Journal of NeuroVirology, 9: 584–593, 2003

ISSN: 1355-0284 print / 1538-2443 online
DOI: 10.1080/13550280390218760

Taylor & Francis
Taylor & Francis Group

Human immunodeficiency virus type 1 Tat-mediated cytotoxicity of human brain microvascular endothelial cells

Naveed Ahmed Khan,[1] Francescopaolo Di Cello,[1] Avi Nath,[2] and Kwang Sik Kim[1]

[1]*Division of Pediatric Infectious Diseases, The Johns Hopkins University School of Medicine, Baltimore, Maryland, USA;*
[2]*Department of Neurology, University of Kentucky Medical Center, Lexington, Kentucky, USA*

Human immunodeficiency virus (HIV)-1 infection is often complicated with neurologic disorders, but the pathogenesis of HIV-1 encephalopathy is incompletely understood. Tat (HIV-1 transactivator protein) is released from HIV-1–infected cells and has been detected in the sera and cerebrospinal fluid of HIV-1–infected patients. Tat, along with increased inflammatory cytokines such as interferon-gamma (IFN-γ), have been implicated in the pathogenesis of HIV-1–associated blood-brain barrier dysfunction. The present study examined the effects of Tat and IFN-γ on human brain microvascular endothelial cells (HBMECs), which constitute the blood-brain barrier. Tat produced cytotoxicity of HBMECs, but required IFN-γ. IFN-γ treatment of HBMECs up-regulates vascular endothelial growth factor receptor-2 (VEGFR2/KDR), which is known to be the receptor for Tat. Tat activated KDR in the presence of IFN-γ, and Tat-mediated cytopathic changes involve its interaction with KDR and phosphatidylinositol 3-kinase (PI3K). Further understanding and characterization of Tat-HBMEC interactions should help us understand HIV-1 neuropathogenesis and develop strategies to prevent HIV-1 encephalopathy. *Journal of NeuroVirology* (2003) **9,** 584–593.

Keywords: human brain microvascular endothelial cells; IFN-gamma; PI3K; Tat; VEGFR2/KDR

Introduction

Central nervous system (CNS) dysfunction is a serious manifestation of human immunodeficiency virsus (HIV)-1 infection, and the development of neurologic disorders is the important cause of morbidity and mortality associated with acquired immunodeficiency syndrome (AIDS) (Koppel, 1998; Janssen, 1997; Corasaniti *et al*, 1998; Glass and Johnson, 1996; Persidsky *et al*, 1997). Several investigators have shown that structural and functional perturbations of the blood-brain barrier occur commonly during HIV-1 infection as shown by increased cerebrospinal fluid (CSF)-serum albumin ratios, demonstration of serum protein extravasation in the brains of patients with HIV-1 infection, increased matrix metalloproteinases, and disruption of tight junctions as determined by the altered patterns of zonula occludens-1 (ZO-1) and occludins (Dallasta *et al*, 1999; Marshall *et al*, 1991; McArthur *et al*, 1992; Petito and Cash, 1992; Power *et al*, 1993; Rhodes, 1991; Sporer *et al*, 1998; Tran Dinh *et al*, 1990). But the mechanisms contributing to this blood-brain barrier dysfunction remain incompletely understood.

Blood-brain barrier dysfunction during HIV-1 infection has been proposed due to many host and viral factors (Annunziata *et al*, 1998; Bussolino *et al*, 2001; Fiorelli *et al*, 1999; Rappaport *et al*, 1999). For example, cytokines, such as tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-1β and IL-6, have been shown to be increased in plasma, brain tissue, and CSF of patients with HIV-1 encephalitis (Graziosi *et al*, 1996; Lahdevirta *et al*, 1988; Westendorp *et al*,

Address correspondence to Kwang Sik Kim, Division of Pediatric Infectious Diseases, The John Hopkins University School of Medicine, 600 N. Wolfe Street, Park 256, Baltimore, MD 21287, USA. E-mail: kwangkim@jhmi.edu

This work was supported by NIH grants R01 HL 61951 and AA 13858.

Received 5 November 2002; revised 6 December 2002; accepted 10 March 2003.

1995) and have been shown to modulate the blood-brain barrier (Claudio *et al*, 1994; De Vries *et al*, 1996). In addition, viral proteins such as gp120 (HIV-1 envelope protein) and Tat (HIV-1 transactivator protein) have been shown to play a role in the vascular endothelial cell dysfunction. For example, gp120 is capable of altering and activating the blood-brain barrier *in vivo*. This was shown in the brains of HIV-1 gp120 transgenic mice by the demonstration of albumin extravasation and up-regulation of intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 in cerebral vessels (Toneatto *et al*, 1999). We have also shown that gp120 activates human brain microvascular endothelial cells (HBMECs) in ICAM-1 and VCAM-1 up-regulation and increased monocyte transmigration across HBMEC monolayers (Stins *et al*, 2001). Tat protein of HIV-1 is a transcriptional activator essential for viral replication and consists of 86 to 104 amino acids encoded by two exons (Jeang *et al*, 1999; Jones, 1993). Tat protein has been detected in the CNS of HIV-1–infected patients (Hofman *et al*, 1999). Tat is secreted by the HIV-1–infected cells and picomolar concentrations of Tat have been detected in the supernatant of HIV-1–infected cells and in the sera of HIV-1–infected individuals (Ensoli *et al*, 1990; Westendorp *et al*, 1995; Zauli *et al*, 1995). Previous studies have shown that extracellular Tat acts as a pleiotropic molecule exhibiting diverse effects on host cells. These include endothelial cell stimulation (Hofman *et al*, 1993, 1999), angiogenic effects in HUVECs (Del Sorbo *et al*, 2001), up-regulating expression of endothelial cell adhesion molecules in HUVECs (Dhawan *et al*, 1997), apoptosis in rat pheochromocytoma (PC12) cells (Gavriil *et al*, 2002), excitotoxicity (Magnuson *et al*, 1995) and apoptosis in neurons (New *et al*, 1997), and the release of neurotoxic substances in glial cells (Power *et al*, 1995).

Our investigations have focused on the role of HBMECs in HIV-1–associated blood-brain barrier dysfunction. We examined whether Tat affects cytotoxicity in HBMECs and whether Tat-induced cytotoxicity requires specific signaling pathways.

Results

Purified Tat

Purity of Tat proteins, Tat86 and ΔTat lacking basic domain region (31 to 61 residues) was estimated to be >95% following sodium dodecyl sulfate–poly acrylamide gel electrophoresis (SDS-PAGE). Purified Tat86 increased long terminal repeat (LTR) activation as we have observed previously (Ma and Nath, 1997), whereas ΔTat, as expected, exhibited no evidence of LTR transactivation (data not shown).

Tat-mediated cytotoxicity in HBMECs

Tat induced cytotoxicity of HBMECs in the presence of IFN-γ (Figure 1). More than 30% cytotoxicity was observed at 72 h incubation. Of interest, Tat or IFN-γ alone did not exhibit any significant cytotoxicity (Figure 1). In order to determine whether Tat-induced cytotoxicity requires the presence of IFN-γ, or pretreatment of HBMECs with IFN-γ is sufficient, cytotoxicity assays were performed using HBMECs that were previously treated with IFN-γ for 24 h and then incubated with Tat alone. We observed that either pretreatment of HBMECs with IFN-γ followed by Tat incubation or coincubation of IFN-γ with Tat gave similar HBMEC cytotoxicity (data not shown). In contrast, ΔTat exhibited significantly less cytotoxicity

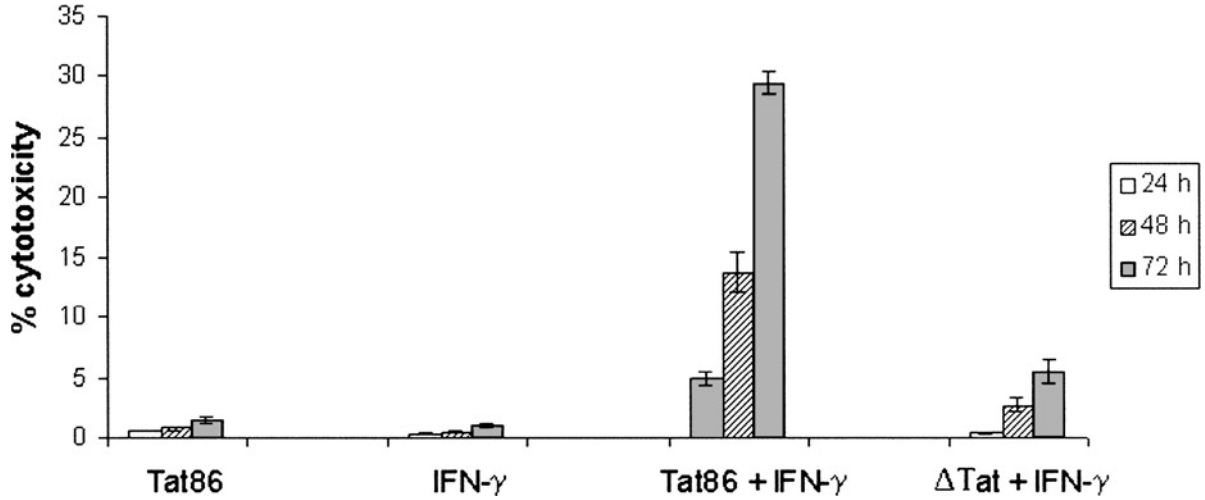

Figure 1 Tat-mediated cytotoxicity in HBMECs.Tat (0.1 μg/ml) and IFN-γ (2 ng/ml) were added to confluent cultures of primary HBMECs. Cultures were incubated at 37°C in a 5% CO_2 incubator for varying periods. At the end of the incubation period, supernatants were collected and analyzed for LDH release and converted into percentage cytotoxicity as described in Materials and Methods. Note that Tat-mediated cytotoxicity was observed only with full length Tat 86 in the presence of IFN-γ, not with ΔTat (Tat lacking basic domain region, 0.1 μg/ml). Results represent mean of three independent experiments. All experiments were performed in triplicates. Error bars represent standard deviation.

(Figure 1), suggesting that HBMEC cytotoxicity requires full length Tat and/or the basic domain of Tat may be involved in HBMEC cytotoxicity. The lack of cytotoxicity with ΔTat also indicates that Tat-mediated HBMEC cytotoxicity is not due to any contamination from the purification procedures in the Tat preparations.

Tat stimulates tyrosine phosphorylation of KDR in HBMECs

To determine whether Tat-mediated cytotoxicity require tyrosine kinase signaling pathways, HBMEC monolayers were pretreated with genistein (25 μM) (a protein tyrosine kinase inhibitor) before stimulation with Tat and IFN-γ. Tat-induced cytotoxicity was inhibited by more than 50% with genistein (data not shown), suggesting the role of tyrosine kinases signaling pathways in Tat-dependent cytotoxicity. Tat has been shown to bind and activate KDR, a 205-kDa vascular endothelial growth factor (VEGF)-A tyrosine kinase receptor (Ganju *et al*, 1998). We therefore examined whether Tat interactions with HBMECs leads to phosphorylation of KDR. As shown in Figure 2, Tat stimulation in the presence of IFN-γ resulted in a clear-cut tyrosine phosphorylation of KDR. Equal amounts of the KDR protein were present in each lane, as shown by stripping and reprobing the membrane with anti-KDR antibody (Santa Cruz Biotech, Santa Cruz, CA) (Figure 2). These results were reproduced in three independent experiments. Of interest, ΔTat incubation with HBMECs in the presence of IFN-γ did not stimulate KDR phosphorylation (data not shown).

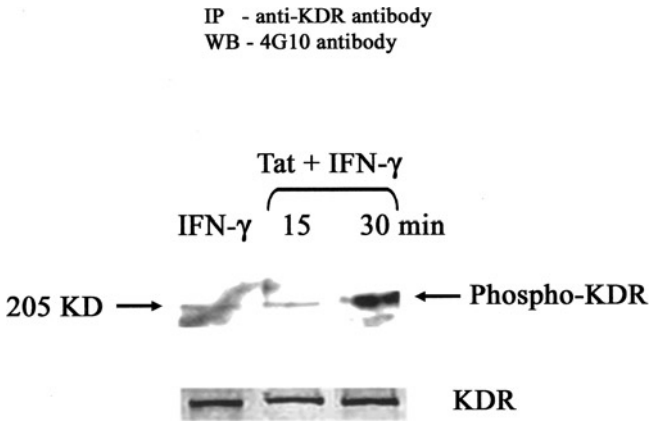

Figure 2 Tat stimulates tyrosine phosphorylation of KDR in HBMECs. Confluent cultures of HBMECs grown in 60-mm dishes were pretreated with IFN-γ (2 ng/ml) for 24 h, followed by stimulation with Tat (0.1 μg/ml) for 15 and 30 min. The cell lysates were prepared, immunoprecipated using anti-KDR antibody, separated by SDS-PAGE, followed by immunoblotting with antiphosphotyrosine antibody 4G10 as described in Materials and Methods. Molecular weight markers are indicated in kilodaltons and shown on the left. A band corresponding to KDR (205 kDa) was identified at the expected molecular weight.

IFN-γ treatment leads to up-regulation of KDR both at the mRNA and protein level in HBMECs: We next used reverse transcriptase–polymerase chain reaction (RT-PCR) to determine whether the expression of *kdr* is affected by IFN-γ treatment. A 490-bp PCR product deriving from *kdr* mRNA was observed when HBMECs were treated with IFN-γ for 24 or 48 h, but not in untreated cells, indicating that *kdr* is expressed in HBMECs only after the cells are exposed to IFN-γ (Figure 3**A**). Amplification of a 294-bp β-actin mRNA fragment gave comparable bands from both IFN-γ–treated and untreated samples, confirming that the same amounts of cDNA were used (Figure 3**A**). To further confirm whether increase in expression of *kdr* leads to increased level of KDR protein, Western blotting was performed using KDR antibody. Briefly, IFN-γ–treated HBMECs were lysed as described above. Lysates were Western blotted using KDR antibody. We observed the expression of KDR is increased in IFN-γ–treated HBMECs (Figure 3**B**), thus further confirming that IFN-γ treatment up-regulates KDR, which can act as a receptor for Tat. Increased expression of KDR in response to IFN-α treatment was not related to loading different concentrations of proteins (Figure 3**C**). Taken together, these findings indicate that IFN-γ increases both transcriptional and translational levels of KDR, which contributes to Tat-mediated effects on HBMECs.

Tat-mediated cytotoxicity is dependent on its basic domain interactions with KDR on HBMECs

To further examine that Tat-induced cytotoxicity is due to Tat-KDR interactions, cytotoxicity assays were performed in the presence of anti-KDR antibody. Tat-induced cytotoxicity was completely inhibited in the presence of anti-KDR antibody, indicating that Tat interaction with KDR contributes to HBMEC cytotoxicity (Figure 4).

Tat can be divided into five domains termed, N-terminal, cysteine-rich, core, basic, and C-terminal. The basic domain (42 to 64 amino acids), similar to the basic sequence of VEGF-A, is the region known to specifically bind to KDR in HUVECs (Jia *et al*, 2001). In an attempt to determine whether Tat interaction with KDR leading to HBMEC cytotoxicity was mediated by the basic domain of Tat, cytotoxicity assays were performed using ΔTat lacking basic domain as described above. The results revealed that Tat lacking basic domain exhibited significantly less cytotoxicity of HBMECs as compared to a full length Tat (Figure 1). Taken together, these data suggested that Tat basic domain is essential for HBMEC cytotoxicity.

Tat-mediated cytotoxicity is dependent on PI3K but FAK independent

Previous studies have shown that KDR activation stimulate downstream signaling events involving phosphatidylinositol 3-kinase (PI3K) and focal adhesion kinase (FAK) activations (Mitola *et al*, 2000;

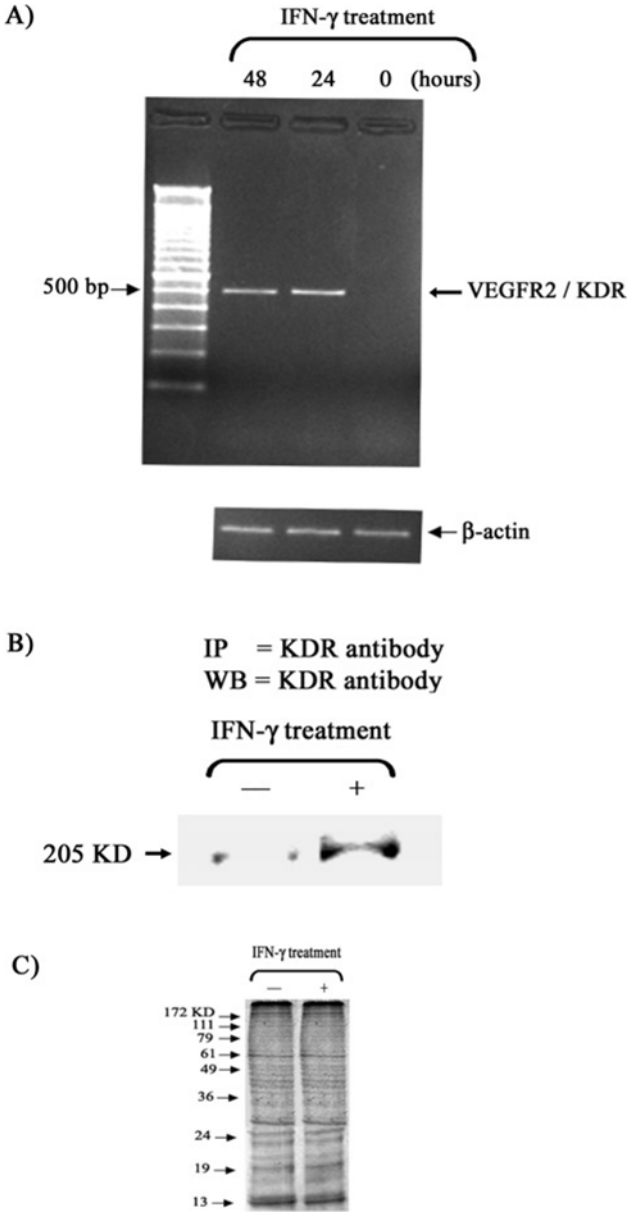

Figure 3 (**A**) Amplification of *kdr* and β-actin by RT-PCR. The expected 490-bp *kdr* fragment was amplified only in HBMECs treated with IFN-γ, whereas β-actin 294-bp fragment was equally amplified from all samples. Note that HBMECs treated with IFN-γ for 24 and 48 h exhibited higher levels of KDR. Results are representative of three independent experiments. (**B**) IFN-γ treatment of HBMECs leads to increased KDR expression at the protein level. HBMECs with or without the prior treatment of IFN-γ for 48 h were lysed. Proteins were immunoprecipitated and immunoblotted with anti-KDR antibody as described in Materials and Methods. Note that KDR expression is increased in IFN-γ–treated HBMECs. (**C**) For loading control, following HBMEC lysis, as in **B**, 2 μl of total proteins were electrophoresed and stained using coomassie blue staining.

Qi and Claesson-Welch, 2001; Thakker *et al,* 1999). To determine whether Tat-mediated cytotoxicity requires PI3K and/or FAK, we performed cytotoxicity assays using PI3K or FAK dominant-negative

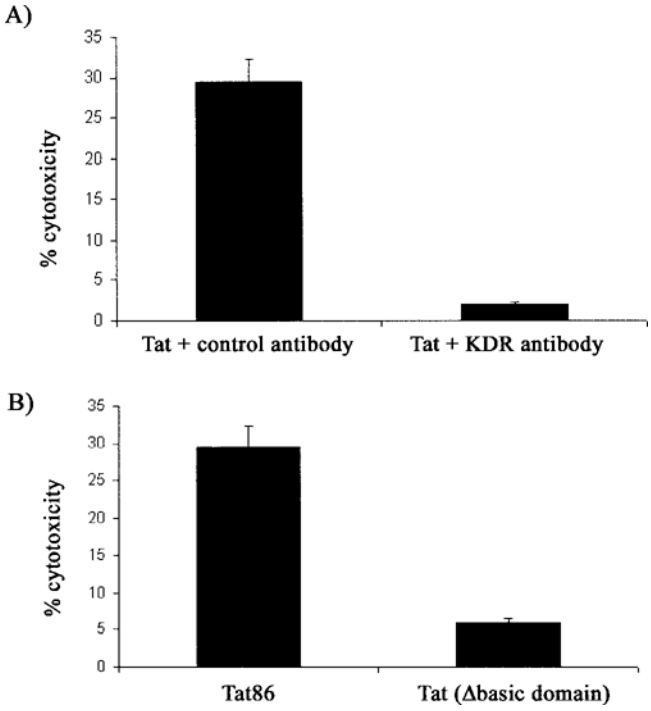

Figure 4 Tat-induced cytotoxicity of HBMECs is mediated by its interactions with KDR on HBMECs. To determine the role of KDR, cytotoxicity assays were performed using Tat and IFN-γ in the presence of anti-KDR antibody (10 μg/ml) or control antibody (anti-His antibody, 10 μg/ml) as described in Figure 1. Note that KDR antibody inhibited Tat-mediated HBMEC cytotoxicity. Results represent mean of three independent experiments. Error bars represent standard deviation.

HBMECs. Tat-mediated cytotoxicity was completely abolished in PI3K dominant-negative cells as compared to HBMECs transfected with pcDNA3 alone (Figure 5). HBMECs expressing either mutant p85

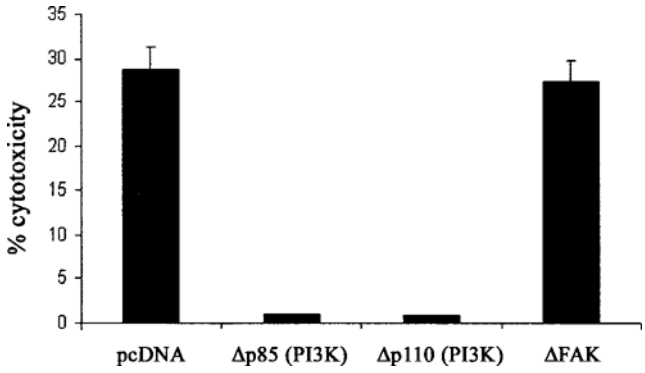

Figure 5 Tat-induced cytotoxicity of HBMECs is PI3K dependent and FAK independent. To determine the role of PI3K and FAK in Tat-mediated cytotoxicity, HBMECs were transfected with pcDNA3, or Δp85, or Δp110, or ΔFAK and used in Tat-induced cytotoxicity assays in the presence of IFN-γ as described in Materials and Methods. Note that Tat-induced cytotoxicity was abolished in HBMECs overexpressing PI3K mutants (Δp85 and Δp110). Results represents mean of three dependent experiments. All experiments were performed in triplicates. Error bars represent standard deviation.

or mutant p110 (PI3K subunits) showed complete inhibition of Tat-induced cytotoxicity. In contrast, Tat-mediated cytotoxicity was not affected in FAK dominant-negative cells, indicating that Tat-induced cytotoxicity in HBMECs is dependent on PI3K but independent of FAK (Figure 5).

Discussion

Several lines of evidence suggest that HIV-1 proteins such as gp120 or Tat and inflammatory cytokines such as TNF-α and IFN-γ released by HIV-1–infected cells contribute to HIV-1–associated neurologic disorders (Annunziata *et al*, 1998; Banks *et al*, 1997; Bussolino *et al*, 2001; Fiorelli *et al*, 1999; Hofman *et al*, 1993; Kolb *et al*, 1999; Kruman *et al*, 1998; Lafrenie *et al*, 1997; Rappaport *et al*, 1999). In this study, we showed that Tat (0.1 μg/ml) in the presence of IFN-γ (2 ng/ml) induced cytotoxicity in primary HBMEC monolayers. These cytopathic effects of Tat at picomolar concentrations are likely to be biologically relevant because similar concentrations of Tat have been detected in the supernatant of HIV-1–infected cells as well as in the sera of some HIV-1–infected individuals (Ensoli *et al*, 1990; Westendorp *et al*, 1995; Zauli *et al*, 1995). In addition, IFN-γ has been shown to be increased in the serum of HIV-1–infected individuals (Benyoucef *et al*, 1997; Graziosi *et al*, 1996). This is the first report describing Tat-induced cytotoxicity in HBMECs, suggesting Tat in the presence of IFN-γ contributes to the blood-brain barrier perturbations that occur commonly in HIV-1–infected patients. These findings also suggest that Tat is a major HIV-1 protein contributing to blood-brain barrier perturbations. The characterization of Tat-mediated HBMEC dysfunction may provide insights into mechanisms involved in CNS injury and paracellular migration of HIV-1 monocytes across the blood-brain barrier, critical events in HIV-1 neuropathogenesis.

We determined the molecular basis for the requirement of IFN-γ for Tat-mediated cytotoxicity in HBMECs. Tat can be divided into five domains termed, N-terminal, cysteine-rich, core, basic, and C-terminal. The C-terminal domain contains an RGD sequence, which represents the major cell attachment moiety recognized by integrin receptors $\alpha_5\beta_1$ and $\alpha_v\beta_3$ for fibronectin and vitronectin, respectively (Barillari *et al*, 1999; Fiorelli *et al*, 1999). The basic domain (42 to 64 amino acids) is similar to the basic sequence of VEGF-A and has been shown to bind $\alpha_v\beta_v$ (Vogel *et al*, 1993) and KDR (Jia *et al*, 2001) in HUVECs, whereas the cysteine-rich domain interacts with chemokine receptors, CCR2 and CCR3 (Albini *et al*, 1998). IFN-γ treatment has been shown to up-regulate the expression of integrins $\alpha_5\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_v$, and *kdr* in HUVECs (Barillari *et al*, 1993; Fiorelli *et al*, 1995, 1998, 1999; Philippon *et al*, 1994). We showed that IFN-γ treatment of HBMECs resulted in up-regulation of KDR both at the mRNA and protein level, as compared to untreated cells. In contrast, expression levels of integrins receptors were unchanged (data not shown), suggesting a possible role of KDR in Tat-mediated cytotoxicity in IFN-γ–treated HBMECs. In support of this possibility, we showed that Tat directly activates KDR (i.e., tyrosine phosphorylation) when incubated with HBMECs in the presence of IFN-γ. We further showed that anti-KDR antibody blocks Tat-mediated HBMEC cytotoxicity. Our findings are consistent with those of other investigators who have shown that vascular endothelial cells become responsive to Tat after exposure to cytokines (e.g., TNF-α, IFN-γ) (Barillari *et al*, 1999; Fiorelli *et al*, 1999; Morini *et al*, 2000). In contrast, several investigators have shown in HUVECs that Tat up-regulates E-selectin expression and IL-6 secretion (Hofman *et al*, 1993), as well as activates KDR, leading to angiogenic effect (Mitola *et al*, 2000) or apoptosis (Jia *et al*, 2001), and this activation does not require IFN-γ. One explanation for this discrepancy could be due to the use of endothelial cells from different origins such as umbilical vein. An alternative explanation may stem from different receptors interacting with different regions of Tat. For example, Tat activates a proangiogenic program in HUVECs by activating KDR and integrins, which requires the basic and cysteine-rich domain as well as the C-terminal region of Tat (Mitola *et al*, 2000). Additional studies are needed to examine whether Tat-mediated diverse effects in different tissues may stem from its interactions with different receptors and/or involvement of different regions of Tat.

KDR, upon interaction with its ligand such as Tat, is autophosphorylated at its cytoplasmic domain. There are four tyrosine phosphorylation sites (Tyr-951, Tyr-996, Tyr-1054, and Tyr-1059) identified to date, which act as docking sites for SH2- and SH3-containing molecules (Dougher-Vermazen *et al*, 1994). Signaling events downstream of KDR have been somewhat controversial in endothelial cells. Thakker *et al* (1999) have shown in HUVECs that upon interaction with VEGF-A, cytoplasmic domain of KDR is phosphorylated and binds p85 (a regulatory subunit of PI3K that contains SH2 domain), which recruits p110 (catalytic subunit of PI3K), thus mediating PI3K signaling cascades. PI3K activation in HUVECs was shown to depend on the interaction of KDR with the basic domain of Tat, not the C-terminal domain (Mitola *et al*, 2000). Of interest, VEGF has been shown to activate FAK and stimulate migration and stress fiber formation in HUVECs, but has no effect on PI3K (Abedi and Zachary, 1997). In contrast, Qi and Claesson-Welch (2001) have shown in porcine aortic endothelial cells that VEGF-A interactions with KDR induced migration (angiogenesis), which was dependent on activation of both FAK and PI3K. These studies were performed using VEGF-A (a well-known ligand for KDR). These findings represent the diverse mechanisms of KDR signaling leading to various phenotypes such as

angiogenesis, but their relevance to Tat-KDR interactions in HBMECs is unclear.

To determine the roles of FAK and PI3K in Tat-mediated cytotoxicity in HBMECs, we performed cytotoxicity assays, using FAK and PI3K dominant-negative cell lines. Tat-mediated cytotoxicity was completely abolished in PI3K dominant-negative cells, whereas no significant change in cytotoxicity was observed in FAK dominant-negative HBMECs. These data suggest that Tat-induced cytotoxicity in HBMECs is dependent on PI3K but independent of FAK. FAK is known to be activated by various integrins (Ivankovic-Dikic *et al*, 2000), suggesting that Tat-integrins interactions may not be critical in Tat-mediated cytotoxicity of HBMECs. These findings are supportive of those of our RNA studies, which showed no up-regulation of integrins in IFN-γ–treated HBMECs. In contrast, other investigators have shown the upregulation of $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins by IFN-γ and Tat (the RGD sequence at the C-terminal region of Tat) interactions with these integrins induces migration and invasion in human umbilical vascular endothelial cells (HUVECs) (Barillari *et al*, 1999). These differences could be due to different cell types (HBMECs versus HUVECs) or different phenotypes (cytotoxicity versus angiogenesis), or involvement of different domains of Tat (basic region versus C-terminal region).

Extracellular Tat has been shown to interact with at least three classes of cell surface receptors present on different target cells, e.g., cell adhesion receptors of the integrin family, VEGF receptors, KDR and Flt-1, and chemokine receptors, CCR2 and CCR3, and different domains of Tat are shown to interact with these receptors (Albini *et al*, 1998; Barillari *et al*, 1999; Bussolino *et al*, 2001; Ganju *et al*, 1998; Mitola *et al*, 2000; Morini *et al*, 2000). It is therefore tempting to speculate that Tat-mediated diverse effects involve its interactions with different receptors and/or different signaling pathways. For example, Tat binding to integrins ($\alpha_5\beta_1$, $\alpha_v\beta_3$) in HUVECs stimulate their proliferation and invasion of extracellular matrix (Fiorelli *et al,* 1999). In our studies, we showed that Tat-mediated cytotoxicity of HBMECs is dependent mainly on its basic domain interaction with KDR and KDR-stimulated signaling events involve PI3K activation. This was shown by inhibition of Tat-induced cytotoxicity using anti-KDR monoclonal antibody and by abolition of cytotoxicity with ΔTat (basic domain deletion mutant) as well as PI3K dominant-negative HBMECs. It is interesting to note that Tat lacking the basic domain retained some cytotoxicity, suggesting the role of other regions of Tat. Our preliminary studies have shown that IFN-γ up-regulates CCR3, a known receptor for the cysteine-rich domain of Tat. Additional studies are needed to determine whether CCR3 plays a role in Tat-mediated HBMEC cytotoxicity. The characterization of the Tat-mediated HBMEC cytotoxicity may provide insights into targeted therapies to prevent HIV-1–associated blood-brain barrier dysfunction.

Materials and methods

Cell cultures and transfections

Primary HBMECs (derived from individuals undergone surgical resections) were routinely grown on rat tail collagen–coated dishes in complete medium (RPMI containing 10% heat inactivated fetal bovine serum, 10% Nu-Serum, 2 mM glutamine, 1 mM pyruvate, penicillin [100 U/ml], streptomycin [100 μg/ml], nonessential amino acids, and vitamins) as previously described (Stins *et al*, 1997). Our primary HBMECs were positive for factor VIII, carbonic anhydrase IV, ulex europaeus agglutinin I, took up fluorescently labeled acetylated low density lipoprotein (DI-AcLDL), and expressed gamma-glutamyltranspeptidase, demonstrating their brain endothelial cell characteristics. More importantly, upon cultivation on collagen-coated Transwell inserts, HBMECs exhibited morphologic and functional properties of tight junctions as well as polar monolayer as shown by the development of high transendothelial electric resistance (TEER) equal to 300 to 600 Ω/cm^2 as compared to TEER values of nonbrain endothelial cells, which are usually less than 15 Ω/cm^2 (Stins *et al*, 1997; Zhang *et al,* 2002). For dominant-negative studies, HBMECs were transfected with plasmid constructs encoding human PI3K and FAK mutants as described previously (Reddy *et al*, 2000a, 2000b). Briefly, DNA-Lipofectamine complex (Invitrogen**,** Carlsbad, CA) in RPMI were added to 50% to 70% confluent HBMEC monolayers. Cultures were incubated for 6 h followed by the addition of complete medium. Three days after the transfection, cells were transferred to medium containing G418 at 400 μg/ml (geneticin; Invitrogen) to establish cell lines that stably expressed mutant PI3K or FAK proteins. For controls, HBMECs were transfected with the vector alone (pcDNA3; Invitrogen) and selected as described above.

Purification of Tat

Tat protein (86 amino acids with six histidine at the C-terminus) was prepared as previously described (Patki and Lederman, 1996). Briefly, *Escherichia coli* (XL1-blue; Stratagene, La Jolla, CA) transfected with plasmid (pTat6xHis, obtained from the National Institute of Health AIDS Research and Reference Reagent Program) were grown in 1-L cultures. Tat expression was induced by incubation of mid-log-phase bacterial cultures with 200 μg/ml isopropyl-D-thiogalactopyranoside (IPTG; Invitrogen) for 2 h. Bacteria were collected and lysed overnight in 6 M guanidine HCl buffered with 0.1 M Na_2HPO_4, 0.1 M NaH_2PO_4, pH 8.0 (sample buffer), at room temperature. Lysates were centrifuged at 20,000 × *g* for

60 min at room temperature. The supernatant was then placed over one ml of Ni^{2+}-nitrilotriacetate-agarose (NTA-agarose; Qiagen, Valencia, CA). Loosely bound proteins were eluted by washing with five column volumes of sample buffer and five column volumes of 6 M guanidine HCl, 0.2 M sodium acetate, pH 6.0. Purified protein was recovered by eluting with 10 ml of 6 M guanidine HCl, 0.2 M sodium acetate, pH 4.0. The eluted recombinant Tat protein was dialyzed against 8 M urea to remove guanidine HCl. Tat protein was then refolded by sequential dialysis against decreasing concentrations of urea. Protein concentration was determined (Bio-Rad, Hercules, CA). Aliquots of protein were stored at −80°C and used for all experiments. Purity of protein was estimated to be >95% after SDS-PAGE. Western blotting was performed using anti-Tat antibody to confirm Tat reactivity. Purified Tat obtained from the National Institute of Health AIDS Research and Reference Reagent Program was used as a reference. Purified Tat86 and ΔTat lacking basic domain region (31 to 61 residues) were examined for their intracellular LTR activity using transactivation assays in D3R5 lymphocytic cells containing HIV-LTR-GFP (green fluorescence protein) as we have previously described (Ma and Nath, 1997).

Cytotoxicity assays

To determine the pathogenic potential of HIV-1 Tat, cytotoxicity assays were performed as previously described (Khan *et al*, 2000). Briefly, HBMECs were grown to monolayers in 24-well plates. Once confluent, cultures were incubated at 37°C with Tat (0.1 μg/ml) and IFN-γ (2 ng/ml; R & D, systems, Minneapolis, MN) in experimental media (5% heat-inactivated fetal bovine serum, 2 mM glutamine, penicillin [100 U/ml], streptomycin [100 μg/ml], and nonessential amino acids) in 5% CO_2 incubator for up to 72 h. Supernatants were collected and cytotoxicity was determined by measuring lactate dehydrogenase (LDH) release using Cytotoxicity Detection Kit (Roche, Indianapolis, IN) and converted into percent cytotoxicity as follows: (sample value − control value/total LDH release − control value) × 100 = % cytotoxicity. Control values were obtained from cells incubated alone. Total LDH release was determined from HBMEC treated with 1% Triton X-100 for 15 min at 37°C. Results represent mean from three independent experiments. In inhibition experiments, antibodies and inhibitors were added 1 h before treatment of HBMECs with Tat and IFN-γ, and cytotoxicity assays were carried out as described above.

RT-PCR analysis of kdr *expression*

Total RNA was extracted from HBMEC cultures grown on 60-mm dishes using RNAqueous-4PCR (Ambion, Austin, TX) according to the manufacturer's instructions. The mRNA fraction was purified from total RNA using Oligotex mRNA mini kit (Qiagen) and ethanol precipitated in 0.5 M ammonium acetate using Linear Acrylamide as coprecipitant (Ambion). The amount and the quality of the mRNA was verified by measuring the absorbance at 260 and 280 nm. Oligo (dT)-primed reverse transcription of RNA was performed using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). Approximately 200 ng of mRNA were used in each reaction. PCR amplification of *kdr* was performed from 2 μl of each cDNA using primers KDR3510f (5′-TGGATGGCCCCAGAAACAAT) and KDR3999r (5′-CAGGCCGGCTCTTTCGCTTA). Primers were designed on different exons using Jellyfish 1.4 (Biowire.com) for the amplification of a 490-bp fragment from cDNA. The PCR program consisted of one preincubation at 94°C for 2 min and 40 cycles at 94°C for 30 s, 55°C for 30 s, and 68°C for 1 min. β-Actin amplification was successively performed from 2 μl of each *kdr* PCR reaction using commercial primers (QuantumRNA β-Actin Internal Standards; Ambion). The PCR program consisted of one preincubation 94°C for 2 min and 20 cycles at 94°C for 30 s, 60°C for 30 s, and 68°C for 1 min. All PCR reactions were performed using a Robocycler Gradient 40 with heated lid (Stratagene, La Jolla, CA) in 50 μl of 1 × PCR buffer—1.5 mM $MgCl_2$, 0.2 μM of each primer, 200 μM of dNTP, and 1 U of Taq DNA polymerase (Invitrogen). Amplification mixtures were analyzed by agarose gel electrophoresis and bands were compared on a Gel Doc 1000 using Quantity One 4.1 software (Bio-Rad, Hercules, CA).

Immunoprecipitation and Immunoblotting

Confluent HBMECs were treated with and without IFN-γ (2 ng/ml) in experimental media for 24 h. Following this treatment, cells were stimulated with Tat (0.1 μg/ml) for the indicated periods and rinsed with ice-cold phosphate-buffered saline (PBS) containing 0.1 mM Na_3VO_4. The monolayers were lysed in lysis buffer composed of 50 mM Tris-HCl (pH 7.4), 0.1% SDS, 0.5% Na deoxycholate, 10 mM Na pyrophosphate, 25 mM β-glycerophosphate, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1% Triton X-100, 1 mM Na_3VO_4, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml aprotonin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin. Cell lysates were centrifuged at 16,000 × g at 4°C and supernatant quantified for protein concentration (Bio-Rad). Equal amounts of protein (500 μg) were incubated with appropriate antibody overnight at 4°C and incubated for 1 h with Protein A-agarose (Roche). The samples were washed four times with lysis buffer without sodium deoxycholate. Samples were eluted by boiling in SDS sample buffer (Invitrogen) containing 10% β-mercaptoethanol (Sigma, St. Louis, MO) and separated by SDS-PAGE. Samples were then electrophoretically transferred onto pure nitrocellulose membrane (Schleicher & Schuell, Keene, NH). The blots were blocked in TBST (25 mM Tris, pH 7.4, 150 mM NaCl, and 0.1% Tween 20)

containing 5% nonfat dry milk at room temperature for 1 h. Blots were probed with specific antibodies overnight at 4°C, washed with TBST, and incubated with horseradish peroxidase–conjugated secondary antibody for 1 h at room temperature. Finally, blots were washed with TBST and immune complexes were visualized through enhanced chemiluminescence (Amersham, La Jolla, CA).

References

Abedi H, Zachary I (1997). Vascular endothelial growth factor stimulates tyrosine phosphorylation and recruitment to new focal adhesions of focal adhesion kinase and paxillin in endothelial cells. *J Biol Chem* **272:** 15442–15451.

Albini A, Ferrini S, Benelli R, Sforzini S, Giunciuglio D, Aluigi MG, Proudfoot AE, Alouani S, Wells TN, Mariani G, Rabin RL, Farber JM, Noonan DM (1998). HIV-1 Tat protein mimicry of chemokines. *Proc Natl Acad Sci U S A* **95:** 13153–13158.

Annunziata P, Cioni C, Toneatto S, Paccagnini E (1998). HIV-1 gp120 increases the permeability of rat brain endothelium cultures by a mechanism involving substance P. *AIDS* **12:** 2377–2385.

Banks WA, Kastin AJ, Akerstrom V (1997). HIV-1 protein gp120 crosses the blood-brain barrier: role of adsorptive endocytosis. *Life Sci* **61:** PL119–PL125.

Barillari G, Gendelman R, Gallo RC, Ensoli B (1993). The Tat protein of human immunodeficiency virus type 1, a growth factor for AIDS Kaposi sarcoma and cytokine-activated vascular cells, induces adhesion of the same cell types by using integrin receptors recognizing the RGD amino acid sequence. *Proc Natl Acad Sci U S A* **90:** 7941–7945.

Barillari G, Sgadari C, Palladino C, Gendelman R, Caputo A, Morris CB, Nair BC, Markham P, Nel A, Sturzl M, Ensoli B (1999). Inflammatory cytokines synergize with the HIV-1 Tat protein to promote angiogenesis and Kaposi's sarcoma via induction of basic fibroblast growth factor and the alpha v beta 3 integrin. *J Immunol* **163:** 1929–1935.

Benyoucef S, Hober D, De Groote D, De La Tribonniere X, Vilain V, Lion G, Bouzidi A, Wattre P (1997). An interferon-gamma (IFN-gamma) based whole blood assay to detect T cell response to antigens in HIV-1 infected patients. *Pathol Biol (Paris)* **5:** 400–403.

Bussolino F, Mitola S, Serini G, Barillari G, Ensoli B (2001). Interactions between endothelial cells and HIV-1. *Int J Biochem Cell Biol* **33:** 371–390.

Claudio L, Martiney JA, Brosnan CF (1994). Ultrastructural studies of the blood-retina barrier after exposure to interleukin-1 beta or tumor necrosis factor-alpha. *Lab Invest* **70:** 850–861.

Corasaniti MT, Bagetta G, Rotiroti D, Nistico G (1998). The HIV envelope protein gp120 in the nervous system: interactions with nitric oxide, interleukin-1beta and nerve growth factor signalling, with pathological implications in vivo and in vitro. *Biochem Pharmacol* **56:** 153–156.

Dallasta LM, Pisarov LA, Esplen JE, Werley JV, Moses AV, Nelson JA, Achim CL (1999). Blood-brain barrier tight junction disruption in human immunodeficiency virus-1 encephalitis. *Am J Pathol* **155:** 1915–1927.

Del Sorbo L, Arese M, Giraudo E, Tizzani M, Biancone L, Bussolino F, Camussi G (2001). Tat-induced platelet-activating factor synthesis contributes to the angiogenic effect of HIV-1 Tat. *Eur J Immunol* **31:** 376–383.

De Vries HE, Blom-Roosemalen MC, van Oosten M, de Boer AG, van Berkel TJ, Breimer DD, Kuiper J (1996). The influence of cytokines on the integrity of the blood-brain barrier in vitro. *J Neuroimmunol* **64:** 37–43.

Dhawan S, Puri RK, Kumar A, Duplan H, Masson JM, Aggarwal BB (1997). Human immunodeficiency virus-1-tat protein induces the cell surface expression of endothelial leukocyte adhesion molecule-1, vascular cell adhesion molecule-1, and intercellular adhesion molecule-1 in human endothelial cells. *Blood* **90:** 1535–1544.

Dougher-Vermazen M, Hulmes JD, Bohlen P, Terman BI (1994). Biological activity and phosphorylation sites of the bacterially expressed cytosolic domain of the KDR VEGF-receptor. *Biochem Biophys Res Commun* **205:** 728–738.

Ensoli B, Barillari G, Salahuddin SZ, Gallo RC, Wong-Staal F (1990). Tat protein of HIV-1 stimulates growth of cells derived from Kaposi's sarcoma lesions of AIDS patients. *Nature* **345:** 84–86.

Fiorelli V, Barillari G, Toschi E, Sgadari C, Monini P, Sturzl M, Ensoli B (1999). IFN-gamma induces endothelial cells to proliferate and to invade the extracellular matrix in response to the HIV-1 Tat protein: implications for AIDS-Kaposi's sarcoma pathogenesis. *J Immunol* **162:** 1165–1170.

Fiorelli V, Gendelman R, Samaniego F, Markham PD, Ensoli B (1995). Cytokines from activated T cells induce normal endothelial cells to acquire the phenotypic and functional features of AIDS-Kaposi's sarcoma spindle cells. *J Clin Invest* **95:** 1723–1734.

Fiorelli V, Gendelmann R, Sirianni MC, Chang HK, Colombini S, Markham PD, Monini P, Sonnabend J, Pintus A, Gallo RC, Ensoli B (1998). gamma-Interferon produced by CD8+ T cells infiltrating Kaposi's sarcoma induces spindle cells with angiogenic phenotype and synergy with human immunodeficiency virus-1 Tat protein: an immune response to human herpesvirus-8 infection? *Blood* **91:** 956–967.

Ganju RK, Munshi N, Nair BC, Liu ZY, Gill P, Groopman JE (1998). Human immunodeficiency virus tat modulates the Flk-1/KDR receptor, mitogen-activated protein kinases, and components of focal adhesion in Kaposi's sarcoma cells. *J Virol* **2:** 6131–6137.

Gavriil ES, Cooney R, Weeks BS (2002). Tat mediates apoptosis in vivo in the rat central nervous system. *Biochem Biophys Res Commun* **67:** 252–256.

Glass JD, Johnson RT (1996). Human immunodeficiency virus and the brain. *Annu Rev Neurosci* **19:** 1–26.

Graziosi C, Gantt KR, Vaccarezza M, Demarest JF, Daucher M, Saag MS, Shaw GM, Quinn TC, Cohen OJ, Welbon CC, Pantaleo G, Fauci AS (1996). Kinetics of cytokine expression during primary human immunodeficiency virus type 1 infection. *Proc Natl Acad Sci U S A* **93:** 4386–4391.

Hofman FM, Chen P, Incardona F, Zidovetzki R, Hinton DR (1999). HIV-1 tat protein induces the production of interleukin-8 by human brain-derived endothelial cells. *J Neuroimmunol* **94:** 28–39.

Hofman FM, Wright AD, Dohadwala MM, Wong-Staal F, Walker SM (1993). Exogenous tat protein activates human endothelial cells. *Blood* **82:** 2774–2780.

Ivankovic-Dikic I, Gronroos E, Blaukat A, Barth BU, Dikic I (2000). Pyk2 and FAK regulate neurite outgrowth induced by growth factors and integrins. *Nat Cell Biol* **2:** 574–581.

Janssen RS (1997). *AIDS and the Nervous System*, 2nd ed. Philadelphia: Lippincott-Raven, pp 13–37.

Jeang KT, Xiao H, Rich EA (1999). Multifaceted activities of the HIV-1 transactivator of transcription, Tat. *J Biol Chem* **274:** 28837–28840.

Jia H, Lohr M, Jezequel S, Davis D, Shaikh S, Selwood D, Zachary I (2001). Cysteine-rich and basic domain HIV-1 Tat peptides inhibit angiogenesis and induce endothelial cell apoptosis. *Biochem Biophys Res Commun* **283:** 469–479.

Jones KA (1993). Tat and the HIV-1 promoter. *Curr Opin Cell Biol* **5:** 461–468.

Khan NA, Jarroll EL, Panjwani N, Cao Z, Paget TA (2000). Proteases as markers for differentiation of pathogenic and nonpathogenic species of *Acanthamoeba*. *J Clin Microbiol* **38:** 2858–2861.

Kolb SA, Sporer B, Lahrtz F, Koedel U, Pfister HW, Fontana A (1999). Identification of a T cell chemotactic factor in the cerebrospinal fluid of HIV-1-infected individuals as interferon-gamma inducible protein 10. *J Neuroimmunol* **93:** 172–181.

Koppel BS (1998). *AIDS and Other Manifestations of HIV Infection*. Philadelphia: Lippincott-Raven, pp 431–474.

Kruman II, Nath A, Mattson MP (1998). HIV-1 protein Tat induces apoptosis of hippocampal neurons by a mechanism involving caspase activation, calcium overload, and oxidative stress. *Exp Neurol* **154:** 276–288.

Lafrenie RM, Wahl LM, Epstein JS, Yamada KM, Dhawan S (1997). Activation of monocytes by HIV-Tat treatment is mediated by cytokine expression. *J Immunol* **159:** 4077–4083.

Lahdevirta J, Maury CPJ, Teppo AM, Repo H (1988). Elevated levels of circulating cachectin/tumor necrosis factor in patients with acquired immunodeficiency syndrome. *Am J Med* **85:** 289–291.

Ma M, Nath A (1997). Molecular determinants for cellular uptake of Tat protein of human immunodeficiency virus type 1 in brain cells. *J Virol* **71:** 2495–2499.

Magnuson DS, Knudsen BE, Geiger JD, Brownstone RM, Nath A (1995). Human immunodeficiency virus type 1 tat activates non-*N*-methyl-D-aspartate excitatory amino acid receptors and causes neurotoxicity. *Ann Neurol* **37:** 373–380.

Marshall DW, Brey RL, Butzin CA, Lucey DR, Abbadessa SM, Boswell RN (1991). CSF changes in a longitudinal study of 124 neurologically normal HIV-1-infected U.S. Air Force personnel. *J Acquir Immun Defic Syndr* **4:** 777–781.

McArthur JC, Nance-Sproson TE, Griffin DE, Hoover D, Selnes OA, Miller EN, Margolick JB, Cohen BA, Farzadegan H, Saah A (1992). The diagnostic utility of elevation in cerebrospinal fluid beta 2-microglobulin in HIV-1 dementia. Multicenter AIDS Cohort Study. *Neurology* **42:** 1707–1712.

Mitola S, Soldi R, Zanon I, Barra L, Gutierrez MI, Berkhout B, Giacca M, Bussolino F (2000). Identification of specific molecular structures of human immunodeficiency virus type 1 Tat relevant for its biological effects on vascular endothelial cells. *J Virol* **74:** 344–353.

Morini M, Benelli R, Giunciuglio D, Carlone S, Arena G, Noonan DM, Albini A (2000). Kaposi's sarcoma cells of different etiologic origins respond to HIV-Tat through the Flk-1/KDR (VEGFR-2): relevance in AIDS-KS pathology. *Biochem Biophys Res Commun* **273:** 267–271.

New DR, Ma M, Epstein LG, Nath A, Gelbard HA (1997). Human immunodeficiency virus type 1 Tat protein induces death by apoptosis in primary human neuron cultures. *J NeuroVirol* **3:** 168–173.

Patki AH, Lederman MM (1996). HIV-1 Tat protein and its inhibitor Ro 24-7429 inhibit lymphocyte proliferation and induce apoptosis in peripheral blood mononuclear cells from healthy donors. *Cell Immunol* **169:** 40–46.

Persidsky Y, Stins M, Way D, Witte MH, Weinand M, Kim KS, Bock P, Gendelman HE, Fiala M (1997). A model for monocyte migration through the blood-brain barrier during HIV-1 encephalitis. *J Immunol* **158:** 3499–3510.

Petito CK, Cash KS (1992). Blood-brain barrier abnormalities in the acquired immunodeficiency syndrome: immunohistochemical localization of serum proteins in postmortem brain. *Ann Neurol* **32:** 658–666.

Philippon V, Vellutini C, Gambarelli D, Harkiss G, Arbuthnott G, Metzger D, Roubin R, Filippi P (1994). The basic domain of the lentiviral Tat protein is responsible for damages in mouse brain: involvement of cytokines. *Virology* **205:** 519–529.

Power C, Kong PA, Crawford CO, Wesselingh S, Glass JD, McArthur JC, Trapp BD (1993). Cerebral white matter changes in acquired immunodeficiency syndrome dementia: alterations of the blood-brain barrier. *Ann Neurol* **34:** 339–350.

Power C, McArthur JC, Johnson RT, Griffin DE, Glass JD, Dewey R, Chesebro B (1995b). Distinct HIV-1 env sequences are associated with neurotropism and neurovirulence. *Curr Top Microbiol Immunol* **202:** 89–104.

Qi JH, Claesson-Welsh L (2001). VEGF-induced activation of phosphoinositide 3-kinase is dependent on focal adhesion kinase. *Exp Cell Res* **263:** 173–182.

Rappaport J, Joseph J, Croul S, Alexander G, Del Valle L, Amini S, Khalili K (1999). Molecular pathway involved in HIV-1-induced CNS pathology: role of viral regulatory protein, Tat. *J Leukoc Biol* **65:** 458–465.

Reddy MA, Prasadarao NV, Wass CA, Kim KS (2000a). Phosphatidylinositol 3-kinase activation and interaction with focal adhesion kinase in *Escherichia coli* K1 invasion of human brain microvascular endothelial cells. *J Biol Chem* **275:** 36769–36774.

Reddy MA, Wass CA, Kim KS, Schlaepfer DD, Prasadarao NV (2000b). Involvement of focal adhesion kinase in *Escherichia coli* invasion of human brain microvascular endothelial cells. *Infect Immun* **68:** 6423–6430.

Rhodes RH (1991). Evidence of serum-protein leakage across the blood-brain barrier in the acquired immunodeficiency syndrome. *J Neuropathol Exp Neurol* **50:** 171–183.

Sporer B, Paul R, Koedel U, Grimm R, Wick M, Goebel FD, Pfister HW (1998). Presence of matrix metalloproteinase-9 activity in the cerebrospinal fluid of human immunodeficiency virus-infected patients. *J Infect Dis* **178:** 854–857.

Stins MF, Gilles F, Kim KS (1997). Selective expression of adhesion molecules on human brain microvascular endothelial cells. *J Neuroimmunol* **76:** 81–90.

Stins MF, Shen Y, Huang SH, Gilles F, Kalra VK, Kim KS (2001). Gp120 activates children's brain endothelial cells via CD4. *J NeuroVirol* **7:** 125–134.

Thakker GD, Hajjar DP, Muller WA, Rosengart TK (1999). The role of phosphatidylinositol 3-kinase in vascular endothelial growth factor signaling. *J Biol Chem* **274:** 10002–10007.

Toneatto S, Finco O, van der Putten H, Abrignani S, Annunziata P (1999). Evidence of blood-brain barrier alteration and activation in HIV-1 gp120 transgenic mice. *AIDS* **13:** 2343–2348.

Tran Dinh YR, Mamo H, Cervoni J, Caulin C, Saimot AC (1990). Disturbances in the cerebral perfusion of human immune deficiency virus-1 seropositive asymptomatic subjects: a quantitative tomography study of 18 cases. *J Nucl Med* **31:** 1601–1607.

Vogel BE, Lee SJ, Hildebrand A, Craig W, Pierschbacher MD, Wong-Staal F, Ruoslahti E (1993). A novel integrin specificity exemplified by binding of the alpha v beta 5 integrin to the basic domain of the HIV Tat protein and vitronectin. *J Cell Biol* **121:** 461–468.

Westendorp MO, Frank R, Ochsenbauer C, Stricker K, Dhein J, Walczak H, Debatin KM, Krammer PH (1995). Sensitization of T cells to CD95-mediated apoptosis by HIV-1 Tat and gp120. *Nature* **375:** 497–500.

Zauli G, La Placa M, Vignoli M, Re MC, Gibellini D, Furlini G, Milani D, Marchisio M, Mazzoni M, Capitani S (1995). An autocrine loop of HIV type-1 Tat protein responsible for the improved survival/proliferation capacity of permanently Tat-transfected cells and required for optimal HIV-1 LTR transactivating activity. *AIDS* **10:** 306–316.

Zhang GW, Khan NA, Kim KJ, Stins MF, Kim KS (2002). TGF-α increases *Escherichia coli* K1 adherence, invasion and transcytosis in human brain microvascular endothelial cells. *Cell Tissue Res* **309:** 281–286.