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Matrix Metalloproteinases in the Normal Human Central Nervous System, Microglial Nodules, and Multiple Sclerosis Lesions

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Abstract. Matrix metalloproteinases (MMPs) comprise a family of proteolytic enzymes that are involved in remodeling of the extracellular matrix (ECM) of many tissues. They have been implicated in degradation of vascular basement membranes thereby facilitating leukocyte migration into inflammatory sites. To determine the cellular localization and levels of MMPs in the normal human central nervous system (CNS), multiple sclerosis (MS) lesions, and other conditions, cryostat sections of CNS samples were immunostained with antisera to MMP-1, -2, -3 and -9. In control white matter the principal cells that express the MMPs were perivascular and parenchymal microglia. Cellular MMP expression was also found in sporadic microglial nodules in MS white matter. Most CNS microvessel endothelial cells expressed MMP-3 and -9 but not MMP-1 or -2. The majority of macrophages in active MS and necrotic lesions were MMP-1-, -2-, -3-, and -9-positive whereas chronic MS lesions had fewer MMP-positive macrophages. Small numbers of astrocytes were MMP-2-, -3- and -9-positive in acute and chronic MS lesions. These data suggest that microglia-derived MMPs may mediate turnover of the CNS ECM under normal conditions and in microglial nodules. In sites of CNS tissue injury there is complex and dynamic regulation of MMP expression by different cell populations. In MS lesions MMP-mediated proteolysis may contribute to breakdown of the blood-brain barrier and leukocyte migration into the CNS, in situ immune activation, demyelination, metabolism of bioactive peptides, and the formation of an ECM that does not promote remyelination or axonal repair.

Key Words: Astrocytes; Endothelial cells; Extracellular matrix; Macrophages; Matrix metalloproteinases; Microglia; Multiple sclerosis.

INTRODUCTION

Matrix metalloproteinases (MMPs) are proteolytic enzymes responsible for maintaining the integrity of the extracellular matrix (ECM) and for remodeling of the ECM in embryogenesis and in many pathological processes (1, 2). The MMPs have been divided into 3 broad families based on their domain structure and their substrate specificities. Interstitial collagenase (MMP-1) and neutrophil collagenase (MMP-8) belong to the collagenase family that have as their major substrates the fibrillar collagens types I, II, and III. Members of the stromelysin family include MMP-3 (stromelysin, transin) and MMP-7 (matrilysin) and act on a wide range of substrates including proteoglycans, laminin, fibronectin, gelatin, and procollagen precursor peptides. The enzymes MMP-2 (72kDa gelatinase; type IV collagenase A) and MMP-9 (92kDa gelatinase; type IV collagenase B) are members of the gelatinase family, the substrates for which include types IV and V collagen, fibronectin, proteoglycans and gelatin.

The synthesis and proteolytic activities of MMPs are tightly regulated by several mechanisms. Cellular MMP gene expression and activation may be influenced by pleiotropic cytokines (3–7), viral infection (8, 9) and through intracellular signaling pathways triggered by β-1 (VLA) and β-3 integrin-mediated recognition of ECM molecules (10–12). The MMPs are secreted in proenzyme forms that are activated in the extracellular space by other enzymes including plasmin (2) and mast cell-derived proteinases (13). The activities of MMPs are also counterbalanced by endogenous tissue inhibitors of metalloproteinases (TIMP) (2, 14). Leukocyte- and endothelial cell-derived MMPs have been implicated in a proteolytic cascade that degrades microvessel wall components, e.g. type IV collagen and laminin, and thereby facilitates leukocyte migration into inflammatory sites (3–7, 15). As understanding of the importance of the maintenance of the normal ECM of many tissues and of alterations in ECM occurring in pathological processes has increased, the potential for therapeutic modulation of MMPs in a wide range of diseases has been recognized (16, 17).

In normal central nervous system (CNS) tissues the collagens, fibronectin, and laminin are generally restricted to vascular and connective tissue stromal elements (18–21) whereas the neuropil ECM contains the glycosaminoglycan hyaluronic acid and proteoglycans (22, 23). The turnover and metabolic activities occurring in the CNS ECM are, however, poorly understood. Recognition of the potential contribution of CNS extracellular fluid drainage pathways to the generation of humoral and cellular immunity (24) suggests that MMP-mediated proteolytic activities in the CNS ECM may be important both in immune surveillance under normal conditions as well as in pathological processes within the CNS.

In the CNS MMPs have been implicated in the pathogenesis of neoplastic, degenerative and inflammatory/demyelinating diseases (25–28). The lesions of both mul-

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tiple sclerosis (MS) and the animal model of MS experimental allergic encephalomyelitis (EAE) contain activated T-cells that may both synthesize MMPs and via secreted cytokines induce their production by other inflammatory and CNS resident cells (29–31). In CNS inflammatory lesions, breakdown of the blood-brain barrier results in leakage into the CNS ECM of plasma proteins such as fibronectin (19, 20) which through integrin-mediated recognition may influence MMP activities and be degraded by them. Furthermore, macrophage-derived neutral and other proteases including the MMPs have long been known to participate in CNS demyelination (32–36) and their inhibition has resulted in suppression of EAE (37–39). Thus, there is considerable evidence suggesting involvement of the MMPs in the pathogenesis of both the inflammatory and demyelinating components of MS lesions.

The specific cell sources of the MMPs in the normal human CNS and in MS lesions are incompletely defined. To understand the potential contributions of members of the different MMP families to CNS ECM homeostasis and to the pathobiology of MS lesions, we determined the cellular localization and assessed relative levels of expression in different cell populations of MMP-1, -2, -3, and -9 using immunohistochemistry in the normal human CNS, in various stages of MS lesions, and in other neurologic disease control samples. Our findings suggest new potential roles for microglia in the metabolism of the ECM under physiologic conditions and indicate widespread, potentially critical involvement of the MMPs derived from several cell sources in active MS lesions and other conditions with activated microglia and phagocytic activity.

TABLE 1
Case Material

Diagnoses	Number of cases	Number of samples studied
Demyelinating diseases		
Acute multiple sclerosis (MS)*	1	9
Chronic MS#	16	72
Other neurological diseases		
Acute herpes simplex encephalitis§	2	2
Necrotizing myelopathy¶	1	5
Recent cerebral cortical infarcts†	2	2
Normal CNS**	12	18

* 30-year-old male, disease duration 11 months.

In cases for which information is available, there were 8 females, 7 males; mean age = 43.5 years (range = 24 to 78 years); clinical disease duration mean = 15 years (range = 7 to 37 years); postmortem intervals mean = 11.6 hours (h) (range = 2.3 to 72 h).

§ 53-year-old female, postmortem interval 5.5 h; 22-year-old male, postmortem interval not known.

¶ 61-year-old male with chronic MS and additional acute necrotizing lesion of uncertain etiology in the thoracic spinal cord.

† 70-year-old male, postmortem interval 11.5 h; 74-year-old male, postmortem interval 18 h.

** Neuropathologically normal samples were from cerebral hemispheres (N = 8), basal ganglia (N = 1), brain stem (N = 4), cerebellum (N = 3), and spinal cord (N = 2). There were 6 females and 6 males; mean age = 61.7 years (range = 6 months to 84 years); mean post-mortem interval = 10.0 h (range = 3 to 20 h).

MATERIALS AND METHODS

Case Material

Samples of CNS tissues were obtained from autopsies performed at the Stanford University Medical Center, Stanford, CA, the Veterans Administration Medical Center, Palo Alto, CA, and Harvard Medical School–affiliated hospitals, Boston, MA (Table 1). Additional samples were obtained from MS brain banks at the University of Colorado, Denver, CO, the National Neurological Research Bank, Veterans Administration Medical Center, University of California, Los Angeles, CA, and the Rocky Mountain MS Tissue Bank, Englewood, CO. The samples had been frozen at the time of autopsy and were stored at −80°C in OCT Compound (Miles Laboratories, Naperville, IL).

Immunohistochemistry

Cryostat sections were stained using immunoperoxidase staining kits for rabbit, sheep, and mouse immunoglobulin (Ig) obtained from Vector Laboratories, Burlingame, CA as described (18). Non-crossreactive rabbit antibodies specific for MMP-1, MMP-2, MMP-3 and MMP-9 were obtained from Biogenesis, Inc, Sandown, NH. Sheep antibodies to MMP-2 and MMP-3 were obtained from Biodesign International, Kennebunk, ME. Rabbit anti-glial fibrillary acidic protein (GFAP) serum was a gift from L.F. Eng, PhD. The microglia/macrophage marker mouse anti-CD68 monoclonal antibody (EBM/11) (40) was obtained from Dako Corp, Carpinteria, CA. Primary antibodies were diluted in phosphate buffered saline (PBS), pH 7.3 as follows: rabbit anti-MMP-2 and -3, 1:50; anti-CD68 and anti-GFAP, 1:200; all others, 1:100.

In brief, six-μm-thick air dried sections were fixed in acetone, washed in PBS, and incubated sequentially in 10% normal serum, primary antibody, 0.03% H_2O_2 in PBS, biotinylated anti-Ig of the appropriate species, and avidin biotin horseradish peroxidase complex, with washes in PBS between each incubation step. Immunoperoxidase reaction product was visualized with 3-amino-9-ethyl carbazole (Aldrich Chemical Co, Milwaukee, WI) and fixed in formol-acetate. The sections were counterstained with hematoxylin. Samples of normal spleen were initially used as positive staining controls and for determinations of optimal staining dilutions for each antibody. Staining of normal and pathological tissues were done concurrently and there was uniform exposure of each batch to chromogen. Negative staining controls included the substitution of PBS or normal rabbit or sheep serum in the same concentrations as the primary antibodies. The majority of specimens used were from the cerebral hemispheres but there were no differences in staining patterns among different CNS anatomic levels. Regions of MS

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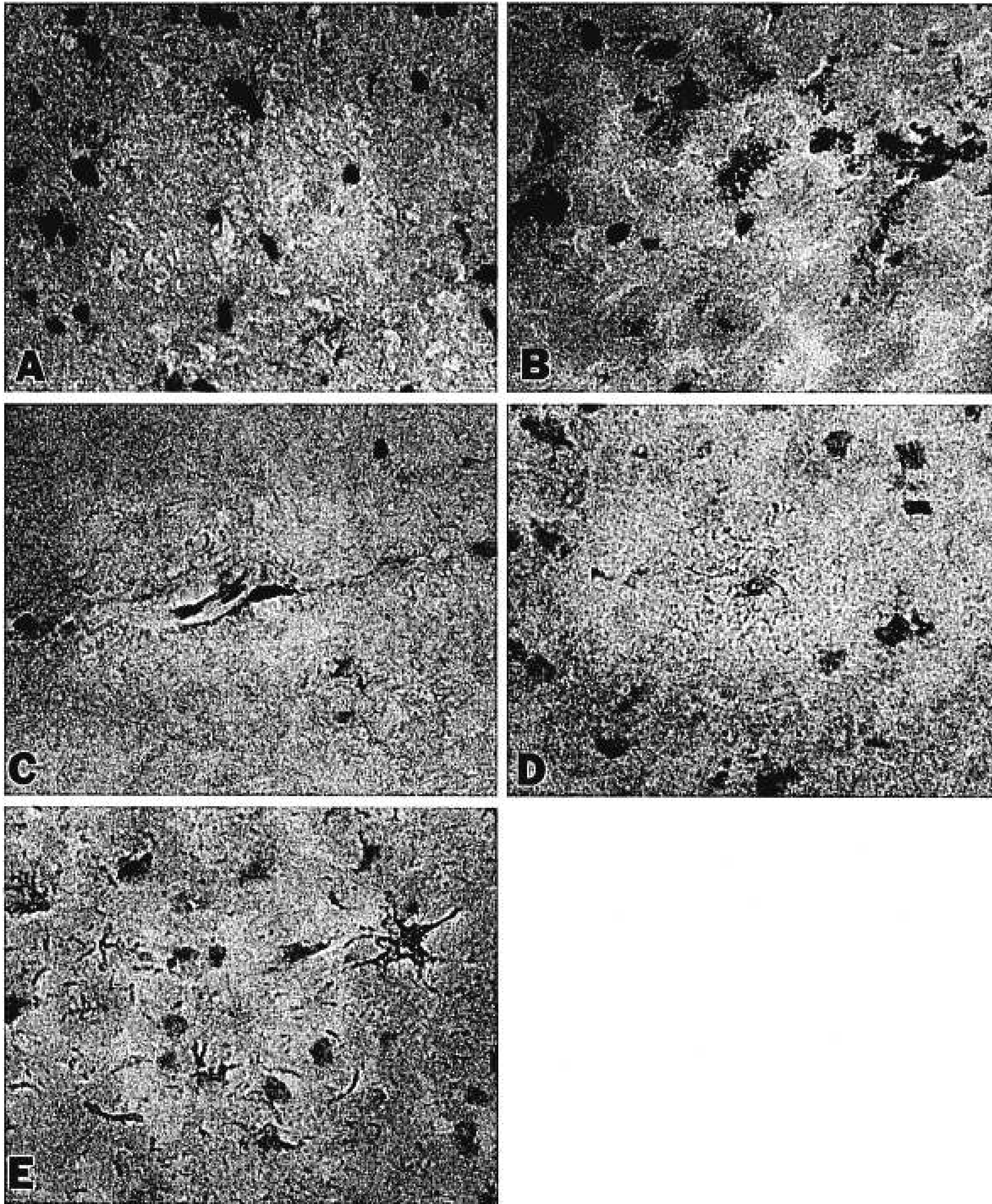

Fig. 1. MMPs in control and MS normal-appearing white matter. All are counterstained with hematoxylin. A. MMP-1. Scattered cells with elongated nuclei and delicate processes (ameboid microglia) in control patient subcortical white matter. Rabbit anti-MMP-1, 217×. B. MMP-2. "Ramified microglia" in control patient subcortical white matter. Rabbit anti-MMP-2, 217×. C. MMP-3. Elongated perivascular microglial cell in normal thoracic spinal cord. The capillary is not stained. Rabbit anti-MMP-3, 217×. D. MMP-9. Elaborate fine processes of a "ramified" microglial cell in the normal-appearing subcortical white matter of a patient with MS. Rabbit anti-MMP-9, 217×. E. GFAP. Astrocytes in an adjacent serial section to that shown in D. The astrocytes have more abundant perinuclear cytoplasm and fewer, longer, and more coarse processes than the microglia in A–D. Rabbit anti-GFAP, 217×.

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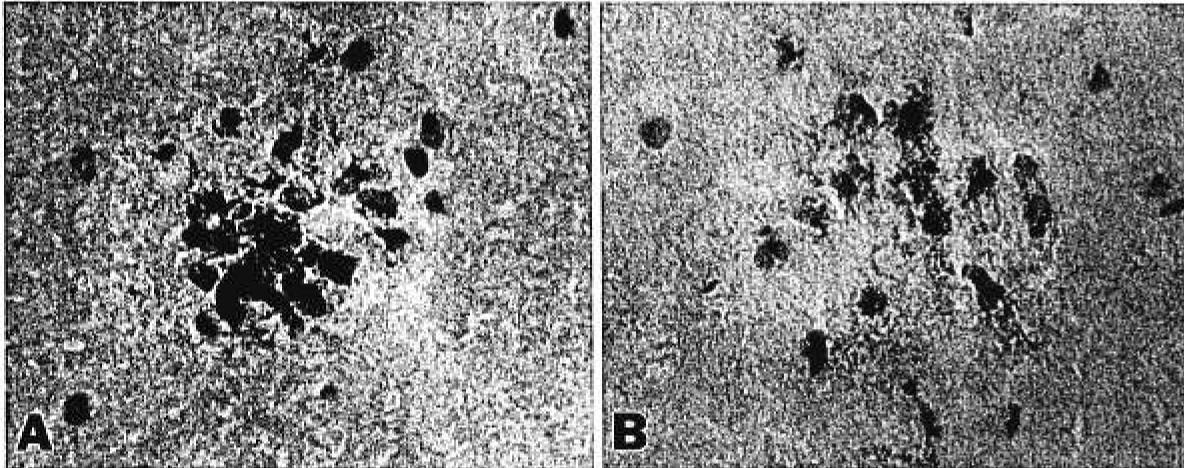

Fig. 2. Microglial nodules. MMPs associated with microglial nodules in the normal-appearing white matter of 2 patients with chronic MS. A. Pons. Rabbit anti-MMP-1, 217×. B. Occipital lobe white matter. Rabbit anti-MMP-9, 217×.

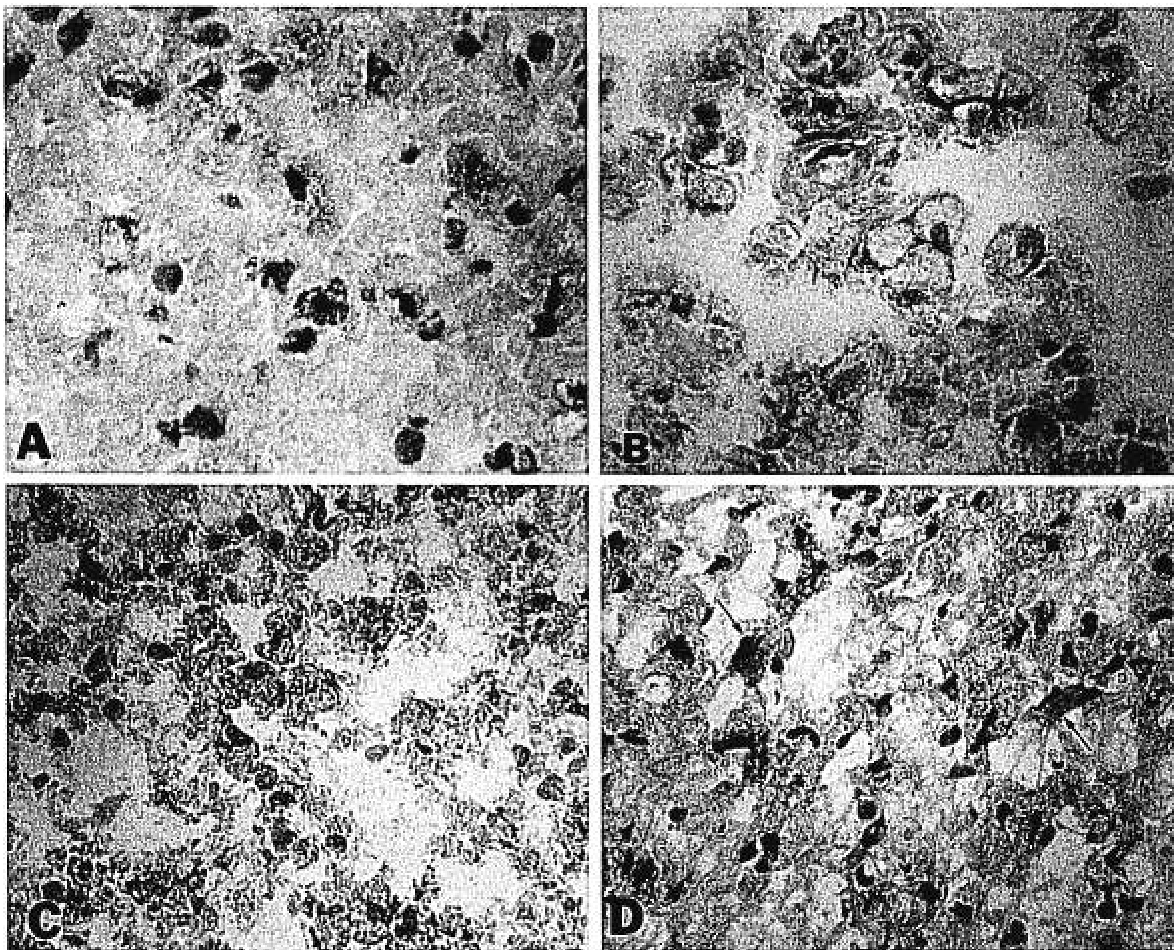

Fig. 3. Macrophages in active MS lesions. All are stained with rabbit antisera and hematoxylin. A. MMP-1. Scattered foamy macrophages near the edge of an active lesion. The neuropil in this field is intact. 217×. B. MMP-2. Foamy macrophages within an active lesion show staining of the peripheral portion of the cytoplasm. 217×. C. MMP-3. Sheets of stained foamy macrophages in an active lesion, 136×. D. MMP-9. Chronic active MS plaque. Macrophages are stained but astrocytes (arrows) in this field show less staining, 135×.

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tissue were characterized as having active demyelination by the use of Oil Red O staining and by routine analysis of adjacent serial sections. For evaluation of the extent of microvessel staining samples were scored as having all (>90%), some (10 to 90%) or rare (<10%) microvessels by both authors.

RESULTS

In sections of normal control white matter stained for the 4 MMPs, microglia were the most numerous immunoreactive cells and there were few differences in microglial staining patterns among the different MMPs (Fig. 1A–D). A range of microglial morphology, i.e. from small cells with elongated nuclei and minimal cytoplasm (Fig. 1A), cells with abundant ramified processes (Fig. 1B, D), and perivascular cells (Fig. 1C), was observed. The stained cells were generally smaller and had fewer large and more numerous shorter processes than the cells stained in adjacent sections for GFAP (Fig. 1E). Cells exhibiting the different morphologic patterns were distributed unevenly throughout the white matter. Normal cerebral cortical and other gray matter areas also had apparently randomly distributed MMP-positive microglia. Occasional MMP-positive neurons were also seen in gray matter, particularly in necrotic foci.

In the normal-appearing white matter (NAWM) of patients with MS, the distribution, numbers, and appearances of MMP-positive microglia were generally similar to those in the controls. In some MS NAWM samples, however, there were fewer stained microglia than in the controls. Adjacent to the borders of active MS plaques there were greater numbers of MMP-positive cells with more abundant cytoplasm, i.e. tending to a macrophage morphology. In the NAWM of 2 of the chronic MS cases there were scattered microglial nodules in which most of the cells were strongly MMP-positive (Fig. 2A, B). Similar distributions and morphological appearances of the microglia were observed in control and MS NAWM samples stained for CD68.

Large numbers of MMP-positive macrophages were present in acute MS lesions and in the active edges of chronic plaques (Fig. 3A–D). In some inflammatory foci there was accentuation of staining in perivascular inflammatory cuffs. Specific mononuclear cell types in these foci could not be determined, but the positively stained cells likely included lymphocytes as well as foamy macrophages. Most macrophages had diffusely positive cytoplasmic staining, although those with abundant cytoplasmic lipid frequently appeared to have a more peripheral cytoplasmic or membrane localization of the immunoperoxidase reaction product (Fig. 3B). Within chronic active plaques there were smaller numbers of MMP-positive macrophages and even fewer in inactive plaques. Large numbers of MMP-positive macrophages were also present in the necrotic areas in the other neurologic disease control samples. There were no clear differences in the numbers of stained macrophages among the various MMP antibodies although staining intensities in sections in which the sheep anti-MMP antibodies had been used were generally less than those in which the rabbit anti-MMP antibodies had been used. Cells with similar macrophage staining patterns were present in adjacent serial sections of MS lesions stained for CD68.

Some MMP-positive gemistocytic astrocytes were seen in several MS lesions. Many of these were so-called Creutzfeldt astrocytes, i.e. cells with very large amounts of cytoplasm and multiple or fragmenting nuclei (31) in the acute MS case (Fig. 4A). MMP-positive astrocytes, particularly MMP-3- and MMP-9-positive astrocytes, were also present in active and inactive lesions (Fig. 4B, C). Many MMP-positive astrocytes surrounded corpora amylacea in periventricular chronic plaques (Fig. 4C, D). In all sections, however, MMP-positive astrocytes were a minority of the astrocytes in the lesions, i.e. in most active and chronic MS plaques astrocytes were MMP-negative (Fig. 3D).

In control and MS samples stained for MMP-1 and MMP-2, gray and white matter vessels were most frequently negative (Figs. 1C, 5A) whereas a greater proportion of microvessels were MMP-3-positive, and most white matter microvessels were MMP-9-positive (Fig. 5B; Table 2). These patterns of vessel staining were not detectably different among control, MS, and other neurologic disease samples. When there were sheets of immunoreactive macrophages in active MS lesions, however, positive vessels might not have been distinguished and the extent of vessel staining may have been underestimated.

DISCUSSION

Previous immunohistochemical localization studies of MMPs in paraffin sections of normal human postmortem CNS tissue samples have identified reactivity for MMP-9 on endothelial cells, vascular smooth muscle cells, cortical neurons, and ependymal cells, whereas immunoreactivity for neither MMP-1, -2 nor -3 was found (26, 41). Using different reagents on cryostat sections, we found perivascular, ameboid, and ramified microglia to be the principal parenchymal cells that express MMP-1, -2, -3 and -9 in normal human white matter. Immunohistochemistry does not, however, necessarily distinguish between MMP precursors and proteolytically processed active forms nor does it directly address extracellular MMP enzymatic activity. On the other hand our observations are consistent with the demonstration of MMP activity in cultured neonatal rat microglia (42). The abundance of MMP immunoreactivity we identified suggests the presence of a large, potentially rapidly mobilized pool of MMPs that might function at relatively low levels normally and could be rapidly activated to act on a wide range of substrates in pathologic conditions.

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TABLE 2
The MMP in CNS Microvessels

	Microvessels stained*			
	All (Percent)	Some (Percent)	Rare (Percent)	Total
MMP-1				
Control	0 (0%)	11 (64.7%)	6 (35.3%)	17
MS NAWM#	0 (0%)	4 (7.0%)	53 (93.0%)	57
MS active plaque	0 (0%)	0 (0%)	13 (100%)	13
MS old plaque	0 (0%)	0 (0%)	8 (100%)	8
MP-2				
Control	0 (0%)	1 (5.9%)	16 (94.1%)	17
MS NAWM	0 (0%)	2 (3.7%)	52 (96.3%)	54
MS active plaque	0 (0%)	0 (0%)	9 (100%)	9
MS old plaque	0 (0%)	0 (0%)	12 (100%)	12
MMP-3				
Control	6 (33%)	11 (61.1%)	1 (5.6%)	18
MS NAWM	9 (12.5%)	47 (65.3%)	16 (22.2%)	72
MS active plaque	1 (14.3%)	6 (85.7%)	0 (0%)	7
MS old plaque	0 (0%)	9 (64.2%)	5 (35.7%)	14
MMP-9				
Control	13 (72.2%)	5 (27.8%)	0 (0%)	18
MS NAWM	48 (67.6%)	23 (32.3%)	0 (0%)	71
MS active plaque	4 (36.6%)	7 (63.6%)	0 (0%)	11
MS old plaque	6 (46.1%)	7 (53.8%)	0 (0%)	13

* Samples were scored as having all (>90%), some (10%–90%), or rare (<10%) microvessels stained in sections stained with rabbit anti-MMP antisera. Data are number of samples with pattern (percent of samples analyzed in each region).
# MS normal-appearing white matter.

The spectrum of morphologic forms of MMP-positive microglia observed in control white matter samples was similar to the range of forms found using other markers for microglia in rodents and humans (43–48). Since alterations in the ECM protein substrates of MMPs affect microglial metabolism (49) and morphology (50), focal variations in the composition of the ECM may contribute to this heterogeneity. Conversely, in view of the known biological functions of the MMPs, variations in microglial morphology and degrees of MMP immunoreactivity suggest that microglia-derived MMPs may be involved in a dynamic and perhaps continuous process of remodeling of the normal CNS ECM. The data also suggest that the MMPs, particularly MMP-1 and -2, which appear to be more restricted to microglia than the other MMPs, might be useful for in situ identification of these cells in the normal CNS and for specific targeting of microglia with molecular probes in vivo.

Microglial nodules are found as a response to diverse types of CNS injury. Thus, the strongly MMP-positive cells observed in sporadic microglial nodules in the NAWM of two MS patients could be related to MS or they may be incidental and secondary to an unrelated process, such as an intercurrent or terminal infection. Increases in proteolytic enzymes have been described in MS NAWM (35), however, and the MMP-positive microglial nodules might therefore represent sites of increased MMP activity in isolated foci of demyelination or axonal injury. A precise correlation between MMP activities in microglial nodules and specific pathologic processes would require a more systematic analysis of conditions such as CNS viral infections (44) in which microglial nodules are more consistently found in large numbers and are more pathognomonic than they are in MS. Nevertheless, the present observation suggests an association of MMP activity and focal microglial "activation" in microglial nodules.

Perivascular mononuclear cell inflammatory cuffs in active MS lesions frequently showed diffuse MMP immunoreactivity, suggesting expression by leukocytes as well as endothelial cells. This observation is therefore consistent with studies implicating leukocyte-derived MMPs in microvascular basement-membrane degradation in cellular immune reactions in the CNS (27, 51). Near the edges and within active MS lesions, however, there was greater and more consistent MMP expression on virtually all parenchymal macrophages. The degree and patterns of MMP expression were similar to those of CD68, β-1 integrins, and MHC class II molecules on microglia and macrophages in MS lesions (40, 45–47). The MMP expression, like that of these cell surface antigens, is also likely induced by the pro-inflammatory cytokines known

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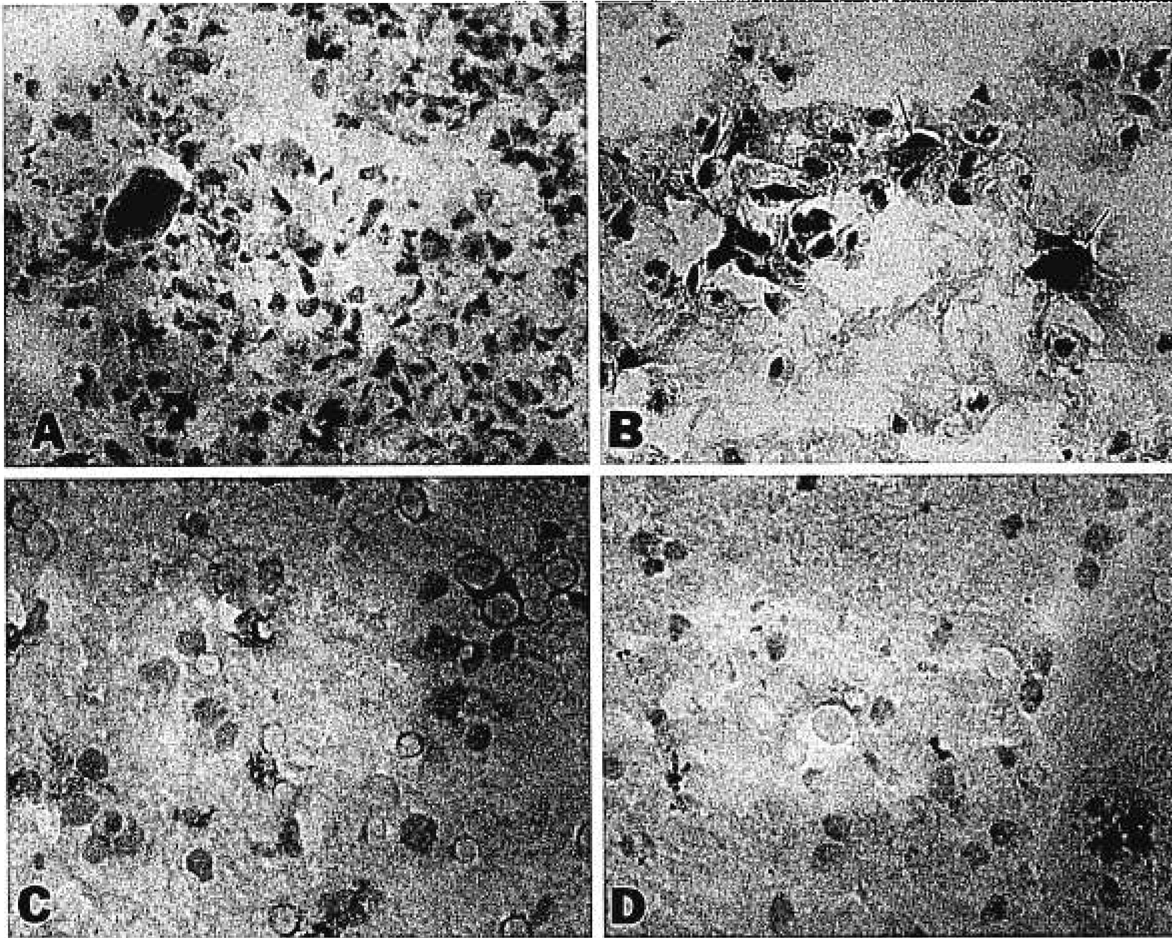

Fig. 4. Astrocytes in acute and chronic MS lesions. A. A large "Creutzfeldt" astrocyte with fragmented nuclei is strongly MMP-2-positive in an active lesion. Sheep anti-MMP-2, 136×. B. Gemistocytic astrocytes (arrows) in a chronic plaque are MMP-3-positive. Sheep anti-MMP-3, X136×. C. MMP-9-positive astrocytes surround corpora amylacea in a chronic periventricular MS plaque. Rabbit anti-MMP-9, 163×. D. Serial section to C stained for CD68. Fewer cells are positive and cells surrounding the corpora amylacea are unstained, indicating that they are not microglia/macrophages. Monoclonal antibody EBM/11, 163×.

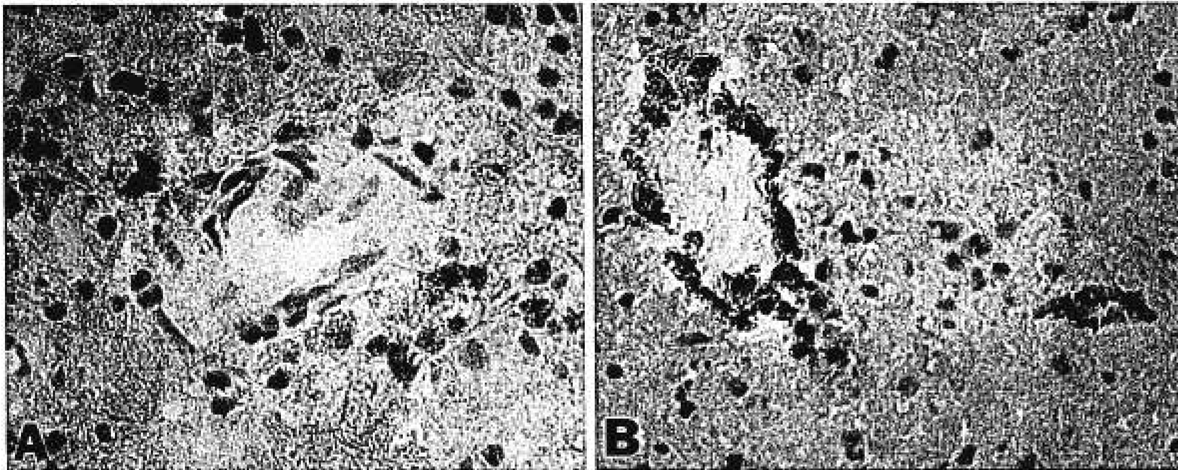

Fig. 5. A. MMP-2. Perivascular foamy macrophages are stained in this chronic active plaque but the vessel wall cells are not stained, 163×. B. MMP-9. Intense staining of microvessels in normal-appearing white matter of the medulla in a patient with MS. Rabbit anti-MMP-9, 136×.

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to be present in active lesions (52–54). Furthermore, the MMPs cleave and activate the cytokine TNF-α, which is believed to be of critical importance in MS lesion pathogenesis (55, 56). Thus, macrophage MMP expression not only correlates with expression of other markers of immunologic activation but MMPs likely also contribute directly to this activation.

Since MMP immunoreactivity was reduced in inactive lesions, MMP expression also correlates with localization of active demyelination. MMPs do mediate proteolysis of myelin components in vitro (57) and it is possible that they contribute to myelin degradation in vivo. Extracellular myelinolysis may result in the generation of multiple immunogenic and encephalitogenic epitopes of myelin components (57, 58) that have the potential to induce anti-myelin immune responses (59). Such responses could contribute to further white matter injury and prolongation of the disease. At present, however, it is unclear whether the widespread macrophage MMP expression observed in these lesions indicates actual extracellular digestion of myelin proteins, an enhanced turnover of the ECM, or both. Furthermore, it is not known whether or how these two apparently distinct processes may be interrelated in vivo. Since comparable patterns of MMP staining were seen on macrophages in herpes encephalitis and other necrotizing lesions, strong macrophage expression by MMPs is not MS-specific and appears to be a general response occurring in association with macrophage phagocytic activity in the CNS.

In contrast to macrophages, gemistocytic astrocytes were inconsistently MMP-positive in some MS lesions. Since many astrocytes in active lesions were MMP-negative and the distribution of MMP-positive astrocytes was irregular, it is possible that positive immunostaining reflects secondary acquisition, i.e. phagocytosis by the astrocytes of extracellular MMPs. On the other hand, reactive astrocytes are known to produce proteases and protease inhibitors (60), and fetal and neoplastic human astrocytes and cultured rat astrocytes do secrete MMPs (26, 29, 30). Furthermore, this secretion is regulated by cytokines, notably TNF-α; MMP-2 and -9 may also contribute to the activation of cultured rat astrocytes (30). Thus, astrocytes may also synthesize MMPs in both active and inactive MS lesions (41).

The numbers of MMP-immunoreactive cells did not return to normal levels in chronic MS plaques. Persistent alterations of the ECM that may impede repair are found in chronic MS plaques following damage to the blood-brain barrier (47, 61), and MMP-mediated alterations of the ECM in active lesions might similarly have long-lasting effects. For example, ECM proteoglycans affect oligodendrocyte proliferation and myelination in vitro (62) and, although analogous effects on oligodendrocytes have not been identified to date, MMP-3-cleaved fibronectin inhibits Schwann cell proliferation (63). Nerve growth factor-induced neurite outgrowth is MMP-2-dependent (64), and MMPs may also metabolize bioactive peptides (65). Thus, several lines of evidence suggest that as a consequence of both MMP activity in active MS lesions and probably decreased MMP activity in chronic lesions, an altered ECM in chronic lesions may not be conducive to oligodendrocyte proliferation, remyelination, neurite regeneration, or the restoration of neural function.

The known mechanisms of MMP gene regulation and modification of MMP activities as well as their substrates overlap among the MMPs and, with the exception of endothelial cell expression, there were generally similar immunostaining patterns among the representative MMPs of the 3 families studied. There are likely important differences, however, among the MMP substrates and in the differential regulation in response to cytokines of MMPs of mononuclear phagocytes (3, 6) and endothelial cells (4). For example, greater immunoreactivities for MMP-3 and -9 than for the other MMPs probably indicate a more significant role for endothelial cell-derived MMPs in blood cell trafficking in inflammation. More precise data on the regulation of expression and specific enzymatic activities of the individual MMPs in the human CNS under normal and pathological conditions are needed for a better understanding of the functional roles of MMPs in ECM metabolism, immune activation, phagocytosis, demyelination, and repair.

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