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Human immunodeficiency virus type 1 TAT protein induces adhesion molecule expression in astrocytes

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AIDS encephalitis is a frequent consequence of CNS HIV infection, especially in children. One of its many characteristics is a leukocyte infiltrate that is believed to contribute to the production of cytokines, chemokines and neurotoxic factors resulting in CNS damage. Entry of such leukocytes into the CNS is mediated in part by the expression of adhesion molecules by blood–brain barrier (BBB) endothelial cells. Expression of these proteins by astrocytes, the other main component of the BBB, also serves to target leukocytes to the CNS parenchyma. We now demonstrate that HIV-1-derived Tat, a soluble protein secreted by infected cells, induced astrocyte VCAM-1 and ICAM-1 expression in a dose- and time-dependent manner. The functional role of Tat in monocyte binding *in vitro* was also demonstrated. These data suggest that the presence of extracellular Tat may be a significant factor in the trafficking of HIV-infected and inflammatory cells into the CNS via its effect on adhesion molecule expression by astrocytes.

Keywords: Tat; astrocytes; HIV-1; ICAM-1; VCAM-1

Introduction

Up to one-third of adults and half of children with AIDS eventually have neurological complications as a direct result of infection by human immunodeficiency virus-1 (HIV-1) (Lipton and Gendelman, 1995). HIV encephalitis (HIVE), is a pathological correlate of AIDS Dementia Complex (ADC), and is characterized by a leukocytic infiltrate (Weidenheim *et al*, 1993; Dickson *et al*, 1994). A mechanism by which leukocytes extravasate out of the vasculature into the CNS parenchyma involves the expression of cell adhesion molecules by the blood–brain barrier (BBB) which is composed both structurally and functionally of endothelial cells and astrocytes (Hurwitz *et al*, 1993). The expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) by both endothelial cells and astrocytes has been demonstrated under inflammatory conditions (Rossler *et al*, 1992; Hurwitz *et al*, 1992; Lee *et al*, 1998; Rosenman *et al*, 1995) such as that exhibited in the milieu of HIVE. This *in vitro* data is supported by the increased density of ICAM-1 and VCAM-1 positive endothelial cells and astrocytes in the brains of ADC patients (Seilhean *et al*, 1997). These adhesion molecules may be involved in the initial seeding of the CNS, as HIV-1 infection of monocytes also results in an upregulation of their adhesion molecule expression (Nottet *et al*, 1996). Thus, astrocyte adhesion molecule expression likely plays an integral role in facilitating the trafficking of leukocytes into the CNS parenchyma subsequent to their initial binding to cerebral vascular endothelial cells. This infiltrate is thought to contribute to the production of cytokines, chemokines, and neurotoxic factors resulting in CNS damage (for review see Kolson *et al*, 1998).

Tat is an HIV-1 encoded transactivating protein involved in upregulating HIV-1 replication (Arya *et al*, 1985). Tat has been shown to be released from infected microglial cells (Tardieu *et al*, 1992) and lymphocytic cell lines (Ensoli *et al*, 1990; Chang *et*

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al, 1997), and therefore may potentially serve as a soluble factor able to act on uninfected cells. Although the amount of extracellular Tat present in HIV-1 infected brains has yet to be determined, its transcripts are elevated in HIV-1 demented brains (Wesselingh *et al*, 1993; Wiley *et al*, 1996), and its presence has been described in the serum of HIV-1 infected individuals (Westendorp *et al*, 1995; Piller *et al*, 1998). Tat has been reported in a variety of non-CNS cell types to modulate the expression of: TNFα, TNFβ, IL-2, IL-6, TGF-β, CXCR4, CCR5, IL-2 receptors, IL-4 receptors, and TNFα receptors (Sastry *et al*, 1990; Lotz and Seth, 1993; Buonaguro *et al*, 1992; Gibellini *et al*, 1994; Scala *et al*, 1994; Opalenik *et al*, 1995; Puri *et al*, 1992; Husain *et al*, 1996; Weiss *et al*, in press). In CNS cells, Tat has been shown to induce the expression of TNFα (Chen *et al*, 1997), TGF-β, (Sawaya *et al*, 1998), IL-6 (Zidovetzki *et al*, 1998), MCP-1, MIP-1α, MIP-1β (McManus *et al*, in press; Conant *et al*, 1998), and MCP-1 induced monocyte transmigration (Weiss *et al*, in press). Interestingly, Tat has also been demonstrated to have CCR2 receptor agonist potential (Albini *et al*, 1998). Tat can have a toxic (Sabatier *et al*, 1991; Nath *et al*, 1996) or apoptotic (New *et al*, 1997) effect on neurons. The induction of adhesion molecules on human endothelial cells after Tat treatment has also been observed (Dhawan *et al*, 1997).

Astrocytes, comprising the majority of the perivascular glia limitans of the BBB, are poised to facilitate leukocyte trafficking into the CNS. As such, this investigation examined the effect of Tat on astrocyte adhesion molecule induction. The results demonstrate that Tat induces astrocyte ICAM-1 and VCAM-1 expression in a time- and dose-dependent manner. This is consistent with the hypothesis that extracellular Tat plays an important role in the CNS pathology of ADC by modulating the expression of a number of proinflammatory molecules.

Results

Adhesion molecule protein and mRNA expression

To determine whether Tat induces ICAM-1 or VCAM-1 protein expression, cells were treated at different time intervals with either 10 or 100 ng/ml of Tat at 37°C, and analyzed by cell-based ELISA. A significant increase ($P<0.05$) in the expression of both ICAM-1 and VCAM-1 was seen after a 16 h treatment with 100 ng/ml of Tat, and a maximal increase ($P<0.05$) in expression for both ICAM-1 (fivefold) and VCAM-1 ($\sim$fourfold) was observed after a 24 h treatment with 100 ng/ml of Tat (Figure 1). Immunoelution of 100 ng/ml of Tat with polyclonal rabbit anti-Tat at a 1 : 50 dilution before treatment abrogated ($P<0.05$) Tat-induced adhesion molecule expression after 24 h (Figure 2). ICAM-1 and VCAM-1 mRNA expression also increased after 100 ng/ml of Tat treatment for 16 h at 37°C, which correlates with the observation of protein expression at 16 and 24 h (Figure 3a,b). Likewise, the concentration of Tat necessary to induce expression was the same at both the protein and mRNA levels. Expression of E-selectin protein was also assayed after 100 ng/ml of Tat treatment

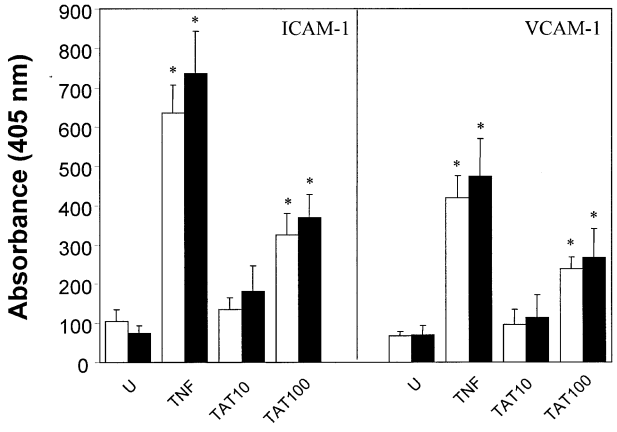

Figure 1 Cell-based ELISA demonstrating Tat-induced ICAM-1 and VCAM-1 expression by astrocytes. Astrocytes were plated in 96 well dishes, grown for 2 days, treated with 10 ng/ml (TAT 10) or 100 ng/ml (TAT 100) of Tat or 400 U/ml of TNFα for 16 h (□) or 24 h (■). Cells were washed, fixed and analyzed as described in Materials and methods. Maximal ICAM-1 and VCAM-1 induction was achieved with 100 ng of Tat (n=3–7, $P<0.05$). Asterisks represent significance as compared to untreated cells.

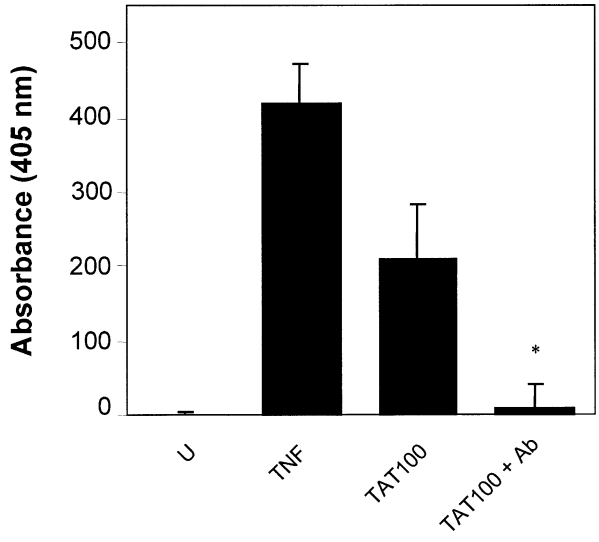

Figure 2 Adhesion molecule expression is not induced after immunoelution of Tat. Astrocytes were treated for 24 h with 100 ng/ml of Tat (TAT100) or 100 ng/ml of Tat after being immunoeluted with anti-Tat antibody (TAT100+Ab) as described in Materials and methods. Adhesion molecule expression was significantly abrogated by immunoelution of Tat (n=3, $P<0.05$). Asterisk represents significance as compared to TAT 100 treated cells.

and no significant increase was observed (data not shown).

Treatment with 10 ng/ml of Tat performed at 16 and 24 h showed a trend toward an increase in ICAM-1 and VCAM-1 protein expression; however, statistical significance was not met (Figure 1). Five, 8 and 12 h treatments with 10 and 100 ng/ml of Tat failed to show a significant increase (data not shown).

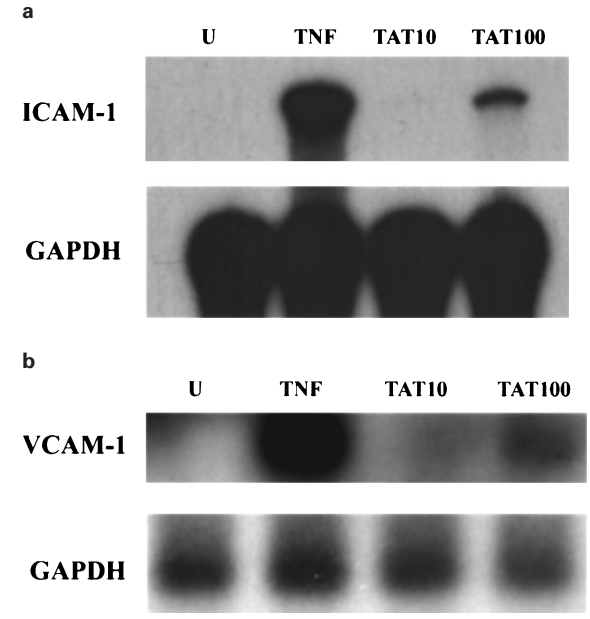

Figure 3 ICAM-1 and VCAM-1 mRNA induced after 16 h of Tat treatment. Astrocytes were treated with 10 ng/ml (TAT 10) and 100 ng/ml (TAT 100) of Tat for 16 h. A significant increase in both ICAM-1 (**a**) and VCAM-1 (**b**) mRNA expression was observed after the 100 ng/ml Tat treatment.

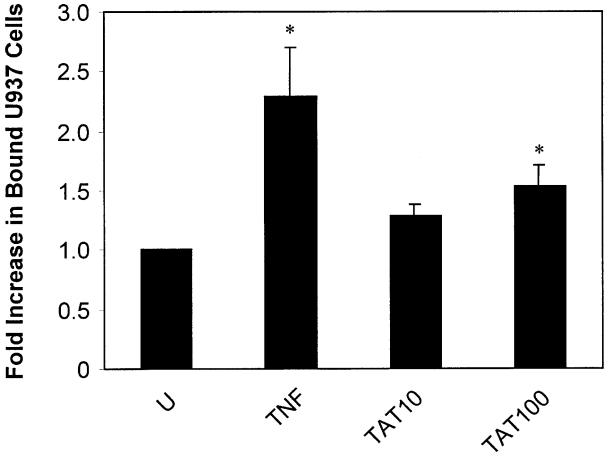

Figure 4 Tat-treated astrocytes bind increased numbers of U937 cells. Astrocytes were treated with 10 ng/ml (TAT 10) or 100 ng/ml (TAT 100) of Tat for 24 h. ^{51}Cr-labeled U937 cells were then added to the cells and allowed to bind for 1 h. A significant increase in U937 cell adhesion to 100 ng/ml Tat-treated cells was observed (n=5, $P<0.05$). Asterisks represent significance as compared to untreated cells.

Monocyte adhesion

To determine if Tat induced expression of astrocyte ICAM-1 or VCAM-1 plays a functional role in monocyte adhesion *in vitro*, binding assays were performed. The adhesion of U937 cells to astrocytes significantly increased ($P<0.05$) after treatment of these glial cells with 100 ng/ml of Tat for 16 h (Figure 4). Treatment with 10 ng/ml failed to significantly increase U937 binding.

Discussion

In this study the effect of HIV-1 Tat on astrocyte adhesion molecule expression was examined. It was demonstrated that both ICAM-1 and VCAM-1 expression was induced in a time- and dose-dependent manner. In addition, treatment of astrocytes with Tat resulted in an increased binding of U937 cells, providing a functional correlate for the observed Tat-induced modulation of expression. These results further implicate Tat as having direct involvement in primary HIV-1 CNS pathology apart from its transactivating role. The importance astrocytes may have in trafficking HIV-1 infected and inflammatory cells into the CNS has also been indicated by this study.

Tat has been demonstrated to induce ICAM-1 and VCAM-1 expression by endothelial cells (Dhawan *et al*, 1997). It was found that endothelial ICAM-1 expression was maximally induced by 6 h with 100 ng/ml Tat treatment, whereas endothelial VCAM-1 expression did not reach maximum even after 12 h and was inducible with 10 ng/ml of Tat. The present study did not demonstrate a statistically significant induction of ICAM-1 expression prior to 16 h treatment with 100 ng/ml of Tat, and peak levels of expression for both adhesion molecules were observed at 24 h with 100 ng/ml of Tat, although relatively high levels of expression were seen at 16 h. This suggests that there may be a cell-type specific adhesion molecule response to Tat exposure.

Expression of ICAM-1 has also been demonstrated on astrocytes in response to IL-1β and TNFα, both of which are increased by INFγ (Hurwitz *et al*, 1992; Lee *et al*, 1998). Likewise, expression of VCAM-1 has been demonstrated on astrocytes in response to TNFα, and is increased by INFγ (Hurwitz *et al*, 1992; Rosenman *et al*, 1995). The study of Hurwitz *et al* (1992) documented TNFα-induced ICAM-1 protein expression as early as 4 h, with peak expression of ICAM-1 and VCAM-1 at 16 h, after TNFα treatment. E-selectin expression was also induced by TNFα. Tat treatment of astrocytes exhibits different kinetics than cytokine treatment and failed to induce E-selectin expression.

The mechanism by which Tat induces astrocyte ICAM-1 and VCAM-1 expression has not yet been determined. The present study demonstrates that only the first 72 amino acids of Tat, comprising the first exon, are necessary to induce astrocyte adhesion molecule expression. It has been proposed that Tat may bind integrin receptors, namely $\alpha_5\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$ (Brake *et al*, 1990; Barillari *et al*, 1993; Vogel *et al*, 1993), possibly resulting in the direct stimulation of cell function. Relevant to the present study, $\alpha_v\beta_5$ has been shown to bind to the basic domain of Tat, located within the first 72 amino acid sequence (Vogel *et al*, 1993). Integrin receptors may not mediate cellular uptake, however, as it has been demonstrated that putative Tat binding sites and antisera to the aforementioned integrins fail to inhibit Tat uptake in astrocytes (Ma and Nath, 1997). The basic domain of Tat has also been shown to bind to and activate the Flk-1/KDR receptor (Albini *et al*, 1996). The possibility of non-receptor mediated endocytic cellular uptake of Tat must also be considered (Mann and Frankel, 1991), although there is evidence for receptor-mediated uptake of Tat in astrocytes (Ma and Nath, 1997).

Treatment of astrocytes with extracellular Tat results in an increase in intracellular calcium (Nath *et al*, 1996), protein kinase C activity and NFκB binding (Conant *et al*, 1996) all of which have been implicated in cytokine production and cell proliferation. Tat may also induce astrocyte adhesion molecule expression by an indirect mechanism through the induction of cytokines (Chen *et al*, 1997), although it has been shown that neither TNFα nor IL-1β can be detected in astrocyte culture supernatants following Tat treatment (Nath *et al*, 1999). Studies to analyze the mechanism of Tat action on astrocyte adhesion molecule expression are a current focus of our research.

Materials and methods

Fetal tissue and cell culture

This study is part of an ongoing research protocol that has been approved by the Albert Einstein College of Medicine Committee on Clinical Investigation and the City of New York Health and Hospitals Corporation. CNS tissue was obtained under the auspices of the Neuropathology Fetal Tissue Bank from 20–23 week-old human fetuses at the time of elective termination of intrauterine pregnancy from healthy females. Informed consent was obtained from all participants. Astrocyte cultures were prepared as previously described (McManus *et al*, 1998). Briefly, human fetal CNS tissue was separated from the meninges, minced, and digested in 1 × trypsin (GibcoBrl, Grand Island, NY, USA), 1 × HBSS (GibcoBrl) and DNase1 (Boehringer-Mannheim, Indianapolis, IN, USA) at 37°C for 45 min. The resulting cell suspension was filtered through 250 and 150 micron nylon mesh (Tetko, Inc., Briar Cliff Manor, NY, USA). Cells were pelleted at 1000 r.p.m., washed, and resuspended in complete DMEM (25 mM HEPES, 10% fetal calf serum, 1% non-essential amino acids, and 1% penicillin-streptomycin). Cells were plated at 1.2×10^8 cells/175 cm^2 tissue culture flask (Falcon, Becton Dickinson, Franklin Lakes, NJ, USA). After 12 days in culture, microglia were removed by shaking the flasks at 4°C for 30 min. Astrocytes, which remain adherent to the flask, were incubated in RPMI (10% fetal calf serum and 1% penicillin-streptomycin), then trypsinized, passaged onto 100 mm dishes, and grown to confluency at least three times. Astrocyte cultures prepared as above are $\geqslant$95% GFAP (an astrocyte marker) positive. Astocytes were treated with 400 U/ml TNFα, as a positive control, 10 or 100 ng/ml Tat protein for 5, 8, 12, 16, 24, and 48 h.

Reagents

Human recombinant TNFα was purchased from R&D Research (Minneapolis, MN, USA). Protein A agarose beads were obtained from GibcoBrl. Recombinant Tat protein was prepared as previously described (Ma and Nath, 1997). Briefly, the first exon of the Tat gene (corresponding to amino acids 1–72) was expressed as a fusion protein in *Escherichia coli* DH5αf'1Q (Gibco-Bri), and purified with a metal chelate affinity column. Tat was diluted in buffer (50 mM Tris, pH 8.0; 100 mM NaCl; 1 mM $CaCl_2$ and 0.5 mM DTT), and endotoxin levels were shown by Limulus assay (Biowhitaker, Walkersville, MD, USA) to be less than 1 ng/ml.

Poly A$^+$ RNA isolation, Northern blot and RNase protection assay (RPA) analysis

Poly A$^+$ RNA was extracted from Tat-treated astrocytes using the Micro-FastTrack mRNA Isolation kit (Invitrogen, San Diego, CA, USA). For Northern analysis, RNA samples were denatured and fractionated on 1% agarose gels prepared in 6.5% formaldehyde/1X MOPS buffer. After electrophoresis, RNA was transferred to a nylon filter and hybridized with a VCAM-1 ^{32}P random primer-labeled (Amersham Corp., Arlington Heights, IL, USA), cDNA probe or with a ^{32}P-labeled GAPDH cDNA probe. For RPA analysis, RNA samples were hybridized with both human ICAM-1 and GAPDH riboprobes (2.5×10^4 c.p.m.) at 42°C overnight in 20 μl of 40 mM PIPES, pH 6.4, 80% deionized formamide, 400 mM NaOAc, and 1 mM EDTA. The hybridized mixture was then treated with RNase A/T1 (1 : 100 dilution in 200 μl of Rnase digestion buffer) at 37°C for 30 min, and analyzed by 5% denaturing (8 M urea) polyacrylamide gel electrophoresis (PAGE). The protected fragments of the ICAM-1 and GAPDH riboprobes are 489 and 230 nucleotides in length, respectively. Quantification was performed by scanning with the Phosphoimager (Molecular Dynamics, Sunnyvale, CA, USA),

and values for ICAM-1 and VCAM-1 were normalized to GAPDH mRNA levels.

Cell-based ELISA analysis
Astrocytes were seeded onto 96 well plates, grown for 2 days, and treated. Tat-treated astrocytes were fixed in 100% methanol at 4°C for 30 min. The cells were then incubated in 1% BSA in PBS for 1 h to block nonspecific binding of antibody. This was followed by the addition of monoclonal mouse anti-human VCAM-1 or monoclonal mouse anti-human ICAM-1 antibodies (DAKO Corp., Carpinteria, CA, USA), each in a 1 : 1000 dilution of 1% BSA in PBS, at 37°C for 1.5 h. Cells were then incubated in goat anti-mouse IgG_1 alkaline phosphatase (Southern Biotechnology, Birmingham, AL, USA), also at a 1 : 1000 dilution, at 37°C for 1.5 h. P-Nitrophenyl Phosphate Disodium Salt at 0.5 mg/ml (Pierce, Rockford, IL, USA) in 1X diethanolamine buffer (Pierce) was used as the substrate. Absorbance was measured at OD 405 nm.

Immunoelution of Tat
Protein A agarose beads were pelleted, washed, and purified rabbit anti-Tat (1 : 50 dilution) was added and incubated for 1 h at room temperature. After washing, Tat was added at the treatment concentration for 1 h at room temperature. Following centrifugation, the supernatant was used in the ELISA studies.

U397 cell binding assay
U937 cells, a promyelomonocytic cell line, were incubated in RPMI media containing 500 μCi of ^{51}Cr (Amersham, Arlington Heights, IL, USA) at 37°C for 1 h, pelleted at 1000 r.p.m., washed and resuspended in RPMI medium. ^{51}Cr labeled U937 cells (2×10^5 cells) were added to Tat-treated astrocytes at 37°C for 1 h. Nonadherent U937 cells were removed by multiple washes with RPMI medium. Cells were then lysed by adding 150 μl/well of 1 N NaOH, and gamma counts were determined for each well. The percentage of binding was assessed as the number of counts in each well divided by the total number of counts originally added to each well.

Statistical analysis
Statistical significance was determined using the Wilcoxan Signed Rank test. Significance was assigned at $P < 0.05$ (StatView, Abacus Concepts, Berkeley, CA, USA).

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