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M- and T-tropic HIVs Promote Apoptosis in Rat Neurons

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Abstract

Neuronal loss, reactive astrocytes, and other abnormalities are seen in the brain of individuals with acquired immune deficiency syndrome-associated Dementia Complex (ADC). Human immunodeficiency virus-1 (HIV-1) is believed to be the main agent causing ADC. However, little is known about the molecular and cellular mechanisms of HIV-1 neurotoxicity considering that HIV-1 does not infect post-mitotic neurons and that viral load does not necessarily correlate with ADC. Various viral proteins, such as the envelope protein gp120 and the transcription activator Tat, have been shown to induce neuronal apoptosis through direct and indirect mechanisms both in vitro and in vivo. Progeny HIV-1 virions can also cause neuronal death. However, it has not been fully established yet whether HIV-1 promotes neuronal apoptosis by a direct mechanism. To explore the neurotoxic effect of HIV-1, we exposed rat cerebellar granule cells and cortical neurons in culture to two different strains of HIV-1, IIIB and BaL, T- and M-tropic strains that utilize CXCR4 and CCR5 coreceptors, respectively, to infect cells. We observed that both viruses elicit a time-dependent apoptotic cell death in these cultures without inducing a productive infection as determined by the absence of the core protein of HIV-1, p24, in cell lysates. Instead, neurons were gp 120 positive, suggesting that the envelope protein is shed by the virus and then subsequently internalized by neurons. The CXCR4 receptor antagonist AMD3100 or the CCR5 receptor inhibitor D-Ala-peptide T-amide blocked HIV IIIB and HIV BaL neurotoxicity, respectively. In contrast, the N-methyl-D-aspartate receptor blocker MK801 failed to protect neurons from HIV-mediated apoptosis, suggesting that HIV-1 neurotoxicity can be initiated by the viral protein gp 120 binding to neuronal chemokine receptors.

Keywords

acquired immune deficiency syndrome-associated dementia complex; gp 120; neuronal apoptosis; CXCR4; CCR5; AMD3100; DAPTA

Introduction

Acquired immune deficiency syndrome-associated dementia complex (ADC) is frequent in human immunodeficiency virus (HIV)-positive patients in the late phase of infection (Navia

et al. 1986) and is characterized by neuronal cell death and white matter abnormalities (Masliah et al. 1996; Gonzalez-Scarano and Martin-Garcia 2005). The consequent neuropsychological alterations of these patients include several degrees of cognitive and motor coordination impairments (Grant 1990; Navia and Rostasy 2005). Nevertheless, it is still unclear how HIV-1 induces neuronal damage. Although HIV can enter the brain through infiltration of infected peripheral monocyte-derived macrophages (Persidsky and Gendelman 2003; Kaul et al. 2005), HIV-1 does not directly infect post-mitotic neurons. This is because neurons do not express CD4, a receptor crucial for HIV-1 fusion with the host membrane and subsequent infection. Yet, immature neurons or progenitors are infected by HIV-1 in vitro (Ensoli et al. 1995; Lawrence et al. 2004). In addition, the number of activated rather infected monocytes was found to correlate with ADC severity (Glass et al. 1993). Thus, while plasma HIV-1 levels is predictive of acquired immune deficiency syndrome (AIDS; Childs et al. 1999), the viral load in the brain necessary to cause ADC remains controversial (Wiley et al. 1998).

HIV-1 infects macrophages and lymphocytes after binding of the envelope glycoprotein 120 (gp120) to CD4 in conjunction with several chemokine receptors (reviewed in Berger et al. 1999). Different viral strains bind to different chemokine receptors. M-tropic HIV-1 strains which infect macrophages use CC-chemokine receptor 5 (CCR5). Individuals lacking CCR5 are highly resistant to primary HIV infection (Cohen 1996; Huang et al. 1996). T-tropic strains which infect T cells use CXCR4-chemokine receptor 4 (CXCR4). CCR5-using viruses predominate in the initial stage of infection, whereas a switch from CCR5 to CXCR4-using viruses occurs in the late stages of infection in a subset of patients (reviewed in Berger et al. 1999). However, CXCR4 and CCR5 distribution is not limited to macrophages or T cells. In fact, both receptors are present in a variety of cells in the brain including neurons and astrocytes (Lazarini et al. 2003), although these cells appear resistant to harbor productive HIV-1 infection. Yet, apoptosis of neurons has been demonstrated in brain tissue from adult (Adle-Biassette et al. 1995) and pediatric AIDS patients (Gelbard and Epstein 1995). On the other hand, chemokine receptors, and in particular CXCR4, are also crucial for gp 120 neurotoxicity even in the absence of the virus (Hesselgesser et al. 1998; Meucci et al. 1998; Kaul and Lipton 1999; Bachis et al. 2003). These data have suggested that neuronal apoptosis may not depend upon direct viral infection. In support to this notion, earlier studies have shown that HIV-1 isolates that use CXCR4 induce, although limited, apoptosis in human fetal cells or astrocytes independently from the replication capacity (Ohagen et al. 1999; Zheng et al. 1999a). In contrast, viruses which use CCR5 produced mild or no apoptosis (Ohagen et al. 1999; Zheng et al. 1999a). These considerations raise the issue as whether productive infection is required for the pathogenesis of ADC.

It has been suggested that neuronal loss and dysfunction in ADC is the consequence of viral proteins released from infected microglial cells. Microglia can also release cellular toxins known to evoke neuronal cell death such as cytokines, glutamate, and nitric oxide (reviewed in Kaul and Lipton 2005). However, it has been shown that CXCR4 activation by the viral protein gp 120 leads to neuronal apoptosis even in the absence of glia. In addition, neuronal apoptosis by gp 120 is seen preceding microglia activation and is blocked by a selective antagonist of CXCR4 (Bachis et al. 2006), suggesting that HIV may be initially neurotoxic irrespective of the glia environment. Therefore, it appears that the virus may cause apoptosis by an indirect mechanism. The current study was undertaken in order to (1) clarify whether HIV-1 causes neuron apoptosis independently from microglia infection, (2) establish the relative neurotoxic property of two strains of HIV, and (3) further understand the role of chemokine receptors in HIV-induced neurotoxicity.

Materials and methods

Preparation of cerebellar granule cells

Cerebellar granule cells (CGC) were prepared from 8-day-old Sprague—Dawley rat pups (Taconic Farms, Germantown, NY, USA) as described previously (Brandoli et al. 1998; Bachis et al. 2001). Briefly, neurons were plated onto poly-L-lysine (1%)-precoated 100 mm plastic dishes at a density of 2.5×10^6 cells/ml and grown in basal medium Eagle (BME; Invitrogen, Grand Island, NY, USA) containing 2 mM glutamine, 10% fetal calf serum, 25 mM KCl, 100 µg/ml gentamicin, and 10,000 U/ml penicillin—streptomycin. Cells were maintained at 37°C in 5% CO₂, and 95% O₂. Cytosine arabinoside (10 µM) was added 24 h after cell plating to inhibit glial proliferation. HIVs, gp 120s, and other compounds were added at 8 days in vitro. Medium was not replaced during the experiments.

Preparation of cortical neurons

Cortical neuronal cultures were obtained from 1-day-old rat cortices (Taconic Farms) as previously described (Nosheny et al. 2005). In brief, cortices were cleaned from meninges and blood vessels in Krebs-Ringers bicarbonate buffer containing 0.3% bovine serum albumin, minced and dissociated in the same buffer with 1,800 U/ml trypsin at 37°C for 20 min. Trypsin was inactivated by the addition of soybean trypsin inhibitor. Cells were then recovered by centrifugation. The final pellet was reconstituted in BME containing glutamine (2 mM), fetal calf serum (10%), and KCl (25 mM). Neurons were plated onto poly-L-Lysine (1%)-precoated 100 mm plastic dishes at a density of 2.5×10^6 cells/ml and grown in the same medium containing gentamicin (100 µg/ml) and penicillin/streptomycin (10,000 U/ml). The medium was replaced 24 h later by the same medium except for KCl concentration that was lowered to 5 mM. Cytosine arabinoside (10 µM) was added 24 h after cell plating to inhibit glial proliferation. Cells were maintained at 37°C in 5% CO₂/95% air. HIVs, gp 120s, and other compounds were added at 8 days in vitro. Medium was not replaced during the experiments.

Characterization of neuronal cultures

CGC and cortical cultures were plated onto 12 mm round, 1 mm thick precoated glass coverslips. Cells were fixed with 4% paraformaldehyde and incubated overnight with a cocktail of antibodies against neurofilament (anti-neurofilament 200 kDa, anti-neurofilament 160 kDa, anti-neurofilament 70 kDa; 1:100 dilution; Millipore, Billerica, MA, USA) to visualize neurons or an antibody against glial fibrillary acidic protein (GFAP; 1:100 dilution; Millipore) to visualize astrocytes or an antibody against either CD68 (1:100 dilution; Millipore) or Iba1 (1:200 dilution; Abcam, Cambridge, MA) to visualize microglia. Cells were washed and incubated with Alexa Fluor® 350, Alexa Fluor® 488, and Alexa Fluor® 594 (1:1,000 dilution, Invitrogen, Carlsbad, CA) to visualize neurons, astrocytes, and microglia, respectively. Coverslips were then mounted using Vectashild mounting medium with 4',6'-diamidino-2-phenylindole (DAPI; Vector, Burlingame, CA, USA). Immunoreactivity (IR) was visualized with a Zeiss fluorescence microscope Axiophot2. Immunoreactive cells were counted using a ×20 objective and MetaMorph® software (Molecular Devices, Downingtown, PA, USA) as previously described (Bachis et al. 2003).

Reagents

Human T-lymphotropic virus (HTLV)-IIIB (HIV_{IIIB}) was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH): from Dr. R. Gallo (Popovic et al. 1984; Ratner et al. 1985). HIV-1_{BaL} (BaL) was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: from Drs. S. Gartner, M. Popovic, and R. Gallo (Gartner et al. 1986). Gp120 was obtained for Immunodiagnostics Inc,

(Woburn, MA, USA); AMD3100 and (+)-5-methyl-10,11-dihydro-5H-dibenzo [*a,d*] cyclohepten-5,10-imine maleate (MK801) from Sigma, St Louis, MO; D-Ala-peptide T-amide (DAPTA) from Tocris Bioscience, St Louis, MO, USA.

Cell death

The activity of mitochondrial dehydrogenases [3 (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) assay was used to determine cell death/survival. This assay was carried out according to the manufacturer's specifications (MTT Kit 1, Boehringer Mannheim, Indianapolis, IN, USA) as described previously (Bachis et al. 2001,2003).

Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling

Apoptotic neurons were examined by in situ terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) and activated caspase-3 as described previously (Bachis et al. 2001, 2003). In brief, neurons were plated onto 12-mm-round, 1-mm-thick precoated glass coverslips. Cells were fixed with 4% paraformaldehyde, post-fixed in 2:1 ethanol/acetic acid, washed, and equilibrated according to the instructions of the manufacturer (ApoTag®; Millipore). Neurons were then incubated with terminal deoxynucleotidyl transferase enzyme in the presence of digoxigenin-labeled deoxyNTP, followed by anti-digoxigenin (fluorescein conjugate) antibody and mounted using Vectashield Mounting Medium (Vector Laboratories) as counterstaining. Reaction was visualized with a Zeiss fluorescence microscope Axiophot2. TUNEL-positive cells were counted using a ×20 objective and MetaMorph® software as previously described (Bachis et al. 2003).

Caspase-3 immunocytochemistry

For neurofilament and caspase-3 staining, cultures were fixed with 4% paraformaldehyde and incubated overnight with a cocktail of antibodies against neurofilament as described above and a cleaved-caspase-3 antibody (1:150 dilution; Cell Signaling, Beverly, MA, USA) for 48 h at 4 °C. Cells were washed and then incubated with Alexa Fluor® 488 and Alexa Fluor® 594 (1:1,000 dilution, Invitrogen) to visualize neurofilament and caspase-3, respectively. Coverslips were then mounted using Vectashield mounting medium with DAPI. IR was visualized with a Zeiss fluorescence microscope Axiophot2. Caspase-3-positive cells were counted using a ×20 objective and MetaMorph® software as previously described (Bachis et al. 2003).

Gp120, Tat, Nef, gp41, and p24 immunoreactivity

CGC were exposed to HIV_{IIIB} for different times, washed, and then fixed with 4% paraformaldehyde for 20 min. Cultures were then immunostained overnight with a cocktail of antibodies against neurofilament as described above along with an antibody against either gp120 (1:100 dilution), Tat (1:150 dilution), Nef (1:50 dilution), gp41 (1:150 dilution), or p24 (1:50 dilution). All antibodies were obtained from Immunodiagnostics Inc. Cells were then incubated with Alexa Fluor® 488 and Alexa Fluor® 594 (1:1,000 dilution, Invitrogen) to visualize viral proteins and neurons. Coverslips were then mounted using Vectashield Mounting Medium with DAPI.

Detection of HIV-1 p24 by enzyme-linked immunosorbent assay

Detection of HIV infection was determined by measuring the amount of the core protein in the virus particle, p24, by enzyme-linked immunosorbent assay (ELISA). p24 has been previously used to determine active HIV infection in human astrocytes (Tornatore et al. 1991). This assay was carried out according to the manufacturer's specification (HIV-1 p24 capture ELISA, Immunodiagnostics Inc.). In brief, cell lysates were prepared and protein content measured in the supernatant by the Bradford Coomassie Blue colorimetric assay (Bio-Rad, Hercules, CA,

USA). A plate holder with solid phase ligand strips (2×96 -well), provided by the manufacturer, was used for the assay. A seven-point standard curve using 2-fold serial dilutions in diluents buffer (DB) was prepared. The highest standard had a concentration of 2 ng of p24 per 100 μ l of DB. One hundred microliters of medium or 100 μ g of each sample in 100 μ l of DB, as well as 100 μ l of each standard, was incubated for 1 h at room temperature (RT) in the plate holder. Each sample was done in triplicate. Wells were then washed three times and incubated with the detection reagent for 1 h at RT. After washing, 100 μ l of tetramethyl benzidine substrate was added to each well and incubated for 10 min at RT. The reaction was then stopped using 100 μ l per well of Stop Solution. The optical density was immediately determined using a microplate reader set to 450 nm. Total p24 levels for each sample were calculated by plotting sample absorbance values against the standard curve.

Results

HIV-1_{BaL} and HIV-1_{IIIB} cause apoptosis in cerebellar granule cells and cortical neurons in culture

Neurons do not express CD4, yet they undergo apoptotic cell death in ADC (Garden et al. 2002). To characterize whether HIV causes cell death even in the absence of CD4, we used rat CGC and cortical neurons in vitro. We first determined the type of cells that compose these cultures at 8 days in vitro. At least 96% of cells were neurofilament positive, indicating neurons (Table 1). The remaining 4% were GFAP positive, indicating astrocytes. Cells were CD68 negative (Table 1). This antibody was used previously to detect microglia in rat brain (Bachis et al. 2006). Moreover, cells were also negative for Iba I, a marker for microglia cells. Thus, no microglia cells were present at the time of the experiments.

Cultures were exposed to virus stock of HIV-1 (1.5 ng/ml of p24) for various times. The concentration of the virus used for these studies was based on previous data showing active infection in human astrocytes (Tornatore et al. 1991). Moreover, to compare the effect of different strains of HIV-1, we used two viruses: HIV-IIIB (IIIB), a laboratory-adapted CXCR4-specific strain of HIV, and HIV-BaL (BaL), a CCR5-specific strain. As a control, neurons were exposed to heat-inactivated HIVs. MTT assay was initially used to determine cell death. We found no difference in cell viability between neurons exposed to medium alone or to heat-inactivated HIVs. Instead, both viral strains caused a time-dependent significant decrease in cell viability starting at 12 h, reaching a plateau around 48 h in both CGC (Fig. 1a) and cortical neurons (Fig. 1b). IIIB appears to have a toxic effect slightly stronger than BaL (Fig. 1).

To characterize the type of cell death, we examined apoptosis by TUNEL and activated caspase-3, a protease that plays a key role in apoptosis. In control CGC cultures (Fig. 2a) and cortical neurons (Fig. 2b), only 5% of cells were TUNEL and caspase-3 positive. Instead, in IIIB- and BaL-exposed neurons, the number of TUNEL- and caspase-3-positive cells was increased within 12 h and reached a plateau by 48 h (Fig. 2). The data obtained with MTT, TUNEL, and caspase-3 assays have a profile both temporally and quantitatively similar, suggesting that HIV-mediated cell death is mainly apoptotic. Overall, our data confirm previous findings that neuronal apoptosis can be induced by progeny HIV-1 virions (Zheng et al. 1999a).

Rat cerebellar granule cells and cortical neurons in culture do not support BaL and IIIB productive infection

It has been previously shown that primaty human astrocytes and neuronal progenitors support HIV replication (Tornatore et al. 1991; Conant et al. 1994; Lawrence et al. 2004). Our cultures may contain progenitor cells. To exclude that HIV-mediated neuronal apoptosis is caused by a productive infection in our experimental model, CGC and cortical neurons were exposed to

IIIB or BaL (1.5 ng/ml of p24) for 24 h. p24 is the HIV Gag protein that forms the viral capsid. The level of intracellular p24 is an indicator of HIV infection progression (Tornatore et al. 1991; Wang et al. 2003). Medium and cell lysates were then collected at various times (Table 2), and cellular and extracellular p24 levels were determined by ELISA. The medium of both cultures contained p24 (Table 2), whereas p24 was undetectable at all time points post-infection in the lysates of both CGC and cortical cultures (Table 2). In addition, a group of cells were exposed to HIV for 24 h, washed, and allowed to survive with control medium from sister cells for additional 96 h. No p24 was detected (data not shown). These data indicate that rat neurons used in this study do not support productive HIV-1 infection.

HIV-1 and chemokine receptors

HIV-1 infection of the central nervous system (CNS) is associated with dendritic pruning and neuronal apoptosis, alterations that are also caused by the HIV-1 envelope glycoprotein gp120 alone in rodents (Toggas et al. 1994). Indeed, considerable experimental data indicate that this protein may be directly responsible for HIV-mediated neurotoxicity through binding to chemokine receptors on the neuron surface (Meucci et al. 1998; Kaul and Lipton 1999; Zheng et al. 1999b). To examine whether chemokine receptors play a role in the toxic effect of HIV-1, CGC were exposed to AMD3100 (5 nM), a bicyclam that precludes HIV-1 entry by antagonizing CXCR4 receptor (Donzella et al. 1998) or D-Ala-peptide T-amide (5 nM), a selective CCR5 inhibitor (Polianova et al. 2005), prior to HIVs or gp120s. The number of caspase-3-positive neurons was determined 12 and 24 h later. AMD3100 prevented IIIB- but not BaL-mediated apoptosis at all time points examined (Fig. 3a). In contrast, DAPTA blocked the neurotoxic effect of BaL but not IIIB (Fig. 3b), suggesting that binding to CXCR4 or CCR5 might be the main event underlying the toxic effect of HIVs.

MK801 does not block HIV-mediated neuronal apoptosis

HIV-associated neuronal damage is thought to be due to an indirect mechanism that involves various toxins including the excitatory amino acid glutamate (Lipton 1992; Ushijima et al. 1995; Bezzi et al. 2001) and the HIV transactivator protein Tat (Chen et al. 1997). Interaction between Tat and the *N*-methyl-D-aspartate (NMDA) receptor has been demonstrated (Wang et al. 1999; Eugenin et al. 2007). On the other hand, preliminary data have shown that NMDA receptor is not implicated in gp120-mediated neurotoxicity (Bachis and Mocchetti 2004). Therefore, we deemed it of importance to examine the role of NMDA in HIV-1 toxicity. CGC were exposed to the noncompetitive NMDA receptor antagonist MK801 (10 μ M) 15 min prior to HIVs, and then cell death was determined 24 and 48 h later by MTT. MK801 failed to prevent the decrease in cell viability by both strains of HIVs (Fig. 4). Overall, these data confirm the role of chemokine receptors in HIV-mediated neuronal cell death.

Gp120, but not Tat or Nef, is internalized by neurons after exposure of cell cultures to IIIB

We have previously shown that internalization of gp 120 is crucial for its neurotoxic effect both in vitro (Bachis et al. 2003) and in vivo (Bachis et al. 2006). CXCR4 plays a key role in these processes (Bachis et al. 2006). Thus, HIV-1 might shed gp 120 which may cause neuronal injury after its internalization into neurons by a CXCR4-mediated mechanism. We tested this hypothesis by determining gp 120 IR in neurons exposed to IIIB (viral stock containing 1.5 ng/ml of p24) for various times. CGC and cortical neurons were also exposed to AMD3100 15 min prior to IIIB. Gp 120 IR was detected in both CGC (Fig. 5a) and cortical neurons (Fig. 5b) intracellularly as early as 6 h and up to 24 h after the addition of the viruses (Figs. 6a,b). In AMD3100- pretreated neurons, we observed a significant reduction in gp120 IR (Fig. 6). No IR for Tat, nef, or gp41 was found at these time points (data not shown), suggesting that Gp120 and no other viral proteins might be released from the virus in vitro and subsequently taken up by neurons.

Discussion

HIV-1 enters cells by a direct fusion mechanism triggered by sequential binding of the gp 120 subunit of the envelope glycoprotein, first to CD4, then to the co-receptor CCR5 or CXCR4. Gp120 is presumed to interact with the extracellular portion, which consists of the N-terminal segment and three extracellular loops. The entry of HIV-1 into the brain is still the subject of intensive studies. The “Trojan horse” hypothesis states that HIV-1 gains access to the brain by infiltration of infected immune cells, including monocytes and lymphocytes. Another current hypothesis suggests that HIV-1 particles can diffuse through brain microvascular endothelial cells into the brain (Bobardt et al. 2004). Irrespective of the way of entry, once inside the brain, HIV-1 may replicate in microglia and macrophages or, alternatively, in astrocytes as revealed by examinations of post-mortem ADC brain tissue (Kure et al. 1991; Trillo-Pazos et al. 2003; Churchill et al. 2006). Thus, direct HIV-1 infection of neurons in vivo is unlikely to be the major cause of neuronal loss in ADC. Nevertheless, several studies indicate that the number of HIV-producing cells do not correlate well with the degree of dementia in ADC (Glass et al. 1995; Masliah et al. 1997). Indeed, it has been shown that HIV disease progression is affected by both viral entry as well as the innate immune response (Dolan et al. 2007). Most importantly, apoptotic neurons are not HIV positive (Shi et al. 1996). The in vitro model used in this study shows that two strains of HIV-1 promote neuronal apoptosis even without a productive infection. This result supports the contention that HIV-1-induced neurodegeneration is probably not caused by direct infection of neurons but by other complex mechanisms.

Glial cells have emerged as playing an important function in ADC. Astrocytes and microglia serve as potential reservoirs for HIV-1 (Tornatore et al. 1994; Takahashi et al. 1996; Trillo-Pazos et al. 2003), although the characteristics of HIV-1 infection are different. Infected microglia/astrocytes increase the production and release of inflammatory cytokines or glutamate (Bezzi et al. 2001; Wang et al. 2003) which are known to evoke neuronal apoptosis. Therefore, it has been suggested that loss of neurons in ADC may be triggered by toxic substances, including glutamate, released from infected glial cells (Kaul and Lipton 2005). We have previously demonstrated that MK801, a potent NMDA receptor inhibitor, does not prevent gp 120-mediated neurotoxicity (Bachis and Mocchetti 2004). Here, we show that HIV-1 causes apoptosis even when neurons were preincubated with MK801, although the majority of CGC in our experimental conditions are NMDA sensitive. In fact, in these neurons, both glutamate and NMDA elicit responses typically associated with NMDA receptor activation such as increases in intracellular calcium concentrations (Brandoli et al. 1998), NMDA receptor channel activation, and apoptosis (Bachis et al. 2001). These data, taken together, suggest that the portion of HIV-1 that interacts with chemokine receptors can initiate neuronal apoptosis without requiring glutamate. However, our data do not exclude the possibility that other soluble viral proteins may act in concert with glutamate to cause neurotoxicity. Indeed, an interaction between the HIV transcription activator protein tat and NMDA receptor has been suggested to be crucial for HIV-mediated neuronal apoptosis (Maggirwar et al. 1999; Haughey et al. 2001). Such an interaction promotes the generation of nitric oxide and causes neuronal apoptosis (Eugenin et al. 2007). On the other hand, tat-induced nitric oxide is brought about a formation of a protein complex of lipoprotein receptor-related protein and NMDA receptor (Eugenin et al. 2007), whereas gp 120-mediated signal transduction occurs through chemokine receptors (Lazarini et al. 2000). Therefore, HIV-1 may promote neuronal loss by two or more mechanisms that are not mutually exclusive.

In this work, we show that both strains of HIV-1 cause a time-dependent neuronal apoptosis. We also presented evidence that exposure of CGC to IIIB results in gp120 accumulation but not p24 (the viral capsid) accumulation inside neurons, suggesting that neurons are capable of internalizing shed/virion-free gp120 and not virions. Intriguingly, neuronal internalization has been described also for tat (Bruce-Keller et al. 2003), which is also neurotoxic (Nath et al.

1999), although tat endocytosis occurs independently from a chemokine receptor-mediated mechanism. Thus, viral proteins can be taken up by neurons even without productive HIV-1 infection. How gp120, and not the virus, enters neurons is still a matter of speculation, although one may predict that the lack of CD4, a receptor crucial for HIV-1 fusion with the host membrane, renders neurons not suitable to HIV infection. Several members of the chemokine receptor family are used together with CD4 for HIV-1 entry into macrophages or T cells. The M-tropic strain binds to CCR5, while the T-tropic strain binds predominantly to CXCR4. These chemokine receptors play a key role in gp120-mediated neurotoxicity (Hesselgesser et al. 1998; Meucci et al. 1998). On the other hand, recent data have shown that CXCR4, and not CD4, is crucial for gp120 entry into neurons (Bachis et al. 2003, 2006). Indeed, the selective CXCR4 receptor antagonist AMD3100 blocks both gp 120 internalization and toxicity (Bachis et al. 2003, 2006). In this study, we have shown that neurons exposed to IIB and AMD3100 exhibited no gp120 immunoreactivity. Remarkably, we were able to prevent IIB- and BaL-mediated neuronal apoptosis by AMD3100 and DAPTA, respectively, further supporting the suggestion that chemokine receptor-mediated gp 120 internalization may initiate neurotoxicity (Bachis et al. 2006). This scenario is reminiscent of previous work showing that inhibition of CXCR4 receptor signal or synthesis in neurons (Meucci et al. 1998) is neuroprotective. Overall, our data suggest that viral envelope protein(s) may be primarily involved in initiating HIV-1 neurotoxicity even when the virus is in limited amount.

In addition to neuronal loss, HIV infection of the CNS is characterized by macrophage infiltration, diffuse microglia activation, and reactive astrogliosis. These pathological features have prompted the suggestion that HIV-1 is neurotoxic by an indirect mechanism whereby nonneuronal cells release neurotoxins. In a previous work, we have shown that gp 120 injection into the rat striatum causes neuronal apoptosis without eliciting an inflammatory response from microglia cells (Nosheny et al. 2004; Bachis et al. 2006). Other investigators have shown that gp120 causes apoptosis of dorsal root ganglia in vitro independently from the presence of glial cells (Melli et al. 2006). In this work, we present evidence that HIV-1 promotes apoptosis even if astrocytes are only a contaminant (~4%) of the cultures and microglia cells are absent. Our data call attention to an alternative hypothesis of HIV neurotoxicity that does not rely solely on an inflammatory event. We suggest that HIV-1 is neurotoxic because of its ability to activate chemokine through gp120. Indeed, chemokine receptor antagonists inhibited HIV-mediated neuronal apoptosis. Nevertheless, we cannot exclude that other neurotoxins released from glia cells in vivo may potentiate the toxic effect of either HIV-1 or gp 120 because other investigators (Kaul and Lipton 1999) have shown that gp120 exhibits more toxic activity in neuron/glia cocultures. Moreover, in vitro studies have shown that gp 120 activation of chemokine receptors in macrophages promotes the synthesis of a variety of pro-inflammatory cytokines (Lee et al. 2003). In addition, gliosis has been found in ADC individuals (Budka 1991) and gp 120 transgenic mice (Toggas et al. 1994). Thus, it is possible that glia-generated neurotoxins may not be required to initiate the apoptotic cascade in neurons but rather that they are involved in propagating and exaggerating the toxic effect of HIV-1.

In conclusion, the data presented here suggest that HIV-1 releases gp 120, which in turn, causes neuronal apoptosis. These findings may be relevant for human cells because they may explain how viral proteins may lead to ADC. Productively infected nonneuronal cells in the brain may release HIV-1 structural proteins, like envelope proteins. Alternatively, free viral particles could be a source of these viral proteins. The presence of chemokine receptors, and in particular CXCR4 and CCR5 (Lavi et al. 1997; van der Meer et al. 2000; Banisadr et al. 2002), on neurons makes these cells particularly at risk because gp 120 binds to these receptors and initiates the apoptotic cycle. Microglia cells change from their resting state to active state migrating toward dying neurons. Under normal circumstances, microglia may help repair neuronal injury through the guided migration of stem cells to the site of neuronal injury (Aarum et al. 2003). However, in the presence of additional HIV proteins, such as tat or gp41 (Sheng et al. 2000), infected

microglia cells produce proneurotoxic inflammatory cytokines that help, rather than inhibit, neuronal injury. This cascade of events, if unchallenged, will lead ultimately to the spread of apoptosis to other neurons and contribute to neuropathogenicity of HIV-1.

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Fig. 1. HIVs decrease neuronal survival. CGC (a) or cortical neurons (b) were exposed to boiled HIVs (control), IIB, or BaL (1.5 ng/ml of p24, each) for the indicated time points. Cell survival was determined by MTT assay. Data are the mean $\pm$ SEM of five independent determinations each point. Pound signs $p < 0.01$ vs control; asterisks $p < 0.001$ vs control

Fig. 2.

HIVs activate caspase-3. CGC (a) or cortical neurons (b) were exposed to IIIB or BaL for the indicated time points. The number of caspase-3-positive neurons was determined as described in "Materials and methods." Data, expressed as percent above control (boiled HIVs), are the mean $\pm$ SEM of five independent determinations each point. Pound sign $p < 0.05$ vs control; asterisks $p < 0.001$ vs control

Fig. 3.

Activation of chemokine receptors underlies the toxic effect of HIVs. CGC were exposed to medium control (heat-inactivated HIVs) or medium containing HIVs alone or in combination with AMD3100 (AMD) or DAPTA. AMD or DAPTA were added 15 min prior to HIVs. The number of caspase-3-positive neurons was then determined 12 and 24 h later. Data are the mean $\pm$ SEM of five independent determinations each point. Pound sign $p < 0.05$, asterisks $p < 0.01$ vs control; caret sign $p < 0.05$ vs HIVs

Fig. 4.

MK801 does not prevent HIV-mediated neuronal apoptosis. CGC were exposed to medium control (boiled HIVs) or medium containing IIIB or BaL, alone or in combination with MK801 (10 μ M). Neuronal survival was then determined at the indicated times. Data are the mean $\pm$ SEM of five independent determinations each point. Pound signs $p < 0.001$ vs control BaL, asterisks $p < 0.05$ vs control IIIB

Fig. 5.

Neurons exposed to IIIB internalize gp120. CGC (**a**) or cortical cultures (**b**) were exposed to IIIB for 6 h. Cells were then washed, fixed, and gp120 IR determined by histological analysis as described in “Materials and methods.” The photomicrograph shows example of a neurofilament (*red*) and gp120- (*green, appears as yellow* due to the overlay of red and green) positive cells. Arrows indicate gp120 in cell bodies, while *arrowheads* indicate gp120 in processes. Blue DAPI. Scale bar 25 μm in **a**, 50 μm in **b**

Fig. 6.

Time-dependent internalization of gp 120 in HIV-treated neurons. CGC (**a**) or cortical cultures (**b**) were exposed to medium control (boiled HIVs) or medium containing IIBB alone or in combination with AMD3100 for the indicated time points. Cells were then fixed, and the number of gp120-positive neurons was determined as described in Fig. 5. Data are the mean $\pm$ SEM of five independent determinations each point. Pound signs $p < 0.05$, double asterisks $p < 0.001$ vs control; asterisks $p < 0.05$, caret signs $p < 0.001$ vs IIBB

Table 1

Characterization of CGC and cortical cultures

	CGC (% of positive cells)	Cortical neurons
Neurons	96±1	95±1.5
Astrocytes	3±0.5	4±1
Microglia	ND	ND
Others	1	1

Cells (at 8 days in vitro) were fixed and stained with a cocktail of antibodies against neurofilament, GFAP, or CD68/Iba 1 to visualize and count neurons, astrocytes, or microglia, respectively. Immunoreactivity was visualized with a Zeiss fluorescence microscope Axiophot2. Immunoreactive cells were counted using a ×20 objective and MetaMorph®

Table 2

Levels of p24 in neuronal cultures exposed to HIV-1

	CGC	Cortical neurons
Medium 12 h	600±180 pg/ml	750±112 pg/ml
Lysates 12 h	N/D	N/D
Medium 24 h	530±110 pg/ml	680±89 pg/ml
Lysates 24 h	N/D	N/D
Medium 7 days	500±80 pg/ml	550±119 pg/ml
Lysates 7 days	N/D	N/D

Neurons were exposed to IIIB for the indicated times. Medium was collected, and cells were washed three times with phosphate-buffered saline and then lysates prepared. p24 was measured in both medium and lysates as described in "Materials and methods." Data are the mean±SEM of four independent determinations N/D below the sensitivity of the assay (~10 pg/ml)