**ORIGINAL ARTICLE**

**HIV-1 gp120–Induced Injury to the Blood-Brain Barrier: Role of Metalloproteinases 2 and 9 and Relationship to Oxidative Stress**

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Abstract

Blood-brain barrier (BBB) disruption occurs during human immunodeficiency virus encephalopathy, but the mechanisms involved are not understood. We studied how acute and ongoing exposure to human immunodeficiency virus 1 envelope gp120 alters BBB structure and permeability. Intravenous Evans blue, given before stereotaxic gp120 injection into the caudate putamen of rats, was rapidly extravasated. Gelatinolytic activity, studied by in situ zymography, was increased after gp120 administration and was localized within cerebral vessel walls. The gp120 increased the expression of matrix metalloproteinases (MMPs) 2 and 9. Laminin and claudin-5, key BBB components and targets of both MMPs, were greatly reduced upon gp120 administration. The gp120 increased lipid peroxidation in the vascular endothelium and in neurons. Prior administration of rSV40 vectors carrying the antioxidant enzymes Cu/Zn superoxide dismutase or glutathione peroxidase protected from gp120-induced BBB damage. N-methyl-D-aspartate receptor activation upregulated pro-MMP-9 and increased MMP-9 gelatinase activity, and memantine, an N-methyl-D-aspartate receptor blocker, mitigated gp120-induced BBB abnormalities. Using intra–caudate putamen SV(gp120) to test the effects of chronic exposure to expressed gp120, we determined that oxidant stress and increased BBB permeability occurred as in acute exposure. These data indicate that both direct administration and cellular expression of gp120 lead to disruption of the BBB by increasing MMPs and reducing vascular tight junction proteins via mechanisms involving reactive oxygen species generation and oxidant injury.

**Key Words:** Blood-brain barrier, HIV-1, HIV encephalopathy, gp120, Matrix metalloproteinases, Oxidative stress.

**INTRODUCTION**

The blood-brain barrier (BBB) is composed of brain microvascular endothelial cells that are tightly apposed to each other and, along with astrocytic end feet, pericytes, basal lamina, and neurons, form the neurovascular unit (1, 2).

The neurovascular unit is important in restricting the passage of soluble and formed elements from the blood into the CNS and in maintaining the immunologic privilege in the CNS (1, 3).

Breaches of the BBB occur in many diseases and, in some conditions, may enhance the damage caused by the initial injury. It has been suggested that BBB disruption mediates some of tissue damage that accompanies human immunodeficiency virus 1 (HIV-1) infection of the brain, thereby facilitating viral entry into the CNS (4).

Loss of BBB integrity may manifest in several ways, including leakage of blood components into the brain parenchyma, loss of key protein components of endothelial cell tight junctions, and loss of vessel structural proteins. Serum protein leakage across the BBB occurs in the brains of patients with HIV-associated dementia (5, 6), 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 (7). At early stages of HIV-1 invasion of the CNS, myelin pallor and gliosis of the white matter have been attributed to opening of the BBB because of vasculitis (8). A weakened BBB is associated with neurocognitive dysfunction and elevated plasma viral load and may increase the risk for development of HIV-associated dementia (9). Absence or fragmentation of occludin and zonula occludens 1, two important structural proteins of tight junctions, was demonstrated in the brains of patients with HIV-1 encephalitis, but no significant changes were observed in the brains of HIV-seronegative control patients or HIV-1–infected patients without encephalitis (10). Moreover, loss of zonula occludens 1 was highly correlated with monocye infiltration and HIV-associated dementia (11). In transgenic mice, the expression of the HIV-1 envelope protein gp120 led to albumin extravasation and to other indices of vascular damage (12–14).

The basal lamina of the BBB contains the extracellular matrix molecules laminin, Type IV collagen, and fibronectin, most of which are substrates for a family of neutral proteases called matrix metalloproteinases (MMPs), especially MMP-2 and MMP-9 (also known as gelatinases). These MMPs can injure the BBB, in part, through their proteolytic activity at the tight junctions of BBB endothelial cells and basal lamina. The MMPs have been implicated in invasion of neural tissue by inflammatory cells and in direct cellular damage in diseases of the central and peripheral nervous systems (15–25), but the mechanisms by which HIV damages the BBB in patients with HIV encephalopathy are still unclear.

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This study was undertaken to identify biochemical and pathological parameters of BBB injury caused by gp120. We examined the effects of its exposure on the cellular and protein constituents of the BBB and analyzed the contributions of MMPs, oxidative injury, and key CNS signaling processes to BBB dysfunction. Because exposure to HIV-1 in HIV/acquired immunodeficiency syndrome (AIDS) patients is protracted, we studied BBB injury in a model system of continuing gp120 exposure expression by CNS cells.

MATERIALS AND METHODS

Animals
Female Sprague-Dawley rats (300–350 g) were purchased from Charles River Laboratories (Wilmington, MA). Protocols for injecting and killing animals were approved by the Thomas Jefferson University Institutional Animal Care and Use Committee and were consistent with Association for Assessment and Accreditation of Laboratory Animal Care standards. Female Sprague-Dawley rats were used to maintain comparability to our prior studies on gp120; all of our preclinical experiments were done in these rats (26–30). Experiments were done in female rats at similar points of their estrous cycle determined by vaginal smears. The animals received a regular standard commercial diet, regular powdered rodent diet without any component that might cause oxidative stress, such as high-fat diet, or high manganese, and was not folate/methyl or iron deficient. Rats had free access to water and diet.

Antibodies
The following primary antibodies were used: rabbit anti-N-acetylaspartate-4-hydroxy-2-nonenal (HNE) immunoglobulin G (IgG), 1:100; Calbiochem, La Jolla, CA), a marker of lipid peroxidation; rabbit anti ionized calcium-binding adaptor molecule 1 (Iba1), a marker of quiescent and active microglia (IgG, 1:100; Wako Chemicals, Osaka, Japan); mouse anti-claudin-5 (IgG1, 1:100; Zymed Laboratories, Inc, San Francisco, CA); rabbit anti-MMP-9 (IgG, 1:100); goat anti-MMP-2 (IgG, 1:100); goat anti-CD31/platelet endothelial cell adhesion molecule 1 (IgG, 1:100); goat anti-α4 integrin (IgG, 1:100) (Santa Cruz Biotechnology, Santa Cruz, CA); mouse anti-β2 integrin (IgG1, kappa, 1:100); mouse anti-neuN (IgG1, 1:100) (Chemicon International, Temecula, CA); mouse anti-glial fibrillary acidic protein (GFAP) IgG2b, 1:100; BD Pharmingen, Franklin Lakes, NJ); mouse anti-α5 integrin (IgG1, 1:100; Serotec, Oxford, UK); and rabbit anti-laminin (IgG, 1:100) (Sigma, St Louis, MO). Secondary antibodies were used at 1:100 dilution: fluorescein isothiocyanate (FITC-) and tetramethyl rhodamine isothiocyanate (TRITC-)conjugated goat anti-mouse IgG (γ-chain specific and against all whole molecule, respectively), 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) and anti-goat IgG (whole molecule; Jackson ImmunoResearch Laboratories, Inc, West Grove, PA), and FITC-conjugated goat anti-rat IgG H&L (F(ab)2 fragment; Abcam, Cambridge, MA).

Vector Production
The general principles for making recombinant, Tag-deleted, replication-defective SV40 viral vectors have been previously reported (31–34). The SV(gp120) is a recombinant Tag-deleted SV40-derived vector that expresses HIV-1NL4-3 gp120 under control of the cytomegalovirus immediate early promoter (20, 22). Superoxide dismutase 1 (SOD1) and glutathione peroxidase 1 (GPX1) transgenes were cloned into pT7[RSVLTR], in which transgene expression is driven by the Rous sarcoma virus long terminal repeat. The SV(human bilirubin-uridine 5′-diphosphate-glucuronosyltransferase [BUGT]), which was used here as negative control vector, with a nontoxic by-product, has been reported (35).

In Vivo Transduction and Injection of gp120
Rats were anesthetized with isoflurane USP (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 Precisions Instruments, Inc, Sarasota, FL) with tip diameters of 15 μm were backfilled with 5 μL of SV(BUGT), SV(gp120), SV(SOD1), or SV(GPX1) viral vector, which contains approximately 10⁶ particles. The vector-filled micropipettes were placed in the caudate putamen (CP) using coordinates obtained from the rat brain atlas of Paxinos and Watson (36). 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 same coordinates were used for injecting 500 ng of gp120 in 1 μL saline, as well as for injecting saline and 1 μL saline containing 500 ng of rat IgG. The gp120, saline, rat IgG, or the vector were given by a Picospritzer II (General Valve Corp, Fairfield, NJ) pulse of compressed N2, duration of 10 milliseconds at 20 psi until the fluid was completely ejected from the pipette. After surgery, animals were housed individually with free access to water and food.

Assessment of BBB Disruption After gp120 Injection
The BBB permeability was studied by intravenous (i.v.) injection of 2% Evans blue (EBF) FW, 960.81; Sigma) in 0.9% saline in the jugular vein 15 minutes before administering 500 ng of gp120. Blood-brain barrier disruption was evaluated 1 and 24 hours after injection of saline or gp120 (n = 5 at each time point for each treatment; total, 20). To test the specificity of the effects of gp120, 1 μL of saline containing 500 ng of rat IgG (Sigma) was injected into the CP as a control unrelated protein (n = 5 for each time point; total, 10). The brains were then removed without prior fixation, divided into ipsilateral and contralateral hemispheres, weighed, homogenized in 400 μL of N,N-dimethylformamide (Sigma), then centrifuged at 21,000 × g for 30 minutes. Evans blue was...
quantified using a spectrophotometer from the absorbance at 620 nm of each supernatant minus the background calculated from the baseline absorbance between 500 and 740 nm. Recombinant HIV-1 BaL gp120 was obtained from the National Institutes of Health AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Germantown, MD. Recombinant HIV-1 BaL gp120 was free of endotoxin, as were the rat IgG and the vehicle used (saline).

Injection of EB was also performed before gp120 administration in a second group of rats from which brains were removed 15 minutes, 30 minutes, 1 hour, and 24 hours after injection of 500 ng of gp120 (n = 5 at each time point for gp120-, saline-, and rat IgG–injected rats; total, 60). The brains were removed without fixation and frozen for histochemistry and in situ zymography in cooled isopentane (−70°C) for 1 minute and stored at −80°C. In addition, to study the relationship between the gp120 concentration and BBB abnormalities, 1 μL of saline containing either 100 ng or 250 ng of gp120 was injected into the CP of rats, the brains of which were harvested without fixation and frozen for histochemistry and 1 and 24 hours after injection (n = 5 rats for each time point for each concentration; total, 20 rats). Evans blue injection was performed before gp120 administration. To study EB extravasation on sections, 10-μm sections of unfixed frozen brains were cut on a cryostat and observed using a fluorescent microscope (Leica Microsystems, Wetzlar, Germany) with a TRITC filter.

MMP Expression and Consequences of gp120 Injection

The MMP-2 and MMP-9 are the major tissue proteases capable of targeting microvascular proteins. Therefore, we assessed the time courses of their expression after intra-CP injection of gp120. One microliter of saline containing 500 ng of gp120 was injected stereotaxically into the CP of rats, and their brains were harvested at different time points after the injection (15 minutes, 30 minutes, 1 hour, 6 hours, 1 day, 2 days, 7 days, 14 days, and 28 days; n = 5 rats at each time point; total, 45). Controls (n = 4 for each time point; total, 36) received saline instead of gp120 in the CP.

Injection of SV(gp120) Into the CP

To assess the effects of more protracted exposure to gp120 on BBB, we used a system in which HIV-1 envelope gp120 is expressed over time in CNS cells (30). Rats were injected intra-CP with SV(gp120), which allows for continued gp120 production by transduced cells. The brains from the animals were then studied for 4 weeks for evidence of BBB injury using immunohistochemical detection of serum IgG leakage from the blood vessels into the brain substance as a marker of vascular permeability. To study the relationship between IgG leakage and vascular breakdown, we studied brain microvessels for the vascular basement membrane protein, laminin. SV(gp120) was injected into the CP of Sprague-Dawley rats, and their brains were harvested 1, 2, and 4 weeks after injection (n = 6 for each time point; total, 18). Controls received saline or SV(BUGT) instead of SV(gp120) in the CP (n = 4 for each time point for SV[BUGT]; total, 12; n = 4 for each time point for saline; total, 12).

Challenge With gp120 After Administration of SV(GPx1)/SV(SOD1)

To study the possible protection of the BBB from gp120-related injury by rSV40-mediated overexpression of SOD1 and GPx1, we first injected the CP of rats with SV(SOD1) (n = 5) and SV(GPx1) (n = 5). One month later, the CP in which SV(SOD1) or SV(GPx1) had been administered was injected with 500 ng of gp120 into the CP. Brains were harvested 1 day after injection and studied for BBB abnormalities and MMP-9 immunoreactivity (IR) (n = 5 for each vector). In all cases, control rats received SV(BUGT) in the CP instead of SV(SOD1) and SV(GPx1) (n = 5; total, 15).

Tissue Harvesting for Histological Studies

After variable survival periods, rats were anesthetized by 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 mol/L phosphate buffer (pH 7.4). Immediately after perfusion-fixation, the brains were removed, placed in 4% paraformaldehyde for 24 hours, then placed in a 30% sucrose solution for 24 hours, then frozen in methyl butane cooled in liquid nitrogen. Samples were cut on a cryostat (10-μm sections).

In Situ Zymography

Gelatinolytic activity was studied in situ on frozen 10-μm-thick brain sections using a commercial kit (EnzCheck Gelatinase Assay Kit; Molecular Probes, Eugene, OR), as described (16, 37). Briefly, sections were incubated with the fluorogenic substrate DQ gelatin conjugate at 37°C overnight then washed in PBS and mounted in mounting medium containing 4′,6-diamidino-2-phenylindole ([DAPI] Vector Laboratories, Burlingame, CA). Cleavage of DQ protein by MMPs resulted in a green fluorescent product (excitation wavelength, 495 nm; emission, 515 nm). Un.injected contralateral sides were compared with injected sides. Controls consisted of brains in which the CP had been injected with saline or saline with rat IgG, and sections of brains injected with gp120 were incubated with the zinc chelator 1,10-phenanthroline (1 mmol/L in dimethyl sulfoxide, Molecular Probes), a nonspecific inhibitor of MMP activity. Sections passing through normal hippocampus, which contains abundant gelatinolytic activity, were used as positive controls (22).

To identify the cell types that expressed gelatinolytic activity, some sections were stained using NeuroTrace (NT), a stain for neurons (26–30, 38–44), or were immunostained for neuN, RECA-1, CD31, and GFAP after the in situ gelatinolytic assay was performed. Specimens were examined under a Leica DMRBE microscope. Combination staining was performed using primary and secondary antibodies staining first, followed by staining with NT staining. All experiments were repeated 3 times, and test and control slides were stained the same day.

Immunocytochemistry

Coronal cryostat sections (10-μm thick) were processed for indirect immunofluorescence, as previously described (30, 45). Detection of BBB disturbances in SV(gp120)-injected CPs was assessed by using a 1-step immunohistochemical
FIGURE 1. Injections of gp120 into the caudate putamen (CP) result in blood-brain barrier breakdown. (A) Evans blue (EB) was injected intravenously before the injection of gp120 into the CP. Extravasation of EB was observed in the CP from 15 minutes after injection of gp120 (arrow). No EB was observed on the contralateral (cont.) side or in controls injected with saline or 1 K saline containing 500 ng rat immunoglobulin G (IgG). (B) Fluorescence corresponding to EB extravasation was observed by microscopy in the injected CP. *Injection site. (C) The EB-positive areas measured by spectrophotometry were higher in gp120-injected brains than in controls (p < 0.01). (D) The EB-positive areas were dependent upon the gp120 doses injected (p < 0.01, 500 ng and 250 ng vs saline, saline containing rat IgG and 100 ng; p < 0.05, 100 ng vs saline, saline containing rat IgG and 500 ng vs 250 ng). Values are mean ± SEM. Scale bars = (B) 100 μm.
detection of IgG (130–140 kD; 0.5 mg/mL) in which sections were incubated for 1 hour with the antibody (1:100). Mounting media contained DAPI to stain nuclei. Specimens were then examined under a Leica DM2500 microscope (Leica Microsystems). 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.

**Morphometry**

The ICAM-1, claudin-5, laminin-positive structures, and MMP-2 and MMP-9-positive cells were counted on injected and uninjected sides of the CP of animals given gp120 or saline throughout the entire area of the CP per section (within at least 5 consecutive sections) using a computerized imaging system (Image-Pro Plus, MediaCybernetics, Bethesda, MD). This procedure was also used for assessing numbers of vessels expressing gelatinolytic activity on injected and uninjected sides in whole CPs of gp-120 animals and controls in at least 5 consecutive sections.

The computerized imaging system was also used to quantitate the EB-positive area. Computer-assisted tracing of the perimeter of the EB-positive area and of the whole CP was conducted on unfixed cryostat sections to determine ratios of the EB-positive areas to the whole CP areas in a total of 20 sections (1 per 100 μm; 10 sections rostral and 10 sections caudal).

**FIGURE 2.** Injection of gp120 into the caudate putamen (CP) increases gelatinolytic activity. (A) Frozen sections of CP injected with 500 ng gp120 and stained by in situ zymography. There was increased gelatinolytic activity in the vicinity of the lesion as early as 30 minutes after injection, which was abolished with an inhibitor (zinc chelator 1,10-phenanthroline). Gelatinolytic activity was strong in the hippocampus; almost no gelatinolytic activity was seen on the contralateral side or in injected controls. (B) The fluorescent product was observed in blood vessel-like structures and in cells. (C) Number of vessels exhibiting gelatinolytic activity were increased in CPs injected (inj.) with gp120 at 1 hour versus CPs injected with saline, saline containing rat immunoglobulin G (IgG), and uninjected (uninj.) contralateral CPs (p < 0.01). In (A) and (B), injection sites are indicated by asterisks (*). Scale bars = (A) 120 μm; (B) left) 100 μm; (B) right) 45 μm.
caudal to the injection site) per animal. Final numbers were average measurements in the different sections for both immunohistochemistry and EB-positive ratios.

**Malondialdehyde Measurement and Pharmacological Studies**

Malondialdehyde was used as an indicator of lipid peroxidation, as previously described (30). The N-methyl-D-aspartate (NMDA) has been reported to upregulate the pro-enzyme form of MMP-9 and to increase MMP-9 gelatinolytic activity (46). The potential involvement of NMDA-type glutamate receptors in the BBB disturbances induced by gp120 was studied using memantine, an NMDA receptor (NMDAR) antagonist, which reduces MMP-9 in some conditions associated with pathological activation of the enzyme (47). Memantine (30 mg/kg; Tocris, Ellisville, MO) was injected intraperitoneally 15 minutes before 500 ng gp120 injection into the CP (n = 5); control rats received saline (n = 5). Brains were

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**FIGURE 3.** Cell types with gelatinolytic activity. (A–E) Cryostat sections of caudate putamen (CP) injected with 500 ng gp120 1 hour earlier, stained by in situ zymography (Z) for neuronal markers NeuroTrace (NT) (A) and neuN (B), rat endothelial cell antigen 1 (RECA-1), a rat brain vessel marker (C), CD31, an endothelial cell marker (D), and glial fibrillary acidic protein (GFAP) for astrocytes (E), show colocalization in merged images. In (C), there was colocalization between in situ zymography and RECA-1 in both longitudinal and transverse (insets) sections of vessels. In ([C] upper inset), some cells are stained by in situ zymography in addition to the vessel. Scale bars = (A, B) 50 μm; (C) 70 μm; (D) 25 μm; (E) 80 μm; ([E] inset) 35 μm.
removed 24 hours after injection and were frozen, stored, and examined for EB areas and ICAM-1 immunocytochemistry, as previously described.

Statistical Analysis

Medians between 2 groups were compared using the Mann-Whitney U test (with a 2-tail p value). Comparison of medians between more than 2 groups was done using the Kruskal-Wallis test. Differences between groups were considered significant when p < 0.05.

RESULTS

Gp120 Increases BBB Permeability

In rats given a prior i.v. injection of EB, leakage of the dye was seen as early as 15 minutes after injection of gp120.

FIGURE 4. Cryostat sections of caudate putamen (CP) at different time points after injection with 500 ng gp120 and immunostained for matrix metalloproteinases (MMP-2 and MMP-9). (A) The expression of MMP-2 and MMP-9 is increased after injection of gp120 into the CP but not on the contralateral (uninjected) side or after injection of saline (not shown). (B) Increased MMP-2 expression is detected in the area of vascular leakage, as evidenced by Evans blue positivity, 1 hour after gp120 injection. (C) At 6 hours after injection, MMP-9 colocalized mainly with neurons, stained by NeuroTrace (NT) and neuN, and rarely with glial fibrillary acidic protein (GFAP)-positive astrocytes. Inset: higher magnifications of the fields. In (A-C), the injection sites are indicated by asterisks (*). Scale bars = (A) 100 μm; (B) 70 μm; (C) first row) 80 μm; (C inset) 70 μm; (C) second row) 40 μm.
into the CP. The extension and intensity of EB extravasation were more pronounced 1 hour after injection and remained so until 24 hours. No EB extravasation was observed on the contralateral side or when saline or saline containing rat IgG were injected instead of gp120 (Fig. 1A). Evans blue extravasation was detected on cryostat sections as a red fluorescent signal; no signal was seen when saline was injected in the CP instead of gp120 or in the contralateral side (Fig. 1B). Similar results were observed when saline containing rat IgG was injected (not shown). Evans blue measurements revealed significantly more extravasation in the CP on the injected sides compared with the contralateral side, saline-injected or

**FIGURE 5.** (A) Cryostat sections of caudate putamen (CP) at 1 hour after injection of 500 ng gp120 and immunostained for rat endothelial cell antigen 1 (RECA-1), a marker of brain vessels in rats; NeuroTrace (NT), a neuronal marker; glial fibrillary acidic protein (GFAP), a marker of astrocytes; and Iba1, a marker of microglial cells. Lower row, Immunostaining for Iba1 and matrix metalloproteinase 2 (MMP-2) were performed using fluorescein isothiocyanate (FITC)- and tetramethyl rhodamine isothiocyanate (TRITC)-conjugated secondary antibodies; insets are from different sections immunostained using TRITC- and FITC-conjugated secondary antibodies for Iba1 and MMP-2, respectively. Arrows indicate colocalizations. (B) The MMP-2 in microvessels immunostained for intercellular adhesion molecule 1 (ICAM-1). Note the absence of MMP-2 immunostaining in the CP injected with saline. In (A) and (B), the injection sites are indicated by asterisks (*). Scale bars = ([A] first row) 60 μm; ([A] second row) 80 μm; ([A] inset) 60 μm; ([A] third and fourth rows) 35 μm; (B) 60 μm.
its contralateral side, and to the CP injected with saline containing rat IgG ($p < 0.01$) (Fig. 1C). The extents of the EB-positive areas were gp120 dose dependent ($p < 0.01$, 500 ng and 250 ng vs saline, saline containing rat IgG and 100 ng; $p < 0.05$, 100 ng vs saline, saline containing rat IgG and 500 ng vs 250 ng) (Fig. 1D).

**Gelatinolytic Activity is Increased by gp120 Administration**

There was increased gelatinolytic activity at the site of gp120 administration within 30 minutes after injection. Almost no gelatinolytic activity was seen in the contralateral uninjected side or after injecting either saline or saline

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**FIGURE 6.** Cryostat sections of brains injected with gp120 after prior intravenous injection with Evans blue (EB) and immunostained for laminin, matrix metalloproteinase 2 (MMP-2), MMP-9, and claudin-5. (A, A') There is colocalization of MMP-2–laminin at 30 minutes (A) and MMP-9–laminin at 24 hours (A') after injection of gp120 in the caudate putamen (CP). (B) There is a decrease in the number of laminin-positive structures over time ($p < 0.01$ gp120 vs saline). (C) There is a decrease in numbers of claudin-5–positive structures in CPs injected with gp120 ($p < 0.05$ gp120 vs saline) (C'). This reduction was more obvious in the EB-positive area. Injections sites are indicated by asterisks (*) in (B), (C), and (C'). Scale bars = (A) 80 μm; (B, C) 100 μm.
containing rat IgG. Nearly all fluorogenic activity was blocked by preincubating the tissue sections with a zinc chelator (1,10-phenanthroline) for 20 minutes (Fig. 2A). At 30 and 60 minutes after injection of gp120, fluorescent product was observed in blood vessel-like structures and in cells (Fig. 2B). There was a significant increase in the number of vessels showing gelatinolytic activity in CPs injected with gp120 1 hour earlier compared with the uninjected contralateral side and compared with the CPs injected with saline or saline containing rat IgG (p < 0.01) (Fig. 2C).

Most gelatinolytic activity was within neurons that were stained by NT (Fig. 3A) or immunostained for neuN.
MMP-2 and MMP-9 Are Expressed Early in the CP After gp120 Injection

Increased MMP-2 IR was first detected 30 minutes to 1 hour after gp120 injection; it had largely subsided by 24 hours. By contrast, MMP-9 IR was first detected 6 hours after gp120 injection.

FIGURE 8. Injection of SV(gp120) into the caudate putamen (CP) increases blood-brain barrier permeability. (A) Cryostat sections of the CP of rats that were uninjected or injected 7 days earlier with SV(gp120) or SV(human bilirubin-uridine 5'-diphosphate-glucuronosyl-transferase [BUGT]) at the striatum level immunostained for immunoglobulin G (IgG). (B) At 7 days after injection of SV(gp120), there are numerous matrix metalloproteinase 2 (MMP-2)-positive and MMP-9-positive cells; most of these are stained with NeuroTrace (NT). Injection site indicated by asterisks (*). (C) At 1 week after injection of SV(gp120), there were fewer laminin-positive structures, whereas after injection of SV(BUGT), laminin immunostaining was normal (p < 0.01). Scale bars = (A) 100 μm; (B) 80 μm; (B inset) 40 μm.
postinjection, which peaked at Day 1 and then decreased at Day 2 (Fig. 4A). No MMP-2 IR or MMP-9 IR was detected at Day 7 or later time points (not shown). Neither enzyme was detected on the contralateral (uninjected) side or in injection controls (not shown). The spatial relationship between EB extravasation and MMP-2 was established by colocalization studies. Increased MMP-2 IR was detected in the area of vascular leakage (indicated by EB positivity) within 1 hour after gp120 injection (Fig. 4B). The MMP-9 IR was colocalized mainly within neurons that were NT positive and

**FIGURE 9.** (A) Injection of memantine (mem) intraperitoneally before the intravenous injection of Evans blue (EB) and administration of gp120 to the caudate putamen (CP) limited the extent of the EB-positive area (p < 0.01, gp120 CP + memantine 30 mg/kg vs gp120 CP + saline). (B) Less reduction in the number of intercellular adhesion molecule 1 (ICAM-1)–positive structures in the CP after injection of gp120 when it was preceded by memantine 30 mg/kg (p < 0.01, gp120 CP + mem vs gp120 CP + saline). Inj. side, injected side; Uninj. side, uninjected side. (C) Gene delivery of antioxidant enzymes by SV40-derived vectors in the CP 1 month before gp120 injection into the same structure mitigated the extent of the blood-brain barrier breakdown after gp120 injection. Morphometry of extent of EB-positive area (p < 0.01 SV[superoxide dismutase 1 (SOD1)] + gp120 and SV[glutathione peroxidase 1 (GPx1)] + gp120 vs SV[human bilirubin-uridine 5’-diphosphate-glucuronosyl-transferase (BUGT)] + gp120). (D) Prior gene delivery of antioxidant enzymes into the CP before injection of gp120 decreased the numbers of matrix metalloproteinase 9 (MMP-9)–positive cells compared with injection of the vector control SV(BUGT) (p < 0.01, SV(SOD1) + gp120 and SV(GPx1) + gp120 vs SV(BUGT) + gp120). Injection sites in (B) and (D) are indicated by asterisks (*). Scale bars = (B) 80 μm.

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neuN positive, and rarely with astrocytes, 6 hours after gp120 injection (Fig. 4C). The MMP-2 was observed within the walls of blood vessels (RECA-1 positive) 1 hour after gp120 injection and colocalized mostly with NT and with neuN (not shown), rarely with astrocytes, and exceptionally with Iba1-positive microgria (Fig. 5A). The MMP-2 IR was also present in the remaining ICAM-1-positive microvessels 1 hour after injection (Fig. 5B). The MMP-2 was not detected in saline control–injected CPs (Fig. 5B). The MMP-2- and MMP-9-positive cells were enumerated at the peak of expression of each MMP (i.e. 1 hour after gp120 administration for MMP-2 and 24 hours after gp120 injection for MMP-9). The average number of MMP-2-positive cells at the peak was 38.7% ± 4.2% and 68.4% ± 7.6% for MMP-9-positive cells.

**gp120 Injection Reduces Laminin and Claudin-5**

The MMP-2 and MMP-9 colocalized with laminin, respectively, 30 minutes and 24 hours after injection of gp120 into the CP (Fig. 6A, A'). At 6 hours after the injection, there was a significant reduction in the number of laminin-positive structures in the injected CP (p < 0.01 gp120 vs saline) (Fig. 6B). No reduction in the number of laminin-positive structures was seen in the contralateral (uninjected) side or when saline was substituted for gp120. The tight junction protein claudin-5 was significantly decreased in CPs injected with gp120 (p < 0.05, gp120 vs saline), and no reduction was observed in CPs injected with saline or on uninjected sides (Fig. 6C). Claudin-5-positive structures were most reduced in EB-positive areas (Fig. 6C).

**Lipid Peroxidation in Brain Vessels After Administration of gp120**

Malondialdehyde levels were significantly higher in CPs injected with gp120 than in control CPs (p < 0.01) (Fig. 7A). Double immunostaining for cell markers and HNE, a marker of lipid peroxidation, demonstrated HNE-positive cells in the walls of large vessels 1 hour after administering gp120 into the CP. The HNE-positive cells expressed the endothelial cell marker CD31 (Figs. 7B, C) and were positive for NT (Fig. 7D) and GFAP (Fig. 7E).

**SV(gp120) Injection Into the CP Leads to BBB Abnormalities**

There were areas of IgG accumulation 7 days after injection of SV(gp120) into the CP (Fig. 8A). No such IgG leakage was detected in the contralateral CP or when SV(BUGT) was injected (Fig. 8A). At 1 week after injection of SV(gp120), there was increased MMP-2 IR and MMP-9 IR in the CPs injected with SV(gp120). The MMP-2–positive and MMP-9–positive cells were mainly neurons (Fig. 8B) and were more numerous close to the site of injection. There were also fewer laminin-positive structures, particularly in areas of IgG accumulation after SV(gp120) injection. This decrease in CPs injected with SV(gp120), compared with contralateral CPs or CPs injected with SV(BUGT), was significant (p < 0.05) (Fig. 8C). Results at 2 and 4 weeks were similar to those at 1 week (not shown).

**Blocking NMDAR and Antioxidant Gene Delivery Protect From gp120-Induced Effects**

Injection of memantine before i.v. injection of EB and intra-CP administration of gp120 limited the extent of the EB-positive area (p < 0.01, gp120 CP + memantine 30 mg/kg vs gp120 CP + saline) (Fig. 9A). Memantine pretreatment also mostly protected ICAM-1–positive structures from gp120 effects (p < 0.01 gp120 CP + memantine vs gp120 CP + saline) (Fig. 9B).

Gross examination showed that antioxidant gene delivery decreased EB leakage. This interpretation was supported by morphometric analysis of the extent of EB-positive areas and spectrophotometric measurements of EB concentrations (p < 0.01 for either SV[SOD1] + gp120 or SV[GPx1] + gp120 vs SV[BUGT] + gp120) (Fig. 9C). Substituting saline for gp120 did not lead to EB leakage. Moreover, intra-CP gene delivery of either of the antioxidant enzymes before injection of gp120 decreased the number of MMP-9–positive cells when compared with injection of the vector control SV(BUGT) (p < 0.01 for either SV[SOD1] + gp120 or SV[GPx1] + gp120 vs SV[BUGT] + gp120) (Fig. 9D).

**DISCUSSION**

We report that gp120 injection into the CP increases levels of MMP-2 and MMP-9 and gelatinolytic activity and leads to sustained changes in vascular permeability. The MMP-2 and MMP-9 are considered to be gelatinases (48), and expression of both MMP-2 and MMP-9 and gelatinolytic activity was detected mainly in neurons, vessel matrix, and endothelial cells. Neuronal localization of both MMPs and gelatinolytic activity has also been described (18, 37). Endothelial cells (24, 49, 50) and vascular smooth muscle cells can release MMP-9 when exposed to stimuli such as thrombin, interleukin-1α, mechanical injury, and 3-nitropropionic acid; and MMP-9 is expressed constitutively in hippocampal blood vessels (51–53). The early-enhanced MMP2/MMP9 expression and gelatinolytic activity after gp120 injection into the CP resemble findings in other experimental brain injury models (16, 24).

Reduction in gp120-induced oxidative stress by gene delivery of antioxidant enzymes was associated with decreased MMP-9–positive cells and protection of the BBB from disruption. We have shown (both here and previously) that injection of gp120 into the CP induces lipid peroxidation and that antioxidant enzyme gene delivery protects from neuronal apoptosis caused by the HIV-1 protein (26, 28, 54). Reactive oxygen species are important in the pathogenesis of HIV-induced CNS injury (55) and can be induced in brain endothelial cells by HIV-1 gp120 and Tat (56, 57). Various factors, such as reactive oxygen species, nitric oxide, and proteases such as plasmin and stromelysin-1, are involved in MMP activation and upregulation in CNS injury (24, 58), and MMPs have been reported in the cerebrospinal fluid of HIV-infected patients (59, 60). In rapidly progressing simian immunodeficiency virus–infected monkeys, MMP-9 levels correlate with motor and cognitive deficits (61). Moreover, cerebrospinal fluid levels of the urokinase-type plasminogen activator receptor, which plays an important role in degradation...
of extracellular matrix, and hence BBB injury, are elevated in patients with HIV dementia (62).

The HIV-1 viral proteins have been implicated in MMP activation. Tat increases release of MMP-1 and MMP-2 in neuron cultures (63) and, in combination with basic fibroblast growth factor, activates MMP-2 and membrane type-1 MMP in endothelial cells (64). After injection of gp120 into the lateral ventricle, levels of MMP-2 and MMP-9 increased rapidly; prior administration of an MMP inhibitor reduced consequent neuronal apoptosis (65). The MMP-2 activity and protein were increased in C6 cells (i.e. stable transfectants for gp120) and in gp120-transgenic mouse brains (66).

We found that laminin and claudin-5 were reduced after gp120 injection, suggesting that MMP activation may lead to abnormalities of the BBB. Indeed, gp120 is toxic to brain endothelial cells in vitro, (67–69) but previous studies of gp120 injection in vivo have not described BBB dysfunction (29, 40, 70). Disruption of the BBB caused by MMP-related reduction in tight junction proteins (e.g. claudin-5) has been previously linked to increased transmigration of normal monocytes, dendritic cells, and HIV-infected human leukocytes through brain vessel endothelium and in an in vitro human BBB model (1, 71, 72). However, in addition to BBB permeability, leukocyte capture from the circulation, as a consequence of upregulation of adhesion molecules, plays an important role in leukocyte movement from the blood to the CNS. Thus, we postulate a vicious cycle in which HIV-1–infected monocytes enter the CNS through the microvascular network, produce HIV-1 proteins (gp120 and Tat) that directly and indirectly injure the BBB, thereby allowing further immigration of HIV-1–infected cells and perpetuating the cycle.

Administration of SV(gp120) leads to more chronic continuing gp120 production (30), and accumulation of extravasated IgG in the CP suggests that ongoing cellular production of gp120 also increases BBB permeability. 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, is associated with biochemical evidence of oxidative cellular injury, caspase activation, microglial cell accumulation (30), and increased vascular permeability. Thus, gene delivery of ongoing gp120 expression, as a model of production of HIV-1 Env by HIV-1–infected cells recapitulates chronic brain vascular injury caused by acute gp120 administration and by HIV-1 infection.

We found that both acute and chronic exposure to HIV envelope gp120 disrupted the BBB via lesions in brain microvessels, MMP activation, and degradation of the vascular basement membrane and vascular tight junctions. All of these factors lead to a loss of BBB integrity. This is a novel model in which normal rat brains are subjected to gp120 damage (at artificially selected doses), and in which gp120–induced injury is associated with loss of BBB integrity. We show that gp120 induces BBB impairment by increasing MMPs, causing a reduction in tight junction proteins associated with oxidative stress. These results suggest that gp120 alone might be sufficient, whether acting directly or indirectly by triggering a cascade of events, to cause BBB damage. It is known that soluble gp120 increases glial cell release of arachidonate, which impairs neuron and astrocyte upregulation of glutamate, in turn leading to prolonged activation of NMDAR with consequent disruption of cellular Ca2+ homeostasis. This causes reactive oxygen species production and leads to neuron cell death after mitochondrial permeabilization, cytochrome c release, and activation of caspases and endonucleases (73). Activation of the NMDAR plays also a role in the BBB injury, as indicated by the protective effects of rSV40-delivered antioxidant enzymes and NMDAR antagonists.

On the other hand, many factors, including other HIV proteins, chemokines, or inflammatory cytokines secondary to infection of monocytes/macrophages by the virus, and so on, may cause oxidative stress and BBB impairment. Involvement of the BBB in human HIV infection mainly involves the white matter in which productive HIV infection with abundant HIV protein is uncommon. It also occurs in asymptomatic pre-AIDS cases, in which there is little or no productive HIV infection. In later stages, secondary effects, other infections, the terminally dysregulated human immune system, and other factors in the months to decades of damage in neuroAIDS have to be considered also. The HIV-1 gene products may act alone or in concert with vasoactive molecules (e.g. prostaglandins, nitric oxide, substance P) to produce this BBB injury in neuroAIDS (4). Model systems that allow study of HIV-1–related CNS and BBB injury, both acutely and over time, may improve our understanding of neuroAIDS pathogenesis and the role of BBB dysfunction in neuroAIDS and, perhaps, lead to new therapeutic approaches for neurological complications of HIV-1 infection.

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