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Tumor necrosis factor alpha leads to increased cell surface expression of CXCR4 in SK-N-MC cells

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Both host and viral factors play an important role in the pathogenesis of human immunodeficiency virus (HIV)-associated bran injury. In this study, the authors examined the interactions between tumor necrosis factor (TNF)-α, CXCR4, the alpha chemokine receptor, and three HIV isolates, including the T-tropic viruses, HIV-1_{MN} and HIV-1_{IIIB}, and the dual tropic virus, HIV-$1_{89.6}$. The authors show by flow cytometry that treatment of differentiated SK-N-MC cells with TNF-α induces a significant increase in the cell surface expression of CXCR4 in a time- and dose-dependent manner. The effect is partly regulated at the level of transcription. To assess the biological significance of this finding, we show that TNF-α potentiates the ability of the above mentioned HIV isolates to induce neuronal apoptosis and that the effect is significantly reduced by pretreating cells with monoclonal antibodies to either CXCR4 and TNF-α. Together these results suggest that TNF-α may render neuronal cells vulnerable to the apoptotic effects of HIV by increasing the cell surface expression of CXCR4 and thus identify another mechanism by which TNF-α contributes to the pathogenesis of HIV-associated brain injury. *Journal of NeuroVirology* (2005) **11,** 247–255.

Keywords: AIDS; chemokine receptors; CXCR4; HIV dementia; TNF-α

Introduction

HIV infection of the central nervous system results in a wide spectrum of neurological and neuropathological abnormalties that include perivascular/parenchymal collections of inflammatory cells, mostly composed of macrophages, white matter pallor, and neuronal apoptosis in the cortex and basal ganglia (Navia *et al*, 1986a, 1986b; Glass *et al*, 1993; Rostasy *et al*, 1999).

As neurons are not directly infected by human immunodeficiency virus (HIV), neuronal injury and apoptosis are likely related to the effects of monocyte/glia-released viral and host factors, which include HIV proteins (gp120, gp41, and Tat), inducible nitric oxide synthase (iNOS), protease resistant molecules, chemokines (e.g., stromal derived factor-1α [SDF-1α], monocyte chemoattractant protein-1) and the cytokines, notably tumor necrosis factor (TNF)-α (Wesselingh *et al*, 1993; Yeung *et al*, 1995; Adamson *et al*, 1996; Chen *et al*, 1997; He *et al*, 1997; Conant *et al*, 1998; New *et al*, 1998; Kaul and Lipton, 1999; Rostasy *et al*, 1999, 2003). CXCR4, the

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cognate receptor for the alpha chemokine SDF-1α mediates the entry of T-tropic viruses (Oberlin *et al*, 1996). Its expression in the mammalian brain is localized to neurons, astrocytes, and microglia cells (Klein *et al*, 1999; Lavi *et al*, 1997). Further, several studies have also shown that CXCR4 can mediate the neurotoxic effects of gp120 and the ability of T-tropic strains of HIV to cause neuronal apoptosis (Hesselgesser *et al*, 1998; Meucci *et al*, 1998; Ohagen *et al*, 1999; Zheng *et al*, 1999a, 1999b; Catani *et al*, 2000; Keswani *et al*, 2003).

TNF-α, which is produced and secreted from inflammatory cells, induces a broad range of cellular responses ranging from induction of other cytokines, up-regulation of HIV, and apoptosis (Talley *et al*, 1995; Shi *et al*, 1998). Levels of protein and mRNA expression correlate with the severity of the Aids Dementia complex (ADC) (Wesselingh *et al*, 1997; Rostasy *et al*, 2000). *In vitro* studies have shown that TNF-α either mediates or potentiates the neurotoxic effects of Tat and gp120 (Yeung *et al*, 1995; Chen *et al*, 1997; Shi *et al*, 1998, New *et al*, 1998). Several studies also suggest that TNF-α can influence the cell surface expression of chemokine receptors such as CXCR4. Depending on the cell type, TNF-α can increase the cell surface density of CXCR4 (U937 cells) or down-regulate CXCR4 receptor expression (endothelial cells) (Biswas *et al*, 2001; Gupta *et al*, 1998). The influence of TNF-α on neuronal expression of CXCR4, however, has not been previously studied.

Results

TNF-α and cell surface expression of CXCR4

To determine the effects of TNF-α on CXCR4 receptor expression, differentiated SK-N-MC cells were treated with increasing concentrations of TNF-α (1 ng, 5 ng, and 10 ng/ml) for 1, 24, and 48 h (Figures 1 and 2). In general, TNF-α led to a concentration- and time-dependent increase of CXCR4 expression compared with untreated cells. A significant increase in cell surface CXCR4 expression was noted for 1 ng/ml, 5 ng/ml, and 10 ng/ml TNF-α after 48 h of treatment ($P = .033$, $P = .048$, $P = .028$, respectively) and after 1 and 24 h of treatment, respectively, with 10 ng/ml ($P = .015$ and $P = .017$; Figure 2). CXCR4 expression was not significantly changed at these two time points with either 1 ng/ml ($P = .09$ and $P = .14$, respectively) or 5 ng/ml TNF-α ($P = .07$ and $P = .15$, respectively). Cells treated with 5 ng/ml TNF-α at 48 h induced greater expression compared to cells treated for 24 h ($P = .003$). There was no significant change in CXCR4 expression when comparing cells from different time points treated with 1 ng/ml TNF-α or between cells treated with 1 ng/ml and those with 5 ng/ml. A trend towards an increase in CXCR4 expression was observed when comparing cells treated with 10 ng/ml at 48 h to cells treated for 1 h ($P = .06$) and for 24 h ($P = .07$) at the same concentration. Further, cell treated at 10 ng/ml for 48 h showed significantly greater CXCR4 expression when compared to cells treated with 1 ng/ml ($P = .011$) or 5 ng/ml ($P = .022$) for 1 h.

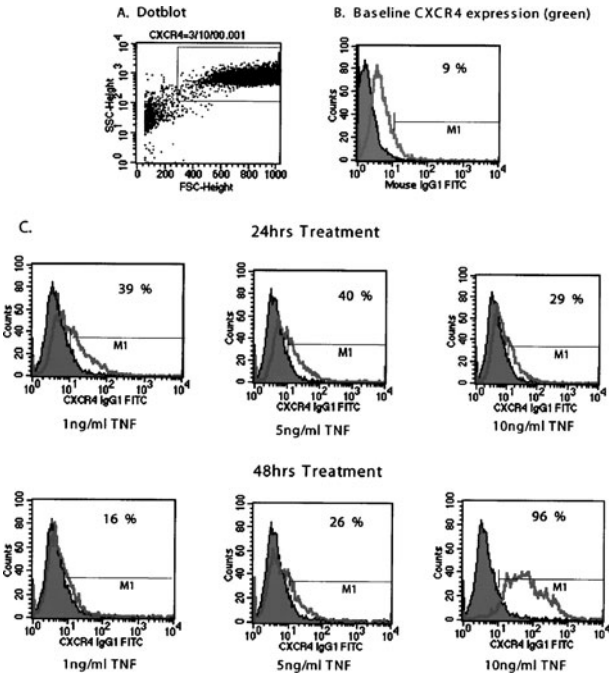

Figure 1 CXCR4 expression in response to three different concentrations of TNF-α (1, 5, 10 ng/ml) at the 24 and 48 h determined by flow cytometry. (**A**) Scattergram and (**B**) Baseline CXCR4 cell surface receptor expression of SK-N-MC cells (grey peak). The grey peak represents unlabeled differentiated cells. (**C**) Increase in CXCR4 expression in response to TNF-α at various concentrations at 24 and 48 h.

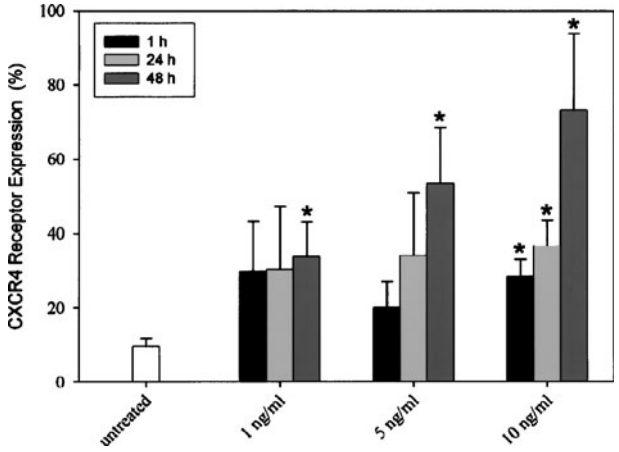

Figure 2 Average cell surface expression of CXCR4 in response to TNF-α treatment as determined by FACS analysis. Increased expression of CXCR4 cell surface receptors was noted for all three TNF-α concentrations and all three time points (1, 24, 48 h). All data represent percentages of CXCR4 expression above controls (untreated, unlabeled) calculated as mean ± SE values from three different experiments (*$P < .05$ by Student *t* test, compared with untreated controls).

CXCR4 mRNA levels measured by RT-PCR
To address whether the expression of CXCR4 is regulated at the level of transcription, messenger RNA was isolated from treated and untreated cells at each time point for the different concentrations tested by fluorescence-activated cell sorting (FACS) and analyzed by semiquantitative reverse transcriptase–polymerase chain reaction (RT-PCR). In general, there was no significant increase of CXCR4 mRNA levels in cells treated with 1 ng/ml or 5 ng/ml. Treatment with 10 ng/ml TNF-α for 48 h induced a trend towards increased CXCR4 transcription ($P = .06$) (data not shown).

TNF-α and HIV-1 isolates
As the above experiments showed that TNF-α increases the cell surface expression of CXCR4, we hypothesized that TNF-α would render cells more susceptible to neuronal injury when exposed to the T-tropic isolates HIV-$1_{MN,}$ HIV-1_{IIIB} and the dual-tropic HIV-$1_{89.6}$. Differentiated SK-N-MC cells were exposed to each of these isolates for 24 h without pretreatment with TNF-α. Exposure to the HIV-1_{MN} isolate resulted in the greatest number of apoptotic cells (14%) compared to untreated cells (2%), ($P = .001$), whereas HIV 1_{IIIB} (8%) and HIV-$1_{89.6}$ (7.5%) caused a similar degree of apoptosis ($P = .04$). The odds of apoptosis was 1.53 greater in the HIV-1_{MN} compared to HIV-1_{IIIb} treated cells (95% confidence interval [CI] 1.02–2.31; $P = .040$, Mantel-Haenszel [M-H] approach) and 1.86 or almost double that observed in the HIV-$1_{89.6}$ exposed cells (95% CI 1.21–2.86; $P = .004$, M-H approach).

In a second set of experiments, differentiated SK-N-MC cells were pretreated with 10 ng/ml TNF-α for 48 h and then exposed to the same panel of HIV isolates for 24 h. As shown in Figure 3, the addition of TNF-α further increased the number of apoptotic neurons but in a strain-dependent manner. The TNF-α/HIV-1_{MN} treatment showed a nearly twofold increase compared to cells treated with virus alone. The odds of apoptosis induced in the TNF-α/HIV-1_{MN} cells was 1.94 compared to that treated with HIV-1_{MN} alone (95% CI 1.38–2.72; $P < .0001$, M-H approach). Exposure to TNF-α led to a similar increase in apoptosis of HIV-$1_{89.6}$–treated cells ($P = .0001$), whereas the increase in TNF-α/HIV-1_{IIIB}–treated cells was less pronounced compared to HIV-1_{IIIB}–treated cells alone (14% versus 8%, $P = .003$). When comparing TNF-α/HIV-1_{MN}–TNF-α/HIV-1_{IIIb}–treated cells, the odds of apoptosis was 1.69 times in the HIV-1_{MN}–versus the HIV-1_{IIIb}–treated cells (95% CI 1.21–2.34, $P = .002$, M-H approach), whereas the odds difference when comparing TNF-α/HIV-1_{MN} to TNF-α/HIV-$1_{89.6}$ treatments was not significant. Further analysis showed a 10% increase in the absolute number of apoptotic cells or an overall increase of 132% in TNF-α/HIV-$1_{89.6}$–treated cells compared to an increase of 9.5% or an overall increase of 80% in response to TNF-α/HIV-1_{MN} treatment. This finding suggests that the effect of TNF-α was greater with HIV-$1_{89.6}$ than with HIV-1_{MN}.

TNF-α, CXCR4, and HIV-1
To further assess the role of TNF-α and CXCR4 in mediating the apoptotic effects of HIV, SK-N-MC cells were treated with monoclonal antibodies (Ab) to CXCR4 or TNF-α followed by exposure to (1) HIV-1_{MN} isolate alone, (2) TNF-α alone, or (3) HIV-1_{MN} and TNF-α as detailed in Methods. We chose the HIV-1_{MN} strain because it proved to be most neurotoxic among the three isolates.

As observed above, the combination of TNF-α and HIV-1_{MN} induced greater apoptosis compared to cells treated with either agent alone (TNF-α/HIV-1_{MN} versus TNF-α alone: $P = .05$; TNF-α/HIV-1_{MN} versus HIV-1_{MN}: $P = .01$) (Figure 4). Pretreatment of SK-N-MC cells with CXCR4 Ab alone prior to exposure to HIV-1_{MN} led to a significant reduction in the number of apoptotic cells (8.3%, $P = .05$) compared to cells exposed to HIV-1_{MN}, suggesting that CXCR4 partly mediates the neurotoxic signal of the virus, consistent with previous reports (see below).

To assess the role of TNF-α in potentiating the neurotoxic effects of HIV-1_{MN}, cells were pretreated with TNF-α and a TNF-α monoclonal antibody for 48 h prior to exposure to HIV-1_{MN}. Cells treated in this manner showed a similar degree of apoptotic cells when compared to cells exposed to HIV-1_{MN} alone (11.6% versus 13%) and when compared to cells treated with TNF-α alone (11.6% versus 10.5%), but showed significantly reduced apoptosis when compared to TNF-α/HIV-1_{MN}–treated cells (11.6% versus 20%, $P = .01$).

The degree of apoptosis in SK-N-MC cells, pretreated with TNF-α and exposed to HIV-1_{MN} virus, was compared to cells preincubated with CXCR4

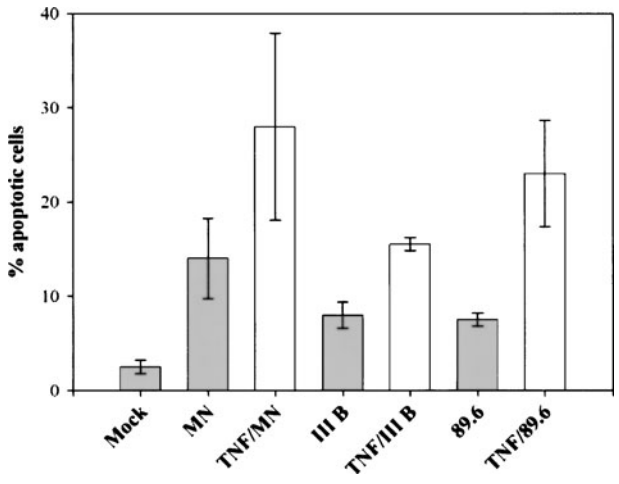

Figure 3 Synergistic effects of HIV and TNF-α on neuronal apoptosis as measured by TUNEL staining in SK-N-MC cells. Pretreatment with TNF-α for 48 h and subsequent exposure to three different HIV isolates (HIV-$1_{MN,89.6,IIIB}$) for 24 h led to a significant increase in neuronal apoptosis. The effect was greatest for HIV-1_{MN}.

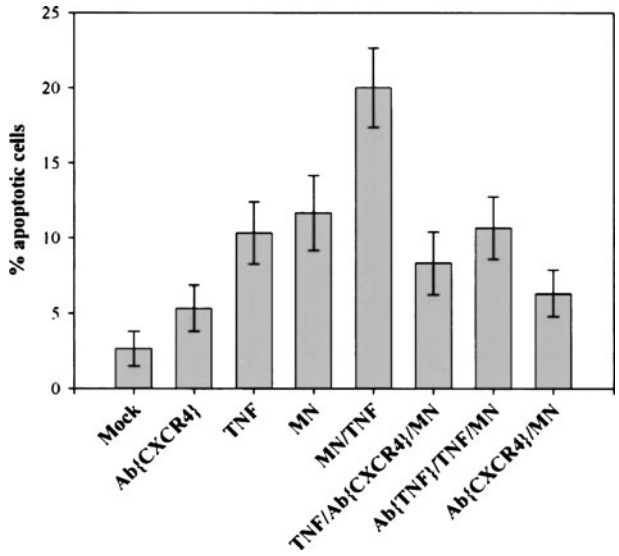

Figure 4 Apoptosis in cells pretreated with $CXCR4_{MAB}$ (lane 2), TNF-α (lane 3), or HIV-1_{MN} alone (lane 4) in cells pretreated for 48 h with TNF-α and then exposed for 24 h to HIV-1_{MN} virus (lane 5), cells pretreated for 48 h with TNF-α and with CXCR4 monoclonal antibody (Ab) for 30 min prior to exposure to HIV-1_{MN} virus for 24 h (lane 6), cells treated with a mixture of TNF-α and TNF-αAb for 30 min and subsequent to exposure to HIV-1_{MN} virus for 24 h (lane 7), cells treated with CXCR4Ab for 30 min prior to the exposure to HIV-1_{MN} virus for 24 h (lane 8). TNF-α pretreatment and subsequent HIV-1_{MN} exposure (lane 4) induced greater apoptosis compared to cells treated with either agent alone. Addition of CXCR4Ab significantly reduces the amount of apoptosis (8%, lane 6) in comparison to cells pretreated with TNF-α and then exposed to HIV-1_{MN} (20%, lane 4). All data represent the average values calculated as mean $\pm$ SE of three different experiments.

Ab prior to exposure with HIV-1_{MN} and TNF-α. As shown in Figure 4, treatment with CXCR4 Ab significantly reduced the number of apoptotic cells (8.0%) compared to TNF-α/HIV-1_{MN}–treated cells (20%, $P = .003$).

Discussion

Both host and viral factors play an important role in the pathogenesis of HIV-associated bran injury. Few studies, however, have addressed the interactions between the two or how they may lead to neuronal injury in the HIV-infected brain. This study demonstrates that exposure to TNF-α leads to an increase in CXCR4 cell surface receptor expression in a human neuronal cell line. We also show that pretreatment with TNF-α renders neuronal cells more susceptible to injury when exposed to HIV isolates, and that this effect is in part due to an increase in CXCR4 receptor expression.

Of particular interest is the role of CXCR4 in the disease process. Expression of CXCR4 has been found in microglia, astrocytes, and subpopulations of neurons (Klein *et al*, 1999; Cota *et al*, 2000; Croitoru-Lamoury *et al*, 2003) and shown to mediate the neurotoxic effects of HIV, gp120, and SDF-1α, its ligand (Hesselgesser *et al*, 1998; Meucci *et al*, 1998; Kaul and Lipton, 1999; Ohagen *et al*, 1999; Zheng *et al*, 1999a; Catani *et al*, 2000). Of relevance to the current study is the observation that up-regulation of CXCR4 correlates with neuronal loss in the hippocampus in patients with advanced ADC (Petito *et al*, 2001). Together these findings suggest that increased expression of CXCR4 may provide a key step in the pathogenesis of HIV-related neuronal loss.

In view of these observations, several investigators have studied the regulation of CXCR4 expression in response to different stimuli. Fibroblast growth factor, for example, decreases neuronal CXCR4 levels in a dose-dependant manner (Sanders *et al*, 2001). Different viral proteins and cytokines (Tat, gp41, interleukin [IL]-10, TNF-α) have been shown to cause an increase or decrease of CXCR4 cell surface expression depending on the cell type (Gupta *et al*, 1998; Speth *et al*, 2000; Han *et al*, 2001; Croitoru-Lamoury *et al*, 2003; Kury *et al*, 2003). Our results extend these observations as they show that TNF-α can increase the expression of CXCR4 in a neuronal cell line in a time- and dose-dependent manner. Most studies have shown that CXCR4 expression is regulated partly at the level of transcription (Gupta *et al*, 1998). We only detected a modest increase of CXCR4 mRNA, suggesting that CXCR4 expression may also be regulated by posttranscriptional mechanisms (Kury *et al*, 2003).

We show that T-tropic and dual-tropic isolates cause neuronal apoptosis and that HIV isolates vary in their ability to cause this effect, which was particularly pronounced with the HIV-1_{MN} and HIV-$1_{89.6}$ strains. One possible limitation in the interpretation of these findings is the use of differentiated SK-N-MC cells, but several studies have shown this cell line to be useful in studying in HIV-related neurotoxicity (Talley *et al*, 1995; New *et al*, 1998). Further, this observation is consistent with recent findings which showed that T-tropic strains via CXCR4/CCR3 induce significantly greater apoptosis in primary brain cultures, independent of HIV replication, when compared to that following infection with M-tropic strains, which utilize CCR5 (Albright *et al*, 1999; Ohagen *et al*, 1999; Zheng *et al*, 1999a). In contrast, Zhang *et al* showed that supernatants of infected macrophages induced varying levels of neurotoxicity depending on the HIV isolate, although the effect was also independent of replicative capability (Zhang *et al*, 2003). Combining these findings would suggest that both direct mechanisms via viral-induced neurotoxicity and indirect mechanisms contribute to HIV neuropathogenesis. Several studies have stressed the emergence of T-tropic strains in the periphery with disease progression (Connor *et al*, 1997), which may provide a potential source for viral protein signaling and neurotoxicity through CXCR4. It is noteworthy that T-tropic and dual-tropic viruses have been detected at a low frequency in the HIV-infected brain (Korber *et al*, 1994; Chang *et al*, 1998), but conceivably, in some patients, these isolates may contribute

to the progression of neurological disease in the later stages of infection.

Although neuronal injury is a prominent feature in the HIV-infected brain (Wiley *et al*, 1991; Masliah *et al*, 1992; Petito and Roberts, 1995; Adle-Biassette *et al*, 1999; Everall *et al*, 1999; Fisher *et al*, 1999; Petito *et al*, 2001), its relationship to the onset and progression of cognitive impairment has remained somewhat unclear. Masliah and colleagues have suggested that synaptic-dendritic injury may provide the cellular substrate for minor motor-cognitive impairment (Masliah *et al*, 1997). Further insight into this relationship has come more recently from studies based on *in vivo* proton magnetic resonance spectroscopy that suggest that decreases in neuronal function may indeed represent a critical event in the onset of HIV-associated cognitive impairment (Chang *et al*, 2004; Yiannoutsos *et al*, 2004).

The consensus based on several lines of evidence is that mechanisms of HIV-associated neuronal injury are largely indirect and implicate both host and viral factors (Kaul *et al*, 2001). The number of activated rather than infected macrophages has been shown to be a stronger correlate of ADC severity, providing a link between the initial event of infection and dementia (Glass *et al*, 1993; Rostasy *et al*, 1999). Multiple signaling pathways have been identified in which activated macrophages/microglia exert a central role in the events leading to neuronal injury. These can be broadly divided into direct and indirect mechanisms that act in concert. Key host-derived molecules include TNF-α, iNOS, quinolinate, platelet-activating factor, SDF-1α, the sole ligand for CXCR4, and protease resistant molecules (Giulian *et al*, 1990; Wesselingh *et al*, 1993; Adamson *et al*, 1996; New *et al*, 1998; Kaul and Lipton, 1999; Rostasy *et al*, 1999; Kaul *et al*, 2001). The secretion of viral proteins (e.g., gp120, gp41, and Tat) by HIV-infected macrophages/microglia have also been shown to cause neuronal injury, but their neurotoxic effects may be mediated by host factors, notably TNF-α (Merrill *et al*, 1992; Yeung *et al*, 1995; New *et al*, 1998; Nath *et al*, 2000). However, as suggested in this study and others, the emergence of T-tropic strains in the periphery with disease progression may provide another source for neuronal injury.

The reason for differences in neurotoxicity among HIV isolates remains unclear. HIV isolates have been shown to vary significantly in their ability to induce TNF-α (Khanna *et al*, 2000). Conceivably, neurovirulence may be associated with greater expression of TNF-α and other host factors in the brain. Determinants of both neurotoxicity and TNF-α production have been shown to reside largely in the V3, suggesting sequence variations in this region may also contribute to differences in HIV-associated neurotoxicity (Powers *et al*, 1998; Ohagen *et al*, 1999; Zheng *et al*, 1999a; Khanna *et al*, 2000; Zhang *et al*, 2003).

Pretreatment of neuronal cells with TNF-α led to a significant increase in apoptosis by T-tropic and dual-tropic HIV isolates although the effect was most pronounced with HIV-1_{MN} and HIV-1_{89}. Gabuzda and colleagues have shown that TNF-α can potentiate the neurotoxic effects of Tat via oxidative stress mechanisms (Shi *et al*, 1998). However, there have been no previous reports examining the interactions of TNF-α and HIV in relationship to neuronal injury. It is noteworthy when the effects for HIV-1_{MN} and HIV-$1_{89.6}$ were compared, HIV-1_{MN}–treated cells caused significantly greater apoptosis compared to HIV-$1_{89.6}$–treated cells. However, when TNF-α/HIV-1_{MN}–treated cells were compared to TNF-α/HIV-$1_{89.6}$–treated cells, the degree of apoptosis was nonsignificant, suggesting a possible TNF-α interaction with HIV-1_{MN} and HIV-$1_{89.6}$. The relative increase in apoptosis in TNF-α–treated HIV-$1_{89.6}$ cells (average 17.5%) compared to TNF-α–negative HIV-$1_{89.6}$ cells (average 7.5%) was significantly greater that what was observed in HIV-1_{MN} cells (22% in the TNF-α/HIV-1_{MN} compared to 12.5% in HIV-1_{MN}–treated cells). These results carry implications for further understanding HIV neuropathogenesis as they suggest that neuronal injury may reflect the combined effects of TNF-α and particular HIV strains, mediated at the CXCR4 receptor.

The fact that monoclonal antibodies to either CXCR4 or TNF-α significantly reduced apoptosis in TNF-α–pretreated, HIV-infected cells suggests that TNF-α may render neuronal cells more vulnerable to the neurotoxic effects of certain HIV isolates. The increase in CXCR4 cell surface expression identifies another mechanism by which TNF-α can lead to neuronal injury in the HIV-infected brain. Further studies are needed to elucidate the precise mechanisms of these interactions and their relationship to neuronal injury.

Methods

Tissue culture

SK-N-MC cells (American Tissue Culture Collection, Rockville, MD), a human neuroblastoma cell line, were differentiated into a neuronal phenotype following 5 days of treatment with retinoic acid (5 μM; Sigma, St. Louis, MO) as previously described (Talley *et al*, 1995). Cells were grown in a humidified incubator at 37°C with 5% CO_2 and minimal essential medium (Life Technology, Rockville, MD) supplemented with 10% fetal bovine serum (FBS) added every 2 to 3 days. Prior to each experiment, cells were cultured in 60 × 15-mm plates for 6 to 8 h and subsequently treated with 1 ng/ml, 5 ng/ml, and 10 ng/ml TNF-α (Boehringer, Mannheim, Germany) in duplicate. Cells were collected at 1, 24, and 48 h and processed for FACS analysis and RNA extraction as outlined below.

Flow cytometry and cell surface expression of CXCR4

SK-N-MC cells were washed twice with phosphate-buffered saline (PBS), treated with 0.25% trypsin/EDTA (Life Technology), and 10% FBS. Cells were centrifuged for 2 min at 2000 $\times$ *g*, and washed twice with cold PBS containing 0.1% bovine serum albumin (BSA; Sigma). The sample was then incubated with a monoclonal antibody against CXCR4 (10 μl/10^6cells; R&D, Minneapolis, MN) for 30 min at 37°C. Cells were centrifuged, washed twice with PBS, and then a secondary anti-mouse antibody coupled with fluorescein isothio cyanate (FITC) (Pharmingen, San Diego, CA) was added for 30 min at room temperature. Flow cytometry measurements were carried out using FACS (Becton-Dickson, San Diego, CA). Experiments were done in triplicate and included the following controls: omission of the primary antibody and FITC; cells not treated with TNF-α; unlabeled and untreated cells to account for nonspecific staining.

RT-PCR

From the second half of the above used SK-N-MC cells RNA was extracted for analysis of CXCR4 mRNA expression by semiquantitative RT-PCR. SK-N-MC cells were washed with PBS twice and centrifuged for 5 min at 2000 $\times$ *g*. Total RNA was isolated using Trizol (GIBCO BRL, Life Technology) extraction. Five microgram of total RNA were reverse transcribed using Superscript cDNA synthesis kit (Life Technology) and random primers. For PCR amplification, an aliquot of the cDNA was amplified using a 'hot start' protocol in a total volume of 25 μl containing 100 μM of each dNTP and 1 μM of specific primer. The sequences of the primers were as follows (S = sense primer and AS = antisense primer). CXCR4-S: 5′CCCGACTTCATCTTTGCCAACG3′ and CXCR4-AS: 5′TGACTGTGGTCTTGAGGGCCTT 3′. β-Actin amplification was used as internal control. β-Actin-S: 5′GTTTCGTGGATGCCACAGGACT3′ and β-actin-AS: 5′ATCTGGCACCACACTTCTACA3′.

After initial denaturation at 95°C for 5 min, 0.5 μl of Taq was added. An aliquot of the PCR reaction was subjected to electrophoresis on a 1.2% percent agarose gel and transferred to Hybond nylon (Amersham Pharmacia Biotech, Little Chalfont, UK) by Southern blotting. Blots were hybridized using standard conditions with oligonucleotide probes labeled with T4 kinase (Labeling system, Life Technology). Probes included β-actin: 5′TGAGACCTT CAACACCCCAGCATGTACGTTGCTATCCAG3′ and CXCR4: 5′TTTCAGCACATCATGGTTGGCCTTATCC TGCCTGGTACT3′. Filters were exposed by autoradiography using XAR-5 film (Kodak, New Haven, CT). The intensity of the bands was determined by phosphorimaging analysis (Biorad, Hercules, CA) and the amount of expressed message calculated by the ratio of the density of CXCR4 expression to β-actin.

Viral stocks

89.6, MN, and IIIB virus stocks were prepared from the supernatants of infected phytohemagglutinin/IL-2–stimulated lymphocytes from healthy donors (HIV-negative subjects) who were infected *in vitro*. All three viral inoculates were obtained from the National Institutes of Health (NIH) AIDS Research Repository Program, Bethesda, MD.

Apoptosis

Apoptosis was assessed using the Deokynucleutidyltransferase (TdT)-mediated dUTP nick end-labeling (TUNEL) method (Apoptag Kit, Life Technology). As above, SK-N-MC cells were treated with retinoic acid (5 μM; Sigma), plated at a density of 3×10^5/ml on glass cover slips and treated with 10 ng/ml TNF-α (Sigma) for 48 h. Following the treatment with TNF-α, cells were exposed for 24 h to three different strains of HIV (MN, IIIB, 89.6) at a multiplicity of infection (m.o.i.) of 0.05. Additionally, cells not pretreated with TNF-α were exposed to each virus mentioned above. DNase-treated cells were included as a positive control. Cells were washed with PBS and fixed in 1% paraformaldehyde for 10 min at room temperature and in 100% ethanol/acetic acid (2:1) for 10 min at −20°C. Cells were then washed twice with cold PBS and treated with a mixture of reaction buffer and TdT (2:1) for 1 h at 37°C, followed by incubation for 30 min in the dark with an anti-digoxin fluorescin conjugate. Coverslips were placed on a slide, mounting medium containing propidium iodide (PI; Sigma) was added, and a second coverslip was placed on the first. Experiments were carried out in duplicate and repeated three times. The percentage of apoptotic cells was determined by counting 250 PI-stained cells using a 40× objective. In the same field, the TUNEL-positive cells were counted and divided by the number of PI-stained cells.

In a second set of experiments, differentitaed SK-N-MC cells were treated with the HIV-1_{MN} virus under the following conditions: (1) untreated cells, (2) cells treated with a CXCR4 monoclonal Ab; (10 ng/ml; R&D); (3) cells treated with TNF-α alone (10 ng/ml; Sigma) for 48 h; (4) cells treated with HIV-1_{MN} virus (0.05 m.o.i.) alone for 24 h; (5) cells pretreated for 48 h with TNF-α (10 ng/ml) and then exposed for 24 h to HIV-1_{MN} virus (0.05 m.o.i.); (6) cells pretreated for 48 h with TNF-α (10 ng/ml) and for 30 min with CXCR4 Ab (10 ng/ml; R&D) prior to exposure to HIV-1_{MN} virus for 24 h (0.05 m.o.i.); (7) cells pretreated for 48 h with a mixture of TNF-α (10 ng/ml) and TNF-α monoclonal antibody (10 ng/ml; R&D) prior to exposure to HIV-1_{MN} virus for 24 h (0.05 m.o.i.); (8) cells treated with CXCR4 Ab (10 ng/ml) for 30 min prior to exposure to HIV-1_{MN} virus for 24 h (0.05 m.o.i.). Apoptosis was measured as described above.

Statistical analysis

Results were analyzed using the Student *t* test and the Mantel-Haenszel (M-H) approach. A P value $<$ .05 was considered significant.

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