Microglial Activation and Matrix Protease Generation During Focal Cerebral Ischemia

Gregory J. del Zoppo, MD; Richard Milner, MD, PhD; Takuma Mabuchi, MD, PhD; Stephanie Hung, MS; Xiaoyun Wang, MS; Greta I. Berg, MA; James A. Koziol, PhD

Abstract—Local environmental conditions contribute to the activation state of cells. Extracellular matrix glycoproteins participate in cell-cell boundaries within the microvascular and extravascular tissues of the central nervous system and provide a scaffold for the local environment. These conditions are altered during focal cerebral ischemia (and other central nervous system disorders) when extracellular matrix boundaries are degraded or when matrix proteins in the vascular circulation enter the neuropil as the microvascular permeability barrier is degraded. Microglia in the resting state become activated after the onset of ischemia. During activation these cells can express a number of factors and proteases, including latent matrix metalloprotease-9 (pro–MMP-9). Whereas MMP-9 and MMP-2 are generated early during focal ischemia in select models, their cellular sources in vivo are still under study. In vitro microglia cells activate and respond to exposure to specific matrix proteins (eg, vitronectin, fibronectin) that circulate. Certain MMP inhibitors, specifically tetracycline derivatives, can modulate microglial activation and reduce injury volume in limited studies. But, the injury reduction relies on preinjury exposure to the tetracycline. Other studies underway suggest the hypothesis that microglial cell activation and pro-MMP-9 generation during focal cerebral ischemia is promoted in part by matrix proteins in the circulation that extravasate into the neuropil when the blood-brain barrier is compromised. These matrix proteins are known to activate microglia through their specific cell surface matrix receptors. (Stroke. 2007;38[part 2]:646-651.)

Key Words: extracellular matrix ■ ischemic stroke ■ matrix metalloproteinases ■ microglia ■ tetracyclines

Extracellular matrix (ECM) glycoproteins participate in the cell-cell boundaries within the microvascular and extravascular tissues of the central nervous system (CNS). Endothelial cells and astrocytes of cerebral capillaries are separated by the basal lamina to whose ECM constituents (eg, collagen type IV, laminins, fibronectin, and heparan sulfate proteoglycans [eg, perlecan]) both cells adhere by specific adhesion receptors. Astrocytes and neurons interact directly and are themselves stabilized by ECM of a different composition. The association of the microvessel endothelial ECM-astrocyte complex with the neurons they serve comprises a theoretical and possibly functional “neurovascular unit.” Recent work has demonstrated that during focal ischemia characteristic and rapid changes in both the ECM and the ultrastructural relationships among the cellular components of the neurovascular unit appear. In addition to focal cerebral ischemia, matrix alterations within the CNS occur (1) during acute (eg, infection) or chronic (eg, multiple sclerosis) inflammatory disorders, (2) during invasion by cells with metastatic potential that display anchorage-independent growth, (3) in association with primary tumors of the CNS (eg, gliomas, arteriovenous malformations), and (4) from direct trauma. All of these conditions disturb the matrix and cellular components of the “neurovascular unit,” causing various alterations in the microvascular permeability barrier and in the function and survival of the neurons, astrocytes, and pericytes. The roles that microglia could play in maintaining and, on activation, altering the neurovascular unit in these conditions, but particularly ischemic stroke, are relatively unexplored. That microglia could contribute substantially to the integrity of the neurovascular unit is underlined by (1) their generation of matrix proteases, including matrix metalloproteinases (MMPs), and (2) their roles in immunomodulation. The possible relationships between microglial activation and the fate of the ECM observed after middle cerebral artery occlusion (MCAO) are the subject of this presentation.

MMPs and Their Sources in the CNS

MMPs comprise a family of endopeptidases which have similar primary structure and require Zn$^{2+}$ in a conserved cage for cleavage of specific ECM proteins. To date, this protease...
family consists of 28 members, of which active MMP-2 (gelatinase A) and MMP-9 (gelatinase B) and their inactive proforms are the most conveniently detected. Within the CNS endothelial cells, astrocytes, neurons, and microglia can generate pro-MMP-2. Each of these cells can also generate pro-MMP-9 although the ability of astrocytes to express pro-MMP-9 appears to depend on the conditions of the study (Table 1). During focal cerebral ischemia in the nonhuman primate, the direct activators of pro-MMP-2, MT1-MMP (MMP-14) and MT3-MMP (MMP-16) are rapidly generated. Evidence exists both directly and indirectly that plasminogen, MMP-1, MMP-2, MMP-3, and perhaps certain serine proteases, known to activate pro-MMP-9, are also generated. However, the detection of either gelatinase in active form depends on the conditions of tissue isolation, the tissue source, and its handling. So far, direct evidence that either MMP actually degrades vascular or extravascular matrix during MCAO is limited. Furthermore, the exact cell sources for the MMP-2 and MMP-9 generation seen during focal cerebral ischemia are not yet defined in vivo.

However, the responses of both microglia and astrocytes (isolated by new standard techniques) to several inflammatory stimuli suggest that one activator, nuclear factor-kB, can promote MMP-2 or MMP-9 expression and secretion (Table 1). When grown on a plastic surface and with exposure to lipopolysaccharide, microglia are stimulated to produce MMP-9, but not MMP-2. In contrast, cultured astrocytes express increased quantities of MMP-2 above constitutive levels, and variable amounts of MMP-9. Hence the controversy that astrocytes can generate MMP-9. Kauppinen et al demonstrated that tumor necrosis factor-α can directly stimulate MMP-9 production by isolated microglia resting on a plastic surface. In sum, those studies indicate that microglial cells can be stimulated by known nuclear factor-kB agonists to express MMP-9, suggesting that these cells could be a source of the increased pro-MMP-9 observed after focal ischemia in some models.

Table 1: Stimulation of MMP Expression in Microglial Cells and Astrocytes

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Microglia</th>
<th>Astrocytes</th>
<th>Species</th>
<th>Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS</td>
<td>↑ MMP-9</td>
<td>↑ MMP-9</td>
<td>rat</td>
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<tr>
<td>Zymosan</td>
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<td>rat</td>
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</tr>
<tr>
<td>LPS</td>
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<td>↑ MMP-2</td>
<td>rat</td>
<td>36</td>
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<tr>
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<td>↑ MMP-9</td>
<td>rat</td>
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</tr>
<tr>
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<td>↑ MMP-9</td>
<td>↓ MMP-2</td>
<td>rat</td>
<td>38</td>
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<tr>
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</tr>
<tr>
<td>PARP-1</td>
<td>↑ MMP-9</td>
<td></td>
<td>mouse</td>
<td>40</td>
</tr>
</tbody>
</table>

LPS indicates lipopolysaccharide; TNF-α, tumor necrosis factor. PARP-1, poly(ADP-ribose)polymerase.

LPS uniformly activates microglial cells to generate MMP-9, whereas astrocytes appear to generate MMP-2.

TABLE 1. Stimulation of MMP Expression in Microglial Cells and Astrocytes

**MMP Expression During Focal Cerebral Ischemia**

Pro–MMP-2 and pro–MMP-9 appear differentially in nonhuman primates and rodents after MCAO. Lo et al have demonstrated that pro–MMP-9 appears in the ischemic hemisphere of C57Bl/6, CD-1, and SV129 mice subject to MCAO for 24 hours (Lo E, Asahi M, personal communication, 2000), but can appear as early as 3 hours after MCAO in CD-1 mice. That observation has been confirmed by a number of other groups using several strains of rats or mice. In contrast, pro–MMP-2, MT1-MMP, and MT3-MMP rapidly appear in the ischemic core after MCAO in the nonhuman primate. Pro–MMP-9 was significantly associated with hemorrhagic transformation in those preparations. Pro–MMP-2 was directly correlated with both the area of core injury and the density of neurons displaying evidence of DNA scission. In addition, both urokinase (u-PA) and the cysteine protease cathepsin L are rapidly generated in the ischemic core, in association with both microvessels and neurons within the ischemic core region. Those findings demonstrate the complexity of ECM protease generation in the setting of focal cerebral ischemia even with limited evidence, and the limitations of MMP-2 and MMP-9 expression imposed by the setting and tissue sources of focal ischemic injury. Hence, the participation of microglia in ischemic injury must take into account differential MMP and non-MMP protease expression, the association with neuron injury, and local environmental settings.

**Cell Environments and Microglial Activation**

In consideration of cellular responses to injury within the CNS, given the variety of cell types and the construction of the neurovascular unit, at least 4 variables could alter the local environmental conditions for microglia: (1) cellular location (eg, gray matter versus white matter, vascular versus extravascular space), (2) extracellular matrix composition, (3) cell-cell associations, (4) time, and (5) the severity of injury. Consideration of both location and matrix composition raises the question whether the local ECM environment can alter microglial responses, as it can for both endothelial cells and astrocytes in the neurovascular unit. As shown for both endothelial cells and astrocytes, cell proximity can and does “instruct” the formation of features of the endothelial permeability barrier. What signals are provided to microglia in response to injury that could determine or shape its responses? Finally, during the time course of microglial activation, when does matrix adhesion most probably affect protease expression?

**Matrix Composition and Cell Adhesion Receptor Expression**

As an environmental feature, ECM composition reflects the status of cells that synthesize the individual matrix glycoproteins and the expression of active matrix proteases by both resident or invading cells. Both intact and partially proteolyzed matrix proteins can have distinct biological activities. Intact ECM proteins found within the CNS can have proliferative activities when disinhibited (eg, perlecan on endothelial cells). Furthermore, fragmentation of certain ECM proteins can produce degradation products with biological activity (eg, laminin).
The matrix composition of the cerebral microvasculature appears to differ from that of the extravascular space.6,84 It is a plausible notion that cells of the neuropil could react characteristically to matrix proteins released from the vascular space into the regions of injury. This notion implies that rapid alterations in microglial environment could facilitate activation.

Vascular Cell Adhesion Responses to Matrix
Endothelial cells and astrocytes of the neurovascular unit both express adhesion receptors of the integrin and dystroglycan families. In vivo, both cell types express select β integrins that mediate cell adhesion to specific ECM proteins present in the cerebrovascular basal lamina. In vitro function-blocking studies indicate that endothelial cell adhesion to collagen IV is mediated by the αβ integrin, adhesion to laminin by the αβ integrin, and adhesion to fibronectin by the αβ integrins.52 Similar function-blocking studies have shown that astrocyte adhesion to collagen IV is mediated by the αβ integrin, adhesion to laminin by the αβ integrins, and adhesion to fibronectin by the αβ integrin.53–55 In addition, vitronectin which is absent from the CNS in the normal state, but induced after ischemia,56 and also deposited with a perivascular pattern in demyelinating disease,57,58 promotes both brain endothelial52 and astrocyte adhesion53–55 through the αβ and αβ integrins, respectively.

The ECM strongly influences the behavior of endothelial cells.59–63 Fibronectin is a promoter of brain endothelial survival and proliferation,64–66 compatible with a role in promoting remodeling and angiogenesis via the αβ integrins.52 Laminin and collagen IV promote endothelial cell differentiation and maturation.67–70 This is entirely consistent with the switch of brain endothelial cells from fibronectin to laminin-mediated signaling observed during CNS development.71 Thus, during development this change in the composition of the adjacent matrix accompanies (promotes) a change in the behavior of the cell.

Effect of ECM on Microglial Activation and Integrin Expression
The activation state of isolated microglia is also strongly regulated by the composition of the matrix they contact. Microglial cells of murine origin can express integrin subunit α, select β integrins (paired with subunits α, α, or α), and typical β integrins (LFA-1 and MAC-1).72 In microglia from murine or rat origin, subunit α is paired with β, suggesting that αβ is important in microglial adhesion interactions.73 Vitronectin and fibronectin stimulate microglial activation, as assessed by a morphological switch from a resting ramified phenotype to an amoeboid phenotype.74 This is accompanied by increased expression of the activation marker, major histocompatibility complex class I, and increased expression of the integrins αβ, αβ, and MAC-1 (αβ), which have been shown to be induced during microglial activation in vitro and in vivo.72–77 Interestingly, laminin, an abundant ECM protein in the cerebrovascular basal lamina, has the opposite effect on microglial activation. On a laminin substrate, microglia adopt a less activated, poorly adhesive phenotype, and show reduced levels of expression of the activation markers major histocompatibility complex class I and the αβ, αβ, and MAC-1 integrins.71 Thus, it is clear that induction or deposition of different ECM proteins within the CNS profoundly affects microglial activation state.

Microglial Cell Activation During Focal Ischemia
After the onset of focal cerebral ischemia, cellular inflammation is initiated. The inflammatory processes are manifest by the endothelial cell adherence and transmigration of polymorphonuclear leukocytes and the accumulation of cells of the macrophage/monocytes lineage.78–80 Mabuchi et al surveyed the responses of immune-competent microglia and oligodendroglia within the ischemic regions as potential contributors to the “secondary injury.”80 By 16 to 24 hours after MCAO, within the ischemic regions of the cortex of the Wistar-Kyoto rat, ameboid microglia express interleukin-1β, consistent with their level of activation.80

Coincident with the evolution of the tissue injury after focal ischemia is focal loss of the microvascular permeability barrier.12,81–83 Okada and colleagues demonstrated the progressive extravasation of fibrinogen from the plasma compartment with time, implying the leakage of coagulation factors and soluble matrix proteins (eg, fibronectin, vitronectin) into the injured neuropil.84 This suggests the hypothesis that microglial cell activation and their generation of (pro-)MMP-9 during focal ischemia results from exposure of resident microglia to stimulatory ligands derived from the plasma compartment attributable to ischemia-dependent leakage in the microvascular permeability barrier or generated by adjacent cells.

Protease Expression in the Neurovascular Unit
Experiments in the nonhuman primate have indicated consistent selective expression of matrix proteases within the neurovascular unit (Table 2).21,22,85 Their exact cellular distribution and coexpression are under study. Expression of MMP-2 and MMP-9 appears to be confined to the microvascular portion of the “unit”21,22 although microglial expression per se could not be detected in those studies.22 However, Mabuchi, del Zoppo, and colleagues have suggested that MMP-9 could appear in association with hemorrhagic trans-

<table>
<thead>
<tr>
<th>Protease*</th>
<th>Microvessel</th>
<th>Neuron</th>
<th>Microglia</th>
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<tbody>
<tr>
<td>Pro-MMP-2</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Pro-MMP-9</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>u-PA*</td>
<td>+</td>
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</tr>
<tr>
<td>u-PAR</td>
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<tr>
<td>t-PA</td>
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<td>PAI-1</td>
<td>+</td>
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<tr>
<td>Heparanase*</td>
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</table>

u-PA indicates urokinase plasminogen activator; u-PAR, urokinase receptor; t-PA, tissue plasminogen activator; PAI-1, plasminogen activator inhibitor-1. Matrix proteases can be expressed by components of the neurovascular unit under conditions of activation (eg, focal ischemia).Microvessels (endothelial cells and astrocytes) and neurons can generate MMP-2, u-PA, cathepsin L, and heparanase variably to focal ischemia. In rodent models and in response to hemorrhage, MMP-9 is generated.

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Table 2. Protease Expression in the Neurovascular Unit

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formation (personal observations, 2003). It is plausible, although unproven, that (pro-)MMP-9 observed in the ischemic regions in this setting is generated by activated microglia.

### Inhibitors of MMPs, in Particular the Modified Tetracyclines

Agents which can modulate or block the activities of the MMP families have been derived from both known endogenous inhibitors (which have site-specific activity) and original or modified exogenous chemical inhibitors (Table 3). The value of the modified tetracyclines as potential inhibitors of microglial MMPs has been raised for consideration. Minocycline or the modified tetracyclines as potential inhibitors (which have site-specific activity) and original or modified tetracyclines have variable selectivity but also several mechanisms of action.

Among the modified tetracyclines, doxycycline (CMT 3) and minocycline (CMT 8) have been used in studies of cerebral injury reduction after focal ischemia. The principal advantage of these compounds is their ease of delivery and relatively low expense. However, the mechanisms of MMP inhibition are poorly understood at the molecular level. The tetracyclines (both the COL and CMT compounds) display variable and multiple actions. MMP inhibition can result from divalent cation chelation, cell penetration, resultant anti-inflammatory effects, freeradical quenching activities, and leukocyte function blockade in addition to direct MMP inhibition. Reduction in MMP-9 activity can also reflect an indirect leukocyte-related effect. In model systems the induction of apoptosis and inhibition of angiogenesis are not connected with known MMP inhibitory activity but have been reported. Importantly, tetracycline analogues display variable IC₅₀ values compared within a class, making specific MMP inhibitors uncertain. For instance, whereas the IC₅₀ for (pro-)MMP-2 by CMT 3 is 56 μmol/L, the IC₅₀ by CMT 8 is >1 mmol/L, and the IC₅₀ for (pro-)MMP-1 or MMP-3 are much different.

In addition to the modulation of microglial activation in either cell culture or in cerebral tissue, minocycline appeared to inhibit injury to neurons (PC12 cells) in culture by nitric oxide or oxygen-glucose deprivation, respectively. No impact on neuron injury by doxycycline during global ischemia was observed in a separate study. Two recent experimental studies have indicated that minocycline could reduce injury volume after MCAO by 2 different methods. However, Koistinaho and colleagues demonstrated an inhibition of pro–MMP-2 and pro–MMP-9 expression with a reduction in injury volume after MCAO when either CMT 3 or CMT 8 were applied before, but not after MCAO. Those studies raise the questions to what degree does microglial cell–associated MMP-9 contribute to the injury caused by focal ischemia in rodent models, and whether it is ischemia per se or the consequences of injury to the neurovasculature that leads to microglial activation.

### Summary and Hypothesis

The association of cerebral microglial cell reactivity and MMP (gelatinase) generation in the setting of focal cerebral ischemia suggests the question of how microglial activation can alter the function and ultrastructure of the neurovascular unit (in the regions of ischemic injury). It remains an unproven hypothesis that MMP-9, particularly in latent form, can initiate or augment neuron or microvascular (endothelial cell-astrocyte) injury during focal ischemia.

These considerations raise a number of queries that prompt incisive study: (1) What is the true environment of resting microglial cells, and how does the time from ischemic injury alter this environment? (2) What is the impact of the exposure to plasma proteins that can occur on increased microvascular permeability? Specifically, how do the circulating matrix proteins fibronectin and vitronectin or activated coagulation proteins alter microglial cell reactivity and why? (3) What is the relevance of microglial activation to neuron injury after focal ischemia? (4) How does this information support an understanding of the requirements for transplantation after MCAO? Relevant to the conflicting findings with the use of tetracycline analogues, does one explain the dependence of microglial MMP-9 expression on the timing of tetracycline exposure? One possible explanation could be that exposure of the ischemic region to plasma matrix proteins (such as plasma fibronectin or vitronectin) can alter the local environment of microglial cells, thereby stimulating their generation of pro–MMP-2 and pro–MMP-9 expression with a reduction in injury volume after MCAO when either CMT 3 or CMT 8 were applied before, but not after MCAO. Those studies raise the questions to what degree does microglial cell–associated MMP-9 contribute to the injury caused by focal ischemia in rodent models, and whether it is ischemia per se or the consequences of injury to the neurovasculature that leads to microglial activation.

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Disclosures
None.

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