Induction of monocyte chemoattractant protein-1 in HIV-1 Tat-stimulated astrocytes and elevation in AIDS dementia

(chemotaxis/central nervous system)

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ABSTRACT Activated monocytes release a number of substances, including inflammatory cytokines and eicosanoids, that are highly toxic to cells of the central nervous system. Because mononuclear infiltration of the central nervous system closely correlates with HIV-1-associated dementia, it has been suggested that monocyte-derived toxins mediate nervous system damage. In the present study, we show that the HIV-1 transactivator protein Tat significantly increases astrocytic expression and release of monocyte chemoattractant protein-1 (MCP-1). Astrocytic release of β-chemokines, which are relatively less selective for monocytes, including RANTES, macrophage inflammatory protein-1α, and macrophage inflammatory protein-1β, was not observed. We also show that MCP-1 is expressed in the brains of patients with HIV-1-associated dementia and that, of the β-chemokines tested, only MCP-1 could be detected in the cerebrospinal fluid of patients with this condition. Together, these data provide a potential link between the presence of HIV-1 in the brain and the monocytic infiltration that may substantially contribute to dementia.

At present, our understanding of the pathogenesis of AIDS-related neurological damage is incomplete. However, there are much data to support the possibility that monocyte-derived cells, including macrophages and microglia, play a critical role in the genesis of this condition. Monocytic infiltration of the central nervous system (CNS) is a cardinal feature of AIDS-related neuropathology (1) and a significant correlate of related neurological damage (2). Monocytic infiltration that may substantially contribute to dementia. We have previously shown that the HIV-1 encoded transactivator Tat, a soluble protein that is released from HIV-1-infected cells (20), can increase NF-κB binding in astrocytes (21). Because NF-κB can influence the expression of MCP-1 (22), in the present study we examined whether Tat could affect astrocytic expression and release of this chemoattractant. We have also examined the possibility that MCP-1 is elevated in the CNS of patients with AIDS dementia, a condition characterized not only by monocytic infiltration of the brain, but by an increase in Tat-encoding transcripts (23).

METHODS

Preparation of Tat Protein and Astrocytes. Highly purified recombinant Tat1–72 was prepared as described previously (7). Tat1–72 contains the epitope that increases NF-κB binding in astrocytes (21) and is similar to Tat1–86 (Intracel) in its ability to increase astrocytic expression of MCP-1.

Cultured Astrocytes. Brain tissue from 12- to 14-week-old human fetuses was obtained in accordance with National Institutes of Health guidelines. The tissue was mechanically disrupted by aspiration through a 19-gauge needle, washed in Eagle’s minimal essential medium (EMEM), and then distributed into tissue culture flasks. Cells were maintained in EMEM containing 10% fetal bovine serum, 2 mM of L-glutamine and 5 μg/ml of gentamicin. Several days later, flasks were placed into an orbital incubator shaker set at 37°C and 210 rpm for 6 h. Non-adherent cells were removed. A portion of the adherent cells were later stained with an antibody to glial fibrillary acidic protein and only those cultures that were >95% positive were used.

RNA Extraction and Northern Blot Analysis. Total RNA was extracted using RNAzol (Tel-Test) according to the manufacturer’s instructions. Before RNA extraction, cells were maintained for 6 hr in serum-free media. Northern blot analysis was performed as described (24).

Abbreviations: CNS, central nervous system; CSF, cerebrospinal fluid; HIVD, HIV-1-associated dementia; HIV(N), HIV-1 positive patient without dementia; MCP-1, monocyte chemoattractant protein-1; MIP-1α, macrophage inflammatory protein-1α; MS, multiple sclerosis; NIN, non-inflammatory neurological; TPCK, n-tosyl-l-phenylalanine chloromethyl ketone; G6-PD, glucose 6-phosphate dehydrogenase.

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Experiments with gp41 and gp120. Astrocytes (10⁶ per 1 ml medium) were stimulated with 100 nM of HIV-1IIIB gp41 (Intracel) or 100 nM of HIV-1IIIB gp120 (Intracel), and supernatants were assessed 24 hr later by immunoassay (R&D Systems).

Proliferation Assays. Astrocytes were grown in 96-well plates. At 70% confluence, cells were treated with varying concentrations of exogenous Tat, in media that contained 1 μCi (1 Ci = 37 Gbq) per well of tritiated thymidine (New England Nuclear). Twenty-four hours later, cells were washed and harvested onto glass fiber filters. Filters were then dried and placed into scintillation fluid for counting in a Betaplate Apparatus. Twenty hours after the administration of Tat, in doses ranging from 10 to 1000 nM, there was no measurable increase in astrocyte proliferation.

Trypsin Digestion. Trypsin digestion of Tat was performed by the addition of 25 μl of 0.25% trypsin per μg of Tat. The mixture was then incubated for 4 hr before the 1:1 addition of soybean trypsin inhibitor (Sigma).

Detection of MIP-1α, MIP-1β, and RANTES. MIP-1α, MIP-1β, and RANTES were detected by ELISA (R&D Systems). These ELISAs could detect concentrations as low as 10 pg/ml. Protein measurements were determined by comparison to a standard curve, run in duplicate with each assay.

Immunoaosorption. Immunoaosorption of Tat was performed as described previously (7). Briefly, a Tat-specific monoclonal antibody (Intracel) was bound to protein A-Sepharose (Pharmacia), washed, and then incubated with Tat for 60 min at room temperature, followed by centrifugation.

Cerebral Spinal Fluid (CSF) and Serum Studies. CSF was obtained from a prospectively characterized population of patients. Computerized tomography or magnetic resonance imaging scans were performed on all patients. Because opportunistic infections of the CNS may influence chemokine expression (25), those patients with such infections were excluded. Similarly excluded were patients with CNS lymphoma. CD4 count <200 and/or dementia were the AIDS-defining illnesses in those patients with HIV-1-associated dementia (HIVD). Of the HIV-1 positive patients without dementia (HIV(N)), five had a diagnosis of AIDS as defined by a CD4 count of less than 200 (n = 4) or non-CNS opportunistic infection (Candida, n = 1). The CD4 cell count (number of cells per cubic millimeter) of the HIVD patients (n = 10, mean ± SE = 125 ± 38) were not significantly different from those of HIV(N) patients (n = 10, mean ± SE = 159 ± 60). The patients ages, in years, were as follows: HIVD, 46 ± SE = 9; HIV(N), 34 ± SE = 8; multiple sclerosis (MS), 40 ± SE = 11; non-inflammatory neurological (NIN) conditions, 39 ± SE = 8. Also, eight patients in each HIV positive group were on antiretroviral therapy at the time of lumbar puncture. Antiretroviral therapy consisted of azidothymidine only [n = 4 HIVD and 8 HIV(N)], azidothymidine + lamivudine [n = 1 HIVD], lamivudine + stavudine + viramid (n = 1 HIVD), or azidothymidine + lamivudine + saquinavir (n = 2 HIVD). In this limited sample size, we could not detect any correlation between triple therapy and CSF MCP-1 values among patients with HIVD. In fact, of the three patients on triple therapy, MCP-1 values were 4279, 1037, and 223 pg/ml, respectively.

In Situ Hybridization Studies. In situ hybridization was performed on paraffin-embedded brain tissue sections from two HIVD patients, one HIV(N) patient, two MS patients, and two normal controls. Patients were autopsied at similar postmortem times (12–18 hr). Cause of death was dementia (n = 2 HIVD) and interstitial pneumonia [n = 1 HIV(N)]. Sections from the frontal cortex, hippocampus, and brainstem were studied.

In situ hybridization was performed using a 32P-labeled MCP-1 riboprobe (I.M.A.G.E. Consortium no. 488534, homologous to GenBank no. M37719). Before use, the probe was sequenced and tested by Northern blot analysis. Negative controls for in situ included hybridization with the MCP-1 sense strand.

RESULTS

MCP-1 Expression. To determine whether Tat could increase astrocytic expression of MCP-1, we stimulated astro-
dependent increase in astrocytic release of MCP-1. This increase
we found that exogenous Tat was associated with a dose-
by ELISA and Western blot. As demonstrated in Figs. 2
lease, we next analyzed the supernatants of Tat-treated astrocytes
MCP-1 would correlate with increased protein synthesis or re-
systems). As compared with untreated astrocytes (Fig. 2B).
In Vivo Studies. We next examined MCP-1 levels in the CSF
of AIDS patients with and without dementia. Both groups of
patients had significantly elevated levels of MCP-1 when
compared with those patients with MS or NIN conditions.
Additionally, patients with HIVD had significantly higher
levels of MCP-1 than did HIV(N) patients (Fig. 4). Of note is
that the levels of MCP-1 in the CSF of HIVD patients were
within the range required to induce monocyte chemotaxis (16).
Also, MCP-1 levels were substantially higher in the CSF as
compared with the serum in HIVD patients. This suggests that
MCP-1 was synthesized intracereally. Simultaneously, we
assayed all samples for RANTES, MIP-1α, and MIP-1β. RAN-
TES was present in serum samples in concentrations of 1–2
ng/ml. However, these chemokines were below detectable
limits (10 pg/ml) in the CSF.

The presence of MCP-1 in the brains of patients with HIVD
was further supported by in situ hybridization. Strongly positive
cells were noted in several brain regions, including CNS white
matter (Fig. 5a and b). Morphologically, cells that expressed
MCP-1 included both astrocytes and neurons. Of interest, cells
expressing MCP-1 RNA were often observed in perivascular
regions (Fig. 5c). In contrast, no positive cells were seen in
normal brain tissue or in tissue from patients without dementia
(Fig. 5d).

**DISCUSSION**

In summary, our results demonstrate that primary cultures of
human astrocytes produce MCP-1 that HIV-1 Tat can increase by
100 nM of Tat and then extracted total RNA 2 and
4 hr later. As demonstrated in Fig. 1, we observed an increase
in MCP-1-encoding RNA as early as 2 hr after stimulation of
astrocytes with Tat. Furthermore, consistent with the possi-
bility that NF-κB is required for Tat’s effect, we observed that
this increase was inhibited by n-tosyl-L-phenylalanine chloro-
ethyl ketone (TPCK). This compound blocks the activa-
tion of NF-κB by interfering with the degradation of I-κBα
(28) (Fig. 1B).

MCP-1 Protein. To determine whether increased expression of
MCP-1 would correlate with increased protein synthesis or re-
lease, we next analyzed the supernatants of Tat-treated astrocytes
ELISA and Western blot. As demonstrated in Figs. 2A and 3,
we found that exogenous Tat was associated with a dose-
dependent increase in astrocytic release of MCP-1. This increase
was specific in that astrocytic release of RANTES, MIP-1α, and
MIP-1β was not observed. We also determined that neither
gp120 nor gp41 had the same effect (data not shown). In addition,
we found that release of MCP-1 was independent of proliferation,
and could be inhibited by either pretreatment of Tat with trypsin
or immunoabsorption of Tat with a specific antibody (Fig. 2B).

**Fig. 2.** MCP-1 ELISA analysis of supernatants from variously
larged human astrocytes. (A) Astrocytes were grown to near conflu-
ency in 35-cm plates. Each well contained 10⁶ cells in 1 ml of medium.
The medium was then changed and astrocytes were stimulated with
exogenous Tat in doses ranging from 0.01 to 1.0 µM. Twenty hours
later, samples were taken for analysis by immunoassay (R&D Sys-
tems). As compared with untreated astrocytes (–) that, when grown
in tissue culture, express MCP-1 in the absence of stimulation,
Tat-stimulated astrocytes showed a significant increase in MCP-1
release. Data are shown as mean ± SE for three replicates. (B) Similar
experiment except that astrocytes were stimulated with 100 nM of Tat
or with an equivalent amount of Tat that had first been either digested
with trypsin (Tat-tr) or immunoabsorbed (Tat-im).

**Fig. 3.** Western blot analysis of MCP-1 in astrocyte supernatants.

In lanes 2–7, 50 µg of protein from variously treated astrocyte
supernatants were run on a 15% Trisglycine denaturing gel. Three
nanograms of non-glycosylated recombinant MCP-1 (R&D Systems)
was run in lane 1 as a control. After protein transfer to nitrocellulose,
the blot was probed with a polyclonal antibody that recognizes human
MCP-1 (R&D Systems). After washing, an appropriate secondary
antibody was applied [horseradish peroxidase conjugated anti-goat
(Santa Cruz Biotechnology)] and electrochemiluminescence (Amer-
sham) was used to visualize the bands. The two bands, which are
specifically increased in association with Tat, are indicated by arrows.
The lower arrow represents a band that runs with an apparent
molecular mass of 9 kDa, whereas the upper band, of slightly higher
molecular mass, is likely to represent MCP-1 that has been altered by
the addition of O-linked carbohydrates. Both forms of MCP-1 are
active in vitro (15).

In summary, our results demonstrate that primary cultures of
human astrocytes produce MCP-1, that this production is
increased by the HIV-1 protein Tat, and that the production
of MCP-1 is increased in the brains of AIDS patients with
dementia.

One might speculate that altered MCP-1 expression could
contribute to HIVD. Unlike RANTES, MIP-1α, or MIP-1β,
MCP-1 does not not significantly neutralizing activity against
primary viral isolates (29) nor does it inhibit HIV-1 infection
of microglia (30). In addition, although MCP-1 may have some
antiviral activity under select in vitro conditions (31), in some
experiments it has been associated with an increase in HIV-1
replication (32). Also, like other β-chemokines, MCP-1 stim-
ulation of select cell types has been associated with increased expression of proinflammatory substances such as interleukin-1β, interleukin-6, and arachidonate (33). Moreover, MCP-1 stimulation of monocytes has been associated with an increase in the release of superoxide (34).

Of additional importance, however, is the possibility that MCP-1 could contribute to the monocytic infiltration that has been observed to correlate with HIVD. Monocytic infiltration would in turn be associated with an increase in the release of neurotoxins. Furthermore, it could contribute to a positive feedback loop whereby more cells in the brain could be infected, leading to increased levels of Tat and hence more of MCP-1. The possibility that MCP-1 contributes to monocytic infiltration in vivo is supported by a number of studies. In transgenic mice, glial-specific expression of MCP-1 is associated with pronounced monocytic infiltration of the CNS (35, ††). MCP-1 injection into the murine hippocampus also leads to the selective recruitment of monocytes (14). Additionally, monocyte chemoattractant activity in the CSF of patients with viral meningitis can be inhibited with antibodies to MCP-1 (36).

The concentrations of MCP-1 in the CSF of patients with HIVD are sufficient to induce monocyte chemotaxis (16), and it is possible that local amounts in brain tissue are even higher.

The possibility that a viral protein such as Tat could up-regulate the expression of MCP-1 in the CNS of patients with HIVD is intriguing. Tat is essential for viral replication and, in comparison to HIV-1 structural proteins, is a relatively small diffusible molecule. Once released from infected cells (20), Tat could increase MCP-1 expression through a number of mechanisms. By its ability to increase NF-kB binding (21), it could directly effect MCP-1 expression. At later time points, Tat might also increase MCP-1 expression through indirect mechanisms. For example, Tat could stimulate the production of cytokines that can also induce MCP-1 expression (39, 40).

In the present study, low nanomolar concentrations of Tat were sufficient to increase astrocytic MCP-1 release. These concentrations are slightly lower than those required by gp41 to increase nitric oxide production in mixed neuroglial cultures (8). Because of the rapid degradation of extracellular protein in autopsy material and the cross reactivity of antiserum to Tat with endogenous brain proteins (41), it is difficult to quantitate Tat protein in vivo. However, it has been shown that tat transcripts are elevated in the CNS of AIDS patients with both dementia (23) and encephalitis (42).

Although astrocytes may not be the only CNS cells to produce MCP-1, nor Tat the only stimulus for such production, it is possible that local amounts in brain tissue are even higher.

the ability of Tat to increase astrocytic expression of this chemokine is likely to be significant. Not only are astrocytes the most abundant cells in the brain, they are in intimate contact with the blood brain barrier. Such astrocytes could be expected to play an important role in the recruitment of monocytes to the CNS.

The finding that MCP-1 is significantly elevated in the CNS of patients with AIDS dementia could direct future therapies toward clinically effective inhibitors of MCP-1 or its principal receptor CCR-2 (43). These compounds might inhibit the attraction of peripheral monocytes to the CNS even in the presence of stimuli that could increase MCP-1 expression. Furthermore, these compounds might also inhibit the activation of monocyte-derived cells not only in the CNS but in the periphery. Of note is that MCP-1 antagonists are presently under investigation for the treatment of other inflammatory conditions, including rheumatoid arthritis (44).

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