Research report

Neurotoxicity of HIV-1 proteins gp120 and Tat in the rat striatum

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Abstract

HIV-associated dementia complex is a serious disabling disease characterized by cognitive, behavioral and motor dysfunction. Basal ganglia involvement in HIV-1 infection may be responsible for some of the psychomotor symptoms associated with HIV dementia. The objectives of the present study were to determine: (1) whether gp120 and Tat produce striatal toxicity, and (2) whether gp120 and Tat show synergistic toxicity in the striatum. In these studies, the recombinant proteins gp120, Tat, or saline (0.9\%) were stereotaxically injected in the striatum of adult male rats. The striatal sections were evaluated for area of tissue loss (Cresyl-violet stained sections) and the number of GFAP immunoreactive cells 7 days after the injections. Doses of gp120 250 ng/\mu l or higher and Tat 5 \mu g/\mu l or higher produced a significant area of tissue loss and significantly increased the number of GFAP reactive cells. We found no toxicity in animals treated with immunoabsorbed gp120 or Tat. Combined gp120 (100 ng/\mu l)+Tat (1 \mu g/\mu l) injections into the rat striatum significantly increased the area of tissue loss and altered morphology and increased number of GFAP reactive cells, as compared to controls. Thus, the present results suggest the involvement of gp120 and Tat in striatal toxicity and provide a model for further studies to fully characterize their role in HIV-1 toxicity and to develop therapeutic strategies for HIV-1 associated dementia complex. © 2000 Elsevier Science B.V.

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1. Introduction

HIV-associated dementia complex is a serious disabling disease developing in approximately 20\% of the patient population with advanced Human Immunodeficiency Virus type 1 (HIV-1) infection [25]. HIV-associated dementia complex manifests in severely immuno-compromised patients and involves cognitive, behavioral and motor dysfunction. With the development of highly active anti-retroviral therapy (HAART) it is now recognized that symptoms of HIV dementia are reversible in some patients [12,37], however these beneficial effects may not be long lasting due to development of drug resistance. Therefore, to effectively treat or prevent HIV dementia, understanding of its pathogenesis is essential. Towards that effort, recently, research has centered on the viral toxins, gp120 and Tat, as potential mediators of pathogenesis of HIV dementia complex [30].

Gp120 is a coat glycoprotein and plays an important role in viral entry and in determining viral tropism [7]. Gp120 may be shed during viral entry or may be released extracellularly by infected cells [30]. Gp120 causes neurotoxicity indirectly through activation of microglia and astrocytes [17]. Glial cells can secrete a variety of toxic substances such as arachidonic acid metabolites, NMDA agonists, platelet activating factor and various cytokines.
such as TNF-alpha and IL-1, which are involved in neuronal injury [11,18]. In studies with the transgenic mice where gp120 is over-expressed the neuronal changes resemble those in post mortem HIV infected human brains [39]. Thus, gp120 is a potential factor in the pathogenesis of HIV dementia; however the exact role of gp120 in the pathogenesis remains unclear.

Tat, a nonstructural protein of HIV, is also implicated in the pathogenesis of HIV dementia. Tat is actively released from infected lymphoid and neuroglial cells [10]. Tat causes neurotoxicity by direct neuronal depolarization [6] and by increasing levels of intracellular calcium and cytokines. Tat, like gp120, may also cause indirect neurotoxicity by activating excitatory amino acid receptors [20]. Even a brief exposure to Tat may stimulate a cascade of events leading to neuronal death [28], suggesting the possibility of an excitotoxic ‘hit and run’ mechanism in the CNS.

The basal ganglia are affected in HIV-1 infection with high viral load [43] and basal ganglia involvement may be responsible for some of the psychomotor symptoms of HIV dementia. When patients with AIDS and symptoms of psychosis received dopamine-blocking drugs, they showed increased frequency and severity of extrapyramidal features [26,15,35]. Reductions in dopamine, and its major metabolite homovanillic acid, have been shown in caudate nucleus specimens of AIDS patients [36] and in the CSF of patients with HIV-associated dementia. The basal ganglia have among the highest levels of HIV RNA in AIDS patients [43]. In vitro treatment of rat midbrain cultures with gp120 produces reduction in uptake of dopamine and reduction in dopamine neuron process length, while not affecting the total number of neuronal cells or dopamine containing cells [3]. In animals, treatment with gp120 reduces the ability of the neurons to transport dopamine [9] and a recent study [45] suggested that Tat may inhibit tyrosine hydroxylase expression in dopaminergic neurons. Collectively, these in vivo and in vitro studies support the hypothesis of gp120 and Tat involvement in basal ganglia damage by HIV.

Much work has been done studying the neurotoxic effects of gp 120 and Tat in cell culture models; relatively few studies have been done with these proteins in animal models focusing on neurotoxicity in dopaminergic brain regions. However, other studies of Tat injection into the brain parenchyma have found it to be a potent neurotoxin. Injection of MVV tat peptide produced toxicity at 10 nmol of peptide [13]. Infusion of basic domains of Tat into the lateral ventricle or into the hippocampus or thalamus produced an inflammatory process [32]. A recent study injected Tat into the striatum (10 μg) and reported reduced staining of neurons expressing TH in the substantia nigra 7 days post-injection [45]; however Tat-induced striatal effects were not described in this study.

In the present study, we examined the effects of gp120 and Tat by microinjecting these proteins in the rat striatum. First, we performed a dose–response study to determine the concentrations of gp120 and Tat in the rat striatum that result in overt tissue loss and reactive astrocytosis. Second, we assessed the specificity of gp120 and Tat toxicity by injecting solutions from which the proteins had been immunoabsorbed. Third, we determined whether gp120 and Tat together produced greater neurotoxicity than either protein alone in the rat striatum. Thus, the goal of these studies was to establish an in vivo rodent model for exploring the mechanisms of HIV-protein induced neurotoxicity within the basal ganglia. The importance of developing such a model of HIV protein neurotoxicity is highlighted by the occurrence of psychomotor deficits and dopamine alterations in HIV-associated dementia complex.

2. Materials and Methods

2.1. Animals

Young (3–4 months old) Sprague–Dawley male rats (approximate weight 300 g) were obtained from Harlan Laboratories (Indianapolis, IN). The animals were placed in quarantine facilities for 1 week and then were moved to the animal colony. Animals were maintained according to NIH guidelines in AAALAC accredited facilities. The animals were group housed (2/cage) under controlled temperature (21°C±2°C), relative humidity (50%±10%) and lighting (12l:12d, lights on at 0700 h) conditions. The animals had ad libitum access to distilled water and a standard rat chow diet (Pro-Lab Rat, Mouse, hamster Chow No. 3000). No gross pathology was found in any animal used in this study.

2.2. HIV proteins

The recombinant gp120 was provided as a gift from Chiron Corporation. Gp120 was produced in CHO cells from HIV-SF2 and was 100% pure. Recombinant Tat protein was produced as previously described [21]. We prepared solutions of gp120 in varying concentrations 400 ng/μl, 250 ng/μl, 100 ng/μl, 50 ng/μl, 10 ng/μl by adding sterile saline (0.9%). The lyophilized fraction of Tat protein was used in varying concentrations of 50 μg/μl, 5 μg/μl, 1 μg/μl.

2.3. Surgical techniques and microinjections

Standard stereotaxic surgery techniques were used for protein injection. The instrument used for stereotaxic surgery was obtained from KOPF® instruments. The syringe was obtained from Hamilton Co., Nevada, USA (Microliter ® 701 RN, 10 μl). The rats were anesthetized with Sodium pentobarbital (50 mg/kg body weight). The head was fixed in the bars of the instrument in the flat skull position and stereotaxic coordinates for injections were
according to a standard rat stereotaxic atlas [31]. The coordinates used for striatum were: 0.3 mm anterior to bregma, 2.5 mm lateral to bregma and 4.8 mm dorsal from dura. The injection volume was 1 μl injected over 1 min. The injection began after a 1-min period letting the tissue come into original conformation, and was followed by a 2-min period of needle removal so as to avoid needle reflux. After recovery from surgical anesthesia the animals were returned to the vivarium.

2.4. Experimental design

Animals were group housed and randomly distributed into gp120 (n=12) and Tat (n=12) treatment groups. Animals in the gp120 group were injected with a concentration of 400 ng/μl, 250 ng/μl, 100 ng/μl, 50 ng/μl or 10 ng/μl. Animals in the Tat group were injected with a concentration of 50 μg/μl, 5 μg/μl or 1 μg/μl doses. Two animals were treated with 0.9 saline (vehicle).

To evaluate the specificity of our lesioning results, gp120 or Tat were immunoadsorbed with respective antisera (goat anti-gp120 antisera or rabbit anti-Tat antisera) bound to protein A beads, and animals intracerebrally microinjected with the supernatants (n=4; Imgp120 and ImTat groups). Additionally, gp120 or Tat was treated with protein A beads bound to either normal goat serum or normal rabbit serum, respectively, and intracerebrally microinjected (n=4; gp120 and Tat).

Additional animals (n=3) were used in the gp120+Tat dosing experiment in which 100 ng gp120+1 μg Tat were mixed (1 μl) and injected in the striatum. The control groups for this latter experiment were injected with 100 ng gp120, 1 μg Tat or saline.

2.5. Tissue processing

Seven days following injections the animals were anesthetized with sodium pentobarbital (50 mg/kg bodyweight). The animals were transcardially perfused with approximately 250 ml of 4% paraformaldehyde solution and perfused brain was extracted. The brain was carefully blocked according to standardized anatomical landmarks and postfixed in 4% paraformaldehyde (pH 7.4) for 24 h. The cortical surface was notched on the non-injected side prior to sectioning to orient the tissue when mounting on the glass slides. Tissue blocks were sectioned using a vibrating microtome (Vibratome, Energy Beam Sciences). The 40-μm thick sections were stored in cryoprotectant solution until processed. Every fourth section was stained with Cresyl-violet Nissl-staining or with anti-Glial Fibrillary Acidic Protein (GFAP) for immunostaining throughout the striatum.

The free-floating tissue sections were washed in Phosphate Buffered Saline (PBS) (NaCl, Na₂HPO₄, H₂O; pH 7.4). The tissue sections to be processed for GFAP staining were placed in horse normal blocking serum for a period of 1 h and then directly transferred into a 1:500 dilution of affinity purified monoclonal mouse anti-Glial Fibrillary Acidic Protein (anti-GFAP) (Boehringer Mannheim, Inc.) and left overnight at room temperature. Following primary antibody incubation, all sections were washed in PBS and incubated for 1 h in mouse biotinylated secondary immunoglobulin (Vector Laboratories, Inc.). The avidin–biotin-complex technique (Elite ABC kit, Vector Labs, Burlingame, CA) was used to stain these tissue sections. Following a brief (approximately 5 min) reaction with 0.05% diaminobenzidine in 0.015% H₂O₂ and enhanced with Nickel chloride (NiCl₂ 8%) the sections were rinsed 3× in PBS. These sections were then mounted on glass slides, air dried, dehydrated through a series of alcohol/xylene solutions and coverslipped with Vectamount (Vector Laboratories, Inc.)

Nissl solution was prepared from 20 mls of Cresyl-violet stock stain (0.1%) and 200 ml of buffer solution (0.1 M sodium acetate/acetic acid, pH 3.9). Tissue sections to be processed with Nissl were rinsed 3× in PBS. These sections were then mounted on glass slides and rinsed in Nissl solution for 5–10 min. The slides were then rinsed in distilled water and dehydrated through a series of alcohol/xylene steps and coverslipped with DPX (Aldrich Chemical Company, Inc.)

2.6. Quantitative anatomical techniques

A computerized imaging system (Bioquant System, R&M Biometrics, Nashville, TN) system was used to systematically identify GFAP-IR cells and determine the area of striatal tissue loss [38]. GFAP-IR cells were identified in those tissue sections showing the maximal area of tissue loss for each animal or, if no tissue loss, the injection tracks (1 section/animal). In vehicle-injected animals, the sections including the injection track were selected for quantitative analysis (1 section/animal). Four different regions of the striatum were counted on the injected side and contralateral control side, excluding the injection track. A computerized grid was visualized over the imaged striatum (approximately 60×60 mm) with 100 intersections and GFAP-IR cells were counted at 400×. Every third square of the grid containing the striatum was counted.

The area of tissue loss was determined from Nissl stained sections by digitizing the tissue sections using the Bioquant imaging system. Subsequently, computer-assisted tracing of the perimeter of the striatal tissue loss surrounding the injection site was conducted to determine area measures.

2.7. Data analysis

Data are expressed as mean±S.E.M. For the GFAP-IR analysis, data are presented as percent control (uninjected side) for each animal. For group analysis, ANOVA was
used (BMDP Statistical Software, Inc Los Angeles, CA) and planned comparisons used to determine specific treatment effects. Regression analysis was used to determine dose–response relationships [44]. In cases of completely non-overlapping distributions (i.e., lesion size data), no statistics were calculated. An alpha level of $P \leq 0.05$ was the significance level for rejection of the null hypothesis.

Similarly, a significant area of tissue loss was found in the animals treated with gp120 doses of 400 and 250 ng/μl. The area of tissue loss was graded in that a greater area of loss was significantly greater in the 400 ng dose, as compared to 250 ng dose (Fig. 1B). In contrast, there was no tissue loss in the 100 ng/μl dose group animals. No tissue loss was observed in striatum of the vehicle-injected control group.

3. Results

3.1. Striatal tissue loss following gp120 and Tat injection

At the highest intracerebral doses, both gp120 and Tat produced an area of tissue loss in the rat striatum (Fig. 1, Panels A and B) immediately surrounding the injection area. Overall, Tat produced a significantly greater area of tissue loss in the rat striatum, relative to the loss produced by gp120. Significant striatal tissue loss occurred with intracerebral Tat doses of 50 and 5 μg. No significant tissue loss was observed in the 1 μg dose group, relative to the contralateral non-injected striatum or vehicle injected controls. The area of tissue loss was dose dependent, as more damage occurred with 50 μg dose as compared to 5 μg dose (Fig. 1A).

Similarly, a significant area of tissue loss was found in the animals treated with gp120 doses of 400 and 250 ng/μl. The area of tissue loss was graded in that a greater area of loss was significantly greater in the 400 ng dose, as compared to 250 ng dose (Fig. 1B). In contrast, there was no tissue loss in the 100 ng/μl dose group animals. No tissue loss was observed in striatum of the vehicle-injected control group.

3.2. Alterations in astrocyte morphology

On GFAP immuno-stained sections the astrocytes in the gp120 and Tat injected striatum demonstrated significant alterations in morphology (Fig. 2). The astrocytes were increased in size, and the number of arborizations were also increased as compared to the control side. The changes became less conspicuous distal from the lesion area. In gp120 treatment group these alterations were observed in 400 ng/μl and 250 ng/μl dose groups, whereas, no changes were observed in animals treated with 100 ng/μl dose. In Tat treatment group similar alterations were seen in 50 μg/μl dose treated animals, whereas, in animals treated with 5 or 1 μg/μl doses no significant alterations were seen as compared to the control side. Similarly, no significant changes in astrocyte morphology were seen in the striatum of vehicle injected animals.

![Graphs showing lesion area and GFAP immunoreactivity](image_url)
3.3. Increase in GFAP reactive cells following gp120 and Tat injections

There was a significant overall increase in GFAP-IR positive cells in the gp120 and Tat injected striatum (Fig. 1, Panels C and D), relative to the contralateral non-injected striatum \[F(2,15)=4.6, P\leq0.03\]. The magnitude of this increase in GFAP-IR is significantly greater with intracerebral injection of gp120 relative to Tat \[F(1,14)=6.0, P\leq0.03\]. For gp120, this was a dose-dependent effect with a significant linear increase in GFAP-IR cell numbers \[F(1,4)=16.6, P\leq0.015\], with the cell numbers significantly different in 400 ng/µl dose group as compared to control, uninjected, striatum \(P\leq0.05\).

In the Tat treatment group, there was a significant dose-dependent linear increase in GFAP-IR cells \[F(1,4)=10.0, P\leq0.034\]. The increase in the number of cells was significant in 50 µg/µl dose group, whereas 5 µg/µl and 1 µg/µl groups were not significantly different from the control or uninjected striatum \(P\leq0.05\). No significant increase in GFAP-IR cells was seen in the striatum of vehicle-injected animals.

3.4. Immunoabsorption of gp120 or Tat

No significant increase in GFAP-IR was seen following injections in which gp120 or Tat had been removed \[F(2,3)=2.6, P\leq0.23\], relative to injection of gp120 and Tat (Fig. 3). Similarly, there was no area of tissue loss in the animals receiving the vehicle injection in which gp120 or Tat had been removed. A significant increase in the GFAP positive cells, and alterations in astrocyte morphology in the animals receiving gp120 (250 ng/µl) and Tat (5 µg/µl) were observed as in the previous experiment.
Moreover, we have observed occasional microglia at the injection site of gp120+Tat (not shown). No tissue loss and no significant changes in astrocytes were seen in the control group receiving serum alone.

4. Discussion

In the present study, we examined the toxic effects of gp120 and Tat by microinjecting these proteins in the rat striatum. A dose–response study was performed to determine the concentrations of gp120 and Tat in the rat striatum that resulted in tissue loss and astrocytosis. We found microinjection of gp120 and Tat produced reactive astrocytosis in a dose-dependent manner in the rat striatum. These effects were specifically mediated by gp120 and Tat proteins, as determined by injecting solutions from which proteins had been immunoabsorbed. Moreover, the independent and synergistic mechanisms of gp120 and Tat toxicity may be addressed. Gp120 and Tat together produced greater neurotoxicity than either protein alone in the rat striatum. Thus, the HIV-protein rodent model may be useful for exploring the mechanisms of HIV-protein induced neurotoxicity in the basal ganglia.

Reactive astrocytosis has been consistently shown to be an early change in the pathogenesis of HIV encephalitis [5,41,42]. Astrocytosis may be a protective mechanism to ward off the potential toxins from reaching the brain, or more significantly, this may compromise the blood brain barrier function [4,8]. The evolving model of HIV-associated dementia suggests the involvement of virotoxins (Tat, gp120, etc.) interacting with various cellular toxins (cytokines, arachidonic acid metabolites, quinolinic acid, NO, NTOX etc.) [27,30]. It is thought that virotoxins activate glial cells to release various cellular toxins, which in turn adversely affect normal physiological functions of the neurons [30]. In this model of HIV-associated dementia astrocytes play an important role. Astrocytes may not only be infected by HIV [22] but uninfected cells may also be activated by gp120 and Tat to induce changes in intracellular calcium [14] and release of cytokines (reviewed in [27]). Thus, activation of astrocytes may be an important early indicator of subsequent neuropathology.

In previous in vitro studies we have shown that gp120 and Tat can cause synergistic neurotoxicity [29]. We now demonstrate that similar synergistic effects can occur in vivo. Moreover, the present study extends these observations to show that gp120 and Tat may also synergize to produce astrocytosis in vivo. These observations are important as they suggest that much smaller concentrations of these proteins may be necessary to induce neuro-pathological changes than previously thought, thereby supporting the role of these viral proteins as being an important factor in the neuropathogenesis of HIV dementia complex.
In the current studies we have developed a rodent model for exploring the mechanisms of HIV-protein induced neurotoxicity in the basal ganglia. Various studies have shown a reduction in basal ganglia and posterior cortex and a more generalized reduction in white matter on magnetic resonance imaging [1,2] in HIV-associated dementia. It has been shown that people with HIV-associated dementia have psychomotor slowing, also suggesting the involvement of basal ganglia [24]. Dopamine alterations in HIV, together with high viral load in the basal ganglia [43], indicate that the basal ganglia may be a preferential target for the virus [19]. For these reasons, substantial energies must be invested in developing in vivo and in vitro models best suited to study the neurotoxicity of HIV infection, especially with reference to the basal ganglia. However, given that humans are the only host for HIV infection, development of an entirely homologous animal model is not possible. Therefore, careful consideration must be given to the inherent problems and advantages of each model system for exploring HIV-associated dementia.

Primates can be infected with related retroviruses such as simian immunodeficiency virus (SIV) and a chimeric strain of SIV and HIV. However, some important differences still exist in the neurological outcome of these animals when compared to humans. For example, the onset of symptoms is much more rapid in macaques and the encephalitis much more fulminant than is typically seen in humans [34]. Furthermore, sample sizes in primates must be limited due to the large amount of resources, expense and technical effort needed and the scarcity of these animals. The feline immunodeficiency (FIV) virus also causes encephalitis with neurodegeneration, although some differences exist with respect to the neuropathology seen in HIV encephalitis. Moreover, substantial differences exist between the FIV and HIV genome. However, this model provides important information for certain targeted questions related to lentiviral infection and to monitor the effects of neuroprotective therapy [33].

Our alternative strategy was to use HIV protein toxins that have been implicated in the neuropathogenesis of HIV infection in a rodent model of striatal neurotoxicity. The knowledge base for the mechanisms of HIV protein toxicity in vitro provides a substantial foundation for examining the proteins in vivo. Numerous in vitro studies have been done to elucidate the mechanisms of neurotoxicity of HIV proteins. For example, gp120 and Tat in vitro produce excitatory amino acids, nitric oxide, arachidonic acid, IL-1, and IL-6 (for review see [27]). However, in vitro techniques cannot provide the same information as that obtained in the whole animal; for example, they cannot provide data on behavioral changes reflecting damage to the basal ganglia. A rodent model to study the neurotoxicity of these proteins can help determine whether these putative in vitro mechanisms are translational to whole animals and ultimately to the pathology of HIV-associated dementia complex.

Direct microinjection of HIV-protein toxins that have been implicated in the neuropathogenesis of HIV infection provides a rodent model in which to examine mechanisms of striatal neurotoxicity as well as toxic interactions with other risk factors. Since IV drug abusers are one of the major groups acquiring HIV infection [40], and most of these drugs act on the dopaminergic systems, the study of basal ganglia becomes even more compelling in animal models. Recent work suggests that Tat alone may regulate key dopaminergic enzymes [45]. Therefore, in future studies this model may be combined with existing models of intravenous drugs of abuse [23] to study the complex interactions of drugs of abuse with HIV in producing the symptomology of HIV-associated dementia complex.

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