Available at http://www.springerlink.com/content/1895-104x/6/4/
DOI: 10.2478/s11535-011-0030-z
Cent. Eur. J. Biol.• 6(4) • 2011 • 471-485

Matrix metalloproteinases at key junctions in the pathomechanism of stroke

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Running title: MMPs in stroke

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ABSTRACT

Matrix metalloproteinases play a crucial role in the remodelling of the extracellular matrix through direct degradation of its structural proteins and control of extracellular signaling. The most common cause of ischemic brain damage is an atherothrombotic lesion in the supplying arteries. The progress of the atherosclerotic plaque development and the related thrombotic complications are mediated in part by matrix metalloproteinases. In addition to their role in the underlying disease, various members of this protease family are upregulated in the acute phase of ischemic brain damage as well as in the post-ischemic brain recovery following stroke. This review summarizes the current understanding of the matrix metalloproteinase-related molecular events at three stages of the ischemic cerebrovascular disease (in the atherosclerotic plaque, in the neurovascular unit of the brain and in the regenerating brain tissue).

Keywords: extracellular matrix, atherosclerosis, blood-brain barrier, ischemic damage, brain regeneration

INTRODUCTION

Cardiovascular and cerebrovascular diseases are the most common cause of mortality in the developed countries (they are responsible for more than 50 % of the death events in Europe) (WHO statistics, Mortality database. http://www.who.int/healthinfo/morttables/en/) and in the majority of cases (more than 90 %) an atherosclerotic plaque is the culprit lesion. The major characteristic of

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atherosclerosis is a plaque formed in the intima layer of the arteries, which develops in the course of decades as a consequence of chronic inflammation. Some of the plaques contain low number of cells, but extensive extracellular matrix (ECM), and these are known as "stable plaques", which resist mechanic forces and in clinical terms remain "silent" for long time. Other plaques are rich in cells and lipids ("unstable plaques", which are ruptured easily and thus provoke thrombosis with eventual blood vessel occlusion and tissue ischemia). The stability of the plaque is tightly related to the proteolysis-dependent re-organization of the ECM based on the activity of matrix-metalloproteinases (MMP).

The maintenance of the special metabolic environment in the central nervous system is based on the function of the neurovascular unit forming the interface between blood and parenchymal tissue in brain microcirculation. The endothelial cells, pericytes and astrocytes together with the ECM of this unit comprise a highly selective barrier between blood and brain, the blood-brain barrier (BBB) (1). The cellular interactions in the neurovascular unit induce unique differentiation of the brain microcirculatory endothelial cells, which are fenestration-free, joined together through tight junctions and anchored through their integrin receptors to the underlying basal lamina. Similarly to the rest of the brain tissue, which accounts for 20 % of the oxygen consumption in the whole body (2), the neurovascular unit is also very sensitive to ischemic insults, which cause cellular dysfunction. The post-ischemic disruption of the BBB results in diffusion of small osmotically active molecules and plasma proteins with consequent brain edema. However, the ECM of the basal lamina composed of type IV collagen, laminin, fibronectin, thrombospondin, entactin, proteoglycans (3) would not allow the leakage of cellular elements from blood. Thus, the frequent complication of the ischemic brain damage, the haemorrhagic transformation of an infarct, which occurs in 15 – 45 % of patients with cerebral infarction (4), can be explained only with concomitant degradation of the ECM structure. The proteases capable of performing this task at this location also belong to the family of MMPs.

The repair process in the penumbra zone of the post-ischemic brain is accompanied by axon regeneration and maturation of progenitor cells. Recent data evidence the contribution of MMPs to this repair process, as well as to the revascularization of the affected tissue. This review discusses the role of MMPs in the abovementioned three stages of the cerebrovascular disease (the culprit lesion in the

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arteries, the immediate post-ischemic brain damage and the post-ischemic tissue remodelling in the brain).

STRUCTURE AND CLASSIFICATION OF MMPs

Since the identification of collagenase-1 in the tail of metamorphizing tadpole (5) 23 different MMPs have been described in humans. All MMPs show structural homology to collagenase-1, which according to the present nomenclature is named MMP-1. Two structural domains are common for all MMPs: the Zn^{2+}-binding sequence (HEXGHXXGXXH) in the catalytic domain and the cystein-lock in the pro-peptide, which binds the active site Zn^{2+} through the PRCGXPD sequence in the zymogens and keeps the pro-MMPs in inactive form. Most of the MMPs contain two more common structures: the hinge region and the hemopexin domain, which participate in the initial binding of collagen fibers, the unwinding of their triple helices and the positioning of the separate polypeptide chains in the active site of the protease. Most MMPs are released in soluble form from the cells, but some of them function in membrane-bound form (membrane-type MMP, MT-MMP). The MMPs can be classified in 6 groups according to their relative substrate specificity, which is related to their primary and domain structure (Table 1) (reviewed in Ref. 6).

REGULATION OF MMP ACTIVITY (gene expression, activation, inhibition)

The common biological functions of MMPs are related to the reorganization of the ECM structure and the cell-matrix interactions in the course of tissue remodelling or regeneration as well as to the activation of signal peptides. These functions require stringent spatiotemporal control of the MMP activity, so that these enzymes can accomplish their tasks, but the detrimental effects of their propagation are restricted. Most MMPs are released immediately following their synthesis without storage and accordingly the regulation at transcriptional level is of primary importance for the appropriate MMP activity in the tissues.

The regulatory region of some MMPs contains an activator protein 1 (AP-1) binding site, where the heterodimers of c-*fos* and c-*jun* gene products bind (7), as well as a nuclear factor κ B (NFκB) binding site (8). The induction and activation of these gene products are under the control of cytokines (interleukins, tumor necrosis

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factor-α, interferons) and growth factors (epithelial, fibroblast growth factor), which can stimulate the expression of MMP-9 through activation of signaling pathways including a cascade of tyrosine and serine/threonine protein kinases as recently reviewed by Dong et al. (9). The expression of MMP-1 depends also on the presence of substrate: type I collagen binds to a cell surface tyrosine-kinase receptor with a discoidin domain, which is activated by native collagen and inhibited by partially digested collagen (10,11). Thus, MMP-1 is repressed by its own product. MMP-8 and MMP-9 in neutrophil granulocytes and MMP-12 in macrophages are exceptions from the general rule, because after their synthesis they are stored in secretion granules and released later by various triggers (e.g. inflammatory mediators, thrombin). Thus, these MMPs are present in the leukocytes, but in sequestered form and can exert their effects only at sites of inflammation.

Because MMPs are produced in zymogenic form, their release does not result in automatically proteolytic activity. The sulfhydryl group of a cystein residue in the propeptide binds the active site Zn^{2+} and blocks it. The Cys-Zn^{2+} interaction should be disrupted for the activation of the proenzyme either by proteolytic or conformational modification, which results in binding of a water molecule as a fourth ligand of the Zn^{2+} for the attack on the target peptide bond in the MMP-substrate. The activation proceeds through two stages. At first, a portion of the propeptide is removed by partial proteolysis (usually catalyzed by a serine protease) or a chemical agent (an *in vivo* relevant molecular species may be peroxinitrite formed in the reaction of NO and O_2^-) which destabilizes the Cys-Zn^{2+} bond allowing water to bind the active site, which gains partial activity in this way (12). In the second stage, this partially active MMP autocatalytically removes the remnant propeptide (alternatively another MMP can catalyze this step) as a result of which the active site adopts its maximally active conformation.

In the case of MT-MMP the initial activation step is catalyzed by the intracellular serine protease furin and thus when these enzymes are translocated to the plasma membrane, they are already fully active. The MT-MMPs take part in the activation of other MMPs in the extracellular space contributing to the tightly controlled pericellular remodelling of the ECM. In the case of soluble MMPs the first activation step occurs out of the cell, but it is also restricted to the pericellular space. For instance, polarized expression of urokinase receptor on the cell surface promotes

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plasminogen activation by urokinase at distinct sites of the plasma membrane and the generated plasmin activates pro-MMPs in the near-by pericellular compartment (13).

The action of MMPs is restricted in time and space by their endogenous inhibitors, which belong to the four-member protein family of tissue inhibitors of metalloproteinases (TIMP-1, 2, 3, 4) present in the extracellular space at much higher concentrations than the MMPs (14). TIMPs are 20 – 29 kDa proteins, the N- and C-terminal domains of which have distinct functions. The free α-amino group of the N-terminal Cys as well as its carbonyl group form coordination bonds with the active site Zn^{2+} of the MMPs, whereas the N-terminal Cys-Thr-Cys-Val-Pro peptide is tightly aligned to the substrate binding site of the enzyme. The C-terminal domain contributes to the specificity of the TIMP, e.g. the C-terminal of TIMP-1 binds with higher affinity to the hemopexin domain of MMP-9 than to MMP-2, whereas TIMP-2 shows a reverse order of affinity. TIMPs restrict the action of the MMPs, but the TIMP-MMP complexes are reversible. Thus, their eventual elimination requires one more inhibitor, α_2-makroglobulin (α_2-M) from blood plasma. MMPs cleave a peptide bond in the reactive site of α_2-M followed by a conformational change in the molecule of the inhibitor, which thus entraps the protease (15). The α_2-M-MMP complexes are then eliminated from circulation through scavenger receptor mediated endocytosis in the macrophages.

If present at low, non-saturating concentrations, TIMPs act as cofactors in the proMMP activation. Playing this role the N-terminal domain of TIMP-2 binds the active site of MT1-MMP, whereas the C-terminal domain of the same TIMP-2 molecule can bind the hemopexin domain of a proMMP-2. In this way TIMP-2 converts some of the MT1-MMP molecules to proMMP-2 receptors, which anchor the zymogens and present them for more efficient activation by other, TIMP-free MT1-MMP molecules, which act as proMMP activators (16). Similarly, TIMP-3 binds to heparan-sulfate and presents the proenzymes to their activators (17).

THE ROLE OF MMPs IN THE ATHEROSCLEROTIC LESIONS

Ischemic stroke is typically provoked by acute thrombosis over ruptured atherosclerotic plaque resulting in complete or partial occlusion of the affected artery. The most common type of atherosclerotic lesion is the atheroma composed of a lipid core and a thin fibrous cap comprising smooth muscle cells and ECM rich in type I

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and III collagen. If the cap is disrupted, blood infiltrates the core region and the activation of platelets by ECM components triggers thrombus formation. Thus, the stability of the atherosclerotic plaques depends primarily on the structural integrity of the fibrous cap. The ECM of the atheroma undergoes continuous remodeling as balanced protein synthesis and degradation with MMPs on the proteolytic side of this balance. Strong evidence supports the contribution of MMPs to the instability of the plaque. Quantitative analysis of the MMP content in human carotid plaques obtained from endarterectomy indicates definite increase in the MMP-9 antigen and mRNA (18). The concentration of MMP-9 correlates with the histological signs of plaque instability (plaque rupture, intraplaque hemorrhage) and the severity of clinical symptoms (spontaneous cerebral particulate embolization detected with transcranial Doppler) (18). MMP-1 protein and mRNA are also detected in carotid endarterectomy specimens (19). The level of MMP-1 correlates with the areas of hemorrhage in the plaques (19). The cleavage of the native triple collagen helix is the unique property of three MMPs (MMP-1, MMP-8 and MMP-13). The overexpression of all three of them has been shown in vulnerable human atheroma. In addition to MMP-1, MMP-13 co-localizes with cleaved collagen in the plaques with macrophages and endothelial cells as potential sources of this protease (20). Although MMP-8 is typically expressed by neutrophils, macrophages in human carotid atheroma have also been shown to produce MMP-8, which co-localizes with cleaved collagen (21).

The atherosclerotic plaque is a focus of chronic inflammation and overexpression of MMPs in the plaque is related to the inducing effects of inflammatory mediators. Tumor necrosis factor-α and interleukin-1 trigger MMP-1, MMP-3 and MMP-9 release in smooth muscle cells (22). Macrophages are also an important source of MMPs and the lipid-load stimulates the production of MMP-1 and 3 in macrophage-derived foam cells (23), whereas tumor necrosis factor-α and interleukin-1 induce MMP-9 (24). In addition to these inducing effects, the local generation of reactive oxygen species by inflammatory cells in the atherosclerotic plaques supports the activation of the pro-enzyme forms of the MMPs (25). Inflammatory mediators contribute not only to the overexpression of MMPs in the vulnerable plaques, but also to the impairment of collagen synthesis in the fibrous cap and thus to plaque instability. Interferon-γ has been shown to suppress the expression of collagen genes in smooth muscle cells (26). In the fibrous cap the amount of type I

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collagen is definitely lower around T-cells producing interferon-γ (27). At basal level the synthesis of ECM molecules by smooth muscle cells is limited, but MMPs may promote a switch from a contractile to a synthetic phenotype in these cells. Collagenase treatment stimulates a 25-30-fold increase in the collagen synthesis (primarily type I) by smooth muscle cells (28). The defect in this phenotypic switch may explain some surprising data that the collagen content in atherosclerotic lesions of MMP-9 knock-out mice is lower than in the wild-type mice (29). In this respect the substrate preferences of the MMPs may be of special importance; the proteolytic activity of MMP-9 on type IV collagen seems to be a prerequisite for basal membrane breakdown and consequently for the migration and phenotypic switch of smooth muscle cells in atherosclerotic lesions. In line with this role of MMPs, the neointimal thickening of carotid arteries in a murine blood-cessation model requires migration of cells that is preceded by increase in the MMP-9 activity (30). Smooth muscles cells from MMP-9 knock-out mice show decreased migratory activity (31). Thus, the role of MMPs in the atherosclerotic plaques should not be oversimplified. Clearly, the MMPs promote instability of the atherosclerotic plaques through degradation of ECM components, but their activity may also favor the penetration of proliferating cell into the neointima. The increase in the cell number in the area of the atheroma, however, favors the synthesis of new ECM components and thus may improve the stability of the plaque.

As discussed in the preceding section, the proteolytic activity of MMPs is under the control of TIMPs. Accordingly, the fate of the atherosclerotic plaque is affected by the local expression of TIMPs, too. TIMP-1 and TIMP-2 are constitutively secreted by smooth muscle cells in normal and atherosclerotic arteries (32). Macrophages in the atherosclerotic plaques express TIMP-1, TIMP-2 and TIMP-3 (33). Importantly, the triggers of TIMP expression differ from those for MMP expression. The constitutive expression of TIMP-1 and TIMP-2 is not affected by the cytokines discussed above as inducers of MMP (tumor necrosis factor-α and interleukin-1). In contrast, growth factors known to promote neointima formation (platelet-derived growth factor, transforming growth factor-β) promote TIMP-1 and TIMP-3 production by atherosclerotic lesion-associated macrophages (33). Thus, these fibrogenic growth factors may mediate a defense mechanism against plaque rupture.

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EVIDENCE FOR THE ROLE OF MMPs IN THE STROKE-RELATED DISRUPTION OF THE BLOOD-BRAIN BARRIER

Solid experimental and clinical observations support the concept for the involvement of the MMPs in the haemorrhagic transformation of the ischemic stroke (34). The earliest data come from direct intracerebral injection of MMP (collagenase), which provokes hematoma and brain edema in rats (35,36). After experimental cerebral vascular occlusion and reperfusion in rats, MMP-2 activity increases in the brain tissue and in parallel the BBB opens to proteins (37). Experimental ischemia causes elevation of the MMP-9 activity in the brain of mice (38) and rats (39), whereas MMP inhibitors reduce the infarct volume in rats (39) and the thrombolysis-induced haemorrhage after thromboembolic stroke in rabbits (40). The activity of MMP-2 and MMP-9 in the brain and of MMP-9 in the blood plasma increases during focal cerebral ischemia in baboons (41). Strong correlation exists between the size of brain lesion and the level of MMP-9 in blood of patients with acute ischemic stroke (recently reviewed in 42), middle cerebral artery occlusion or cardioembolic stroke independently of the applied fibrinolytic therapy (43,44). In addition, the increased MMP-9 level in blood is a predictor for poor outcome and hemorrhagic complications following tissue-type plasminogen activator (tPA) treatment of ischemic stroke (45). MMP-9 knock-out mice are protected against injury in transient focal ischemia and the BBB disruption following ischemic insult is attenuated in the MMP-9 knock-out mice compared to the wild-type (46,47), but MMP-2 gene knock-out does not provide neuroprotection in mouse models of permanent and transient middle cerebral artery occlusion (48). MMP-2 and MMP-9 have been shown to differentially mediate disruption of the BBB and neuronal damage after cerebral ischemia. These two proteases may play different roles in human ischemic stroke. Increase in plasma MMP-2 is observed only in patients with lacunar stroke early (within 12 hours), and is related to better outcome. In contrast, increase in plasma MMP-9 seems to be late (at day 7) and related to more severe stroke (49).

BBB opening during focal cerebral ischemia/reperfusion has long been considered to follow a biphasic time course. In a transient middle cerebral artery occlusion rat model, the initial opening of the BBB occurred 3 hours after reperfusion, and increased activation of MMP-2 correlated with the early opening of the BBB. The early degradation of occludin and claudin-5 seems to be associated with MMP-2

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activity (50). Experimental data demonstrate greater increase in MMP-2 than in MMP-9 at 3 hours, with increased expression of furin and MT1-MMP, the activators of MMP-2. In contrast, there was no increase in MMP-2 mRNA and activity, while MMP-9 mRNA and activity increased at 24 hours of reperfusion, suggesting a role of MMP-9 in mediating the second, delayed opening of BBB after ischemic stroke.

Although MMP activity is minimal at basal level in brain, multiple cellular sources exist in the central nervous system. Immunohistochemistry data evidence localization of MMP-2 in astrocytes, MMP-3 in microglial cells and MMP-9 in endothelial cells around experimentally induced ischemic core in rat brain (51). Data from cell culture of human capillary endothelial cells (HBEC) evidence that these cells produce and secrete MMP-9 (52). It seems that the type of MMP expressed in different brain cells shows species specificity. Our data (52) on the gelatinolytic activity in the supernatants of human brain capillary endothelial cells and their immunoreactivity confirm that these cells express MMP-9, but not MMP-1, -2, or -3 in contrast to the reported expression of MMP-2 in rat brain capillary endothelial cells (53) and in agreement with the MMP-2 localisation in astrocytes around experimentally induced ischemic foci in rat brain (51). Although extrapolation of data from knock-out mice to the role of MMPs in human disease should be treated with due caution, mice data indicate that inflammatory cells, leukocytes and bone marrow-derived cells appear to be an important alternative source of MMP-9 in stroke. Neutrophil leukocytes contain pro-MMP-9 in secretory granules, and the factors that promote degranulation of these cells are available in the ischemic brain. With the use of MMP-9 knockout and chimeric knockout (lacking MMP-9 in leukocytes or in resident brain cells) mice, the loss of BBB integrity and neuronal injury can be attributed to leukocyte MMP-9 during the initial 8 hours of reperfusion after focal stroke (54). Another similar study using chimera knockout mice showed that bone marrow cells derived MMP-9 contributes to BBB disruption and increase in infarct volume after reperfusion (55).

TRIGGERS OF MMP ACTIVITY IN THE ISCHEMIC BRAIN

Hypoxia on its own is a strong trigger of gelatinolytic activity in human brain endothelial cells, which according to the immunoblotting evaluation can be attributed to MMP-9 release. This effect is almost maximal at 4-h exposure to mild hypoxia and at this early time it is matrix-dependent (no effect in the absence of

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collagen). The collagen-dependence of the MMP-9 expression is not surprising; similar dependence has been shown for other metalloproteinases (MMP-2, MT1-MMP) from endothelial cells of different origin (56). Following reoxygenation for a period of 24 h and even 5 days the MMP-9 activity expressed early after the exposure to hypoxia is moderated down to the normoxic basal level independently of the matrix. Additional factors, however, may prolong the action of MMP-9: the specific activity in the conditioned media of cultured human brain endothelial cells indicates that the strongest trigger of the MMP-9 expression for the 24-h incubation period is the exposure to reactive oxygen (H_2O_2) after hypoxia (a 6-fold increase compared to the basal level) (52). H_2O_2 can be used as a stable reactive oxygen species to model the reperfusion phase after ischemic brain injury. Excessive production of H_2O_2 has been shown in postischemic reperfusion (57,58) and this H_2O_2 causes ATP-depletion and severe abnormalities in the ion (Na^+, Ca^{2+}) homeostasis of the affected neurons (59). At concentrations corresponding to the *in vivo* data reported for reperfused areas of ischemic rat brain (up to 100 μM) (60), but still below the cytotoxicity threshold of H_2O_2 (61), H_2O_2 exerts its effects on the MMP-9 expression. The time-course of this hypoxia-induced and active oxygen-maintained MMP-9 expression in HBECs is in good agreement with the reported temporal pattern in ischemic brain (62) and this supports the concept that the microvascular endothelial cells can be the source of MMP-9 activity in the ischemic brain.

The effect of MMPs depends strongly on the inhibitor potential in the extracellular space. The major natural inhibitor of MMP-9 is TIMP-1 and according to our data (52) the constitutive expression of this inhibitor is not affected by the triggers that stimulate the MMP-9 activity. Thus, it is justified to regard the changes in the MMP-9 activity as a result of changed expression or activation (against the background of constant inhibitor level). The other potential inhibitor, TIMP-2 is present in increased amounts in the conditioned media of HBECs following hypoxia and exposure to hydrogen-peroxide. Even in this situation, however, the concentration of the inhibitor in the medium (up to 2 nM) is an order of magnitude lower than the K_i for MMP-9 (40 nM), thus TIMP-2 cannot be taken into consideration as a relevant factor influencing MMP-9 in the medium. The profile of TIMPs produced in human brain microcirculatory endothelial cells resembles that in mouse (63) in contrast to rat, where only the expression of TIMP-2 has been reported (53). The constitutive

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presence of TIMP-1 provides an additional link to the control of MMP-9 activity in posthypoxic inflammation loci. This inhibitor is preferentially degraded by polymorphonuclear leukocyte elastase (PMN-elastase) (64), a protease released by inflammatory cells. Thus, this leukocyte-derived protease can promote extracellular matrix degradation in the cerebral microvasculature in at least three ways: through its own proteolytic action, induction of MMP-9 (see later) and modulation of the MMP-9 inhibitory system.

The oxidative stress-dependent MMP-9 induction is accompanied by a significant increase in the amount of NF-κB localized in the nuclei. Hypoxia or H_2O_2 separately and their combination significantly elevate the nuclear level of NF-κB. The variations in the effects of these three triggers can be attributed to their controversial impact on the activation of NF-κB and the redox-state of the intracellular glutathione. The DNA-binding of the activated NF-κB is inhibited by oxidized glutathione, but the concentration of the latter decreases in hypoxia (65) with consequent optimal NF-κB response. H_2O_2 directly activates NF-κB, but when applied in the post-hypoxic state it oxidizes glutathione and this shift in the redox state partially reverses the observed stimulation of NF-κB in hypoxia. The functional role of the NF-κB in the transcription is supported by the decreased susceptibility of NF-κB to the monoclonal antibody specific for the transactivation domain of NF-κB (52). Under certain post-hypoxic conditions the COOH-transactivation domain is functionally involved (and thus prevented from binding to detection antibodies) in interactions with the transcriptional machinery resulting in sustained MMP-9 gene expression. At basal state the NF-κB is present in a latent form in the cytosol as a heterodimer with an inhibitory protein (IκB) and the destruction of the latter by the 20S proteasome allows the nuclear translocation of NF-κB. When the activation of NF-κB is blocked with an inhibitor of IκB degradation (MG132), the post-hypoxic MMP-9 response to H_2O_2 is blunted in parallel to a decrease in the NF-κB localized in the nucleus. It is notable that the effect of hypoxia/H_2O_2 on the nuclear NF-κB level and the cytoplasmic IκB is sustained for a period as long as 24 h after 4-h hypoxia followed by a bolus of H_2O_2. These temporal changes in the NF-κB/IκBα levels and the accompanying MMP-9 activity derived from human brain capillary endothelial cells are in good agreement with the time-course of the MMP-9 changes observed in the animal models and

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patients discussed above. This supports the role of the described NF-κB signal transduction circuit in the control of the oxidative stress-dependent MMP activity in brain after ischemic insults.

Data from our laboratory (66,67) show that a set of serine proteases (thrombin, plasmin, miniplasmin and PMN-elastase) cause extravasation of circulating phase-borne tracer in a rat brain-perfusion model indicating that these proteases definitely change the structure of the BBB. This effect is related to the protease activity; if the active site of the enzymes is irreversibly blocked, the integrity of the BBB is not disturbed. These proteases are presumably active participants in the ischemic insult in brain. Thrombin is involved in the acute thrombus formation over ruptured atherosclerotic plaques or it is entrapped in active form in the fibrin mesh of cardioemboli and thus it can be carried downstream to the cerebral microvasculature. The ischemic brain injury is accompanied by inflammatory response in the ischemic locus and penumbrial area, a part of which is the infiltration by leukocytes releasing PMN-elastase and MMPs. In brain microcirculation plasmin may be generated from blood-borne plasminogen by tPA or urokinase produced in the microvascular endothelial cells. Alternatively, treatment of ischemic stroke with plasminogen activators is a potential source of plasmin. Only fibrin-bound proteases are efficient in fibrin solubilization, since free enzymes are not protected against blood plasma protease inhibitors. Thus, lysis of the occluding thrombus requires massive generation of fibrinolytic enzymes to escape the regulation by the plasma protease inhibitors at least in the immediate vicinity of the fibrin clot. If the amount of fibrinolytic enzymes increases over the locally available concentration of plasma protease inhibitors, they may bind to endothelial cells in the downstream vascular bed, where they are also protected against the plasma protease inhibitors. Reversible, specific and saturable binding of plasmin, thrombin and PMN-elastase to the brain capillary endothelial cells has been documented (66,67). The affinity (in terms of dissociation equilibrium constant) of these proteases to the capillary endothelial cells is in the range of 10^{-7} M. The molecular nature of the cellular binding site has not been identified. The fact that thrombin, plasmin and PMN-elastase affect the morphology of the brain capillary endothelial cells in a similar way (all of them cause rapid contraction) raises the possibility for a common event in their action. Interference of plasmin with the thrombin effects on a different cell-type (glioma cells) has been reported (68) and this

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has been interpreted as a result of the cleavage of the thrombin-receptor by plasmin. Similar interplay of enzymes on a single molecular target is a possibility for the phenomena observed in brain capillary endothelial cells (the number of binding sites for the different proteases varies within a very narrow range). The family of proteolytically activated cellular receptors (PAR) is a potential candidate target for the action of the discussed serine proteases.

A highly specific serine protease, tPA is found mostly in blood, its main function is to convert plasminogen to plasmin. In the brain, tPA has been shown in the endothelial cells of the BBB, where it may regulate BBB permeability and vascular tone. This enzyme is also expressed by neurons and microglia, and causes microglial activation and neuronal death after injury (69). In the brain, tPA is regulated by neuroserpin, a member of the serine proteinase inhibitor family. In rodent stroke models, endogenous tPA activity in the brain is induced as early as 1 hour after focal cerebral ischemia, and is present in the perivascular tissue and the vessel wall. tPA inhibition by neuroserpin decreases BBB disruption, edema, neuronal death. Levels of MMP-9 in ischemic brain are significantly reduced in tPA knock-out mice compared to wild-type animals, exogenous tPA administration increases MMP-9 response to wild-type levels (70). tPA increases MMP-9 expression and activity resulting in the degradation of claudin-5 and development of cerebral edema (71). It has been shown that MMP-3 plays a role in the intracranial bleeding after tPA treatment of stroke in mice. MMP-3 expression is enhanced in the neurons of the ischemic hemisphere, but upregulation can be seen in the endothelial cells after tPA treatment. In vitro, tPA induces MMP-3 in murine brain endothelial cells (72,73). tPA can bind to LRP (LDL receptor related protein), which can be found in the brain neurons and in perivascular astrocytes. Injection of recombinant tPA into the cerebrospinal fluid increase BBB permeability, and this effect is reversed by blocking LRP using anti-LRP antibodies (74). Recombinant tPA stimulates MMP-9 expression in cultured human brain endothelial cells, and this effect is reduced when the cells were treated with siRNA to suppress LRP. tPA induces MMP-3 in cultured murine brain endothelial cells, and this induction is prevented by inhibition either of LRP or NF-κB activation (73). These data suggest that tPA can induce MMP-9 and MMP-3 via LRP/NF-κB with consequent BBB damage.

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The enzyme-induced endothelial cell contraction and the consequent gap formation in the endothelial lining of the brain capillaries could essentially modify the permeability of the BBB. In addition to their direct effect on cell morphology thrombin and PMN-elastase are strong triggers of the MMP-9 expression in HBECs. Plasmin does not induce MMP-9 in brain endothelium, but its role in the partial proteolytic activation of proenzyme is known (6). The role of these proteases in the hemostatic balance helps the interpretation of their effects on the expression of MMP activity in brain microcirculation. Fibrinogen is converted by thrombin to form fibrin, the solid matrix of hemostatic plugs, but it is not digested by catalytic amount of MMP-9 in solution (75). Thus, *in vivo* MMP-9 is not expected to interfere with the basic haemostatic function of thrombin and to contribute to the propagation of the haemorrhage. In contrast, higher pericellular concentrations of MMP-9 could contribute to the removal of fibrin deposits (76) and consequently counteract the clot-forming action of thrombin. Altogether, the thrombin-induced MMP-9 response of brain endothelium may serve the protection of the cerebral microvasculature against fibrin deposition under physiological conditions, but in ischemic stroke especially of cardioembolic origin thrombin reaching the capillary endothelium may contribute to the disruption of the vascular integrity with consequent haemorrhagic transformation. Thus, paradoxically under hypoxic conditions thrombin generates a pericellular zone of enhanced fibrinolysis in the BBB. This pathophysiological impact of thrombin is further enhanced by the locally formed plasmin and the PMN-elastase released from inflammatory cells through their direct fibrinolytic action and induction/activation of MMP-9.

In addition to the serine proteases discussed above, activation of MMPs in the brain is related to the effects of NO (12). As a part of the nitrosative and oxidative stress following cerebral ischemia and reperfusion, the level of NO in the affected brain areas is high (77). NO reacts with cysteine residues in proteins and the resulting S-nitrosylated derivative modifies the function of the proteins. In the case of proMMPs this nitrosylation is followed by oxidation of the Cys thiol group to sulfonic acid, which disrupts the bond between the pro-peptide Cys and the active site Zn^{2+} with subsequent activation of the enzyme. In experimental mouse model of ischemic stroke and reperfusion elevated MMP-9 activity can be detected, which is abrogated

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in neuronal NO synthase knock-out mice or in wild-type mice treated with NO synthase inhibitor (12).

TARGETS OF MMPs IN THE CNS

The pericellular proteolysis targets a large range of matrix structural proteins, but it also affects the availability of the active form of signal molecules and the function of cellular receptors. The disruption of the extracellular matrix causes cell detachment and a special form of apoptotic cell death called anoikis (78, reviewed in 79).

An essential element of the barrier function of brain capillary endothelium is the tight junctions between the cells, the structure of which is based on zonae occludens-1 (ZO-1) protein (80). ZO-1 is a substrate of MMP-9 and following ischemic brain injury its extensive degradation can be detected, 24 h after the ischemic insult the ZO-1 level in the affected area is less than 30 % of the baseline (46). MMP-9 was demonstrated to degrade also occludin and claudin-5 in focal cerebral ischemia (50). The role of MMP-9 in the post-ischemic disruption of the brain endothelial tight junctions is supported by the lack of ZO-1 degradation in MMP-9 knock-out mice under the same conditions of ischemic insult. Several proteins of the basal lamina in brain microcirculation are also degraded following ischemic damage (laminin, collagen type IV and cellular fibronectin) (81), all of which are substrates of MMP-9. Collagen degradation after cerebral ischemia might be in part caused by MMP-13 (43). Another matrix metalloproteinase, MMP-3 can also be activated after ischemia in rat brain, causing cleavage the cerebral matrix agrin (82). MMP-3 can contribute to BBB opening during neuroinflammation after intra-cerebral lipopolysaccharide injection in mice. MMP-3 knock-out mice showed less degradation of tight junction proteins (occludin, claudin-5, laminin-alpha-1), compared to wild-type animals (83).

Although the endothelial cells are a major source of MMP-9 in the brain and so the tight-junction and basal lamina proteins are easily accessible to the protease, the role of MMP-9 in the degradation of brain extracellular proteins is not restricted to the BBB. The myelin basic protein is a known substrate of MMP-9 and its

DOI: 10.2478/s11535-011-0030-z
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degradation accompanies the ischemic damage of the white matter. MMP-9 knock-out mice, which have reduced lesion size after transient ischemia, are protected against degradation of the myelin basic protein too (46). Similarly to the situation in BBB the proteolysis in the white matter is not general, but has specific targets; two other myelin proteins (PLP and DM20) are not affected in the ischemic regions.

The MMP-9 induced neuronal apoptosis (12) may be related to the degradation of basement membrane proteins in brain (84). MMP-9 activity is co-localized with laminin early after transient ischemia in mouse brain and progressive loss of laminin immunoreactivity is observed later, which is prevented by MMP inhibitors. The disruption of the cell-matrix connection caused by the laminin degradation is suggested as a trigger of neuronal apoptosis (84). This hypothesis is supported by the effect of a laminin-blocking antibody, which abrogates the protective effects of MMP-inhibitors in the early post-ischemic brain damage. Detachment from the matrix proteins triggers a suicide cell program called anoikis (85). The intercellular junctions and the matrix-cell interactions definitely act as a survival signal in capillary endothelial cells (86) and thus the degradation of ZO-1 and laminin by MMP-9 can trigger programmed cell death in the post-ischemic brain areas.

An additional role of MMPs at the site of ischemic brain injury is the local control of the inflammatory reaction. MMP-9 cleaves the 6 N-terminal amino acids of interleukin-8 and thus converts it to a ten-fold more potent chemoattractant of neutrophil leukocytes (87). On their own neutrophils represent a source of MMP-9 and MMP-8, thus forming a self-accelerating circuit promoting inflammation and further increase in the local MMP activity. Another chemokine, stromal cell-derived factor 1 (SDF-1) is cleaved at position 4-5 by MMP-1 -,2, -3, -9, -13 and 14, following which it loses its chemoattractant activity (88). The resultant des(1-4)-SDF-1, however, adopts strong neurotoxicity (89). The cleaved SDF-1 triggers apoptosis in neurons *in vitro* and following stereotactic implantation in the striatum of mice it provokes massive neuronal loss accompanied by hypertrophy of microglia and astrocytes (gliosis) seen phenotypically as behavioral and motor abnormalities.

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The upregulation of MMPs in the early (up to 24 h) period after an ischemic insult has detrimental effects on the BBB and the affected neurons, but their function may be beneficial later, in the stage of tissue regeneration and remodelling.

The subventricular zone of the lateral ventricles and the subgranular zone of the hippocampus are critical structures for neurogenesis in adult brain. Increased neurogenesis can be triggered by central nervous system insults such as stroke or trauma. Stroke leads to the expansion of subventricular zone and produces BrdU-labeled immature cells and doublecortin-positive neural precursors. At 2 weeks after stroke in mice, MMP-9 was enhanced in the subventricular zone and colocalized with BrdU and doublecortin-positive neuroblast cells (90). MMP-3 also participated in neurogenesis, and together with MMP-9 mediated the differentiation and chemokine-induced cell migration of adult neural or stem progenitors (91). Inhibition of MMPs reduced the extension of neuroblast signals that extended from the subventricular zone into the damaged striatum. After middle cerebral artery occlusion/reperfusion in rat, the number of MMP-13 positive neurons, mainly in the border zone of the infarct, increased at day 3, peaked after a week, and 14 days later it was still higher than in normal tissue (92). The observed co-localization of the proliferation marker BrdU and MMP-13 suggests that the presence of newborn neurons is related to MMP-13 activity. The post-ischemic tissue regeneration involves not only a cellular response, but also reorganization of the ECM proteins, as seen in the accumulation of aggrecan, a histomorphogenetic proteoglycan in brain, around MMP-13 positive cells in the periinfarct zone (92). These data indicate that MMPs may contribute to endogenous repair mechanisms by helping the migration and proliferation of neuroblasts after stroke.

In contrast to the situation in the acute (up to 24-48 h) post-ischemic stage, when the increase in MMP-9 activity is associated with the endothelial cells of the BBB, on days 7-14 after transient focal brain ischemia in rats MMP-9 expression is localized to neurons and astrocytes in the peri-infarct zone (93). Rat endothelial cell antigen-1 (RECA-1) is a marker of microvessel endothelial cells and it is upregulated in the peri-infarct area on days 10-14 after a 2-hour transient focal ischemia. The protein product of the *Egr1* immediate early gene is associated with neuron sprouting

DOI: 10.2478/s11535-011-0030-z
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and its expression is also stimulated in this later stage after a stroke. Both RECA-1 and Egr1 responses are abrogated by MMP inhibitors applied at the time preceding their maximal expression suggesting a causative relation between the MMP-9 expression and the tissue recovery after an ischemic insult. MMP inhibitors applied later than day 7 after the transient focal brain ischemia significantly increase the infraction size and the occurrence of delayed gross cerebral hemorrhage (93). MMP-9 is not neuroprotective on its own; through the disruption of the cell-matrix connections it even induces anoikis as discussed above. Thus, its beneficial effects are presumably related to its role in the delayed neurovascular remodelling. An important step in brain recovery is the reformation of damaged myelin, which is based on the oligodendrocyte precursor migration, process extension and ensheathment of axons by these processes. Cultured oligodendrocytes from MMP-9 knock-out mice have diminished capacity to reform such processes (94). The NG2 chondroitin sulfate proteoglycan is inhibitory on axon regeneration, because the NG2-rich matrix is unfavourable for the maturation of progenitor cells into oligodendrocytes (95). MMP-9 degrades NG2 and thus it promotes remyelination as a part of the brain recovery process (95). Phosphorylated neurofilament M is a marker of regenerative elongation of axons and it is induced by MMP-9 treatment in a neuron cell-culture model, whereas anti-MMP-9 antibodies inhibit it, supporting the role of MMP-9 in neuron regeneration (96).

In addition to the neuronal recovery, MMP-9 is engaged in the vascular remodelling through its role in the regulation of angiogenic factors. Vascular endothelial growth factor (VEGF) is a potent inducer of angiogenesis. MMP-9 provokes angiogenesis in organ culture (97) and in parallel VEGF is released in the medium. A proposed mechanism for this effect is the mobilization of VEGF from its bound form to an extracellular proteoglycan following proteolysis by MMP-9. The cerebral vascular perfusion is improved in the penumbra of the cortex as a result of VEGF infusion at 48 h after the ischemic insult in a rat stroke model (VEGF infusion at the onset of the acute stage, 1 h after the artery occlusion has detrimental effects, causing BBB leakage and gross hemorrhage) (98). This improved perfusion is due to the appearance of newly grown vessels in the penumbra. The functional neurological recovery of the rats exposed to VEGF infusion shows better results compared to non-treated animals in the same rat stroke model. VEGF levels are elevated in the

DOI: 10.2478/s11535-011-0030-z
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penumbra 14 days after transient focal ischemia, coinciding with MMP-9 elevation (93). MMP inhibitors abrogate this VEGF response and increase the infarct volume, whereas exogenous VEGF treatment restricts the infarct size, even if MMP inhibitors are also applied. All these data support the concept that MMP-9 is an essential factor in the vascular recovery following ischemic stroke through VEGF-mediated effects. Not only MMP-9, but TIMPs can also affect angiogenesis. TIMPs can inhibit angiogenesis through MMP inhibition, as well as MMP-independent mechanisms such as interference with VEGF signaling. TIMP-2 has been shown to disturb intracellular phosphorylation and activation of VEGFR-2 (vascular endothelial growth factor receptor-2) (99), and TIMP-3 is able to block VEGF binding to VEGFR-2 (100).

In summary, the data accumulated in the last decade confirm well-defined functions of MMPs in the pathomechanism of ischemic stroke. In the atherosclerotic lesion the basic effect of MMPs is the degradation of ECM components with consequent instability of the plaques. Within the brain MMP activity is generally detrimental in the initial acute phase (within 24 h after the ischemic insult), but beneficial in the delayed recovery period (several days after the acute event) (Fig.1). The interplay of ischemia-related chemical factors (hypoxia, reactive oxygen species, NO), inflammatory mediators (IL-1β, TNF-α) and regulatory proteolytic activity (MMP-3, MT1-MMP, plasminogen/plasminogen activator system) results in massive activation of effector MMPs (MMP-9, MMP-13, MMP-2) in the ischemic brain areas. However, the same proteolytic actions appear essential in the regenerative remodeling of the post-ischemic brain tissue. This spatial and temporal pattern of MMP action should be considered with respect to the design of pharmacological intervention targeting these proteases.

Acknowledgements

This work was supported by a grant from the Hungarian Research Fund OTKA [K83023].

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DOI: 10.2478/s11535-011-0030-z
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Table 1. Major MMP classes based on structural characteristics and substrate preferences.

MMP class	Representative enzyme	Structural features	Substrates
Collagenases	MMP-1 MMP-8 MMP-13 MMP-18	PCHH	type I, II and III interstitial collagen
Gelatinases	MMP-2 (gelatinase-A) MMP-9 (gelatinase-B)	PCHH + three fibronectin-like domains for gelatin and laminin binding	type IV collagen gelatin (denatured and partially degraded collagen)
Stromelysins	MMP-3 (stromelysin-1) MMP-10 (stromelysin-2) MMP-11 (stromelysin-3)	PCHH	proMMP activation ECM proteins
Matrilysins	MMP-7 (matrilysin-1) MMP-26 (matrilysin-2)	PCHH without a hemopexin domain	ECM proteins, pro-α-defensin, Fas-ligand, pro-TNF-α, cadherin E
Membrane-type MMPs	MMP-14 MMP-15 MMP-16 MMP-24	PCHH + 4 TM domains	type I, II, III collagens proMMP-2
	MMP-17 MMP-25	PCHH + GPI anchor	
Other MMPs	MMP-12 (macrophage metalloelastase)	PCHH	elastin other ECM proteins

Abbreviations: TM, transmembrane; GPI, glycosyl-phosphatidylinositol; PCHH, propeptide – catalytic – hinge – hemopexin domains

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Figure legend

Fig. 1. Mechanisms of activation and biphasic roles of MMPs in stroke.

Ischemia enhances proMMP-9 gene transcription through hypoxia, IL-1β, TNF-α and tPA. The proenzyme can be activated by multiple proteases, such as other MMPs, plasmin and by free radicals (ROS, NO). The early activation of the MMPs after stroke degenerates neurovascular matrix and BBB, disrupts cell-matrix homeostasis, and causes neuronal death, edema, hemorrhage. Delayed actions of these enzymes involve neural and vascular remodeling, that may contribute to brain recovery after stroke.
Abbreviations: IL-1β, interleukin-1β; TNF-α, tumor necrosis factor- α; tPA, tissue plasminogen activator; LRP, LDL receptor related protein; NF-κB, nuclear factor κB, ROS, reactive oxygen species; NO, nitric oxide; (pro)MMP, (pro) matrix metalloproteinase.

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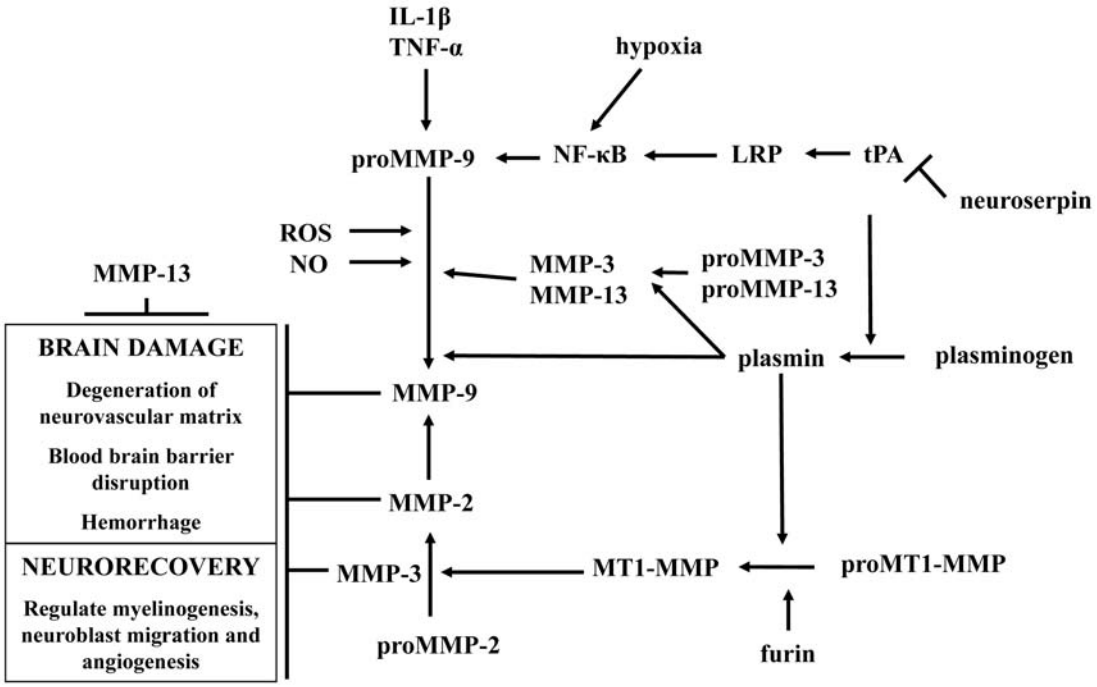
IL-1β
TNF-α
hypoxia
proMMP-9
NF-κB
LRP
tPA
neuroserpin
ROS
NO
MMP-3
MMP-13
proMMP-3
proMMP-13
MMP-13
plasmin
plasminogen
BRAIN DAMAGE
Degeneration of neurovascular matrix
Blood brain barrier disruption
Hemorrhage
MMP-9
MMP-2
NEURORECOVERY
Regulate myelinogenesis, neuroblast migration and angiogenesis
MMP-3
MT1-MMP
proMT1-MMP
proMMP-2
furin

Figure 1