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Up-Regulation of Proteinase-Activated Receptor 1 Expression in Astrocytes During HIV Encephalitis

Leonie A. Boven,* Nathalie Vergnolle,† Scot D. Henry,* Claudia Silva,* Yoshinori Imai,§ Janet Holden,‖ Kenneth Warren,‖ Morley D. Hollenberg,‡‡ and Christopher Power*†‡

Proteinase-activated receptor 1 (PAR-1) is a G protein-coupled receptor that is activated by thrombin and is implicated in the pathogenesis of inflammation. Although PAR-1 is expressed on immunocompetent cells within the brain such as astrocytes, little is known about its role in the pathogenesis of inflammatory brain diseases. Herein, we investigated PAR-1 regulation of brain inflammation by stimulating human astrocytic cells with thrombin or the selective PAR-1-activating peptide. Activated cells expressed significantly increased levels of IL-1β, inducible NO synthase, and PAR-1 mRNA. Moreover, supernatants of these same cells were neurotoxic, which was inhibited by an N-methyl-D-aspartate receptor antagonist. Striatal implantation of the PAR-1-activating peptide significantly induced brain inflammation and neurobehavioral deficits in mice compared with mice implanted with the control peptide or saline. Since HIV-related neurological disease is predicated on brain inflammation and neuronal injury, the expression of PAR-1 in HIV encephalitis (HIVE) was investigated. Immunohistochemical analysis revealed that PAR-1 and (pro)-thrombin protein expression was low in control brains, but intense immunoreactivity was observed on astrocytes in HIVE brains. Similarly, PAR-1 and thrombin mRNA levels were significantly increased in HIVE brains compared with control and multiple sclerosis brains. These data indicated that activation and up-regulation of PAR-1 probably contribute to brain inflammation and neuronal damage during HIV-1 infection, thus providing new therapeutic targets for the treatment of HIV-related neurodegeneration. The Journal of Immunology, 2003, 170: 2638–2646.

Abbreviations used in this paper: PAR, proteinase-activated receptor; AD, Alzheimer’s disease; Aβ, amyloid-β protein; ALZ-50, a monoclonal antibody against Aβ; Aβ1–42, Aβ1–40; AR-A, anti-Aβ receptor antagonist; ASO, antisense oligonucleotide; BDNF, brain-derived neurotrophic factor; B-27, a conditioned medium from non-neuronal cells; BDNF, brain-derived neurotrophic factor; BDNF, brain-derived neurotrophic factor; FGF, fibroblast growth factor; Ha, human astrocytes; HIV-1, human immunodeficiency virus type 1; HERV, human endogenous retrovirus; IL-1β, interleukin-1β; MAP4, microtubule-associated protein 4; MEK, mitogen-activated protein kinase; MDM, monocyte-derived macrophage; NMDA-R1, N-methyl-D-aspartate receptor 1; NO, nitric oxide; PAR-1, proteinase-activated receptor 1; PPAR, peroxisome proliferator-activated receptors; PKC, protein kinase C; SCG, superior cervical ganglia; SNAP-25, synaptosomal-associated protein 25; STAT, signal transducer and activator of transcription; T20, a protease inhibitor; TNF, tumor necrosis factor; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling.
also expressed by cells within the CNS (29, 30), and an increase in thrombin expression could also lead to increased PAR-1 activation. Accumulation of thrombin was found in senile plaques in brain tissue of individuals with Alzheimer’s disease (AD) (31), possibly contributing to the immune activation observed in AD brains. Infusion of thrombin into the brain was found to cause infiltration of inflammatory cells, proliferation of mesenchymal cells, induction of angiogenesis, and an increase in reactive astrocytes (32). This process mimics the inflammation, scar formation, and reactive gliosis found in neuroimmune diseases. Infiltration of monocytes and lymphocytes into brain parenchyma and activation of inflammatory cells are cardinal features of diseases such as MS and HIV (26, 33).

In this study we investigated whether activation of PAR-1 could contribute to inflammation and neuronal injury and death within the brain. In particular, its role in HIV was studied. HIV is a neurodegenerative disease that affects ~25% of adults with AIDS (34) and is characterized by the loss of select neuronal populations, infiltration of monocytic cells, astroglialosis, induction of proinflammatory molecules such as cytokines and chemokines, and breakdown of the blood–brain barrier (35, 36). Although it has been well established that monocytic cells contribute to neurodegeneration by secreting a wide variety of neurotoxic molecules (37), dysregulation of astrocyte function also appears to be involved (38). Thus, as PAR-1 activation results in many of the events associated with HIV, we hypothesized that PAR-1 may play an important role in the neuropathogenesis of HIV infection.

Materials and Methods

Cells

U373 astrocytoma cells were obtained from American Type Culture Collection (Manassas, VA) and were subcultured without using trypsin to avoid stimulation or desensitization of PAR-1. Cells were seeded and grown in serum-free AIM-V (Life Technologies, Burlington, Canada) to prevent activation by serum-derived thrombin. Primary human fetal neurons and astrocytes were provided by Dr. W. Yong (39) and cultured in polyornithine-coated plates in serum-free AIM-V. Fetal cells were obtained and used in accordance with the University of Calgary ethics approval committee.

Neuronal survival assay

Primary human fetal neurons were cultured for 6 h under various conditions. A neuron-specific ELISA using an Ab against microtubule-associated protein 2 was used to measure neuronal viability (40). Following treatment with astrocyte-conditioned medium for 6 h, cells were fixed using 2% formalin, washed with PBS, and preincubated with PBS, 0.5% Triton X-100, and 10% normal goat serum. All slides were incubated with various primary Abs at a dilution of 1/500, except for PAR-1, anti-angiogenin, and (pro)thrombin. Following washing, sections were preincubated with a biotinylated secondary Ab for 1 h. After washing, sections were incubated with avidin-biotin-peroxidase complexes (Vector Laboratories) for 1 h at room temperature and with isotype-matched IgG (1/100 dilution). Immunolabeling with primary Abs was detected by biotinylated goat anti-rabbit or biotinylated goat anti-mouse (Vector Laboratories) for 1 h at room temperature and with avidin-biotin-peroxidase complexes (Vector Laboratories) for 1 h at room temperature. Peroxidase activity was demonstrated with 0.05% 3,3’-diaminobenzidine tetrachloride (Vector Laboratories) in 0.05 M Tris-HCl buffer (pH 7.6) containing 0.03% H2O2. Sections were counterstained with hematoxylin (Vector Laboratories), dehydrated, and mounted. An examiner unaware of the slide identity performed cell counts of Iba-1- and GFAP-immunopositive cells adjacent to, but not within, the implantation site.

Animal experiments

Three-week-old male CD-1 mice were obtained from Charles River Laboratories (Wilmington, MA) and housed in a biocentrainment facility according to the guidelines of the Canadian Animal Care Committee. Animals were placed in a stereotaxic frame under anesthesia. Peptides were delivered into the striatum. In vivo neurological injury was assessed according to the Ungerstedt model (44). In short, ipsiversive rotations as well as total rotations were monitored over 10 min after i.p. injection of amphetamine (1 mg/kg) on days 3 and 7 following striatal injection. More ipsiversive rotations or less total rotations are both indicative of neurological injury. Animals were sacrificed after 7 days, and brain sections were prepared for immunohistochemical analysis.

Brain tissue samples

Brain tissue (frontal white matter) was collected at autopsy from all experimental groups and stored at −80°C as described previously (45). Control subjects included four male and two female patients (mean age, 56 ± 16.4 years) who were all sero-negative for HIV-1 and had been diagnosed with cerebral arteriosclerosis (n = 2), anoxic encephalopathy (n = 1), or normal brain pathology (n = 5). MS patients included one male and seven female patients (age, 63.3 ± 13.4 years) who had been classified as primary progressive (n = 1), secondary progressive (n = 5), and relapsing-remitting (n = 2) and had who had Estimated Disability Status Scale scores ranging from 7 to 10 before death. Brain issue was also obtained from six male patients with HIV (age, 41.5 ± 10.1 years). All HIV-infected patients died of AIDS-related illnesses (CD4 count, <200/mm3), and brain pathology demonstrated multinucleated giant cells, microglial nodules, and astrogliosis. Brain samples from control and HIV-infected patients were

Immunohistochemistry

Rabbit anti-human PAR-1 antiserum, generated against a peptide (ORN-NATLDP/RSLRNPNDKY-AMIDE) representing the cleavage/activation sequence of human PAR-1, with an N-terminal ornithine added for N-terminal coupling as a hapten to keyhole limpet hemocyanin, was used in immunohistochemistry. Monoclonal antibodies were used for Iba-1 and GFAP. A monoclonal Ab raised against the C-terminal sequence of anti-oxidized-cysteine-binding molecule 1 (anti-(pro)thrombin) (43) was used. Anti-iNOS and anti-STAT-1 were obtained from Transduction Laboratories (Lexington, KY).

Paraffin-embedded sections (5 μm) of human or mouse brain tissue were deparaffinized and hydrated using decreasing concentrations of ethanol. Sections were boiled in 0.01 M citrate buffer, pH 6.0, for 10 min for PAR-1, iNOS, and STAT-1 staining. Endogenous peroxidases were blocked by preincubating sections in 0.5% hydrogen peroxide for 20 min. To prevent nonspecific binding, sections were preincubated with 10% normal goat serum for 30 min. Control sections were incubated with isotype-matched IgG (1/100 dilution). Immunolabeling with primary Abs was detected with biotinylated goat anti-rabbit or biotinylated goat anti-mouse (Vector Laboratories) for 1 h at room temperature and with avidin-biotin-peroxidase complexes (Vector Laboratories) for 1 h at room temperature. Peroxidase activity was demonstrated with 0.05 M Tris-HCl buffer (pH 7.6) containing 0.03% H2O2. Sections were counterstained with hematoxylin (Vector Laboratories), dehydrated, and mounted. An examiner unaware of the slide identity performed cell counts of Iba-1- and GFAP-immunopositive cells adjacent to, but not within, the implantation site.

RT-PCR detection

Brain tissue or astrocytic cells were homogenized and lysed in TRIzol (Life Technologies Gaithersburg, MD) according to the manufacturer’s guidelines. Total RNA was isolated and dissolved in diethylpyrocarbonate-treated water, 1 μg of RNA was used for the synthesis of cDNA, and PCR reactions were performed as described previously (41). Primers were as follows: GAPDH: 5’ primer, CAC TCG TCT GGA CGG GCG TCC, 3’ primer, CAA AGT TCT CAT GGA TGA CC, 5’ primer, GCA TCC ACC AGG CTG CTC C; CAC 5’ primer, TCC TTT CTA CAT CTT CCA C; 3’ primer, GTT CAG GGC TAA ACT CTA CC; (pro)thrombin: 5’ primer, TCA AGT CAC TGT AGT GAT G, 3’ primer, GCA GTG GGC GGC GGT GAA GAC; and inducible nitric oxide synthase (iNOS): 5’ primer, ACC ACT GGC AGA TGC TGG CCT, 3’ primer, CAA AGG CTG TGA GTC TGG CAC. Semiquantitative analysis was performed by monitoring in real-time the increase in fluorescence of SYBR-Green dye on an i-Cycler (Bio-Rad, Hercules, CA). To confirm single-band production, melt curve analysis was performed, and in addition, reactions were subjected to 40 cycles of amplification and subsequently analyzed by electrophoresis and ethidium bromide staining. All data were normalized against the GAPDH mRNA level and expressed relative to controls.
obtained from the AIDS Brain Bank at St. Paul’s Hospital (Vancouver, Canada). Brain tissue from MS patients was obtained from the Multiple Sclerosis Patient Care and Research Clinic (Edmonton, Canada).

Statistical analysis

For all data analysis a nonparametric Mann-Whitney test was performed using GraphPad InStat version 3.00 for Windows 95 (GraphPad Software, San Diego CA; www.graphpad.com). A value of \( p < 0.05 \) was considered significant.

Results

Stimulation of human astrocytic U373 cells and primary astrocytes with thrombin or a PAR-1-activating peptide results in up-regulation of IL-1\( \beta \), iNOS, and PAR-1

Since PAR-1 has been shown to be expressed by astrocytes (11), and astrocytes are important modulators of brain inflammation (46), we determined whether astrocytes could be immune-activated by thrombin and if this effect was dependent on PAR-1 specifically by also testing the selective PAR-1-activating peptide (TF). The reverse peptide, RL, was used as a control for TF, and cells were treated with the selective PAR-4-activating AY peptide (47) to ensure that thrombin-induced effects were not PAR-4 mediated. After culturing U373 cells and human fetal astrocytes (HFA) in the presence of different concentrations of thrombin, TF peptide, RL peptide, or AY peptide, mRNA levels of PAR-1, iNOS, and IL-1\( \beta \) were determined by real-time RT-PCR (Fig. 1).

After stimulation with thrombin (25 U/ml) and TF (25 \( \mu \)M), a significant increase in PAR-1 was observed in both U373 (36- and 21-fold, respectively) and HFA (7.5- and 22-fold, respectively) compared with untreated cells. The RL peptide slightly increased PAR-1 mRNA levels in U373 cells, but not in HFA (Fig. 1, A and B). Both thrombin and the TF peptide also induced significant increases in iNOS mRNA levels in U373 cells (18- and 13-fold, respectively; Fig. 1C), while similar results were observed in HFA (Fig. 1D) for both stimuli (7- and 13-fold, respectively). The RL peptide induced a modest increase in iNOS mRNA in HFA, but not U373; however, the AY peptide, which activates PAR-4 (47, 48), induced iNOS mRNA (9-fold) in HFA (Fig. 1D). In U373 cells (Fig. 1E) and HFA (Fig. 1F), both concentrations of thrombin significantly induced IL-1\( \beta \) mRNA in dose-dependent manner. In contrast, the TF peptide was a highly potent stimulant of IL-1\( \beta \) mRNA in U373 cells (70-fold; Fig. 1E), but nonsignificantly stimulated HFA (3-fold) at the higher concentration (Fig. 1F). These results showed that U373 astrocytoma cells and HFA express PAR-1 and can be immune-activated by thrombin. This activation is very likely mediated by PAR-1, since the PAR-1-activating TF peptide shows similar effects.

Supernatants of astrocytic cells stimulated with thrombin or TF peptide induce neurotoxicity in human fetal neurons via an N-methyl-D-aspartate (NMDA) receptor-mediated mechanism

Earlier studies have shown that stimulated astrocytes release glutamate, which mediates the excitotoxicity of neurons through

![FIGURE 1.](http://www.jimmunol.org/Downloadedfrom)
activation of glutamate receptors, such as the NMDA receptor (49). To investigate this mechanism of neurotoxicity in relation to PAR-1 activation, U373 cells were stimulated with 5 or 25 U/ml thrombin or 30 or 100 µM TF peptide for 2 h and washed, and supernatants were harvested after 48 h. Supernatants from thrombin-stimulated U373 cells were highly neurotoxic and resulted in 66% (p < 0.05) and 40% (p < 0.05) neuronal survival for 5 and 25 U/ml thrombin, respectively (Fig. 2A). Supernatants of TF peptide-stimulated cells were less neurotoxic: 78% (p > 0.05) and 73% (p < 0.05) neuronal survival for 30 and 100 µM TF peptide, respectively. Diluting the supernatant from U373 cells treated with 25 U/ml thrombin resulted in a dose-dependent decrease in neurotoxicity. In addition, neurotoxicity could be partially blocked by 100 µM MK-801, a specific NMDA receptor, for up to 10-fold diluted supernatant, and complete blocking was observed for 30- and 100-fold diluted supernatant (Fig. 2B). These findings suggested that neuronal survival was mediated by a ligand for the NMDA receptor released by astrocytes.

**Implantation of the PAR-1-activating peptide in mouse striatum results in local immune activation and behavioral changes**

Since PAR-1 agonists could induce immune activation of astrocytes in vitro, we tested their ability to induce inflammation and neuronal damage in vivo in a mouse model. Although thrombin has been shown to be directly neurotoxic in vivo (50), the extent to which the effect was dependent on PAR-1 activation remains unknown. To determine the in vivo effects of TF and RL, each peptide was implanted in the right striatum of CD1 mice. Mice were sacrificed, and paraffin-embedded brain sections were immunostained for GFAP. Iba-1, STAT-1 (which is involved in gene transcription), and iNOS, which is regulated by STAT-1 (51). Brain sections from TF mice showed increased and more widespread astrocyte activation, as indicated by GFAP immunoreactivity (arrows, Fig. 3B) compared with RL-implanted mice (arrows, Fig. 3A). Iba-1 expression on microglia was present in brains of RL mice (Fig. 3C), but more intense and frequent immunostaining was found in brain tissue of TF mice (Fig. 3D). For both groups, Iba-1-positive cells consisted of cells with both microglial morphology (arrows and inset, Fig. 3D) and infiltrating mononuclear cells (arrowheads and inset, Fig. 3C). STAT-1 immunoreactivity was detected in nuclei (arrows) and cytoplasm (inset, Fig. 3E) of cells in RL mice (Fig. 3E, needletrack on the far left side). However, STAT-1-positive cells were more frequently found in the needletrack in TF-treated mice and demonstrated greater nuclear localization (Fig. 3F). Nuclei that were STAT-1 immunopositive belonged to infiltrating mononuclear cells (open arrows), glial cells (arrows and inset, Fig. 3F), and endothelial cells (arrowheads). Inducible NOS-positive cells were rarely observed within the needletrack in RL-implanted mice (Fig. 3G), but were more frequently detected within and outside the needletrack among TF-implanted mice (Fig. 3H). To quantify immune activation in the brains of animals implanted with the TF and RL peptides, GFAP- and Iba-1-immunopositive cells in the ipsilateral hemisphere to the implantation site were counted (Fig. 4A). These studies revealed significant increases in both GFAP- and Iba-1-immunoreactive cells in the ipsilateral hemisphere (right) implanted with the TF peptide (Fig. 4A). Conversely, in animals implanted with PBS or the RL peptide, there was no increase in immunoreactive cells in either the ipsilateral or contralateral hemisphere. Neurobehavioral analysis revealed that after 3 days no significant changes were observed in either ipsiversive rotary (Fig. 4B) or total rotary (Fig. 4C) behavior among PBS, TF, or control RL peptide-implanted animals. In contrast, after 7 days, mice receiving the TF peptide demonstrated significantly more ipsiversive rotations (Fig. 4C) and significantly fewer total rotations (Fig. 4D) compared with mice that received the RL (control) peptide or PBS, indicating that the active TF peptide caused neurological damage. These results demonstrated that activation of PAR-1 results in CNS inflammation, as illustrated by activation of astrocytes and microglial together with increased expression of inflammatory markers and neurobehavioral deficits.

**Proteinase-activated receptor is up-regulated in brain tissue of individuals with HIV encephalitis compared with control and MS cases**

Since activation of PAR-1 resulted in immune activation and neuronal damage in vitro as well as in the in vivo mouse model, we studied PAR-1 and (pro)thrombin expression in the brain tissue of individuals with HIV encephalitis, a neurodegenerative disease characterized by inflammation and astrogliosis. The relative abundance of PAR-1 and thrombin mRNA was assessed by real-time RT-PCR in the brain tissue of patients with HIV infection, MS, and other disease controls (Fig. 5). PAR-1 mRNA levels were significantly increased in the brain tissue of individuals with HIV infection compared with control cases (p < 0.01) and MS cases (p < 0.05; Fig. 5A). In addition, (pro)thrombin levels in the brains of HIV cases were also significantly higher than those in control (p < 0.05) or MS cases (p < 0.05; Fig. 5B). No significant differences were observed between control and MS groups for PAR-1.
PAR-1 and thrombin expression was selectively induced among individuals with HIV infection. PAR-1 is expressed on astrocytes in brain tissue of HIV cases.

To identify PAR-1- and thrombin-expressing cells in the CNS, brain sections of HIVE and control cases were immunostained for GFAP, PAR-1, and (pro)thrombin (Fig. 6). Whereas GFAP-positive cells were occasionally present in gray matter in control cases (Fig. 6A), widespread astrogliosis was observed in gray matter of the brain tissue of HIVE cases, as indicated by intense and more frequent GFAP immunodetection (Fig. 6B). Rarely, PAR-1-positive cells resembling mononuclear cells were identified in close proximity to blood vessels in control cases (arrow, Fig. 6C). In contrast, HIVE cases demonstrated intense PAR-1 immunoreactivity in cells (Fig. 6D) that were identified as astrocytes using double-label immunostaining for GFAP (blue/gray) and PAR-1 (brown; arrow, Fig. 6E). However, PAR-1-reactive cells were also (infrequently) observed that were immunonegative for GFAP (arrowhead, Fig. 6F). We detected PAR-1 staining on neurons, endothelial cells, and mononuclear cells, but not to the same extent as on astrocytes (data not shown). Weak (pro)thrombin staining was observed on neurons in control cases (Fig. 6F), but more intense and frequent staining was detected on both neurons (Fig. 6G) and astrocytes (Fig. 6H) in HIVE cases. Thus, although PAR-1 and thrombin were only detected at minimal levels in control brain tissue, the immunoreactivity of both proteins in astrocytes was markedly enhanced and more widespread in HIVE brains.
Discussion

Thrombin, in large part due to activation of PAR-1, has been reported to mediate immune responses throughout the body (7, 52, 53). As both thrombin and PAR-1 levels in the CNS are low under normal conditions, little research has focused on investigating the role of PAR-1/thrombin in inflammatory diseases of the CNS. Herein, we demonstrate that the selective PAR-1 agonist, TF, induced the expression of proinflammatory genes and the release of neurotoxic molecules from astrocytic U373 cells cultured in vitro, and caused in vivo neuroinflammation and neurobehavioral deficits when administered intrastrially. Moreover, this is the first study to characterize thrombin/PAR-1 expression patterns in human neuroinflammatory diseases. We show that PAR-1 and (pro)thrombin mRNA and protein expression are significantly increased in brain tissue derived from individuals with HIVE, but not those with MS. The concurrent expression of increased amounts of the receptor and its potential activator (thrombin) has major pathogenic implications, since PAR-1 regulates many inflammatory and neurotoxic pathways. In this regard, the process of activation of prothrombin in the CNS will bear further scrutiny, as will the production of other serine proteinases (e.g., granzyme A) that can potentially activate PAR-1 on neuronal cells and astrocytes (54). Therefore, activation of PAR-1 may well be an important contributor to the innate immune activation and neurodegeneration that characterize HIVE.

Astrocytes are important mediators of inflammation in the brain (46) and have been shown to express PAR-1 (reviewed in Ref. 55). However, previous studies that investigated PAR-1 on astrocytes focused on the effect of PAR-1 activation on calcium signaling and morphology (11, 14, 56) and signaling through mitogen-activated protein kinase (57), but many of the downstream effects on gene expression remain largely unknown. This is the first report to show that PAR-1-mediated activation of astrocytes results in the induction of inflammatory mediators. Treatment in vitro with thrombin or the PAR-1-activating peptide resulted in the induction of IL-1β, iNOS, and PAR-1 mRNA in human astrocytoma cells and primary human astrocytes. Although small in magnitude relative to the TF peptide, the control RL peptide also had a minor effect on PAR-1 expression, but its induced response was not concentration dependent (not shown) and could not be due to PAR-1 activation, since the RL peptide is inactive in this regard. It is possible that the strongly basic arginine guanidinium side chain in the RL peptide may be responsible for this PAR-1-independent effect. The finding of increased expression of iNOS and IL-1β mRNA in HIV/AIDS cases compared with control or MS patients may be due to the innate immune activation and neurodegeneration that characterize HIVE.

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determining neuronal death (59). IL-1β may also induce the release of neurotropins (60), but it can also initiate the production of toxic reactive oxygen species, such as NO, through the induction of iNOS (61). Of interest, U373 cells exhibited greater reactivity than primary astrocytes in response to thrombin and TF, especially for IL-1β, which probably reflected residual effects of thrombin in serum and the need to culture primary astrocytes in high levels of serum for survival before experimental use. The present observation of increased iNOS expression in primary astrocytes following treatment with the AY peptide, which activates PAR-4, is intriguing and will be examined in future studies. Nonetheless, further studies are warranted to investigate whether the thrombin-induced increase in iNOS mRNA levels is indirectly mediated via the up-regulation of IL-1β or is caused by a direct activation of iNOS transcription by PAR-1 signal transduction.

In addition to participating in innate immunity, astrocytes are critical for maintaining homeostasis within the brain. One of their chief functions is scavenging excessive levels of glutamate, which causes neurotoxicity via several receptors (62). Dysregulation of astrocyte function and NMDA receptor-mediated toxicity are believed to be responsible in part for the neuronal damage observed in HIV (63). Proinflammatory cytokines such as IL-1β and TNF-α, inhibit astrocyte glutamate uptake (38) by a mechanism involving NO (64, 65), and indeed, glutamate levels have been shown to be elevated in CSF and plasma of individuals with AIDS-related neurological disease (66). Here, we show that supernatants from thrombin-stimulated astrocytes contain molecules that cause neurotoxicity via an NMDA-mediated pathway that may be regulated by increased levels of IL-1β and iNOS. Moreover, it has been reported that thrombin renders neurons more susceptible to glutamate-induced toxicity by potentiating the NMDA receptor (67), which could result in an amplification of glutamate toxicity in vivo.

Advanced HIV infection is frequently associated with cognitive impairment and movement disorders (68), which include psychomotor slowing, tremor, and altered posture and gait similar to those observed in Parkinson’s disease, implicating dysfunction of striatal dopaminergic systems (69). Thus, striatal implantation of a PAR-activating peptide into the mouse brain and subsequently monitoring motor functions provided us with a reliable and clinically relevant model to study PAR-1 activation in vivo. After striatal implantation of the PAR-1 agonist, mice showed a significant

FIGURE 6. PAR-1 and (pro)thrombin immunoreactivity was detected in astrocytes and neurons, respectively, in brain tissue of HIVE cases. Immunohistochemical staining of brain tissue sections obtained from representative control (A, C, and F) and HIVE cases (B, D, E, G, and H). Few GFAP-immunopositive astrocytes were observed in control sections (A) compared with HIVE tissue (B). Infrequent PAR-1 immunostaining was observed in control tissue (C), whereas HIVE tissue showed intense and widespread PAR-1 immunoreactivity on cells with astrocytic morphology (D). PAR-1 staining colocalized with GFAP immunoreactivity (E). Weak (pro)thrombin expression was detected on neurons in control brains (F), whereas concentrated (pro)thrombin immunoreactivity was detected on neurons (G) and astrocytes (H) in HIVE cases. Magnification: A–C, ×200; D–H, ×400.
deficit in neurobehavioral function, which corresponds with substantial brain inflammation, as demonstrated by the presence of astrogliaosis and increased immunoreactivity for microglia/macrophages. The iNOS- and STAT-1-expressing cells were up-regulated to a greater extent in tissue from agonist-treated mice compared with brain tissue of control mice. It is likely that this immune activation contributed to the observed behavioral abnormalities. In fact, iNOS is under control of IL-1β via STAT-1 and NF-κB-mediated pathways (51, 70). Besides being directly neurotoxic, cytokines can also contribute to neurodegeneration in indirect ways. IL-1β is known to induce chemokines (71, 72) and adhesion molecules (73), which will both lead to enhanced leukocyte infiltration into the brain. It has been reported that thrombin can induce leukocyte recruitment, also a key feature of HIV infection, MS, and stroke (33, 36, 74), through the induction of chemokines and adhesion molecules (16, 75). These effects may very well be mediated by IL-1β.

It is likely that thrombin-mediated activation of PAR-1 and the subsequent inflammation and neuronal damage are a mechanism that occurs during HIV, given that both PAR-1 and (pro)thrombin are up-regulated in brain tissue of individuals with HIV. The fact that PAR-1 and (pro)thrombin mRNA levels were increased in the HIV group compared with the MS group suggests that this increase is not solely an effect of inflammation, but is possibly related to a virus-induced effect. Although little is known about the control of PAR-1 mRNA expression, its expression is up-regulated by thrombin via activation of the Ras/mitogen-activated protein kinase pathway in endothelial cells (15), and both thrombin and PAR-1 are overexpressed after spinal cord injury (76). We demonstrate that PAR-1 expression in astrocytoma cells is up-regulated by thrombin, but the signaling pathways remain to be elucidated. PAR-1 and thrombin protein expression was predominantly detected on reactive astrocytes in HIV brains, suggesting a potential important autocrine loop leading to immune activation, calcium signaling (77), and astrogliaosis (14). PAR-1 immunoreactivity was also observed on neurons, endothelial cells, and infiltrating monocytes, although to a much lesser extent. However, this finding suggests that elevated levels of thrombin may activate PAR-1 on different cell types throughout the brain and result in a wide array of effects. These effects may range from direct neurotoxic or neuroprotective effects (8, 78) to enhancing recruitment of inflammatory cells into the brain via induction of chemokines and adhesion molecules in endothelial and monocytes (16, 19).

Inflammation of the brain occurs in many neurodegenerative diseases, such as AD, MS, stroke, and HIV-associated dementia (58, 79–82). Although the underlying cause differs for each disease, neuroimmune activation may be a common pathway that leads to neuronal damage and loss. We show that PAR-1 up-regulation is tightly coupled to brain inflammation and neurodegeneration, suggesting that PAR-1 may be a promising target for the development of anti-inflammatory therapeutic strategies for CNS diseases.

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References


