A Rat Model of Human Immunodeficiency Virus 1 Encephalopathy Using Envelope Glycoprotein gp120 Expression Delivered by SV40 Vectors

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Abstract
Human immunodeficiency virus 1 (HIV-1) encephalopathy is thought to result in part from the toxicity of HIV-1 envelope glycoprotein gp120 for neurons. Experimental systems for studying the effects of gp120 and other HIV proteins on the brain have been limited to the acute effects of recombinant proteins in vitro or in vivo in simian immunodeficiency virus–infected monkeys. We describe an experimental rodent model of ongoing gp120-induced neurotoxicity in which HIV-1 envelope is expressed in the brain using an SV40-derived gene delivery vector, SV(gp120). When it is inoculated stereotaxically into the rat caudate putamen, SV(gp120) caused a partly hemorrhagic lesion in which neuron and other cell apoptosis continues for at least 12 weeks. Human immunodeficiency virus gp120 is expressed throughout this time, and some apoptotic cells are gp120 positive. Malondialdehyde and 4-hydroxynonenal assays indicated that there was lipid peroxidation in these lesions. Prior administration of recombinant SV40 vectors carrying antioxidant enzymes, copper/zinc superoxide dismutase or glutathione peroxidase, was protective against SV(gp120)-induced oxidative injury and apoptosis. Thus, in vivo inoculation of SV(gp120) into the rat caudate putamen causes ongoing oxidative stress and apoptosis in neurons and may therefore represent a useful animal model for studying the pathogenesis and treatment of HIV-1 envelope–related brain damage.

Key Words: Brain, gp120, HIV-1, HIV dementia, SV40.

INTRODUCTION
The human immunodeficiency virus (HIV) enters the brain very early after its initial entry into the body. The principal manifestations of central nervous system (CNS) HIV infection result from neuronal injury and loss and from extensive damage to the dendritic and synaptic structures in the absence of neuronal loss. Neurons are rarely infected by HIV-1, and damage to neurons is felt mainly to be indirect (1, 2). The HIV-1 infects resident microglia, periventricular macrophages, and some astrocytes (3), leading to increased production of cytokines and the release of cytoxic HIV-1 proteins (4). Several HIV gene products have been identified as neurotoxins; these include the envelope (Env) proteins gp120 and gp41 and the nonstructural proteins Tat, Nef, Vpr, and Rev. Glycoprotein gp120–induced apoptosis has been demonstrated in cortical cell cultures, in rat hippocampal slices, and by intracerebral injections in vivo (5). Soluble gp120 triggers oxidative stress and leads to neuronal cell death after mitochondrial permeabilization, cytochrome c release, and activation of caspases and endonucleases (6).

Several animal models have been used to study the pathogenesis of HIV-1–induced neurological disease. Many of them are based on other lentiviruses. Feline immunodeficiency virus infection of cats (7, 8), Visna-Maedi virus infection of sheep (9), and simian immunodeficiency virus infection of macaques (10, 11) have all greatly contributed to our present knowledge. In some of these models, however, only small percentages of animals develop neurological manifestations despite the development of more reproducibly neurovirulent strains (12); moreover, animal costs for these species may be high (13). Rodent models have also been developed. Transgenic expression of gp120 (14) has been used, but gp120 in transgenic mice is chiefly expressed in astrocytes, whereas HIV-1 mainly infects microglial cells in humans. The introduction of HIV-infected macrophages into severe combined immunodeficient mice brains induces gliosis (15), microglial activation, and neuronal death (16), but this model suffers from the issue of the incompatibility of human macrophages delivered into a murine brain. Transplanted human cells into immunosuppressed mice and the use of hybrid viruses have also led to experimental CNS infections that resemble neuroAIDS (17–20).

We (21–23) and others (24, 25) have used model systems in which recombinant gp120 or Tat proteins are directly injected into the striatum. The neurotoxicity of such recombinant proteins is highly reproducible but is too acute to be useful either for studying chronic HIV-related tissue injury or for testing most therapeutic interventions. The lesions of HIV-associated dementia (HAD) reflect chronic injury caused by the ongoing production of gp120 and other substances by HIV-1–infected cells. Here, we report an experimental rodent model of ongoing gp120-induced neurotoxicity in which HIV-1 envelope is expressed in the brain using an SV40-derived gene delivery vector, SV(gp120). When it is inoculated stereotaxically into the rat caudate putamen, SV(gp120) caused a partly hemorrhagic lesion in which neuron and other cell apoptosis continues for at least 12 weeks. Human immunodeficiency virus gp120 is expressed throughout this time, and some apoptotic cells are gp120 positive. Malondialdehyde and 4-hydroxynonenal assays indicated that there was lipid peroxidation in these lesions. Prior administration of recombinant SV40 vectors carrying antioxidant enzymes, copper/zinc superoxide dismutase or glutathione peroxidase, was protective against SV(gp120)-induced oxidative injury and apoptosis. Thus, in vivo inoculation of SV(gp120) into the rat caudate putamen causes ongoing oxidative stress and apoptosis in neurons and may therefore represent a useful animal model for studying the pathogenesis and treatment of HIV-1 envelope–related brain damage.

Key Words: Brain, gp120, HIV-1, HIV dementia, SV40.
experimental model of chronic HIV-1 Env-induced neurotoxicity based on recombinant SV40 (rSV40) vector–mediated expression of gp120 in the brain.

MATERIALS AND METHODS

Animals
Female Sprague-Dawley rats (200–250 g) were purchased from Charles River Laboratories (Wilmington, MA). Nude rats were kindly provided by the National Institutes of Health. Protocols for injecting and killing animals were approved by the Thomas Jefferson University Institutional Animal Care and Use Committee and are consistent with the Association for Assessment and Accreditation of Laboratory Animal Care standards. Because estrogens can regulate microglial activation in some conditions, experiments were done in female rats at similar points of their estrous cycle. The diet that the animals received specifically avoided components that might cause oxidative stress. Numbers of animals used in experiments are indicated in the Experimental Design section.

Antibodies
Different primary antibodies were used: mouse anti-rat CD68/ED1 (immunoglobulin G1 [IgG1]; 1:50), a marker of activated microglial cells in a phagocytic state (Serotec, Oxford, UK), rabbit anti-iba-1 (IgG; 1:100), a marker of quiescent and active microglia (Waco Chemicals, Osaka, Japan), mouse anti-glial fibrillary acidic protein ([GFAP] IgG2b; 1:100; Becton Dickinson, Franklin Lakes, NJ), rabbit anti-N-acetyllysine-4-hydroxy-2-nonenal ([HNE] IgG; 1:50), a marker of lipid peroxidation (Calbiochem, La Jolla, CA), rabbit anti-caspase 3 (IgG; 1:100 [this antibody detects the active form of caspase 3]; Santa Cruz Biotechnology, Santa Cruz, CA), rat anti-dinitrophenol ([DNP] IgG1; 1:50; Zymed Laboratories, San Francisco, CA), mouse anti-proliferating cell nuclear antigen (IgG2a; 1:100), goat anti-CD68 (IgG; 1:100; Santa Cruz Biotechnology), rabbit anti-laminin (IgG; 1:100; Sigma), and mouse anti-NeuN (IgG1; 1:100; Chemicon International, Temecula, CA). The monoclonal antibody against gp120 (monoclonal antibody to HIV-1 V3, 257-D IV; IgG1; 1:100) was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health, and was a generous gift from Dr S. Zolla-Pazner. This antibody reacts with HIV-1 MN V3 epitope KRIJI (26). Secondary antibodies were used at 1:100 dilution: fluorescein isothiocyanate (FITC)– and tetramethylrhodamine isothiocyanate (TRITC)–conjugated anti-mouse IgG (γ-chain specific and against whole molecule, respectively), FITC-conjugated mouse anti-human IgG (γ-chain specific), TRITC-conjugated goat anti-rabbit IgG (whole molecule), FITC-conjugated sheep anti-rabbit IgG (whole molecule), FITC-conjugated rabbit anti-goat IgG (whole molecule), Cy3-conjugated rabbit anti-goat IgG (whole molecule) (Sigma), FITC- and TRITC-conjugated donkey anti-mouse IgG (whole molecule), Cy3-conjugated donkey anti-rabbit IgG (whole molecule), goat anti-IgG (whole molecule) (Jackson ImmunoResearch Laboratories, Inc, West Grove, PA), and FITC-conjugated goat anti-rat IgG H&L ([Fab]2 fragment; Abcam, Cambridge, MA).

Vector Production
The general principles for making recombinant Tag-deleted replication-defective SV40 viral vectors have been previously reported (27). The SV(gp120) is a recombinant Tag-deleted SV40-derived vector that expresses HIV-1 NL4-3 gp120 under the control of the cytomegalovirus immediate early promoter (28). Copper/zinc superoxide dismutase 1 (SOD-1) and glutathione peroxidase 1 (GPx-1) transgenes were subcloned into pT7[RSVLTR], in which transgene expression is controlled by the Rous sarcoma virus long terminal repeat as a promoter. The cloned rSV40 genome was excised from its carrier plasmid, gel purified, recircularized, and then transfected into COS-7 cells. These cells supply large T-antigen (Tag) and SV40 capsid proteins in trans, which are needed to produce recombinant replication-defective SV40 viral vectors (29). Crude virus stocks were prepared as cell lysates, band-purified by discontinuous sucrose density gradient ultracentrifugation, and then titered by quantitative polymerase chain reaction (30). The SV (human bilirubin-uridine 5'-diphosphate-glucuronosyl-transferase) (SV[BUGT]) was used as negative control vector with a nontoxic by-product (31).

Experimental Design
Injection of SV(gp120) Into the Caudate Putamen
SV(gp120) was injected into the caudate putamen (CP) of Sprague-Dawley rats, and the brains were harvested 1, 2, 4, and 12 weeks after injection, with 6 rats at each time point (n = 24). Controls received saline or SV(BUGT) instead of SV(gp120) in the CP (3 controls for each time point, n = 12 for SV[BUGT]; n = 12 for saline). The SV(gp120), SV(BUGT), or saline was also injected into the CP of nude rats. Brains of nude rats were harvested 1, 2, 4, and 12 weeks after the injection into the CP (6 rats for each time point for the animals injected with SV[gp120] [n = 24], 3 rats at each time point for the animals injected with SV[BUGT] [n = 12] and saline [n = 12]).

Challenge With SV(gp120) After Administration of SV(GPx-1)/SV(SOD-1)
To study possible neuroprotection by rSV40-delivered expression of SOD-1 and GPx-1 from SV(gp120)-related apoptosis, we first injected the CP of rats with SV(SOD-1) (n = 6) and SV(GPx-1) (n = 6). One month later, the CP in which SV(SOD-1) or SV(GPx-1) had been administered was injected with 5 µL of SV(gp120). Brains were harvested 1 week after injection and studied for apoptosis by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay and lipid peroxidation. Controls received SV(BUGT) in the CP instead of SV(SOD-1) and SV(GPx-1) (n = 6).

In Vivo Transduction
Rats were anesthetized with isoflurane UPS (Baxter-Healthcare Corp, Deerfield, IL) (1.0 unit isoflurane/1.5 L O2 per minute) and placed in a stereotaxic apparatus (Stoelting Corp, Wood Dale, IL) for cranial surgery. Body temperature was maintained at 37°C using a feedback-controlled heater (Harvard Apparatus, Boston, MA). Glass micropipettes (1.2-mm outer diameter, World Precision Instruments, Inc, Sarasota, FL) with
A) SV(BUGT)- 1 week  SV(gp120) 1 week  SV(gp120) 2 weeks  SV(gp120) 4 weeks

B) SV(BUGT) d7  SV(gp120) d7  SV(gp120) d14  SV(gp120) d28  SV(gp120) d84

H&E  NT  Iba1  GFAP

C) DAPI  NeuN  NT  NeuN + NT

D) Extent of the lesion (% of CP area)

E) Neuronal loss (% compared to controlateral side)

F) Number of loss-pos.

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tip diameters of 15 \( \mu m \) were backfilled with either 5 \( \mu L \) of SV(gp120), SV(SOD-1), or SV(GPx-1) viral vector, which contains approximately 10^7 infectious units. The vector-filled micropipettes were placed in the CP using coordinates obtained from the rat brain atlas of Paxinos and Watson (32). For injection into the CP, a burr hole was placed +0.48-mm anterior to bregma and −3.0 mm lateral to the sagittal suture. Once centered, the micropipette was placed 6.0 mm ventral from the top of the brain. The vector was given by a Picospritzer II (General Valve Corp, Fairfield, NJ) pulse of compressed N2 for 10 milliseconds at 20 psi until the fluid was completely ejected from the pipette. As positive control for immunocytochemistry for gp120, 500 ng/\( \mu L \) recombinant gp120 were injected into the CP, as described, and the brains were harvested 4 days after injection and analyzed for gp120 immunoreactivity. After surgery, animals were individually housed with free access to water and food.

After variable survival periods, rats were anesthetized via intraperitoneal injection of sodium pentobarbital (Abbott Laboratories, North Chicago, IL) at 60 mg/kg and perfused transcardially through the ascending aorta with 10 mL of heparinized saline followed by ice-cold 4% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA) in 0.1 M phosphate buffer (pH 7.4). Immediately after perfusion-fixation, the rat brains were dissected out, placed in 4% paraformaldehyde for 24 hours, then in a 30% sucrose solution for 24 hours, and then frozen in methyl butane cooled in liquid nitrogen. The samples were cut on a cryostat (10-\( \mu m \)-thick sections).

**Immunocytochemistry**

For immunofluorescence, coronal cryostat sections were processed for indirect immunofluorescence. Blocking was performed by incubating for 60 minutes with 10% goat serum or 10% donkey serum in PBS (pH 7.4). Sections were then incubated with antibodies diluted according to manufacturer’s recommendations: 1 hour with primary antibody then 1 hour with secondary antibody, all at room temperature. Double immunofluorescence was performed as previously described (33). Detection of blood-brain barrier (BBB) dysfunction was assessed by using a 1-step immunohistochemical detection of IgG in which sections were incubated for 1 hour with the antibody (1:100). All incubations were followed by extensive washing with PBS. Medium containing 4’,6-diamidino-2-phenylindole ([DAPI] Vector Laboratories, Burlingame, CA) was used to stain nuclei. Specimens were examined under a Leica DMRBE microscope (Leica Microsystems, Wetzlar, Germany). Negative controls consisted of preincubation with PBS, substitution of nonimmune isotype-matched control antibodies for the primary antibody, and/or omission of the primary antibody.

**TUNEL Assay**

The TUNEL assay was performed according to the manufacturer’s recommendations (Roche Diagnostics, Indianapolis, IN) and following a previously described protocol (21, 23).

**Staining of Neurons Using NeuroTrace**

NeuroTrace ([NT] Molecular Probes, Inc, Eugene, OR) staining has been used as a neuronal marker in numerous studies focusing on neuron characterization (18, 25, 34–36) and was performed as previously reported (21, 23, 37, 38). After rehydration in 0.1 M of PBS, pH 7.4, sections were treated with PBS plus 0.1% Triton X-100 for 10 minutes, washed twice for 5 minutes in PBS, then stained by NT (1:100) for 20 minutes at room temperature. Sections were washed in PBS plus 0.1% Triton X-100 then 2 times with PBS, then let stand for 2 hours at room temperature in PBS before being counterstained with DAPI. Combination NT and antibody staining was performed using primary and secondary antibodies staining first (as previously described), followed by staining with the NT fluorescent Nissl stain. For antibody, TUNEL, and NT staining, immunohistochemistry...
was the first step, followed by TUNEL assay, then by NT staining. All experiments were repeated 3 times, and test and control slides were stained the same day.

**General Morphology**

Microscopic morphology of the brains was assessed by hematoxylin and eosin staining of cryostat sections (10-μm thick). Briefly, cryostat sections were stained with hematoxylin for 3 minutes, rinsed in deionized and tap water, dipped in acid ethanol, and rinsed in tap and deionized water before being stained by eosin for 30 seconds. Sections were dehydrated in 95% then 100% ethanol before being cleared in xylene and mounted in Permount (Fischer Scientific, Pittsburgh, PA). Morphometric study of the brain lesion was assessed by neutral red (NR) staining of the sections. Briefly, the sections were stained by NR for 5 minutes, then washed, dehydrated in alcohols, and finally cleared in xylene.

**Morphometry**

Transduction was assessed for each injected brain (CP injections) by serial cryosectioning of the whole brain (10-μm-thick coronal sections), each slide being numbered, then by immunostaining of every 10th section for gp120 (i.e. at 100-μm intervals, spanning at least 3 mm of tissue rostral and 3 mm caudal to the injection site). This step determined the most anterior and posterior extents of the transduced area. Between 50 and 60 sections were immunostained for each rat. The total number (and not the number in random areas) of gp120-positive cells in 1 hemisphere for every 10th section was counted and summed using a computerized imaging system (Image-Pro Plus, MediaCybernetics, Bethesda, MD). The final number presented was an average of the results measured in the different sections examined. The total number of positive cells in a brain could be estimated by multiplying the cell counts by the length of the transduced area, assuming that the number of positive cells in the sections spanned between the first and 10th one, for example, will be close to the one measured on these first and 10th sections. This procedure, already described for assessment of numbers of transgene-positive cells in the brain (38, 39), allows quantitative and relative comparisons among different time points, although it does not reflect the total number of transduced cells in vivo. If these calculations of cell counts do not conform to unbiased stereological methods, however, they have been realized on 10-μm-thick sections and the total number of positive cells is counted in each section, while stereological methods are usually applied to 40- to 50-μm-thick sections and random fields of the sections are analyzed. Moreover, we used this method for enumerating total neurons in the rat normal striatum by immunocytochemistry (using anti-NeuN antibodies) and staining of neurons by NT. Preliminary results showed a total number of neurons close to the one measured using the optical fractionator method reported in the literature.

The longitudinal distribution of the transgene away from the injection site was assessed as the distance between the most distant sections anterior and posterior to the injection site demonstrating transgene expression.

The TUNEL-, Iba-1-, and CD68-positive cells were counted using Image-ProPlus in the whole CP (and not randomly chosen fields) after TUNEL assay or immunostaining with anti-CD68 and anti-Iba-1 antibodies, respectively, in at least 5 consecutive section. The final number was an average of results measured in the different sections. The NT-positive cells in the CP were counted, as previously described, and neuronal loss was estimated by calculating the ratio of the number of NT-positive cells on the injected side compared with the number of NT-positive cells on the un.injected side.

The lesion areas were determined in NR-stained sections using the imaging system. Computer-assisted tracing of the perimeter of the lesion area surrounding the injection site as well as the whole CP was conducted to determine area measures. The ratio of the lesion area compared with the whole CP area was determined. A total of 20 sections (1 section every 100 μm; 10 sections rostral and 10 sections caudal to the injection site) per animal were used. The length of the extent of tissue damage was determined on serial sections of the whole brain stained by NR. This procedure was similar to one previously described (25).

**Measurement of Malondialdehyde**

Measurement of malondialdehyde (MDA) was used as an indicator of lipid peroxidation. The MDA assay was performed using lipid peroxidation kit (Oxford Biomedical Research, Oxford, MI). The assay is based on reaction of chromogenic...
RESULTS

Injection of SV(gp120) Into the CP Induces a Lesion With Neuronal Loss

Examination of brains at 1, 2, and 4 weeks after injection of SV(gp120) into the CP showed partly hemorrhagic lesions (Fig. 1A). No lesions were seen in the CP of rats injected with SV(BUGT), a control vector, at any time point after the injection. Hematoxylin and eosin staining showed a progressive loss of tissue and cells (particularly neurons) in the area injected with SV(gp120) as well as in the periphery of it. Numerous cells with fragmented nuclei were observed 7 days after injection. Features suggestive of inflammation were seen 14 days after injection. A significant reduction in neurons was observed 28 and 84 days after injection of SV(gp120) into the CP. Loss of tissue led to the development of pseudocystic lesions at the later time points. No abnormalities were observed after injection of SV(BUGT) into the CP or in the contralateral side in SV(gp120) recipients (not shown) (Fig. 1B, first row). Sections stained with the neuronal marker NT showed a progressive loss of neurons not only in the area directly injected with SV(gp120), but also at the periphery; almost no NT cell loss was seen after injection of SV(gp120) (Fig. 1B, second row). Iba-1 identifies both quiescent and activated microglial cells. An appearance suggestive of reactive microglial cells was observed in numerous Iba-1-positive cells from Day 14 to Day 84 after SV(gp120) injection (insets), whereas 7 days after injection, reactive microglial cells coexisted with Iba-1-positive cells with more normal morphology (inset). Iba-1-positive cells were increased to 84 days after injection of SV(gp120) into the CP, with a peak 14 days after injection. No augmentation in the number of microglial cells was seen after injection of SV(BUGT) into the CP or on the contralateral side (not shown) (Fig. 1B, third row). The numbers of GFAP-positive astrocytes were also increased to 84 days after SV(gp120) injection; there was a peak at 2 weeks after injection (Fig. 1B, fourth row). Almost all remaining neurons stained by NT immunostained for NeuN, another neuronal marker, at the different time points (Fig. 1C). The area of the lesion caused by injection of SV(gp120) and measured on NR-stained sections was between 12.9% and 20.6% of total CP area 1 and 12 weeks after injection; this was significantly higher than after injection of SV(BUGT) at the different time points (p = 0.0007) (Fig. 1D). Tissue damage measured on NR-stained sections extended several millimeters rostral and caudal to the injection site (range, 2.1 ± 0.3 to 3.2 ± 0.3 mm, with minimal differences between the different time points). The neuronal loss, expressed as a percentage of NT-positive cells compared with the contralateral side, was between 34.1% and 42.6% after injection of SV(gp120) in the CP and was significantly higher than after the injection of SV(BUGT) in the same area (p = 0.0009) (Fig. 1E). The number of Iba-1-positive cells peaked 2 weeks after injection of SV(gp120) in the CP and remained.

FIGURE 3. Injection of SV(gp120) into the caudate putamen (CP) induces apoptosis. (A) Cryostat brain sections of SV(gp120)- or (control) SV (human bilirubin-uridine 5’-diphosphate-glucuronosyl-transferase) (SV(BUGT))-injected rats assayed for apoptosis by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) at the indicated time points postinjection. (B) Left column: Cryostat sections of brains 12 weeks postinjection analyzed by TUNEL assay and stained with NeuroTrace (NT). Cells that are both TUNEL positive and NT positive are highlighted (arrows). Right column is a higher magnification of an area of the left column. (C) Number of TUNEL-positive cells in the CP after injection of SV(gp120) as a function of time postinjection. (D) Brains at 2 weeks postinjection assayed for TUNEL, stained for NT (NTB), and immunostained for gp120. Overlays demonstrate NT-, gp120-, and TUNEL-positive cells. (E) Injected brains at 2 weeks analyzed by TUNEL, stained by NT, and immunostained for caspase 3. Overlay shows colocalization of caspase 3 and TUNEL in neuronal cells. (E) Panel shows on combined images an NT-positive cell colocalizing with caspase 3 and TUNEL (magnification of a field from Fig. 3E). Scale bars = (A) 80 μm; (B) 60 μm in left column and 20 μm in right column; (C) 45 μm; (D) 30 μm; (E) 25 μm; (F) 8 μm. DAPI, 4’,6-diamidino-2-phenylindole.
significant (p = 0.001) higher than after injection of SV[BUGT] (Fig. 1F) or compared with the uninjected contralateral side (p = 0.001) (not shown).

**Transgene Expression of gp120 by SV(gp120) Injection Into the CP**

Expression of gp120 protein, which is produced as a result of transduction, was demonstrated by immunocytochemistry at different time points after injection. Glycoprotein gp120 was expressed in the cytoplasm. One month after SV(gp120) injection, gp120-positive cells were still present near the site of injection (Fig. 2A). A decrease in numbers of gp120-positive cells was seen at 12 weeks after the injections (Fig. 2B). For a positive control, we used sections of brains harvested 4 days after injection of 300 ng/μL of the recombinant protein gp120 into the CP and immuno-stained them for gp120; they showed that neurons of the somato-sensory cortex took up the protein, probably by retrograde transport (Fig. 2A). No gp120 was detected after injection of SV(BUGT) in the CP (Fig. 2A) or on the contralateral side of SV(gp120)-injected brains, nor was there any positive staining with an isotype-matched control primary antibody or by substitution of the antibody with PBS (not shown).

Western blotting of brain samples harvested 2 weeks after injection of SV(gp120) demonstrated expression of gp120 (Fig. 2C). Glycoprotein gp120 was localized mainly within neurons in the first 2 weeks after injection (Fig. 2D), and in cells of neuronal and nonneuronal origin, such as microglia (Fig. 2E), at later time points (see later).

**Injection of SV(gp120) Into the CP Induces Apoptosis**

Apoptotic cells were demonstrated by TUNEL assay at different times after injection of SV(gp120) into the CP. Rare apoptotic cells were seen after injection of SV(BUGT) (Fig. 3A) (p = 0.001, compared with SV(gp120)). No apoptotic cells were observed in the contralateral uninjected side (not shown). Three months after injection, some TUNEL staining colocalized with NT, whereas other staining did not (Fig. 3B). By 4 weeks postinjection, the numbers of apoptotic cells decreased (Fig. 3C). At 2 weeks postinjection, some apoptotic cells were NT positive, but some apoptotic cells were not NT positive (Fig. 3D). Caspases are cysteine proteases often activated during apoptosis; activated caspase 3 has been described in HAD, as well in cortical or striatal cell cultures exposed to gp120 (40, 41). At 2 weeks after SV(gp120) injection, TUNEL-positive cells also stained for the active form of caspase 3 (Fig. 3E). Figure 3E shows an NT-positive cell colocalizing with caspase 3 and TUNEL.

**Activation of Microglial Cells After Injection of SV(gp120) Into the CP**

To assess microglial activation, we used an antibody against ED1/CD68, a marker of activated microglial cells. The ED1/CD68-positive cells were observed at different time points after injection of SV(gp120) (Figs. 4A, B); some CD68-positive cells were also immunopositive for gp120 (not shown). Very few CD68-positive cells were seen in the area of inoculation when SV(BUGT) was substituted for SV(gp120) (Figs. 4A, B) (p = 0.0005 compared with SV[gp120]) or in the contralateral side (p = 0.0001 compared with SV[gp120]). To assess the type of cells undergoing apoptosis, we enumerated TUNEL-positive cells that were either NT positive or CD68 positive at different time points. The TUNEL-positive cells that were not NT or CD68 positive were considered to belong to another cell type. Apoptotic cells in the area of SV(gp120) injection were mainly of neuronal origin through 2 weeks postinjection. After that time point, microglia predominated (Fig. 4C). Because the numbers of cells varied with time after the injection of SV(gp120) (i.e. loss of neurons, increase of microglial cells), we measured the ratios of the number of TUNEL-positive cells to the number of cells for a given cell type. The results confirmed that the percentage of TUNEL-positive neuronal cells decreased after the first 2 weeks, whereas the percentage of apoptotic CD68-positive cells increased with time (Fig. 4D). One month after gp120 injection, TUNEL staining was observed essentially in DAPI-positive nuclei of CD68-positive cells (Fig. 4E, arrows). The TUNEL-positive material was exceptionally seen outside of the nucleus in CD68-positive cells, suggesting that engulfment of apoptotic bodies by these cells occurred but was a rare event (Fig. 4F). Several CD68-positive cells are also immunostained for the proliferation marker proliferating cell nuclear antigen at different time points after the injections, suggesting that CD68-positive

FIGURE 4. Microglial activation elicited by SV(gp120) injection into the caudate putamen (CP). (A) Brain sections were immunostained for CD68 at 7, 14, and 28 days after intra-CP inoculation of SV(gp120), or control, SV (human bilirubin-uridine 5'-diphosphate-glucuronosyl-transferase) (SV[BUGT]). (B) Numbers of CD68-positive cells per section of CP after injection of SV(gp120) in the CP. (C) Distribution of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive cells of different lineages at different times after injection of SV(gp120) in the CP. The TUNEL-positive cells are expressed as total numbers in the CP. (D) Repartition of TUNEL-positive cells of different lineages at different times after injection of SV(gp120) in the CP. The TUNEL-positive cells are expressed as percentages related to the number of existing cell types in the CP. (E) One month after gp120 injection, TUNEL staining was primarily observed in nuclei (4',6-diamidino-2-phenylindole [DAPI] positive) of CD68-positive cells (arrows). (F) The TUNEL-positive material was exceptionally seen outside of the nucleus in CD68-positive cells, suggesting that engulfment of apoptotic bodies by CD68-positive cells was a rare event. Note that secondary antibodies were tetramethylrhodamine isothiocyanate– and fluorescein isothiocyanate–conjugated in (E) and (F), respectively, and that TUNEL labels were fluorescein and tetramethyl rhodamine conjugated, respectively, in (E) and (F). (G) Cryostat brain sections at 2 weeks after injection of SV(gp120) into the CP immunostained for CD68 and proliferating cell nuclear antigen (PCNA), a marker of cell proliferation. Several CD68-positive cells show immunostaining for PCNA, suggesting that they are proliferating. Insert shows a magnification of 1 area of the field. Scale bars = (A) 150 μm; (E) 15 μm; (F) 4 μm; (G) 80 μm, with 30 μm for insert. NT, NeuroTrace.
cells were proliferating (Fig. 4G). No proliferating cell nuclear antigen–positive cells were seen in the CP injected with saline or SV(BUGT) (not shown).

Injection of SV(gp120) Induces Oxidative Damage

Because oxidative injury is seen in the brains of patients with HAD (42), we tested for oxidative damage after SV(gp120) injection. Lipid peroxidation was measured by calorimetric MDA assay (43) and was assessed by immunocytochemistry for HNE. The HNE-positive neurons were seen in SV(gp120)-injected brains (Fig. 5A), and HNE-positive cells were detected by TUNEL assay (Fig. 5B), suggesting that, as in HAD, neuronal damage caused by SV(gp120) is associated with oxidative stress. No HNE-positive cells were observed in the CP injected with SV(BUGT) or in the contralateral side (not shown). The SV(gp120) elicited more MDA than did the control rSV40 (p = 0.0007) (Fig. 5C).

Protein oxidation was also assessed by immunohistochemistry using antibodies against DNP to identify oxidized proteins (44). The DNP-positive cells were detected in brains injected with SV(gp120) mainly 1 and 2 weeks after SV(gp120) injection. The DNP-positive cells were not seen in the control brains (Fig. 5D), nor when PBS or an isotype-matched control primary antibody was used (not shown). The DNP-positive cells were mainly NT positive at the different time points assessed (Fig. 5E).

Injection of SV(gp120) in the CP of Nude Rats

To determine whether cytotoxic or other T lymphocytes were involved in the CNS lesions caused by SV(gp120), we injected SV(gp120) into the CP of athymic nude rats. These rats lack T cells but have normal B and natural killer cells. The SV(gp120) elicited the same type of lesions previously described in nude rats with comparable numbers of TUNEL-positive cells (Figs. 6A, B) and microglial cells (Figs. 6C, D) after the injection of SV(gp120). Likewise, injecting the control vector SV(BUGT) into the CP of nude rats was not different from injecting the control vector into the normal rats; significantly fewer TUNEL-positive cells (p = 0.0004) and CD68-positive cells (p = 0.0005) were seen in the control injected rats than were found after the injection of SV(gp120).

Blood-Brain Barrier Abnormalities After SV(gp120) Injection Into the CP

Increased BBB permeability has been reported in brains of patients with HAD and in gp120-transgenic mice (45, 46). Immunohistochemical detection of serum IgG leakage from the blood vessels into the brain substance was used as a marker of vascular permeability in sections of rat brains injected with SV(gp120). Areas of IgG accumulation were observed 7 and 14 days after SV(gp120) injection into the CP of normal and nude rats (Fig. 7A). No IgG accumulation was seen in the contralateral side (not shown). The extent of such leakage was less at later time points. No IgG leakage was detected when SV(BUGT) was used instead of SV(gp120) (Fig. 7A). Results were similar in normal and nude rats.

To study the relationship with IgG leakage and vascular breakdown, we immunostained brain microvessels for laminin, a basement membrane protein. At 7 days after injection of SV(BUGT) in the CP, laminin immunostaining was normal and no IgG leakage was observed. By contrast, after injection of SV(gp120) into the CP, few laminin-positive structures were seen, and those that remained were surrounded by areas of IgG accumulation (Fig. 7B). Abnormalities observed in the CP after injection of SV(gp120) are summarized in the Table.

Overexpression of Antioxidant Enzymes Protects Against SV(gp120)-Induced Lesions

The rSV40-delivered antioxidant enzymes copper/zinc SOD-1 and GPx-1 strongly protect from the neurotoxicity of recombinant gp120 injected into the CP (21, 23). To determine whether SOD-1 or GPx-1 could protect from SV(gp120)-induced injury, we administered SV(SOD-1) and SV(GPx-1) to the CP 1 month before SV(gp120), then assayed for apoptotic cells. Prior administration of SV(SOD-1) or SV(GPx-1) significantly protected the brains from the ongoing damage elicited by HIV-1 Env glycoprotein (Fig. 8). Fewer apoptotic cells were observed when SV(SOD-1) or SV(GPx-1) (reduction by 84% and 71% compared with untreated rats, respectively) were administered 1 month before SV(gp120) was injected into the CP (p = 0.01) (Figs. 8A, B). Prior rSV40 delivery of these antioxidant enzymes also reduced SV(gp120)-induced lipid peroxidation (p = 0.003 for both SV[SOD-1] and SV[GPx-1]) (Fig. 8C), although the magnitude of decrease in MDA was less than the level of protection from apoptosis.

DISCUSSION

We describe a model system for HIV-1 Env–induced neurotoxicity in which injection of SV(gp120) into the CP of rats leads to chronic expression of gp120 in microglia and neurons, ongoing apoptosis of these cell types, neuronal loss, oxidative stress, and increased BBB permeability. The rSV40 vectors were used to deliver the gp120 gene into the brain, leading to the long-term expression of the transgene in neurons and microglial cells. In many respects, the consequences of rSV40-delivered gp120 expression in this system resemble the pathological and biochemical alterations observed in neuroAIDS. Ongoing HIV-1 Env–induced apoptosis, especially neuronal apoptosis, in this system is associated with biochemical evidence of oxidative cellular injury, caspase activation, microglial cell accumulation, and increased vascular permeability.

The rSV40-delivered expression of HIV-1 gp120 led to ongoing apoptosis of neurons and microglia. Because inflammatory cells naturally disappear by apoptosis, however, it is also possible that microglia/macrophage apoptosis observed in the present study represents to some extent a physiological phenomenon. Apoptosis caused by SV(gp120) was associated with caspase 3 activation, as has been described in HAD (40), and when cortical or striatal cell cultures were exposed to gp120 (40, 41).

The NT fluorescent stain binds to the Nissl substance of neuron cell bodies and exhibits bright fluorescence that is visible with filters appropriate for fluorescein or TRITC; it is
considerably more sensitive than cresyl violet stain. The NT has been used as a neuronal marker in numerous studies focusing on the characterization of neurons (34–36) and has been used to delineate the lineage of apoptotic cells in murine models of CNS HIV infection and of gp120 neurotoxicity (18, 25). It does not bind to astrocytes or microglial cells.

**FIGURE 5.** Administration of SV(gp120) elicits oxidative stress. (A) Cryostat brain sections at 2 weeks after SV(gp120) injection immunostained for hydroxynonenal esters (HNE) and stained for NeuroTrace (NT) show colocalization of NT and HNE in some but not all HNE-positive cells (arrows). (B) The HNE-positive cells may also be terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) positive; all HNE-positive cells were not TUNEL positive (arrows). (C) Increased brain malondialdehyde (MDA) levels after injection of SV(gp120) assayed 1, 2, and 4 weeks postinjection. (D) Protein oxidation, demonstrated by immunostaining for dinitrophenol (DNP) in brain cryosections, 1 week after injection of SV(gp120) or SV (human bilirubin-uridine 5'-diphosphate-glucuronosyl-transferase) (SV[BUGT]) in the caudate putamen. Insert: a DNP-positive cell (arrow) centered by a 4',6-diamidino-2-phenylindole (DAPI)-positive nucleus. (E) Higher magnification of cryosections as in (D), identifying some DNP-positive cells as neurons. Scale bars = (A) 60 μm; (B) 30 μm; (D) 50 μm, with 20 μm for the insert; (E) 20 μm.
(36). In previous studies, we found that NT staining correlated well with immunostaining using antibodies to other neuronal markers such as NeuN and microtubule-associated protein 2 (MAP-2) (21, 23, 37, 38). We show here that almost all remaining neurons stained by NT immunostained for NeuN at the different times. Injection of SV(gp120) into the CP caused an increase in neuron loss with time.

We used antibodies against Iba-1 and ED1/CD68 to stain microglial cells. An antibody against ED1 was chosen because ED1 is expressed in activated microglial cells, whereas Iba-1 can be present in quiescent as well as in activated microglia. Even if ED1/CD68 is not entirely specific for activated microglial cells, antibodies against ED1/CD68 have been repeatedly used for recognizing activated microglial cells, particularly phagocytic microglia (47). Numbers of ED1/CD68-positive cells in normal brains are typically very low (47), whereas Iba-1–positive cells are frequently observed in normal brains. The numbers of Iba-1– and ED1/CD68-positive cells were both increased after injection of SV(gp120) into the CP, suggesting either microglial proliferation or attraction of monocytes/macrophages to the lesion from the circulation. The TUNEL staining was seen in the cytoplasm of a CD68-positive cell, suggesting that DNA can be phagocytosed as apoptosis occurs. In this regard, it should be mentioned that Gelbard et al (48) described TUNEL-positive microglial cell cytoplasm in brains from children with HIV-1 encephalitis, postulating that microglia might phagocytose DNA in this condition.

Soluble gp120 induces apoptosis in many cell types including cardiomyocytes (49), lymphocytes (50), and neurons (51, 52). At high concentrations, HIV gp120 may be directly neurotoxic (53). Among other effects, gp120 binds neuron cell membrane coreceptors (CCR3, CCR5, and CXCR4) and elicits apoptosis that is signaled via these G protein–coupled pathways (54, 55). When we previously injected recombinant gp120 directly into the CP (21, 23), gp120 was detected in the CP by immunocytchemistry only at early time points after injection (from 1 hour to 2 days), and then disappeared. Apoptosis peaked 1 day after the injection of recombinant gp120 protein, and oxidative stress was seen at early postinjection time points as well. By 4 days after the injection of gp120, ongoing apoptosis and oxidative damage were no longer detectable in the CP. In contrast, gp120 was still present 3 months after inoculation of SV(gp120), and apoptotic cells were still seen. Oxidative stress continued to be observed at least 1 month after injection of SV(gp120). Direct injection of recombinant gp120 induces acute lesions, which are reproducible but are unlike the chronic injury observed in AIDS. For this reason, we tried to develop an in vivo model of chronic HIV-1 Env–induced neurotoxicity that recapitulated some of the chronicity and pathogenetic features of Human Immunodeficiency Virus Encephalopathy (HIVE) not generally seen when recombinant HIV-1 proteins are inoculated. It should be pointed out that although the gp120 used in these studies was from the X4-tropic strain HIV-1 NL4-3, the pathology and biochemical changes are indistinguishable from those caused by gp120 from R5-tropic HIV-1 (21, 23).

The lesions caused by SV(gp120) were virtually identical in nude rats and their nonnude counterparts. Neuron apoptosis, vascular leakage, and accumulation of microglial cells were all similar in both types of rats. Thus, effective T cell–mediated immune responsiveness does not seem to play a role in these CNS lesions elicited by gp120 expression. This conclusion may be significant for understanding the pathogenesis of the CNS lesions seen in HIVEL.

Injection of SV(gp120) in the CP of rats induced oxidative stress, as assessed by increased MDA levels in the brain, HNE production, and immunolocalization of DNP indicative of protein oxidation in neuronal cells. Oxidative stress can play a role in HIV-1–associated dementia in several ways. Oxidation of polyunsaturated fatty acids results in production of multiple aldehydes with different carbon chains including hydroxyxenonenal esters (HNE) (56) that can mediate oxidative stress–induced apoptosis of cultured neurons (57) and damage neurons and cause cognitive dysfunction in vivo (58). The HNE-positive neurons, as well as increases in ceramide, have been demonstrated in the brains of patients with active progressive HIV-1 encephalitis (42, 59). It has also previously been shown that HIV-1 gp120 can cause lipid peroxidation (60).

Accumulation of extravasated IgG in the CP suggested that SV(gp120) increased BBB permeability. Abnormalities in the BBB are important in mediating some of the tissue damage that accompanies HIV-1 infection of the brain, as well as facilitating viral entry into the CNS (45). Evidence of serum-protein leakage across the BBB has been demonstrated in the brains of patients with HAD (46, 61), and accumulation of serum proteins in subcortical neurons and glia has been observed more frequently in HIV-1–positive patients with dementia than in those with no cognitive impairment (62). The BBB compromise is associated with neurocognitive impairment, and elevated plasma viral load in the presence of BBB compromise may increase the risk for development of HAD (63). Absence or fragmentation of occludin and zona occludens 1, 2 important structural proteins of tight junctions, was demonstrated in brains of patients who died with HIVEL, but in contrast, no significant changes were observed in tissues from HIV-seronegative control patients and from HIV-1–infected patients without encephalitis (64). It is

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**FIGURE 6.** The SV(gp120)-induced apoptosis and microglial cell accumulation do not require T cell–mediated immunity. (A) Cryostat brain sections from nude rats that had been injected with SV(gp120) or (control) SV (human bilirubin-uridine 5′-diphosphate-glucuronosyl-transferase) (SV[BUGT]) analyzed by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. (B) Numbers of TUNEL-positive cells in the caudate putamen (CP) after injection of SV[BUGT] or SV(gp120) in the striatum as a function of time postinjection. (C) Microglial cells accumulate in response to SV(gp120) in nude rats. Cryostat brain sections from injected nude rats immunostained for CD68. The cortex, far from the inoculation point, was also examined. (D) Numbers of CD68-positive cells in the CP after injection of SV[BUGT] or SV(gp120) in the CP. Scale bars = (A) 100 μm; (C) 150 μm in 3 columns on the left and 100 μm in the right column. DAPI, 4′,6-diamidino-2-phenylindole.
reported that gp120 leads to extravasation of albumin, increased numbers of vessels immunostained for intercellular adhesion molecule 1 and vascular cell adhesion molecule 1, as well as immunoreactivity for substance P at the endothelial cell surface, as demonstrated in gp120-transgenic mice (65, 66).

The lesion induced by the injection of SV(gp120) into the CP was partly hemorrhagic, whereas we did not observe hemorrhagic lesions in previous studies when we injected either saline or other SV40-derived vectors into the CP (23, 38). On the other side, direct injection of recombinant gp120 protein into the CP was associated with hemorrhage, the extent of the lesion being dependent on the concentration of gp120 injected (see Figure, Supplemental Digital Content 1, http://links.lww.com/A1072). Hemorrhage has been considered to be a rare feature of HIV-related brain damage, occurring mainly in children. Recently, however, subarachnoid or multiple intracerebral hemorrhages associated with diffuse vasculopathy have been described in adults (67–72). It has also been reported that the introduction of highly active antiretroviral therapy in children with HIV-associated multiple intracranial aneurysms resulted in the nonprogression, or resolution, of the aneurysms (73, 74). Postmortem histology in some patients showed intimal hyperplasia, fibrosis, and thickened beaded internal elastic lamina with fragmentation, suggesting that the focus of the insult in the intracranial vasculopathy might be the intima (71). The mechanism of development of vascular abnormalities in HIV-infected

**FIGURE 7.** Intracerebral injection of SV(gp120) increases blood-brain barrier (BBB) permeability. (A) Cryostat brain sections of the caudate putamen (CP) from normal rats (upper panels) and nude rats (lower panels) injected with SV(gp120) or SV (human bilirubin-uridine 5'-diphosphate-glucuronosyl-transferase) (SV[BUGT]) at the level of the striatum were immunostained for immunoglobulin G (IgG) to evaluate BBB leakage of plasma protein. (B) Seven days (d) after injection of SV(gp120) into the CP, few laminin-positive structures were seen, and those remaining were surrounded by areas of IgG accumulation, whereas after injection of SV(BUGT), laminin immunostaining was normal, and no IgG leakage was observed. Scale bars = (A) and (B) 100 μm. DAPI, 4',6-diamidino-2-phenylindole.
TABLE. Parameters Studied Chronologically After Injection of SV(gp120) Into the Caudate Putamen

<table>
<thead>
<tr>
<th>Postinjection</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 28</th>
<th>Day 84</th>
</tr>
</thead>
<tbody>
<tr>
<td>gp120 positivity</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Apoptosis</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Neuron loss</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Microglial activation</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Astrocyte proliferation</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BBB abnormalities</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

The frequency of the abnormalities observed in the caudate putamen after injection of SV(gp120) was graded from low (+) to high (++++). BBB, blood-brain barrier.

patients remains unknown. Vascular changes might be caused by any number of factors, including membrane changes in the endothelial cells in response to cytokines, or direct toxic effects of a viral protein on the endothelial cells. In this respect, we have found that recombinant gp120 protein alone can induce apoptosis of brain microvascular endothelial cells, breakdown of the BBB (unpublished data), and hemorrhage (see Figure, Supplemental Digital Content 1, http://links.lww.com/A1072). It is, therefore, not surprising to observe hemorrhage associated with the disruption of the BBB after injection of gp120 into the CP (see Figure, Supplemental Digital Content 1, http://links.lww.com/A1072).

The hemorrhagic lesions disappear 7 days after injection of recombinant gp120 into the CP, whereas they still can be observed more than 1 month after inoculation of SV(gp120) into the same structure; this underscores our contention that SV(gp120)-related damage is more chronic.

A potential therapeutic strategy for the treatment of HAD might be to limit oxidative stress–induced neurotoxicity. Gene transfer of antioxidant enzymes has been achieved in a gene therapy perspective in numerous models of neurological disorders using diverse viral vectors: adenovirus (75), lentiviruses (76), herpes simplex (77). We previously showed that SV40 vectors mediated overexpression of antioxidant enzymes in vitro and in vivo protects against gp120- and Tat-related neuronal apoptosis (21–23). Because the lesions caused by recombinant gp120 were very brief and thus different from the ongoing ones in this report, our present findings emphasize the role of antioxidant molecules in the neuroprotection against gp120 in a chronic model of HAD. They also indirectly reinforce the putative link between oxidative stress and genesis of the lesions because fewer apoptotic cells were achieved when a reduction of the MDA levels was obtained after gene transfer of antioxidant enzymes. It is important to note that the protection from apoptosis afforded by SV(SOD-1) and SV(GPx-1) was substantially greater than the percentage decrease in MDA. These findings have potential implications for therapies of other neurological diseases in which protein, lipid, and DNA oxidation are thought to play key roles.

One of the potential uses of this experimental system is to facilitate development of experimental therapeutics. Interesting information could also be provided by neuropsychological tests (e.g. water maze testing), as well as behavioral assays focused on the basal ganglia (rotational behavior [39]; behavioral testing of the sensitivity to psychostimulants [78]). SV(gp120) inoculation into the CNS does not perfectly recapitulate all aspects of neuroAIDS pathogenesis. However, its reproducibility, relative accessibility, and low cost, as well as its chronicity and susceptibility to inhibition by antioxidant gene delivery, all suggest that this model may readily lend itself to screening and analysis of experimental neuroAIDS therapeutics.

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