Tumor Necrosis Factor α Inhibits Glutamate Uptake by Primary Human Astrocytes

IMPLICATIONS FOR PATHOGENESIS OF HIV-1 DEMENTIA*

(Received for publication, April 25, 1996)

Steven M. Finet, Robert A. Angel§, Seth W. Perry², Leon G. Epstein§†, J effrey D. Rothstein**, Stephen Dewhurst††, and Harris A. Gelbard§§

From the Departments of ††Medicine (Infectious Diseases), §Neurology, ¶Microbiology and Immunology, ¶¶Pediatrics, §§Pharmacology and Physiology, and the Cancer Center, University of Rochester Medical Center, Rochester, New York 14642 and the **Department of Neurology, The Johns Hopkins University, Baltimore, Maryland 21287

Human immunodeficiency virus (HIV) infection is commonly associated with neurological disease that occurs in the apparent absence of extensive infection of brain cells by HIV, suggesting that indirect mechanisms account for neuropathogenesis in the CNS, perhaps including changes in the normal neuroprotective functions of astrocytes. To test this hypothesis, we examined the effect of the pro-inflammatory cytokine, tumor necrosis factor α (TNFα), produced by HIV-1-infected macrophages and microglia, on glutamate transport by primary human fetal astrocytes (PHFAs). A dose-dependent inhibition of high affinity glutamate uptake sites was observed 12–24 h after addition of exogenous recombinant human TNFα to PHFAs. This effect was specific since it was blocked by a neutralizing monoclonal antibody directed against TNFα. Furthermore, the inhibitory effect was reproduced by a monoclonal antibody that is an agonist at the 55-kDa TNF receptor. These results suggest that the neurotoxic effects of TNFα may be due in part to its ability to inhibit glutamate uptake by astrocytes, which in turn may result in excitotoxic concentrations of glutamate in synapses.

Human immunodeficiency virus type 1 (HIV-1), like other lentiviruses, is able to efficiently enter the central nervous system, and cause “primary” neurological disease that is not attributable to opportunistic pathogens in a high percentage of infected persons. Indeed, HIV-1 is estimated to be the most frequent cause of dementia in young adults in the United States (1).

It is clear that the extent of HIV-1 infection within the central nervous system is much more limited than might be expected in light of the severity of neurological impairment which occurs in HIV-1 dementia (2). Furthermore, productive viral infection of CNS cells is limited almost exclusively to cells of monocytic lineage (i.e. brain macrophages and microglia) (reviewed in Ref. 3). While other cell types, including astrocytes, may undergo nonproductive or “restricted” infection in some cases, it is apparent that HIV-1-induced neurological disease must be mediated in large measure via indirect mechanisms (3). Thus, several investigators have proposed that soluble factors released by HIV-1-infected brain macrophages and microglia may cause neuronal damage (3–5). Candidate neurotoxins in HIV-1 dementia include viral gene products such as gp120 and Tat, as well as pro-inflammatory cytokines such as tumor necrosis factor α (TNFα) and other soluble mediators released by activated macrophages and microglia, such as platelet-activating factor (3–5).

Astrocytes are the most common glial cells within the central nervous system and are crucial to the normal homeostatic regulation of the neuronal microenvironment (6), in large part because of their ability to selectively regulate extracellular levels of glutamate (7), which is the primary excitatory neurotransmitter in the brain. Glutamate also competitively inhibits neuronal uptake of cysteine, which is the limiting precursor for glutathione (8), and high extracellular levels of glutamate may render neurons more vulnerable to oxidative injury. In the present report, we show that addition of exogenous recombinant human TNFα, at levels equivalent to those that are produced by activated, HIV-1-infected, monocytes (4), inhibits glutamate uptake by primary human fetal astrocytes. In light of earlier reports that the HIV-1 envelope glycoprotein, gp120, enhances glutamate efflux from human astrocytes (9), these data suggest a novel mechanism of neurotoxicity whereby HIV-1 infection may promote an increase in extracellular glutamate within the brain, thereby leading to neuronal excitotoxicity and increased neuronal sensitivity to oxidative stress.

EXPERIMENTAL PROCEDURES

Purified Human Fetal Astrocyte Cultures (PHFA)—Primary human astrocytes were isolated from second-trimester human fetal brain tissue obtained from elective abortions (performed in full compliance with both NIH and University of Rochester guidelines) using methods described previously (4). The purity of human fetal astrocyte preparations was then determined by immunostaining for glial fibrillary acidic protein, and cultures were used for experiments only if >95% of cells were glial fibrillary acidic protein-immunoreactive.

[3H]Glutamate Uptake—PHFA were plated at 1 × 10⁶ per well in 24-well tissue culture plates. When confluent, cells were equilibrated in incubation medium (125 mM NaCl, 4.5 mM KCl, 1.2 mM CaCl₂, 1.2 mM MgCl₂, 5 mM glucose, buffered to pH 7.4 with sodium phosphate). Preliminary experiments established that glutamate uptake was maximal after a 15-min incubation with 1 nM [3H]glutamate and 1 μM cold L-glutamate (data not shown) based on methods described previously (10). Subsequently, experiments were performed in triplicate with medium containing 1 nM [3H]glutamic acid (DuPont NEN, specific activity 57.4 Ci/mmol) and 1 μM unlabeled L-glutamate, added to PHFAs for 15 min at 25 °C, after which uptake was terminated by washing the cells twice with excess cold incubation medium or phosphate-buffered saline. After aspiration of medium, cells were solubilized overnight in 0.25 mM NaOH, and lysates were then added to scintillation fluid for quantitation of cell-associated radioactivity as...
RESULTS

Initial experiments were conducted to determine whether cultured primary human fetal astrocytes (PHFA) possess a functional high affinity transporter for glutamate. Pilot studies revealed that this was indeed the case, and PHFA were reproducibly induced a decrease in glutamate uptake by primary human astrocytes, we next examined the time course of this effect (Fig. 2A). Little inhibition of glutamate uptake was found to occur within the first 12 h after exposure of cells to TNF-α, but maximal levels of inhibition (30%) were achieved within 16 to 24 h post-TNF-α treatment (a statistically significant inhibition in glutamate uptake was first evident at 16 h post-TNF-α treatment (p < 0.05) and the magnitude of this effect increased to a maximum at 24 h (p < 0.01); see Fig. 2A). No further inhibition was obtained at a 48-h time point (data not shown). This inhibitory effect of TNF-α on astrocyte glutamate uptake was shown to be dose-dependent (Fig. 2B) and reached peak levels at a concentration of between 1 and 10 ng/ml TNF-α, which is equivalent to the level of TNF-α that is produced by activated, HIV-1-infected, monocytes in vitro (4).

In order to address the possibility that the results obtained...
FIG. 3. **TNFα inhibition of glutamate uptake is specific.** Glutamate uptake by cultured PHFA was measured after incubation of cells for 48 h in the presence of the indicated reagents. TNFα, exogenous recombinant human TNFα (Genzyme) at 1 ng/ml, TNFα + cA2, TNFα plus anti-TNFα monoclonal antibody cA2 (10 μg/ml, Centocor Inc.); TNFα + SF25, TNFα plus isotype-matched anti-hepatoma mAb SF25 (10 μg/ml, Centocor Inc.); htr-9, agonist mAb htr-9, directed against 55-kDa TNF receptor (10 μg/ml, Hoffman-La Roche); utr-1, antagonist mAb utr-1, directed against 75-kDa TNF receptor (10 μg/ml, Hoffman-La Roche); Tja, α-threo-β-hydroxyaspartate (1 mM, Sigma). In all experiments, data represent the means of at least three experimental replicates; bars denote the S.E. values. 100% glutamate uptake (0% inhibition) was defined as the level of glutamate uptake by untreated astrocytes. (★, statistically significant difference, relative to glutamate uptake by control PHFAs (media); p < 0.05, paired t test).

using exogenous recombinant TNFα reflected a nonspecific phenomenon, due to the presence of trace amounts of contaminating proteins within this product, additional control experiments were conducted. First, PHFA were incubated with TNFα in the presence or absence of a neutralizing monoclonal antibody directed against human TNFα (clone cA2; Ref. 13). As shown in Fig. 3, the cA2 monoclonal antibody completely reversed the effect of TNFα on glutamate uptake whereas an isotype-matched control antibody directed against a human hepatoma antigen (clone SF25; Centocor Inc.) had no such effect. Second, a monoclonal antibody that is known to act as an agonist of the 55-kDa TNFα receptor (clone htr-9; Refs. 14 and 15) was determined to inhibit glutamate uptake in PHFA, to an extent similar to TNFα (Fig. 3), whereas an antibody which acts as a partial antagonist of the 75-kDa TNF receptor (clone utr-1; Refs. 14 and 16) had no effect on glutamate uptake. Note that α-threo-β-hydroxyaspartate, a known inhibitor of glutamate uptake (10), was found to decrease high affinity glutamate uptake by approximately 60% in our experiments, when added to cells at 1 mM (Fig. 3). In contrast, the addition of various additional candidate HIV-1 neurotoxins to PHFAs, including recombinant, mammalian (CHO)-cell-derived HIV-1p24gp120 (25 nM, AIDS Research and Reference Reagent Program (ARRRP)), recombinant HIV-1 Tat (0.01–0.1 μM; ARRRP), and platelet-activating factor (1–10 μM, Sigma), did not result in any significant alteration in glutamate uptake (data not shown).

Finally, immunoblot analyses confirmed the presence of the GLAST/EAAT1 form of the glutamate transporter in PHFAs and, to a lesser degree, the EAAC1/EAAT3 form of the transporter (Fig. 4). In contrast, expression of the GLT1/EAAT2 and EAAT4 forms of the transporter could not be detected in human fetal astrocytes (17, 18) and in no case did TNFα treatment result in any gross alteration in cellular expression of these transporters, as determined by densitometric analysis of immunoblots.

**DISCUSSION**

It has been suggested that glutamate may be a key contributor to the neuropathogenesis of AIDS (3) and other neurodegenerative conditions, such as amyotrophic lateral sclerosis (12). In light of its central role in neuronal signaling and its potential neurotoxicity in elevated concentrations, it is not surprising that glutamate is tightly regulated within the CNS. Previous studies have shown that glutamate uptake by rodent astrocytes is inhibited by arachidonic acid (19) and reactive oxygen intermediates (20), both of which are known to be produced by activated, HIV-1-infected, monocytes (4), and both of which have been implicated as candidate neurotoxins in AIDS. Here, we show that human TNFα, another candidate HIV-1 neurotoxin (21, 22), also inhibits glutamate uptake by cultured primary human fetal astrocytes.

The inhibitory effect of TNFα on glutamate uptake by human astrocytes was shown to be dose-dependent (Fig. 2) and specific (Fig. 3) since (i) this effect was reproduced by an agonist monoclonal antibody (htr-9) directed against the 55-kDa TNF receptor (TNF-R1), and (ii) the inhibitory effect of TNFα was blocked by a neutralizing antibody (cA2). Furthermore, this effect of TNFα was not the result of cytotoxicity, since even high levels (10 ng/ml) of TNFα had no apparent effect on astrocyte viability as measured by the 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide method, trypan blue exclusion, or DNA fragmentation (data not shown) in astrocytes. It is therefore striking that the present findings stand in marked contrast to previous studies on the effect of murine TNFα on glutamate uptake by rat astrocytes, which failed to show any change in astrocyte glutamate uptake following exposure to TNFα (20). The reason for this apparent discrepancy is uncertain, although it may reflect species-specific differences in the biology of astrocyte responses to TNFα, which may be analogous to previously reported species-specific differences in neuronal responses to TNFα (21). Alternatively, rodent astrocytes may express a different subset of glutamate transporters than do human fetal astrocytes. This could result in different responses to TNFα, since molecularly cloned glutamate transporters have been shown to exhibit markedly different responses to arachidonic acid (e.g. transport via EAAT1/GLAST1 is inhibited by arachidonic acid, while transport via EAAT2/GLT1 is actually enhanced; Ref. 23).

The mechanism by which TNFα inhibits glutamate uptake by human astrocytes is at present uncertain. However, the delayed kinetics of the astrocyte response to TNFα (Fig. 2B) strongly suggests that TNFα does not interfere directly with the astrocyte glutamate transporter. This stands in contrast to arachidonic acid and reactive oxygen species, which both induce a very rapid decline in astrocyte glutamate uptake (this occurs within 10 min of addition of these compounds to astrocytes) and which interfere directly with the transporter mole-
cule (19, 20, 24). Furthermore, incubation of PHFA with TNFα in the presence of the anti-oxidant N-acetylcysteine (up to 0.5 mM) had no detectable effect on TNFα-mediated inhibition of glutamate uptake. It seems likely, then, that TNFα may alter astrocyte glutamate uptake either (i) by inducing the synthesis of arachidonic acid (25), or (ii) by selectively regulating the post-translational modification of the glutamate transporter, for example, by altering its phosphorylation state (26). However, the role of cyclooxygenase in TNFα-mediated decrease of high affinity glutamate transporter sites can be eliminated because co-incubation with the cyclooxygenase inhibitor indo-methacin over a wide range of doses (0.4–40 μg/ml) did not reverse the inhibition of glutamate uptake induced by TNFα (data not shown).

Taken together with previous reports on the ability of glutamate receptor antagonists to block the neurotoxic effects of several candidate HIV-1 neurotoxins (3, 5, 27, 28), the present studies suggest that one factor which may render neurons vulnerable to excitotoxic (3) or oxidative (8) damage in AIDS may be a selective deficit in the normal homeostatic regulation of extracellular glutamate by astrocytes. In particular, virally encoded proteins such as gp120 may trigger an increase in extracellular glutamate by astrocytes. In particular, virally encoded proteins such as gp120 may trigger an increase in extracellular glutamate by astrocytes. In particular, virally encoded proteins such as gp120 may trigger an increase in extracellular glutamate by astrocytes. In particular, virally encoded proteins such as gp120 may trigger an increase in extracellular glutamate by astrocytes.
**Tumor Necrosis Factor α Inhibits Glutamate Uptake by Primary Human Astrocytes: IMPLICATIONS FOR PATHOGENESIS OF HIV-1 DEMENTIA**

Steven M. Fine, Robert A. Angel, Seth W. Perry, Leon G. Epstein, Jeffrey D. Rothstein, Stephen Dewhurst and Harris A. Gelbard

*J. Biol. Chem. 1996, 271:15303-15306.*

doi: 10.1074/jbc.271.26.15303

Access the most updated version of this article at [http://www.jbc.org/content/271/26/15303](http://www.jbc.org/content/271/26/15303)

**Alerts:**
- When this article is cited
- When a correction for this article is posted

[Click here](http://www.jbc.org/content/271/26/15303.full.html#ref-list-1) to choose from all of JBC's e-mail alerts

This article cites 27 references, 13 of which can be accessed free at [http://www.jbc.org/content/271/26/15303.full.html#ref-list-1](http://www.jbc.org/content/271/26/15303.full.html#ref-list-1)