Interaction of HIV Tat and matrix metalloproteinase in HIV neuropathogenesis: a new host defense mechanism

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ABSTRACT Tat, the HIV transactivating protein, and matrix metalloproteinases (MMPs), a family of extracellular matrix (ECM) endopeptidases, have been implicated in the pathogenesis of HIV-associated dementia. However, the possibility that MMPs interact with viral proteins has remained unexplored. We therefore treated mixed human fetal neuronal cultures with recombinant Tat and select MMPs. Neurotoxicity was determined by measuring mitochondrial membrane potential and neuronal cell death. Previous studies have shown that Tat and MMP independently cause neurotoxicity. Surprisingly, we found the combination of Tat and MMP produced significant attenuation of neurotoxicity. To determine whether there was a physical interaction between Tat and MMP, we used protein electrophoresis and Western blot techniques, and found that MMP-1 can degrade Tat. This effect was blocked by MMP inhibitors. Furthermore, MMP-1 decreased Tat-mediated transactivation of the HIV long terminal repeat region, and this functionality was restored when MMP-1 activity was inhibited. These results suggest that the decrease in Tat-induced neurotoxicity and HIV transactivation is due to Tat's enzymatic cleavage by MMP-1. The direct interaction of human MMPs with viral proteins has now been demonstrated, with resultant modulation of Tat-mediated neurotoxicity and transactivation. This study elucidates a unique viral-host interaction that may serve as an innate host defense mechanism.—Rumbaugh, J., Turchan-Cholewo, J., Galey, D., St. Hillaire, C., Anderson, C., Conant, K., Nath, A. Interaction of HIV Tat and matrix metalloproteinase in HIV neuropathogenesis: a new host defense mechanism. *FASEB J.* 20, E1114–E1123 (2006)

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Up to 30% of individuals infected with the HIV will develop some form of cognitive impairment during the course of their infection, and the prevalence of HIV associated dementia is ~5 to 15% (1). HIV dementia thus affects ~500,000 people in the U.S. alone. Incidence after onset of acquired immunodeficiency syndrome (AIDS) is ~7% per year (2). Though the use of highly active antiretroviral therapy (HAART) over the past several years has significantly reduced the incidence of severe HIV dementia, mild forms persist. In fact, with better treatment of HIV in the HAART era, patients are living longer with the infection and the prevalence of HIV dementia is actually rising (3–6). It has thus become clear that, in addition to HAART, neuroprotective strategies must be employed to effectively combat this condition (7), and, therefore, the neuropathogenesis must be better understood.

Matrix metalloproteinases (MMPs) have recently been implicated in the neuropathogenesis of HIV infection. MMPs are a family of structurally similar, zinc containing endopeptidases that enzymatically degrade extracellular matrix (ECM) proteins and thus can disrupt the blood-brain barrier and neuronal synapses (8–12). However, it has only recently been recognized that MMPs can cleave other host proteins, such as chemokines (13), and that these cleavage products can cause neurotoxicity. Further, MMPs may directly interact with integrin receptors on neurons and initiate a cascade of events leading to neuronal cell death (14). Recent studies have also shown that MMPs can become nitrosylated and persist in a hyperactive state, perhaps contributing to neurotoxicity under conditions of oxidative stress (15). Based on these and other observations, a significant effort is being devoted to developing clinically useful, broad spectrum MMP inhibitors. There have even been clinical trials of some of these agents in patients with advanced cancers, but neither efficacy nor side effect profiles have been encouraging (16). The failure of these studies may be partly due to the use of broad spectrum inhibitors disrupting the beneficial, as well as deleterious, effects of MMPs. For example, cleavage of the chemokine, MCP-3, by MMP-2 has been shown to decrease the inflammatory response (17).
The HIV transactivator protein, Tat, also has prominent neurotoxic properties, and likely plays a significant role in the pathogenesis of HIV dementia. Tat is a nonstructural protein but, due to its transactivating properties, is essential for viral replication. It is the earliest protein produced by the viral genome and is released extracellularly by an energy-dependent process (18–22). Tat’s production by HIV-infected cells is not affected by currently available antiretroviral drugs, which target the reverse transcriptase and protease enzymes of HIV. The role of Tat in producing neurotoxicity and its potential as a therapeutic target may therefore be especially important in the current HAART era. Tat is known to cause neurotoxicity by some mechanisms that overlap with those implicated in MMP-induced neurotoxicity. Such mechanisms include binding to integrin receptors, induction of oxidative stress, production of mitochondrial injury, and initiation of inflammatory cascades (23). It was also demonstrated recently that Tat can interact with astrocytes, microglia, and brain endothelial cells to increase expression of inducible NOS and release NO (24–26).

Although the interaction of MMPs with other human proteins has been extensively researched, the possibility that MMPs may interact with viral proteins has not previously been studied. We chose to study MMPs-1, -2, and -9 because of their known association with central nervous system (CNS) infection or inflammation (27). We have now demonstrated for the first time direct interactions between the viral proteins Tat and p24, and human MMPs-1, -2, and -9. Our data show that the different MMPs have different specificities in their interactions with viral proteins.

MATERIALS AND METHODS

Tat

Recombinant Tat1–72 and Tat1–101 were produced in our laboratory. Details of Tat production and purification have been published (22, 28). Briefly, since Tat is a nonglycosylated protein, we inserted the genes into an E. coli vector, PinPoint Xa-2 (Promega, Madison, WI, USA), which allowed expression of Tat as a fusion protein naturally biotinylated at the NH2 terminus. The fusion protein was then desalted and endotoxin was removed. It was >99% pure as analyzed by HPLC and silver-stained gel electrophoresis. Each batch is monitored for purity by Western blot analysis and for endotoxin contamination by Limulus amebocyte lysate assay (Associates of Cape Cod, Inc., East Falmouth, MA, USA).

MMPs

Recombinant active MMP-1, -2, and -9 were obtained from R&D Systems (Minneapolis, MN, USA). They were stored in aliquots at −70°C and dissolved in MMP buffer (50 mM Tris, 10 mM CaCl₂, 150 mM NaCl, 0.00008% Brij, 1 mM ZnCl₂, pH 7.5). This was used as a reaction buffer for all experiments utilizing MMPs. The neurotoxicity of Tat alone was not affected by this buffer.

To confirm bioactivity of MMPs prior to use, zymography was performed (data not shown) using reagents from Bio-Rad (Hercules, CA, USA). MMP samples were mixed 1:2 with sample buffer (62.5 mM Tris-Cl, pH 6.8, 4% SDS, 25% glycerol, and 0.01% bromphenol blue) and run at 100 V through a gel (10% polyacrylamide [w/v], 0.1% SDS, 0.1% gelatin). The gel was then placed in 2.5% Triton X-100 to allow for renaturation of the embedded proteins. Subsequently, the gel was placed in development buffer (50 mM Tris-Cl, pH 7.5, 200 mM NaCl, 5 mM CaCl₂, and 0.02% Brij-35) at 37°C for 16 h to optimize metalloproteinase activity. The gel was then stained for 1 h in 40% methanol/10% acetic acid/0.5% (w/v) Coomassie brilliant blue G-250, then destained in the same buffer without Coomassie brilliant blue. Proteinase activity was inferred by the presence of clear bands appearing against a blue background. Molecular weights were determined by comparison to protein MW standards (Bio-Rad).

Other reagents

Batimastat (also known as BB-94; 4-(N-hydroxyamino)-2R-isobutyl-3S-(thienyl thiomethyl)-succinyl-(L-phenylalanine)-N-methylamide; relative molecular mass (M,) 478) was provided by British Biotech Pharmaceuticals, Ltd (Cowley, Oxford, UK). FN-439 was obtained from Calbiochem (La Jolla, CA, USA). p24 was obtained from the NIH AIDS Repository.

Cultures of human fetal brain cells

Mixed neuronal-glial cultures were prepared, as described previously (29), from human fetal brain specimens of 12 to 15 wk gestational age, with consent from women undergoing elective termination of pregnancy, as approved by the Johns Hopkins University Institutional Review Board. Briefly, meninges and blood vessels were removed and the specimens were washed in OptiMEM (GIBCO, Gaithersburg, MD, USA). The tissue was dissociated with a 20 gauge needle and syringe, then pelleted at 270 g for 10 min. Cells were resuspended in OptiMEM with 5% heat-inactivated FBS and 0.2% N-2 supplement to encourage neuronal growth and prevent glial proliferation. Antimicrobial solution (1000 U penicillin G per milliliter, 10 μg streptomycin/ml, and 25 μg amphotericin B/ml in 0.9% NaCl) was added at a final concentration of 1%. Cells were maintained in tissue culture flasks for at least 4 wk, then plated in 96-well plates for 3–7 days before use in neurotoxicity assays. These cells contained ~70% neurons as determined by immunostaining for microtubule associated protein-2, 30% astrocytes by immunostaining for glial fibrillary acidic protein, and 1% microglia by immunostaining for CD68.

Cytotoxicity assays

Human fetal mixed neuronal-glial cell cultures were exposed to recombinant Tat and select MMPs (at concentrations detailed in the figure legends) individually and in combination. Controls included untreated cultures and cultures treated with 3-nitropropionic acid (Aldrich Chemical Company, Milwaukee, WI, USA), a highly neurotoxic compound (30).

Cell death assay

When performing the experiment, culture medium was replaced by Locke’s buffer (154 mM NaCl, 5.6 mM KCl, 2.3 mM
CaCl₂, 1 mM MgCl₂, 3.6 mM NaHCO₃, 5 mM glucose, and 5 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid, pH 7.2). Neuronal cell death was assayed by staining with 0.4% trypan blue 16 h after exposure to MMP and Tat, a time point chosen based on previous work (31). We used trypan blue exclusion as a measure of neuronal cell death because it measures both apoptotic and necrotic cell death, and we have extensive experience in using this assay for characterizing Tat-induced neurotoxicity. The dead cells were quantitated as described previously (22, 29, 32). Briefly, cultures were assayed by neuronal cell counts determined from 10 fields at predetermined coordinates. Each field was photographed, coded, and counted without knowledge of its experimental identity, and each experiment was done in triplicate. The means ± s.e.m were calculated and the data analyzed by 1-way ANOVA and the Bonferroni post-test.

**Mitochondrial assay**

Similarly, Locke’s buffer was used to replace the culture media at the time of performing the assay. Mitochondrial potential was monitored in the cultures by using 5,5’,6,6’-tetramethyl-1,3,3’-tetrachlorobenzidazole carboxanilide iodide (JC-1) dye (Molecular Probes, Eugene, OR, USA) as we have described (22). We performed this assay 6 h after exposure to Tat and MMP, a time period we have shown to have optimal kinetics for consistent measurements (31). In the absence of mitochondrial damage, membrane potential remains high and JC-1 forms aggregates with a red fluorescence. With mitochondrial damage, membrane potential decreases and JC-1 remains in monomers, giving a green fluorescence. Measurements were therefore made using a fluorescent plate reader (Molecular Dynamics, Sunnyvale, CA, USA) with excitation at 485 nM and emission at 538 nM and 590 nM. A ratio of fluorescence at 590 nM to that at 538 nM provides an indication of mitochondrial health. Results were normalized so that control mean values were equal to 100. The means ± s.e.m were calculated and the data analyzed by 1-way ANOVA and the Bonferroni post-test.

**Western blot analysis**

Appropriate reagents were coincubated in MMP buffer (50 mM Tris, 10 mM CaCl₂, 150 mM NaCl, 0.00008% Brij, 1 mM ZnCl₂, pH 7.5) at indicated concentrations for 2 h at 37°C. Controls included Tat without MMP and Tat degraded with trypsin. In reactions that included an MMP inhibitor, the inhibitor and MMP-1 were preincubated at 37°C for 30 min before adding the Tat. Reactions were inactivated by addition of tricine sample buffer (Bio-Rad) and heating to 95°C for 5 min. Samples were then run on a 16.5% tris-tricine Ready gel with a 4% stacking gel (Bio-Rad). The gel was stained with colloidal Coomassie Simply Blue Safe Stain (Invitrogen) or the Silver Stain Plus System (Bio-Rad), according to the manufacturer’s protocol.

**Tat transactivation activity**

The ability of Tat to transactivate the long terminal repeat (LTR) domain of the HIV genome was assayed using human SVGA-LTR-GFP cells. This astrocyte-derived cell line is stably transfected with HIV-LTR linked to green fluorescent protein (18). The cells were seeded onto 12-well plates 3–7 days before use in this assay. Reagents including Tat, MMPs, and MMP inhibitors were added to the wells as appropriate. For this assay, Tat1–101 was used because it enters astrocytic cells more efficiently than Tat1–72 (28). Transactivation of the LTR leads to increased expression of the green fluorescent protein, and this was measured using a fluorescent plate reader (Molecular Dynamics) with excitation at 488 nM and emission at 507 nM. Background measurements were made just prior to addition of the reagents, and experimental measurements were made 24 h after exposure. Results are expressed as relative fluorescent units with background readings subtracted from experimental readings. The means ± s.e.m were calculated and the data analyzed by 1-way ANOVA and the Bonferroni post-test.

**RESULTS**

**Neurotoxic properties of Tat and MMP-1**

Previous work has shown that both Tat (24–26, 29, 33–39) and MMP-1 (10, 11, 14, 40) independently can cause neurotoxicity. Furthermore, Tat can synergize with gp120, glutamate, and drugs of abuse such as morphine, cocaine, and methamphetamine to cause increased neurotoxicity (22, 31, 41–46). Hence, we coincubated Tat and MMP-1 to determine whether they too would similarly cause synergistic neurotoxicity. Surprisingly, we found that when mixed neuronal cultures were exposed to Tat and MMP-1 together, there was a decreased amount of neurotoxicity, as measured by changes in mitochondrial membrane potential (Fig. 1A). At lower MMP-1 concentrations, the neurotoxic effect of Tat was predominant but, with increasing amounts of MMP-1, a reverse dose response curve was noted, leading to a progressively decreasing amount of neurotoxicity. At higher concentrations of MMP-1, the neuroprotective effect was lost presumably because the neurotoxic effect of MMP-1 alone became predominant. Similar effects were seen when MMP-1 and Tat were preincubated together prior to adding them to the cell culture (Fig. 1) and when they were added simultaneously to the cultures without preincubation (not shown).

The combination of Tat and MMP-1 was also protective against cell death as measured by trypan blue exclusion (Fig. 1B). Furthermore, MMP inhibitors block the neuroprotective effect of MMP-1. We recognized that mitochondrial membrane potential is an
intermediate measurement of neuronal viability, and so tested MMP-1’s ability to protect against Tat induced cell death. We incubated Tat and MMP-1 alone or together in mixed neuronal cultures with or without the MMP inhibitor, FN-439. Results demonstrate that coinubcation of Tat and MMP-1 prevents the cell death observed with either Tat or MMP alone. Furthermore, addition of FN-439 reversed this protective effect.

To further explore the relatively narrow dose window in which MMP-1 is neuroprotective, we decided to examine the effect of FN-439 on the combination of Tat and the higher nonprotective dose of MMP-1 (Fig. 1C). We found that FN-439 had no significant effect on the toxicity caused by the combination of high-dose MMP-1 and Tat. Since MMP-related neurotoxicity is not mediated through the enzymatically active site, this result further supports our conclusion that MMP-1 is not protective at high doses because the direct toxicity of MMP-1 dominates at these doses over the ability to cleave Tat, which is seen at lower doses.

Cleavage of HIV-Tat by MMP-1

We hypothesized that the observed protective effect could occur if Tat and MMP-1 bind to one another and interfere with each other’s neurotoxic potential or if MMP-1’s endopeptidase activity cleaves Tat and thus decreases its neurotoxicity. To determine whether MMP-1 could directly interact with Tat, we coinubcated Tat and MMP-1 in an MMP reaction buffer and ana-

brane potential was measured 6 h later. Both Tat and MMP-1, when incubated alone, caused toxicity. However, when incubated together, intermediate concentrations of MMP-1 showed a significant decrease in amounts of toxicity compared to either protein alone (*P<0.05). At low or high MMP-1 concentrations, the respective effects seen with Tat or MMP alone seem to predominate and the protective effect is lost. Toxicity was measured as a loss of mitochondrial potential compared to untreated control cultures. Data represent mean ± se. B) Mixed human neuronal cultures were exposed to Tat and MMP-1, either alone or in combination, with or without the broad spectrum MMP inhibitor, FN-439. Cell death was measured by uptake of trypan blue 16 h later. Both Tat and MMP-1, when incubated alone, caused significant cell death. However, when incubated together, levels of cell death returned to that seen with no treatment or with FN-439 alone (P<0.001). When Tat, MMP-1, and FN-439 were all incubated together, the MMP-1 protective effect was inhibited and toxicity reappeared (P<0.001). FN-439 had no effect on the toxicity of MMP-1 or Tat alone. Concentrations: Tat 200 nM, MMP-1 20 ng/μL, FN439 0.5 μg/μL. Data represent mean ± se. C) Mixed human neuronal cultures were exposed to Tat, high-dose MMP-1, and/or FN-439. Mitochondrial membrane potential was measured 6 h later. Tat and high-dose MMP-1 caused toxicity compared to untreated control cultures (P<0.05). FN-439 alone caused no significant change from control conditions (P>0.05). Furthermore, when FN-439 was added to the combination of Tat and high-dose MMP-1, it had no significant effect (P>0.05). Toxicity was measured as a loss of mitochondrial potential compared to untreated control cultures. Data represent mean ± se.
Figure 2. Cleavage of Tat is specific to the endopeptidase activity of MMP-1. A) Tat was coincubated with MMP-1 at 37°C for 2 h. Reaction products were separated by tris-tricine gel and analyzed by colloidal Coomassie blue staining. Lanes 1–6 contain 0.5 μg of Tat 1–72. Lanes 2 and 3 respectively contain 5 and 10 ng/μl, and lanes 4–7 contain 20 ng/μl of MMP-1. For lanes 5 and 6, the MMP was preincubated with an MMP inhibitor for 30 min, prior to the addition of Tat. Lane 5: 0.05 ng/μl batimastat. Lane 6: 0.5 μg/μl FN-439. B) Tat was coincubated with MMP-1 at 37°C for 0 (lane 3), 15 (lane 4), 30 (lane 5), 60 (lane 6), 90 (lane 7), or 120 (lane 8) min. Reaction products were separated by tris-tricine gel and analyzed by silver staining. Lanes 1 and 3–8 contain 0.5 μg of Tat 1–72. Lanes 2–8 contain 20 ng/μl of MMP-1. For lanes 1 and 2, the Tat and MMP-1, respectively, were each incubated alone for 2 h. The higher molecular mass bands present with the Tat-MMP cleavage reaction, Tat and MMP-1, were each incubated for various periods, resolved by SDS-PAGE, and the gels were silver stained (Fig. 2B). Degradation of Tat was complete within 60 min. The extra bands present with the Tat were not degraded even after 120 min. This serves as an internal control, showing that our MMP-1 preparation did not nonspecifically degrade all proteins. On the other hand, since all of the bands on the Western blot (Fig. 2C) must represent Tat, it is not surprising that all of these bands degrade.

Next, we determined whether cleavage of Tat was itself neuroprotective or whether cleavage might produce Tat fragments that have neuroprotective properties. We therefore created nine segments of Tat. Each segment was 15 amino acids in length and overlaps the previous segment by 10 amino acids. Together, they span the entire Tat protein sequence. Each segment was incubated with full-length Tat in our neuronal cell cultures and neurotoxicity was measured by mitochondrial potential, using JC-1 dye. None of the segments had any neuroprotective effect (data not shown).

As another control, we coincubated the HIV core protein, p24, with MMPs-1, -2, and -9 and analyzed it by Western blot. Surprisingly, MMPs-1 and -2 both appeared to interact with p24, causing attenuation of the p24 band, whereas MMP-9 did not (data not shown). These results suggest that MMP interaction is not specific for the viral Tat protein. The possibility that MMPs might interact with other viral proteins, thus potentially serving a more general antiviral role, requires future study.

Interaction of MMP-1 with Tat inhibits HIV-LTR transactivation

Based on these results, we concluded that the observed neuroprotective effect, as measured by mitochondrial membrane potential and cell death, was due to cleavage of Tat by MMP's endopeptidase activity. Also, both cleavage and the neuroprotective effect are reversible by addition of an MMP inhibitor. To further confirm this observation and to evaluate MMP's effects on Tat functions beyond neurotoxicity, we utilized a functional assay of LTR transactivation. We hypothesized that Tat incubated with MMP-1 would not efficiently transactivate LTR because it would be cleaved. MMP-1, Tat, and/or FN-439 were coincubated, as appropriate,
MMP enzymatically active cleavage site. Similar results to reverse a protective effect that is not mediated by the not cleave Tat. MMP inhibitors would not be expected effect, consistent with the observation that MMP-2 does not cleave Tat. Addition of FN-439 did not reverse the protective neurotoxicity and cell death observed with Tat alone. Coincubation of Tat and MMP-2 prevents the neurotoxicity and cell death observed with Tat alone. Concentrations: Tat 200 nM, MMP-1 40 ng/µl, FN-439 0.5 µg/µl. Data represent mean ± se.

MMP-2 and -9 also have protective properties against Tat neurotoxicity

We next determined whether MMP-1’s cleavage and neuroprotective properties were specific to MMP-1 or more generalizable to other MMPs. MMPs-2 and -9 were unable to cleave Tat (Fig. 4A, D). However, we further determined whether MMP-2 and -9 could produce synergistic neurotoxicity, neuroprotection, or have no effect on Tat activity. MMP-2 and Tat were coincubated in mixed neuronal cultures, and neurotoxicity was measured by change in mitochondrial potential (Fig. 4B) and by neuronal cell death (Fig. 4C). Results demonstrate that MMP-2 and Tat were independently toxic when compared to untreated controls and that coincubation of Tat and MMP-2 prevents the neurotoxicity and cell death observed with Tat alone. Addition of FN-439 did not reverse the protective effect, consistent with the observation that MMP-2 does not cleave Tat. MMP inhibitors would not be expected to reverse a protective effect that is not mediated by the MMP enzymatically active cleavage site. Similar results were seen for MMP-9 (Fig. 4E, F). MMP-9 and Tat were toxic vs. untreated controls, the combination was protective vs. either protein alone, and the MMP inhibitor failed to reverse this protection.

We have hypothesized that MMPs and Tat might interact by various mechanisms. One such possible mechanism would be influences on oxidative stress. Such mechanisms are important topics for future exploration.

**DISCUSSION**

Neuronal loss of up to 90% has been observed in certain brain regions of patients with HIV dementia, yet actual infection of neurons by HIV is rare (47, 48). The perivascular macrophages and microglia are the cell types productively infected in brain (49), suggesting that the observed neurotoxicity is an indirect effect of HIV-infected cells produced by factors that are released into the ECM of the CNS and trigger inflammatory cascades and oxidative stress (50). These mediators likely include two main groups—viral proteins, like Tat, and host proteins, such as matrix metalloproteinases (51).

We chose to focus the current work on Tat for several reasons. Tat is critical for viral replication and it is released by infected cells, including monocytes (21, 22) and glial cells (18, 20), into the ECM, where it can interact with MMPs. In fact, Tat release is optimal in low serum conditions, such as that provided to the brain by the blood-brain barrier (52), and Tat can also cross the blood-brain barrier bidirectionally (53), so that Tat released in the brain might have effects beyond the brain, or *vice versa*. Both full-length Tat and Tat 1–72, from the first exon alone, are released (19).

We have previously shown that MMP-1 can cause neurotoxicity through direct interaction with neuronal membrane integrins, leading to dephosphorylation of Akt kinase and activation of caspases (14). Furthermore, glial cell activation is associated with increased production of MMP-1 (40). Tat has synergistic neurotoxic effects with multiple other toxins (22, 31, 41–46), and Tat’s neurotoxic mechanisms seem to overlap with those of MMPs. We thus expected to find synergistic, or at least additive, effects.

Our work demonstrates that MMP-1 can cleave Tat protein and protect against its neurotoxic and HIV-LTR transactivation properties. It is important to note that this cleavage effect is seen only at intermediate MMP doses; at low MMP doses, Tat-mediated toxicity seems to predominate, whereas at higher MMP doses, MMP-mediated toxicity predominates. This observation emphasizes the need to fully characterize the interaction of the MMPs with viral proteins if we are to successfully manipulate that interaction to the benefit of patients.

Proteolysis of specific proteins with resultant accumulation of neurotoxic cleavage products is a common pathogenic mechanism in many neurodegenerative conditions, including Alzheimer’s, Pick’s, Parkinson’s,
and Huntington’s diseases (54, 55). Cleavage of Tat by another human protease, furin, was recently shown as well (56). However, this is the first demonstration of interaction between MMPs and viral proteins; surprisingly, our data imply that MMP-1 cleavage of Tat may actually reduce overall neurotoxicity by reducing Tat levels.

It is well known that MMPs have overlapping, but different, substrate specificities (27). MMPs do not have specific cleavage sequences on their target molecules, and cleavage sites are based on the tertiary structure of the protein and not on the primary amino acid sequence. It is therefore very difficult to predict a priori which proteins may be cleaved by which MMPs. Additionally, MMPs may interact with Tat and other viral proteins by mechanisms other than cleavage. For example, both viral proteins and MMPs can initiate cell signaling cascades that may influence each other downstream. Furthermore, MMPs may participate in host defense without specifically targeting infectious molecules, as does the MMP, matrilysin, in mouse. Matrilysin

Figure 4. Modulation of Tat-induced neurotoxicity by MMP-2 and -9. A, D) Tat was coincubated with MMP-2 or -9 at 37°C for 2 h. Reaction products were separated by SDS-PAGE gel and analyzed by Western blot using an mAb to Tat and chemiluminescence for visualization. Each lane contains 0.5 μg of Tat 1–72. Lanes 1–3 contain 0, 1.25, and 2.5 ng/μl, and lanes 4–6 contain 5 ng/μl of MMP-2 and -9, respectively. For lanes 5 and 6, the MMP was preincubated with an MMP inhibitor for 30 min prior to the addition of Tat. Lane 5: 0.05 ng/μl batimastat. Lane 6: 0.5 μg/μl FN-439. B, E) Mixed human neuronal cultures were exposed to Tat and MMP-2 or -9, with or without FN-439. Mitochondrial membrane potential was measured 6 h later. MMP and Tat incubated together was significantly less toxic than Tat incubated alone (P<0.01). FN-439 did not reverse this protective effect, nor did it have a statistically significant effect on the toxicity of MMP-2, -9, or Tat alone. Concentrations: Tat 200 nM, MMP-2 or -9 5 ng/μl, FN-439 0.5 μg/μl. Toxicity (or protection) was measured as a change in mitochondrial potential compared to untreated control cultures. Data represent mean ± se. C, F) Mixed human neuronal cultures were exposed to Tat and MMP-2 or -9, either alone or in combination, with or without FN-439. Cell death was measured by uptake of trypan blue 16 h later. Tat, MMP-2, and MMP-9, when incubated alone, each caused toxicity (P<0.05). When Tat and MMP were incubated together, levels of cell death were significantly less than that seen with Tat alone (P<0.001). FN-439 did not reverse the protective effect, nor did it have any effect on the toxicity of MMP-2, -9, or Tat alone. Concentrations: Tat 200 nM, panel C and 400 nM, panel F, MMP-2 or -9 5 ng/μl, FN-439 0.5 μg/μl. Data represent mean ± se.
cleaves an intestinal protein, called cryptdin, thus activating the cryptdin’s antibacterial properties (57).

Unlike MMP-1, neither MMP-2 nor -9 cleaves Tat. However, like MMP-1, we show that MMP-2 and -9 have neuroprotective effects against Tat-induced neurotoxicity. This neuroprotective effect must be mediated by a mechanism other than cleavage; in the case of MMP-2, we have shown this mechanism apparently involves reduction in reactive oxygen species. The data generated from this research will guide future studies of other host-viral protein interactions. We now hypothesize that MMP production is an innate host response to HIV infection that plays a dual role in HIV neuropathogenesis. MMPs alone are well documented to have neurotoxic potential. Alternatively, our work now shows that MMPs may bind, cleave, or antagonize viral proteins, neutralizing their effects on brain cells.

Though MMPs clearly have many other roles, it is possible that they have evolved from a primitive host defense mechanism. Plants, without a specific adaptive immune system, may use MMPs, along with other innate defense mechanisms, to combat infection. For example, the MMP2 gene of the soybean, Glycine max, is up-regulated in response to a variety of infections (58). While hosts may use MMPs to defend against infectious molecules, microorganisms may use MMPs to evade host defense mechanisms. The parasite, Nector americanus, uses a metalloprotease to cleave and neutralize host eotaxin, a potent eosinophil chemoattractant important in the control of helminthic infections (59). It is thus clear that the complex interactions between MMPs, infectious proteins, and host defense mechanisms need to be fully understood to develop a better rationale for MMP-directed therapeutic approaches. Since neurons themselves are not typically infected by HIV, but rather die due to secondary mediators, there is an excellent opportunity to prevent HIV dementia by blocking these mediator pathways (60). MMPs are prime candidates as therapeutic targets. However, it may not be possible to identify a single target pathway or molecule; combination therapy may be necessary.

Future work in this field should explore other mechanisms of Tat-MMP interaction beyond cleavage, such as the oxidative stress mechanisms at which we have hinted. Then, utilizing the framework provided by knowledge of these interactions, MMP-based therapeutic strategies can be explored in vivo, using currently available murine Tat transgenic and MMP knockout model systems.

In addition to HIV-associated dementia, MMPs have been implicated in several other disease conditions, including tumor metastasis (27, 61), atherosclerosis (62), emphysema (63), arthritis, and neurodegenerative disorders such as Alzheimer’s, multiple sclerosis, and even stroke (64–66). Optimal treatment of these conditions may require the knowledge and ability to choose appropriate MMP selective inhibitors, or even MMP-selective augmentors, while broad spectrum MMP modulation may actually be harmful.

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Interaction of HIV Tat and matrix metalloproteinase in HIV neuropathogenesis: a new host defense mechanism

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SPECIFIC AIMS
The possibility that human matrix metalloproteinases (MMPs) interact with viral proteins has not been explored. Hence, we coincubated the HIV transactivating protein, Tat, with select MMPs to determine if they would cause synergistic or additive neurotoxicity.

PRINCIPAL FINDINGS
Neurotoxic properties of Tat and MMP-1

Previous work has shown that both Tat and MMP-1 independently can cause neurotoxicity. Furthermore, Tat can synergize with gp120, glutamate, and drugs of abuse such as morphine, cocaine, and methamphetamine to cause increased neurotoxicity. Hence, we coincubated Tat and MMP-1 to determine whether they too would similarly cause synergistic neurotoxicity. Surprisingly, we found that when mixed neuronal cultures were exposed to Tat and MMP-1 together, there was a decreased amount of neurotoxicity as measured by changes in mitochondrial membrane potential (Fig. 1A). At lower MMP-1 concentrations, the neurotoxic effect of Tat was predominant, but with increasing amounts of MMP-1 a reverse dose response curve was noted, leading to a progressively decreasing amount of neurotoxicity. At higher concentrations of MMP-1, the neuroprotective effect was lost presumably because the neurotoxic effect of MMP-1 alone became predominant. Similar effects were seen when MMP-1 and Tat were preincubated together prior to adding them to the cell culture (Fig. 1) and when they were added simultaneously to the cultures without preincubation (not shown).

The combination of Tat and MMP-1 was also protective against cell death as measured by trypan blue exclusion (Fig. 1B). Furthermore, MMP inhibitors block the neuroprotective effect of MMP-1. We recognized that mitochondrial membrane potential is an intermediate measurement of neuronal viability, and so tested MMP-1’s ability to protect against Tat-induced cell death. We incubated Tat and MMP-1 alone or together in mixed neuronal cultures with or without the MMP inhibitor, FN-439. Results demonstrate that coincubation of Tat and MMP-1 prevents the cell death observed with either Tat or MMP alone. Furthermore, addition of FN-439 reversed this protective effect.

Cleavage of HIV-Tat by MMP-1

We hypothesized that the observed protective effect could occur if Tat and MMP-1 bind to one another and interfere with each other’s neurotoxic potential or if MMP-1’s endopeptidase activity cleaves Tat and thus decreases its neurotoxicity. To determine whether MMP-1 could directly interact with Tat, we coincubated Tat and MMP-1 in an MMP reaction buffer and analyzed them by colloidal Coomassie blue staining, silver staining, and Western blot (not shown). We found that MMP-1 could cleave Tat, as demonstrated by disappearance of the Tat band from the gel, utilizing all three techniques.

Furthermore, when either batimastat or FN-439, both broad spectrum MMP inhibitors, were added to the reaction mixture, the Tat band reappeared, strongly suggesting that Tat was indeed cleaved by MMP-1’s endopeptidase activity.

To test the kinetics of the Tat-MMP cleavage reaction, Tat and MMP-1 were coincubated for various periods, resolved by SDS-PAGE, and the gels were silver stained (not shown). Degradation of Tat was complete within 60 min. There is a higher running band present with the Tat, which is either a contaminant or a
multimer of Tat. It was not degraded and therefore serves as an internal control, showing that our MMP-1 preparation did not nonspecifically degrade all proteins.

**Interaction of MMP-1 with Tat inhibits HIV-long terminal repeat transactivation**

Based on these results, we concluded that the observed neuroprotective effect, as measured by mitochondrial membrane potential and cell death, was due to cleavage of Tat by MMP’s endopeptidase activity. Also, both cleavage and the neuroprotective effect are reversible by addition of an MMP inhibitor. To further confirm this observation, we hypothesized that Tat incubated with MMP-1 would not efficiently transactivate long terminal repeat (LTR) because it would be cleaved. MMP-1, Tat, and/or FN-439 were coincubated, as appropriate, with SVGA-long terminal repeat-GFP cells in culture and transactivation activity was measured by assessing the change in green fluorescence under each experimental condition (Fig. 2). Tat alone produced significant transactivation compared to untreated controls, but transactivation returned to untreated levels when MMP-1 was added. Further addition of FN-439, thus inhibiting MMP endopeptidase activity, returned transactivation to levels seen with Tat alone. Neither FN-439 alone nor MMP-1 alone caused significant transactivation.

**MMP-2 and -9 also have protective properties against Tat neurotoxicity**

We next determined whether MMP-1’s cleavage and neuroprotective properties were specific to MMP-1 or

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**Figure 1.** Modulation of Tat neurotoxicity by MMP-1. A) Mixed human neuronal cultures were exposed to Tat and MMP-1 either alone or in combination. Mitochondrial membrane potential was measured 6 h later. Both Tat and MMP-1, when incubated alone, caused toxicity. However, when incubated together, intermediate concentrations of MMP-1 showed a significant decrease in amounts of toxicity compared to either protein alone (\( P < 0.05 \)). At low or high MMP-1 concentrations, the respective effects seen with Tat or MMP-1 alone seem to predominate, and the protective effect is lost. Toxicity was measured as a loss of mitochondrial potential compared to untreated control cultures. Data represent mean \( \pm \) se. B) Mixed human neuronal cultures were exposed to Tat and MMP-1, either alone or in combination, with or without the broad spectrum MMP inhibitor, FN-439. Cell death was measured by uptake of trypan blue 16 h later. When incubated alone, both Tat and MMP-1 caused significant cell death. When incubated together, however, levels of cell death returned to that seen with no treatment or with FN-439 alone (\( P < 0.001 \)). When Tat, MMP-1, and FN-439 were all incubated together, the MMP-1 protective effect was inhibited and toxicity reappeared (\( P < 0.001 \)). FN-439 had no effect on the toxicity of MMP-1 or Tat alone. Concentrations: Tat (200 nM), MMP-1 (20 ng/μl), FN439 (0.5 μg/μl). Data represent mean \( \pm \) se.

**Figure 2.** Effect of MMP-1 on Tat-induced LTR transactivation. SVGA-long terminal repeat-GFP cell cultures were exposed to Tat and MMP-1, either alone or in combination, with or without FN-439. Transactivation of LTR was measured 24 h later. Tat alone caused significant transactivation compared to untreated controls (\( P < 0.05 \)). When Tat was incubated with MMP-1, transactivation returned to that seen with no treatment (\( P < 0.05 \)). When Tat, MMP-1, and FN-439 were all incubated together, transactivation reappeared (\( P < 0.05 \)). MMP-1 alone and FN-439 alone had no effect on the toxicity of MMP-1 or Tat alone. Concentrations: Tat (200 nM), MMP-1 (40 ng/μl), FN439 (0.5 μg/μl). Data represent mean \( \pm \) se.
more generalizable to other MMPs. MMPs-2 and -9 were unable to cleave Tat (not shown). However, we further determined whether MMP-2 and -9 could produce synergistic neurotoxicity, neuroprotection, or have no effect on Tat activity. MMP-2 and Tat were coinubicated in mixed neuronal cultures and neurotoxicity was measured by a change in mitochondrial potential and by neuronal cell death (not shown). Results demonstrate that MMP-2 and Tat were independently toxic when compared to untreated controls and that coinubation of Tat and MMP-2 prevents the neurotoxicity and cell death observed with Tat alone. Addition of FN-439 did not reverse the protective effect, consistent with the observation that MMP-2 does not cleave Tat. Similar results were seen for MMP-9. MMP-9 and Tat were toxic vs. untreated controls; the combination was protective vs. either protein alone and the MMP inhibitor failed to reverse this protection.

**CONCLUSIONS AND SIGNIFICANCE**

Neuronal loss of up to 90% has been observed in certain brain regions of patients with HIV dementia, yet actual infection of neurons by HIV is rare. The perivascular macrophages and microglia are the cell types productively infected in brain, suggesting that the observed neurotoxicity is an indirect effect of HIV-infected cells produced by factors that are released into the extracellular matrix of the central nervous system and trigger inflammatory cascades and oxidative stress. These mediators likely include two main groups—viral proteins, like Tat, and host proteins, such as matrix metalloproteinases (Fig. 3).

Tat has synergistic neurotoxic effects with multiple other toxins, and Tat’s neurotoxic mechanisms seem to overlap with those of MMPs. We thus expected to find synergistic, or at least additive, effects. Our work demonstrates that MMP-1 can cleave Tat protein and protect against its neurotoxicity and HIV-long terminal repeat transactivation properties (Fig. 3). Cleavage of Tat by another human protease, furin, was recently shown as well. However, this is the first demonstration of interaction between MMPs and viral proteins, and, surprisingly, our data imply that MMP-1 cleavage of Tat may actually reduce overall neurotoxicity by reducing Tat levels.

Unlike MMP-1, neither MMP-2 nor -9 cleaves Tat. However, like MMP-1, we show that MMP-2 and -9 have neuroprotective effects against Tat-induced neurotoxicity. This neuroprotective effect must be mediated by a mechanism other than cleavage. The data generated from this research will guide future studies of other host-viral protein interactions. We now hypothesize that MMP production is an innate host response to HIV infection that plays a dual role in HIV neuropathogenesis. MMPs alone are well documented to have neurotoxic potential. Alternatively, our work now shows that MMPs may bind, cleave, or antagonize viral proteins, neutralizing their effects on brain cells.

Though MMPs clearly have many other roles, it is possible they have evolved from a primitive host defense mechanism. Plants without a specific adaptive immune system may use MMPs, along with other innate defense mechanisms, to combat infection. For example, the MMP2 gene of the soybean, *Glycine max*, is up-regulated in response to a variety of infections. It is thus clear that the complex interactions between MMPs, infectious proteins, and host defense mechanisms need to be fully understood to develop a better rationale for MMP-directed therapeutic approaches. Since neurons themselves are not typically infected by HIV, but rather die due to secondary mediators, there is an excellent opportunity to prevent HIV dementia by blocking these mediator pathways. MMPs are prime candidates as therapeutic targets. However, it may not be possible to identify a single target pathway or molecule; combination therapy may be necessary. In addition to HIV-associated dementia, MMPs have been implicated in several other disease conditions, including tumor metastasis, atherosclerosis, emphysema, arthritis, and neurodegenerative disorders such as Alzheimer’s, multiple sclerosis, and even stroke. Optimal treatment of these conditions may require the knowledge and ability to choose appropriate MMP selective inhibitors, or even MMP selective augmentors, while broad spectrum MMP modulation may actually be harmful.