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*J Immunol* 2001; 167:2766-2771; doi: 10.4049/jimmunol.167.5.2766

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The American Association of Immunologists, Inc.,
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
HIV-1 Tat Induces Microvascular Endothelial Apoptosis Through Caspase Activation

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HIV-1 Tat, in addition to its critical role in viral transcription, is secreted from infected cells and can act as a proto-cytokine. We studied the effects of HIV-1 Tat in primary human microvascular endothelial cells of lung origin and found that it caused apoptosis. This apoptosis occurred without induction of either Fas or TNF, known mediators of programmed cell death. Tat, like Fas ligand, induced cleavage of chromatin structure, as evidenced by changes in DNA ladder, incorporation of fluorescein into the nicked chromosomal DNA (TUNEL assay), and mono- or oligonucleosomes. Furthermore, Tat treatment caused cleavage of poly(A/DP)-ribose polymerase, a substrate of caspases. Caspase-3, but not caspase-9, was activated following treatment of primary human microvascular endothelial cells of lung origin with either Tat or anti-Fas agonist Ab (anti-Fas). Inhibition of caspase-3 activity markedly reduced apoptosis. Although Fas-mediated apoptosis involved changes in Bcl-2, Bax, and Bad regulatory proteins, such alterations were not observed with Tat. Taken together, these data demonstrate that HIV-1 Tat is able to activate apoptosis in microvascular endothelium by a mechanism distinct from TNF secretion or the Fas pathway. The Journal of Immunology, 2001, 167: 2766–2771.

The effects of Tat on small vessel endothelium have not been well characterized. We now report that HIV-1 Tat can induce apoptosis in primary human microvascular endothelial cells (HMVEC) through activation of specific caspases. This effect in small vessel endothelium would enhance transit of virally infected cells and cell-free viral particles from the circulation into the peripheral tissues. Furthermore, HIV-1 Tat as an apoptotic ligand may contribute to endothelial injury syndromes associated with AIDS, such as thrombotic thrombocytopenic purpura.

Materials and Methods

Antibodies

The Abs used were anti-poly(A/DP)-ribose polymerase (anti-PARP) (Boehringer Mannheim, Indianapolis, IN); anti-Bcl-2 (Dako, Glostrup, Denmark, and Transduction Laboratories, Lexington, KY); anti-Bax, anti-Fas, and anti-Fas ligand (anti-Fas-L; Santa Cruz Biotechnology, Santa Cruz, CA); anti-caspase-3 and caspase-9 (PharMingen, San Diego, CA); anti-Bcl-xL, and anti-Bad (Transduction Laboratories).

HIV-1 Tat protein

HIV-1 Tat protein was purified, lyophilized, and reconstituted in Tat buffer (PBS containing 1 mg of BSA and 0.1 mM/ml DTT) as described (3). The purified Tat protein was endotoxin-free, judging from the timed gel formation assay using the Limulus amebocyte lysate reagent (Sigma, St. Louis, MO). This protein was biologically active, as assessed by its rescue of tat-defective provirus replication in HLM-1 cells (3). Synthetic RGD-containing peptides, HQVSLSKQPTSQRGD, and basic-rich peptides, SYGRKKRRQRRPPQ, of Tat were obtained from Intracel (Issaquah, WA).

Cells

HMVEC of lung origin (HMVEC-L) were obtained from Clonetics (San Diego, CA) and cultured in EGM-2 mv medium, containing microvascular endothelial cell growth factors, antimicrobials, and 5% FBS. To avoid phenotypic drift with decreasing expression of various surface receptor molecules, HMVEC-L were not used beyond passage 4.

Stimulation of cells

At 80% confluence, HMVEC-L were starved overnight by placing them in culture medium with the endothelial basal medium, EBM-2 (Clonetics) supplemented with 0.5% FBS. The cells were then stimulated with 10 U/ml heparin with or without 25 ng/ml of HIV-1 Tat protein in fresh EBM-2 medium containing 0.5% FBS for the indicated time periods. It is known that heparin augments the biologic activities of Tat, such as the induction of endothelial cell growth, migration, and invasion in vitro (7). For controls, the cells were stimulated with known apoptotic ligands: LPS (1 µg/ml; Sigma) or with anti-Fas (DX2, 2 µg/ml; PharMingen) as a Fas-L with or without protein G’ (2 µg/ml; Sigma) for the indicated time periods.
Protein G is known to enhance anti-Fas-mediated apoptosis according to the manufacturer’s protocol (PharMingen).

**Detection of apoptosis**

**Quantitation of mono- and oligonucleosomes generated from apoptotic cells.** Relative amounts of mono- and oligonucleosomes generated from apoptotic HMVEC-L were quantitated with a cell death ELISA kit (Boehringer Mannheim), according to the manufacturer’s protocol. Briefly, Tat-treated, anti-Fas-treated, or control cells were lysed with the manufacturer’s buffer. After centrifugation of the lysates at 200,000 × g for 10 min, an aliquot of supernatant (cytoplasmic fraction) was transferred onto a streptavidin-coated microtiter plate and incubated for 2 h at room temperature. The plate was then thoroughly washed three times, and 100 µl of substrate solution was added to each plate to develop color, measured at 405 nm.

**DNA fragmentation assay.** DNA generated from Tat-treated, anti-Fas-treated, or control HMVEC-L was analyzed for evidence of fragmentation. Briefly, cells were lysed with lysis buffer containing 10 mM Tris-HCl (pH 7.6), 1 mM EDTA, and 0.2% Triton X-100, and the fragmented DNA in the lysates was separated from the unfragmented chromosomal DNA by precipitation at 12,000 × g for 30 min. The fragmented DNAs in the supernatants were then digested with 100 ng/ml ribonuclease A (Invitrogen, Carlsbad, CA), 20 ng/ml protease K (Invitrogen), and 1% SDS at 37°C for 45 min, purified by the phenol/chloroform extraction method, and precipitated with ethanol/ammonium acetate. The DNA was then electrophoresed on a 1.6% agarose gel containing 0.5 µg/ml ethidium bromide.

**Microscopic analysis of apoptosis.** HMVEC-L were grown on chamber slides and stained with the fluorescein-labeled fragmented DNA was detected by microscopic analysis, and the percentage of apoptotic cells was derived by counting the cells from four different fields based on two independent experiments.

**Western blot analysis**

Cells stimulated with Tat plus heparin or heparin alone were lysed in radiolabeled precipitation assay buffer (3). Total cell lysates were clarified by centrifugation at 12,000 × g for 20 min. Protein from the clarified supernatants was quantitated by the Lowry method with a Bio-Rad (Herlev, Denmark) reagent, according to the manufacturer’s instructions. Brieﬂy, 20 µg protein from each cell extract was added to the microtiter wells, and the reaction was initiated by adding 200 μM Ac-DEVD-pNA substrate. In parallel, the samples were reacted with this substrate in the clariﬁed supernatants was quantitated by the Lowry method with a Bio-Rad (Herlev, Denmark) reagent, according to the manufacturer’s instructions.

**Measurement of caspase-3 activity**

Caspase-3 activity was assayed using a caspase-3 cellular activity assay kit (Biomol, Plymouth Meeting, PA), according to the manufacturer’s protocol. Brieﬂy, 20 µg protein from each cell extract was added to the microtiter wells, and the reaction was initiated by adding 200 μM Ac-DEVD-pNA substrate. In parallel, the samples were reacted with this substrate in the presence of 0.1 µM Ac-DEVD-CHO, a specific caspase-3 inhibitor, to measure the nonspecific hydrolysis of the substrate. Absorbance was read at 37°C at 405 nm in a microtiter plate reader at the indicated time intervals. To examine the effect of the caspase-3 inhibitor on mono- and oligonucleosome release by Tat, HMVEC-L were incubated with serially diluted cell-permeable DEVD-CHO (Biomol) for 1 h, followed by stimulation with 50 ng/ml of Tat plus 10 µM heparin for 24 h. The level of apoptotic HMVEC-L was quantitated by measuring mono- and oligonucleosomes with a cell death ELISA kit (Boehringer Mannheim) as described above.

**Results**

**Tat induces apoptosis of HMVEC-L**

Internucleosomal fragmentation of cellular DNA is a hallmark of apoptosis. Thus we evaluated the effects of HIV-1 Tat on the incorporation of fluorescein into the 3′-OH of nicked chromosomal DNA (TUNEL analysis), on the fragmentation of chromosomal DNA, and on the release of mono- and oligonucleosomes from chromosomes. Microscopic analysis of fluorescein-dUTP-labeled cleaved DNA showed that treatment of HMVEC-L with Tat plus heparin induced apoptosis (Fig. 1A). When the percentage of apoptotic cells was derived by averaging the counts from four different fields based on two independent experiments, the amount of intracellular fluorescein-labeled fragmented DNA was shown to peak at 25 ng/ml (Fig. 1A). The level of cleavage at this concentration of Tat was at least 7-fold higher than in the cells treated with heparin alone, indicating that the fragmentation was due specifically to Tat. This induction of apoptosis was apparent when the cells were treated for 24 h, but did not appear before 12 h (Fig. 1B). Furthermore, treatment of HMVEC-L with Tat protein generated a significant amount of fragmented DNA, a hallmark of apoptosis (Fig. 1C), confirming that stimulation of the cells with Tat induces apoptosis.

To assess the comparative potency of Tat with a well characterized apoptotic ligand, we stimulated HMVEC-L with anti-Fas and assessed apoptosis in parallel with the Tat-treated cells. Similar to the Tat activation, the anti-Fas treatment caused DNA fragmentation (Fig. 1D) and fluorescein incorporation into the nicked chromosomal DNA (Fig. 1A). Quantitation of fluorescein-labeled cells by microscopy indicated that ~40% of the cells were stained within 6 h of anti-Fas treatment, whereas 16% of the cells were stained within 24 h of Tat treatment (Fig. 1B). Taken together, the assays indicate that HIV-1 Tat protein specifically induces apoptotic cell death in HMVEC-L.

**Tat does not induce Fas or TNF in HMVEC-L**

Activation of Fas- or TNF-receptors by Fas-L and TNF, respectively, is a well characterized trigger of apoptosis (8–11). We examined the possible role of Fas or TNF in mediating Tat-induced apoptosis. Western blot analysis indicated that HMVEC-L express Fas but not Fas-L, and that Fas expression was not significantly altered by Tat treatment (Fig. 2A). Similarly, secretion of TNF into the culture supernatants was not induced by Tat treatment of HMVEC-L, using a highly specific ELISA (R&D Systems) (data not shown). These data indicate that Tat-induced apoptosis was not mediated by Fas or TNF.

**Expression of Bcl family molecules in Tat-treated cells**

Changes in the concentration-dependent homo- or heterodimer formation of Bcl family molecules play a key role in apoptosis. Thus we examined Bcl expression after Tat treatment, by using Western blot analysis. Bcl-2 protein was not detected by this method using two different anti-Bcl-2 Abs (Fig. 2B), although these Abs were proficient in Bcl-2 detection in other tested cell lines (12–14). Interestingly, expression of Bcl-xL, another known apoptotic suppressor, was detected in HMVEC-L, but was not altered after Tat treatment (Fig. 2B). This suggested that removal of the Bcl-xL suppressor is not essential for Tat-induced apoptosis. Similarly, expression of Bax, a pro-apoptotic molecule, was unaltered by Tat. Bad, which can replace Bax in the Bax-Bcl-xL complex (thereby enhancing apoptosis), was also not detected in HMVEC-L by Western blotting (Fig. 2B). Because some of the Bcl family molecules were not detected in the Tat-treated cells but were apparent in HMVEC-L treated for 1 h with anti-Fas, we repeated this experiment by stripping and reprobing the same membrane with the indicated Abs to confirm the absence of these molecules. Consistent with the results in Fig. 2, A and B, Fas-L, Bcl-2, and Bad proteins were not detected in HMVEC-L treated with Tat for 1 h, whereas those proteins were detected in anti-Fas-treated HMVEC-L and in untreated Jurkat cells (Fig. 2C). Stimulation of the cells with either Tat or anti-Fas did not significantly change the amount of Bcl-xL. These data confirm the
results shown in Fig. 2B, in which Bcl and Bax family members do not participate in Tat-induced apoptosis in HMVEC-L.

Effect of Fas stimulation on the expression of Bcl family molecules
To further confirm the specific lack of Bcl family expression and induction by Tat, the expression of Bcl family members was examined after stimulation of HMVEC-L with anti-Fas plus protein G'. Interestingly, expression of Bcl-2 and Bad, which was not detected upon Tat stimulation, was found to be induced by anti-Fas plus protein G'. Expression of each protein was detectable after 1 h of anti-Fas stimulation (Fig. 3). It is noteworthy that the observed apoptosis by Fas ligation occurred despite appreciable amounts of induced Bcl-2, an anti-apoptotic molecule. It is possible that Fas

FIGURE 1. A. TUNEL staining of apoptotic HMVEC-L. The panels exhibit fluorescein-labeled apoptotic cells generated from HMVEC-L treated with Tat plus heparin, anti-Fas plus protein G', heparin alone, and culture medium (EGM-2 mv). B. Percentage of apoptotic cells upon treatment with Tat (a and b) or anti-Fas (c) over the indicated concentrations or time periods was derived by averaging the counts from four different fields based on two independent experiments. C. DNA fragmentation assay. Fragmented DNA separated from unfragmented chromosomal DNA was analyzed on a 1.6% agarose gel. Lanes 1–3, Electrophoretic patterns of the fragmented DNAs from HMVEC-L treated with heparin alone, HIV-1 Tat plus heparin, and anti-Fas, respectively, in low serum (EBM-2 supplemented with 0.5% FBS).
ligation gives rise to an excess of the pro-apoptotic molecule, Bax, so that Bax exhausts the available Bcl-2 (by heterodimerizing with the latter), thus resulting in a molar excess of Bax-Bax homodimers. To test this possibility, Bax expression in response to Fas stimulation was investigated. Activation of HMVEC-L with anti-Fas caused augmented Bax expression, peaking at 3 h of treatment (Fig. 3). Expression of Bcl-xL was not changed upon Fas stimulation. Taken together, these data show that Bcl-2 and Bad were not expressed in Tat-treated HMVEC-L (Fig. 2B) but were induced by anti-Fas treatment (Fig. 3), and that the relative molar concentrations of homo- and heterodimers of the Bcl family may regulate Fas-mediated apoptosis.

PARP cleavage assay

Several different pathways can cause apoptosis; some involve activation of specific caspases, whereas others do not. To test whether the caspase pathway participates in Tat-induced apoptosis in HMVEC-L, we measured apoptosis using a PARP cleavage assay. We observed basal level cleavage of the PARP precursor protein (116 kDa) into a smaller 23-kDa fragment in the unstimulated cells (Fig. 4, A and B), which might be due to the starvation of HMVEC-L in low serum culture. The amount of full-length PARP was strongly increased following Tat treatment, and the subsequent cleavage of the precursor protein was dependent in part upon the duration of Tat stimulation (Fig. 4A). Similar results were obtained when cells were treated with LPS, a known inducer of PARP degradation and apoptosis in endothelial cells (Fig. 4B). Thus, Tat-induced HMVEC-L apoptosis correlated with the extent of biochemical cleavage of PARP, a known substrate of caspases.

Analysis of caspase activity

Because PARP cleavage is activated by caspases, particularly caspase-9 and -3, we further investigated specific caspase involvement in this process. Caspase-9 expression was evaluated upon Tat treatment by immunoblot analysis. No expression of this enzyme was detected (data not shown). However, Western analysis showed that caspase-3 (32 kDa) was expressed and cleaved into 17- to 19-kDa molecules upon treatment with Tat (Fig. 5A). These data indicate that PARP cleavage resulted from activated caspase-3 but not activated caspase-9. Thus, we directly quantitated caspase-3 activity in the Tat-treated cell lysates using a caspase-3 specific activity assay kit. We found that caspase-3 activity increased in response to Tat treatment, peaking at 3 h (Fig. 5B), a corroboration of the Western analysis (Fig. 5A). However, it is unclear why a
FIGURE 5. A, Cleavage of caspase-3 by Western blot analysis. HMVEC-L were exposed to Tat plus heparin for the indicated time points. Cell extracts were subjected to Western blot analysis with an anti-caspase-3-specific Ab. The bands of caspase-3 and its cleaved product are indicated. B, Enzymatic activity of caspase-3 in Tat-treated HMVEC-L. The enzymatic activities of caspase-3 in the cell lysates were quantitated after incubation of HMVEC-L with Tat over various time periods by measuring absorbance at 405 nm in a microtiter plate reader. Data represent the fold increase in enzymatic activity over the heparin control.

Effect of the caspase-3 inhibitor, DEVD-CHO, on nucleosomal cleavage

Because Tat treatment increased caspase-3 activity, we examined whether the cell-permeable caspase-3 specific inhibitor, DEVD-CHO, abrogated Tat-induced apoptosis. To this end, endothelial cells were exposed to the indicated concentrations of this inhibitor before treatment with Tat plus heparin. The amount of mono- and oligonucleosomes generated from each sample was quantitated with a cell death ELISA kit. Nucleosomal cleavage was significantly reduced in a dose-dependent manner upon pretreatment of HMVEC-L with cell-permeable DEVD-CHO (Fig. 6). These results indicate that caspase-3 plays a major role in Tat-mediated apoptosis in HMVEC-L.

Discussion

We used HMVEC as a model to study the effects of HIV-1 Tat on small vessel endothelium. We observed significant induction of apoptosis, both by measurement of internucleosomal fragmentation and by PARP degradation. The degree of apoptosis was comparable to that induced by known apoptotic ligands such as LPS, but less than that by anti-Fas. Caspase-3, a potent “executioner” enzyme in apoptotic signaling, appeared to mediate the Tat effects on HMVEC-L.

The basic domain of Tat has homology with heparin-binding growth factors such as VEGF (15, 16). Tat can co-opt VEGF signaling pathways by its specific binding to cognate VEGF receptors. In addition, a classical RGD integrin-binding domain is found in the amino terminus of HIV-1 Tat. This moiety can activate β integrins, which normally bind to extracellular matrix proteins like fibronectin (3, 17). Therefore, cell adhesion properties can be altered by these receptor interactions with HIV-1 Tat.

HIV-1 Tat activation of Flk-1/KDR (1), FLT-1 (1, 3), and α5β1 and αvβ3 integrins (4, 16, 17) may cause cells to be “confused” by the multiplicity of pathways simultaneously activated, whereas these phenomena do not normally occur in the presence of physiological ligands. Alterations in normal growth factor receptor and adhesion receptor signaling can lead to apoptosis by such confused cell signaling (18). To test whether a profusion of inappropriate signals by the RGD motif or basic peptide of Tat causes apoptosis, the levels of apoptosis of HMVEC-L treated with these peptides were measured by quantitating mono- and oligonucleosomes released from the treated cells. Our data showed that significant chromosomal cleavage occurred when the cells were treated with high concentrations of the RGD-containing peptide (data not shown). However, at the corresponding concentrations of Tat (picomolar unit), these peptides did not induce a significant level of apoptosis (data not shown). These data suggest that the contributions of the peptide to the observed Tat-mediated apoptosis are nominal. This result implies that the signaling cascades leading to Tat-mediated apoptosis might be distinct from those leading to RGD-mediated angiogenesis and inflammation in vascular cells (1, 3, 7, 15–18).

Recent reports demonstrate that several members of the caspase family play important roles as effector molecules in endothelial apoptosis (19). For instance, TL-1, a novel tumor necrosis factor-like cytokine, induces a pathway activating caspase-3. Caspase-3 is a central component of the proteolytic cascade, which culminates in apoptosis in large vessel aortic endothelial cells (20–21). Participation of caspases has also been demonstrated in the LPS-induced apoptosis of HMVEC (22). Our study demonstrates that HIV-1 Tat induces apoptosis in HMVEC-L through PARP cleavage upon activation of caspase-3, and not through the modulation in expression of several Bcl family molecules. However, it is unclear why a second peak of caspase-3 activity appeared at 24-h treatment, a result that did not parallel with the Western analysis of procaspase-3 cleavage (Fig. 5). It is possible that if the caspase blocker lacks specificity for an individual caspase and therefore titrates other caspases, the concentration of the caspase-3 inhibitor...
would not be sufficient to maintain the observed inhibitory effect at 24 h. Additional study is required to confirm this possibility.

Interestingly, Bcl-2, Bad, and Bax proteins were detected in anti-Fas-treated, but not untreated, HMVEC-L cultures (Fig. 3). The levels of precursor PARP were also significantly higher in HMVEC-L cultures stimulated for 1 h with Tat or LPS. However, these findings must be considered in light of 1) the brevity of the stimulation period relative to the magnitude of the results, and 2) earlier reports in which the solubility (and therefore the recovery) of the relevant factors may have been affected by apoptotic subcellular redistribution (23–26), a phenomenon that could account for the increases observed in the present study.

Endothelial cell injury, teleologically, would act in the interests of the virus by disturbing a component of innate immunity, that of the endothelial barrier, thus allowing the passage of pathogens between the circulation and tissue compartments. In the particular type of microvascular endothelium that we examined (derived from the lung), this Tat-mediated apoptosis could facilitate the egress of HIV virions and HIV-infected cells as they take residence in lung parenchyma. Because different forms of endothelium vary with regard to expression of important cell surface receptors as well as growth properties, it will be necessary to examine microvascular cells derived from other tissue sources, particularly brain, to elucidate mechanisms whereby HIV may transit into other tissue sites. Data (from T. Kim and S. Avraham, unpublished observations) indicate that brain endothelium, but not bone marrow endothelium, respectively, is susceptible to Tat-mediated apoptosis but not in cell cycle progression. J. Immunol. 160:3562.


Acknowledgments
We thank Janet Delahanty for editing this manuscript and Dan Kelley for preparation of the figures.

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