T Lymphocytes Activated by Persistent Viral Infection Differentially Modify the Expression of Metalloproteinases and Their Endogenous Inhibitors, TIMPs, in Human Astrocytes: Relevance to HTLV-I-Induced Neurological Disease[1]

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Activation of T lymphocytes by human pathogens is a key step in the development of immune-mediated neurologic diseases. Because of their ability to invade the CNS and their increased secretion of proinflammatory cytokines, activated $CD4^+$ T cells are thought to play a crucial role in pathogenesis. In the present study, we examined the expression of inflammatory mediators the cytokine-induced metalloproteinases (MMP-2, -3, and -9) and their endogenous inhibitors, tissue inhibitors of metalloproteinases (TIMP-1, -2, and -3), in human astrocytes in response to activated T cells. We used a model system of $CD4^+$ T lymphocytes activated by persistent viral infection (human T lymphotropic virus, HTLV-I) in transient contact with human astrocytes. Interaction with T cells resulted in increased production of MMP-3 and active MMP-9 in astrocytes despite increased expression of endogenous inhibitors, TIMP-1 and TIMP-3. These data suggest perturbation of the MMP/TIMP balance. These changes in MMP and TIMP expression were mediated, in part, by soluble factors (presumably cytokines) secreted by activated T cells. Integrin-mediated cell adhesion is also involved in the change in MMP level, since blockade of integrin subunits (α_1, α_3, α_5, and β_1) on T cells resulted in less astrocytic MMP-9-induced expression. Interestingly, in CNS tissues from neurological HTLV-I-infected patients, MMP-9 was detected in neural cells within the perivascular space, which is infiltrated by mononuclear cells. Altogether, these data emphasize the importance of the MMP-TIMP axis in the complex interaction between the CNS and invading immune cells in the context of virally mediated T cell activation. *The Journal of Immunology*, **2000, 164: 2718–2727.**

Several immune-mediated neurological diseases associated with viral infection are characterized by a chronic inflammatory process in which T cells infiltrate the CNS (1, 2). Irrespective of their specificity, T lymphocytes can indeed cross the blood-brain barrier (BBB)[3] and traffic into the CNS when activated in periphery by antigenic stimulation or viral infection (3, 4). T cell activation leading to modulation of their surface phenotype and cytokine secretion pattern (5, 6) is a crucial event in CNS inflammation (7, 8). Overproduction of proinflammatory soluble mediators, including cytokines and chemokines, in the CNS influences its immune response and thus the functional outcome of immune attack (9, 10). In addition to the part played by infiltrating T lymphocytes and monocytes/macrophages, recent studies have emphasized the crucial role of the neural cells themselves in the immune response. In particular, activation of astrocytes following interaction with activated T cells may be decisive in pathogenesis since these glial cells, which are essential for survival and operation of other neural cells, facilitate and locally amplify the inflammatory process initiated by immune effectors within the CNS (11).

In multiple sclerosis (MS), the prototype inflammatory demyelinating disease, the presence of infiltrated T lymphocytes within lesions is associated with enhanced expression of cytokines and metalloproteinases in glial cells (12, 13). Similar hallmarks are observed in a virally induced demyelinating disease, human T lymphotropic virus (HTLV)-I-associated-myelopathy, also termed tropical spastic paraparesis (TSP/HAM), which can affect patients infected by HTLV-I, a retrovirus with T cell tropism (14). HTLV-I causes persistent infection of T lymphocytes, resulting in phenotypic shift from the resting to the activated state (15). As shown by mRNA and protein studies, proinflammatory cytokines are expressed in T cells infiltrating CNS lesions in TSP/HAM patients, and reactive astrocytes (gliosis) in the same area also express cytokines (16).

We postulate that, in HTLV-I-infected patients, the interaction between astrocytes and CNS-infiltrating T lymphocytes may play a crucial role in inflammatory damage via a mechanism involving cytokines and cytokine-induced molecules, such as proteases, produced by activated T cells and astrocytes. The involvement of matrix metalloproteinases (MMP) in inflammatory damage is suspected in TSP/HAM, since the balance between pro-/antiinflammatory cytokines and proteases/antiproteases probably determines the outcome of inflammatory insult, as shown in other diseases

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[3] Abbreviations used in this paper: BBB, blood-brain barrier; MS, multiple sclerosis; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; HTLV, human T lymphotropic virus; TSP, tropical spastic paraparesis; HAM, HTLV-1-associated myelopathy; GFAP, glial fibrillary acidic protein; CSF, cerebrospinal fluid; APMA 4-aminophenylmercuric acetate; PDTC, pyrolidine dithiocarbamate; ECM, extracellular matrix.

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Table I. *Secretion of cytokine by HTLV-1-infected T cells and Dev cells after contact with T cells*[a]

	TNF-α (pg/ml)	TNF-β (pg/ml)	IL-1α (pg/ml)	IL-6 (ng/ml)
C91PL T cells	149–814	7,500	41–116	32
C8166-45 T cells	138–438	17,500	41–61	0.3–2.5
CEM T cells	3–13	<7	<3–10	<0.003
Stimulated Dev cells	16–33	11–55	<5	1.5–43.8
Stimulated-infected Dev cells	2,044–14,441	218–3,430	8–59	56.6–208.5
Dev cells with T cell control	<5	<7	<5	2–34

[a] Cytokine levels were quantified by EIA (Immunotech, France) in the supernatant of stimulated and stimulated-infected Dev cells on days 4 and 8 after transient contact with activated T lymphocytes. Cytokine expression by T lymphocytes was quantified in the cell supernatant of 0.5×10^6 T cells/ml. The lowest and highest values are shown (four experiments).

(17, 18). Using a model system of the T cell-astrocyte interaction, we have shown that transient contact between astrocytes and T lymphocytes activated by persistent HTLV-I infection leads to activation of the astrocytes (cytokine secretion and up-regulation of glial fibrillary acidic protein (GFAP) and MHC class I molecules) (19). Interestingly, induction of MMP-9 is seen in parallel with astrocytic cytokine expression (20). In addition, we detected presence of MMP-9 and tissue inhibitor of metalloproteinase (TIMP)-3 in cerebrospinal fluid (CSF) from HTLV-I-infected patients suffering from TSP/HAM and not from healthy virus carriers or patients suffering from noninflammatory neurological diseases. This observation emphasizes the clinical relevance of the studies on the role of MMPs/TIMPs in TSP/HAM (21, 22, 62).

The aim of the present work was to determine whether, and how, interaction with T lymphocytes activated by persistent HTLV-I infection affects the human astrocyte MMP-TIMP network. Changes in the expression and activity of MMP-2, MMP-3, and MMP-9 and TIMP-1, TIMP-2, and TIMP-3 were analyzed by coculturing human astrocytes with human HTLV-I-activated T cell lines. The specific differential modulation of MMPs and TIMPs detected in these astrocytes was shown to be mediated via T cell-produced cytokines and integrins. The MMP-9 expression pattern in postmortem brain specimens from TSP/HAM patients showed induction of this protease in the vicinity of vessels, but only in the area containing reactive astrocytes and infiltrated immune cells. These data indicate that T lymphocytes activated by persistent viral infection may disturb the concerted expression of MMPs and TIMPs in neural cells.

Materials and Methods

Cell lines and treatments

The Dev cell subline used in this work derived from a cell line established from a primitive neuroectodermic tumor (23). These cells exhibit astrocytic markers, GFAP, and glutamate transporters GLT1 and GLAST. They were cultured as a monolayer in DMEM medium (Life Technologies, Gaithersburg, MD) supplemented with 10% FCS. The following $CD4^+$ T cell lines persistently infected with HTLV-I were used for transient contact with the Dev: 1) the HTLV-I-producing T cell line C91PL (24) and 2) the HTLV-I-nonproducing T cell line C8166/45 (25) and H36 (gift of Dr. Desgranges, U271 Institut. National de la Santé et de la Recherche Médicale). C91PL and C8166/45 T cell lines express the Tax-1 protein (26) and cytokines (Table I). Concentration of IL-1β, TNF-α, TNF-β, and IL-6 were determined using commercial EIA kits according to the manufacturer's instructions (Immunotech, Luminy-Marseille, France). The human $CD4^+$ T cell line CEM was used as a control. The human HTLV-I-infected T lymphocytes were cultured in suspension, gamma-irradiated (136 Gy) to prevent further proliferation, then cocultured with Dev cells (T cell/Dev cell ratio 1:10) for 20 h before being removed from the monolayer by three medium washes. The complete elimination of these $CD4^+$ T cells from the culture was verified by the absence of CD4 Ag (flow cytometry). In previous studies, we have also shown the effectiveness of this procedure to eliminate T cells from cocultures with rat astrocytes: no human GAPDH and TNF-α transcripts could be detected (19–21). Alternatively, HTLV-I-infected T lymphocytes were cultured in an upper chamber (Costar, Cambridge, MA) separated from the adherent Dev cells by a membrane allowing the passage of soluble factors, but not of cells.

To investigate the involvement of integrins in MMP expression, the HTLV-I-infected T lymphocytes were treated, before contact with the Dev cells, with anti-integrin subunit blocking Abs (anti-α_1, -α_3, -α_5, and -β_1) or anti-VCAM-1 Abs as a control (Immunotech, 2–10 μg/5×10^4 T cells for 1 h at 37°C, six experiments). Expression of MMP-9 was evaluated on zymogram by NIH Image software, the data obtained with treated T lymphocytes being standardized with respect to the MMP-9 level induced by untreated T lymphocytes.

To analyze the effect of soluble factors on fibronectin release, supernatants from 4-day cultures of 2×10^6 stimulated/infected Dev cells (Dev cells secreting cytokine but no viral progeny) were used to treat Dev cells. After 20 h of treatment, the cells were washed and fed with fresh medium; then, cell supernatants were examined three days later for presence of fibronectin by Western-blotting.

RNA purification and analysis by RT-PCR

Total RNAs from various treated cells were prepared by homogenization with RNAzol (Bioprobe, Montreuil s/Bois, France) and extraction as described by Chomczynski and Sacchi (27). The concentration and purity were determined spectrophotometrically (Beckman), and the integrity was verified by denaturating agarose gel electrophoresis and ethidium bromide staining. The oligonucleotide primers for the human MMPs and TIMPs were chosen from their mRNA sequences using GeneJockey software, and their specificity was verified by GenBank analysis. The following primers were used: for MMP-2 (sense, GCA ATA CCT GAA CAC CTT CTA TGG; antisense, TCA CAT CGC TCC AGA CTT GG; probe, GGT CAA GAT CAC CTG TCT); for MMP-3 (sense, TGA CTC CAC TCA CAT TCT CCA GGC; antisense, GGT CTG TGA GTG AGT GAT AGA GTG G; probe, TTA ATC CCT GGC CCA GGG GCA TAG); for MMP-9 (sense, GGA GTG AGT TGA ACC AGG; antisense, AAG GTT AGA GAA TCC; probe, GGA TTT ACA TGG CAC TGC); for TIMP-1 (sense, CAC TCA TTG CTT GTG GAC GG; antisense, GCA GGA TTC AGG CTA TCT GG; probe, TTT CAG AGC CTT GGA GGA GC); for TIMP-2 (sense, CTC TGC GAC TTC ATC GTG CC; antisense, AGG AGA TGT AGC ACG; probe, CCT GAG CAC CAC CCA GAA G); for TIMP-3 (sense, AGG CTT CAC CAA GAT GCC; antisense, GTC CAG AGA CAC TCG TTC TTG G; probe, GCA GGT ACT GGT ACT TGT TGA C).

Reverse transcription and PCR amplification were performed as described previously (19). Briefly, first strand cDNAs were synthesized by reverse transcription of 1 μg of total denatured RNA (10 min at 70°C), using 100 ng of oligo(dT) 12–18 (Pharmacia) and 800 U of μ-LV RTase (Life Technologies). The cDNAs for MMP-2, MMP-3, MMP-9, TIMP-1, TIMP-2, TIMP-3, and the housekeeping gene GAPDH (used as control) were amplified by PCR (AmpliTaq Perkin-Elmer), using specific primers. Each specific PCR was optimized by testing different numbers of cycles, temperatures, and $MgCl_2$ concentrations. Amplifications were conducted on the same sample of reverse-transcribed RNA (1/20 RT volume), under the conditions described by Mohler and Butler (28) to allow a semiquantitative estimate to be made. Contamination by genomic DNA was shown to be negligible by performing PCR on the RNAs, but omitting the RT step. The PCR products were migrated on a 2% agarose gel by electrophoresis with m.w. markers and analyzed by Southern blotting using appropriate [^{32}P]dATP-5′end-labeled internal oligonucleotide probes. An autoradiographic film was exposed to the membranes; once developed, the membrane was used to quantify the labeled bands, which were excised and counted in a liquid scintillation counter (Beckman). Counting was sometimes conducted several days after Southern blotting, which may explain the low level of radiation detected by scintillation even in the presence of high autoradiographic signal.

Detection of MMPs by zymography and TIMPs by ELISA

Analysis by zymography on gelatin gel allows detection of enzymatic activity of the secreted type IV collagenases MMP-2 and MMP-9. This was performed as described previously (19). Briefly, an aliquot (10 μl) of each sample of culture supernatant (18 h conditioned serum-free medium) was subjected to electrophoresis on an SDS-polyacrylamide gel (10%) containing gelatin (0.4%), a substrate for MMP-2 and MMP-9. Molecular mass markers (20 to 200 kDa) used were obtained from Life Technologies. After electrophoresis, the gel was incubated in a buffer containing 2.5% Triton (2×15 min), rinsed with distilled water, then incubated overnight at 37°C in enzyme activation buffer (10 mM Tris-HCl, 15 mM $CaCl_2$ (pH 7.4)) with gentle rocking. After the gels were stained with Coomassie blue (0.1% in acetic acid-methanol 1:3), MMP-9 and MMP-2 activity was seen as clear bands of gelatin degradation. Note that zymography technique classically evidences two bands for MMP-2, corresponding to proMMP-2 (released as inactive proenzyme) and active MMP-2 (after cleavage of regulatory domain). Incubation of the gel in enzyme activation buffer containing 10 mM EDTA or 10 mM *O*-phenanthroline inhibited the activity of these gelatinases/type IV collagenases.

Secretion of the stromelysin MMP-3 was quantified using an ELISA kit (Biotrack, Amersham), as we were unable to detect MMP-3 on SDS-polyacrylamide gel-containing casein. In some experiments, TIMP-1 secretion was also quantified by ELISA (Biotrack, Amersham) according to the manufacturer's instructions (no TIMP-3 detection kit was available).

Human brain specimens

Paraffin-embedded human brain specimens (mesencephalon) were obtained from two TSP/HAM patients (Hospidal del Salvador, Santiago, Chile) and one patient with noninflammatory neurological disease (Hôpital Neurologique, Lyon, France). TSP/HAM case 1, a 57-yr-old woman, initially experienced heaviness of her legs, and, 3 yr later, spastic paraparesis was noticed. She had spastic hyperefletic tetraparesis and bilateral Babinski. She also had a neurogenic bladder. A CSF study showed 5 lymphocytes/mm2. She was seropositive for HTLV-I and seronegative for HIV. Eight years after the initial symptoms, she had severe bronchopneumonia and died in severe respiratory failure. Neuropathological findings were dorsal atrophy of the spinal cord, demyelination and axonal loss in the dorsal and lumbar segments of the corticospinal tract, mild adventitial thickening of the spinal cord vessels, and lymphocytic cuffing of the vessels. TSP/HAM case 2, a 35-yr-old man, developed weakness in his legs and urinary urgency. On examination, he showed paraparesis, increased reflexes, and a bilateral Babinski response. He was seropositive for HTLV-I and seronegative for HIV. Four years after the appearance of the first symptoms, he died from a mesenteric thrombosis. Neuropathological findings were thickening and gross lymphocytic infiltrates of the meninges, demyelination in the cervical segment of Goll's and Burdach tracts, and moderate adventitial thickening and gross lymphocytic cuffing of the vessels in the white matter and gray substance of the spinal cord, medulla, midbrain, thalamus, cortex, and subcortical areas. No other structural changes were found in the brain.

Immunodetection of fibronectin and MMP-9

Expression of fibronectin was analyzed by Western blotting in Dev cell culture supernatants (2-, 4- and 8-day cultures for stimulated/infected cells and 3-day cultures for Dev cells treated with conditioned medium). Twenty microliters of supernatant were loaded on each lane, separated on a 10% acrylamide-bisacrylamide gel (29), and electrotransferred onto nitrocellulose membranes (BA85S, Schleicher and Schuell, 150 mA, 1 h). After the membranes were saturated by incubation for 10 min at room temperature with 10% BSA, fibronectin was detected using polyclonal rabbit anti-fibronectin (Chemicon) and the peroxidase-3,3′-diaminobenzidine system (Vectastain ABC kit, Vector, Biosys-France).

Polyclonal rabbit anti-MMP-9 (AB805, Chemicon) and monoclonal mouse anti-GFAP (M761, Dakopatts, Denmark) Abs were used in indirect immunofluorescence or immunocytochemistry studies to detect the presence of MMP-9 and reactive astrocytes in brain sections of postmortem specimen from TSP/HAM patients and control patient with noninflammatory neurological disease. Brain sections were deparaffined and rehydratated in graded ethanol. When HRP system was used, endogenous peroxidases were blocked by incubation with 0.03% hydrogen peroxide prior to Ab incubation. After blocking with 2% normal bovine serum, the sections were incubated with first Ab for 18 h at 4°C. After three rinses, the sections were incubated for 2 h at room temperature with Cya3-conjugated secondary Abs against mouse IgG for GFAP detection or with biotinylated anti-rabbit IgG secondary Abs for MMP-9 detection. After a further three rinses, they were incubated for 2 h at room temperature with either Alexa 488 Dye (green fluorescence; Molecular Probe laboratories) or avidin-coupled HRP (brown color; Vectastain ABC kit, Vector Laboratories). When the primary Abs were omitted, no signal was seen.

Results

Model of interaction between activated T cells and human astrocytes to investigate MMP and TIMP expression

We investigated whether, and how, activated T lymphocytes alter the MMP/TIMP balance in astrocytes. As a model for interaction of astrocytes and T cells that may occur within the CNS, we used transient coculture of the human astrocytic cell line Dev with T lymphocyte cell lines activated by persistent HTLV-I infection. The T cell lines C91PL and C8166/45 are, respectively, virus producers and nonproducers, but evaluation of cytokine secretion showed that both secrete proinflammatory cytokines, including TNF-α, TNF-β, IL-1α, and IL-6 (Table I), as already suggested by Buckle et al. (15). In the light of previous observations (19), contact with the virus-producing T lymphocytes (C91PL) was considered as stimulation-infection of the Dev cells, since marked cytokine secretion is seen in these cells and virus replication may take place in a few astrocytes but without virus particle. Contact with the non-virus-producing T cell line C8166-45 was used to discriminate the effects of viral infection per se from those due to secreted factors and contact with T lymphocytes. Contact with C8166-45 T lymphocytes did not lead to viral infection, but to stimulation of Dev cells subsequently releasing cytokines. Both stimulation and stimulation-infection induced secretion of TNF-α, TNF-β, and IL-1α in Dev cells (Table I), whereas these cytokines were not detected after contact of the Dev cells with nonactivated T lymphocytes (the CEM T cell line).

Contact with activated T lymphocytes differentially modifies expression of MMP-2, MMP-3, and MMP-9 in human astrocytes

We investigated the presence of various MMPs at the secreted protein and mRNA levels in Dev cells after transient contact with the activated T lymphocytes, C91PL and C8166/45 cell lines. Analysis by zymography indicated that collagenases type IV, MMP-2, and MMP-9 were not or only faintly secreted by untreated Dev cells or the activated T cell lines (Fig. 1*a*). Following transient contact of Dev cells with activated T lymphocytes, either virus producers or nonproducers, MMP-9 secretion was markedly induced and maintained at a high level in stimulated-infected (day 2 to 9) and stimulated (day 2 to 7) Dev cells. Qualitatively similar results were obtained from all of the four sets of experiments realized. MMP-2 secretion was also increased in both sets of Dev cells on days 3 and 4, then returned to the pretreatment level. In contrast, MMP-9 and MMP-2 secretion was never altered in Dev cells cocultured with nonactivated T lymphocytes (CEM). The molecular mass of MMP-9 and MMP-2 secreted in stimulated/infected and stimulated Dev cells (85 and 65 kDa, respectively) indicated that these enzymes were present in the medium in the active form (before maturation through pro-peptide cleavage, pro-MMP9 and pro-MMP2 have molecular masses of 92 kDa and 72 kDa, respectively). Moreover, addition of APMA (4-aminophenylmercuric acetate), a pro-MMP activator (30), to the cell medium before electrophoresis did not result in additional bands of smaller m.w. Proteoglycan-degrading stromelysin MMP-3 could be detected only by ELISA but not by zymography on casein-gel, which was found less sensitive. Contact with activated T lymphocytes increased the secretion of MMP-3 in stimulated Dev cells. A stronger effect was observed in stimulated-infected Dev cells (Fig. 1).

Levels of the mRNAs coding for MMP-2, MMP-9, or MMP-3 were monitored by RT-PCR on days 2–9 (Fig. 1). In control Dev cells, MMP-9 was undetectable, whereas the expression of MMP-2

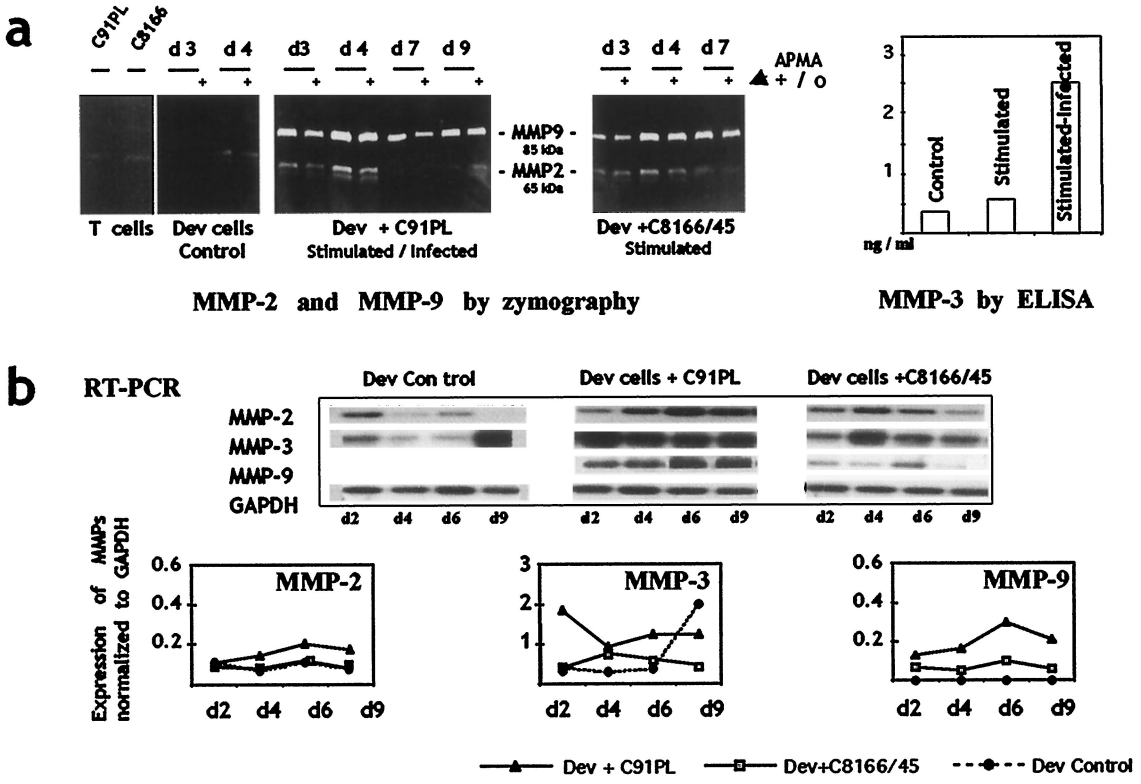

FIGURE 1. Modulation of MMP expression in human astrocytes (Dev cell line) after transient contact with persistently infected/activated T lymphocytes. *a*, Typical time course of MMP-2 and MMP-9 secretion in the supernatant of stimulated/infected and stimulated Dev cells (cocultured with virus-producing C91PL T cell line or the nonproducing C8166-45 T cell line, respectively), as examined by gelatin zymography. Transient contact of Dev cells with these T cells (20 h) induced a sustained expression of MMP-9 and a transient increase in MMP-2 content. Active (i.e., cleaved) MMP-2 and -9 are secreted by the Dev cells transiently exposed to infected T lymphocytes, since no additional bands were obtained by APMA, which cleaves proMMPs. The histogram on the *right* shows an increased secretion of MMP-3 by Dev cells as detected by ELISA. The largest effect was observed after stimulation/infection (C91PL T cell line on day 8 postcontact). *b*, Typical time course of the expression of mRNAs coding for MMP-2, MMP-3, and MMP-9 in cell lysates from stimulated and stimulated/infected Dev cells. The *upper panel* shows Southern blots of MMPs as detected by RT-PCR using specific ^{32}P-labeled internal probes. The same RT products were used to amplify the four target sequences. In control, MMP-9 was never detected. Transcripts were quantitated from these blots, and GAPDH counts were used to standardize MMP expression. All presented data were obtained from four sets of experiments that gave consistent and reproducible results.

and MMP-3 mRNAs was constitutively expressed, this being progressively abolished by serum deprivation. Intriguingly, MMP-3 mRNAs were again expressed on day 9. Contact of Dev cells with activated T lymphocytes, either virus producers or nonproducers, modified the expression of these mRNAs, but not that for the housekeeping gene, GAPDH, analyzed in the same RNA samples. Expression of mRNAs coding for MMP-9 was clearly induced in both stimulated-infected and stimulated Dev cells, this effect being maintained up to day 9 post T cell contact. An increase in the levels of mRNAs coding for MMP-3 was seen in both stimulated-infected and stimulated Dev cells. The transcriptional activity of MMP-3 and MMP-9 correlated with their secretion. Intriguingly, the expression of MMP-2 mRNAs was increased but did not correlate with the decreased activity of the enzyme as seen by zymography.

Globally, increased/induced levels of MMPs were detected at the translational and transcriptional levels in both stimulated-infected and stimulated Dev cells, suggesting that contact with activated T lymphocytes (cell-cell adhesion and soluble factors) modified MMP expression in astrocytes. The higher MMP levels seen in stimulated-infected cells suggest that persistent viral infection in a few astrocytes had an additional bystander effect.

The levels of endogenous MMP inhibitors are modulated concomitantly with those of their ligands

The MMPs form heterocomplexes with the TIMPs, which inhibits their enzyme activity (31). The balance between MMPs and TIMPs determines the net proteolytic activity and thus the integrity of the cells and tissues (32). TIMP expression was therefore studied in the same cell cultures tested for MMPs ($n = 4$). Examination at the transcriptional level using RT-PCR demonstrated mRNAs coding for TIMP-1, TIMP-2 to be constitutively expressed in Dev cells whereas TIMP-3 mRNA was undetectable (Fig. 2). Expression of TIMP-2 mRNA was unchanged in stimulated and stimulated-infected Dev cells. In contrast, expression of TIMP-3 mRNA was clearly induced, but quantification at the protein level was not possible since there is no TIMP-3 ELISA kit available to date. We detected a slight but consistent increase in TIMP-1 mRNA expression, which was similar to the increased secretion of TIMP-1 in stimulated and stimulated/infected Dev cells as detected by ELISA (49 and 113 ng/ml, respectively, on day 6 postcontact; control value of 39 ng/ml in untreated Dev cells). The levels of TIMP-1 and TIMP-3 mRNAs remained augmented up to day 9 after contact with activated T lymphocytes (viral producers or not), demonstrating that TIMP up-regulation in glial cells can persist for several days

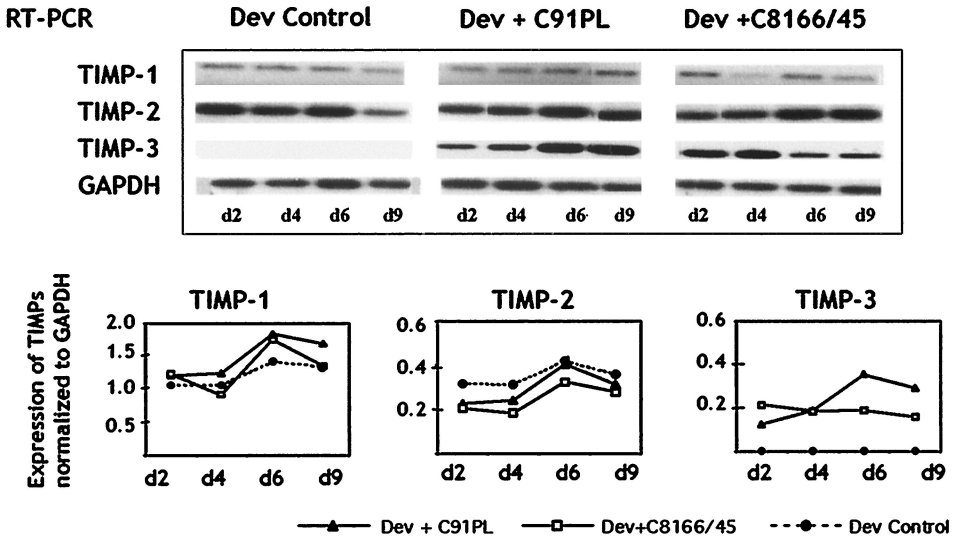

FIGURE 2. Modulation of expression of TIMPs in Dev cells by persistently infected/activated T lymphocytes. Dev cells were transiently exposed to infected T cells (C91PL or C8166-45) for 20 h. Southern blots of RT-PCR products are shown in the *upper panel*. In the *lower row*, graphs depicting the time course of TIMP transcript expression were realized using quantification procedure as described in Fig. 1, with respect to GAPDH mRNAs. The most prominent increase in mRNA expression was observed for TIMP-3. All four sets of experiments yielded reproducible effects.

following such contact. Taken together, these results indicate that transient contact with T lymphocytes that are activated by persistent viral infection dramatically modifies the balance between MMPs and TIMPs in human astrocytes.

Integrin-mediated lymphocyte-astrocyte adhesion is involved in the modulation of MMP expression in astrocytes

Cell-cell interactions occurring via integrin-mediated adhesion may trigger MMP gene expression (33). Thus, the involvement of T lymphocyte integrins in the changes in MMP expression seen in astrocytes was evaluated by blocking specific integrin subunits on the activated T lymphocytes before their contact with Dev cells. To avoid possible effect of viral infection on MMP expression, the non-virus-producing T cell line C8166-45 was chosen for this experiment. These T cells were treated with blocking Abs (2 to 10 μg/5 $\times$ 10^4 T cells, 1 h, 37°C) before coculture with Dev cells (20 h; two to three experiments for each Ab). These Abs are directed against the integrin subunits (α_1, α_3, α_5, and β_1) that we have preliminarily observed on the T cell surface by flow cytometry. Secretion of MMP-2 and MMP-9 was evaluated in Dev cell supernatants by zymography on day 3 postcontact. As shown in Fig. 3, treatment of T lymphocytes with irrelevant Abs (anti-endothelial marker VCAM-1) did not affect MMP-9 secretion induced by contact with activated T lymphocytes. In contrast, anti-integrin treatment clearly decreased the capacity of T lymphocytes to induce MMP-9 expression in Dev cells.

These observations indicate that an integrin-mediated interaction of activated T lymphocytes and astrocytes is involved in the alteration of glial MMP expression.

Soluble factors secreted by activated T lymphocytes are implicated in the MMP and TIMP changes in astrocytes

To evaluate the effect of factors secreted by activated T lymphocytes on MMP expression in Dev cells, conditioned medium from various T cell lines activated by chronic HTLV-I infection (C91PL, C8166-45, and H36) were tested for their ability to induce MMP-9 expression in astrocytes. One day after treatment, Dev cells were incubated in fresh medium for a further day; then, the Dev cell-conditioned medium was examined by zymography for MMP-9 secretion. As shown in Fig. 4, treatment with conditioned media from activated T cells always induced MMP-9 production in Dev cells whereas conditioned medium from nonactivated T lymphocytes (CEM) had no effect. We then determined whether cytokine secretion by activated lymphocytes was involved in the induction of MMP-9 in astrocytes by treating the T cell line C8166-45, which does not produce virus but secretes high levels of cytokines (Table I), with pyrolidine dithiocarbamate (PDTC, 1 nM), a molecule known to decrease cytokine expression (34) and shown by us to cause a 22–50% decrease in TNF-α and IL-6 secretion (ELISA detection, data not shown). The treated C8166-45 cells were then placed for 1 day above Dev cells in a culture chamber insert that prevented cell-cell contact and allowed only the passage of soluble factors (3-μm pore membrane). Then, the culture medium was changed (serum deprived), and MMP-9 secretion was examined in the Dev cell supernatant on day 2 by zymography. As shown in Fig. 4, the MMP-9 secretion induced in

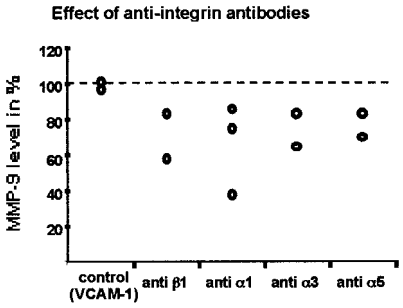

FIGURE 3. Effect of anti-integrin subunit-blocking Abs on MMP-9 induction in Dev cells. Treatment of T lymphocytes (C8166-45 T cell line) with anti-integrin subunit-blocking Abs (α_1, α_3, α_5, and β_1) before contact with human astrocytes decreased the magnitude of the effect of transient coculture with infected T lymphocytes (20 h). The control used Abs directed against endothelial marker (VCAM-1) and was without effect on MMP-9 secretion. MMP-9 was detected by zymography. Each point represents one experiment, and these values were normalized with those obtained in naive Dev cells.

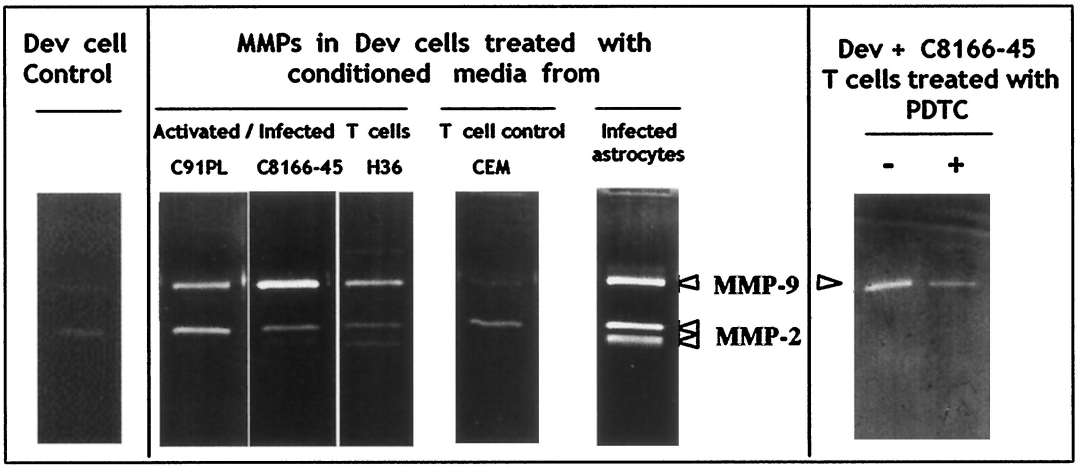

FIGURE 4. Effect of soluble factors secreted by activated T lymphocytes on the secretion of MMP-9 by human astrocytes. Zymographies were made on day 2 after treatment. These data were obtained from six sets of experiments. *Left panel*, Naive Dev cells do not secrete MMP-2 and -9. *Middle panel*, Dev cells were treated with conditioned medium from activated T lymphocytes (C91Pl, C8166-45, and H36), or from infected astrocytes. These supernatants enhanced the expression of MMP-9 and MMP-2 in Dev cells; the supernatant from control T cells (CEM) only slightly increased MMP-2 expression. Note that supernatant from infected astrocyte culture induced two prominent bands corresponding to proMMP-9 and active MMP-9 (cleavage of regulatory domain). *Right panel*, Pretreatment of T lymphocytes (C8166-45) with PDTC, which inhibits cytokine secretion, prevented the increased secretion of MMP-2 and -9 by astrocytes induced by C8166-45 cells.

Dev cells by soluble factors released from C8166-45 T lymphocytes was clearly decreased when T cells were treated with PDTC, suggesting that secreted cytokines were involved in MMP-9 production. Interestingly, treatment under the same conditions with medium conditioned by HTLV-I-infected astrocytes that contains proinflammatory cytokines (TNF-α, IL-1α, and IL-6 as reported in Ref. 19; see Table I) also resulted in the induction of MMP-9 in Dev cells (Fig. 4), indicating that cytokines secreted by infected astrocytes can amplify the effect of activated T lymphocytes.

The proinflammatory cytokines IL-1α and TNF-α mediate MMP and TIMP changes in astrocytes

Since the proinflammatory cytokines IL-1α and TNF-α were secreted by the activated T lymphocytes used in the present study (Table I), these two cytokines were candidates for the soluble factors regulating MMP and TIMP expression. Dev cells were therefore treated with TNF-α or IL-1α, and the antiinflammatory cytokine TGF-β (10 ng/ml on day 0), and the expression of MMPs and TIMPs was examined on days 2, 3, and 5 posttreatment. We observed similar results from all of the six experimental sets. MMP-2 and MMP-9 enzyme activity in cell supernatant was assessed by zymography, MMP-3 level was quantified by ELISA, and their mRNA levels by RT-PCR.

As shown in Fig. 5, treatment with the proinflammatory cytokines TNF-α and IL1α resulted in a progressive increase in MMP-9 secretion, whereas TGF-β treatment had no effect. Identical MMP-2 secretion was seen in control and cytokine-treated Dev cells. Further analysis using RT-PCR confirmed the results obtained by zymography, namely that TNF-α and IL-1α, but not TGF-β, induced expression of MMP-9 mRNA (Fig. 5). As already observed in infected glial cells (see *Results* above), TNF-α, IL-α, or TGF-β treatment increased MMP-2 mRNA levels, but not MMP-2 activity. TNF-α or IL-1α treatment also resulted in an increased MMP-3 mRNA expression (Fig. 5) and increased MMP-3 protein as detected by ELISA (15 and 20 ng/ml, respectively, vs 6 ng/ml in untreated Dev cells). In contrast, TGF-β decreased the constitutive expression of MMP-3 mRNA. Globally, the two proinflammatory cytokines tested, TNF-α and IL-1α, consistently increased MMP-9 and MMP-3 levels, whereas the antiinflammatory cytokine TGF-β did not. IL-1α was the most potent MMP inducer in Dev cells.

The expression of TIMP mRNAs was studied in the same cell samples using RT-PCR. As shown in Fig. 5, the expression of TIMP-3 mRNAs was clearly increased in Dev cells treated with TNF-α and IL-1α; the effect of TGF-β was weaker. Expression of TIMP-1 mRNA was also increased by treatment with cytokines, TGF-β being the most potent. TIMP-2 mRNA expression was not affected by TNF-α or IL-1α but was clearly reduced by TGF-β. Taken together, these results indicate that proinflammatory and antiinflammatory cytokines secreted by persistently infected T lymphocytes differentially modulate the MMP/TIMP network of astrocytes.

Release of extracellular matrix component by glial cells after contact with activated T lymphocytes

Our next aim was to examine the functional outcome of the overexpression of MMPs on glial cells. Since MMP-9 is able to cleave extracellular matrix (ECM) components, culture supernatants from stimulated-infected Dev cells or Dev cells treated with conditioned medium from infected Dev cells (24-h treatment, 4-day cultures) were screened for ECM cleavage products. Western blotting was used to detect fibronectin, a molecule previously detected on the surface of Dev cells. As shown in Fig. 6, treatment with conditioned medium or contact with T lymphocytes activated by persistent viral infection resulted in the release from Dev cells of a 230-kDa protein corresponding to fibronectin, as already reported (35). This observation suggests that remodeling of the glial cell ECM occurs in presence of MMP-rich medium.

MMP-9 is detected in neural cells within inflamed CNS areas in the brain of neurological patients infected by HTLV-I

On the assumption that inflammation within the CNS may promote its expression, we then examined whether MMP-9 was present within the CNS of TSP/HAM patients, in areas containing infiltrated lymphocytes. Immunocytochemistry was performed on postmortem CNS specimens from TSP/HAM patients using specific Abs directed against MMP-9, CNS tissue from non-HTLV-I-infected patient being used as a control. As shown in Fig. 7,

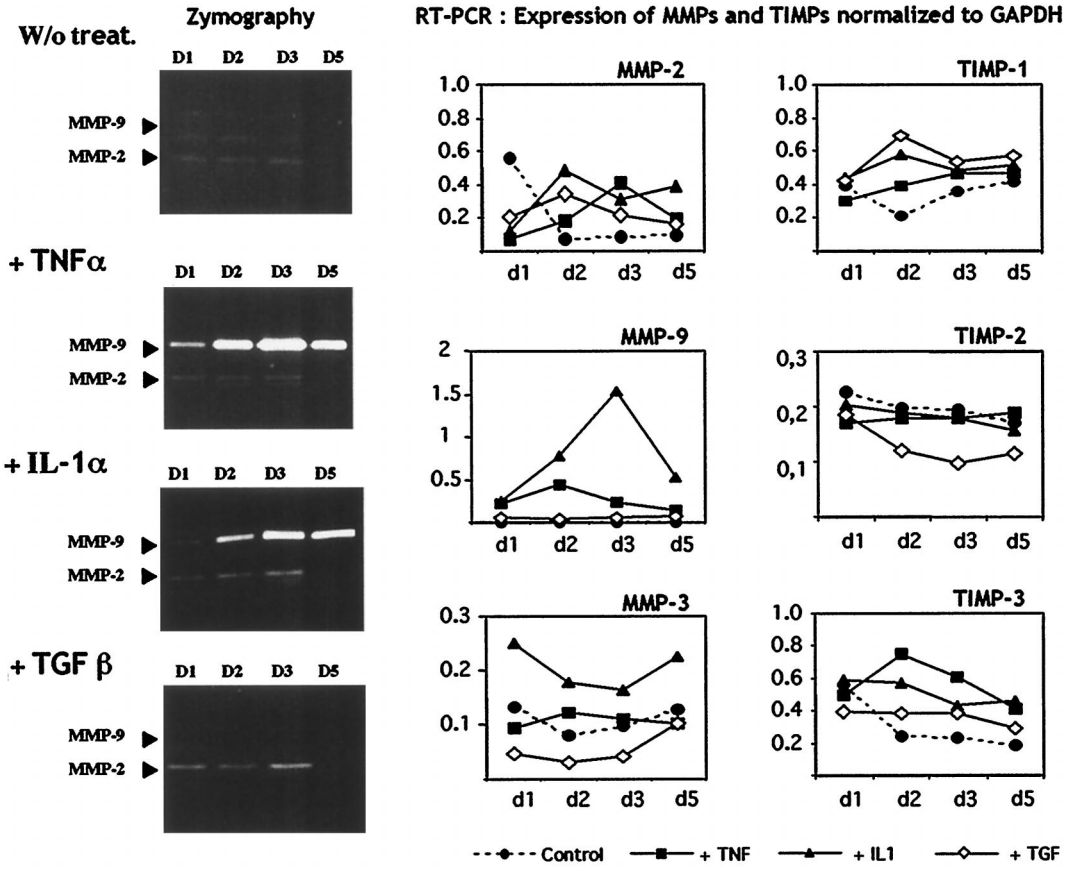

FIGURE 5. Effects of cytokines in the modulation of MMP and TIMP expressions in human astrocytes. *Left panel,* MMP-9 is induced in Dev cells by TNF-α and IL-1α but not by TGF-β (10 ng/ml). Zymographies were realized with cell supernatants collected from day 1 to 5 after treatment. *Right panel,* Modulation of MMPs and TIMPs encoding mRNAs by cytokines. The effects of cytokines on MMP/TIMP transcripts were analyzed in cell lysates by RT-PCR, and subsequent quantification of target sequences was realized using ^{32}P-labeled specific internal probes. MMP/TIMP mRNAs values were normalized with respect to GAPDH transcripts.

MMP-9 was not detectable in the control brain specimen, but was detected in the perivascular space, both in the vascular wall and in CNS cells of TSP/HAM brain tissues that showed infiltration by mononuclear cells. MMP-9-positive cells were localized in areas containing reactive astrocytes (strong GFAP labeling), and comparison with control specimen indicated that the number of reactive astrocytes was clearly increased in TSP/HAM tissues. These observations indicate that, in HTLV-I-infected neurological patients, MMP-9 expression in inflamed brain areas is associated with the presence of infiltrating immune cells and gliosis (astrocytosis).

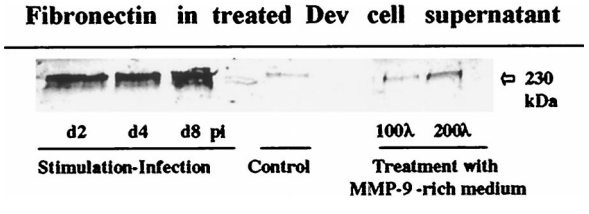

FIGURE 6. Release of the cleaved extracellular component fibronectin from Dev cells. Fibronectin was assayed in culture supernatants using Western blots (20 μl/lane). The three left lanes show a sustained release of fibronectin from Dev cell culture transiently exposed to C91PL T lymphocytes (stimulated/infected Dev cells). The *right* two lanes illustrate a dose-dependent increase in fibronectin after treatment with MMP-9-rich conditioned medium from infected Dev cells (100 and 200 μl/ml). The level of fibronectin in control Dev cell culture supernatant was barely detectable.

Discussion

MMPs are implicated in ECM degradation (36) and the maturation of a variety of cell surface molecules (32). MMPs are expressed by neural cells (30, 37) and are involved in the modeling and operation of the CNS (38, 39). MMP activity is controlled by their endogenous inhibitors TIMPs (31), and the balance between MMPs and TIMPs determines the net proteolytic activity, which constitutes an indication of tissue integrity (40). The central question of the present study was how T lymphocytes activated by persistent viral infection can influence the protease/antiprotease balance in the CNS. Using an in vitro model mimicking the T cell/astrocyte interaction, we demonstrate that T lymphocytes activated by persistent HTLV-I infection perturb the MMP/TIMP network, i.e., enhanced secretion of MMP-3 and active MMP-9 from astrocytes, together with ECM degradation. Such MMP production, which is initiated by the virally activated T cells and amplified in astrocytes, may constitute a peculiar pathway promoting CNS damage in TSP/HAM.

The crucial role of MMPs in inflammation (41) indicates that perturbation of MMP/TIMP axis may be decisive in the pathogenesis of TSP/HAM, since the alteration of the MMP/TIMP balance in TSP/HAM patients is associated with CNS inflammation. Indeed, MMP-9 was expressed in neural cells in areas exhibiting astrocytosis, whereas in the CSF, the presence of MMP-9 and TIMP-3 at substantial levels was associated with high levels of neopterin, an intrathecal marker of inflammation (present study

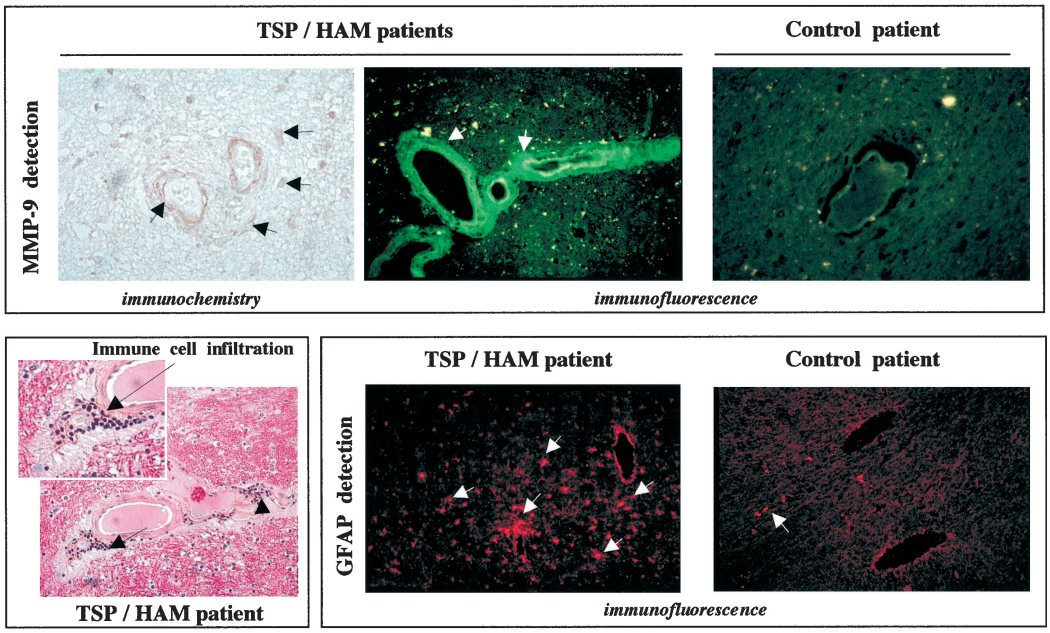

FIGURE 7. Presence of MMP-9 in inflamed CNS tissue of two HTLV-I-infected patients with TSP/HAM. *Upper row,* MMP-9 immunostaining using rabbit anti-MMP-9 Abs raised against human sequence. *Upper left* (×400), In a TSP/HAM patient, MMP-9 immunoreactivity (brown staining) is found in the wall of blood vessels and in some neighboring cells; the immunoreactivity was revealed with DAB/peroxidase technique. *Upper middle* (×400), Vessel walls in another TSP/HAM patient are strongly labeled by MMP-9 immunofluorescence (fluorescein); note that yellow punctates are lipofuscin pigments (lipofescin is endogenous pigment naturally expressed in human brain). *Upper right* (×400), There was no MMP-9 staining in control section obtained from a noninflammatory neurological patient. *Lower left* (×400) and *inset* (×600), This micrography (hematoxylin-phloxin-safran staining) illustrates CNS invasion by numerous immune cells (stained in gray) in the vicinity of blood vessels of the TSP/HAM patient examined for MMP-9 immunoreactivity in the *upper left* picture; note that a similarly conspicuous infiltration of immune cells was observed in the other TSP/HAM patient. *Lower middle* and *right* (×200), Many reactive astrocytes, as evidenced by the typical morphology of GFAP-positive cells, are present in nearby vessels only in the tissue of the TSP/HAM patient but not in the control noninflammatory neurological patient; note that this TSP/HAM patient was also positive for MMP-9 immunoreactivity (*upper middle*).

and Ref. 62). In fact, MMPs secreted by activated T lymphocytes (42) confer on these cells the ability to migrate through the vascular basement membrane, hence to infiltrate the CNS (43). Our data strongly suggest that these invading T cells induce MMP expression in astrocytes of inflamed CNS, as shown in other tissues (44, 45). Such maintenance and amplification of MMP production in astrocytes is also consistent with the overexpression of MMPs described in other immune-mediated demyelinating diseases, including MS (46). In addition to the possible pathological role of MMPs/TIMPs, their increase in the CSF of TSP/HAM patients may result from a remodeling of damaged CNS, as proposed for MS (13).

Overall, our present data strongly indicate that mechanisms underlying the modulation of MMP and TIMP expression in astrocytes involve signals from persistently activated/infected T lymphocytes, in particular integrin-mediated transient adhesion and inflammatory cytokines, as shown in other tissues (33, 41, 47). The T cell integrin subunits involved in MMP-9 induction in astrocytes are those shown to be increased in HTLV-I-infected lymphocytes. Such overexpression presumably confers on these T cells an increased adhesiveness (48), and underscores the importance of cell-to-cell contact. On the other hand, we show that diffusible substances such as cytokines secreted by activated/infected T lymphocytes modulate MMP/TIMP expression in astrocytes. The clinical relevance of such cytokines expressed within the CNS is supported by the fact that the profile of cytokine expression reported in TSP/HAM patients (16) is similar to those obtained with the infected T lymphocytes used in our model as well as with astrocytes stimulated by these T-lymphocytes: both cells produce proinflammatory (TNF-α, TNF-β, IL-1α), but not antiinflammatory (IL-4, IL-5) cytokines (19). These observations are also consistent with the specific effects of individual cytokines on MMP/TIMP expression in astrocytes. Our unpublished data on IFN-γ, IL-4, IL-10, and IFN-β show that proinflammatory cytokines up-regulate MMP-3, MMP-9, and TIMP-3 expression whereas the antiinflammatory cytokines up-regulate TIMP-1 expression. In the present study, TGF-β, considered as a immunosuppressive cytokine, had no effect on MMP-9 expression, decreased the expression of MMP-3 and TIMP-2, but increased that of TIMP-1. Taken together, these results suggest a link between Th1/Th2 and MMP/TIMP balances; proinflammatory cytokines would induce MMP overexpression, but with little counteraction by TIMPs.

This relationship between a Th1-dominated response and MMP overexpression in the CNS is seen in a variety of immune-mediated demyelinating diseases, including MS and its animal models. In MS, MMP-9 is strongly expressed in reactive astrocytes and macrophages within demyelinating lesions (13), whereas MMP-9 and TIMP-3 are found in CSF (49). In animals developing acute experimental allergic encephalitis (EAE), MMP expression is increased in the CNS (50). In addition, differential expression of MMPs and TIMPs in neighboring cells of the CNS of experimental allergic encephalitis developing mice suggest an interplay between these molecules, which presumably determines the outcome of the inflammatory process (50).

The ability of activated astrocytes to secrete TIMPs upon stimulation by T lymphocytes suggests that TIMPs may control the activity of the concomitantly up-regulated MMPs. However, in our model of astrocyte activation by T lymphocytes, increased levels of TIMP-1 and TIMP-3 did not inhibit protease activity, as demonstrated by fibronectin cleavage by stimulated-infected astrocytes

or astrocytes treated with MMP-9-rich medium. This could be due to the concomitant induction of MMP-3, involved in the activation of pro-MMP-9 (51). In addition, TIMPs not only function as MMP inhibitors, but are also true growth and differentiation factors, regulating cell proliferation and death (31, 52). The fact that TIMP-3 overexpression induces programmed cell death (53) provides new clues as to the potential relevance of TIMPs in neurological disease.

Clinically, the changes in MMP/TIMP balance seem highly relevant in immune-mediated neurologic diseases involving activated T lymphocytes. The permeability of the BBB may be prominently increased by the changes in the basement membrane of CNS blood vessels via astrocytes expressing MMP-9 and their feet directly in contact with these vessels. Such augmented permeability of the BBB presumably leads to the amplification of CNS inflammation via extravasation of inflammatory cells regardless of their specificity, as seen in TSP/HAM (54) and MS (55) patients. In MS, the demyelination process has been proposed to be the consequence of immune cell infiltration in white matter (56), and part of the deleterious effect of invading cells may involve a myelin proteolysis by MMPs (57) and a subsequent alteration of oligodendrocytes forming myelin. On the other hand, by generating encephalitogen peptides and neoepitopes (58, 59), MMP-9 and MMP-3 may be involved in the immune-mediated destruction of oligodendrocytes, in particular by the so-called "epitope spreading" mechanism (60). Finally, MMPs and TIMPs may play a critical role in the intricate cellular interaction within the CNS (40) by remodeling the ECM, which constitutes the interface between neural cells (38, 61).

In conclusion, our data emphasize the importance of the MMP-TIMP axis in the complex interactions between the CNS and invading immune cells that determine the degree of tissue damage and the possibility of repair in the CNS of patients with virally mediated T cell activation.

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