HIV-1 Tat-Mediated Apoptosis in Human Brain Microvascular Endothelial Cells

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The integrity of the blood-brain barrier (BBB) is critical for normal brain function. Neuropathological abnormalities in AIDS patients have been associated with perivasculare HIV-infected macrophages, gliosis, and abnormalities in the permeability of the BBB. The processes by which HIV causes these pathological conditions are not well understood. To characterize the mechanism by which HIV-1 Tat protein modulates human brain microvascular endothelial cell (HBMEC) functions, we studied the effects of HIV-1 Tat in modulating HBMEC apoptosis and permeability. Treatment of HBMEC with HIV-1 Tat led to Flk-1/KDR and Flt-4 receptor activation and the release of NO. The protein levels of endothelial NO synthase (NOS) and inducible NOS were increased by HIV-1 Tat stimulation. Importantly, HIV-1 Tat caused apoptosis of HBMEC, as evidenced by changes in the cleavage of poly(A)DP-ribose polymerase, DNA laddering, and incorporation of fluorescein into the nicked chromosomal DNA (TUNEL assay). HIV-1 Tat-mediated apoptosis in HBMEC was significantly inhibited in the presence of N-nitro-l-arginine methyl ester (an inhibitor of NOS) and wortmannin (a phosphoinositol 3-kinase inhibitor). Furthermore, HIV-1 Tat treatment significantly increased HBMEC permeability, and pretreatment with both N-nitro-l-arginine methyl ester and wortmannin inhibited the Tat-induced permeability. Taken together, these results indicate that dysregulated production of NO by HIV-1 Tat plays a pivotal role in brain endothelial injury, resulting in the irreversible loss of BBB integrity, which may lead to enhanced infiltration of virus-carrying cells across the BBB.

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ome 30% of adult and 50% of pediatric AIDS patients develop neurological disorders. HIV enters the CNS early after primary infection (1, 2). Despite extensive investigation of HIV-1 neuropinvasion, the mechanisms of initial entry into the CNS are not well defined. “Trojan” transport of the virus by monocytes and T cells across the blood-brain barrier (BBB) is considered to be of crucial importance for HIV-1 import into the CNS in the late stage of AIDS (3, 4). In addition, HIV-1 Tat and gp120 have been shown to elicit necrotic substances, induce apoptosis in neurons, and increase the migration of monocytes across the BBB (5–10).

Under normal conditions, the mammalian BBB represents a strong barrier to invasion of the CNS by microbial agents. This barrier is based on the paucity of vesicular activity within human brain microvascular endothelial cells (HBMEC), which limits endocytosis and transcytosis, and on tight junctional complexes that are modulated by vasoactive substances, such as thrombin and bradykinin, as well as cytokines (for review, see Refs. 11–13). HIV-1 infection can lead to severe CNS dysfunction (14–16). A syndrome consisting of cognitive and motor function abnormalities, termed HIV-1-associated dementia (HAD), occurs in a significant portion of individuals infected with HIV-1. The pathogenesis of HAD is incompletely understood. Molecular mechanisms leading to neuronal and glial cell damage and death may involve toxic effects from viral proteins and proinflammatory cytokines. Viral proteins themselves may be neurotoxic by virtue of their ability to be either directly toxic to brain cells and/or indirectly toxic through their actions on glial cells or macrophages, thereby releasing neurotoxic products coded by the host genome. Several factors have been proposed to mediate HIV-1-induced neuronal injury. These include the soluble forms of the HIV-1 gp120 and HIV-1 Tat proteins, which are toxic to neurons in vitro (17, 18).

The BBB represents a complex cellular system consisting of endothelial cells (EC), pericytes, perivascular microglia, astrocytes, and basal lamina. While the EC layer forms the barrier, the interaction of all cells seems to be necessary for the induction or maintenance of the specialized functions of this barrier. HBMEC form a unique, tightly interconnected, cellular monolayer. This monolayer together with the closely associated astrocyte processes contribute to the stability of the brain parenchymal microenvironment by forming a high selectively impermeable barrier that strictly controls the exchange of material between the blood circulation and brain (for review, see Refs. 19 and 20). The capillaries that form the BBB express a diverse array of functional characteristics that permit the regulation of cell, protein, and macromolecule passage between blood and brain (21). Special characteristics of HBMEC include the presence of tight junctions (between adjacent EC), adherens junctions, cell-to-cell junctions, and the high expression of zon-1 and occludins. In addition, HBMEC have high resistance (1000–2000 ohm-cm²), compared with 10–20 ohm-cm².

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3 Abbreviations used in this paper: BBB, blood-brain barrier; EC, endothelial cell; eNOS, endothelial NO synthase; HAD, HIV-1-associated dementia; HBMEC, human brain microvascular endothelial cell; HMVEC-Ls, human microvascular endothelial cells-lung; iNOS, inducible NO synthase; KS, Kaposi’s sarcoma; t, NEM, N-nitro-l-arginine methyl ester; MAPK, mitogen-activated protein kinase; eNOS, neuronal NOS; PARP, poly(A)DP-ribose polymerase; PDTC, 1-pyrollidinediacarbodiic acid, ammonium salt; PI3 kinase, phosphoinositol 3-kinase; PKC, protein kinase C; VEGF, vascular endothelial growth factor.
for peripheral EC. Such properties of HBMEC account for the impermeability of the BBB to most macromolecules.

Endothelial dysfunction and injury have been observed in HIV infection. EC participate in inflammatory and immune reactions by producing and responding to soluble mediators (for review, see Refs. 22 and 23). HIV-1 profoundly alters the features of EC. Certain EC, such as those lining liver sinusoids, HUVEC, bone marrow stromal EC, or HBMEC, may be variably permissive for HIV infection (22). Activation of EC appears to occur in HIV infection. This activation is either by cytokines secreted in response to mononuclear cell activation by the virus or by the effects of the secreted HIV-associated proteins, particularly gp120 envelope glycoprotein and Tat (trans-activator of viral replication), on endothelium (23). Indeed, the enhanced adhesiveness of EC, EC proliferation and apoptosis, as well as activation of cytokine secretion have been observed. Furthermore, synergy between select inflammatory cytokines and viral proteins in inducing endothelial injury has been demonstrated (23). In HIV infection, dysfunctional or injured EC potentiate tissue injury and inflammation as well as accelerate the development of inflammatory pathology.

The cytotoxic viral product envelope glycoprotein, gp120, and HIV-1 Tat protein are potent neurotoxins causing neurotoxicity in the picomolar range (24–34). Soluble gp120 and HIV-1 Tat protein have been detected in the serum of HIV-infected patients, and mRNA encoding gp120 was found to be elevated in the brains of patients with HIV encephalitis. Tat at 16 ng/ml was also detected in the cerebrospinal fluid of HIV-1-infected patients (35).

The HIV-1 envelope protein, gp120, and HIV-1 Tat have been proposed to modify host cell gene expression in a manner that facilitates viral replication and have also been proposed as likely etiologic agents of neuronal loss as well as for the development of the HAD complex (29). As mentioned above, gp120 and HIV-1 Tat shed from HIV-1 and HIV-1-infected cells at concentrations of 0.1–1 nM, were shown to be present in the blood of HIV-1-infected patients (30–36). In addition to its intracellular effects, several studies have shown that Tat can be secreted extracellularly by infected cells (31, 35). Furthermore, Tat has been detected in the serum of HIV-1-infected individuals (32, 35). In that study the concentration of Tat in the serum was ~1–3 ng/ml. Recently, Tat protein was reported to be present in HIV-1-infected human and macaque monkey brains (32). Specifically, Tat was present in close proximity to infected cells and neurons.

Tat may alter cellular behavior when released by infected cells in the microenvironment (32, 37). Tat easily enters different cell types and contributes to trans-activation of the HIV-1 long terminal repeat promoter in latently infected cells (32) or acts as a soluble mediator acting on T and B cells and on those of the CNS (38, 39), thus favoring the progression of AIDS and its associated brain damage. Moreover, Tat profoundly affects the functions of vascular EC and the monocytic/macrophage system. It is an angiogenic inducer through activation of vascular endothelial growth factor (VEGF) receptor 2, also known as Flk-1/KDR, and VEGF receptor 1 (Flt-1) (38–44). Tat also induces a proinflammatory program characterized by the release of proteolytic enzymes, the up-regulation of adhesion molecules, and vasopermeability. This results in leukocyte extravasation essential for the homing of infected lymphomononuclear cells into lymphoid organs and for the tissue injury typical of some of the features that characterize the progression of AIDS.

HIV-1 Tat was also shown to induce microvascular endothelial apoptosis through caspase activation and to inhibit angiogenesis (33, 45). In addition, HIV-1 Tat protein induced the production of IL-8 by human brain-derived EC (33, 46), and increased endothelial permeability through tyrosine kinase- and mitogen-activated protein kinase-dependent pathways (34, 47).

NO is a messenger molecule with complex biological activities, including the modulation of immunoregulation and inflammation (48–51). NO released from activated cultured EC induced signaling for apoptosis in thymocytes. NO-mediated, DNA damage-induced apoptotic cell death in hemopoietic cells was recently reported after the induction of inducible NO synthase (iNOS) or the exogenous application of an NO donor. Generation of NO, either from cellular sources or derived from chemical donors, has been shown to induce apoptotic or even necrotic cell death (52–56), while other studies have shown that NO can have a protective effect on apoptosis (57, 58). Exposure of B lymphocytes to an NO donor inhibits caspase activation in vitro, while cellular expression of NOS has been proposed to inhibit both the apoptosis of EBV-transformed B lymphocytes and cell death in other systems induced by TNF-α or Fas (48, 53, 54, 59). Recently, we have shown that VEGF up-regulates ICAM-1 expression and NO secretion in BMEC through the phosphoinositol 3-kinase (PI3 kinase)/Akt signaling pathway (60). HIV-1 Tat was also shown to induce microvascular endothelial apoptosis through caspase activation (61). The precise molecular mechanisms mediating NO-induced tissue injury and the breakdown of the BBB are complex and not completely understood.

The effect of HIV-1 Tat on brain microvascular EC is not well elucidated. In this study we report that HIV-1 Tat induced apoptosis in the unique system of primary HBMEC through the up-regulation of NO and the PI3 kinase pathway, resulting in increased EC permeability. These changes may facilitate HIV-entry into the brain.

Materials and Methods

Reagents

The signaling molecule inhibitors wortmannin, N-nitro-l-arginine methyl ester (l-NNAME; an NO inhibitor), PD98059 (a mitogen-activated protein kinase (MAPK) inhibitor), GF109203X (GFX, a protein kinase C (PKC) inhibitor), and 1-pyrolidinocarbodiithioic acid, ammonium salt (PDTC; an NF-κB inhibitor) were obtained from Calbiochem (La Jolla, CA). The NO assay kit was purchased from Calbiochem.

HIV-1 Tat protein

Recombinant HIV-1 Tat protein was purified, lyophilized, and reconstituted in Tat buffer (50 mM Tris-HCl (pH 7.6), 20 mM KCl, and 7 mM DTT in 20% glycerol) as previously described (36). The purified recombinant HIV-1 Tat was endotoxin free using the Limulus amebocyte lysate assay (Sigma-Aldrich, St. Louis, MO). This protein was biologically active, as assessed by its rescue of Tat-defective provirus replication in HLM-1 cells (61).

Cultures of human BMEC

HBMEC were obtained from Cell Systems (Kirkland, WA). Cells were cultured in CSC complete medium containing 10% FCS (Cell Systems), which was supplemented with the EC growth factor, Endrogo (100 ng/ml; Cell Systems), as described in the manufacturer’s manual. HBMEC cultures were stained with anti-factor VIII, a marker for EC, and expressed by guest on May 1, 2017 http://www.jimmunol.org/ Downloaded from

DNA fragmentation assay

Tat-treated cells were lysed with buffer containing 10 mM Tris-HCl (pH 7.6), 1 mM EDTA, and 0.2% Triton X-100, and the fragmented DNA in the lysate was separated from the unfragmented chromosomal DNA by precipitation at 12,000 × g for 30 min. Fragmented DNA in the supernatant was then digested with 100 ng/ml ribonuclease A (Invitrogen, San Diego,
CA), 20 ng/ml protease K (Invitrogen), and 1% SDS at 37°C for 45 min, purified by the phenol/chloroform extraction method, and precipitated with ethanol/sodium deoxycholate. The DNA was then electrophoresed on a 1.6% agarose gel containing 0.5 µg/ml ethidium bromide.

**TUNEL assay**

Detection of apoptosis in BMEC was performed by the TUNEL method using the in situ cell death detection kit (Roche, Indianapolis, IN) according to the manufacturer’s directions. Cells were grown in fibronectin-coated chamber slides. Intracellular fluorescent-labeled fragmented DNA was detected by microscopic analysis.

**NO release and NOS expression**

To assess NO production, spectrophotometric quantitation of nitrate using the Griess reagent was used. Conditioned medium was subjected to nitrate reductase reaction using the NO assay kit (Calbiochem, La Jolla, CA). All reactions were duplicated and repeated three times. NOS levels were measured by NOS mAbs anti-iNOS, anti-endothelial NO synthase (anti-eNOS), and anti-neuronal NOS (anti-nNOS) Abs, respectively (Transduction Laboratory, Lexington, KY).

**Western blot analysis**

For Western blot analysis, cells were lysed, and the lysates were measured for protein concentration using a bicinchoninic acid protein assay reagent (Pierce, Rockford, IL). Approximately 30 µg of protein was separated by SDS-PAGE. After electrotransfer to polyvinylidene difluoride membranes, the membranes were blocked in 5% nonfat milk in PBS/Tris (PBST) for 2 h at room temperature and then incubated overnight at 4°C with primary Abs. Membranes were washed four times with PBST over 40 min and incubated with the secondary HRP-conjugated Abs. After three washes with PBST, detection was conducted using the ECL reagent (Amersham Pharmacia Biotech, Piscataway, NJ).

**Detection of apoptotic cells**

The relative amounts of mono- and oligonucleosomes generated from apoptotic BMEC were quantitated with a cell death ELISA kit (Roche) according to the manufacturer’s protocol. Briefly, RGD-containing or basic Tat peptide- and control peptide-treated cells were lysed and centrifuged at 200 × g for 10 min. Aliquots of supernatants (cytoplasmic fractions) were transferred onto a streptavidin-coated microtiter plate and incubated for 2 h at room temperature. The plate was thoroughly washed three times, and 100 µl of substrate solution was added and measured at 405 nm.

**Permeability assay**

HBMEC (105 cells) were seeded onto fibronectin-coated polycarbonate membranes with a pore size of 0.4 µm in a two-chamber system (Collaborative Biomedical Products, Bedford, MA) and maintained confluent for 7 days. The cells on the upper chamber were starved in 1% FCS EC medium overnight and then treated with the test sample. After 16 h the lower chamber was loaded with 0.8 ml of fresh medium, and the upper chamber was filled with 0.5 ml of prewarmed medium containing Lucifer Yellow (at 1.0 mg/ml) and test sample. After incubation for 2 h, 50-µl aliquots of medium were withdrawn from the lower chamber. Samples were diluted with 0.1 ml of distilled H2O and 100 µl of 0.15% deoxycholate, and the protein was precipitated by the addition of 150 µl of 50% TCA. The tubes were kept on ice for at least 1 h. The supernatants were centrifuged and diluted with 1 ml of HBSS to a final volume of 2.0 ml. Measurements were performed in a Luminescence spectrometer (LS-5B; PerkinElmer, Palo Alto, CA) at an excitation of 428 nm. Paired Student’s t test was used to determine the statistical difference.

**Results**

**Morphological changes in HBMEC upon HIV-1 Tat treatment**

HIV-1 Tat was reported to cause apoptosis in CD4+ T cells and neurons (63, 64). To test whether HIV-1 Tat induces apoptosis of brain EC, HBMEC were used. The morphology of HBMEC without HIV-1 Tat treatment showed a uniform monolayer of EC with tight cell-cell contact growth (Fig. 1A). Treatment with HIV-1 Tat (25–50 ng/ml) resulted in ~70–80% of the cells dying over a period of 2–3 days, while control immunoabsorbed HIV-1 Tat (data not shown) as well as heparin (10 U/ml; Fig. 1C) had no effect on HBMEC viability. Following treatment with HIV-1 Tat, the first morphological signs of cell death at the light microscopic level occurred after ~12 h, and by 24 h the cell body had lost its typical smooth round shape (Fig. 1B). Active protrusion and retraction of apoptotic-like bodies occurred as indicated in Fig. 1B.

**HIV-1 Tat phosphorylates Flik-1/KDR and Flt-4 receptors**

The HIV-1 Tat molecule has basic and Arg-Gly-Asp (RGD)-containing domains. The basic domain of HIV-1 Tat can bind to and signal via the VEGF receptors, Flik-1/KDR in EC and Flt-1 in monocyte-macrophages (65). The RGD domain of HIV-1 Tat can activate the surface integrins αvβ3 and αvβ5 (40–43). Important consequences of HIV-1 Tat interaction with these multiple surface receptors on umbilical vein EC are the up-regulation of leukocyte adhesion molecules and IL-6 release (44). This up-regulation of adhesion molecules enhances inflammatory cell attachment and has been postulated to contribute to endothelial damage in certain HIV-related syndromes. Clinical and pathological studies have suggested that HAD is dependent in part upon the transmigration of leukocytes across the BBB into the CNS (45).

The possibility that HIV-1 Tat might interact with Flik-1/KDR and other VEGF-binding surface structures on HBMEC was addressed. We observed potent receptor phosphorylation of VEGF receptor 2 (Flik-1/KDR) upon VEGF or HIV-1 Tat treatment (Fig. 2, A and C). In addition, phosphorylation of another VEGF receptor, VEGF receptor 3 (Flt-4), was observed in HBMEC treated with VEGF or HIV-1 Tat (Fig. 2, B and D). Heat-inactivated HIV-1 Tat had no effect on tyrosine phosphorylation of the Flik-1/KDR or Flt-4 receptor (Fig. 2, E and F). These results indicate that Tat ligation of both specific surface molecules, Flik-1/KDR and Flt-4, activates the phosphorylation of these receptors, which,
in turn, forms the initial step of HIV-1 Tat-mediated signal transduction in HBMEC.

**HIV-1 Tat induces NOS regulation and NO release from HBMEC**

NO synthesized by vascular EC is a potent vasodilatory substance. The actions of NO extend well beyond its vasodilatory properties, and increasingly, NO has been recognized as an important signaling regulator for intercellular and intracellular communication in vascular and BBB permeability (66, 67). NO has also been shown to modulate ion channels in cells. Endothelial NOS, nNOS, and iNOS, respectively, are the rate-limiting enzymes in the synthesis of NO, a gas with numerous properties (68), including the modulation of vascular tone, neurotransmission, and cytotoxicity. Thus, we investigated the possibility of induction of NOS and NO secretion in HBMEC. The expressions of iNOS and eNOS were up-regulated by HIV-1 Tat stimulation in HBMEC (Fig. 3A). However, the level of iNOS was decreased in human lung microvascular EC (HMVEC-L), and the expression of eNOS was also very low in these pulmonary cells (Fig. 3A). Expression of nNOS was not observed in HBMEC (Fig. 3, A and B), while its expression was found in rat pituitary and hippocampal neurons (Fig. 3B). In parallel to the increased levels of iNOS and eNOS observed, the kinetics of nitrate production showed that HBMEC significantly released nitrate 3–6 h after HIV-1 Tat treatment (Fig. 4A). In addition to the kinetics study, we analyzed the possibility of a concentration-dependent effect of HIV-1 Tat, and found that the amount of nitrate released peaked at 50 ng/ml HIV-1 Tat (Fig. 4B). To understand the mechanism of HIV-1 Tat-mediated NO secretion, we used several intracellular signaling inhibitors: L-NAME (NO synthase inhibitor), wortmannin (PI3 kinase inhibitor), PDTC (NF-κB inhibitor), GFX (a PKC inhibitor), and PD98059 (a MAPK inhibitor). Among these inhibitors, L-NAME (5 mM), wortmannin (100 nM), or both inhibitors combined significantly inhibited nitrate production by 29, 43, and 70%, respectively (Fig. 4B). L-arginine (NO donor) was used as a positive control. Furthermore, HIV-1 Tat-induced nitrate induction was inhibited in a dose-dependent manner by L-NAME (Fig. 4C). Taken together, these results indicate that Tat-induced NO secretion may play a role in the integrity of HBMEC.

**NO induced apoptosis of HBMEC following treatment with HIV-1 Tat**

Recent evidence suggests that apoptosis of vascular EC regulates angiogenesis and promotes vascular regression (69, 70). Vascular EC undergo apoptotic cell death when cultured under specific conditions. Therefore, we investigated whether HIV-1 Tat treatment could induce apoptosis in HBMEC. As shown in Fig. 3, A and B, HIV-1 Tat induced apoptosis of HBMEC in a dose-dependent manner. The effects of HIV-1 Tat on apoptosis were confirmed by immunofluorescence microscopy and flow cytometry. Additionally, we used Annexin V and propidium iodide staining to confirm the apoptosis induced by HIV-1 Tat. The results showed that apoptosis was significantly induced in HBMEC treated with HIV-1 Tat for 24 h. Furthermore, we investigated the role of caspase-3 and PARP in HIV-1 Tat-induced apoptosis. The results showed that HIV-1 Tat significantly increased the activity of caspase-3 and PARP in HBMEC. These findings suggest that HIV-1 Tat induces apoptosis in HBMEC through a caspase-dependent mechanism.

**FIGURE 2.** Tyrosine phosphorylation of Flk-1/KDR and Flt-4 upon HIV-1 Tat stimulation. HBMEC were treated with VEGF (30 ng/ml; A and B) for 5 min or with HIV-1 Tat (50 ng/ml; C and D) or heat-inactivated HIV-1 Tat (50 ng/ml; E and F) for 5 min. Samples were immunoprecipitated with polyclonal anti-Flk-1/KDR Ab (A, C, and E) or anti-Flt-4 Ab (B, D, and F) and were blotted with 4G10 monoclonal anti-phosphotyrosine Ab (upper panels). Blots were then reprobed with anti-Flk-1 or anti-Flt-4 Abs (bottom panels). This is a representative experiment of three experiments.

**FIGURE 3.** HIV-1 Tat up-regulates NOS expression. A. Regulation of NOS expression by HIV-1 Tat in HBMEC and HMVEC-L. Post-confluent HBMEC and HMVEC-L, as a positive control, were serum-deprived for 4 h. Cells were treated with HIV-1 Tat for the indicated times and lysed in RIPA buffer. Blots were probed with mouse anti-iNOS, eNOS, or nNOS Abs (1/3000–5000). Erk was used as an internal control for loading. B. Expression of nNOS. Total cell lysates were obtained from rat hippocampal neurons, rat pituitary cells, and HBMEC. Western blot analysis was performed using anti-nNOS mAb. This is a representative experiment of three experiments.
growth factor-deprived conditions. Therefore, we studied the role of HIV-1 Tat in the apoptotic process in HBMEC. HBMEC at 85% confluence on fibronectin-coated plates were starved for 4 h and treated with three different concentrations of HIV-1 Tat plus heparin for 12 h. Apoptosis was then measured by analyzing poly-(A)DP-ribose polymerase (PARP) cleavage. The dose-dependent experiment with HIV-1 Tat indicated that the p89 fragment of PARP was increased upon treatment with 50 ng/ml HIV-1 Tat compared with lower concentrations of Tat protein (Fig. 5A). No cleavage of PARP (the p89 fragment) was observed in HBMEC treated with immunoabsorbed HIV-1 Tat buffer plus heparin (lane J). Western blot analysis showed that HIV-1 Tat strongly activated caspase-mediated PARP (p89) cleavage in a time-dependent manner (Fig. 5B). Expression of parental PARP (113 kDa) was decreased by cleavage 12 h after Tat stimulation. In parallel, the cleaved product (89 kDa) started increasing within 1 h, and the highest expression was observed after 24 h (Fig. 5B). To elucidate whether Tat-mediated apoptosis is conferred by the up-regulation of NO secretion, HBMEC were pretreated with the NOS inhibitor, L-NAME (1, 5, and 10 µM) in serum-reduced (0.5%) medium for 30 min and then incubated with HIV-1 Tat for 18 h. Control cultures were treated with heparin (10 U/ml) alone. A reduction in PARP cleavage (p89) was observed during HIV-1 Tat-mediated apoptosis upon NO inhibition by L-NAME (Fig. 5C). Similarly, we observed that inhibition of PI3 kinase by wortmannin or both L-NAME and wortmannin also resulted in a reduction of PARP cleavage (Fig. 5C), while PDTC (NF-κB inhibitor), GFX (PKC inhibitor), and PD98059 (MAPK inhibitor) had no such effect (data not shown). These results suggest that HIV-1 Tat-mediated apoptosis is partly regulated by NO and PI3 kinase.

In addition, we studied the effects of HIV-1 Tat on the release of mono- and oligonucleosomes from chromosomes and on the incorporation of fluorescein into the 3'-OH of nicked chromosomal DNA using the TUNEL assay. As shown in Fig. 6, A and B, treatment of HBMEC with HIV-1 Tat induced apoptosis in 13% more cells compared with the control (heparin alone). L-NAME or wortmannin in the absence of HIV-1 Tat had little effect on apoptosis (Fig. 6, A and C). Tat-induced apoptosis was significantly inhibited by 9–11% in the presence of L-NAME or wortmannin, but was not inhibited in the presence of GFX (a PKC inhibitor), PDTC (an NF-κB inhibitor), or PD98059 (a MAPK inhibitor; Fig. 6C).

**FIGURE 4.** HIV-1 Tat-mediated NO secretion. A, HIV-1 Tat induced NO secretion in HBMEC. Postconfluent HBMEC were plated on fibronectin-coated, six-well plates and were serum-deprived for 4 h. Cells were treated with HIV-1 Tat (50 ng/ml) and heparin (10 U/ml) for the indicated times. Conditioned medium obtained from each culture was applied to the reaction of the nitrate reductase. The y-axis indicates the measurement of nitrate production. B, Secretion of NO induced by different concentrations of HIV-1 Tat protein and by inhibitors (NM, l-NAME; WM, wortmannin). HBMEC were treated with the indicated amounts of HIV-1 Tat protein and l-arginine (l-Arg; 100 µg/ml) for 18 h. In the same experiment HBMEC were pretreated with l-NAME (NM; 5 µM) and/or wortmannin (WM; 100 nM) for 30 min and were treated with HIV-1 Tat. Nitrate secretion was measured as described above. C, Inhibition of HIV-1 Tat-mediated NO secretion by l-NAME. HBMEC grown on 24-well plates were pretreated with different concentrations of l-NAME for 30 min and then treated with HIV-1 Tat (50 ng/ml) for 18 h. Conditioned medium was subjected to nitrate assay. Experiments were repeated at least four times.
FIGURE 5. HIV-1 Tat induced cleavage of PARP in HBMEC. A, Cleavage of PARP by HIV-1 Tat. Postconfluent HBMEC grown on six-well plates were treated with three different concentrations of HIV-1 Tat for 15 h. Cleavage of PARP was analyzed by Western blotting. p113 kDa, parental PARP; p89, the cleaved product, as shown by arrows. Bottom panel, The blot was reprobed with anti-Erk Ab for the gel loading control. B, Kinetics of PARP cleavage by HIV-1 Tat stimulation in HBMEC. Postconfluent HBMEC were preincubated in 1% serum-containing medium for 4 h and then stimulated with 50 ng/ml HIV-1 Tat for the indicated times. The blot was probed with mouse anti-human PARP Ab (BD PharMingen, San Diego, CA), and then reprobed with anti-actin for the loading control. Control at 0 h indicates heparin alone. C, Cleavage of PARP by HIV-1 Tat in the presence of L-NAME and/or wortmannin (WM). HBMEC were exposed to HIV-1 Tat plus heparin for 18 h in the presence of various concentrations of L-NAME and/or wortmannin as indicated. Cell lysates were prepared, and the cleavage of PARP (p89) was assayed by Western blot analysis. The blot was reprobed with anti-Erk Ab for the gel loading control. This is a representative experiment of four experiments.

FIGURE 6. TUNEL staining of apoptotic HBMEC. Panels demonstrate fluorescein-labeled apoptotic HBMEC treated with heparin (control; A) or HIV-1 Tat (50 ng/ml) plus heparin (B). HBMEC untreated (Aa) or treated with L-NAME (5 μM; Ab) or wortmannin (WM; 100 nM; Ac) were analyzed. Similarly, HBMEC were treated with HIV-1 Tat (50 ng/ml) without inhibitor (Ba) or in the presence of the following inhibitors: L-NAME (5 μM; Bb), wortmannin (100 nM; Bc), PDTC (2 μM; Bd), PD98059 (10 μM; Be), and GFX (3 μM; Bf), as indicated. Apoptotic cells were observed by TUNEL assay (Roche). C, Quantitation of apoptotic cells. Two hundred cells from the above experiment (see A and B) were counted on three different fields of TUNEL staining. Experiments were repeated twice.
upper chamber. As shown in Fig. 8B, a significant increase (~2-fold) in transcytosis was observed 12 h after HIV-1 Tat treatment. This indicated that HIV-1 Tat treatment resulted in an increase in HBMEC permeability. These results were comparable with the observed increase in transcytosis upon TNF-α stimulation of HBMEC, which was used as a positive control (Fig. 8A). The Tat-induced permeability was inhibited by l-NAME and/or wortmannin by 30–40% (Fig. 8C), indicating that NO induced by HIV-1 Tat affects HBMEC stability and permeability via the PI3 kinase pathway. Tat-induced permeability was not affected by anti-TNF Abs (Fig. 8D), indicating that HIV-1 Tat-mediated effects are TNF-α independent. Thus, HIV-1 Tat injures the integrity of the HBMEC lining and thereby may enhance the spread of the disease to peripheral tissues.

Discussion
In this study, we demonstrated that HIV-1 Tat induced the apoptosis of BMEC, resulting in the increased permeability of the brain microvascular endothelial monolayer. HIV-1 Tat activates Flik-1/KDR and Flt-4 receptors in HBMEC (Fig. 2). The involvement of the RGD region within the VEGF receptors, Flik-1/KDR and Flt-4, in the in vivo angiogenic effect of Tat (69) is consistent with in vitro studies. These studies showed that binding of the Tat-RGD region to αβ3 provides EC with the adhesion signal required for various activities, including proliferation in response to mitogens, promotion of cellular migration, and activation of the expression of collagenase IV, a protease that plays a key role in angiogenesis and tumor progression (70).

Our findings indicate that activation of HBMEC with HIV-1 Tat for 16 h resulted in increased NO secretion and the up-regulation of eNOS and iNOS expression (Fig. 3). In addition, our results demonstrated that NO induced the apoptosis of HBMEC following HIV-1 Tat treatment, as shown by the cleavage of PARP (Fig. 5), TUNEL assay (Fig. 6), and the fragmentation of chromosomal DNA (Fig. 7). Furthermore, the NOS inhibitor, l-NAME, and the PI3 kinase inhibitor, wortmannin, blocked Tat-induced endothelial apoptosis. Indeed, the PI3 kinase pathway has been reported to mediate NO secretion in brain EC (60, 71).

EC of the BBB are known to possess the inducible and constitutive forms of NOS, as we also observed (Fig. 3). Inflammatory mediators released in the CNS during viral or bacterial infections are able to induce iNOS that is present in EC, astrocytes, and brain macrophages (72, 73).

Evidence of endothelial damage that might underlie the excessive monocyte infiltration into the brain and even contribute to the severe stages of HIV-1 dementia has been documented (74). For example, HIV-1 infection of monocytes/macrophages in vitro resulted in superoxide anion production (75). Endothelial NOS was also induced in cocultures of monocytes/macrophages and EC. Immunohistochemical staining for nitrotyrosine, the footprint of peroxynitrite, indicated extensive immunoreactivity in the perivascular area of brain tissues from HIV-associated dementia patients. Furthermore, the architecture of many blood vessels was damaged, as shown by immunohistochemical staining for zonula occludens-1, a tight junction protein (76). This observation suggested that during the intimate contact with HIV-infected monocytes/macrophages, EC may contribute to their own damage by the production of NO.

Studies of BBB function and permeability during infections revealed the involvement of NO in this process. Increased permeability of the BBB after endotoxin administration to rats was blocked in the presence of the NO inhibitor, l-NAME. On the other hand, this effect could be potentiated after administration of L-arginine, a substrate for NOS (77). We also observed that...
Tat-induced permeability was inhibited by 1-NAME (5 μM) in HBMEC. Tat-induced apoptosis was also inhibited by 1-NAME (Fig. 5–7). These results suggest that HIV-1 Tat-induced NO secretion is critical for inducing damage of the endothelial lining in the BBB. Higher amounts of NO, such as produced by iNOS, are known inducers of programmed cell death. It is generally accepted that apoptosis induction represents a mode of action for the cytotoxic effects of NO. These studies, including our current study, indicated that NO generated by BBB EC could play an important role in the response to injury or inflammation in the CNS.

In the current study, NO appeared to be proapoptotic, since NO inhibitors blocked HIV-1 Tat-triggered programmed cell death (Figs. 5–7). NO is important in signal transduction leading to the activation of PI3 kinase. Interestingly, our studies indicated that treatment with both 1-NAME and wortmannin inhibited HIV-1 Tat-mediated apoptosis, as shown by inhibition of PARP cleavage (Fig. 5) and cleavage of chromosomal DNA by TUNEL assay (Fig. 6). However, various concentrations of the inhibitors PDTC, PD98059 and GFX had no effect on PARP cleavage (data not shown). These data indicated that PKC and MAPK inhibitors had no effect on HIV-1 Tat-mediated apoptosis, while HIV-1 Tat-induced apoptosis of HBMEC was mediated by the PI3 kinase pathway (Figs. 5–7).

The published studies on HIV-1 Tat and endothelium were mostly examined in HUVEC and Kaposi’s sarcoma cells (derived from lymphatic endothelium) (78). HIV-1 Tat was shown to dysregulate the production of TNF-α and to lead to the activation of N-methyl-D-aspartate receptors and neuronal cell death during HIV-1 infection of the CNS. Although HIV-1 Tat has been shown to cause apoptosis of human PBMC, T cells, and neuronal cells (79), the molecular and biochemical pathways activated by HIV-1 Tat to induce apoptosis are not well elucidated. Tat has been reported to both inhibit and induce apoptosis (80, 81). Previous studies noted Tat-induced apoptosis in HMVEC-L through caspase 3 activation (61). There is a similar molecular mechanism between HBMEC and HMVEC-L of using caspase 3 activation for the induction of apoptosis. However, Tat-induced NO secretion is unique in mediating apoptosis in HBMEC, as presented in this study.

Interestingly, the RGD-containing peptide of Tat demonstrated no significant effect on apoptosis (data not shown). A similar effect of the RGD-containing peptide on HMVEC-L apoptosis through the caspase pathway was reported (61). Therefore, the effect of HIV-1 Tat protein on apoptosis, as shown in this study and by Park et al. (61), may not be mediated through the interaction of its RGD domain with integrin, although the RGD-containing Tat protein has biological significance in angiogenesis and in the activation of related adhesion focal tyrosine kinase in Kaposi’s sarcoma cells. The intact BBB represents an important protection against most other virus infections, but not in AIDS patients. The breakdown of in vitro and in vivo barriers is due to the induction of IL-6, TNF-α, and other inflammatory cytokines upon heavy exposure of EC to HIV-1 Tat (82) (Fig. 8). In this study we demonstrated that HIV-1 Tat induced the apoptosis of HBMEC by the up-regulation of NO, thereby increasing HBMEC permeability. Tat was reported to increase endothelial albumin permeability in vitro through the tyrosine kinase and MAPK, but not protein kinase G pathway (34). Elucidating the detailed molecular mechanisms of apoptosis upon HIV-endothelial interaction may lead to novel strategies that will inhibit virus infection in the host.

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References

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