (measured by BIA) in supernatants of unstimulated HMC-1 cells. rhSCF caused a slight (but insignificant) increase in measurable tPA (protein and activity) in HMC-1 supernatants, and a slight decrease in cellular tPA levels (not shown).

The amount of tPA protein in the 2-h supernatants of unstimulated lung MC (10⁶ cells in 1 ml) ranged between 1.8–17.8 ng (mean, 9.4). SCF augmented the baseline release of tPA from MC, although a decrease in tPA mRNA expression was detectable in the same cells (Fig. 1C).

**Functional characterization of MC-derived tPA**

The functional significance of MC tPA was made visible in a clot lysis assay. In this assay various cells (lysates or supernatants) were added to a synthetic fibrin meshwork. Of all cell types tested, tissue MC (lysates or supernatants of lung MC, >98% pure, n = 3; Fig. 4) and HMC-1 (lysates or supernatants, n = 4) induced clot lysis within 24 h. No clot dissolution was seen with supernatants of HUVEC, smooth muscle cells, fibroblasts, or leukocytes (Table II). The clot lysis effect of MC (and rtPA) was completely inhibited by addition of either PAI-1 or a neutralizing Ab against tPA (no clot dissolution seen after 24 h; Fig. 4).

The sp. act. of MC tPA was analyzed in a plasminogen activation assay. Both rtPA and purified HMC-1-tPA were able to induce significant (rapid) plasmin formation in the presence, but not the absence, of fibrin or heparin. Fig. 5 shows the specific tPA activities of crude HMC-1 supernatant, purified HMC-1 tPA, and rtPA in the absence of fibrin (and heparin). Remarkably, in contrast to purified HMC-1 tPA or rtPA, the crude HMC-1 supernatant induced rapid plasmin formation in the absence of fibrin or (exogenous) heparin (Fig. 5). Interestingly, heparin was able to function as a costimulant of purified MC tPA, in that the specific tPA activity of purified HMC-1 tPA increased to the level of the crude supernatant after addition of heparin (Fig. 5). Overall, in the absence of fibrin or heparin, the specific tPA activities obtained for...
CRUDE HMC-1 supernatants, purified HMC-1 tPA, and rtPA were all in the same range (not shown).

Comparative quantitative analysis of tPA and PAI-1 in various cells

The lysates and supernatants of various cells were analyzed for the presence of tPA Ag (ELISA), tPA activity (BIA), and PAI-1 Ag (ELISA). Primary MC and HMC-1 were found to contain and release tPA Ag and tPA activity, but did not express detectable amounts of PAI-1 (Table II). As assessed by BIA, the calculated tPA activity was 530 ± 450 mU/10⁶ HMC-1 cells (lysates; n = 5). The 2-h supernatants of MC and HMC-1 also contained measurable amounts of tPA activity. All other cells tested (including EC, smooth muscle cells, and various leukocytes) did not contain or release measurable amounts of tPA activity (<10 mU/10⁶ cells) although several of these cells expressed the tPA protein (Table II).

These cells coexpressed PAI-1 in excess over tPA, explaining the lack of detectable PA activity. Exposure of such cells to heparin was not followed by a detectable expression of tPA activity (not shown). In a consecutive series of experiments, supernatants and lysates of HMC-1 were supplemented with increasing concentrations of PAI-1. In these experiments, the tPA activity of HMC-1 could be neutralized by addition of PAI-1 in a dose-dependent manner (Fig. 6).

We next asked whether the contribution of MC-derived tPA would be sufficient to induce profibrinolytic activity in a cell mixture composed of MC and EC, even if MC represent the minor cell population. For this purpose, MC (HMC-1) and EC (HUVEC) were mixed to various dilutions (but a constant total cell number), and the tPA activities in the lysates of the cell mixtures were measured. As visible in Fig. 7, HMC-1-derived tPA was able to antagonize and overcome HUVEC-derived PAI even if the ratio between EC and MC was 32 (∼32-fold excess of HUVEC over HMC-1).
In situ expression of tPA and PAIs

To study in situ expression of tPA, double-staining experiments using Abs against tPA, PAI-1, PAI-2, and tryptase were performed. A clear reactivity of tissue MC (identified by tryptase Ab) with anti-tPA Ab was found in all organs tested (lung, skin, and heart). Fig. 8 shows the reactivity of cardiac MC with anti-tPA Ab. A granular staining pattern was obtained for MC analyzed in the tissue sections. Abs against PAI-1 and PAI-2 were found to bind to vascular cells, but not to tissue MC (not shown). The intensity of staining with anti-tPA varied from MC to MC, and a subpopulation of tissue MC (10–50%) in normal physiologic tissue sites (i.e., no tumor cells or signs of inflammation seen) appeared to be tPA-negative. Neither the tPA+ MC nor the tPA− MC appeared to be located in distinct anatomical regions. In a separate set of experiments, isolated lung MC and HMC-1 were analyzed for binding to anti-tPA Ab. In these experiments both cell types produced a clear staining reaction. Fig. 9 shows the reactivity of lung MC with anti-tPA Ab.

Discussion

MC are equipped with several mediators and enzymes that play a role in inflammatory or other biologic reactions (26–30). Likewise, MC-dependent compounds induce EC activation in vitro (29, 30) and supposedly contribute to capillary leak and edema formation in vivo (51, 52). On the other hand, MC are considered to contribute to tissue repair following tissue injury. One of the MC-derived "repair molecules" that may play a role in thrombosis and fibrin deposition following edema formation, is heparin. However, heparin is unable to (directly) induce fibrin or clot lysis per se. More recent data suggest that MC-dependent compounds are also involved in the regulation of fibrinolysis (53–55). Likewise, Stack and Johnson (53) have shown that MC tryptase is able to activate single chain uPA (pro-urokinase). In the present study we show that human MC express and release tPA without producing PAIs. Moreover, we show that MC, unlike other cells (isolated from the microvasculature or blood), can induce fibrinolysis in vitro. In addition, heparin apparently cooperates with MC-derived tPA in plasminogen activation. These observations strongly suggest that human MC are involved in the process of endogenous fibrinolysis.

The expression of tPA in MC was demonstrable by mRNA analysis, protein analysis, in situ staining experiments, as well as functional assay. Both human tissue (lung) MC and the continuous human MC line HMC-1 were found to express tPA. The most significant finding was that human MC not only express the tPA protein, but also tPA (plasminogen-plasmin-converting) activity and clot lysis activity. Interestingly, the second PA, uPA, was not detectable in tissue MC or HMC-1.

A number of different cells are known to express and release tPA (11–23). The molecular basis of the unique properties of MC (as opposed to other physiologic cells) with regard to plasminogen activation are of particular interest and seem to have several explanations. First, MC (both mature tissue MC and HMC-1) apparently produce, express, and release the tPA protein in a constitutive manner. This is a significant observation, because many MC mediators are not constitutively expressed by (mature) MC at the RNA level (56). The second reason for the potent profibrinolytic effect of MC is their lack of inhibitors (PAIs). Thus, in contrast to all other tPA-producing microenvironmental cells tested, MC did
not express detectable amounts of PAI-1, PAI-2, or PAI-3. Even when stimulated with rhSCF for 2 h (significant MC activation), lung MC did not express detectable PAI mRNA, and the same result was obtained with HMC-1 cells. Also, in the disease model analyzed to date (auricular thrombosis), MC did not react with Abs against PAI-1 or PAI-2 by in situ staining experiments (54).

Usually, the tPA-induced conversion of plasminogen into plasmin requires the presence of fibrin or heparin (57). Therefore, the observation that MC (conditioned medium of HMC-1) induce fibrin-independent plasmin activation is remarkable. Thus, crude supernatants of HMC-1 induced rapid plasminogen activation in either the absence or the presence of fibrin, whereas purified HMC-1 tPA (like rtPA) induced plasmin activation only in the presence, but not in the absence, of fibrin or heparin. The underlying molecular basis of this phenomenon is not known. One reasonable explanation may be the action of heparin or a similar proteoglycan (produced by MC) as a tPA cofactor(s). The possibility that mediators other than tPA in the conditioned medium were active (plasminogen activation) seems unlikely. Thus, the plasminogen-activating effect of MC- or HMC-1-conditioned medium could be neutralized by an Ab against tPA (as well as by addition of PAI-1). An effect of the MC protease trypase also seems unlikely, since this enzyme reportedly cleaves fibrinogen as specific substrate, but does not degrade fibrin (55).

The c-Kit ligand SCF is a major regulator of human MC (and MC in other species). In particular, SCF induces differentiation of MC from their progenitor cells as well as MC chemotaxis and mediator secretion (58–63). In the present study rhSCF was found to induce the release of tPA from lung MC after 2 h. However, in the same cells, a decrease in tPA mRNA expression was seen. This divergence may represent a feedback mechanism preventing excess accumulation of tPA in the tissues. In the HMC-1 cell line, rhSCF also induced down-regulation of expression of tPA mRNA. However, the effect of SCF on tPA release was too small to reach statistical significance. The reason for this weak response of HMC-1 to SCF is not known, but may be related to a general intrinsic “release defect” of these cells. It is also noteworthy in this respect that HMC-1 cells exhibit activating point mutations in the kinase domain of the SCF receptor c-Kit (64). Therefore, one possibility could be that the receptor is intrinsically activated by the mutation, so that the additional release response to the ligand (c-Kit ligand) was insignificant.

The amounts of tPA in HMC-1 cells exceeded by far the amounts of tPA in the enriched primary MC (>98% pure). The reason for this discrepancy is not known. One possibility could be that tPA production in MC is (also) associated with proliferation or differentiation processes, explaining the lower tPA levels in mature cells compared with those in the continuously proliferating immature cell line (HMC-1). Alternatively, the MC purification procedure (flow cytometry) caused loss of cellular tPA. In this respect it is noteworthy that the levels of measurable tPA were lower in sorted MC compared with elutriated MC (not shown).

Substantial evidence exists for MC heterogeneity. In particular, depending on the organ or tissue sites examined, MC express varying amounts of the proteolytic enzymes trypase and chymase (32). We therefore were interested to know whether tPA is expressed in various types of MC in different anatomical regions or organs. To date we have been able to detect tPA in MC in all organs tested (lung, skin, and heart). The reactivity of MC with the anti-tPA Ab varied, but no significant correlation between tPA expression and the organ or tissue sites analyzed was detectable in normal tissues. However, we found a weaker reactivity of MC with anti-tPA Abs in the upper endocardium (where SCF immunoreactivity was detected) in patients with auricular thrombosis compared with MC in the myocardium of the same patients (54). This observation may be explained by the release of tPA or by its decreased production. In light of our mRNA and ELISA data for SCF-activated MC, both possibilities seem likely.

The biologic relevance of MC tPA is not yet known. One important question is whether MC-derived tPA can really overcome a massive PAI production by neighboring EC or other activated microenvironmental cells in the case of a thromboembolic event or an inflammatory process. Thus, although MC seem to accumulate in areas of ongoing thrombosis (50) and migrate against thrombin-activated EC in vitro (65), no proof is available for the contribution of MC-derived tPA to endogenous thrombolysis. To address the question of whether tPA derived from a minority of MC can overcome EC-derived PAIs, we performed experiments using mixtures of HUVEC and HMC-1. The results of these analyses suggest that indeed MC-derived tPA can overcome EC-derived PAI in vitro even if MC (HMC-1) represent a minor cell population. However, whether the same holds true for tissue MC and the situation in vivo remains to be shown. It also remains unknown whether MC play a unique role as profibrinolytic cells in diverse microenvironmental processes. Thus, under various conditions, activated macrophages or EC may also produce significant amounts of PAs (11–14, 17, 19, 20). Therefore, it is tempting to speculate that a small amount of MC-derived uncomplexed tPA can regulate tissue homeostasis by influencing the balance between tPA/uPA and PAIs produced by microenvironmental cells.

The identification of MC as a source of active tPA may have several implications. Since MC promote extravasation of fibrinogen to tissues through delivery of vasoactive molecules (51, 52), MC-tPA may function as a “repair molecule,” preventing fibrin deposition during inflammatory or other MC-dependent reactions. Interestingly, an inverse relationship between the number of MC and the amount of tissue fibrin was found in rheumatoid arthritis (66). The ideas that tPA deficiency can predispose for tissue fibrin deposition (6) and that allergic reactions are associated with transient, but not prolonged, fibrin deposition are also in line with the MC tPA concept. Another aspect of the concept is that MC accumulate in areas of thrombus formation (50, 54) and that MC-deficient W/Wv mice show increased susceptibility to thrombogenic stimuli (67). Whether MC-derived tPA does indeed provide an essential contribution to the process of endogenous fibrinolysis is now under investigation.

In summary, our study provides evidence that the MC is a unique site of PA expression and fibrinolysis.

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