Extracellular HIV-1 Tat enhances monocyte adhesion by up-regulation of ICAM-1 and VCAM-1 gene expression *via* ROS-dependent NF- κ B activation in astrocytes

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Abbreviations: ADC, AIDS dementia complex; ICAM-1, intercellular adhesion molecule-1; ROS, reactive oxygen species; Tat, trans-activator of transcription; VCAM-1, vascular cell adhesion molecule-1

Abstract

One of characteristic features of AIDS-related encephalitis and dementia is the infiltration of monocytes into the CNS. HIV-1 Tat was demonstrated to facilitate monocyte entry into the CNS. In this study, we examined the effect of HIV-1 Tat on the expression of adhesion molecules, generation of reactive oxygen species (ROS) and NF- κ B activation in CRT-MG human astrogloma cells. Treatment of CRT-MG cells with HIV-1 Tat protein significantly increased protein and mRNA levels of ICAM-1 and VCAM-1, as measured by Western blot analysis and RT-PCR, indicating that Tat increases these protein levels at an mRNA level. In addition, Tat induced the activation of NF- κ B in astrocytes. Treatment of CRT-MG with NF- κ B inhibitors led to decrease in Tat-induced protein and mRNA expression of ICAM-1 and VCAM-1. Furthermore, HIV-1 Tat protein increased ROS generation. Inhibition of Tat-induced ROS generation by *N*-acetyl cysteine, vitamin C and diphenyl iodonium suppressed Tat-induced NF- κ B activation, ICAM-1 and VCAM-1 expression, and monocyte adhesion in CRT-MG. These data indicate that HIV-1 Tat can modulate monocyte adhesiveness by in-

creasing expression of adhesion molecules such as ICAM-1 and VCAM-1 *via* ROS- and NF- κ B-dependent mechanisms in astrocytes.

Keywords: astrocytes; cell adhesion molecules; inflammation; intercellular adhesion molecule-1; NF- κ B; reactive oxygen species; Tat protein, HIV-1; vascular cell adhesion molecule-1

Introduction

The most common neurological disorder associated with HIV infection is AIDS dementia complex (ADC) (Ozdener, 2005; Rumbaugh and Nath, 2006). Infiltration of monocytes into the CNS is an important step in the development of ADC. Although the precise mechanism by which monocytes infiltrate into the CNS during HIV disease progression is not yet understood, it is considered that abnormal expression of cell adhesion molecules and cytokine dysregulation facilitate the entry of the monocytes into the CNS (Weiss *et al.*, 1999; Pu *et al.*, 2003; D'Aversa *et al.*, 2004). Blood-brain barrier (BBB) endothelial cells express adhesion molecules which mediate entry of monocyte into the CNS. Astrocytes, the major glial cells in the CNS and also one of major components of BBB, play an important role in leukocyte infiltration, acting as a source of chemokines, cytokines and adhesion molecules (for review, see Dong and Benveniste, 2001). When stimulated by pro-inflammatory cytokines (e.g., IL-1 and TNF- α), astrocytes can express the increased level of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), which can contribute to inflammatory events within the CNS.

The HIV-1 Tat (transactivator of transcription) protein is one of the viral gene products essential for the expression and replication of the viral genome (Morrow *et al.*, 1994). Previous study demonstrated that Tat mRNA and protein were present in the brains of patients with ADC (Wiley *et al.*, 1996; Hudson *et al.*, 2000). This Tat protein, which can be secreted from the infected cells, has the ability to enter uninfected cells and exert its activity upon the responsive genes (Ensoli *et al.*, 1993; Reviewed in Ref. Watson and Edwards, 1999). Therefore, HIV-1

Tat is considered as one of major regulators involved in HIV-associated pathologies including ADC. Previous studies have shown that HIV-1 Tat exerts its activity on the expression of various adhesion molecules including VCAM-1, endothelial leukocyte adhesion molecule-1 (ELAM-1) and ICAM-1 in human endothelial cells (Dhawan *et al.*, 1997). We also previously reported that HIV-1 Tat protein induced VCAM-1 expression in human umbilical vein endothelial cells (HUVECs) (Park *et al.*, 2000). In addition, HIV-1 Tat has been shown to induce expression of adhesion molecules such as VCAM-1 and ICAM-1 in astrocytes and increase monocyte binding to astrocytes (Woodman *et al.*, 1999). Previous observations indicated that astrocytic adhesion molecules were increased in HIV-1-associated cognitive/motor complex (Seilhean *et al.*, 1997). Therefore, HIV-1 Tat may be an important regulator in the trafficking of monocytes into the CNS which may contribute to the development of HIV-1-associated dementia (Rappaport *et al.*, 1999).

Oxidative stress has been implicated in many aspects of HIV disease pathogenesis, including the onset of dementia (Mollace *et al.*, 2001; Pocernich *et al.*, 2005). Involvement of oxidative stress in the development of ADC was suggested by demonstrating the presence of increased protein and lipid peroxidation in the brain and cerebrospinal fluid compared to HIV non-demented patients. HIV-1 Tat protein has been shown to induce oxidative stress directly and indirectly. Previous results demonstrated that the injection of HIV-1 Tat protein in the rat striatum induced increased oxidative modification of proteins in the brain (Aksenov *et al.*, 2001). These data indicate that increased levels of reactive oxygen species (ROS) in the brain can induce neuronal damage that leads to neurodegeneration. ROS can also act as second messengers in the intracellular signaling pathways involved in expression of many cellular genes such as matrix metalloproteinases (Schulze-Osthoff *et al.*, 1997; Brenneisen *et al.*, 2002).

Further understanding of the role of ROS generation in Tat mediated infiltration of monocyte into the CNS might help to develop a therapeutic tool for ADC. While it has been reported that Tat may generate ROS, the possible roles of ROS involved in Tat-induced ICAM-1 expression have not been studied. In this study, we further examined the molecular mechanism responsible for HIV-1 Tat-induced expression of adhesion molecules such as ICAM-1 and VCAM-1, and subsequent monocyte adhesion by astrocyte. We show that HIV-1 Tat increased the intracellular level of ROS which is involved in the translocation of NF- κ B and subsequent expression of adhesion molecules and monocyte adhesion in human astrocytes.

Materials and Methods

Cell culture

CRT-MG human astroglial cells and THP-1 human monocytic cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated FBS, penicillin G (100 U/ml), streptomycin (100 μ g/ml) and L-glutamine (2 mM) at 37°C in a humidified atmosphere containing 5% CO₂ and 95% air (Choi *et al.*, 2002; Park *et al.*, 2003).

Reagents

N- α -p-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK), diphenyl iodonium (DPI), vitamin C (L-ascorbic acid) and 2',7'-dichlorofluorescein diacetate (DCF-DA) were obtained from Sigma (St. Louis, MO). N-Acetyl-L-cysteine (NAC) and BAY 11-7082 were purchased from Calbiochem (La Jolla, CA). Calcein acetoxymethyl ester (calcein-AM) was purchased from Molecular Probe (Eugene, OR). Primary antibodies to ICAM-1, VCAM-1, actin and p65 were supplied by Santa Cruz (Santa Cruz, CA). HRP-conjugated anti-rabbit or goat antibodies were supplied by Sigma (St. Louis, MO).

Purification of recombinant HIV-1 Tat protein

Recombinant protein containing an 86 amino acids of HIV-1 Tat at the C terminus and six histidine residues at the N-terminus was purified by Ni²⁺ affinity chromatography under native conditions as described previously (Park *et al.*, 2000). The Tat preparation eluted from column was subjected to a Detoxi-Gel Endotoxin Removing Gel (Pierce, Rockford, IL) to remove endotoxin. The purified Tat protein dissolved in 7 mM DTT and 20% glycerol was aliquoted and stored at -80°C. The protein concentration was determined with Bradford protein assay (Bio-Rad) by using BSA as a standard. The integrity and purity of the HIV-1 Tat proteins were assessed by SDS-PAGE followed by Coomassie blue staining. The biological activity of Tat was confirmed by a transactivation assay in HeLa cells transfected with a plasmid containing an HIV long terminal repeat (LTR)-luciferase gene. Endotoxin levels for the Tat preparation were below the detection limit (<0.1 EU/ml) as tested by Limulus Amoebocyte Lysate assay (BioWhittaker, Walkersville, MD).

Western blot analysis

Cell lysates were prepared by lysing monolayer cells on a six-well plate with a lysis buffer containing 125 mM Tris-HCl, pH 6.8, 2% SDS, 10% v/v glycerol. Fifteen micrograms of the protein from each whole

cell lysate were fractionated by electrophoresis on 10% SDS-polyacrylamide gel. Proteins were electrotransferred to a nitrocellulose membrane, which was then blocked with 10% (w/v) dry milk in PBS. The membrane was probed with rabbit anti-VCAM-1 polyclonal antibody or rabbit anti-ICAM-1 polyclonal antibody diluted 1:1,000, followed by incubation with horseradish peroxidase-conjugated secondary antibodies diluted 1:10,000. The bound antibodies were visualized by enhanced chemiluminescence (ECL; Amersham) as recommended by the manufacturer. The same membrane was stripped and reprobed with an anti-actin antibody (internal control).

Reverse transcription-PCR analysis

Total RNA was isolated from CRT-MG cells using a Trizol reagent kit (Invitrogen, Gaithersburg, MD) according to the manufacturer's instructions (Zhang *et al.*, 2005). The RNA (2 µg) was reversibly transcribed with 10,000 U of reverse transcriptase and 0.5 µg/µl oligo-(dT)15 primer (Promega, Madison, WI). PCR amplification of cDNA aliquots was performed with the following sense and antisense primers (5' → 3'): VCAM-1 sense, ATGCCTGGGAAG-ATGGTCGTGA; VCAM-1 antisense, TGGAGCTGG-TAGACCCTCGTG; ICAM-1 sense, GGTGACGCT-GAATGGGGTTCC; ICAM-1 antisense, GTCCTC-ATGGTGGGGCTATGACTC; beta-actin sense, GCG-GGAAATCGTGCGTGACATT; and beta-actin antisense, GATGGAGTTGAAGGTAGTTTCGTG. PCR was performed in 50 µl of 10 mmol/l Tris-HCl (pH 8.3), 25 mmol/l MgCl₂, 10 mmol/l dNTP, 100 U of *Taq* DNA polymerase, and 0.1 µmol/l of each primer and was terminated by heating at 70°C for 15 min. PCR products were resolved on a 1% agarose gel and visualized with UV light after ethidium bromide.

Measurement of intracellular ROS levels

Intracellular ROS levels were determined using DCF-DA, which is converted by ROS into fluorescent DCF. CRT-MG cells (1×10^5 cells) were maintained in serum free media for 16 h, and then treated with HIV-1 Tat protein (100 ng/ml to 1 µg/ml) for varying time periods (5 min to 24 h). To investigate the effect of NAC, DPI and vitamin C on HIV-1 Tat-induced ROS production, CRT-MG cells were incubated in the absence or presence of these inhibitors for 1 h, and then treated with HIV-1 Tat protein (500 ng/ml) for 15 min. Cells were washed twice with PBS and incubated with DCF-DA (10 µM) for 30 min. The cellular fluorescent images were obtained using a Zeiss Axiovert S100 microscope with a confocal laser-scanning system (Bio-Rad MRC-1024ES) by using laser excitation at 494 nm as previously described (Park *et al.*, 2004).

NF-κB protein localization

The NF-κB p65 nuclear localization was detected by indirect immunofluorescence assay using confocal microscopy as described previously (Kim *et al.*, 2003). Briefly, CRT-MG cells grown on a glass slide in a 12-well plate were treated with HIV-1 Tat for 1 h. After being washed in PBS, the cells were fixed with 4% ice-cold formaldehyde for 30 min and permeabilized with 0.2% Triton X-100. Rabbit polyclonal antibody to human NF-κB p65 (1:500) was applied for 1 h followed by incubation with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (1:500) for 1 h. The cells were transferred to a chamber on the stage of a Zeiss Axiovert S100 microscope and observed with a confocal laser-scanning system (Bio-Rad MRC-1024ES). The fluorescence images (excitation 494 nm/emission 518 nm) of the cells were recorded every 0.25 s ($\times 640$).

Nuclear extract preparation

Nuclear protein extracts were prepared using a detergent lysis procedure from CRT-MG cells to assay the nuclear translocation of NF-κB. Cells were lysed in a buffer of 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF and 6.25% Nonidet P-40 (NP-40) on ice for 10 min. Proteins were extracted from nucleus pellets by

Figure 1. Induction of VCAM-1 and ICAM-1 protein expression in CRT-MG cells by HIV-1 Tat protein. CRT-MG cells were treated with 500 ng/ml HIV-1 Tat protein for varying times (A) and various concentration of HIV-1 Tat protein for 12 h (B). Cell extracts were prepared and analyzed for VCAM-1 and ICAM-1 protein expression. Actin protein was used as an internal control. TNF-α (10 ng/ml for 12 h) was used as a positive control.

incubation with a high-salt buffer containing 0.4 M NaCl, 20 mM HEPES (pH 7.9), 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 1% NP-40 with vigorous shaking. The nuclear debris was pelleted by a brief centrifugation (13,000 rpm for 10 min) and the supernatant was stored at -70°C. For the determination of NF- κ B localization, Western blot analysis was carried out with nuclear protein extracts using anti-human NF- κ B primary antibody (1:1,000). The Western blot analytical procedures were described above.

Electrophoretic mobility shift assay (EMSA)

CRT-MG cells were treated with HIV-1 Tat (500 ng/ml) for 1 h, then nuclear extracts of CRT-MG cells were prepared and analyzed for NF- κ B binding activity by EMSA as described previously (Park *et*

al., 2003; 2004). An NF- κ B consensus oligonucleotide (Promega) was used in the EMSA. The complementary oligonucleotide was annealed and end-labeled with [α - 32 P]ATP using T4 polynucleotide kinase. EMSA was performed in a total volume of 20 μ l at 4°C. Five micrograms of nuclear extracts were equilibrated for 15 min in binding buffer (10 mM Tris-HCl, pH 8.0, 75 mM KCl, 2.5 mM MgCl₂, 0.1 mM EDTA, 10% glycerol, 0.25 mM dithiothreitol) and 1 μ g of poly dI/dC. 32 P-labeled oligonucleotide probe (20,000 cpm) was then added and the reaction was incubated on ice for an additional 20 min. Bound and free DNA were then resolved by electrophoresis on a 6% native polyacrylamide gel in TBE buffer (89 mM Tris-HCl, 89 mM boric acid, and 2 mM EDTA).

Figure 2. Induction of monocyte adhesion to CRT-MG cells by HIV-1 Tat protein. (A) time- and (B) dose-dependency of monocytes adhesion to CRT-MG cells induced by HIV-1 Tat protein. CRT-MG cells were treated with 500 ng/ml HIV-1 Tat protein for various times and various concentrations of HIV-1 Tat protein for 12 h. CRT-MG cells were cocultured with calcein-AM-labeled THP-1 monocytes. Microphotographs were obtained using fluorescence microscopy (original magnification, $\times 40$). (C) The calcein-AM fluorescent intensity was measured by an ELISA plate reader. Values are means $\pm$ SEM, $n = 3$. TNF- α (10 ng/ml for 12 h) was used as positive control.

Cell adhesion assay

Adherence of THP-1 cells to CRT-MG cells was assayed using a cell-cell adhesion assay as described elsewhere (Choi *et al.*, 2004). Briefly, CRT-MG cells were cultured at a density of 7.0×10^4 cells on a 24-well glass chamber slide. CRT-MG cells were pre-treated with the various antioxidants (20 mM NAC, 10 μ M DPI, 10 μ M vitamin C) and NF- κ B inhibitors (100 μ M TLCK, 1 μ M BAY 11-7082) for 1 h and followed by exposure to HIV-1 Tat protein (500 ng/ml) for 12 h. CRT-MG cells were washed three times with PBS before cell-cell adhesion assay. THP-1 cells were labeled for 30 min with 5 μ mol/l calcein-AM (Molecular Probes, Inc.). The labeled THP-1 (7.0×10^5) cells were co-cultured with CRT-MG cells for 1 h. Co-cultured cells were washed three times with PBS. The fluorescence images were obtained at 485 nm excitation and 538 nm emission using a SPOT II digital camera-attached fluorescence microscope with Spot II data acquisition software (Diagnostic Instrument). For the adhesion quantification, the calcein-AM fluorescent intensity was measured at 485 nm excitation and 538 nm emission by a Fluoroskan ELISA plate reader (Labsystems Oy).

Results

HIV-1 Tat induces ICAM-1 and VCAM-1 expression and increases monocyte adhesion to astrocytes

HIV-1 Tat has been shown to induce expression of ICAM-1 and VCAM-1 in human astrocytes (Woodman *et al.*, 1999), suggesting the possible role of HIV-1 Tat in infiltration of monocytes into the CNS, which is important in HIV pathogenesis. To better understand the molecular mechanisms underlying in HIV-1 Tat-induced ICAM-1 and VCAM-1 expression, we analyzed HIV-1 Tat induction of ICAM-1 and VCAM-1 expression in the human astrocytic cell line CRT-MG. HIV-1 Tat induced ICAM-1 and VCAM-1 expression in time- and dose-dependent manners (Figure 1A and B). Next, we examined the effect of HIV-1 Tat on monocyte adhesion to astrocytes. CRT-MG cells were treated with HIV-1 Tat, and then analyzed for monocyte adhesion to astrocytes. As shown in Figure 2, adhesion of monocytes to CRT-MG cells was significantly increased following HIV-1 Tat treatment of CRT-MG cells in time- and dose-dependent manners (Figure 2).

HIV-1 Tat activates transcription of ICAM-1 and VCAM-1 gene in astrocytes

To examine whether HIV-1 Tat activates transcription of ICAM-1 and VCAM-1 in CRT-MG cells, we ana-

lyzed the induction of ICAM-1 and VCAM-1 mRNA expression in Tat-treated CRT-MG cells (Figure 3). Cells were treated with HIV-1 Tat and mRNA levels of ICAM-1 and VCAM-1 were measured by RT-PCR. As shown in Figure 3, time- and dose-dependent increase in levels of ICAM-1 and VCAM-1 mRNA was observed in CRT-MG cells following HIV-1 Tat treatment.

NF- κ B is responsible for induction of ICAM-1 and VCAM-1 expression by HIV-1 Tat in human astrocyte cells

ICAM-1 and VCAM-1 expression is regulated by different transcription factors including NF- κ B in astrocytes (Lee *et al.*, 1998). Previous studies demonstrated that extracellular HIV-1 Tat protein is associated with an increase in NF- κ B binding activity in primary human astrocytes (Conant *et al.*, 1996). To examine the involvement of NF- κ B in ICAM-1 and VCAM-1 expression and monocyte adhesion to astrocytes, CRT-MG cells were treated with HIV-1 Tat, and the translocation of NF- κ B was monitored by indirect immunofluorescence. Nuclear accumulation of NF- κ B p65 in HIV-1 Tat treated CRT-MG cells was strongly induced (Figure 4). Pharmacological NF- κ B

Figure 3. Induction of VCAM-1 and ICAM-1 gene expression in CRT-MG by HIV-1 Tat protein. CRT-MG cells were treated with 500 ng/ml HIV-1 Tat protein for various times (A) and various concentrations of HIV-1 Tat protein for 8 h (B). Total RNA was extracted. VCAM-1, ICAM-1 and β -actin mRNA were quantitated by RT-PCR using specific primers. RT-PCR products were analyzed by 1% agarose gel electrophoresis. TNF- α (10 ng/ml for 6 h) was used as positive control.

Figure 4. NF- κ B dependency of adhesion molecules (VCAM-1 and ICAM-1) expression and monocyte adhesion in CRT-MG cells induced by HIV-1 Tat protein. (A) CRT-MG cells were treated with HIV-1 Tat protein (500 ng/ml) for 1 h with or without pretreatment with NF- κ B inhibitors (100 μ M TLCK, 1 μ M BAY 11-7082) for 1 h. NF- κ B nuclear localization was visualized with a confocal microscopy by indirect immunofluorescence using anti-NF- κ B p65 antibody. TNF- α (10 ng/ml for 30 min) was used as positive control. (B) CRT-MG cells were treated with HIV-1 Tat protein (500 ng/ml) for 12 h with or without pretreatment with NF- κ B inhibitors (100 μ M TLCK, 1 μ M BAY 11-7082) for 1 h. Cell extracts were prepared and analyzed for VCAM-1 and ICAM-1 protein expression. (C) CRT-MG cells were treated with HIV-1 Tat protein (500 ng/ml) for 12 h with or without pretreatment with NF- κ B inhibitors (100 μ M TLCK, 1 μ M BAY 11-7082) for 1 h. CRT-MG cells were cocultured with calcein-AM-labeled THP-1 monocytes. Microphotographs were obtained using fluorescence microscopy (original magnification, $\times$ 40).

inhibitors, TLCK, and BAY 11-7082, prevented Tat-induced translocation of p65. In addition, pretreatment with TLCK, or BAY 11-7082 suppressed Tat-induced expression of ICAM-1 and VCAM-1 protein (Figure 4B) and monocyte adhesion to CRT-MG cells (Figure 4C). These results indicate that Tat-induced NF- κ B activity is necessary for inducing ICAM-1 and VCAM-1 expression in CRT-MG cells as well as monocyte adhesion to astrocytes.

Generation of ROS by HIV-1 Tat in astrocyte cells

Since NF- κ B is one of redox-dependent transcription factors (Sen and Packer, 1996; Flohe *et al.*, 1997),

ROS may be involved in Tat-induced increase of ICAM-1 and VCAM-1 expression by human astrocytes. We examined whether ROS can be generated in CRT-MG cells treated with HIV-1 Tat. After treatment with Tat for various time periods, CRT-MG cells were incubated with DCF-DA, which has been used as a general marker of intracellular ROS production (Park *et al.*, 2004). As shown in Figure 5A, a time-dependent increase in ROS levels was observed in CRT-MG. ROS production was detected as early as 5 min and was maximal at 15 min after treatment with Tat. ROS production was induced in a dose-dependent manner by CRT-MG cells exposed to HIV-1 Tat (100-1,000 ng/ml) (Figure 5B). To

Figure 5. Analysis of ROS generation in the CRT-MG cells exposed to HIV-1 Tat protein. CRT-MG cells were treated with 500 ng/ml HIV-1 Tat protein for various times (A) and various concentrations of HIV-1 Tat protein for 1 h (B). Intracellular ROS levels were measured after staining with DCF-DA. Visualization of the ROS of the treated cells was measured by confocal microscopy (original magnification, $\times 200$). To analyze the effect of NAC, DPI and vitamin C on HIV-1 Tat-induced ROS production, CRT-MG cells were pretreated with NAC (20 mM), DPI (10 μ M) or vitamin C (10 μ M) for 1 h, and incubated with HIV-1 Tat protein (500 ng/ml) for 1 h and then intracellular ROS were measured (C).

further characterize Tat-induced ROS production in CRT-MG cells, we utilized antioxidants, NAC, vitamin C and a flavoprotein inhibitor, DPI. DPI has been used widely to inhibit non-phagocytic NADPH oxidase (Cross and Jones, 1986; Doussiere and Vignais, 1992). Pretreatment with NAC, DPI and vitamin C inhibited Tat-induced increase of intracellular ROS levels in CRT-MG cells (Figure 5C). These results indicate that HIV-1 Tat induces generation of ROS in CRT-MG cells in time- and dose-dependent manners.

ROS are responsible for HIV-1 Tat-induced activation of NF- κ B and ICAM-1/VCAM-1 expression

Next, we examined the possible role of ROS in Tat-induced nuclear translocation and DNA binding ability of NF- κ B in CRT-MG cells. CRT-MG cells were incubated in the absence or presence of NAC, DPI or Vitamin C for 1 h, followed by exposure to HIV-1 Tat (500 ng/ml) for 1 h. Nuclear extracts were prepared and the nuclear translocation of NF- κ B was analyzed by fluorescence microscopy and Western blot technique. In addition, nuclear extracts were used for mobility shift assays with a radioactive

labelled NF- κ B probe. Tat-induced NF- κ B DNA binding complex composed of primarily both p50 and p65, as analyzed by supershift assay using antibodies against the NF- κ B subunits p50 and p65 (data not shown). As an indicator of NF- κ B activation, p65 subunit was chosen and analyzed in confocal microscopy and Western blot analysis. As shown in Figure 6, pretreatment with NAC, DPI and vitamin C suppressed Tat-induced nuclear translocation (Figure 6A and 6B) as well as DNA binding activity (Figure 6C) of NF- κ B. We further used these inhibitors to analyze the role of ROS on Tat-induced up-regulation of ICAM-1 and VCAM-1 expression in CRT-MG cells. Cells were incubated in the absence or presence of DPI or NAC for 1 h, treated with Tat (500 ng/ml), and then ICAM-1 and VCAM-1 expression was analyzed by RT-PCR and Western blot analysis. Pretreatment with DPI or NAC inhibited markedly ICAM-1 and VCAM-1 mRNA (Figure 7A) and protein (Figure 7B) expression.

ROS are responsible for Tat-induced monocyte adhesion to astrocytes

To further confirm the relevance between ROS

HIV-1 Tat has been found to induce abnormal expression of cell adhesion molecules and cytokine dysregulation, which are believed to initiate the inflammation that causes damage in/of the CNS in

patients with ADC (Rappaport *et al.*, 1999). Elevated levels of Tat mRNA and protein in the brains of patients with ADC implicate that HIV-1 Tat is involved in HIV-associated pathologies including ADC (Wiley *et al.*, 1996; Hudson *et al.*, 2000). Intracerebral injection of HIV-1 Tat protein in the rat and the mouse resulted in infiltration of inflammatory cells, reactive astrogliosis, and neurotoxicity (Rappaport *et al.*, 1999; Aksenov *et al.*, 2001), demonstrating direct role of HIV-1 Tat in the CNS damage. Direct and indirect neurotoxic activities of HIV-1 Tat have been demonstrated in various *in vivo* and *in vitro* studies (Pocernich *et al.*, 2005). Especially, up-regulation of expression of adhesion molecules such as ICAM-1 and VCAM-1 by Tat may induce a series of interactions between monocytes, endothelial cells, and astrocytes, resulting in enhanced infiltration of monocytes into the CNS (Rappaport *et al.*, 1999; Langford and Masliah, 2001). The molecular mechanism by which HIV-1 Tat induces expression of adhesion molecules such as ICAM-1 and VCAM-1 in

Figure 7. Effects of antioxidants on HIV-1 Tat-induced VCAM-1 and ICAM-1 expression in CRT-MG cells. **A:** CRT-MG cells were treated with 20 mM NAC, 10 μ M DPI and 10 μ M vitamin C for 1 h, then treated with HIV-1 Tat protein (500 ng/ml) for 8 h. Total RNA was extracted. VCAM-1, ICAM-1 and β -actin mRNA were analyzed by RT-PCR using specific primers. **B:** CRT-MG cells were treated with HIV-1 Tat protein (500 ng/ml) for 12 h with or without pretreatment with various antioxidants for 1 h. Cell extracts were prepared and analyzed for VCAM-1 and ICAM-1 protein expression.

astrocyte has not been fully understood. In this study, we demonstrate that ROS generated by HIV-1 Tat mediates activation of NF- κ B, up-regulation of ICAM-1 and VCAM-1 and subsequent increased monocyte adhesion to astrocytes. Pretreatment of CRT-MG cells with antioxidants inhibited significantly Tat-induced NF- κ B activation, ROS generation and subsequently expression of adhesion molecules, suggesting that prevention of ROS generation by Tat is one of strategies to control monocyte infiltration into the CNS.

Astrocytes can be induced by HIV-1 Tat as well as various proinflammatory cytokines such as TNF- α to express the adhesion molecules. Enhanced expression of adhesion molecules such as ICAM-1 on the surface of astrocytes is required for monocyte-astrocyte interaction which increases infiltration of monocytes into the CNS observed in the patients with HIV dementia (Wu *et al.*, 2000). Our results are consistent with previous report that HIV-1 Tat increased monocyte binding to astrocyte by affecting expression of adhesion molecules such as ICAM-1 and VCAM-1 in astrocytes (Woodman *et al.*, 1999; Pu *et al.*, 2003). ICAM-1 and VCAM-1 are regulated primarily at the level of gene transcription upon a variety of stimuli. We showed that HIV-1 Tat-induced increase in ICAM-1 expression is regulated at the transcriptional level in an NF- κ B-dependent path-

Figure 8. Inhibition of HIV-1 Tat-induced monocyte adhesion to CRT-MG by antioxidants. CRT-MG cells were treated with various antioxidants (20 mM NAC, 10 μ M DPI, 10 μ M vitamin C) for 1 h and followed by exposure to HIV-1 Tat protein (500 ng/ml) for 12 h. CRT-MG cells were co-cultured with calcein-AM-labeled THP-1 monocytes. **(A)** Microphotographs were obtained using fluorescence microscopy (original magnification, $\times 40$). **(B)** The calcein-AM fluorescent intensity was measured by an ELISA plate reader. Values are means $\pm$ SEM, $n = 3$.

way. NF- κ B inhibitors inhibited the expression of ICAM-1 and VCAM-1 gene and monocyte adhesion to astrocyte in response to HIV-1 Tat *via* suppressing NF- κ B activation (Figure 4), suggesting the functional relevance of Tat-mediated activation of NF- κ B. The molecular mechanism by which HIV-1 Tat induces NF- κ B activation which is responsible for adhesion molecule expression such as ICAM-1 and VCAM-1, and subsequent monocyte adhesion on astrocyte is not clear. Conant *et al.* demonstrated that extracellular HIV-1 Tat protein is associated with NF- κ B activation that is independent of new protein

synthesis in primary human astrocytes (1996), suggesting the involvement of the possible signaling pathways that lead to the NF- κ B activation.

We performed experiments to explore the mechanisms of NF- κ B activation by HIV-1 Tat. As shown in Figure 5, ROS generation was induced following HIV-1 Tat treatment of astrocytes. Previous results indicate that HIV-1 Tat induces cellular oxidative stress which potentiates TNF-induced NF- κ B activation (Westendorp *et al.*, 1995). ROS mediate many of the pathophysiological events that lead to development of several neurodegenerative disorders, including HIV-related dementia. HIV-1 Tat-induced NF- κ B activation was significantly inhibited in the cells treated with HIV-1 Tat in the presence of antioxidants (Figure 6), indicating that HIV-1 Tat-mediated ROS generation was associated with activation of NF- κ B in astrocytes. The functional role of ROS generation by Tat was demonstrated by our results showing that up-regulation of mRNA and protein expression of ICAM-1 and VCAM-1 diminished in the astrocytes treated with antioxidants. Furthermore, treatment of CRT-MG cells with antioxidants before exposure to HIV-1 Tat significantly inhibited monocyte adhesion to astrocyte. These results indicate that ROS is involved in the signal transduction pathway leading to activation of NF- κ B and to NF- κ B-activated ICAM-1 and VCAM-1 expression in astrocytes. The exact mechanism by which HIV-1 Tat induces the generation of ROS in astrocyte is not clear. As shown in Figure 5C, HIV-1 Tat-induced generation of ROS was inhibited by DPI, suggesting that a NADPH oxidase is involved in the generation of ROS by Tat. Because DPI is not specific for NADPH oxidase, we can not rule out the involvement of non-NADPH oxidases in HIV-1 Tat-induced ROS generation. Further study is required to define the exact source of ROS generation induced by HIV-1 Tat. In conclusion, understanding of the role of ROS in Tat-mediated adhesion molecule expression and subsequent increased adhesion of monocyte to astrocyte provides a rationale for development of antioxidant compounds that may be useful as therapeutic agents for Tat-induced ROS-associated neurotoxicity.

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