Increased Peroxynitrite Activity in AIDS Dementia Complex: Implications for the Neuropathogenesis of HIV-1 Infection

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Increased Peroxynitrite Activity in AIDS Dementia Complex: Implications for the Neuropathogenesis of HIV-1 Infection

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Oxidative stress is suggested to be involved in several neurodegenerative diseases. One mechanism of oxidative damage is mediated by peroxynitrite, a neurotoxic reaction product of superoxide anion and nitric oxide. Expression of two cytokines and two key enzymes that are indicative of the presence of reactive oxygen intermediates and peroxynitrite was investigated in brain tissue of AIDS patients with and without AIDS dementia complex and HIV-seronegative controls. RNA expression of IL-1β, IL-10, inducible nitric oxide synthase, and superoxide dismutase (SOD) was found to be significantly higher in demented compared with nondemented patients. Immunohistochemical analysis showed that SOD was expressed in CD68-positive microglial cells while inducible nitric oxide synthase was detected in glial fibrillary acidic protein (GFAP)-positive astrocytes and in equal amounts in microglial cells. Approximately 70% of the HIV p24-Ag-positive macrophages did express SOD, suggesting a direct HIV-induced intracellular event. HIV-1 infection of macrophages resulted in both increased superoxide anion production and elevated SOD mRNA levels, compared with uninfected macrophages. Finally, we show that nitrotyrosine, the footprint of peroxynitrite, was found more intense and frequent in brain sections of demented patients compared with nondemented patients. These results indicate that, as a result of simultaneous production of superoxide anion and nitric oxide, peroxynitrite may contribute to the neuropathogenesis of HIV-1 infection.

in brain tissue of demented and nondemented AIDS patients. In addition, we examined the localization of these two enzymes by double immunohistochemical staining on brain slices of demented AIDS patients. To confirm our in vivo results, we investigated whether HIV-1 infection of macrophages in vitro also resulted in changes in oxidative processes, by looking at superoxide anion production and SOD mRNA expression. Finally, immunohistochemical staining for nitrotyrosine, a footprint for peroxynitrite, was performed to investigate whether peroxynitrite was present in the brains of demented and nondemented AIDS patients.

Materials and Methods

Brain tissue

Tissue specimens of the frontal cortex was obtained from autopsied brain of thirteen HIV-1-infected and five control cases (those who died of causes not related to HIV-1 infection). All the HIV-1-infected individuals had developed AIDS at the time of death and showed decreased levels of CD4 T cells (&lt;300). Seven of them developed cognitive and motor impairments. None of the patients received any antiretroviral therapy. The clinical data on all individual patients are shown in Table I. AIDS or HIV-associated dementia was a premortem clinical diagnosis made by a neurologist. The severity of dementia was scored on the Memorial Sloan-Kettering (MSK) scale. Patients 1–6 and 14–18 did not suffer from motor or cognitive impairments. ND, not determined.

<table>
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<tr>
<th>Patient</th>
<th>Age (yr)/Sex</th>
<th>Postmortem Delay (h)</th>
<th>Clinical Status</th>
<th>MSK*</th>
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* HIV-associated dementia was a premortem clinical diagnosis made by a neurologist. The severity of dementia was scored on the Memorial Sloan-Kettering (MSK) scale. Patients 1–6 and 14–18 did not suffer from motor or cognitive impairments. ND, not determined.

HIV-1 infection of macrophages

PBMC were isolated from heparinized blood from HIV-1-, HIV-2-, and hepatitis B-seronegative donors and obtained on Ficoll-Hypaque density gradients. Cells were washed twice, and monocytes were purified by countercurrent centrifugal elutriation. Cells were &gt;98% monocytes by criteria of cell morphology on May-Grünwald-Giemsa-stained cytomorphs and by nonspecific esterase staining using α-naphylacetate (Sigma, St. Louis, MO) as substrate. Monocytes were cultured in suspension at a concentration of 2 × 10^6 cells/ml in Teflon flasks (Nalgene, Rochester, NY) in DMEM with 10% heat-inactivated human AB serum negative for anti-HIV Abs, 10 mg/ml gentamicin, and 10 mg/ml ciprofloxacin (Sigma). As previously described, HIV-1 infection of nonadherent macrophages, especially when using a low multiplicity of infection, appears much more reproducible than infection of macrophages that were first allowed to adhere (30). After 7 days, monocyte-derived macrophages (MDM) were recovered from the Teflon flasks and infected with HIV-1, at a multiplicity of infection of 0.01. Two hours later, macrophages were removed from the Teflon flasks, washed twice to remove unbound virus, and used for studies to determine levels of superoxide anion production by chemiluminescence and SOD expression by RNA PCR in HIV-infected macrophages.

Chemiluminescence

Cells were recovered from Teflon flasks and washed twice with HBSS containing 1% FCS (Life Technologies, Grand Island, NY). A total of 2 × 10^6 cells was exposed to an equal volume of HBSS in the presence of 250 nM bis-N-methylacridinium (Lucigenin, Sigma). Superoxide dismutase (SOD, Sigma) was added to HIV-infected macrophages to demonstrate that chemiluminescence was superoxide anion specific. Lucigenin reacts with superoxide anion (31), and this reaction is accompanied by photon emission. The number of photons emitted during stimulation of the macrophages was measured as light emission in a luminometer (Packard Instruments, Brussels, Belgium) and expressed as cpm, as described previously (32).

RNA PCR detection of cytokines and enzymes

Brain tissue and 2 × 10^6 macrophages were homogenized and lysed, respectively, in 1 ml and 0.4 ml Trizol (Life Technologies) according to the manufacturer’s guidelines. In experiments where the levels of SOD expression of macrophages were determined, the lysed cells were stored in Trizol at −70°C. When lysates of all time points were obtained, total RNA was isolated. Total RNA was dissolved in diethylpyrocarbonate (DEPC)-treated water, and 1 μg of RNA was used for the synthesis of complementary DNA. The RNA was previously heated for 5 min at 70°C, chilled on ice, and added to a mixture containing 1× reverse transcriptase (RT) buffer (Promega, Madison, WI), 200 U of reverse transcriptase, 0.1 M DTT (Life Technologies), 2.5 mM dNTP (Boehringer Mannheim, Indianapolis, IN), 80 U random hexamer oligonucleotides (Boehringer Mannheim), and 10 U
RNAsin (Promega). The complete mixture was now incubated for 60 min at 37°C and then heated for 5 min at 90°C. The final reaction volume was diluted 1:8 by adding distilled water. Amplification of the cDNA was accomplished using one primer biotinylated on the 5' terminal nucleotide to facilitate later capture using streptavidin. The PCR primer pair was chosen to span at least one intron. To the PCR reaction mixture the following components were added: 0.25 mM dNTP mix (Boehringer Mannheim), 1/3 PCR buffer (50 mM KCl, 10 mM Tris-HCl, 1.5 mM MgCl₂; Promega), 0.2 mM sense and antisense primers (Table II), 5 μl cDNA, and 1 U Taq polymerase (Promega). Denaturation, annealing, and elongation temperatures for PCR were 94°C, 60°C, and 72°C for 1, 1, and 2 min each, using a DNA thermal cycler (Perkin-Elmer, Norwalk, CT). Negative controls were included in each assay to confirm that none of the reagents were contaminated with cDNA or previous PCR products. PCR was also performed on RNA samples to exclude genomic DNA contamination. To confirm single band product, positive reactions were subjected to 40 cycles amplification and electrophoresis, followed by ethidium bromide staining. Then, for semiquantification, every primer pair was tested at different cycle numbers to determine the linear range. GAPDH, SOD, and IL-1β mRNA levels were high, and 20 cycles was enough to measure the PCR product in its linear range, whereas iNOS cDNA had to be subjected to 30 cycles and IL-10 even to 40 cycles to be in the linear range.

Aliquots of 5 μl of the biotinylated PCR product were semiquantitatively analyzed using a fluorescent digoxigenin detection ELISA kit (Boehringer Mannheim) according to manufacturer’s protocol. In short, the biotinylated strand of denatured PCR product is captured by immobilized streptavidin. Then, a digoxigenin-labeled probe is added, followed by an alkaline phosphatase-labeled Ab against digoxigenin. After addition of the

Table II. Sequences of the oligonucleotide primers and probes in RT-PCR

<table>
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<th>Target</th>
<th>Primers</th>
<th>Sequence</th>
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<td>Probe</td>
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FIGURE 1. Cytokine and enzyme mRNA levels in the frontal cortex of postmortem brain tissue of HIV-infected nondemented patients (H), HIV-infected demented patients (HD), and seronegative control patients (C) expressed as RFU. Significantly elevated gene expression for IL-1β (A, p < 0.025), IL-10 (B, p < 0.01), iNOS (C, p < 0.05), and SOD (D, p < 0.01) was found in demented patients compared with nondemented HIV-infected patients. p values were calculated using a Student’s t test. Results are representative of at least three independent PCR experiments.
substrate, fluorescence was measured in relative fluorescence units (RFU) in a fluorescence multiwell plate reader (Perceptive Biosystems, Framingham, MA) at excitation 450 nm/emission 550 nm. All data were normalized against GAPDH mRNA level, which was used as an internal standard.

**Statistical analysis**

Data were compared, and a two-tailed Student’s t test was used to determine p values.

**Immunohistochemical analysis of brain tissue**

Frozen sections of brain tissue were analyzed for SOD, iNOS, nitrotyrosine (NT), and HIV p24 Ag expression. Brain slices were first incubated for 18 h at 4°C with the first Ab (anti-SOD human liver, Calbiochem, San Diego, CA; anti-iNOS mac NOS, Transduction Laboratories, Lexington, KY; anti-HIV-1 p24, Dupont-NEN, Boston, MA; anti-NT polyclonal, Upstate, Biotechnology, Lake Placid, NY). Astrocytes and microglial cells were stained with anti-glial fibrillary acidic protein (GFAP; Amersham Life Science, Rainham, England) and anti-human phagocyte macrophage/microglia CD68/Ki-M7 (BMA, Valbiotech, France), respectively. The binding was subsequently revealed after another incubation of 45 min at 18°C with a corresponding alkaline phosphatase conjugated anti-IgG Ab and fast red substrate (Boehringer Mannheim).

**Results**

**Expression of IL-1β, IL-10, iNOS, and SOD mRNA in brain tissue of demented and nondemented HIV patients and control patients**

A semiquantitative fluorescence assay was used to study the levels of expression of IL-1β, IL-10, iNOS, and SOD in brain tissue of the frontal cortex of the individual patients described in Table I. The mRNA levels of all gene products, expressed in RFU, are depicted in Fig. 1, A-D. To detect all PCR products in their linear range, cDNA was subjected to 30, 40, 20, and 20 cycles for iNOS, IL-10, SOD, and IL-1β, respectively, indicating that high levels of IL-1β and SOD were present in all patients. IL-1β, a proinflammatory cytokine, was detected in the HIV-demented group at significantly higher levels than in the groups of control (p = 0.025) and nondemented HIV patients (p = 0.025). This finding confirms that, also in these demented patients, cerebral immune activation seems to occur (10–12), which may eventually prove to be a crucial event in the neuropathogenesis of HIV-1 infection (33–37).

Interestingly, IL-10 was also expressed at significantly higher levels in the HIV-demented group compared with the groups of control (p < 0.01) and nondemented HIV patients (p < 0.01).
SOD mRNA levels were also expressed significantly more in the HIV-demented patients compared with the control patients \((p < 0.02)\) and the nondemented HIV-infected patients \((p < 0.01)\). Finally, iNOS expression was also detected at higher levels in the demented HIV-infected patients when compared with nondemented HIV-infected patients \((p < 0.05)\), but not to the control group. When the individual levels of enzyme RNAs were compared, a trend between the levels of SOD and iNOS can be observed in the group of demented AIDS patients (Fig. 2). With the exception of case 12, it was found that the moderate to high levels of SOD in patients 7, 8, 9, and 11 (Fig. 2A) correspond to moderate and high levels of iNOS (Fig. 2B). This trend is not observed in the nondemented patients or in the control group. Brain specimens of most nondemented patients even do not have detectable SOD levels. In the two cases (cases 3 and 6) where SOD expression was detected, only in one (case 3) iNOS was found. Thus, these data suggest that the presence of both SOD and iNOS seems to be highly associated with the occurrence of clinical dementia.

**Immunohistochemical localization of increased SOD and iNOS gene products in brain tissue of AIDS patients**

The expression of SOD, iNOS, and HIV-1 p24 Ags was analyzed in frozen sections of frontal lobes of five of the studied patients (patients 1, 3, 7, 9, and 14). Expression of SOD and iNOS Ags was detected in patients 3, 7, and 9, whereas they were barely detectable in patient 1 and control patient 14, a result that paralleled that of the RNA detection method. Since staining patterns between the different patients did not differ substantially, only single stains of patient 9 for SOD and iNOS are shown in Fig. 3. A and C, respectively. In general, SOD labeling was more intense than that of iNOS, which was also observed by the RNA detection method. SOD was found in cells of both perivascular and parenchymal areas. To better define the SOD-positive cells, double immunohistochemical staining was performed on cases 3 and 9. SOD immunoreactivity patterns did not differ for both patients, except for minor differences in staining intensity. Therefore, only the result of the double staining of brain tissue of patient 9 is shown in Fig. 3B. SOD expression was mostly localized in CD68-positive microglial cells in the parenchyma (Fig. 3B) and in the perivascular areas where the frequency of SOD-positive cells was less (data not shown). SOD staining was infrequent and faint in GFAP-positive astrocytes (data not shown). To better define the iNOS-positive cells, double labeling experiments were performed on the same cases. By double labeling, we found that both CD68-positive microglial cells (Fig. 3D) and GFAP-positive astrocytes (data not shown) expressed iNOS Ags with roughly the same frequency and intensity in patient 9, whereas iNOS reactivity was detectable but faint in patient 3 (data not shown). Subsequently, double immunohistochemical staining was performed on case 9 with SOD and HIV-1 p24-specific mAbs. More than 60% of the p24 Ag-positive cells also contained SOD Ag (Fig. 3E). Since HIV-1 productively replicates only in brain macrophages (3–5), these findings suggest that SOD expression and possibly superoxide anion production occurred mostly in HIV-1-infected brain macrophages.

**HIV-1 infection of macrophages results in increased SOD expression**

Since elevated SOD levels were detected in brain macrophages in tissue obtained from demented AIDS patients, the ability of HIV-1 to affect SOD expression in macrophages was investigated in vitro. Therefore SOD expression in HIV-infected macrophages was compared with replicate uninfected cultures by RT-PCR analysis. As shown in Fig. 4A, the expression of SOD mRNA in uninfected macrophage cultures decreased in time, whereas the HIV-infected macrophages continued to express SOD. Importantly, the time course pattern of HIV mRNA levels was similar to that of SOD mRNA levels (Fig. 4B). To confirm that HIV RNA expression indeed led to HIV-1 production, the release of p24 Ag in the culture supernatants was detected by ELISA (Fig. 4C).
HIV-1 infection of macrophages results in enhanced superoxide anion production

To investigate whether the relative increased levels of SOD expression in HIV-infected macrophages could be a consequence of increases in superoxide anion production, the production of superoxide anion by HIV-1-infected macrophages was compared with that of uninfected macrophages using chemiluminescence. Immediately after HIV-1 infection, the ratio of the amount of superoxide anion production between HIV-infected and uninfected macrophages measured after 30 min was 1.2 (Fig. 5A). Four days after HIV-1 infection, the amount of superoxide anion production by HIV-infected macrophages increased when compared with that of the uninfected cells (Fig. 5C). The ratio of the amount of superoxide anion production between HIV-infected and uninfected macrophages measured after 30 min was 2.9. To demonstrate that the chemiluminescence signal was indeed superoxide anion specific, we added SOD, resulting in a dose-dependent decrease of the signal (Fig. 6). The chemiluminescence signal was completely abolished when 100 μg/ml SOD was added (data not shown), indicating that, in our experiments, lucigenin indeed reacts specifically with superoxide anion.

Brain sections of patients suffering from AIDS dementia complex show intense parenchymal and perivascular NT staining

Since the reaction between NO and superoxide anion results in the formation of peroxynitrite and both these molecules appear to be present in the brains of demented AIDS patients, elevated levels of peroxynitrite are to be expected. Therefore, brain sections of patients 4, 9, and 11 were stained for NT, which is observed when large amounts of peroxynitrite were produced. Whereas the nondemented AIDS patient 4 did not show any substantial reactivity with the NT polyclonal, the demented AIDS patient 11 showed strong staining for NT parenchymal as well as perivascular (Fig. 7). In addition, patient 9 stained heavily for NT (data not shown) although less than patient 11. Interestingly, patient 9 showed lower
expression of iNOS and SOD than patient 11 (Fig. 2), suggesting a possible relation between the degree of iNOS/SOD expression and the presence of nitrosylated proteins. As a control for nonspecific staining, the primary Ab was omitted. In addition, as a control for NT staining, the primary Ab was preincubated with NT. Both control experiments resulted in inhibition of staining (data not shown).

**Discussion**

It is generally assumed that AIDS dementia complex is a disease in which immune activation of glial cells plays an important role (10–12, 33–37). This study confirms earlier observations that IL-1β mRNA levels are increased in brain tissue of demented AIDS patients. Surprisingly, we also detected elevated expression of IL-10 in these brain samples. IL-10 is a potent inhibitor of cytokine secretion by macrophages/microglia and was not to be expected in the group of patients with elevated IL-1β levels. However, this further supports the concept of the existence of immune activation in the brains of demented AIDS patients, despite the presence of endogenous antiinflammatory mediators.

In this study, it is demonstrated that, although the ability of macrophages to produce superoxide anion in vitro decreases in time, elevated amounts of superoxide anion are produced upon in vitro HIV-1 infection of macrophages compared with control macrophages. Since macrophages and microglia function as a long-term reservoirs for HIV-1 (38), these cells apparently possess a mechanism to protect themselves against the toxic effects of superoxide anion. Indeed, we show here that elevated levels of superoxide anion coincide with elevated levels of cytosolic copper-zinc SOD, an important intracellular scavenger of superoxide anion, in HIV-infected macrophages compared with control macrophages. Since these in vitro data demonstrate that HIV infection of macrophages result in both increased superoxide anion production and in increased SOD expression, changes in SOD mRNA expression in vivo may also be indicative of changes in superoxide anion production. In vivo, SOD mRNA levels were found to be elevated in demented AIDS patients, and immunohistochemical analysis of brain tissue revealed that SOD is localized mostly in HIV-infected brain macrophages. These data suggest that superoxide anion production by HIV-infected macrophages may also be increased in vivo.

SOD is known to be involved in several other neurodegenerative diseases, like amyotrophic lateral sclerosis, Down’s syndrome, and Alzheimer’s disease (39–43). Although SOD can scavenge superoxide anion, this reaction will not take place in the presence of NO. When produced in large amounts, iNOS is the only molecule that can effectively out-compete SOD for superoxide anion by generating NO, a highly diffusible molecule that is able to react with superoxide anion to form peroxynitrite (20–24). Recently, iNOS has been shown to be involved in the pathogenesis of AIDS dementia complex (16, 19) and to be directly or indirectly responsible for neuronal damage (9, 44, 45). In addition to the elevated mRNA levels of SOD, we here also show that iNOS mRNA levels are significantly elevated in brain tissue of demented AIDS patients compared with nondemented AIDS patients. This suggests that, besides superoxide anion, levels of NO may be elevated as well in brains of demented AIDS patients.

In general, cellular interactions between astrocytes and immune-activated macrophages/microglia are believed to be responsible for the production of neurotoxic as well as neurotrophic factors (46–48). Although we show that in vivo microglia are able to express iNOS, the ability of human macrophages to produce NO remains highly controversial. Despite the presence of iNOS in human macrophages, the production of NO by these cells in vitro is presumably very low (49) or even absent (13, 15). Indeed, we were also not able to demonstrate any NO production or expression of iNOS by HIV-infected macrophages in vitro (data not shown). However, recently it was demonstrated that macrophages isolated from active multiple sclerosis lesions showed immunoreactivity for iNOS and were able to produce NO (50). In addition, it has been reported that IFN-α can induce iNOS and NO in human monocytes (51). This implicates that there might be a trigger involved in vivo that is not present in vitro. NO has been detected in primary human astrocytes, and, for these cells, IL-1β is the key proinflammatory
cytokine involved in the induction of this molecule (13, 14). Interestingly, elevated levels of macrophage-derived IL-1β have been detected in brain tissue of demented AIDS patients (11, 12), suggesting that, in AIDS dementia complex, immune-activated macrophages are able to evoke the release of NO from astrocytes (Fig. 8). Together with the macrophage-derived superoxide anion, this astrocyte-derived NO may result in the formation of the highly neurotoxic peroxynitrite. Importantly, we show that NT, the footprint of peroxynitrite, is detected more frequently and more intensely in brain sections of demented AIDS patients compared with nondemented AIDS patients, indicating that peroxynitrite was indeed present in the brains of these patients. In conclusion, neuronal damage and death may be the result of interactions between both immune-activated microglia and astrocytes and the subsequent production of combined toxic reactive oxygen intermediates like peroxynitrite (Fig. 8). Thus, although HIV-1 replicates in macrophages, astrocytes might also participate in the neuropathogenesis of HIV-1 infection.

Acknowledgments

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References


