Transfection of human endothelial cells with HIV-1 tat gene activates NF-κB and enhances monocyte adhesion

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Pieper, Galen M., Cara L. Olds, Jeffrey D. Bub, and Paul F. Lindholm. Transfection of human endothelial cells with HIV-1 tat gene activates NF-κB and enhances monocyte adhesion. Am J Physiol Heart Circ Physiol 283: H2315–H2321, 2002; 10.1152/ajpheart.00469.2002.—Human immunodeficiency virus (HIV)-1 Tat released from HIV-1-infected monocytes is believed to enter other cells via an integrin-facilitated pathway, resulting in altered gene expression. Indeed, exogenous Tat protein can increase cell adhesion molecule gene expression in human endothelial cells. Signaling pathways initiated by Tat in endothelial cells are not known. We evaluated the ability of endogenous tat to stimulate monocyte adhesion via activation of nuclear factor-κB (NF-κB) within human umbilical vein endothelial cells. Transfection with pcTat, but not control vector DNA, increased NF-κB binding activity, NF-κB luciferase reporter activity, and monocyte adhesion. pcTat also increased NF-κB-dependent HIV-1LTR-CAT reporter activity 28-fold compared with a 3-fold increase produced by transfection with an equivalent amount of pcTax (from human leukemia virus). The pcTat-induced increase in pNF-κB-Luc activity and monocyte adhesion to endothelial cells was blocked by cotransfection with dominant-negative mutant IκBα and by incubation with 10 mM aspirin. We conclude that monocyte adhesion to human endothelial cells stimulated by pcTat is mediated via an NF-κB-dependent mechanism. Furthermore, inhibition studies using aspirin suggest that pcTat-stimulated NF-κB activation and monocyte adhesion occur via a redox-sensitive mechanism.

nuclear factor-κB; signal transduction; cell adhesion; endothelium; human immunodeficiency virus; viral protein

THE MECHANISM OF SPREAD of human immunodeficiency virus (HIV) infection to adjacent cells or tissues is not completely understood. It has been concluded that soluble proteins likely contribute to the early stages of infection. One candidate molecule is the HIV-1 viral protein known as the transactivator of transcription (HIV-1 Tat). This protein is believed to play a critical role in the progression of HIV disease (11). The HIV-1 Tat protein released by HIV-infected monocytes is believed to be bioactive. Tat has been identified in brain and other tissue of HIV-infected patients (37, 39). The mechanism by which Tat protein is expressed in nonmonocytic cells during infection is not understood. Cellular uptake of Tat protein, a potentially important part in this process, has been documented in neuronal cells and astrocytes (15, 22). The cellular uptake of Tat protein during HIV infection is believed to modify gene expression within adjacent cells. The alteration of gene expression in parenchymal cells has become an area of heightened interest to delineate a possible mechanism to explain the progression of acquired immunodeficiency syndrome (AIDS) infection and its complications and even primary vascular disease, atherosclerosis, and hypertension associated with AIDS infection (34).

It is likely (but not fully tested) that activation of endothelial cells is an early event leading to monocyte adhesion, transendothelial migration of HIV-1-infected monocytes into adjacent parenchyma, and subsequent infection of tissue. We believe that HIV-1 Tat protein is a key player in this initial stage. Indeed, exogenous HIV-1 Tat protein has been shown to induce expression of vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 in human astrocytes (40). Vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 are also upregulated in transgenic mice expressing the envelope protein HIV-1 gp120 (35).

Previous studies indicated that exogenous HIV-1 Tat protein increases expression of E-selectin and enhances production of interleukin-6 and interleukin-8 in cultured human endothelial cells (12–14). These actions of exogenous Tat protein might lead to activation of endothelial cells and increased endothelial cell permeability and might even facilitate the development of Kaposi’s sarcoma, an aggressive vascular tumor frequently found in AIDS-infected patients (1). Indeed, HIV-1 tat gene expression in transgenic mice is known to induce endothelial cell proliferation and tumors resembling Kaposi’s sarcoma (7). Transgene expression of HIV Tat is also known to induce cardiomyopathy (26), suggesting that cardiovascular complications may involve more than just vascular complications. Adhesion molecule gene expression and enhanced monocyte

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adhesion may lead to accelerated atherosclerosis. Indeed, there is emerging evidence that HIV-1 infection is a risk factor for vasculopathy and atherosclerotic disease (4, 20). It is not understood whether this is due to drug treatment or an underlying complication of infection. The strong association of monocyte adhesion with atherosclerosis and the stimulation of cell adhesion molecule gene expression by exogenous Tat protein suggest that viral proteins may play a significant role in the development of vascular disease.

Despite these findings, no information is available regarding the proximal signaling events in the regulation of cell adhesion to endothelial cells activated by HIV-1 Tat. There is ample evidence that activation of the transcription factor nuclear factor κB (NF-κB) plays a significant role in cytokine-stimulated cell adhesion molecule expression in endothelial cells owing to known binding sites for NF-κB in the promoter regions for genes encoding cell adhesion molecules (27). Although exogenous Tat protein is known to activate NF-κB in immune cells, including monocytes and T lymphocytes (8, 17, 24), it is not known whether exogenous Tat or transfection to express endogenous tat is able to activate this pathway in endothelial cells.

To our knowledge, this is the first report documenting the role of NF-κB activation in initiation of monocyte adhesion to human endothelial cells transfected with pcTat.

**MATERIALS AND METHODS**

**Endothelial cells and culture media.** Human umbilical vein endothelial cells were obtained from Clonetics (Walkersville, MD). Cells were initially cultured in 75% endothelial basal medium (EBM, Clonetics) supplemented with EGM-2 growth components + 25% RPMI 1640 (per company specifications). Confluent cells were passaged in 50% EBM + 50% RPMI 1640, converted to 25% EBM + 75% RPMI, and finally converted to 100% RPMI 1640 containing l-glutamine, fibroblast growth factor, and 5% fetal bovine serum. Media replacement was designed to minimize potential complicating components in EBM and to achieve a physiological glucose concentration of 5.5 mM to prevent activation of NF-κB due to elevated glucose concentrations, which we have shown can independently activate NF-κB (25). Cells were cultured up to passages 5–7.

**Transfections and reporter assays.** Optimal transfection conditions for 24 or 48 h were determined using various concentrations of Lipofectin and green fluorescence protein plasmid (Promega, Middleton, WI). Fluorescence was viewed on a fluorescence microscope (Olympus, Lake Success, NY). A plasmid construct, pcTat, was used to transfect endothelial cells with HIV-1 tat gene. For control purposes, we used p12S,FS empty vector. For the reporter assays, endothelial cells were cotransfected with 0.3–1.0 μg of the NF-κB-dependent luciferase vector pNF-B-Luc (Clontech Labs, Palo Alto, CA) and compared with the NF-κB null vector pTA-Luc (Clontech Labs). Cells were transfected for 48 h with RPMI 1640 as described above. Normalized luciferase activity was determined using an E4030 luciferase assay system and reporter lysis buffer (Promega, Madison, WI).

For assay of HIV-1 transactivation, cells were transfected with a chloramphenical acetyltransferase (CAT) reporter linked to a wild-type κB promoter for HIV-1 long terminal repeat (pHIV-CAT; National Institutes of Health AIDS Research and Reference Reagent Program). For a negative control, a mutated κB reporter, pκB-HIV-CAT, was used (National Institutes of Health AIDS Research and Reference Reagent Program). These results were also compared with CAT reporter activity stimulated with pCTax for expression of human leukemia virus Tax protein (21).

**Electrophoretic mobility shift assay.** Nuclear extracts were prepared, and NF-κB binding activity was analyzed by electrophoretic mobility shift assay after the extracts were run on 4% gels using a consensus NF-κB oligonucleotide (Promega, Madison, WI), as previously described in detail (6, 28).

**Prevention of NF-κB activation.** Cells were incubated with SN-50 peptide (30 μg/ml; Biomol, Plymouth Meeting, PA) during transfection with pcTat. This peptide contains the nuclear localization sequence for NF-κB dimers linked to a cell-permeable motif of Kaposi’s growth factor and functions to block nuclear translocation of NF-κB dimers. In other studies, cells were transfected with pCMV4, IκBα, S32/36A, a dominant-negative mutant IκBα plasmid recently denoted the superrepressor IκBα (provided by Dr. Warner C. Greene, Gladstone Institute of Virology and Immunology, University of San Francisco, San Francisco, CA). This plasmid, in which alamine is substituted at serine residues 32 and 36, does not allow phosphorylation of IκBα and subsequent release and translocation of NF-κB dimer subunits (32). To assess a role of reactive oxygen, some cells were incubated with 10 mM aspirin. This high concentration of aspirin possesses antioxidant efficacy and has been shown previously to block NF-κB activation and monocyte adhesion to human umbilical vein endothelial cells stimulated with tumor necrosis factor-α (36).

**Monocyte adhesion.** U-937 monocyte cells were cultured in RPMI 1640 containing 10 mM HEPES, 1 mM sodium pyruvate, 2 mM l-glutamine, 4.5 g/l glucose, and 10% fetal bovine serum. Cell adhesion was analyzed by fluorescence labeled with 50 μg of 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxyethyl ester (Molecular Probes, Eugene, OR). Washed-labeled U-937 cells were counted (Coulter, Opa Locka, FL) and resuspended in medium at 5 × 10⁶ cells/ml. A 1.0-ml suspension of U-937 cells was added to transfected endothelial cells and incubated for 37°C for 30 min. Plates were rinsed three times, and 250 μl of lysis buffer (i.e., 1 mM Tris buffer, pH 8.0, and 1% Triton X-100) were added to each well. Samples were transferred to a black DynaTech 96-well plate and read on a CytoFluor II fluorescent plate reader (PerSeptive Biosystems, Foster City, CA) at 485 nm (excitation) and 530 nm (emission). Fluorescence was calibrated against 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxyethyl ester-labeled U-937-derived standards.

**Statistical analysis.** Values are means ± SE. ANOVA or Student’s t-test, as appropriate, was used for statistical analysis. P < 0.05 was selected to denote statistical significance.

**RESULTS**

Electrophoretic mobility shift assay indicated increased NF-κB binding activity in nuclear extracts of endothelial cells transfected with pcTat (Fig. 1, lanes 5 and 6) compared with control DNA (lane 7). The increase in NF-κB binding activity was greater than that achieved by transfection with the human leukemia virus Tax protein (lanes 3 and 4) or by acute stimulation for 1 h with 100 ng/ml phorbol 12-myristate 13-acetate (PMA; lane 8). The specificity for NF-κB was verified using 100-fold excess cold wild-type or mutant
Functional NF-κB activation was also determined in experiments using the HIV-LTR-CAT reporter assay system. Transfection with pcTat caused a potent activation of NF-κB-dependent CAT activity compared with control cDNA (Fig. 3). pcTat produced a calculated 28-fold increase in CAT activity compared with only a 3-fold increase in CAT activity produced by pcTax (Fig. 3). CAT activity was markedly lower (i.e., only a 13-fold increase) in pcTat-stimulated cells co-transfected with mutant pΔκB-HIV-CAT. In contrast to pcTat, pcTax did not stimulate CAT activity in cells transfected with pΔκB-HIV-CAT.

Finally, luciferase reporter assays were used in studies with cotransfection with mutant IkBα S32/36A. Transfection with mutant IkBα completely blocked pcTat-induced NF-κB luciferase activity (Fig. 4). As a positive control, similar findings were obtained in PMA-stimulated cells. As an additional control performed originally in PMA-stimulated cells, using a control empty vector, we determined that dominant-negative mutant IkBα blocked NF-κB luciferase activity by 94.6 ± 0.8% compared with only marginal changes (12.2 ± 2.2% inhibition, n = 2).
Endothelial cells that were transfected with pcTat showed increased monocyte adhesion compared with Lipofectin alone (Fig. 5) or compared with a control DNA vector lacking NF-κB activity (not shown). The increase in monocyte adhesion stimulated by endogenous tat gene was inhibited by 50% when cells were coincubated with SN-50 peptide (Fig. 6) and completely blocked by cotransfection with the dominant-negative mutant IκBα superrepressor (Fig. 5).

Incubation with 10 mM aspirin blocked the increase in NF-κB luciferase activity by 90% in endothelial cells stimulated with pcTat (Fig. 7). In addition, aspirin normalized the increase in monocyte adhesion in pcTat-stimulated endothelial cells (Fig. 8).

DISCUSSION

The new findings in this study are that endogenous expression of tat gene stimulated monocyte adhesion to human endothelial cells and that this process occurred via a redox-sensitive, NF-κB-dependent pathway. Recently, one laboratory has performed peripheral measurements that indicate significantly increased levels of plasma soluble cell adhesion molecules in HIV-positive patients (5, 31). In addition, increased cell adhesion to endothelium has been reported in HIV-1-infected patients. These observations indirectly suggest activation of endothelial cells in vivo but do not prove a causal link, the signaling events involved in endothelial cell activation, or the specific involvement of HIV-1-derived soluble factors, including viral proteins.

A clue to the potential direct actions of HIV-1 viral proteins is provided by studies that show increased interleukin-6 production and increased expression of E-selectin, a key cell adhesion molecule, as a result of incubation of human endothelial cells with exogenous Tat protein (12–14). The molecular events involved in signaling of gene expression by HIV-1 Tat within endothelial cells are unknown. Activation of NF-κB is a possible pathway that has not been examined in detail. We hypothesized that this pathway was important, because the genes for cell adhesion molecules contain an NF-κB binding site in the promoter region for these genes. A role for NF-κB activation in HIV-1 is indirectly supported by a report of increased immunoreactive NF-κB in brains of children with HIV-1 encephalitis (10). Also, exogenous HIV-1 Tat has been shown to cause activation of NF-κB in Jurkat T cells (2, 29, 38) and in macrophages (3). Despite these findings, the potential involvement of HIV-1 Tat-induced activation of NF-κB specifically within endothelial cells per se and the association with increased monocyte adhesion...
were not previously known. Thus the present study provides novel information in this area.

To understand the process of Tat-induced activation of endothelial cells more fully, we elected to examine the signaling events due to endogenous tat gene expression to eliminate the confounding actions of exogenous Tat protein interaction with plasma membrane receptors that might also induce NF-κB activation. To our knowledge, our findings are the very first report of human endothelial cell activation of NF-κB and subsequent monocyte adhesion by endogenous expression of tat.

We have documented NF-κB activation by several criteria. First, gel shift assays confirmed increased NF-κB binding activity after transfection with pcTat. Studies conducted with control cDNA confirm that this increase is due to pcTat and not simply to vector transfection in general. Second, NF-κB activation was also confirmed in protocols designed to assess functional NF-κB activity using NF-κB-dependent luciferase reporter assays. In this instance, pcTat produced a pronounced increase in luciferase activity that was equipotent to PMA, a well-known stimulant of NF-κB activation, within a variety of endothelial cells. Third, luciferase activity was not enhanced in pcTat-transfected endothelial cells that were cotransfected instead with the pTA-Luc reporter that lacks κB elements. These findings indicate that NF-κB activation by pcTat cannot be accounted for by the transfection process alone. Fourth, the pcTat-induced increase in NF-κB luciferase activity was completely blocked by cotransfection to express the dominant-negative mutant IκBα or superrepressor. This mutant contains point mutations for critical serine residues that prevent phosphorylation of IκBα, which in turn would prevent dissociation of the active NF-κB dimer subunits and subsequent nuclear translocation and DNA binding. These findings imply that IκBα phosphorylation is required for pcTat-induced activation of endothelial cell NF-κB. These latter findings represent yet another independent confirmation that pcTat stimulates functional NF-κB-dependent transcriptional activity.

Finally, we performed additional studies of functional NF-κB activation using the pHIV-1-LTR-CAT reporter, which is used to assess potential for viral replication. HIV-1 Tat is believed to cause transactivation of viral gene expression via binding of Tat protein to stem-loop structures denoted as transactivation-responsive (TAR) elements (16, 23). Our studies indicate marked increases in CAT activity by pcTat but not by a vector control DNA. Unlike endogenous expression of HTLV-1-Tat protein, endogenous tat gene was a more potent stimulant of HIV-1-LTR-CAT reporter activity. We observed that a significant portion of this activity was NF-κB dependent, because pcTat produced diminished activity via transfection with a mutant plasmid construct that lacked κB sequences.

Interestingly, a lesser but significant amount of CAT activity remained after stimulation with pΔκB-HIV-CAT, a mutated κB reporter of pcTat. This finding suggests that endogenous tat may also cause transactivation via an NF-κB-independent, TAR-dependent transactivation. In other cell lines, it is known that Tat can regulate gene expression via TAR-dependent and TAR-independent pathways (33). A conclusion from these studies is that a significant portion of viral replication of HIV-1 within endothelial cells, as denoted in the HIV-LTR-CAT vs. pΔκB-HIV-CAT assays, may not require TAR interaction. Accordingly, our studies are new, in that they describe NF-κB-independent and NF-κB-dependent components of HIV-1 transactivation within human vascular endothelial cells.

Another new finding of our study is that transfection of normal human endothelial cells with pcTat results in activation of endothelial cell NF-κB and enhanced monocyte adhesion. Previously, it was shown that HIV infection of monocytes stimulated adhesion to human endothelial cells (9). Additional studies revealed that incubation with extracellular HIV-1 Tat protein is capable of activating purified monocytes and, in turn, stimulating monocyte adhesion to unstimulated endothelium (19). Our new findings using the pcTat vector indicate that HIV-1 Tat protein may also directly activate endothelial cells to facilitate monocyte adhesion via a mechanism involving activation of endothelial cell NF-κB. That HIV-1 Tat-activated monocyte adhesion occurs via an NF-κB-dependent mechanism was verified in our studies showing that cotransfection with dominant-negative mutant IκBα under conditions that block NF-κB-linked reporter activity also blocks monocyte adhesion to pcTat-stimulated endothelial cells.

Additional implications and potential limitations for protein transduction. Because of the known efficient uptake of Tat protein by various cell types, there has been a heightened focus on the potential use of fusion proteins to Tat protein for protein transduction (18, 30). Because of potential limitations of adenoviral transfection, development of protein transduction technology is a novel pharmacological alternative for the delivery of proteins, compounds, and DNA to cells. The present study using transfection with pcTat suggests caution in the use of this potentially exciting pharmacological approach. Accordingly, future studies are warranted to more completely understand the actions and impact of truncated and full-length intracellular...
HIV-1 Tat protein on endothelial cell activation, gene expression, and function.

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REFERENCES


