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Activation of Matrix-Metalloproteinase-2 and Membrane-Type-1-Matrix-Metalloproteinase in Endothelial Cells and Induction of Vascular Permeability In Vivo by Human Immunodeficiency Virus-1 Tat Protein and Basic Fibroblast Growth Factor

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Previous studies indicated that the Tat protein of human immunodeficiency virus type-1 (HIV-1) is a progression factor for Kaposi's sarcoma (KS). Specifically, extracellular Tat cooperates with basic fibroblast growth factor (bFGF) in promoting KS and endothelial cell growth and locomotion and in inducing KS-like lesions in vivo. Here we show that Tat and bFGF combined increase matrix-metalloproteinase-2 (MMP-2) secretion and activation in endothelial cells in an additive/synergistic manner. These effects are due to the activation of the membrane-type-1-matrix-metalloproteinase and to the induction of the membrane-bound tissue inhibitor of metalloproteinase-2 (TIMP-2) by Tat and bFGF combined, but also to Tat-mediated inhibition of both basal or bFGF-induced TIMP-1 and -2 secretion. Consistent with this, Tat and bFGF promote vascular permeability and edema in vivo that are blocked by a synthetic MMP inhibitor. Finally, high MMP-2 expression is detected in acquired immunodeficiency virus syndrome (AIDS)-KS lesions, and increased levels of MMP-2 are found in plasma from patients with AIDS-KS compared with HIV-uninfected individuals with classic KS, indicating that these mechanisms are operative in AIDS-KS. This suggests a novel pathway by which Tat can increase KS aggressiveness or induce vasculopathy in the setting of HIV-1 infection.

INTRODUCTION

Kaposi's sarcoma (KS) is a tumor of vascular origin that is found in four different clinical, epidemiological forms: classical KS that arises in elderly men of eastern Mediterranean origin, endemic KS that is found in subequatorial Africa, posttransplant KS that develops after organ transplantation and immune-suppressive therapy, and acquired immunodeficiency virus syndrome (AIDS)-associated KS (AIDS-KS) that is the most frequent and aggressive form of KS and occurs in the setting of human immunodeficiency virus type-1 (HIV-1) infection (Safai *et al.*, 1985; Ensoli and Stürzl, 1998).

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However, all KS forms show the same histological features, including inflammatory cell infiltration, edema, angiogenesis, and the proliferation of spindle-shaped cells of endothelial origin, considered to be the tumor cells of KS (KS cells) (Ensoli and Stürzl, 1998). In addition, all forms of KS are associated with the infection with the human herpesvirus-8 (HHV-8) (Huang *et al.*, 1995; Moore and Chang, 1995; Rezza *et al.*, 1999).

Previous studies indicated that inflammatory cytokines (ICs) released by immune cells of individuals with KS or at risk of KS, perhaps in response to (or amplified by) HHV-8 (Fiorelli *et al.*, 1998; Sirianni *et al.*, 1998), may function as KS-initiating factors. In fact, ICs induce normal endothelial cells to acquire the KS cell phenotype and to synthesize and release angiogenic factors such as basic fibroblast growth

factor (bFGF) and vascular endothelial growth factor (VEGF) that are highly expressed in primary KS lesions (Ensoli *et al.*, 1989; Fiorelli *et al.*, 1995; Samaniego *et al.*, 1995, 1997, 1998; Cornali *et al.*, 1996). These angiogenic molecules, in turn, mediate KS and endothelial cell growth and induce in mice the development of angioproliferative lesions closely resembling primary KS lesions (Ensoli *et al.*, 1989, 1994b; Fiorelli *et al.*, 1995; Samaniego *et al.*, 1995, 1997, 1998). However, the presence of ICs, angiogenic factors, and HHV-8 is observed in all forms of KS, whereas KS is more frequent and aggressive in HIV-1–infected individuals. Previous and more recent data indicate that this is due to the Tat protein of HIV-1 that acts as a KS progression factor.

Tat, a transactivator of HIV-1 gene expression (Arya *et al.*, 1985), is released by acutely HIV-1–infected T cells (Ensoli *et al.*, 1990, 1993; Chang *et al.*, 1997). In this extracellular form, Tat promotes the migration, invasion, growth, and adhesion of KS and IC-activated endothelial cells in vitro (Ensoli *et al.*, 1990, 1993; Barillari *et al.*, 1992, 1993; Albini *et al.*, 1995; Fiorelli *et al.*, 1995, 1999) and enhances the angiogenic, KS-promoting effect of bFGF in vivo (Ensoli *et al.*, 1994a). In contrast, Tat has no effects when combined with VEGF (Barillari *et al.*, 1999b).

Further studies indicated that Tat binds the extracellular matrix (ECM) and activates different receptors and signal transduction pathways (Barillari *et al.*, 1993; Albini *et al.*, 1996; Ganju *et al.*, 1998; Milani *et al.*, 1998). Specifically, Tat arginine-glycine-aspartic acid region (RGD) binds and activates $\alpha v\beta 3$ and $\alpha 5\beta 1$ (Barillari *et al.*, 1993; Ganju *et al.*, 1998; Milani *et al.*, 1998), two integrins that are highly expressed in AIDS-KS lesions (Ensoli *et al.*, 1994a) and that mediate Tat-induced KS and endothelial cell migration, invasion, and adhesion (Barillari *et al.*, 1999a). Of note, $\alpha 5\beta 1$ and $\alpha v\beta 3$ expression is induced by IC or bFGF in vitro and in vivo (Barillari *et al.*, 1993, 1999a,b; Sepp *et al.*, 1994; Fiorelli *et al.*, 1995, 1999). In addition, Tat basic region binds to heparan sulfate proteoglycans of the ECM and can release and maintain into a soluble form sequestered bFGF, which then mediates Tat-induced cell growth (Barillari *et al.*, 1999a).

Either Tat or bFGF induces the mRNA expression of the matrix metalloproteinase-2 (MMP-2), the 72-kDa type IV collagenase that is involved in tumor growth and angiogenesis (Ensoli *et al.* 1994a; Ray and Stetler-Stevenson, 1994; Barillari *et al.*, 1999a). In addition, when Tat and bFGF are combined, their effects on MMP-2 mRNA expression are additive or synergistic (Ensoli *et al.*, 1994a; Barillari *et al.*, 1999a). Tat-RGD region promotes MMP-2 RNA expression by triggering the $\alpha 5\beta 1$ and $\alpha v\beta 3$ integrins (Barillari *et al.*, 1993, 1999a), mimicking the effects of ECM molecules such as fibronectin and vitronectin (Seftor *et al.*, 1992; Bafetti *et al.*, 1998; Esparza *et al.*, 1999). Tat is also capable of up-regulating in monocyte the synthesis and release of MMP-9 (92-kDa type IV collagenase), providing a potential mechanism to explain the endothelial cell/basement membrane detachment and the monocytes extravasation into underlying tissues (Lafrenie *et al.*, 1996).

VEGF, another angiogenic factor expressed in KS lesions (Cornali *et al.*, 1996, Samaniego *et al.*, 1998), is also known to induce MMP production in endothelial cells (Lamoreaux *et al.*, 1998; Zucker *et al.*, 1998).

Proteolytic degradation of the ECM by MMP-2 and MMP-9 is blocked by the tissue inhibitors of metalloproteinase-1 and -2 (TIMP-1 and TIMP-2), which are important participants in various physiological and pathological processes (Gomez *et al.*, 1997). Recent data have shown that TIMP-2 forms a receptor by complex formation with the membrane-type-1-matrix-metalloproteinase (MT1-MMP), which regulates the generation of functionally active MMP-2 (Murphy *et al.*, 1999), suggesting a bimodal action of this metalloproteinase inhibitor. Furthermore, it has been recently demonstrated that only the active 60-kDa form of MT1-MMP binds MMP-2 through TIMP-2 at the cell surface, and a second unbound MT1-MMP adjacent to this ternary complex may initiate a second step, leading to the activation of pro-MMP-2 (Strongin *et al.*, 1995; Butler *et al.*, 1998; Lehti *et al.*, 1998).

Because MMPs are among the most potent inducers of vascular permeability (Partridge *et al.*, 1993; Zucker *et al.*, 1998), together these results suggested that bFGF, VEGF, and Tat may play a role in the formation of the edema, one important cause of mortality in AIDS-KS patients (Safai *et al.*, 1985; Ensoli *et al.*, 1991). Although bFGF and VEGF are known to cooperate in vascular permeability and edema (Samaniego *et al.*, 1998), nothing is known about Tat, alone or combined, with these angiogenic factors.

In this study we investigated whether Tat could cooperate with bFGF or VEGF in inducing MMP activation and vascular permeability and edema characterizing AIDS-KS. The results indicate that Tat cooperates with bFGF, but not with VEGF, in enhancing MMP-2 and MMP-9 protein production and secretion in endothelial cells. Combined Tat and bFGF augment active MMP-2 release by increasing the levels of both activated MT1-MMP and cell membrane-associated TIMP-2. In addition, Tat decreases the amount of secreted TIMP-1 and -2 produced by endothelial cells under basal conditions or after stimulation with bFGF. These in vitro effects are associated with the induction of vascular permeability and edema in vivo by combined Tat and bFGF, which are blocked by a specific MMP inhibitor. Because high MMP-2 mRNA expression is present in AIDS-KS lesions and increased levels of MMP-2 are found in plasma from patients with AIDS-KS compared with HIV-1-uninfected individuals with C-KS, the results suggest that Tat and bFGF are the key modulator of MMP-2 functions in KS and may participate in the vasculopathy of HIV-1–infected individuals.

MATERIALS AND METHODS

Reagents and Cell Cultures

Recombinant HIV-1 Tat protein (from the III B isolate) was expressed, purified, and handled as previously described (Ensoli *et al.*, 1990, 1993, 1994a; Chang *et al.*, 1997). The CTTHWGFTLC cyclic peptide selectively inhibiting MMP-2 and MMP-9 activity (Koivunen *et al.*, 1999) and the control peptide (GACFSIAHECGA) have been synthesized and purified by Neosystem (Strasbourg, France) as described (Koivunen *et al.*, 1999). The fluorogenic MMP substrate III [DABCYL-GABA-PQGL-E-(EDANS)-AK-NH2; TN0211] (Beekman *et al.*, 1996) was obtained from Calbiochem (San Diego, CA). Human recombinant bFGF and VEGF were purchased from Collaborative Research (Bedford, MA) and R & D Systems (Minneapolis, MN), respectively. The monoclonal antibody (mAb) increased against TIMP-2 (T2–101) was purchased from Chemicon (Temecula, CA). The mAb directed against the catalytic domain of MT1-MMP was obtained from Oncogene Research (Cambridge, MA). The rab-

bit polyclonal anti-MMP-2 antibody (antibody 45) was kindly provided by Dr. Stetler-Stevenson (National Institutes of Health, Bethesda, MD). Goat polyclonal anti-TIMP-1 and anti-TIMP-2 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). The enzyme-linked immunosorbent assay (ELISA) system for the determination of 72-kDa (latent) MMP-2 levels in body fluids was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Human umbilical vein endothelial cells and human macrovascular and microvascular endothelial cells of lung origin were obtained and cultured as described elsewhere (Samaniego *et al.*, 1997; Barillari *et al.*, 1999a; Fiorelli *et al.*, 1999) and used between passages 4 and 10.

Cell Treatments and Zymography

Cells were incubated with 0.1 μg/ml, 1 μg/ml bFGF or VEGF, 10 ng/ml Tat, alone or combined, or with the resuspension buffer (phosphate-buffered saline [PBS]-0.1% bovine serum albumin [BSA]) that was used as the negative control. After stimulation, cells were rinsed twice with serum-free medium and incubated for 18 h in serum-free medium. Culture supernatants were collected and concentrated with the use of Centricon-10 (Amicon, Beverly, MA). Protein concentration was determined by the Bradford assay (Bio-Rad, Hercules, CA) with the use of BSA as a standard. To detect collagenolytic activity, equal amounts of total proteins from concentrated supernatants were subjected to zymographic analysis as described previously (Kleiner and Stetler-Stevenson, 1994; Toschi *et al.*, 2000). Densitometry of destained areas was quantified with the use of an Imaging Densitometer GS-700 connected to a Macintosh Performa computer with the Multi-Analyst software (Bio-Rad).

Immunoprecipitation

Cells were treated with 0.1 μg/ml, 1 μg/ml bFGF, 10 ng/ml Tat, alone or combined, for 24 h or with the resuspension buffer (PBS-0.1% BSA) that was used as the negative control. After stimulation, cells were rinsed twice with serum-free medium and incubated for 18 h in serum-free medium. Culture supernatants were collected and concentrated up to 10 times and protein concentration was detected as described above.

Protein-adjusted aliquots of concentrated culture supernatants were incubated with the anti-TIMP-2 mAb (10 μg/ml) at 4°C for 18 h followed by incubation with Ultralink immobilized protein G Sepharose (Pierce, Rockford, IL) at 4°C for 2 h. This step was repeated twice, until the TIMP-2 protein disappeared from the cell supernatants as controlled by Western blot analysis. The residual supernatants from the previous TIMP-2 immunoprecipitation were then incubated with a rabbit polyclonal anti-MMP-2 antibody (10 μg/ml) at 4°C for 18 h followed by incubation with Ultralink immobilized protein A agarose (Pierce) at 4°C for 2 h. At each step of immunoprecipitation the samples were run in parallel by both zymogram and Western blot to detect MMP-2 activity and TIMP-2 depletion.

Determination of Net MMP-2 Activity in Cell Supernatants

Concentrated supernatants from cells treated with bFGF, Tat, bFGF and Tat combined, or resuspension buffer (5 μg of total protein) were incubated in sterile 96-well Black View Plates (Packard, Meriden, CT) overnight at 37°C with the TNO211 fluorogenic substrate (5 μM) in a buffer containing 50 mM Tris-HCl pH 7.60, 150 mM NaCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 0.01% Brij 35 in the presence or absence of EDTA (10 mM). Fluorometric measures (λ_{ex} = 340 nm; λ_{em} = 485 nm) were done with a Fusion Universal microplate analyzer (Packard) both immediately after the addition of the fluorogenic substrate and at the end of the incubation.

Western Blot Analysis

To detect MT1-MMP protein expression, cells were lysed in modified RIPA buffer (Toschi *et al.*, 2000) and 20–60 μg of total proteins from each sample were electrophoresed onto 9% SDS-PAGE. For TIMP-1 or TIMP-2 protein detection, the cell supernatants were collected, concentrated, and quantified as described above. Forty micrograms of total proteins from each sample was then separated by 12% SDS-PAGE. After protein transferring, filters were incubated in blocking buffer (5% nonfat dry milk, 0.1% Tween 20, PBS) and probed with the mouse monoclonal anti-human MT1-MMP (Oncogene, Cambridge, MA) or with anti-human TIMP-1 or TIMP-2 goat polyclonal antibodies (Santa Cruz Biotechnology), respectively. Membranes were then washed in PBS containing 0.1% Tween 20, incubated with the specific secondary horseradish peroxidase-conjugated antibody, and developed with the use of chemiluminescence ECL kit (Amersham Pharmacia Biotech UK, Little Chalfont, Buckinghamshire, United Kingdom), according to the manufacturer's instructions. Densitometry of the bands was performed as described above.

Fluorescence-activated Cell Sorter Analysis

Flow cytometric analysis was performed on human umbilical vein endothelial cells harvested with the use of 0.1% EDTA/PBS. Cells (5×10^6) were resuspended in 100 μl of PBS/0.1% fetal bovine serum and incubated for 30 min in ice with anti-TIMP2 mAb (Chemicon), followed by incubation with goat anti-mouse fluorescein isothiocyanate-conjugated antibody (Jackson Immunoresearch, West Grove PA) and fixed in 1% paraformaldehyde. All steps were separated by washes in PBS/0.1% fetal bovine serum. Nonspecific binding of the antibody was assessed by incubating cells with the isotype-matched control antibody (PharMingen, San Diego, CA). The relative amount of cell surface fluorescence was analyzed by an FACscan flow cytometer (Becton Dickinson, San Jose, CA).

Vascular Permeability Assay in Guinea Pigs

Guinea pigs (250–300 g) were inoculated subcutaneously into the flanks with serial dilutions of bFGF or Tat, alone or combined. PBS (Invitrogen, Carlsbad, CA) was used as the negative control. Late-phase vascular permeability, nonhistamine-dependent, and edema were then measured by determining the amount of extravasated Evans blue (Sigma, St. Louis, MO) administered 1 h after the injection of bFGF and/or Tat by established procedures (Kim *et al.*, 1992; Samaniego *et al.*, 1998). Briefly, animals were sacrificed 30 min after bFGF and/or Tat inoculation, and the injection sites were excised, minced, and incubated in formamide for 24–36 h at 56°C. The formamide solution was then filtered through a glass filter (Millipore, Bedford, MA) and optical density of the filtrates measured at 500 nm, as described (Kim *et al.*, 1992). To determine the effect of histamine release due to injection, two or three animals per experiment were treated with triprolidine (200 μg/kg i.v.) (Sigma) (Kamata *et al.*, 1985). This antihistamine drug did not inhibit the enhancement of vascular permeability induced by bFGF and Tat.

Vascular Permeability Assay in Nude Mice and Blocking Experiments with Cyclic MMP-inhibiting Peptides

BALB/c nude mice (male, 5–6 wk old; Charles River Breeding Laboratories, Calco, Italy) were inoculated subcutaneously into the lower back with bFGF (0.1 μg) and Tat (1 μg), alone or combined, in 50 μl of 0.1% BSA/PBS mixed with 50 μl of Matrigel (Collaborative Research) before the inoculation (Nakamura *et al.*, 1992). The resuspension buffer was used as the negative control. Two additional groups of mice have been inoculated with the combination of bFGF and Tat in the presence of the CTTHWGFTLC cyclic peptide (50 μg) or a control peptide (GACFSIAHECGA, 50 μg), 6 h after one sys-

temic dose of the inhibitor peptide or the control peptide (100 μg each, in 0.5 ml of saline solution, i.p.) (Koivunen *et al.*, 1999). To avoid interference of histamine release due to injection, all the mice received a single dose of pyrilamine (Sigma) (80 μg i.m.) 1 h before the injection of bFGF and/or Tat or peptides. Eighteen hours later the late-phase vascular permeability was evaluated as described for guinea pigs, by determining the amount of extravasated Evans blue given intravenously immediately before the injection of bFGF and/or Tat or peptides. The care and use of animals were in accordance with the European Community guidelines.

In Situ Hybridization

Bioptic samples from AIDS-KS lesions and control skins obtained after informed consent and for diagnostic purposes were fixed in PBS-buffered 4% paraformaldehyde at 4°C, dehydrated, and paraffin-embedded as previously described (Stürzl *et al.*, 1999). Thin tissue sections (5–10 μm) were hybridized with the antisense MMP-2 RNA probe (1747–2733) (nucleotide enumeration as in GenBank J03210) or sense MMP-2 RNA probe used as control. The probe was cloned in pBSSK$^+$. Briefly, the ^{35}S-radiolabeled RNA probe solution (10–15 μl) was applied to the deparaffinized tissue sections at an adjusted activity of 50,000 cpm/μl in hybridization buffer (50% deionized formamide, 0.3 M NaCl, 20 mM Tris-HCl pH 7.4, 5 mM EDTA, 10 mM $NaPO_4$ pH 8.0, 10% dextran sulfate, 1× Denhardt's solution, 50 μg/ml total yeast RNA). Hybridization was carried out at 50°C for 16 h. At the end of the hybridization step, tissue sections were washed in 5× SSC (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate) containing 10 mM dithiotreitol at 50°C followed by stringent washing at 60°C in a solution containing 50% formamide, 2× SSC, and 0.1 M dithiothreitol, covered with film emulsion and exposed for 14 d.

Determination of MMP-2 Plasmatic Levels

Plasma levels of 72-kDa (latent) MMP-2 were determined with the matrix metalloproteinase-2 (MMP-2), human, ELISA system according to the instructions of the manufacturer. Aliquots of diluted plasma (1:50) from 11 patients with AIDS-KS that were not under treatment with HIV-1 protease inhibitors and 11 HIV-negative patients with C-KS, obtained upon informed consent, were analyzed. The plasma levels of MMP-2 in the two groups of patients were compared by the Student's *t* test (one-tailed).

RESULTS

Induction of MMP-2 and MMP-9 Secretion in Endothelial Cells by Tat and bFGF

Tat and bFGF are both capable of promoting MMP-2 RNA expression in endothelial cells and when combined increase the RNA expression of this collagenase in an additive or synergistic manner (Ensoli *et al.*, 1994a). To evaluate the effect of Tat and bFGF, alone or combined, in the induction of MMP-2 release and activation, kinetic analysis was first performed with human umbilical vein endothelial cells (HUVEC). Results indicated a significant response to the treatment at 24 and 48 h. Therefore, further analyses were performed with supernatants at 24 h of culture and with both macrovascular (HUVEC) and human microvascular endothelial cells of lung origin (HMEC-L).

With HUVEC, stimulation with bFGF or Tat alone resulted in no or little increase of the secreted latent form of MMP-2 (72 kDa) compared with untreated cells, whereas their combination (bFGF 0.1 μg/ml and Tat 10 ng/ml) induced an increase of the amount of secreted latent MMP-2 (Figure 1A). On the other hand, bFGF (at either 0.1 or 1 μg/ml) enhanced the release of the active/cleaved MMP-2 form (62 kDa), whereas Tat alone had no effect. However, the combination of bFGF (0.1 μg/ml) and Tat (10 ng/ml) resulted in an increase of activated MMP-2 compared with untreated cells (Figure 1A).

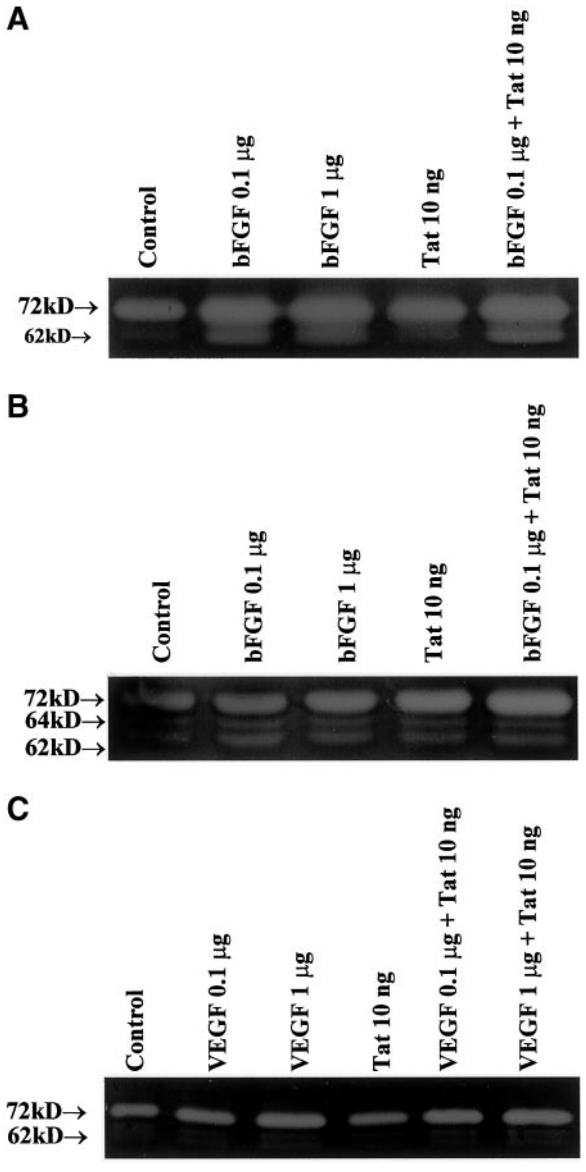

Figure 1. Induction of MMP-2 release by bFGF, VEGF, or Tat, alone or combined, in HUVEC and in human microvascular endothelial cells. Shown are the different total amounts of latent (72-kDa) and activated (62-kDa) MMP-2 or the intermediate form (64 kDa) of MMP-2 released by HUVEC (A) or HMVE-L (B) after stimulation with bFGF and Tat alone or combined, or by HUVEC stimulated with VEGF and Tat, alone or combined (C). The enzymes activity was detected by zymography in the cell supernatants after stimulation with 0.1 or 1 μg/ml bFGF, 0.1 or 1 μg/ml VEGF, with 10 ng/ml Tat, alone or combined, or control buffer (control) for 24 h. Shown are representative experiments. Each experiment was repeated at least three times.

With HMEC-L, bFGF or Tat alone already induced the release of both latent and activated MMP-2 forms compared with the untreated control (Figure 1B). Furthermore, when bFGF and Tat were combined they had additive effects on the increase of both latent and activated MMP-2 compared with the untreated control (Figure 1B). In addition, in HMEC-L the 64-kDa form of MMP-2 was also clearly detected (Figure 1B). This is an intermediate form of active MMP-2 resulting from a first cleavage of the propeptide that is then followed by a second cleavage to give the final 62-kDa active enzyme (Strongin *et al*., 1993).

An induction of the extracellular MMP-9 was also detected by zymography in these experiments. In addition, Tat alone was capable of inducing MMP-9 secretion compared with untreated control cells, as previously observed by others with monocytes (Lafrenie *et al*., 1996). bFGF (0.1 μg or 1 μg/ml) had little effect on MMP-9 release; however, in these experiments, the combination of Tat and bFGF resulted in an increase of the released latent MMP-9 form compared with the control cells (data not shown). Nevertheless, no active form of MMP-9 was detected under these experimental conditions, therefore, no further studies on MMP-9 were conducted.

In contrast to combined bFGF and Tat, VEGF and Tat had no additive or synergistic effects on MMP secretion on HUVEC. Specifically, Tat did not enhance the production and release of MMP-2 that was induced by VEGF (0.1 or 1 μg/ml), and MMP-2 levels remained similar in the presence or absence of Tat (Figure 1C). Similarly, MMP-9 production and secretion were not affected by the treatment of the cells with VEGF alone or in combination with Tat. These results are in agreement with those of other studies indicating that Tat enhances the angiogenic effects of bFGF, but not those of VEGF (Ensoli *et al*., 1994a; Barillari *et al*., 1999a,b), therefore, no further studies were conducted with VEGF.

Induction of Non-TIMP-2–bound MMP-2 by Tat and bFGF

Most of the MMP-2 released in the cell supernatants is complexed with TIMP-2 and it is therefore inactive (Yu *et al*., 1997). To evaluate the amount of non-TIMP-2–bound MMP-2, experiments of TIMP-2 depletion were performed on concentrated (10-fold) supernatants from HUVEC treated with Tat (10 ng/ml) and bFGF (0.1 and 1 μg/ml), alone or combined. After two rounds of immunoprecipitation of both free and MMP-2–complexed TIMP-2, net MMP-2 expression was measured by zymographic analysis (Figure 2, A and B). Depletion of free and MMP-2–complexed TIMP-2 from the cell supernatants after immunoprecipitation was controlled by Western blot (data not shown). The levels of both latent and activated MMP-2 were significantly reduced after TIMP-2 depletion (Figure 2B) in all samples compared with the initial amount of total MMP-2 (Figure 2A). Nevertheless, bFGF alone determined a dose-dependent increase of released latent and activated non-TIMP-2–bound MMP-2, whereas Tat alone had little or no effect compared with the control. However, the combination of Tat (10 ng/ml) and bFGF (0.1 μg/ml) increased both latent and activated non-TIMP-2–bound MMP-2 in an additive or synergistic manner

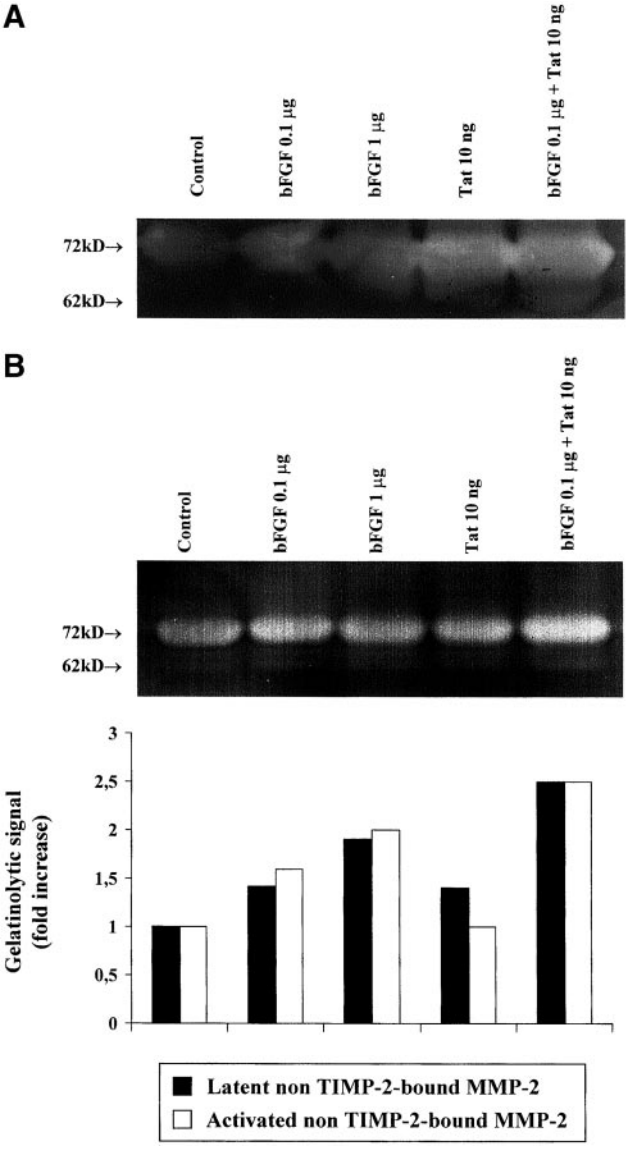

Figure 2. Net MMP-2 activation induced by bFGF and Tat, alone or combined, after TIMP-2 depletion in HUVEC. Shown are the total amounts of MMP-2 (both free and TIMP-2-bound) (A) and the final non-TIMP-2–bound (B) MMP-2 detected after stimulation with Tat and bFGF, alone or combined, or control buffer (control). The net levels of non-TIMP-2–complexed MMP-2 were detected by zymography after two rounds of immunoprecipitation with anti-TIMP-2 mAbs and a final round of immunoprecipitation with anti-MMP-2 polyclonal antibodies as described in MATERIALS AND METHODS. Zymogram bands were quantitated by densitometric analysis. Results are expressed as fold-increase compared with untreated control cells that were given a value of 1. Because the zymogram might have not been in the linear range, the fold-increase of MMP-2 may be underestimated. Western blot analysis to detect TIMP-2 depletion was performed in parallel as described in MATERIALS AND METHODS.

(Figure 2B). These results demonstrate that activated non-TIMP-2–bound MMP-2 is induced synergistically by combined Tat and bFGF

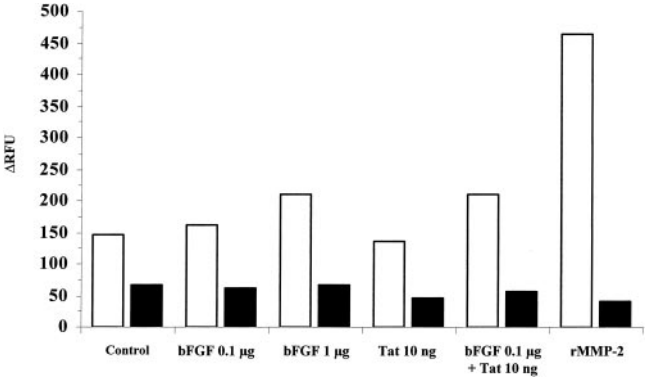

Figure 3. MMP-specific catalytic activity induced by bFGF and Tat, alone or combined. Supernatants from HUVEC treated with 0.1 or 1 μg/ml bFGF, or 10 ng/ml Tat, alone or combined, control buffer (control), or activated recombinant MMP-2 (rMMP-2) (Chemicon) were added to aliquots of the reaction mixture and the levels of fluorescence produced upon substrate cleavage determined. The figure shows the difference between relative fluorescence units (ΔRFU) measured both immediately after the addition of the fluorogenic substrate and at the end of the incubation. White bars indicate the reactions performed in the absence of EDTA and black bars the reactions performed in the presence of EDTA (10 mM), which is known to block MMP activity.

Induction of MMP-2 Net Activity by bFGF and Tat

To determine whether the increased expression of non-TIMP-2–bound MMP-2 induced by bFGF and Tat corresponded to an increase in net MMP-2 activity, HUVEC supernatants were analyzed for the capability of cleaving the fluorogenic peptide TNO211. This peptide is a collagenase substrate that is cleaved with the highest catalytic efficiency by MMP-2 (Beekman *et al.*, 1996). As shown in Figure 3, the catalytic activity present in the supernatants was consistent with the amount of non-TIMP-2–bound MMP-2 detected upon TIMP-2 depletion. In fact, bFGF alone induced a dose-dependent increase of catalytic activity, whereas Tat had no effect. In contrast, the combination of Tat (10 ng/ml) and bFGF (0.1 μg/ml) increased in a more than additive manner the catalytic activity of the cell supernatants. The addition to the supernatants of EDTA, which is known to inactivate MMPs (Beekman *et al.*, 1996), abolished these affects (Figure 3), indicating that the catalytic activity of the cell supernatants was MMP specific.

Induction of MT1-MMP Expression and Activation by Tat and bFGF

MT1-MMP is a transmembrane matrix-metalloproteinase known to bind and activate MMP-2 at the cell surface (Murphy *et al.*, 1999). The expression and activity of this metalloproteinase are regulated by growth factors in other model systems (Lohi *et al.*, 1996). Because we found an increase of the secretion of the proteolitically active MMP-2, Western blot analyses were performed to determine whether Tat and/or bFGF could modulate latent (66-kDa) or activated (60-kDa) MT1-MMP, which determines the catalytic cleavage of MMP-2 (Pei and Weiss, 1996).

No increase of both MT1-MMP forms was observed after endothelial cell incubation with 0.1 or 1 μg/ml bFGF compared with the control (Figure 4, A and B). In contrast, a 5- and 2.5-fold-increase of latent and activated MT1-MMP, respectively, was detected in lysates from HUVEC treated with 10 ng/ml Tat compared with control cells (Figure 4, A and B). Finally, combined bFGF and Tat enhanced by 8-fold the latent MT1-MMP and by 10.5-fold the activated MT1-MMP forms compared with cell lysates from untreated HUVEC, respectively (Figure 4, A and B). These data show that Tat and bFGF combined not only up-regulate MT1-MMP expression but also induce its activation, which is required to mediate cell-surface pro-MMP-2 activation (Pei and Weiss, 1996).

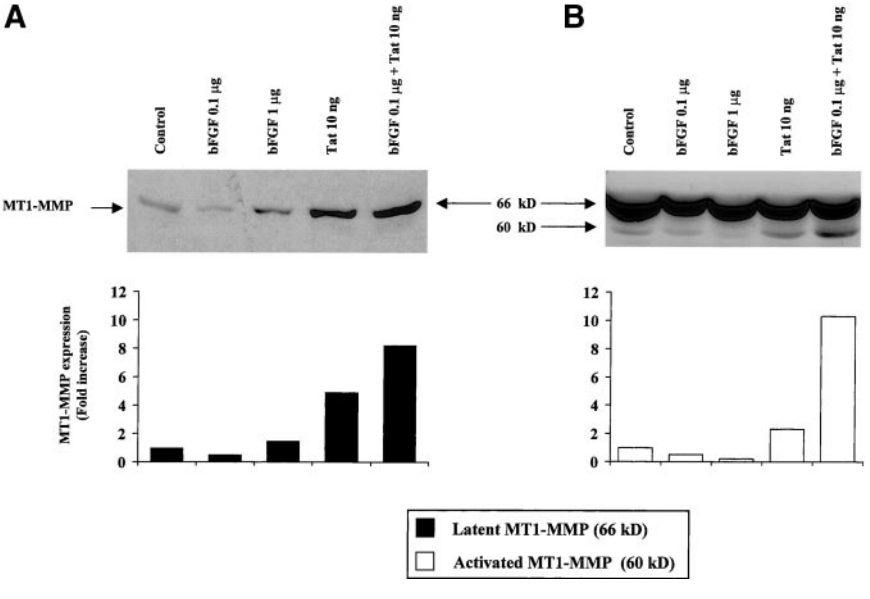

Figure 4. Induction of MT1-MMP expression and activation by bFGF and Tat, alone or combined, in HUVEC. Shown are representative Western blots of latent (66 kDa) and activated (60 kDa) MT1-MMP and the relative densitometric analysis after 24 h of cell incubation with 0.1 μg/ml, 1 μg/ml bFGF, or 10 ng/ml Tat, alone or combined, or control buffer (control). To better quantify latent and activated MT1-MMP, two different gels were run with different amounts of cell lysates because larger amounts of total proteins were required to detect the 60-kDa form and this did not allow an optimal separation of the 66-kDa band of the enzyme. Results are expressed as fold-increase over the control buffer (onefold).

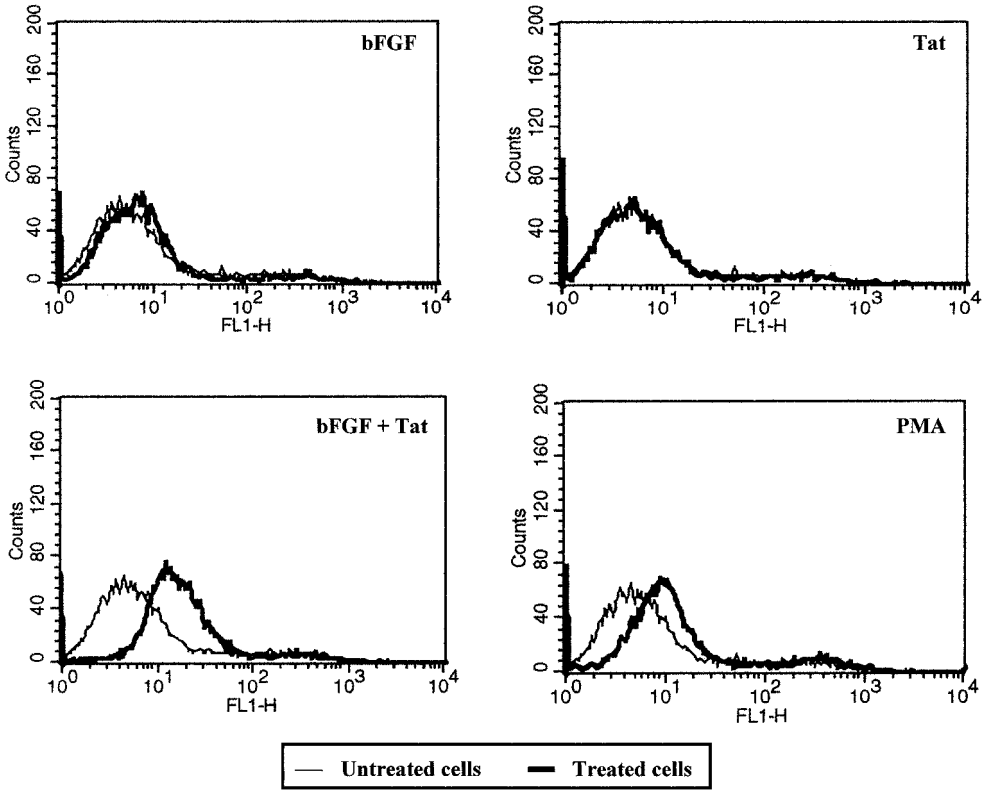

Figure 5. Effects of Tat and bFGF, alone or combined, on the cell membrane-bound form of TIMP-2. Shown is the flow cytometric analysis of the cell surface-bound TIMP-2 induced by 0.1 μg/ml bFGF or 10 ng/ml Tat, alone or combined, or 20 nM phorbol-12-myristate-13-acetate (positive control). The expression of TIMP-2 by the untreated control (−) was normalized on the value obtained with the isotype-matched control.

Induction of Cell Surface-Bound TIMP-2 in Endothelial Cells by Tat and bFGF

Several studies have demonstrated that activation of pro-MMP-2 by MT1-MMP depends upon the presence of critical amounts of cell surface-bound TIMP-2, which is required for the formation of the ternary complex that leads to the activation of MMP-2 (Strongin *et al.*, 1995; Butler *et al.*, 1998).

To study the effects of bFGF and Tat on the cell membrane-associated form of TIMP-2, flow cytometric analysis was performed with specific antibodies on nonpermeabilized cells. A baseline membrane expression of TIMP-2 was found in HUVEC incubated with the control buffer and neither bFGF (0.1 μg/ml) nor Tat (10 ng/ml) alone was able to modify its expression (Figure 5). However, combined Tat and bFGF had a clear up-regulatory effect on the cell surface expression of TIMP-2 at levels observed with phorbol-12-myristate-13-acetate (20 nM) that was used as the positive control (Lehti *et al.*, 1998) (Figure 5). These data indicated that Tat and bFGF combined can enhance MMP-2 activation by increasing the levels of cell-surface bound TIMP-2 which, in turn, allows MMP-2 activation by MT1-MMP.

Inhibition of TIMP-1 and TIMP-2 Release in Endothelial Cells by Tat

To determine whether Tat or bFGF, alone or combined, was also capable of modulating the level of secreted TIMP-1 and -2, Western blots analyses were performed on supernatants collected from HUVEC incubated with Tat and/or bFGF. Both TIMP-1 and TIMP-2 were induced by bFGF (Figure 6, A and B), confirming previous reports (Andersen *et al.*, 1998). In particular, TIMP-1 levels were increased by 3.3- and 4.5-fold with 0.1 and 1 μg/ml bFGF, respectively, compared with untreated control cells (Figure 6A). Similarly, 0.1 and 1 μg of bFGF determined an 8- and 7.5-fold increase of secreted TIMP-2, respectively, compared with control cells (Figure 6B). In contrast, Tat (10 ng/ml) reduced TIMP-1 secretion and blocked almost completely TIMP-2 secretion produced by the cells under basal conditions (Figure 6, A and B). In addition, Tat inhibited the release of both TIMP-1 and TIMP-2 stimulated by bFGF (Figure 6, A and B). Thus, Tat inhibits the secretion of the inhibitors of MMP-2 and MMP-9 activity, favoring the proteolytic degradation of the ECM.

Tat Synergizes with bFGF in Inducing Vascular Permeability and Edema In Vivo

The results from the in vitro experiments showed that combined bFGF and Tat determine an additive/synergistic enhancement of MMP-2 activity that is known to induce vascular permeability (Partridge *et al.*, 1993; Zucker *et al.*, 1998). To evaluate bFGF and Tat effects on vascular permeability in vivo, different amounts (0.1 or 1 μg) of these two proteins were injected into guinea pigs alone or in combination. When bFGF or Tat were injected alone they induced little or no vascular permeability at all the concentrations tested (Figure 7A). However, the combination of the two proteins had a dramatic effect on edema formation at all the doses used. In particular, the combination of 0.1 μg of bFGF and 1

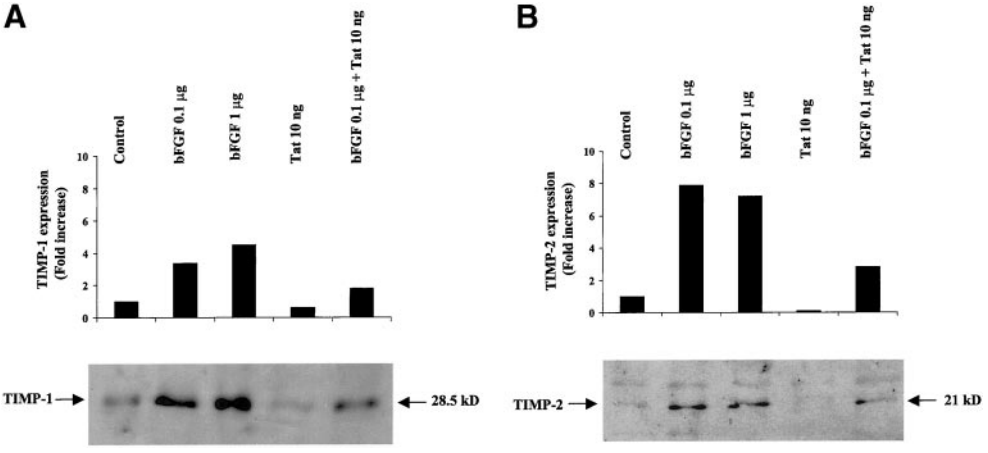

Figure 6. Effect of Tat and bFGF, alone or combined, on TIMP-1 and TIMP-2 secretion. Shown are representative Western blots and densitometric analysis (expressed as fold-increase over the control buffer, onefold) of extracellular TIMP-1 (A) and TIMP-2 (B) detected in supernatants from HUVEC after 24 h of incubation with 0.1 or 1 μg/ml bFGF, or 10 ng/ml Tat, alone or combined, or control buffer (control).

μg of Tat induced a 200% increase of vascular permeability over the control (0%) (Figure 7A). Similar effects were observed with nude mice although edema induction was lower than with the guinea pig model (see below). These data indicate that the combination of Tat and bFGF up-regulates synergistically the vascular permeability effect that characterizes AIDS-KS lesions in vivo.

Inhibition of Vascular Permeability Induced by bFGF and Tat In Vivo with Synthetic MMPs Inhibitor

To determine whether the vascular permeability induced by bFGF and Tat combined was due to MMP activation, a synthetic cyclic peptide (CTTHWGFTLC) known to be a specific inhibitor of MMP-2 and MMP-9 activity in mice (Koivunen *et al.*, 1999) was used for in vivo blocking experiments with the use of a murine model of vascular permeability (Nakamura *et al.*, 1992). The cyclic peptide GACFSIAHECGA was used as control (Koivunen *et al.*, 1999). Blocking studies were performed in nude mice and not in guinea pigs, which are a more sensitive model for vascular permeability, due to the previous use of these peptides in mice models that have shown inhibition of KS growth and a selective targeting of angiogenic blood vessels (Nakamura *et al.*, 1992; Koivunen *et al.*, 1999).

When the inhibitor peptide was administered to the mice a 60% inhibition of the vascular permeability effects promoted by bFGF and Tat combined was observed compared with the control peptide that had a little or no effect (Figure 7B). These data demonstrate that vascular permeability promoted in vivo by bFGF and Tat combined is at least partially due to MMP-2 and MMP-9 induction.

Detection of MMP-2 in AIDS-KS Human Tissues and in Plasma from Patients with AIDS-KS or CKS

To verify the biological relevance of these in vitro and in vivo data, tissues from seven AIDS-KS lesions were examined for MMP-2 expression by in situ hybridization and compared with uninvolved skin from the same donors. In uninvolved skin MMP-2 mRNA expression was very low

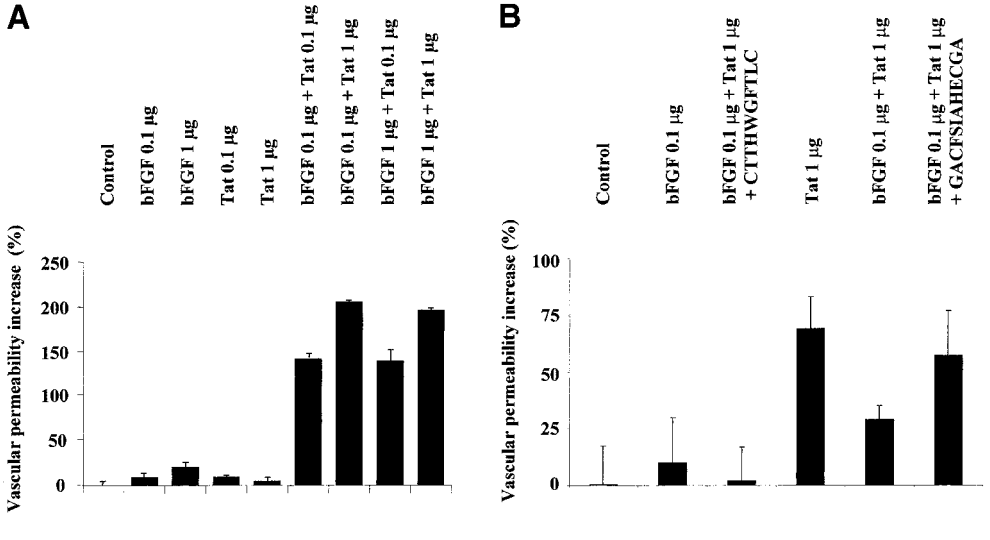

Figure 7. bFGF and Tat synergize in inducing vascular permeability in guinea pigs. (A) Shown are the results after injection of 0.1 or 1 μg of bFGF and 0.1 or 1 μg of Tat alone or combined, or buffer alone, in six animals per experimental condition. (B) Block of vascular permeability induced in mice by combined bFGF and Tat with an MMP-2 synthetic inhibitor. Shown are the results obtained after injection of bFGF and Tat combined (0.1 and 1 μg, respectively), alone or with the CTTHWGFTLC cyclic or the control (GACFSIAHECGA) peptide administered systemically and locally (6 mice per experimental condition). Vascular permeability is expressed as the percentage of increase ($\pm$ SD) over the control that was given a 0% value.

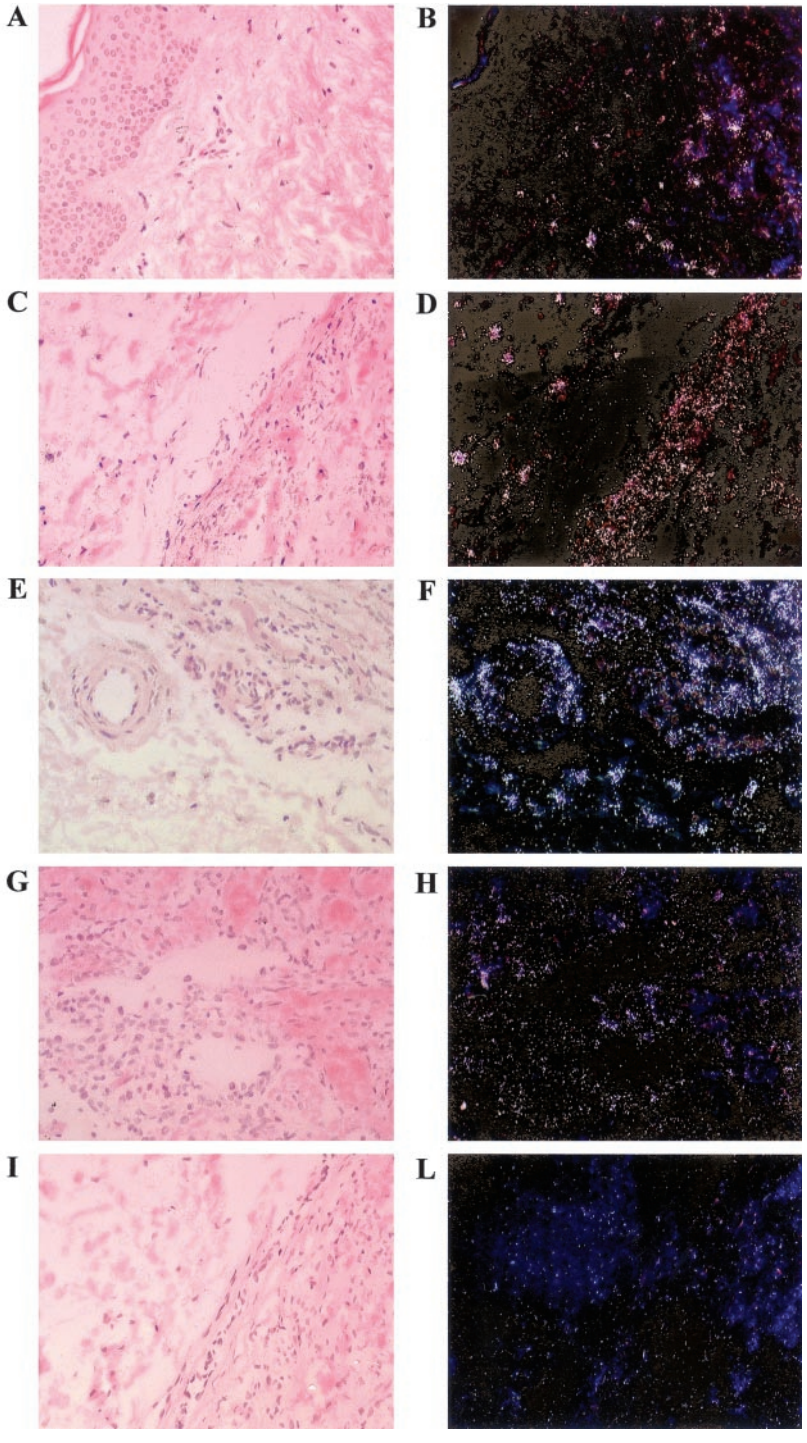

Figure 8. MMP-2 expression in AIDS-KS lesions by in situ hybridization analysis. In situ hybridization was performed on human AIDS-KS lesions as described in MATERIALS AND METHODS. Bright field (A) and dark field (B) show the expression of MMP-2 in healthy skin. Bright field (C and E) and dark field (D and F) show MMP-2 expression at the tumor sheath and in perivascular cells of vessels present in peritumoral tissue. Bright field (G) and dark field (H) show the expression of MMP-2 in the center of the nodular lesion. Bright field (I) and dark field (L) show the staining of an AIDS-KS lesion after use of the sense probe (control). Seven AIDS-KS lesions and corresponding healthy skins were examined with similar results.

and mostly concentrated in fibrocytes (Figure 8, A and B). In contrast, AIDS-KS lesions showed a very strong MMP-2 expression in the tumor sheath and in fibrocytes (Figure 8, C and D), as well as in perivascular cells of vessels present in peritumoral tissue (Figure 8, E and F). In addition, in the center of the nodular lesion MMP-2 was expressed by spindle cells, fibrocytes, and perivascular cells (Figure 8, G and H). As expected, no MMP-2–specific signals were detected in KS lesions by with the use of the sense MMP-2 probe (Figure 8, I and L).

To further support these data, plasma samples from 11 patients with AIDS-KS, which were not under therapy with HIV protease inhibitors, and from 11 subjects with C-KS were analyzed by ELISA for the levels of circulating latent

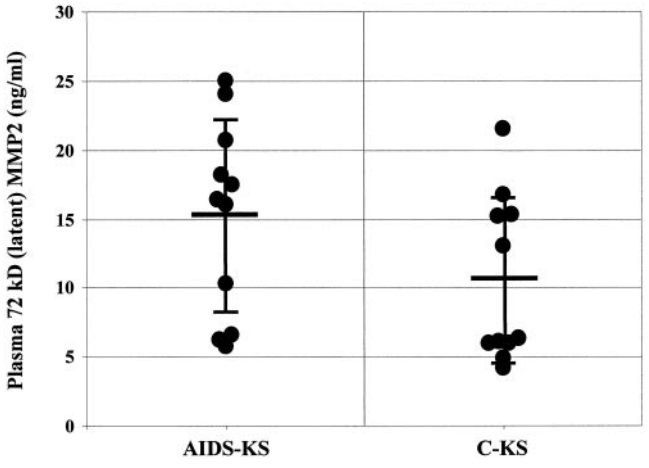

Figure 9. Detection of circulating 72-kDa (latent) MMP-2 in plasma from patients with AIDS-KS and C-KS. Plasma samples collected from 11 subjects with AIDS-KS not under therapy with HIV protease inhibitors, and 11 individuals with C-KS were analyzed by ELISA for MMP-2 levels. Data are expressed as nanograms per milliliter of MMP-2 found in plasma samples diluted 1:50. Bars represent average and SDs. The difference between the MMP-2 plasma levels in AIDS-KS patients compared with patients with C-KS was at the limit of the statistical significance (Student's *t* test, one-tailed; $p = 0.0541$).

MMP-2. Higher levels of circulating MMP-2 were found in plasma from AIDS-KS patients compared with HIV uninfected subjects with C-KS (Figure 9). Although this assay as well as in situ hybridization measure the level of MMP-2 synthesis and not its activation, the data further suggest that Tat and bFGF cooperate in vivo in the induction of MMP-2 production, as observed in vitro.

Thus, MMP-2 is highly produced and expressed in AIDS-KS lesions. Because Tat and bFGF are also highly expressed in these tissues (Ensoli *et al.*, 1994a), they are likely to be the principal modulators of MMP-2 in AIDS-KS patients.

DISCUSSION

Here we have shown that Tat synergizes with bFGF in inducing MMP-2 synthesis, secretion, and activation. These effects are due to the increased expression and activation of MT1-MMP and to the up-regulation of the cell membrane-associated TIMP-2 levels that are induced by Tat and bFGF combined, but also to the Tat-mediated inhibition of TIMP secretion. These effects of Tat and bFGF are associated with the induction of vascular permeability in vivo, which is little or absent when Tat or bFGF is injected alone. As a consequence, combined Tat and bFGF promote the formation of edema, a typical histological and clinical feature of KS (Safai *et al.*, 1985). This is blocked by a specific MMP-2 and MMP-9 synthetic antagonist. Finally, these results appear to be relevant in the disease because MMP-2 is highly expressed in AIDS-KS lesions.

Previous studies indicated that Tat and bFGF synergize in inducing the mRNA expression of MMP-2 (Ensoli *et al.*, 1994a). The fact that type IV collagenases are potent inducers of vascular permeability suggested to us that the up-regulation of MMP expression by Tat and bFGF could play a major role in the pathogenesis of the edema and angiogenesis associated with AIDS-KS. Consistent with this hypothesis, here we have shown that Tat and bFGF combined significantly enhance MMP-2 release and act in a synergistic manner to increase in both macro- and microvascular endothelial cells the secretion of MMP-2 mature/active form (Figure 1), which is essential to express proteolytic activity (Lewalle *et al.*, 1995). The two endothelial cell types did not show an identical pattern of induction of MMP-2 after stimulation with Tat and bFGF. In particular, the abundant presence of the MMP-2 64-kDa intermediate in the HMEC-L cell supernatants suggests that a total conversion of this form to the fully activated MMP-2 protein may require a longer time for HMEC-L compared with HUVEC. However, a synergistic up-regulation of the activated MMP-2 form was similarly determined by Tat and bFGF in both HUVEC and HMVEC-L compared with the single treatments (Figure 1).

The increase of activated MMP-2 in the cell supernatants was observed even after TIMP-2 depletion, indicating that bFGF and Tat induce a significant enhancement of the net MMP-2 activity (non-TIMP-2 bound) as shown by measuring both the non-TIMP-2–complexed MMP-2 and the net MMP-specific catalytic activity present in the cell supernatants (Figures 2 and 3). This active protein has been previously demonstrated to colocalize with $\alpha v\beta 3$ on angiogenic blood vessels and tumor cells in vivo, defining a single cell-surface receptor that regulates both matrix degradation and motility favoring vascular permeability (Brooks *et al.*, 1996). Recent studies have also shown that $\alpha v\beta 3$ and MT1-MMP are colocalized on the cell membrane of tumor cells, suggesting a role of active MT1-MMP in the activation of $\alpha v\beta 3$-bound MMP-2 (Hofmann *et al.*, 2000).

Activation of MMP-2 by Tat and bFGF appears to be due to the induction of MT1-MMP that by forming a complex with TIMP-2 on the cell surface, binds and activates pro-MMP-2 (Strongin *et al.*, 1995; Butler *et al.*, 1998; Lehti *et al.*, 1998; Murphy *et al.*, 1999). In fact, the combination of Tat and bFGF first enhances MT1-MMP expression and activation (Figure 4) and, second, induces critical levels of the cell-surface bound TIMP-2, which in turn favors MMP-2 activation (Figure 5). Furthermore, Tat alone inhibits both TIMP-1 and TIMP-2 secretion, either baseline or bFGF induced (Figure 6). This latter result suggests an additional role of Tat in causing an unbalance between free activated MMP-2 and its specific inhibitors. This unbalance favors both the cell movement and the proteolytic degradation of the ECM. This result is consistent with the fact that Tat mimics the action of ECM proteins (Barillari *et al.*, 1993, 1999a,b; Ensoli *et al.*, 1994a) such as fibronectin, which has been recently shown to promote not only the processing of latent MMP-2 but also MT1-MMP expression (Stanton *et al.*, 1998; Esparza *et al.*, 1999).

Tat alone can also induce in endothelial cells MMP-9 expression, as previously demonstrated in monocytes (Lafrenie *et al.*, 1996). In addition, the combination of Tat and bFGF further increases the release of this gelatinase that is generally undetectable in these cells (Hanemaaijer *et al.*, 1993). However, no activated MMP-9 form was detected, therefore, no further studies were performed on MMP-9. In contrast to bFGF, Tat does not enhance VEGF-promoted

MMP-2 or MMP-9 production and release in endothelial cells (Figure 1).

Previous work indicated that bFGF simultaneously promotes the expression of MMP-2 (Ensoli *et al*., 1994a; Ray and Stetler-Stevenson, 1994) and of the $\alpha5\beta1$ and $\alpha v\beta3$ integrins (Sepp *et al*., 1994; Ensoli *et al*., 1994a; Barillari *et al*., 1999b). The triggering of these two receptors is known to increase MMP-2 synthesis and release (Seftor *et al*., 1992; Ensoli *et al*., 1994a). This is consistent with the finding that Tat synergizes with bFGF, but not with VEGF, in promoting endothelial cell growth and in vivo angiogenesis (Ensoli *et al*., 1994a; Barillari *et al*., 1999b), and with the fact that Tat angiogenic effects correlate with the expression of $\alpha v\beta3$, which is induced by bFGF and binds Tat RGD region, and not with the expression of $\alpha v\beta5$ that is promoted by VEGF (Barillari *et al*., 1999b). In fact, RGD, but not KGE-mutated peptides, block the effects of Tat in vitro and in vivo (Barillari *et al*., 1999a,b).

The effects of bFGF and Tat on MMP-2 appear to be relevant in vivo because Tat synergizes with bFGF in inducing vascular permeability and edema in guinea pigs (Figure 7A) or nude mice (Figure 7B). In addition, vascular permeability induced by Tat and bFGF is reduced (by 60%) by a selective synthetic inhibitor of MMP-2 and MMP-9 (Figure 7B). This is a recently discovered component of a novel class of cyclic peptides containing an HWGF motif, which has been shown to block KS lesion growth and to target angiogenic blood vessels in nude mice (Koivunen *et al*., 1999). The high affinity of this motif for the proliferating/activated endothelium in an angiogenic site suggests that MMP-2 and MMP-9 are preferentially anchored to active endothelial cells but not to quiescent endothelial cells (Koivunen *et al*., 1999). Consistent with this, the synthetic peptide may act on the anchorage complex formed by MMP-2 and $\alpha v\beta3$ integrin (Brooks *et al*., 1996), which are both induced and activated by Tat and bFGF as demonstrated by our present and previous data (Barillari *et al*., 1993, 1999a,b; Sepp *et al*., 1994; Fiorelli *et al*., 1995, 1999). Finally, a strong MMP-2 mRNA expression is detected in AIDS-KS lesions and increased levels of latent MMP-2 are found in plasma from patients with AIDS-KS compared with HIV-1-uninfected individuals with C-KS (Figures 8 and 9). Taken together, these data suggest a key role of bFGF and Tat in the induction of MMP-2 in vivo, which in turn favors the vascular permeability and the angiogenesis characterizing KS lesions and may participate in the vasculopathy of HIV-1 infected individuals. In fact, bFGF, Tat, $\alpha v\beta3$ and $\alpha5\beta1$ and MMP-2 are highly expressed in AIDS-KS lesions (Ensoli *et al*., 1994a) suggesting that these mechanisms are operating in vivo in increasing the angiogenesis and edema present in AIDS-KS patients.

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