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Matrix Metalloproteinases in Neuroinflammation

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ABSTRACT Matrix metalloproteinases (MMPs) are a gene family of neutral proteases that are important in normal development, wound healing, and a wide variety of pathological processes, including the spread of metastatic cancer cells, arthritic destruction of joints, atherosclerosis, and neuroinflammation. In the central nervous system (CNS), MMPs have been shown to degrade components of the basal lamina, leading to disruption of the blood-brain barrier (BBB), and to contribute to the neuroinflammatory response in many neurological diseases. Brain cells express both constitutive and inducible MMPs in response to cellular stress. MMPs are tightly regulated to avoid unwanted proteolysis. Secreted as inactive enzymes, the MMPs require activation by other proteases and free radicals. The MMPs are part of a larger class of metalloproteinases (MPs), which includes the recently discovered ADAMs (a disintegrin and metalloproteinase domain) and ADAMTS (a disintegrin and metalloproteinase thrombospondin) families. MPs have complex roles at the cell surface and within the extracellular matrix. At the cell surface, they act as sheddases, releasing growth factors, death receptors, and death-inducing ligands, making them important in cell survival and death. Tissue inhibitors of metalloproteinases (TIMPs) are endogenous inhibitors that regulate the activity of the MMPs. Synthetic inhibitors have been developed for the treatment of arthritis and cancer. These hydroxymate-based compounds have been shown to reduce injury in experimental allergic encephalomyelitis (EAE), experimental allergic neuritis (EAN), cerebral ischemia, intracerebral hemorrhage, and viral and bacterial infections. MPs have both beneficial and detrimental roles; understanding their expression in various CNS insults will allow for the use of MMP inhibitors in the treatment of neurological disorders. *GLIA 39:279–291, 2002.*

INTRODUCTION

Proteases take apart cellular structures as part of the neuroinflammatory response in many neurological diseases. Neutral proteases, including cysteine, serine, and metalloproteinase (MP) gene families, have complex functions under normal and pathological conditions. Caspases facilitate cell death through DNA fragmentation. Serine proteases, including urokinase-type plasminogen activator and tissue-type plasminogen activator, have important roles in the vasculature and coagulation systems. MPs have multiple roles at the cell surface and in the extracellular matrix (ECM). The MPs include the matrix metalloproteinases (MMPs), the ADAMs (a disintegrin and metalloproteinase), and the ADAMTS (thrombospondin) families. During cellular stress, cell surface signaling activates kinases and forms immediate-early genes, followed by the induction of cytokines and chemokines that induce neutral proteases. The highly toxic MP molecules are tightly regulated at a series of steps, including transcription, translation, activation, and inhibition; they are secreted as latent enzymes, and activation is an impor-

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TABLE 1. Major Classes of the Matrix Metalloproteinases

Classes	MMP	Substrate
I. Interstitial collagenases	Collagenase-1 (MMP-1)	Collagen I, II, III, VII, VIII, X, aggrecan, serpins
II. Stromelysins	Stromelysin-1 (MMP-3)	Collagen IV, V, IX, X, fibronectin, elastin, laminin, aggrecan, nidogen, fibrillin, osteonectin
	Matrilysin (MMP-7)	Elastin, fibronectin, laminin, nidrogen, collagen IV, tenascin, versican
III. Gelatinases	Gelatinase A (72 kDa) (MMP-2)	Collagen I, II, V, VII, X, fibronectin, tenascin, fibrillin, osteonectin
	Gelatinase B (92 kDa) (MMP-9)	Collagen IV, V, VII, X, XIV, elastin, fibrillin, osteonectin
IV. Membrane-type MMP	MT1-MMP (MMP-14)	Collagen I, II, III, IV, laminin, aggrecan, tenascin, nidrogen, fibrillin, fibrin

tant regulatory step. Much has been learned recently about the role of the MPs in neurological diseases, particularly with regard to the MMPs. In the active form, these enzymes have a number of important roles in normal development, but they are highly destructive in inflammation of the central nervous system (CNS). They affect the function of the neurovascular structures by attacking the basal lamina around the cerebral vessels, resulting in hemorrhage and cerebral edema.

The MMPs have many roles in the CNS ranging from remodeling of tissues during development to a critical role in multiple phases of neuroinflammation. The intense study of these enzymes in the brain has provided a clearer, but still incomplete, picture of their multiple roles. The action of MMPs is balanced by that of the endogenous tissue inhibitors of metalloproteinases (TIMPs) and by α_2-macroglobulin. The balance between production, activation, and inhibition prevents excessive proteolysis or inhibition. When the proteolytic activity is greater than the inhibition by the TIMPs, ECM breakdown occurs. Conversely, when the inhibitors are excessively expressed, and proteolysis is restricted, there is a buildup of the ECM proteins with fibrosis. Several recent reviews have been written on the role of the MMPs in brain, including information on the ADAMs family (Romanic and Madri, 1994; Rosenberg, 1995; Yong et al., 1998, 2001; Mun-Bryce and Rosenberg, 1998b; Lukes et al., 1999). The focus of this review is on studies of the role of MPs in the neuroinflammation of brain ischemia.

INDUCTION AND ACTIVATION OF THE MMPs

MMPs fall into four major classes, depending on the components that make up the enzymes (Nagase and Woessner, 1999). They are divided into interstitial collagenases, stromelysins, gelatinases, and membrane-type metalloproteinases (MT-MMPs) based on protein structure (Table 1). The protein structure of the MMPs follows a basic pattern (Fig. 1A). At the N-terminus is the signal peptide and the propeptide segment that maintains the latency state of the enzyme. In the middle is a zinc-binding catalytic site controlled by the cysteine-rich propeptide segment. Removal of the signal peptide and propeptide domains activates the enzymes. Activation can also occur through a change in configuration. The catalytic domain is the minimal element, which contains fibronectin-binding sites and the zinc. Hemopexins are joined by a hinge region and are attached to a transmembrane domain. Collagenases and stromelysins are missing the furin-binding domain and the fibronectin inserts. Matrilysin, the smallest of the MMPs, is missing a hemopexin domain. Stromelysins attack multiple ECM molecules and activate other MMPs. Gelatinases, so named because they break down gelatin, lack the furin-binding region; they have fibronectin-binding regions that allow them to interact with macromolecules in the basal lamina. The transmembrane domain is found only in the MT-MMPs. In the inactive state, an unpaired cysteine sulfhydryl group near the C-terminal end binds with the zinc site. Proteolytic removal or reconfiguration of the propeptide region activates the enzymes. Exposure of the catalytic site is referred to as the "cysteine switch" (Van Wart and Birkedal Hansen, 1990). Gelatinase A (MMP-2) is a constitutively expressed molecule with a molecular weight of 72 kDa; it is normally present in brain tissue and in the cerebrospinal fluid (CSF). Gelatinase B (MMP-9), which has a molecular weight of 92 kDa and attacks similar substrates, is normally only present at low levels but is markedly upregulated in inflammation in many disease states.

A new family of MPs was recently discovered that appear to have important roles in brain function. The ADAMs gene family of proteases have both MP and disintegrin domains (Schlondorff and Blobel, 1999). The disintegrin region binds them to the membrane integrins, while the metalloproteinase domain provides the protease function. They are important in events at the cell surface because they attach to the integrins on the cell surface and carry out metalloproteinase functions. One of the first ADAMs to be described that had implications in brain function was the tumor necrosis factor-α-converting enzyme, or TACE (ADAM-17). TNF-α is bound to the membrane as an inactive 26-kDa form and is processed to an active 17-kDa entity by TACE. Hydroxymate-based metalloproteinase inhibitors blocked the processing of inactive TNF-α to the active form (McGeehan et al., 1994; Gearing et al., 1994). The growing ADAMs family includes enzymes that cleave a number of ECM molecules; they also act as "sheddases" in removing ectodomain molecules from the cell surface. Shedding of the TNF-α receptor, interleukin-6 (IL-6), L-selectin, and syndecans has been shown to be a function of ADAMs (Yong et al., 2001).

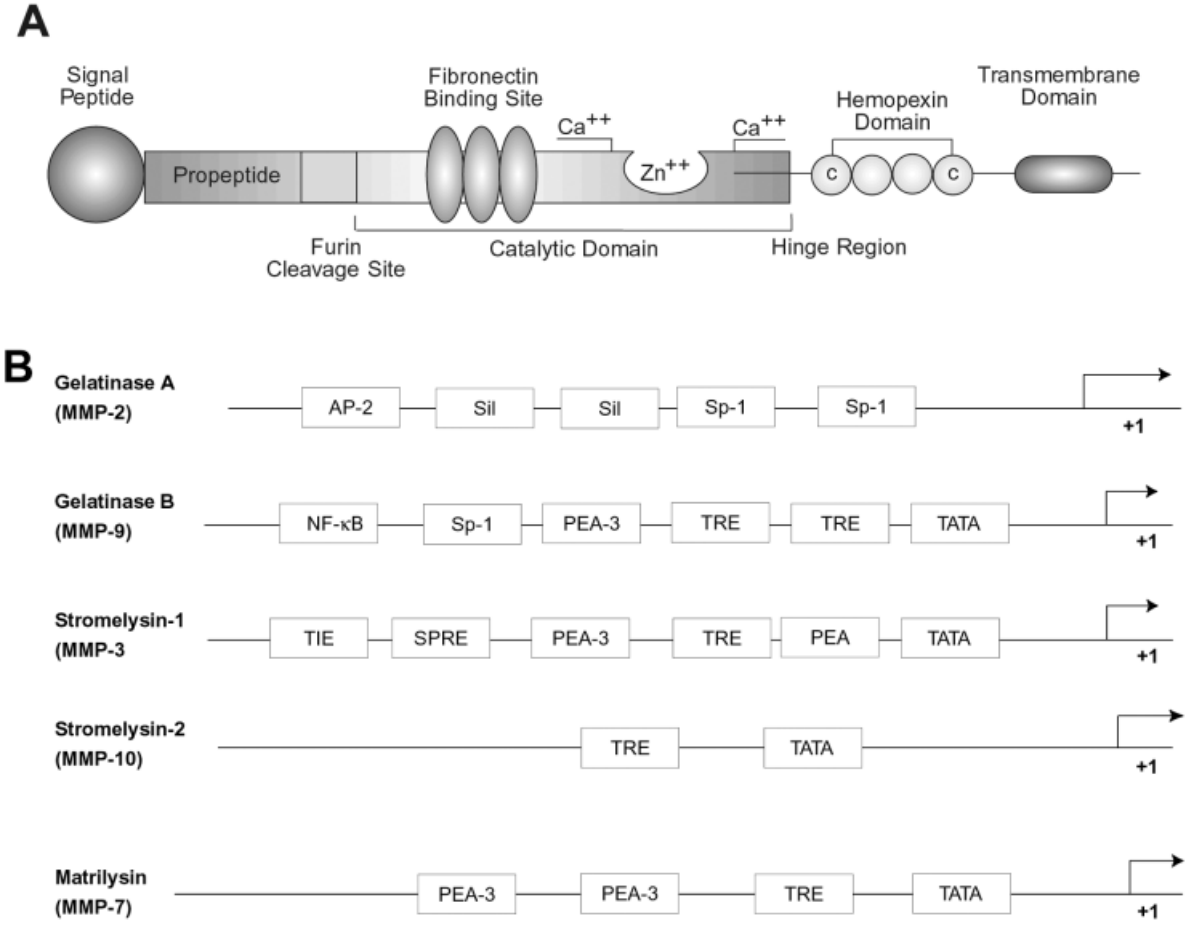

Fig. 1. **A:** Domain structure of the matrix metalloproteinases (MMPs) for a composite molecule. All the MMPs contain the signal peptide region that is attached to the propeptide region, as well as the catalytic domains with the zinc and calcium atoms. Furin and fibronectin binding sites are present in gelatinases and in the membrane-bound MMPs. A hemopexin domain is attached to the transmembrane domain in all of the MMPs except matrilysin (MMP-7). The four classes presented in Table 1 have various components of the composite molecule. **B:** Gene promoter regions of the main MMPs found in the brain. The constitutively produced MMP-2 has AP-2 and Sp-1 sites. The factors controlling the formation of MMP-2 are not well understood. In the inducible MMPs, the promoter regions contain AP-1, NF-κB, and PEA sites. These common sites are induced by the cytokines and immediate-early genes.

ADAMs with a thrombospondin domain (ADAMTS) forms another group of MPs. ADAMTS4 has been shown to degrade aggrecan, and to be involved in spinal cord injury (Lemons et al., 2001).

Mammalian gelatinase A was isolated from metastatic melanoma cells, where it cooperated with the plasminogen/plasmin system in the spread of metastatic cells (Liotta et al., 1980). Plasminogen/plasmin system enzymes interact with the MMPs (Cuzner et al., 1996; Cuzner and Opdenakker, 1999). Serine proteases and urokinase-type and tissue-type plasminogen activators (uPA and tPA, respectively) participate in the activation of the MMPs (Mazzieri et al., 1997; Carmeliet et al., 1997). They are involved in normal extracellular remodeling and angiogenesis and in the pathological processes associated with tumor growth (Mignatti and Rifkin, 1996).

Constitutive production of MMP-2 is controlled at the transcriptional level by activator protein-2 (AP-2) and Sp-1 sites in the promoter region of the gene (Fig. 1B). MT-MMPs are also constitutively produced in brain. Inflammatory processes lead to the induction of collagenase (MMP-1), stromelysin-1 (MMP-3), matrilysin (MMP-7), and MMP-9. Control of the inducible MMPs occurs at multiple sites, including activator protein-1 (AP-1), nuclear factor-κB (NF-κB), and PEA-3 (Ets-1) sites (Nelson et al., 2000) (Fig. 2). Immediate-early genes, free radicals, protein kinases, and cytokines control the sites in the promoter regions of the gene (Fig. 3). Several MMPs perform the same function, complicating analysis of MMP function in genetically manipulated animals (Rudolph-Owen et al., 1997).

An important feature of the MMPs is their latency. Secreted in a proform, they require activation by a variety of mechanisms before they can act. ProMMP-2 is activated by the membrane-bound MT-MMP (Sato et al., 1994). TIMP-2 binds with MT-MMP and proMMP-2 to facilitate activation of MMP-2. Low concentrations of TIMP-2 facilitate activation, while higher concentra-

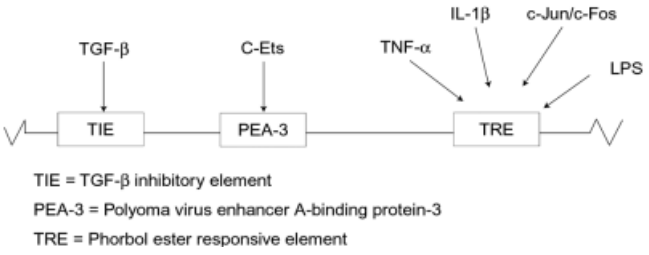

Fig. 2. Some of the factors leading to the induction of the matrix metalloproteinase (MMP) genes are shown. The genes containing the sites shown are given in Fig. 1. Transforming growth factor-β (TGF-β) stimulates the TIE site, C-Ets the PEA-3 site, and multiple cytokines and immediate-early genes the NF-κB and AP-1 sites.

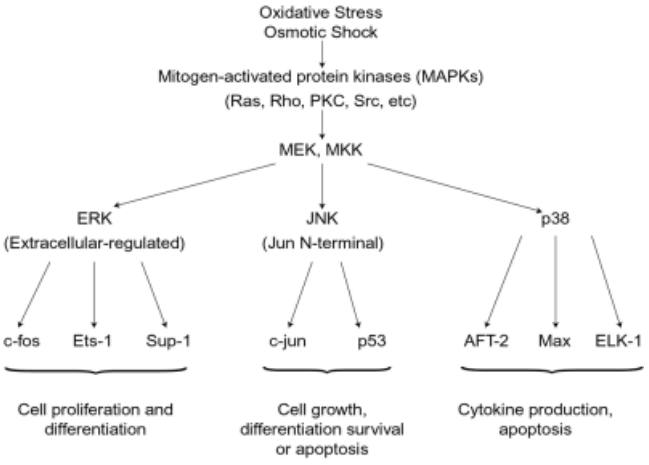

Fig. 3. Signaling pathways involved in the induction of the factors that stimulate the promoter regions of the matrix metalloproteinase (MMP) genes. These are triggered by a variety of stresses, including hypoxia, and act through the mitogen-activated protein kinases. Oxidative injury leads to the activation of the MAPKs that trigger to cascades of other kinases (MEK, MKK), and the ERK, JNK, and p38 systems. Further activation of the immediate-early genes, c-fos and c-jun, Ets-1, Sup-1, p53, AFT-2, Max and ELK-1 occurs. The balance of the complex signaling systems determines the cell proliferation, growth and death cycles.

tions inhibit the processing of proMMP-2 (Strongin et al., 1995). Plasmin activates proMMP-3 (Baricos et al., 1995; Nagase, 1997). ProMMP-9 is activated by MMP-3 (Ramos-DeSimone et al., 1999). The interlocking cascades of activation are important in maintaining the latency of the MMPs, and they provide a means for the MMPs to relate to the plasminogen/plasmin system. This interaction is important in many normal functions, such as angiogenesis. However, in pathological conditions, the interaction between the two systems is deleterious to the organism.

STUDIES OF MMPs IN BRAIN CELL CULTURES

Much has been learned about the function of the MMPs in brain by the use of cell cultures. In an early study of MMPs, rabbit cerebral endothelial cell cultures produced procollagenase (MMP-1) and prostromelysin (MMP-3) after stimulation of the cultures with 12-O-tetradecanoylphorbol-13-acetate (TPA) (Herron et al., 1986b). An important observation in that early study was the absence of enzymatic activity in the supernatant due to the simultaneous production of inhibitors (Herron et al., 1986a). Interestingly, the amounts of procollagenase and prostromelysin were similar to that produced by rabbit fibroblasts, suggesting a commonality of function with other cell types in the body. Recently, rat brain endothelial cells in culture that were stimulated with lipopolysaccharide (LPS) showed the induction of MMP-9, which could be inhibited by dexamethasone (Harkness et al., 2000).

Fetal astrocytes stimulated with phorbol esters produced two gelatinases that could be detected in the supernatants by zymography: one with a molecular weight of 92 kDa representing MMP-9, and the other with a molecular weight of 65 kDa, representing MMP-2 (Apodaca et al., 1990). Inhibitors to MMPs were again found in the conditioned medium, making the extent of activity uncertain. Gelatinases were the first substances to be studied extensively in the brain because of the ease of detection by gelatin-substrate zymography; gelatin, which is embedded in the electrophoretic gel, is dissolved by the gelatinases, leaving a white region in the Coomassie blue-stained gel. The amount of enzyme can be quantified by zymography or enzyme-linked immunosorbent assay (ELISA) (Kleiner and Stetler-Stevenson, 1994).

Enriched astrocyte cultures display normal secretion of MMP-2 and produce inactive proMMP-9 when stimulated with LPS, IL-1β, or TNF-α for 24 h (Gottschall and Yu, 1995). A fragment of amyloid, Aβ (1–40), induced the production of MMP-9 and of stromelysin-1 (MMP-3), suggesting that MMPs may be involved in the processing of amyloid in Alzheimer's disease (Deb and Gottschall, 1996). Astrocytes released MMP-1 when stimulated by IL-1β, and MMP-1 has been shown to be toxic to human neurons in culture (Vos et al., 2000).

Microglia are an important source of MMPs, as shown in cultures of microglia that were grown in a fibronectin–gelatin matrix with a fluorescent marker. Stimulation of MMP production by LPS in rat cortical astrocytes leads to the induction of proMMP-9 but fails to produce the active form of MMP-9. Active forms of MMP-2 appear in the supernatant after LPS or IL-1 stimulation and significantly increased the substrate degradation, resulting in the release of MMP-2 and MMP-9 into the conditioned medium (Colton et al., 1993). Mixed microglia and astrocytes in culture produce an active form of MMP-9, when stimulated by LPS (Rosenberg et al., 2001a). Immunostaining of mixed glial cultures with an antibody to MMP-3 showed that it was expressed by microglia, but not by astrocytes. This finding suggested that the microglia-derived MMP-3 was critical for activation of the MMP-9 during the LPS-induced inflammatory response. Thus, astrocytes, neurons, oligodendroglia, en-

dothelial cells, pericytes, and microglia produce MMPs, but the types of MMPs and the stimuli that induce the MMPs are different for the various cell types.

Several studies at the level of mRNA have been performed in astrocytes in culture. Using a competitive semiquantitative polymerase chain reaction (PCR) method in LPS-stimulated astrocyte cultures, constitutive expression of MMP-2, stromelysin-3 (MMP-11), and MT1-MMP (MMP-14) occurred. LPS treatment strongly induced (>1,000 fold) the production of MMP-9 and MMP-3. Smaller increases were seen in stromelysin-2 (MMP-10), rat collagenase (MMP-13), and macrophage metalloelastase (MMP-12) (Wells et al., 1996).

CELLULAR LOCALIZATION OF THE MMPs IN BRAIN

A number of immunohistochemistry studies have been conducted to identify the cell types expressing the MMPs. In frozen autopsy tissue from one patient with multiple sclerosis, MMP-1, -3, and -9 immunohistochemistry staining was noted; MMP-3 was localized to macrophage-like cells (Maeda and Sobel, 1996). In paraffin-embedded brain tissues from patients with multiple sclerosis and cerebral infarction, antibodies to MMP-9 were localized to blood vessels and neutrophils in those with recent exacerbations, but in the more chronic lesions, MMP-2 and MMP-7 were the prominent species with MMP-7 seen in the inflammatory cells (Anthony et al., 1997). In patients with stroke, neutrophils in acute lesions displayed immunoreactivity for MMP-9, while the chronic lesions were more likely to show MMP-2 and -7. Vascular dementia was shown to lead to an increase in MMPs. When multiple strokes were the cause of the dementia, reactive gemistocytic astrocytes around the infarctions were immunostained for MMP-2; MMP-9 was rarely seen in the chronic tissues. In the progressive form of dementia due to arteriosclerosis of the blood vessels with demyelination of the white matter, often referred to as Binswanger's disease, tissue macrophages were noted in the damaged white matter regions that stained for MMP-3 (Rosenberg et al., 2001b).

More information is available in animal models. In normal rat tissues, MMP-2 was present in an astrocyte population near the ventricular walls, the pial surface, and the blood vessels (Rosenberg et al., 2001a). The MMP-2 was seen in the normal brain in the astrocytic processes, particularly those abutting blood vessels, ependymal, and pial cells. The presence of MMP-2-containing astrocytic foot processes near the fluid-controlling surfaces of the brain suggests a role for MMP-2 in the normal fluid balance in the brain or in the regulation of the BBB. In a rat model of permanent ischemia, MMP-9 was identified in cerebral blood vessels and in inflammatory cells (Romanic et al., 1998). The MMPs have been studied in temporary ischemia caused by 90-min occlusion of the middle cerebral artery by a suture thread with reperfusion for various times (Rosenberg et al., 2001a). Inflammatory MMPs, MMP-3, and MMP-9, were seen at 16 h of reperfusion. MMP-9 was seen in the extracellular space of the infracted region, and was prominent in the blood vessels and in some neurons. MMP-3 was mainly in the neurons and microglia, which was demonstrated by dual-labeling studies. Pericytes around the blood vessels contained MMP-3 in the ischemic regions. Co-localization studies showed MMP-3 in microglia that were Ox-42 positive. At 5 days after reperfusion gemistocytic, astrocytes that lined the necrotic cyst wall were intensely immunopositive for MMP-2. Blood vessels remaining, or regrowing, into the necrotic core, contained MMP-3 after 5 days, possibly related to angiogenesis. By 3 weeks, the main MMP noted was MMP-2 in astrocytes around the cyst. MMP-2 is spatially constrained because it is activated by the MT-MMP on the cell surface, while MMP-3 and MMP-9 are released into the extracellular space, where they can do more damage.

Immunohistochemistry is excellent for the identification and spatial localization of MMP protein. However, it is unable to distinguish active from inactive MMPs. Identification of active MMPs has proved difficult. Recently, a method was developed for in situ zymography, which involves the use of a highly decorated fluorescent gelatin molecule that is quenched until it is cleaved. Incubating a solution of fluorescent-labeled gelatin onto a frozen tissue section shows fluorescence in the regions of gelatinase activity. The method has been used to show gelatinolytic activity in cultured oligodendrocytes, where it can be blocked by MMP inhibitors (Oh et al., 1999). It has also been used to demonstrate increased proteolytic activity in mouse brain after ischemia with reperfusion. Superoxide dismutase null mice have larger strokes, and they have greater activity against gelatin, as shown by gelatin-substrate zymography and the new method. MMP activity co-localized with regions of free radical formation and break down of the BBB (Gasche et al., 2001a).

MMPs IN NEUROINFLAMMATION IN THE ABSENCE OF HYPOXIA

Inflammation in the CNS behaves differently than in the rest of the body (Perry and Andersson, 1992). Because resident microglial cells are activated and mononuclear cells and neutrophils are recruited from the blood, the separation of endogenous from blood-borne inflammatory cells in the nervous system has been difficult (Lassmann et al., 1991). Transfer of white blood cells across the BBB is facilitated by MMPs released by the white blood cells (Leppert et al., 1995). Neuroinflammation, which involves the cytokines and free radicals, can be studied in vivo by the intracerebral injection of LPS or TNF-α. TNF-α injected intracerebrally into rat brain induced the production of MMP-9 after 24 h and was associated at that time with

BBB disruption; a hydroxymate inhibitor of the MMPs, Batimastat (BB-94), blocked the opening of the BBB (Rosenberg et al., 1995). Similarly, LPS was shown to open the barrier at 8 h after LPS injection with the production of MMP-9 at the same time; another hydroxymate-type MMP inhibitor, BB-1101, blocked the opening (Mun-Bryce and Rosenberg, 1998a). Immunohistochemistry of LPS-injected brains showed MMP-3 and -9 production in the LPS lesion region; MMP-2 was present constitutively, but was not increased by LPS. Studies of mRNA production after the injection of LPS showed an elevation in mRNA for MMP-2, -3, and -7 as early as 3 hours (Mun-Bryce et al., 2002).

Experimental allergic encephalomyelitis (EAE) is an animal model of a monophasic inflammatory demyelinating illness. Elevated levels of MMP-9 are found around the time of onset of symptoms and the MMP inhibitor, GM6001, blocked the BBB injury and improved the clinical condition (Gijbels et al., 1994). Other studies, using different synthetic MMP inhibitors, have shown similar results (Hewson et al., 1995; Liedtke et al., 1998).

Multiple sclerosis is a demyelinating disorder that is associated with an increase in the levels of MMP-9 in the CSF (Gijbels et al., 1992). Treatment with high-dose methylprednisolone for 3 days dramatically reduced the levels of MMP-9 in patients with evidence of BBB damage on Gadolinium-enhanced magnetic resonance imaging (MRI) (Rosenberg et al., 1996a). During an acute attack of multiple sclerosis, there is an increase in the levels of MMP-9 in the serum, but the source of the MMP-9 in the serum is uncertain (Waubant et al., 1999; Trojano et al., 1999; Lee et al., 1999). Treatment with interferon-β (IFN-β) reduces the levels of mRNA for MMPs in the serum (Galboiz et al., 2001).

Production of MMPs has been associated with the BBB damage in experimental models of bacterial meningitis induced by intracisternal injection of bacteria (Leib et al., 2001). The opening of the BBB could be blocked by the use of the MMP inhibitor, BB-1101. In another animal model, experimental allergic neuritis, which is a model for the Guillain-Barré syndrome, there was expression of MMPs. Treatment with an inhibitor of MMPs improved electrophysiologic nerve conduction and motor performance (Redford et al., 1997; Hughes et al., 1998; Leppert et al., 1999).

ROLE OF THE MMPs IN NEUROINFLAMMATION IN CEREBRAL ISCHEMIA

Biochemical studies of MMPs in permanent and temporary ischemia have shown that the MMPs contribute to the disruption of the BBB that leads to vasogenic cerebral edema and hemorrhage (Mun-Bryce and Rosenberg,1998b). Permanent middle cerebral artery occlusion in spontaneously hypertensive rats (SHR) resulted in the production of MMP-9 by 24 h and a marked increase in MMP-2 by 5 days (Rosenberg et al., 1996b). The increase in MMP-9 correlates with the time of maximal damage, while the later increase in MMP-2 is related to the increase in reactive astrocytes around the cyst. Middle cerebral artery occlusion for 90 min with reperfusion in SHR caused a biphasic opening of the BBB with a transient opening at 3 h and a second more severe injury at 48 h (Rosenberg et al., 1998). The early opening at 3 h correlated with an increase in MMP-2, while a later opening at 48 h correlated with increased expression of MMP-9. A hydroxymate MMP inhibitor, BB-1101, blocked the edema at 24 h and the early opening of the BBB at 3 h but failed to alter the secondary opening of the BBB at 48 h. Similar changes in MMPs have been seen in the mouse stroke model with reperfusion, using a more sensitive method of extraction of MMPs from small amounts of tissues (Gasche et al., 1999). A MMP-9 knockout mouse was found to have reduced infarct size and less BBB damage (Asahi et al., 2000, 2001). Mice that overexpress superoxide dismutase have reduced production of proMMP-9 (Gasche et al., 2001b). Hydroxymate MMP inhibitors have been shown to reduce cerebral edema in intracerebral hemorrhage secondary to bacterial collagenase in rat brain (Rosenberg and Navratil, 1997). In nonhuman primates, the early opening of the BBB correlated with increased levels of MMP-2 (Heo et al., 1999). Thus, there is considerable evidence to support a role for the MMPs in pathological changes associated with cerebral ischemia, particularly to the changes that occur in the cerebral vasculature.

Many factors most likely contribute to the final common pathway for disruption of the BBB. Our studies and those of other investigators have implicated the plasminogen/plasmin system and the MMPs in this pathological process. Although some aspects remain unresolved, a model of the action of the neutral proteases at the neurovascular unit is emerging. Astrocytic end feet and tight-junctioned endothelial cells act in collaboration with the basal lamina and the pericytes/microglia to form a neurovascular unit (Fig. 4). The tight junctions of the endothelial cells comprise the first line of defense along with enzymes that metabolize substances before they can enter the brain. Basal lamina forms a layer around the endothelial cells, which provides a charge barrier and may impede diffusion of larger molecules. Glial limitans of the astrocytic foot processes are in close proximity to the endothelial cells and basal lamina. MMPs degrade the components of the basal lamina, namely, heparan sulfate, laminin, fibronectin, and type IV collagen. During cellular stress from ischemia/hypoxia, the macromolecules of the basal lamina are reduced, presumably by the action of the ECM-degrading proteases, and loss of the matrix increases the rate of hemorrhage (Hamann et al., 1995). Pericytes are macrophage-like cells that are found next to the endothelial cells and are surrounded by basal lamina. Proteolysis of the ECM could contribute to brain tissue damage either by directly attacking the lining of the blood vessel and increasing the per-

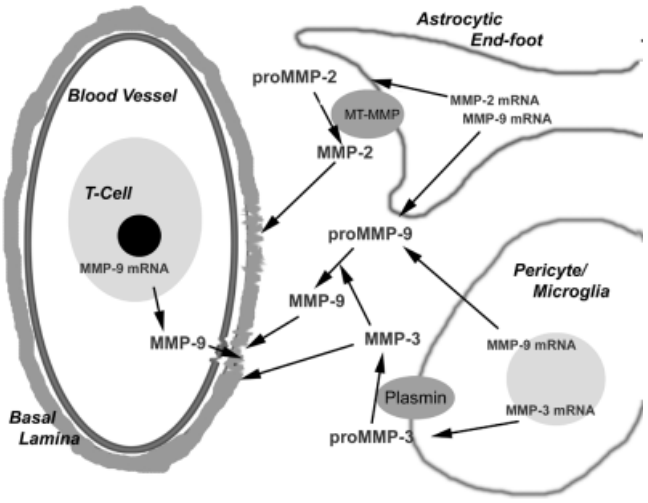

Fig. 4. Schematic representation of the interaction of the matrix metalloproteinases (MMPs) in the theoretical "neurovascular unit." Other proteases and free radicals are also involved but have been excluded to focus on the possible mechanisms for the MMPs. Under normal circumstances, there is constitutive expression of MMP-2 by the astrocytic endfeet with the potential for activation by the membrane-type MMP (MT-MMP). During cellular stress, several additional mechanisms could come into action. T cells circulating in the blood secrete MMP-9 to cross the blood-brain barrier formed by the endothelial cells and the basal lamina. Pericytes/microglia secrete MMP-2, -3, and -9. From cell culture studies (see text for discussion), there is evidence for the activation of the proMMP-3, possibly by the plasminogen/plasmin system in the membrane. Although still speculative, the activated MMP-3 could activate the proMMP-9 that is released by the endothelial cells, the astrocytes, the pericytes/microglia, and leukocytes. Cumulative action of the activated MMPs would lead to amplification of the damage to the blood-brain barrier (BBB) (Rosenberg, 2001).

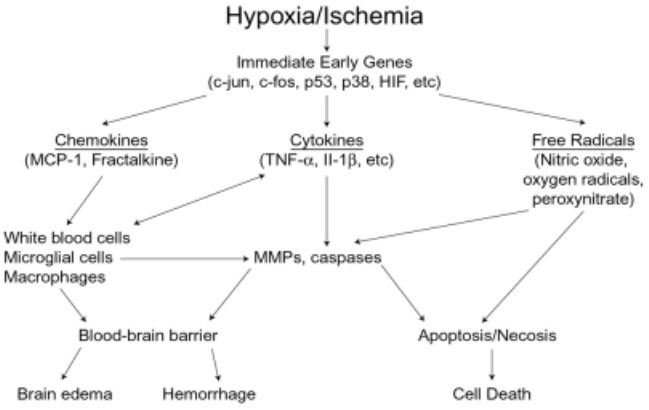

Fig. 5. Schematic representation of the multiple pathways activated by hypoxia/ischemia that lead to brain edema, hemorrhage, and cell death. As shown in Fig. 3, there is formation of the immediate-early gene products, cytokines and chemokines along with the formation of the free radicals. White blood cells, microglia, and macrophages release a series of proteases, including the matrix metalloproteinases (MMPs) and caspases, which directly participate in the opening of the blood-brain barrier (BBB) with edema and hemorrhage and in the apoptosis/necrosis that results in cell death.

meability or by loosening the connections between the cells, breaking communication channels for signaling molecules and nutrients, and causing the cell to die. Another way in which the changes in the ECM could potentially lead to the death of the cells is through the buildup of ECM with fibrosis, which would interfere with transport of nutrients to the cells and the exchange of trophic factors necessary to maintain cellular integrity.

Activation of the proMMPs is a critical step in the proteolytic process that remains poorly understood. In cell cultures, microglia interact with the pericytes, endothelia cells, and astrocytes to activate the MMPs. As shown above, the microglia are necessary for the activation of the proMMP-9. This could be done by MMP-3, which is seen by immunohistochemistry for MMP-3 in cell cultures and in tissues. In addition, there is mRNA evidence for the production of MMP-3. An ischemic insult releases inflammatory mediators, such as TNF-α and IL-1β, which induce the production of the MMPs. The plasminogen/plasmin system could contribute to the normal process by the activation of proMT1-MMP in the presence of TIMP-2. Activation of the MT1-MMP could be done by plasmin, which may be a site of interaction of the plasminogen/plasmin system and the MMPs. As the inflammatory process intensifies MMP-3 and MMP-9 genes are induced, forming the latent forms of the proinflammatory MMPs, which may also include MMP-1 and -7.

In patients who have sustained a stroke, there is an increase in the levels of MMPs in the blood. Serum levels of MMP-2 and MMP-9 were measured by ELISA in patients with cardioembolic stroke. National Institutes of Health (NIH) stroke scores were used to separate the patients into those with severe strokes (NIHSS $\geq$8) and those with milder scores. Those with severe strokes had significantly higher levels of MMP-9 at baseline, and the elevated levels persisted for 48 h (Montaner et al., 2001a). When hemorrhagic transformation or parenchymal hemorrhages were present, the highest levels of MMP-9 were found in those patients with late hemorrhagic transformation (Montaner et al., 2001b). Studies in nonhuman primates have shown that the levels of MMP-9 were associated with hemorrhagic transformation, while MMP-2 correlated with neuronal injury (Heo et al., 1999). A recent study showed that hemorrhagic infarctions, induced in rabbits by the injection of autologous blood clots into the carotid, were reduced by treatment with the MMP inhibitor, Batimistat (BB-94) (Lapchak et al., 2000).

MMP INVOLVEMENT IN STROKE DIFFERS FROM INFLAMMATION

From the studies in animals and humans, a consistent pattern of protease-mediated cell damage is emerging (Fig. 5). Normally, MMP-2 is present in the brain around the cerebral vessels in the astrocytic end feet and at the ependymal and pial surfaces. Astrocytes constitutively produce latent MMP-2. With the onset of an ischemic insult, MMP-2 becomes activated. This may involve conversion of plasminogen to plasmin by

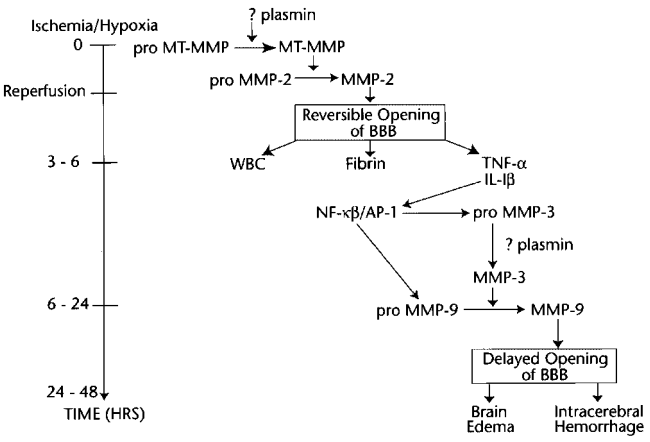

Fig. 6. Summary of the potential reactions in the ischemic brain. The scenario depicted involved transient occlusion of the blood vessel with reperfusion. The time course is depicted on the y-axis with time of occlusion at the top, and onset of reperfusion shown at a later time. There is a biphasic opening of the blood-brain barrier (BBB) with the initial injury thought to be related to the activation of the constitutively expressed matrix metalloproteinase-2 (MMP-2) by MT-MMP, which is activated by plasmin. If the ischemic injury is prolonged, a secondary reaction begins through the white blood cells (WBCs), fibrin deposition, and cytokines. Stimulation of the AP-1/NF-κB sites in the promoter regions of the proinflammatory MMP genes leads to MMP-3 and -9 production, and the opening of the BBB with edema and hemorrhage (Rosenberg et al., 2001a).

plasminogen activators. Plasmin activates MT-MMP, the main activator of the proMMP-2. The early opening of the BBB secondary to the activation of MMP-2 can be blocked by treatment with a MMP inhibitor but, in the absence of treatment, the permeability reverts to normal in several hours. After 24–48 h, depending on the strain of rats studied, there is a second, more severe disruption of the BBB. The second opening of the biphasic BBB pattern of injury may last for several days, and may be associated with hemorrhage and vasogenic edema (Kuroiwa et al., 1985). During the secondary phase of vessel injury, the levels of MMP-9 are markedly increased. Induction of MMP-9 is mediated by cytokines and immediate-early genes. Activation of the proforms occurs by the action of other proteases and of free radicals. In cell culture, microglial cells produce an activator of proMMP-9, possibly MMP-3 or free radicals, and less likely plasmin. Pericytes that are contiguous with the MMP-9-containing blood vessels show immunostaining for MMP-3, suggesting that they are involved in activating MMP-9. During the secondary phase of BBB disruption, which occurs at 48 h after a 90-min occlusion of the middle cerebral artery in the rat, the risk of hemorrhage is increased. Vasogenic edema contributes to the cytotoxic edema already present. Figure 6 shows a possible cascade of effects that lead to MMP-mediated injury with edema and hemorrhage.

Disruption of the BBB by proteases occurs early in inflammation, where the white blood cells use MMPs to enter the brain, setting in motion the release of MMPs by astrocytes and pericytes (Fig. 7). In the absence of hypoxia, the cells remain intact, and once the inflammatory response subsides, recovery is possible. During an ischemic/hypoxic insult, the endothelial cells are damaged secondary to the induction of MMPs by endogenous brain cells. There is an increased risk of bleeding, and recovery is impaired due to the extensive hypoxic injury to the cells.

ENDOGENOUS MMP INHIBITORS: TISSUE INHIBITORS OF METALLOPROTEINASES

Four endogenously produced TIMPs have been described; they inhibit both the MMPs and the ADAMs families (Brew et al., 2000). There is considerable homology in the TIMP molecules. TIMP-1 is a 28-kDa molecule that forms a strong bond with MMP-3 and -9. It is expressed in ischemic infarcts and in damaged peripheral nerve, where it appears to protect the axon from damage (La Fleur et al., 1996; Wang et al., 1998). TIMP-2 is a 21-kDa molecule that binds most strongly with MMP-2, facilitating activation by MT-MMP. At low concentrations, the TIMP-2 facilitates the activation of MMP-2, but at higher concentrations, it is inhibitory. TIMP-3 is unique among the TIMPs in that it is bound to the ECM (Pavloff et al., 1992; Leco et al., 1994) Both TIMP-1 and -3 are multifunctional molecules with roles besides inhibition of MMPs. TIMP-1 has growth factor effects, and is antiapoptotic (Guedez et al., 1998). On the contrary, overexpression of the TIMP-3 gene in cells increases apoptotic cell death (Baker et al., 1998). A number of MMPs and ADAMs are inhibited by TIMP-3, including MMP-3 and -7, and ADAM-10 and -17 (TACE). ADAMs remove ectodomain proteins from the cell surface. Death receptors and ligands are cleaved by sheddases from the cell surface, preventing cell death. Death receptors of the TNF superfamily, such as Fas (APO-1/CD95) and TNF receptor are released from the cell surface by sheddases. TIMP-3 may facilitate cell death by interfering with the action of the sheddases (Smith et al., 1997).

Apoptosis is a form of cell death that has been found in animals with cerebral ischemia (Kerr et al., 1972; Du et al., 1996; Endres et al., 1998). Since both necrosis and apoptosis are ongoing in ischemia, the separation of the two has been controversial (Colbourne et al., 1999) Caspases have been shown to be critical for programmed cell death in brain (Graham and Chen, 2001). Recently, we found that MPs may play an important role in facilitating the apoptotic process in brain. These data are emerging from studies showing the expression of TIMP-3 in ischemic brain tissue in rats and mice, and from cell culture studies of neurons.

When it restricts the growth of cancer cells, TIMP-3-facilitated apoptosis is beneficial. However, in cerebral ischemia, where the goal is to prevent apoptosis, TIMP-3 may be detrimental. We have found expression of TIMP-3 in ischemic neurons were it is co-localized in cells immunostained with markers of cell death (Wallace et al, unpublished data). Furthermore, neurons in

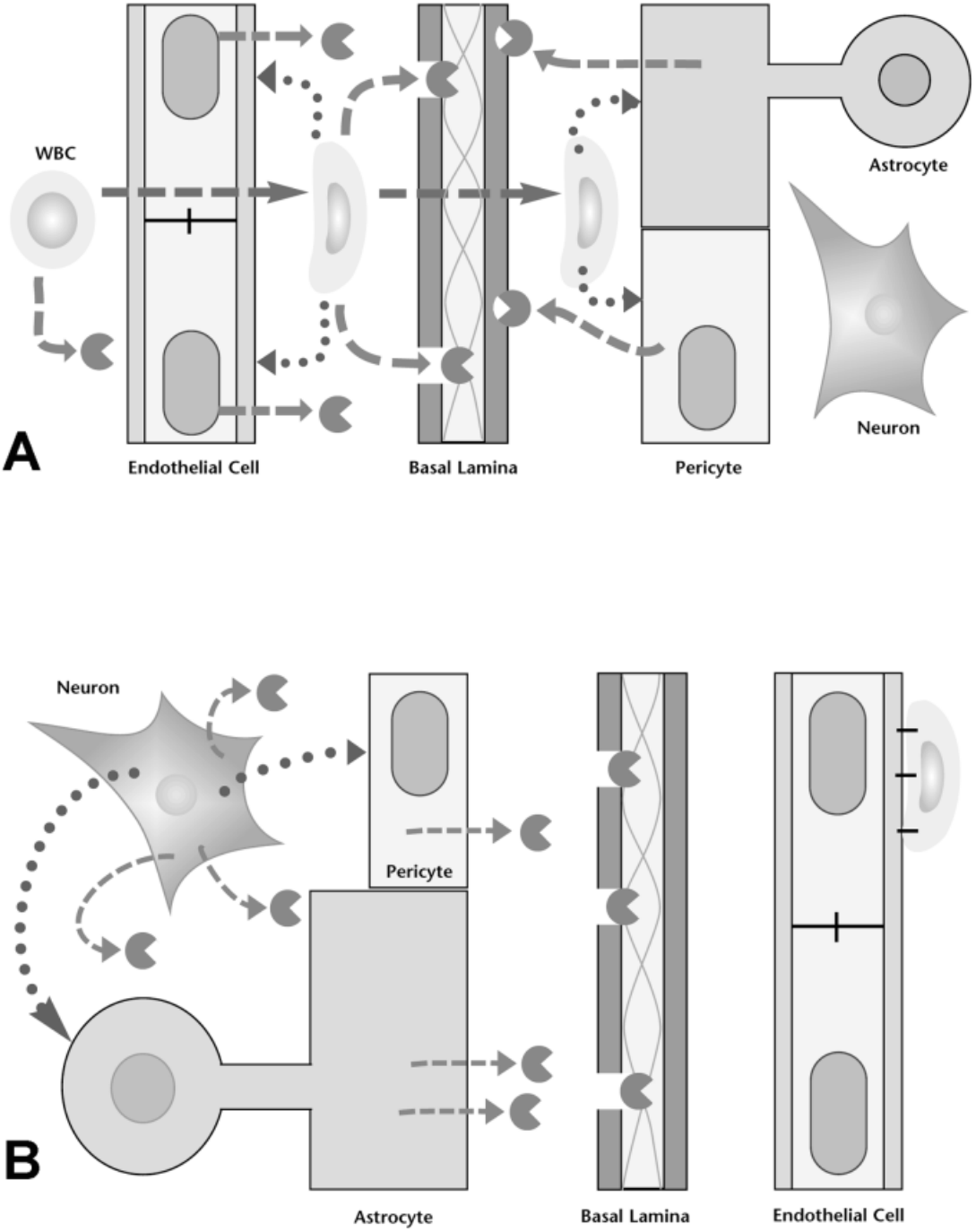

Fig. 7. Schematic drawing of the possible differences between the action of matrix metalloproteinases (MMPs) in inflammation and hypoxia/ischemia. **A:** Inflammation, such as is seen multiple sclerosis or bacterial infection, causes the white blood cells (WBC) to release MMPs, which enables them to move through the capillary. Once in the brain, cytokines stimulate further MMPs, which amplify the damage. **B:** In hypoxia/ischemia, the reaction begins in the brains cells. Neurons (or microglial) release MMPs, and cytokines amplify the response. White blood cells adhere to the cell surface and eventually enter the brain. Damage to the endothelial cells is a secondary event.

culture express TIMP-3, which is not toxic by itself, but its presence facilitates cell death. Antibodies to TIMP-3 block cell death induced by the anticancer drug, Doxorubicin (Wetzel et al., unpublished data). The factors inducing TIMP-3 mRNA in the injured brain are unknown, but it has an AP-1-binding site and a p53 consensus sequence in the promoter region. Mutant p53 suppressed the expression of TIMP-3 by 10-fold (Loging and Reisman, 1999). Other investigators have not found a role for p53 in TIMP-3 expression, however, and further studies will be needed to resolve this issue (Bian et al., 1996).

TABLE 2. Matrix Metalloproteinase Inhibitors Used to Treat Animal Models of Neurological Disease

Model	Species	Agent	Effect	References
Intracerebral injection				
MMP-2	Rat	TIMP-2	Blocked BBB opening	Rosenberg et al., 1992; Rosenberg et al., 1995; Mun-Bryce and Rosenberg, 1998a
TNF-α		BB-94		
LPS		BB-1101		
Experimental allergic encephalomyelitis	Mouse	GM 6001	BBB injury reduced; reduced clinical severity	Gijbels et al., 1994; Hewson et al., 1995
(EAE)	Rat	R031-9790		
Experimental allergic neuritis (EAN)	Rat	BB-1101	Reduced clinical severity	Redford et al., 1997
Cerebral ischemia				
Ischemia/reperfusion	Rat	BB-1101	Reduced BBB opening and brain edema	Rosenberg et al., 1998
	Mouse	BB-94	Reduced infarct size	Asahi et al., 2000
Permanent ischemia	Rat	Antibody to MMP-9	Reduced infarct size	Romanic et al., 1998
Collagenase-induced hemorrhage	Rat	BB-1101	Reduced cerebral edema	Rosenberg and Navratil, 1997
Hemorrhagic transformation	Rabbit	BB-94	Reduced tPA-induced bleeding	Lapchak et al., 2000
Bacterial meningitis	Rat	GM 6001 BB-1101	Improved outcome	Leib et al., 2000, 2001

MMP, matrix metalloproteinase; TNF-α, tumor necrosis factor-α; BBB, blood-brain barrier; tPA, tissue plasminogen activator; TIMP, tissue inhibitor of metalloproteinase.

SYNTHETIC MMP INHIBITORS

Because of the involvement of MMPs in diseases such as arthritis, cancer, multiple sclerosis, stroke, and meningitis, many pharmaceutical companies are actively searching for compounds that can be used to block their action. The major synthetic inhibitors of MMPs, some of which have been used in clinical trials, are based on a hydroxymate structure (Brown, 1998). The hydroxymates interfere with the action of the zinc catalytic domain in the MMP molecule. Current inhibitors are broad spectrum, blocking the actions of several MMPs. These inhibitors tend to be poorly soluble. Clinical trials of MMP inhibitors in cancer have been disappointing (Nelson et al., 2000).

More encouraging results have been obtained with the use of MMP inhibitors in the treatment of animal models of diseases of the CNS (Table 2). TIMP-2 blocked the MMP-2-induced BBB opening in MMP-induced injury in the brain (Rosenberg et al., 1992). Damage to the BBB was blocked, and symptomatic improvement occurred in experimental allergic encephalomyelitis (EAE) with hydroxymate treatment (Gijbels et al., 1994; Hewson et al., 1995; Clements et al., 1997). BB-94 blocked the BBB opening after intracerebral injection of TNF-α, and BB-1101 was effective in LPS-induced BBB injury (Rosenberg et al., 1995; Mun-Bryce and Rosenberg,1998a). Treatments in several animal models of neurological diseases have produced encouraging results. These include intracerebral hemorrhage, experimental allergic neuritis, delayed hypersensitivity, and bacterial meningitis (Rosenberg and Navratil, 1997; Hughes et al., 1998; Lapchak et al., 2000; Leib et al., 2001).

Selective inhibitors of the MMPs have been difficult to synthesize because of the similarity in structure of the essential parts of the MMP molecule. These agents may interfere with the beneficial actions of the MMPs. Because of the broad range of actions of the MMPs in brain, which may be either beneficial or detrimental, study of these agents will be time-consuming. Furthermore, the critical MMPs in the disease processes under investigation are currently unknown. As this information emerges, the use of selective inhibitors will be of great importance. Another major hurdle to the use of these agents in neurological diseases is the side effects identified during clinical trials in cancer treatment. Chronic use of these agents has resulted in side effects in the joints, probably related to the inhibition of important remodeling functions of the MMPs that has lead to fibrosis. Targeting the MMP inhibitors for the treatment of acute injury may overcome the problems associated with long-term use.

CONCLUSIONS AND FUTURE STUDIES

The various roles of the MPs and TIMPs in brain include tissue damage during inflammation and tissue remodeling and repair during development and recovery from the injury. On the one hand, the MMPs break down the ECM around blood vessels, leading to the disruption of the BBB. This action is initially mediated by the constitutively expressed MMP-2, and subsequently by the inducible enzymes, MMP-1, -3, and -9. Different cell types appear to secrete different MMPs, providing additional safeguards against unwanted proteolysis. Activation processes involve proteases and free radicals, providing a link between the production of free radicals in the site of the injury and the activation of the MMPs. Microglia and tissue macrophages play a critical role in the inflammatory response both by releasing the MMPs and by forming the substances that activate them. Inflammation without ischemia damages the blood vessels in a reversible manner that responds to antiinflammatory agents, such as high-

dose steroids. Use of steroids in multiple sclerosis, Guillain-Barré, and bacterial meningitis controls the inflammatory response and reduces the production of the MMPs. Ischemic injuries are complicated by hypoxia, which induces cell pathways that lead to cell death, making ischemic injury more challenging to treat. Therefore, the therapeutic goal in the ischemic lesion is to block at an early stage the factors that are leading to the production of MMPs. Because parallel processes, such as the induction of multiple proteases and formation of free radicals, are occurring at the same time, additional blockers may be required. The rapid onset of cell death mechanisms adds an additional burden on the treatment of the ischemic injury, which is not found with inflammation alone.

The ADAMs branch of the MPs includes members that overlap with the functions of the MMPs and have additional ones related to their attachment to the cell surface. An important function, about which very little is known in the CNS, is the regulation of the shedding of the cell surface-related proteins. The interaction of the TIMPs with the ADAMs and MMPs is largely unexplored in the brain, and should be an area of fruitful research in the future. Genetically modified animals that underexpress or overexpress the MMP, ADAM, and TIMP genes are being developed or are already available. These animal models coupled with the use of selective inhibitors should aid in unraveling the critical roles they play in the brain, and ultimately provide information that will be useful in designing novel agents to reduce injury or promote healing.

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