Cerebrospinal Fluid Levels of MMP-2, 7, and 9 Are Elevated in Association with Human Immunodeficiency Virus Dementia

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Pathological evidence suggests that alterations of the blood–brain barrier (BBB) may occur in association with human immunodeficiency virus (HIV) dementia (HIVD). Increased BBB permeability could contribute to the development of dementia by facilitating the entry of activated and infected monocytes, as well as potentially toxic serum proteins, into the central nervous system. One mechanism by which BBB permeability may be altered is through increased activity of select matrix metalloproteinases (MMPs). In the present study, we examined the possibility that MMPs that target critical BBB proteins, including laminin, entactin, and collagen type IV, are elevated in the cerebrospinal fluid (CSF) of patients with HIVD. We also examined the possibility that such MMPs could be produced by brain-derived cells, and that MMP production by these cells might be increased by tumor necrosis factor-α, an inflammatory cytokine that is produced by HIV-infected monocytes/microglia and is elevated in HIVD. By using western blot and enzyme-linked immunosorbent assay, we observed that CSF levels of pro–MMP-2 and pro–MMP-7 were increased in association with HIVD. In addition, through the use of gelatin substrate zymography, a sensitive functional assay for MMP-2 and MMP-9, we observed that MMP-2 or pro–MMP-9 activity was more frequently detectable in the CSF of individuals with HIV dementia (9/16) than in the CSF from either nondemented seropositive (2/11) or seronegative (0/11) controls. Although the presence of MMPs in the serum could contribute to elevated levels in the CSF, we also show that brain-derived cells release MMP-2, 7, and 9, and that such release is increased after their stimulation with tumor necrosis factor-α. Together, these results suggest that elevated CSF levels of select MMPs may reflect immune activation within the central nervous system. They also suggest that further studies may be warranted to determine whether these proteins may play a role in the development of symptomatic neurological disease.

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Despite recent advances in the treatment of human immunodeficiency virus (HIV) infection, HIV dementia (HIVD) remains a significant cause of morbidity and mortality. Antiretroviral treatment failures may occur because of resistance mutations, and moreover, several patients cannot tolerate select therapeutics.[1,2]

Although our understanding of events that are critical to the development of HIVD is incomplete, monocyte-derived cells are likely to play an important role. Increased numbers of activated brain macrophages are associated with HIVD, as are increased quantities of macrophage-derived proteins.[3–5] Monocytes can also transport virus to the brain and, within the brain, monocyte-derived cells are the predominant sites of viral replication.[6–10] Monocyte-derived cells can also release several potent neurotoxins including viral gene products, and cellular gene products such as TNF-α and nitric oxide.[11–17]

Multiple mechanisms are likely to be responsible for monocyte entry into the brain during HIV infection, including changes in the expression of specific endothelial cell adhesion molecules,[18] the activation status of circulating monocytes,[19] and the production of monocyte-specific chemoattractants within the central nervous system (CNS).[20–24] Alterations in blood–brain barrier (BBB) integrity, however, could also play an important role.

The endothelial BBB is composed of endothelial cell tight junctions, astrocyte foot processes, and a common basal lamina that covers the opposed membranes of the endothelial cells on one side and the astrocyte foot processes on the other. Like other basal laminae, the BBB

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lamina is predominantly composed of type IV collagen, heparin sulfate proteoglycan, laminin, and entactin.

Pathological evidence suggests that the BBB is altered in association with HIV or HIVD. Immunoreactivity for BBB proteins, including laminin and collagen type IV, is reduced in association with HIV infection, as is the mean thickness of the capillary endothelial cell basal lamina.[25,26] In addition, neurons that are immunopositive for serum proteins are more frequently detected in the frontal cortex of patients with HIVD than in that of nondemented controls.[27] Furthermore, cerebrospinal fluid (CSF)–to–serum albumin ratios increase with the duration of HIV infection and become abnormally high in patients with HIVD.[28,29]

One mechanism that may contribute to altered BBB function in the setting of HIVD is increased activity of select matrix metalloproteinase (MMPs). MMPs belong to a family of zinc-containing endopeptidases that can degrade specific components of the extracellular matrix including collagen type IV.[30] Typically, MMPs are secreted as inactive precursors that are subsequently activated by physiological stimuli, including plasmin and other members of the MMP family. The activity of MMPs is also affected by endogenous, differentially inducible tissue inhibitors of metalloproteinases.

Although there is some overlap in substrate specificity, MMPs differ not only with respect to cellular source and inducibility, but with respect to the efficiency with which they can act on specific substrates. Compared with other MMPs, MMP-2 and 9 are especially efficient in their ability to cleave type IV collagen. MMP-7, also known as matrilysin, can efficiently cleave other proteins of the BBB including laminin and entactin.[31]

Previous studies have demonstrated that select MMPs may not only cleave specific BBB proteins, but that they may do so in a manner that is biologically significant. For example, select MMP inhibitors can reduce BBB disruption in animal models of CNS inflammation.[32,33] In addition, it has been shown that intraparenchymal injection of select MMPs, including MMP-7 and 9, is associated with breakdown of the BBB and leukocyte infiltration of the CNS.[34,35]

In the present study, we have examined the possibility that MMP-2, 7 and 9, because they are especially efficient in their ability to degrade critical proteins of the BBB, might be elevated in the CSF of individuals with HIVD. In addition, because examination of fixed tissue and brain homogenates is not ideal for the detection of secreted enzymes or their activity, we have investigated the possibility that BBB-degrading metalloproteinases may be produced by primary cultures of human brain–derived cells.

Materials and Methods

Acquisition of Cerebrospinal Fluid

CSF was obtained by lumbar puncture, after informed consent, from a prospectively followed, clinically characterized, population of patients. Samples were stored in aliquots at −70°C. HIVD was diagnosed according to the American Academy of Neurology criteria and severity was scored using the Memorial Sloan-Kettering (MSK) criteria.[36] Samples from patients with opportunistic infections or CNS malignancies were excluded from our analyses. CSF samples from HIV-seronegative patients with noninflammatory conditions of the CNS were similarly obtained and stored. Of note, the average time samples had been stored at −70°C was similar for each group.

Cell Cultures

Brain-derived cells were prepared from 12- to 14-week human fetuses or from adults undergoing temporal lobe resections for intractable epilepsy. Myelin was dissected from biopsy specimens. Phosphate-buffered saline–washed tissue was triturated through a 19-gauge needle. Biopsy-derived tissues were subsequently placed in 0.05% trypsin and stirred for 45 minutes. After trituration, trypsinization, or both, cells were resuspended in Eagle's minimal essential medium (EMEM) supplemented with 10% fetal calf serum. Where indicated, cultured cells were characterized by immunocytochemistry, using specific antibodies (Dako, Carpinteria, CA).

Enzyme-Linked Immunosorbent Assay of CSF Samples

Enzyme-linked immunosorbent assays were performed by using the BioTrak assay for pro–MMP-2 and pro-MMP-9 (Amersham, Piscataway, NJ), in accordance with the manufacturer's instructions.

Statistical Studies

Sample groups that were evaluated by enzyme-linked immunosorbent assay were first compared with the Kruskal–Wallis test. Pairwise comparisons were then performed with the Mann–Whitney *U* test, using a Bonferroni correction for multiple comparisons. χ^2 analyses were used to assess differences in the proportions of patients with detectable MMP immunoreactivity by western blot.

Recombinant Proteins

Tumor necrosis factor-α (TNF-α) was obtained from R&D Systems, Minneapolis, MN. MMP-7 was obtained from Chemicon, Temecula, CA.

Western Blot

Western blot, using 30 ml of CSF or cell culture supernatant per well, was performed as described[20]; 1 mg/ml of a monoclonal antibody to MMP-7 (Calbiochem, San Diego, CA) was used in these studies. Where indicated, densitometric analysis of these studies was performed by using a personal densitometer (Molecular Dynamics, Sunnyvale, CA).

Gelatin Substrate Zymography

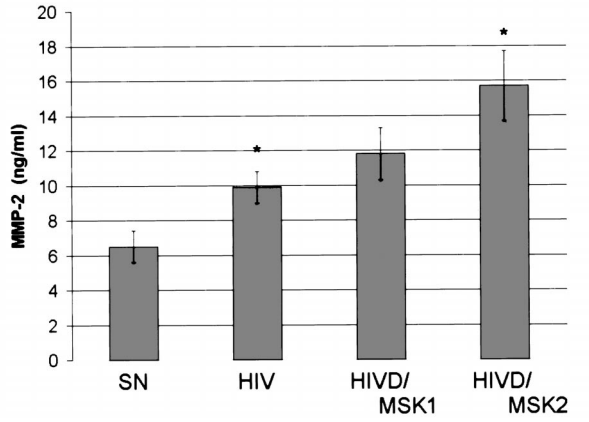
Zymography, using 20 ml of CSF per well, was performed with precast gels (Bio-Rad) according to the manufacturer's instructions. Molecular weights of the proteinases were determined by comparison with protein molecular weight standards (Bio-Rad).

Results

CSF Levels of Pro–MMP-2 Are Elevated in Association with HIVD

To determine whether CSF levels of pro–MMP-2 or pro–MMP-9 might be elevated in association with HIVD, we analyzed 12 samples from seronegative individuals, 17 from neurologically normal HIV-seropositive individuals, and 37 samples from individuals with HIVD by enzyme-linked immunosorbent assay. Pro–MMP-9 was below detectable limits (2 ng/ml) in most samples from each group (not shown). Pro–MMP-2, however, was detectable in all samples tested and was elevated in association with HIV infection (Fig 1, $p < 0.001$). The average pro–MMP-2 value in samples from patients with moderate dementia (HIVD/MSK2, 15.7 ± 2.0) was greater than that in samples from patients with mild dementia (HIVD/MSK1, 11.8 ± 1.5). Although this difference was not statistically significant ($p = 0.158$), the difference between samples from neurologically normal seropositive patients (HIV) and those from patients with moderate dementia (HIVD/MSK2) was significant ($p = 0.017$).

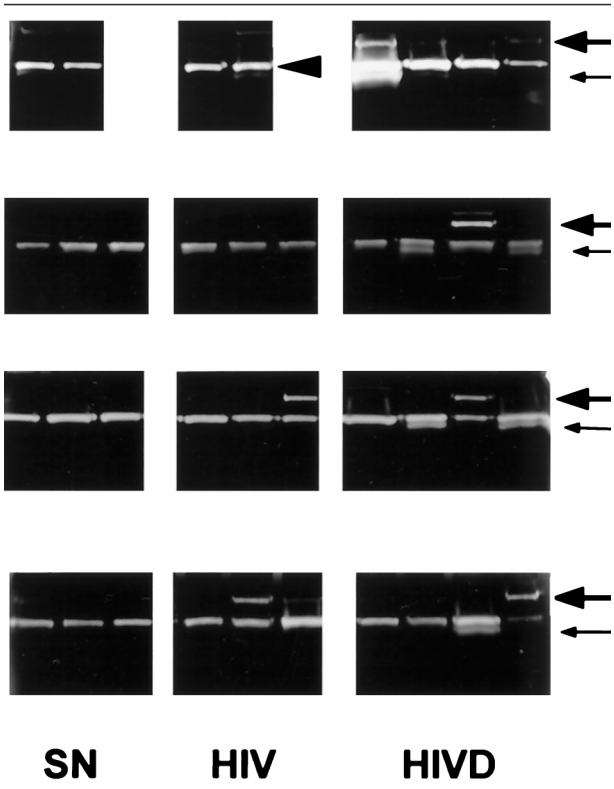
Fig 1. Cerebrospinal fluid (CSF) levels of matrix metalloproteinase type 2 (MMP-2) are increased in association with human immunodeficiency virus (HIV) dementia (HIVD). MMP-2 levels were quantified by enzyme-linked immunosorbent assay in CSF samples from 12 HIV-seronegative controls (SN), 17 neurologically normal HIV-seropositive controls (HIV), 24 HIVD patients with mild dementia as determined by a Memorial Sloan-Kettering (MSK) score of 1 (HIVD/MSK1), and 13 HIVD patients with moderate dementia as determined by an MSK score of 2 (HIVD/MSK2). All data are represented as mean ± SE values. The comparisons of SN with HIV (asterisk at left) *and HIV with HIVD/MSK2* (asterisk at right) *were significant at* $p < 0.001$ *and* $p < 0.017$, *respectively. There was no significant difference between the HIV and HIVD/MSK1 groups.*



Enzymatically Active Forms of MMP-2 and MMP-9 Are Frequently Detected in Association with HIVD

Many of the same CSF samples (11 seronegative, 11 HIV, and 16 HIVD) were assessed by gelatin substrate zymography. In these assays, the functional activity of several distinct gelatinases can be demonstrated based on differences in molecular mass. In all of the samples we tested, gelatinase activity at 72 kd could be detected (Fig 2); this migrates identically to purified pro–MMP-2 (data not shown). It is interesting that renaturation in Triton X allows the precursor form of MMP-2 to exert gelatinase activity.[37] Fully active MMP-2, however, migrates at 65 kd and can be distinguished in many of the samples as a slightly lower band (see Fig 2). The form of MMP-9 that is fully active in vivo typically migrates at 85 kd but, perhaps

Fig 2. Zymographic analysis of cerebrospinal fluid samples from human immunodeficiency virus (HIV)-seronegative patients (SN), neurologically normal HIV-seropositive patients (HIV), and HIV dementia (HIVD) patients (HIVD). Data in each row are from a single experiment, with spaces added for clarity. Pro–MMP-9 activity is observed at 92 kd (large arrows), *pro–MMP-2 is observed at 72 kd* (arrowhead), *and fully active matrix metalloproteinase type 2 (MMP-2) is seen at 65 kd* (small arrows). *MMP-9 = matrix metalloproteinase type 9.*



because of its tight association with extracellular matrix proteins, could not be detected in any of the samples that were run. The pro form of MMP-9 that migrates at 92 kd can, however, be observed in several samples (see Fig 2).

None of the 11 CSF samples from HIV-seronegative patients showed easily discernible pro–MMP-9 activity. In contrast, 2 of 11 (18%) CSF samples from neurologically normal individuals with HIV infection, and 4 of 16 (25%) from those with HIVD showed detectable pro–MMP-9. Furthermore, although none of the CSF samples from HIV-1–seronegative or neurologically normal seropositive individuals contained 65-kd MMP-2 activity, 6 of 16 (38%) from individuals with HIVD were positive. Together, 9 of 16 (56%) of CSF samples from patients with HIVD were positive for either MMP-2 or pro–MMP-9 activity, compared with 2 of 11 (18%) of neurologically normal seropositive and 0 of 11 (0%) of seronegative controls. It is noteworthy that neither of the neurologically normal seropositive patients whose CSF samples demonstrated increased MMP-2 activity by zymography went on to develop dementia within the 6 months after sample acquisition, although undetected clinical differences could explain at least some of the variability in our results.

CSF MMP-7 Immunoreactivity Is Increased in Association with HIVD

Like MMP-2 and 9, MMP-7 (matrilysin) may also degrade critical BBB proteins. Therefore, CSF samples were analyzed by western blot, using an antibody to matrilysin (Fig 3). By using the densitometric reading obtained from the left-most band on the third gel as a low cutoff, MMP-7 immunoreactivity was easily detectable in 2 of 12 CSF samples from seronegative individuals, 6 of 12 CSF samples from HIV-seropositive patients without dementia, and 15 of 16 samples from those with HIVD. The difference between groups was significant (χ^2, $p = 0.001$). Unfortunately, we could not demonstrate increased activity of MMP-7 in samples from patients with HIVD. Gelatin substrate zymography is not a particularly sensitive functional assay for MMP-7, and casein substrate zymography[38] was not sensitive enough to detect MMP-7 activity in CSF samples or controls (1–5 ng/ml recombinant human MMP-7).

MMP-2 and MMP-9 Can Be Detected in the Supernatants of TNF-α–Stimulated Human Brain–Derived Cells

Gelatin substrate zymography was also used to examine MMP release from unstimulated and TNF-α–stimulated primary cultures of human brain–derived cells. Previous studies that have examined MMP production by such cells have typically relied on the use of non-

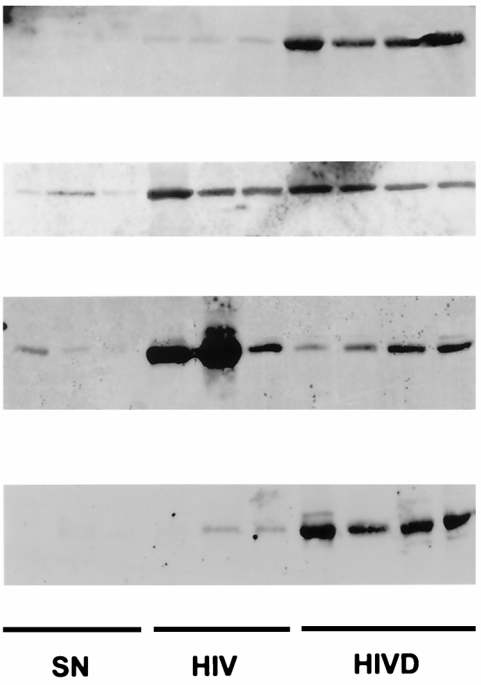


Fig 3. Western blot analysis of matrix metalloproteinase type 7 (MMP-7) in cerebrospinal fluid samples from human immunodeficiency virus (HIV)-seronegative patients (SN), neurologically normal HIV-seropositive patients (HIV), and HIV dementia (HIVD) patients (HIVD). A single band, which runs with an apparent molecular mass equal to that of pro–MMP-7 (29 kd), is shown. With the densitometric reading of the left-most band on the third gel as a low cutoff, there was significant difference in the proportion of samples per group that showed positivity on western blot (χ^2, p = 0.001).

human cells, cell lines, or nonphysiological stimuli such as PMA.[39,40] Figure 4 shows the results of gelatin substrate zymography on various cell culture supernatants. In Figure 4A, supernatants from unstimulated cultures of human fetal brain–derived cells (1 ml of serum-free media on 10^5 cells for 16 hours) were run in lanes 1 and 3, and supernatants from TNF-α–stimulated (10 ng/ml in serum-free media on 10^5 cells for 16 hours) were run in lanes 2 and 4. Figure 4B represents a similar experiment except that mixed cultures of adult human brain–derived cells (containing both astrocytes and CD68-positive microglia) were used. Also, 5 ng of pro–MMP-9 was run in lane 5 as a positive control. In Figure 4C, the same experiment was run except that supernatants were derived from relatively pure cultures of adult-derived human astrocytes (>98% glial fibrillary acidic protein–positive). In this experiment, pro–MMP-9 activity was not increased after stimulation with TNF-α. This suggests that in mixed cultures, microglia are the principal source of this MMP.

Taken together, these data show increased release of pro–MMP-9 after TNF-α stimulation of microglial

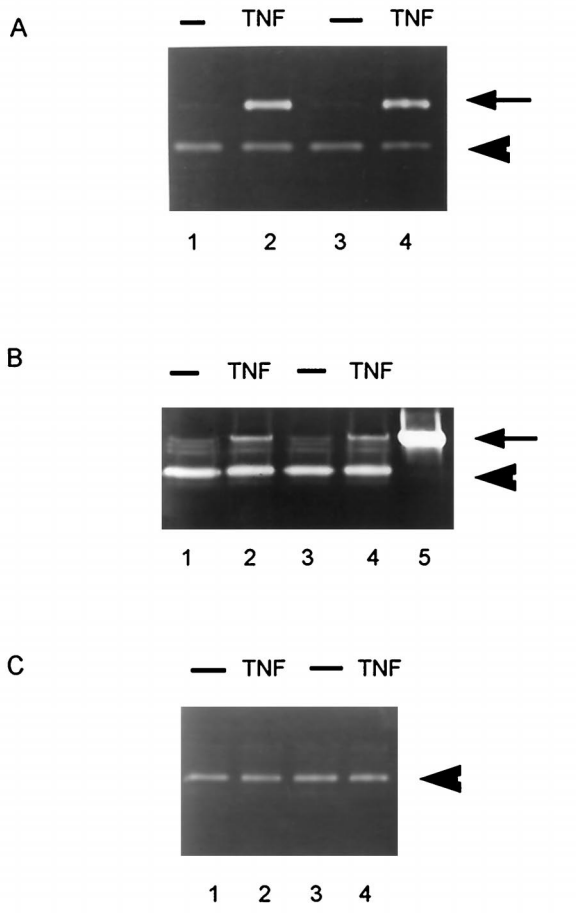


Fig 4. Zymographic analysis of supernatants from cultures of fetal brain–derived cells (A), cultures of adult brain–derived cells (B), or cultures of adult astrocytes (C). Pro–MMP-9 activity is indicated by the arrows *and pro–MMP-2 activity by the* arrowheads. *A, B, and C are each representative of an experiment that was done on three separate occasions. MMP-9 and MMP-2 = matrix metalloproteinase types 9 and 2; TNF = tumor necrosis factor.*

cell–containing brain-derived cell cultures. In addition, they show that astrocytes produce pro–MMP-2, the activity of which is not increased after stimulation with TNF-α.

MMP-7 Immunoreactivity Can Also Be Detected in Supernatants from Brain-Derived Cells, and Is Increased after Their Stimulation with TNF-α

As previously discussed, gelatin substrate zymography is not particularly sensitive with respect to the detection of MMP-7. Therefore, supernatants from mixed cultures of human fetal brain–derived cells (10^5 cells/ml) were also examined by western blot. As shown in Figure 5, supernatants from these cultures demonstrated MMP-7 immunoreactivity, and this immunoreactivity was increased after a 16-hour stimulation with 10 ng/ml TNF-α (R&D Systems).

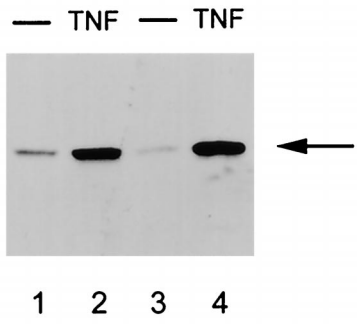


Fig 5. Western blot analysis of matrix metalloproteinase type 7 (MMP-7) in supernatants from separate cultures (1 and 2 vs 3 and 4) of fetal brain–derived cells. A single band, which runs with an apparent molecular mass of 29 kd, is indicated by the arrow. *TNF = tumor necrosis factor.*

Discussion

In this study, we have shown an increase in the amount or activity of MMP-2, MMP-7, and MMP-9 in the CSF of individuals with HIVD, compared with both seronegative controls and HIV-positive individuals without dementia. These MMPs, in particular, are known to target critical components of the BBB.

Elevated CNS levels of select MMPs have been detected in association with other inflammatory diseases including human T-cell lymphotropic virus type I infection and multiple sclerosis.[41–45] In addition, increased CSF activity of MMP-9 has recently been detected in association with HIVD.[46] Increases in the quantity or activity of MMP-7 and 2, however, have not been described in this disorder.

Proteins within the CSF compartment may be derived from the serum or the brain parenchyma. It is noteworthy that inflammatory cells secrete MMPs during their migration through the BBB. MMPs made within the brain parenchyma, however, could alter the BBB from the extraluminal side, and thereby further facilitate the CNS ingress of inflammatory cells. This latter possibility is supported by experiments in which the intraparenchymal injection of MMP-7 and 9 was followed by leukocyte infiltration of the CNS.[34,35] Moreover, MMPs produced within the brain parenchyma could have direct effects on CNS-derived cells.

To address the question of MMP production by resident cells of the CNS, we examined the release of MMPs from primary cultures of human brain–derived cells. Furthermore, we tested the ability of TNF-α, which is elevated in the CNS in association with HIVD,[5] to effect this release. We observed that primary cultures of brain-derived cells release MMPs (MMP-2, 7, and 9), and that TNF-α augments the release of MMP-7 and 9 from such cells. We also found that despite increased activity of MMP-2 in CSF samples from several patients with HIVD, neither unstimulated nor TNF-α–stimulated brain-derived cells

released the active form of this enzyme as detected by zymography. Extracellular stimuli that may be physiologically important to the activation of MMP-2, such as MT1-MMP (membrane type 1 matrix metalloproteinase),[37] may be increased within the HIVD CNS but not within our TNF-α–stimulated culture system. The quantity of select tissue inhibitors of metalloproteinases, which affect the activity of MMP-2, may also differ between the in vivo and in vitro systems we have examined.

Although a focus of our in vitro studies, TNF-α is not likely to be the only HIVD-related stimulus that could potentially affect MMP production by brain-derived cells. Previous studies have shown that MCP-1, HIV-1 Tat, interleukin-1β, and prostaglandin E_2 may influence the production of MMP-9 by monocytes.[47–49] More recently, the HIV-1 envelope proteins gp41 and gp120 have been shown to induce MMP-2 in tumor-derived cells.[50,51]

Although increased CSF levels of select MMPs in association with HIVD could simply reflect generalized CNS inflammation, these enzymes may also contribute to disease activity and progression. MMPs, including MMP-2 and 9, have been shown to affect BBB permeability and may therefore allow inflammatory cells to more easily enter the CNS. MMP-7, through its ability to degrade laminin and entactin, could also compromise this barrier.[31]

In addition to potential effects on the transmigratory ability of inflammatory cells, altered BBB permeability may allow potentially toxic serum proteins, including thrombin, to enter the CNS.[52–55] It is interesting that thrombin has been shown to affect dendritic arborization,[54] which is reduced in association with HIVD.[56]

Although similarly speculative, it is also possible that MMPs may have more direct effects on brain-derived cells. For example, MMP-7, through its ability to degrade laminin, may affect neurite outgrowth,[57] synaptic morphology,[58] and, possibly, neuronal survival.[59] That MMPs may directly affect the function and survival of CNS-derived cells is also supported by the finding that MMP inhibitors can inhibit truncation of the low-affinity nerve growth factor receptor,[60] as well as the finding that MMP-2 and 9 may affect the activity of interleukin-1β.[61]

At present, clinical trials are under way to determine whether MMP antagonists may alter the progression of multiple sclerosis. The results presented herein suggest that these antagonists might also be tested in HIVD and other diseases in which proinflammatory stimuli such as TNF-α are thought to play an especially important role.

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