HIV-1 Tat protein-induced VCAM-1 expression in human pulmonary artery endothelial cells and its signaling

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HIV-1 Tat protein-activated VCAM-1 expression in human pulmonary artery endothelial cells and its signaling. Am J Physiol Lung Cell Mol Physiol 289: L252–L260, 2005. First published April 1, 2005; doi:10.1152/ajplung.00200.2004.—Expression of cell adhesion molecule in endothelial cells upon activation by human immunodeficiency virus (HIV) infection is associated with the development of atherosclerotic vasculopathy. We postulated that induction of vascular cell adhesion molecule-1 (VCAM-1) by HIV-1 Tat protein in endothelial cells might represent an early event that could culminate in inflammatory cell recruitment and vascular injury. We determined the role of HIV-1 Tat protein in VCAM-1 expression in human pulmonary artery endothelial cells (HPAEC). HIV-1 Tat protein treatment significantly increased cell-surface expression of VCAM-1 in HPAEC. Consistently, mRNA expression of VCAM-1 was also increased by HIV-1 Tat protein as measured by RT-PCR. HIV-1 Tat protein-induced VCAM-1 expression was abolished by the NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) and the p38 MAPK inhibitor SB-203580. Furthermore, HIV-1 Tat protein enhanced DNA binding activity of NF-κB, facilitated nuclear translocation of NF-κB subunit p65, and increased production of reactive oxygen species (ROS). Similarly to VCAM-1 expression, HIV-1 Tat protein-induced NF-κB activation and ROS generation were abrogated by PDTC and SB-203580. These data indicate that HIV-1 Tat protein is able to induce VCAM-1 expression in HPAEC, which may represent a pivotal early molecular event in HIV-induced vascular/pulmonary injury. These data also suggest that the molecular mechanism underlying the HIV-1 Tat protein-induced VCAM-1 expression may involve ROS generation, p38 MAPK activation, and NF-κB translocation, which are the characteristics of pulmonary endothelial cell activation.

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human immunodeficiency virus type 1; vascular cell adhesion molecule-1; reactive oxygen species; nuclear factor-κB; mitogen-activated protein kinase

Human immunodeficiency virus type 1 (HIV-1) infection and acquired immunodeficiency syndrome (AIDS) are pandemic and pose one of the greatest challenges to global public health. With effective antiretroviral therapy, HIV-1 infection has become a chronic disease, and the cardiovascular morbidity and mortality are becoming more prevalent and obvious in HIV-1-infected individuals (2, 5, 27, 28). HIV-1 infection has been associated with the development of endocarditis, myocarditis, dilated cardiomyopathy, coronary heart disease, pericardial effusion, pulmonary hypertension, and vasculopathy (2, 27). Recent reports on the appearance of premature atherosclerosis in young patients have raised serious concerns regarding the role of the virus and other factors in atherogenesis (5, 27, 28, 33). However, the mechanisms responsible for the development of atherosclerosis in AIDS remain unclear. Localized between blood and tissues, endothelium represents a dynamic barrier that regulates cellular trafficking and inflammation. Endothelial dysfunction and/or injury are pivotal to the development of cardiovascular and inflammatory pathology. HIV-1 infection causes vascular disorders characterized by an evident activation and perturbation of endothelial cells. This endothelial dysfunction and/or injury have been well described (7, 9). The involvement of endothelial cells in AIDS pathogenesis may be propelled by HIV-1 infection-induced viral proteins or pathogens that cause opportunistic infections resulting in altered endothelial cell behavior.

HIV-1 infection is linked with endothelial activation, further atherosclerotic vasculopathy, an inflammatory disorder mediated by adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1), and by recruited mononuclear cells. We postulated that a secreted HIV-associated protein, transactivator of viral replication or Tat, may play a crucial role in endothelial activation, adhesion molecule induction, monocyte recruitment, and subsequent inflammatory events regulated by endothelium (7). HIV-1 Tat protein is a transcriptional activator of viral gene expression produced early after virus infection and plays an essential role in viral replication. HIV-1 Tat protein upregulates viral gene expression in infected cells by increasing the rates of transcriptional initiation and elongation by DNA polymerase II (1, 23). The protein is composed of 86–104 amino acids (according to viral isolation) encoded by two exons. The product of the first exon possesses full trans-activating activity (16). HIV-1 Tat protein can be secreted from infected cells and circulates in the bloodstream of infected individuals. From the circulation, HIV-1 Tat protein enters uninfected cells and alters cellular biological behavior by positively or negatively affecting gene expression. HIV-1 Tat protein is also able to trans-activate other cellular genes. One of the most relevant targets for HIV-1 Tat protein is the vascular system. It is well recognized that human atherosclerosis is a chronic, inflammatory, fibroproliferative disease involving the blood vessels (18, 42, 45) and cell adhesion molecules [E-selectin, intercellular adhesion molecule-1 (ICAM-1), and VCAM-1] that can be induced during the initiation of atherosclerosis to facilitate the formation of atherosclerotic plaques (20). VCAM-1 is considered as an early marker for the detection of early atherosclerosis; therefore, an early therapeutic intervention targeting VCAM-1 could prevent...
progression of disease (39). VCAM-1 has been shown to be upregulated by HIV-1 infection (12, 46), which may be related to premature atherosclerosis. However, the mechanisms underlying this are not well elucidated.

In this study, we report the regulation of VCAM-1 in primary human pulmonary artery endothelial cells (HPAEC) exposed to HIV-1 Tat protein. Reactive oxygen species (ROS) production, NF-κB translocation, and p38 mitogen-activated protein kinase (p38 MAPK) activation seem to play important roles in the induction of VCAM-1 expression by HIV-1 Tat protein. This study may point to novel mechanisms regulating HIV-induced inflammatory injury involving the vasculature.

MATERIALS AND METHODS

Reagents. Recombinant HIV-1 Tat protein was obtained from National Institutes of Health AIDS Research and Reference Reagent Program (cat. no. 2222; lot no. 899235) (4), dissolved in buffer solution (PBS with 1 mg/ml of BSA and 0.1 mM DTT), and frozen in aliquots at –80°C until use. Endotoxin assay of the HIV-1 Tat protein reagent was conducted with Quantitative Chromogenic LAL Kit (cat. no. 50-647U; Cambrex, Walkersville, MD), and the endotoxin levels were below the detection limit on this assay. IL-1β was kindly provided by National Cancer Institute’s Biological Resources Branch (Rockville, MD). LPS was purchased from Sigma (St. Louis, MO). Pyrrolidine dithiocarbamate (PDTC) was purchased from Sigma and dissolved in PBS. SB-203580 was purchased from Calbiochem (San Diego, CA) and made up with DMSO at 1 mM stock solution. VCAM-1 antibody conjugated with FITC was purchased from Ancigenix America (Huntington Station, NY). 2',7'-Dichlorodihydrofluorescein (DCFH) diacetate was purchased from Alexis (San Diego, CA).

Culture of endothelial cells. HPAEC were obtained from Clonetics (San Diego, CA). HPAEC were cultured in endothelial basal medium (Clonetics) supplemented with SingleQuots (Clonetics) containing human recombinant epidermal growth factor (10 ng/ml), hydrocortisone (1.0 μg/ml), gentamycin (50 μg/ml), amphotericin B (50 μg/ml), bovine brain extract (12 μg/ml), and 5% FBS. Cells were grown in T-75 flasks, petri dishes of 100 × 20 mm, or 96-well culture plates at 37°C in a humidified incubator with a 5% CO2 atmosphere. Confluent monolayers were detached with trypsin/EDTA (Clonetics) and subcultured at a 1:3 ratio. Cell viability always exceeded 95% as determined by the trypan blue exclusion test.

Flow cytometry. HPAEC were cultured in T-75 flasks, treated with PDTC at 100 μM or SB-203580 at 10 μM for 1.5 h, and then stimulated with IL-1β at 10 ng/ml and HIV-1 Tat protein at 250 ng/ml for 12 and 24 h. The cells were harvested by a brief exposure to trypsin/EDTA, washed three times with PBS, and resuspended in fluorescence-activated cell sorting buffer (PBS, pH 7.4, containing 2% FCS, 0.1% Na3VO4, 0.1% BSA, and 1 ng/ml human IgG). Cells were incubated with FITC-conjugated VCAM-1 antibody at 4°C for 20 min and washed three times with PBS. HPAEC were then fixed in 1% paraformaldehyde solution. Isootype-matched IgG was applied as a negative control. The fluorescence intensities of cells were determined with flow cytometer (FACSCalibur E4445, Becton Dickinson), and data were analyzed using FACSComp 4.2 program.

Cellular oxidative stress measurement. Cellular oxidative stress was determined by monitoring ROS-mediated conversion of DCHF into 2',7'-dichlorofluorescein (DCF) that emits fluorescence (37). HPAEC were incubated in 96-well culture plates and treated with conditioned media for 1 h. The cells were washed with PBS and loaded with 200 μl of 5 μM DCHF diacetate. The relative fluorescence unit was measured with a multiplate reader (FLUOstar Galaxy, BMG Lab Technologies) using excitation and emission wavelengths of 485 and 520 nm, respectively. The fluorescence was read every 10 min with 20 cycles.

EMSA. Cells were cultured in petri dishes of 100 × 20 mm. After being treated, the cells (~2.0 × 10⁶ cells/dish) were washed once with cold PBS and collected with cell scrapers. Nuclear proteins were prepared by the method previously described with modifications (34). Briefly, cells were resuspended in 100 μl of hypotonic buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 1 μM aprotinin, 14 μM leupeptin, 1 μM pepstatin, 80 μg/ml benzamidine, 20 mM p-nitrophenyl phosphate, 40 mM β-glycerol phosphate, 1 mM Na3VO4, and 50 mM NaF). Cells were incubated on ice for 30 min and then vortexed for 10 s after 6.5 μl of 10% Nonidet P-40 were added. Nuclei were collected following centrifugation at 12,000 rpm for 1 min. The nuclear pellets were resuspended in 50 μl of buffer C (20 mM HEPES, pH 7.9, 25% glycerol, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 12 mM DTT, 1 M PMSF, 1 mM aprotinin, 14 μM leupeptin, 1 μM pepstatin, 80 μg/ml benzamidine, 20 mM p-nitrophenyl phosphate, 40 mM β-glycerol phosphate, 1 mM Na3VO4, and 50 mM NaF) for 60 min on ice with vigorous vortexing every 20 min. The supernatants containing nuclear proteins were collected after centrifugation at 12,000 rpm for 15 min at 4°C and were stored at −80°C. Protein concentrations were determined with the bicinchoninic acid protein assay (Pierce).

NF-κB DNA binding activity was assayed by EMSA. Briefly, 10 μg of nuclear proteins were added to 1X binding buffer [50 μg/ml of double-stranded poly(dI-dC), 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, 1 mM MgCl2, and 10% glycerol] and 35 fmol of double-stranded NF-κB consensus oligonucleotide (5'-AGT TGA GGG GAC TTT CCC AGG C-3') was added at room temperature for 20 min and analyzed by electrophoresis on 5% nondenaturing polyacrylamide gels. The gels were dried and exposed to Kodak X-ray film at −80°C. The bands were quantitated by scanning densitometry with a Bio-Image Analysis System (Millipore Imaging System, Ann Arbor, MI). The results were expressed as relative integrated intensities compared with control.

NF-κB subunits p50/p65 assay. Nuclear proteins were isolated as previously described. The activation of NF-κB p50 and p65 subunits was assayed by ELISA using a commercially available kit (Chemicon, Temecula, CA). Briefly, the nuclear extracts were incubated with the appropriate capture probes. The mixture was then transferred to a streptavidin-coated plate. The bound NF-κB transcription factor subunits p50 and p65 were detected with specific primary antibodies. A horseradish peroxidase-conjugated secondary antibody was then used for colorimetric detection. The absorbance of the samples at 450/650-nm dual wavelengths was measured using a Dynatech MR5000 spectrophotometric plate reader (Dynatech Laboratories, Chantilly, VA).

RT-PCR. RT-PCR was employed to determine VCAM-1 gene expression in HPAEC as previously described (29, 30). Total RNA was extracted using RNAzol B reagent (Tel-Test, Friendswood, Texas) according to the manufacturer’s instructions with modifications. Briefly, cultured endothelial cells in a petri dish of 100 × 20 mm (~2.0 × 10⁶ cells/dish) were lysed in 1.1 ml of RNAzol B solution followed by the addition of 0.2 ml of cold chloroform. The mixture was shaken vigorously for 1 min and centrifuged at 12,000 g for 15 min at 4°C. The top aqueous part was precipitated in an equal volume of isopropanol for 15 min and then washed once with 0.8 ml of phenol-chloroform and twice with 0.8 ml of cold chloroform. The top aqueous part was precipitated in an equal volume of isopropanol for 15 min at 4°C. The RNA pellet was obtained by centrifugation at 12,000 g for 30 min at 4°C and washed with 75% ethanol. The RNA pellet was air-dried and resuspended in 20 μl of DEPC-treated water. The RNA was stored at −80°C until use.
Reverse transcription with 1 μg of total RNA was carried out at 42°C for 20 min, 99°C for 10 min, and 5°C for 5 min (DNA Thermal Cycler 480, Perkin Elmer) in 20 μl of 5 mM MgCl2, 1 mM dNTP, 1 μg RNase inhibitor, 2.5 μl cloned murine leukemia virus reverse transcriptase, and 2.5 μM oligo(dT)16 as a primer. For amplification of VCAM-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the following primer combinations were used: 5′-GGC TGG AGA TAG ACT TAC TGA-3′ and 5′-CTC AGG GGA GAT CTC AAC AG-3′ (VCAM-1; expecting a 521-bp fragment, Georgia Univ.) and 5′-CAG GTG GCA AAT TCC ATG GCA-3′ and 5′-TCT AGA CGG CAG GTC AGG TCC ACC-3′ (GAPDH; expecting a 593-bp fragment, Johns Hopkins Univ.). GAPDH mRNA was detected as a housekeeping gene. The PCR mixture consisted of 4 μl of the reverse transcriptase reaction product, 1.8 mM MgCl2, 0.2 mM dNTP, 1 μg/50 μl of AmpliTaq DNA polymerase, and 0.2 μM paired specific primers for VCAM-1 or GAPDH in a total volume of 50 μl. The following conditions were used: 95°C for 2 min; 95°C for 45 s; 45 s for 45 s, 72°C for 90 s, 40 cycles; and 72°C for 10 min. PCR products were separated by 2% agarose gel electrophoresis and stained with ethidium bromide (0.5 μg/ml). Semiquantitative mRNA expression of VCAM-1 was obtained by normalizing the band intensities of GAPDH. DNA of VCAM-1 or PCR product was purified with QIAEX II Gel Extraction Kit (QIAGEN, Valencia, CA). The DNA sequence was analyzed with ABI PRISM 377 DNA Sequencer (Perkin Elmer).

Statistics. Data are expressed as means ± SD. Statistical significance was determined by Student's t-test, and the results were considered significant if P values were <0.05.

RESULTS

HIV-1 Tat protein induces protein and gene expression of VCAM-1 in HPAEC. To determine the effect of HIV-1 Tat protein on VCAM-1 protein expression at the cell surface, flow cytometry was conducted. HPAEC were treated with media alone, IL-1β (10 ng/ml), and HIV-1 Tat protein (250 ng/ml) for 12 (Fig. 1A) and 24 h (Fig. 1B), detached by a brief exposure to trypsin/EDTA, stained using the specific antibody against VCAM-1, and examined for the cell surface expression of VCAM-1 protein by flow cytometry. The results showed that both IL-1β and HIV-1 Tat protein induced the cell surface expression of VCAM-1 protein in HPAEC after 12 h of incubation (Fig. 1A). To study the time response, cells were further activated for 24 h (Fig. 1B), and the cell surface expression of VCAM-1 protein was analyzed by flow cytometry. Similar and comparable results were observed for 24-h treatment with IL-1β; however, HIV-1 Tat protein-induced expression of VCAM-1 protein was decreased compared with 12-h treatment (Fig. 1B). An ongoing study is being carried out to further understand the time response of VCAM-1 regulation by HIV-1 Tat protein.

The induction of gene expression of VCAM-1 by HIV-1 Tat protein was determined by RT-PCR. HPAEC were exposed to IL-1β (10 ng/ml) and HIV-1 Tat protein (250 ng/ml) for 2, 4, 6, and 9 h. The optimal concentrations of HIV-1 Tat protein derived from our laboratory were between 100 and 500 ng/ml. We have therefore used these concentrations in this study. RNA was extracted and RT-PCR was performed. Figure 2 shows the induction of VCAM-1 mRNA transcripts following treatment with IL-1β and HIV-1 Tat protein over time. IL-1β and HIV-1 Tat protein-induced VCAM-1 mRNA transcriptions were apparent at 2 h and continued to be induced at up to 9 h of stimulation (Fig. 2). We quantified the band intensities by densitometry, normalized the VCAM-1 mRNA expression by the ratio of VCAM-1 to GAPDH, and compared the mRNA expression levels at different time points by the fold of control. VCAM-1 mRNA transcription induced by IL-1β increased 2.1-, 3.2-, 3.5-, and 4-fold for 2, 4, 6, and 9 h, respectively. Similar results were obtained when HPAEC were stimulated with HIV-1 Tat protein. VCAM-1 mRNA transcription induced by HIV-1 Tat protein increased 1.7-, 2.3-, 2.5-, and 3-fold for 2, 4, 6, and 9 h, respectively. Consistency of results was achieved in the repeated experiments.

HIV-1 Tat protein induces NF-κB DNA binding activity and ROS. To understand the mechanism of VCAM-1 protein and gene expression induced by HIV-1 Tat protein, we examined NF-κB transcriptional activity after exposing HPAEC to IL-1β (10 ng/ml) and HIV-1 Tat protein (250 ng/ml) for 1, 2, and 4 h. NF-κB DNA binding activity was determined by EMSA. It
representative of 3 independent experiments. VCAM-1 gene expression was quantitated by RT-PCR using specific primers. RT-PCR products were analyzed by 2% agarose gel electrophoresis. VCAM-1 and GAPDH mRNA were treated with IL-1β/H9252 protein (250 ng/ml) significantly induced NF-

A B DNA binding activity induced by IL-1β/H9260 that NF-

B nuclear translocation of the NF-

B subunits p65 and p50 was clearly shown that both IL-1β (10 ng/ml) and HIV-1 Tat protein (250 ng/ml) significantly induced NF-κB transcriptional activity compared with control in a time-dependent fashion (Fig. 3A). The analysis of integrated intensities showed that NF-κB DNA binding activity induced by IL-1β and HIV-1 Tat protein at 4 h was significantly increased (both \( P < 0.05 \)) compared with control (Fig. 3B).

The regulation of endothelial cell adhesion molecule expression, particularly VCAM-1, is mediated by oxidation-reduction (redox)-coupled signaling mechanisms. To better understand the mechanism underlying the HIV-1 Tat protein-induced VCAM-1 expression and NF-κB DNA binding activity, we monitored the ROS production by detecting the fluorescent intensities of DCF transformed from DCFH in response to HIV-1 Tat protein treatment. After HPAEC incubation with LPS (100 ng/ml) and HIV-1 Tat protein at different concentrations (100, 250, and 500 ng/ml) for 1 h, ROS production was significantly increased \( (P < 0.05) \) for all treatments, Fig. 4. However, no dose response of ROS production was found for increasing Tat concentrations. These data suggest that one possible mechanism by which HIV-1 Tat protein induces VCAM-1 expression and NF-κB nuclear translocation is mediated through ROS production.

**PDTC and SB-203580 inhibit protein and gene expression of VCAM-1 induced by HIV-1 Tat protein in HPAEC.** To investigate whether NF-κB activation and ROS production were involved in the increased VCAM-1 expressions induced by HIV-1 Tat protein, we pretreated HPAEC with the antioxidant PDTC before stimulation with HIV-1 Tat protein. For VCAM-1 protein expression at the cell surface, HPAEC were incubated with PDTC (100 μM) for 1.5 h and then treated with HIV-1 Tat protein (250 ng/ml) for 12 and 24 h and analyzed by flow cytometry. The changes in mean fluorescence intensity at the 12- and 24-h time points are shown in Fig. 5A. The results showed that PDTC pretreatment markedly inhibited the VCAM-1 expression at the cell surface induced by HIV-1 Tat protein at both the 12- and 24-h time points (Fig. 5A) compared with HIV-1 Tat protein alone. To determine whether the increased VCAM-1 protein and gene expression induced by HIV-1 Tat protein were related to p38 MAPK activation, we also utilized the p38 MAPK-specific inhibitor SB-203580 to pretreat the cells for 1.5 h, followed by activating the cells with HIV-1 Tat protein (250 ng/ml) for 12 and 24 h. The results of flow cytometry showed that SB-203580 (10 μM) pretreatment inhibited VCAM-1 protein expression at the cell surface induced by HIV-1 Tat protein at both the 12- and 24-h time points (Fig. 5A) compared with HIV-1 Tat protein alone. After 12 h, HIV-1 Tat protein-induced VCAM-1 expression was comparable to the IL-1β positive control, and both PDTC and SB-203580 reduced the VCAM-1 expression to the baseline. These data suggest that NF-κB and p38 MAPK may be involved in the regulation of HIV-1 Tat protein-induced VCAM-1.

For VCAM-1 gene expression, HPAEC were also pretreated with PDTC (100 μM) or SB-203580 (10 μM) for 1.5 h and then treated with HIV-1 Tat protein (250 ng/ml) for 6 h, and RT-PCR was performed using VCAM-1 specific primers. The results showed that both PDTC and SB-203580 pretreatment abrogated the VCAM-1 mRNA expression by HIV-1 Tat protein (Fig. 5B) compared with HIV-1 Tat protein-alone treatment. These data further suggest the involvement of both NF-κB and p38 MAPK.

**PDTC and SB-203580 inhibit NF-κB DNA binding activity and ROS production induced by HIV-1 Tat protein.** To determine the relationship between NF-κB and p38 MAPK pathways in the regulation of HIV-1 Tat protein-induced VCAM-1 expression, we used PDTC (100 μM) and SB-203580 (10 μM) to pretreat the HPAEC for 1.5 h and then exposed the cells to HIV-1 Tat protein (250 ng/ml) for 4 h. Nuclear extracts were obtained, and EMSA was carried out for NF-κB DNA binding activity. The results showed that PDTC and SB-203580 pretreatment noticeably decreased HIV-1 Tat protein-induced NF-κB DNA binding activity compared with treatment with HIV-1 Tat protein alone (Fig. 6A). To further demonstrate the effect of HIV-1 Tat protein on the translocation of the NF-κB p50/p65 heteroduplex and the relationship between the NF-κB and p38 MAPK pathways, we performed an ELISA to measure the nuclear translocation of the NF-κB subunits p65 and p50 (Fig. 6B). HPAEC were incubated with IL-1β and HIV-1 Tat...
protein with or without PDTC (100 μM) and SB-203580 (10 μM) pretreatment for 1 h. The results demonstrated that HIV-1 Tat protein induced the nuclear translocation of the NF-κB subunit p65, which was significantly inhibited by PDTC and SB-203580 (both \( P < 0.05 \)). In contrast, no significant difference was observed for the activation of the p50 subunit.

Additionally, the assay of ROS production induced by HIV-1 Tat protein (250 ng/ml, 500 ng/ml) for 1 h with PDTC (100 μM) pretreatment for 1.5 h was also conducted. ROS induction by HIV-1 Tat protein at both concentrations was significantly reduced by PDTC (\( P < 0.05 \), Fig. 4).

**DISCUSSION**

The vascular endothelium serves as a dynamic interface between blood elements and the interstitial tissues. Any responses to injurious agents could lead to pathological changes. Atherosclerosis can be viewed as a specialized type of chronic inflammation (18, 42, 45), and its development involves three stages: initiation, progression, and complications (36). Recruitment of mononuclear leukocytes to the intima characterizes the initiation of the atherosclerotic lesion. In response to atherogenic factors, mononuclear cells in blood attach and adhere to endothelial cells using cell adhesion molecules. These cells then emigrate across the endothelium and accumulate within the intima to induce the formation of the atherosclerotic plaque. The increased specific adhesion molecules at the vascular endothelial cell surface mediating the leukocyte adhesion include members of the immunoglobulin gene superfamily (such as ICAM and VCAM) and the selectins (8, 15). Selectins mediate rolling or transitory contact of leukocytes with the endothelium, whereas the immunoglobulin gene superfamily mediates more sustained adhesion of leukocytes to the endothelium (36). In this study we have concentrated on one pivotal member of the immunoglobulin gene superfamily, VCAM-1. VCAM-1 binds to the cognate ligand VLA-4 (very late antigen-4) expressed on monocytes and lymphocytes that are recruited to the intima during early atherogenesis (11, 36). VCAM-1 activates signals within endothelial cells resulting in the opening of an “endothelial cell gate” through which leukocytes migrate (10). In experimental rabbits with hypercholesterolemia, focal expression of VCAM-1 on aortic endothelial cells occurs before monocyte adhesion. By transfecting the resting endothelium with adenovirus carrying VCAM-1, investigators showed that VCAM-1 is capable of supporting lymphocyte adhesion and mediates monocyte rolling, firm adhesion, and transmigration (17). Blocking VCAM-1 on endothelium with an antibody reduced adhesion by 75% (40). Mice deficient in VCAM-1 have major developmental abnormalities, thus frustrating attempts to study atherogenesis in these animals (19). These studies suggested that VCAM-1 might play a role in mediating monocyte recruitment and atheroma initiation.

A number of investigators have reported that the biological functions of mesenchymal cells, such as monocytes and endo-
endothelial cells, can be modulated by HIV-1 Tat protein (9, 22, 31). It is likely that HIV-1 Tat protein modifies the adhesion molecule profile on endothelial cells. As mentioned before, HIV-1 Tat protein induced the expression of E-selectin and upregulated ICAM-1 and VCAM-1 in macrovascular and microvascular endothelial cells (12, 22). In the present report, we demonstrated the upregulation of VCAM-1 gene and protein expression in pulmonary endothelium by IL-1β and HIV-1 Tat protein. We also demonstrated that this upregulation was mediated through a p38 MAPK-NF-κB pathway. These may

Fig. 4. Effect of HIV-1 Tat protein on reactive oxygen species (ROS) production in HPAEC. HPAEC were cultured in 96-well plates and treated in quadruplicate with LPS (100 ng/ml) and HIV-1 Tat protein (100, 250, and 500 ng/ml) for 1 h with or without pretreatment with the NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC; 100 μM) for 1.5 h. The cells were loaded with 200 μl of 5 μM 2′,7′-dichlorodihydrofluorescein diacetate, and the fluorescence was read with a microplate reader at excitation of 485 nm and emission of 520 nm. The data are expressed as means ± SD. The data shown are representative of 3 independent experiments. *P < 0.05 vs. control; **P < 0.05 vs. HIV-1 Tat protein alone.

Fig. 5. Inhibition of HIV-1 Tat protein-induced VCAM-1 expression by PDTC and SB-203580 in HPAEC. A: inhibition of HIV-1 Tat protein-induced VCAM-1 protein expression at cell surface by PDTC and SB-203580. HPAEC were cultured in T-75 flasks and treated with HIV-1 Tat protein (250 ng/ml) with or without pretreatment with the NF-κB inhibitor PDTC (100 μM) and the p38 MAPK inhibitor SB-203580 (10 μM) for 1.5 h. The VCAM-1 expression at the cell surface was quantitated by flow cytometry. The data shown are representative of 3 independent experiments. B: inhibition of HIV-1 Tat protein-induced VCAM-1 gene expression by PDTC and SB-203580. HPAEC were cultured in 100 × 20-mm petri dishes and treated with IL-1β (10 ng/ml) and HIV-1 Tat protein (250 ng/ml) for 6 h with or without pretreatment with PDTC (100 μM) or SB-203580 (10 μM) for 1.5 h. Total RNA was extracted. VCAM-1 and GAPDH mRNA were quantitated by RT-PCR using specific primers. RT-PCR products were analyzed by 2% agarose gel electrophoresis. The data shown are representative of 3 independent experiments.
represent pivotal early molecular events in HIV-induced vascular/pulmonary injury and cellular recruitment. In our system, both IL-1β and HIV-1 Tat protein can contribute to VCAM-1 induction on endothelium. IL-1β-induced VCAM-1 expression and the importance of p38 MAPK in IL-1-induced endothelial activation have been well documented (13, 14, 43). It is thought that IL-1β facilitates early lesion formation by increasing mononuclear cell adhesion to endothelial cells and mediating mononuclear cell transmigration (44). HIV-1 infection increases IL-1β production in HIV-infected cells, thus enhancing atherogenesis (3). We also used human umbilical vein endothelial cells (HUVEC) to determine the effect of IL-1β, TNF-α, and HIV-1 Tat protein on VCAM-1 and ICAM-1 expression. We failed to find significant effects of HIV-1 Tat protein on adhesion molecule expressions in these cells as opposed to pulmonary endothelial cells. The difference in responses to HIV-1 Tat protein between HPAEC and HUVEC deserves further study. To eliminate the possibility that these observed results were the effect of endotoxin in the Tat stock, endotoxin assay of the HIV-1 Tat protein reagent was conducted with a Quantitative Chromogenic LAL Kit according to the instructions of the manufacturer. Endotoxin levels were
below the detection limit on this assay, suggesting that the observed effects were most likely mediated by HIV-1 Tat protein itself.

A number of studies strongly indicate that the inducible transcription factor NF-κB is involved in the pathogenesis of atherosclerosis (6). Activation of NF-κB plays a significant role in cytokine-stimulated cell adhesion molecule expression in endothelial cells due to known binding sites for NF-κB in the promoter regions for genes encoding cell adhesion molecules (41). NF-κB is composed of members of Rel family that share a 300-amino acid region. This Rel homology domain mediates dimerization, nuclear translocation, DNA binding, and interaction with NF-κB inhibitors (25). Activation of NF-κB is controlled by a family of inhibitors, or IκB, that bind to NF-κB dimers (p65/p50) and mask the nuclear localization sequence of NF-κB, thus retaining the entire complex in the cytoplasm. Upon stimulation, IκB is phosphorylated and degraded by the proteosome. After the release of active complex p65/p50 of NF-κB from the inhibitor, NF-κB dimers translocate from the cytoplasm to the nucleus, where they bind target genes and stimulate transcription. Although exogenous HIV-1 Tat protein is known to activate NF-κB in immune cells, including monocytes and T lymphocytes, it is not well known whether exogenous HIV-1 Tat protein is able to activate the NF-κB pathway in endothelial cells. We carried out EMSA for the measurement of NF-κB DNA binding activity. We found an increase in NF-κB DNA binding activity in nuclear extracts from HIV-1 Tat protein-treated HPAEC (Fig. 3). The application of PDTC, a specific NF-κB inhibitor, abolished the NF-κB activation induced by HIV-1 Tat protein (Fig. 6). Also, PDTC inhibited the protein and gene expression of VCAM-1 induced by HIV-1 Tat protein (Fig. 5), thus indicating that the induction of VCAM-1 expression on HPAEC by HIV-1 Tat protein is NF-κB dependent.

The various risk factors for atherosclerosis induce intracellular oxidative stress. NF-κB is one of the transcription factors that may be controlled by the redox status of the cells (35). Generation of ROS may be a common step in all of the signaling pathways that lead to IκB degradation and NF-κB nuclear accumulation. We showed that HIV-1 Tat protein can induce the ROS production, which may be a mechanism for IκB phosphorylation/degradation leading to further NF-κB activation. ROS production is an early event in signaling pathways; therefore, we also studied the effect of PDTC on HIV-1 Tat-induced ROS (Fig. 4). PDTC reduced the ROS production to the control level. These indicate that ROS production is involved in HIV-1 Tat protein-induced VCAM-1 expression and is a possible upstream event of NF-κB activation.

Inflammatory mediators released during acute and chronic diseases activate multiple intracellular signaling cascades, of which MAPK signal transduction pathway plays a significant role in the recruitment of leukocytes to sites of inflammation. Stimulation of leukocytes by proinflammatory cytokines activates MAPK isoform p38. However, the functional consequences of p38 MAPK activation during leukocyte recruitment, including adhesion, migration, and effector functions such as oxidative burst and degranulation, are just beginning to be elucidated. Specific inhibitors of p38 MAPK aimed at reducing the production of inflammatory mediators are now being developed and might in the future provide a more effective treatment for inflammatory diseases (21, 32). Our present report shows that p38 MAPK pathway is involved in HIV-1 Tat protein-induced VCAM-1 expression (Fig. 5).

Our results showed that both NF-κB and p38 MAPK were involved in the increased expression of VCAM-1 induced by HIV-1 Tat protein. Generally, NF-κB is neither the downstream substrate nor the transcription factor activated by p38 MAPK (38). However, some reports indicate that p38 MAPK is an important activator of NF-κB in thrombin-induced NF-κB-dependent upregulation of E-selectin and subsequent leukocyte recruitment (26). It has also been reported that HIV-1 glycoprotein 120 enhances nitric oxide production by cardiac myocytes through p38 MAPK-mediated NF-κB activation (24). Our study indicates that the NF-κB activation by HIV-1 Tat protein was abolished by the specific inhibitor of p38 MAPK, SB-203580 (Fig. 6). It implies that NF-κB acts as a downstream substrate of p38 MAPK during VCAM-1 expression induced by HIV-1 Tat protein.

Together, our study demonstrates that HIV-1 Tat protein induces VCAM-1 expression in HPAEC. This is a possible mechanism by which HIV-1 infection can contribute to accelerated atherogenesis and/or pulmonary vasculopathy (Fig. 7). The increased VCAM-1 expression induced by HIV-1 Tat protein may be mediated by ROS production, p38 MAPK activation, and NF-κB translocation, which may represent potential targets for novel therapeutic approaches for the vasculopathy induced by HIV infection.

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REFERENCES


