Cerebral Mast Cells Mediate Blood-Brain Barrier Disruption in Acute Experimental Ischemic Stroke Through Perivascular Gelatinase Activation

Olli S. Mattila, MB*; Daniel Strbian, MD, PhD*; Jani Saksi, BSc*; Tero O. Pikkarainen, BSc; Ville Rantanen, MSc; Turgut Tatlisumak, MD, PhD; Perttu J. Lindsberg, MD, PhD

Background and Purpose—Perivascularly positioned cerebral mast cells (MC) have been shown to participate in acute blood-brain barrier disruption and expansive brain edema following experimental transient cerebral ischemia. However, the underlying molecular mechanisms remain unknown. Because proteolytic gelatinase enzymes, matrix metalloproteinases (MMP)-2 and MMP-9, are thought to have a central role in compromising the integrity of the blood-brain barrier following ischemia, we examined whether cerebral MCs influence gelatinase activity in ischemic cerebral microvasculature.

Methods—Rats underwent 60 minutes of middle cerebral artery occlusion followed by 3-hour reperfusion, and were treated with a MC-stabilizing (cromoglycate), or MC-degranulating (compound 48/80) agent, or vehicle. Genetically manipulated, MC-deficient WsRc/Nugw/r rats and their wild-type littermates (WT) underwent the same procedures. Cerebral edema and extravasation of Evans blue albumin were measured. Gelatinase activity was visualized by in situ zymography and was quantified with computerized high-throughput image and data analysis.

Results—Activated MCs showed secretion of gelatinase-positive granules. Genetic MC deficiency decreased global gelatinase-active area (−69%, compared with WT; P<0.001) and the mean gelatinase activity of the ischemic microvasculature (−57% compared with WT; P=0.002). MC stabilization with cromoglycate decreased the percentage of microvessels with high gelatinase activity (−36% compared with saline; P<0.05). Compound 48/80 showed increased area of in situ zymography activity in the ischemic lesion (+55% compared with saline; P<0.001). Microvascular gelatinase activity correlated with brain swelling (r=0.84; P<0.001; and r=0.61; P=0.02).

Conclusions—Our data demonstrate that cerebral MCs participate in regulation of acute microvascular gelatinase activation and consequent blood-brain barrier disruption following transient cerebral ischemia. (Stroke. 2011;42:00-00.)

Key Words: mast cells ■ gelatinases ■ blood-brain barrier

Expansive brain edema is a frequent cause of death after large ischemic strokes, as it reduces blood flow in the ischemic penumbra and can lead to herniation of brain structures. Edema is predominantly caused by functional and structural disturbance of the blood-brain barrier (BBB). Proteolytic gelatinase enzymes, most importantly matrix metalloproteinases (MMP)-2 and MMP-9, are considered to be central mediators of ischemic BBB disruption; this is because of their ability to degrade components of microvascular basal lamina, especially collagen type IV, and to disrupt tight junction proteins. The mechanisms triggering early activation of gelatinases in ischemic brain remain under active debate.

Mast cells (MC) are resident inflammatory cells positioned in the outer layers of various tissues (eg, mucosa, epithelia, and vasculature) in mammals, including the central nervous system. We and others have previously shown that MCs have a role in the pathophysiology of ischemic, hypoxic-ischemic, and hemorrhagic brain injury. Both pharmacological inhibition and genetic deficiency of MCs led to a significant reduction in posts ischemic BBB disruption, cerebral edema, and neutrophil infiltration, presenting MC inhibition as a potential therapeutic avenue to prevent inflammatory damage to the neurovasculature, especially in conjunction with thrombolytics.

We envisaged a molecular pathway for ischemic BBB disruption where factors liberated from MCs might fuel gelatinase activation within the targeted neurovascular unit and lead to degradation of the microvascular wall, especially...
tight junctions and basement membrane.\textsuperscript{12,13} This led us to investigate whether MCs are involved in regulation of microvascular gelatinase activity following ischemia-reperfusion injury, with a focus on the early onset of BBB failure and expansive brain edema, which progressively degrade blood circulation in the tissue at risk, leading to acute dysfunction of the neurovascular unit.\textsuperscript{4}

**Materials and Methods**

**Animals**

The in vivo methodology has been described in detail previously.\textsuperscript{8} Briefly, adult, male, Wistar rats (Harlan Nederland) and WsRc\textsuperscript{Ws}/Ws\textsuperscript{Rs} rats (Japan SLC, Inc), 290 to 340g, were anesthetized with ketamin hydrochloride (intraperitoneal, 50 mg/kg, Ketalar, Parke-Davis) and medetomidine hydrochloride (subcutaneous, 0.5 mg/kg, Domitor, Orion). The left femoral artery and vein were cannulated for measurements of blood pressure, arterial pH, blood gases, and blood glucose and drug and/or vehicle infusions. Cerebral blood flow was measured on-line with laser-Doppler flowmetry (Oxy-Flow, Oxford Optronix) as described.\textsuperscript{9} Middle cerebral artery occlusion (MCAo) was induced with an intravascular suture for 60 minutes followed by reperfusion period, animals were euthanized with an overdose of pentobarbital (intraperitoneal, 60 mg/kg; Mebunat, Orion) and perfused intracardially with ice-cold saline. The brains were cut into 6-2 mm coronal slices, of which the third was further divided into 1-mm-thick rostral and caudal slices. The rostral section was immersed in Tissue-Tek (Sakura Finetek Inc) and snap-frozen with liquid nitrogen. Frozen 1-mm sections were stored at $-80^\circ\text{C}$ and sectioned into 8-μm cryosections for staining. For quantification of infarct volumes, all remaining slices were incubated for 15 minutes in 2,3,5-triphenyltetrazolium chloride at 37°C and subsequently fixed with 10% formaldehyde. Infarct volumes, brain swelling, and EBA leakage were calculated as described.\textsuperscript{8} A Typhoon9400 fluorescence scanner (Amersham Biosciences) was used for quantitative EBA imaging.

**In Situ Zymography**

Tissue gelatinase activity was visualized using in situ zymography (ISZ).\textsuperscript{10} Cryosections were air-dried for 5 minutes in room temperature and washed with phosphate-buffered saline. Sections were incubated for 2 hours in 37°C covered with DQ-gelatin (0.1 μg/μL in reaction buffer, EnzChek gelatinase/collagenase assay kit, Invitrogen). After incubation, the sections were washed twice in phosphate-buffered saline and once in dH\textsubscript{2}O. Control sections were incubated with MMP inhibitors ilomastate (500 μmol/L, GM6001, Millipore) or tissue inhibitor of metalloproteinase-1 (500 nmol/L, R&D Biosystems) 1 hour before and during incubation with DQ-gelatin 37°C, and showed blunted ISZ activity (data not shown). ISZ sections were imaged with a fluorescent microscope (Stallion, Carl Zeiss) and montage images of the coronal sections (10× magnification) were constructed (Slidebook v.4.1, Olympus/3i). Sections incubated without DQ-gelatin lacked fluorescent activity.

**Immunohistochemistry**

For automated quantification of microvascular ISZ-activity and colocalization with cellular markers, ISZ-sections were further stained with immunohistochemistry. We used primary antibodies for neurons (NeuN, A60 Millipore, 0.5 μg/mL), astrocytes (glial fibrillary acidic protein, G-A-S Sigma-Aldrich, 1:1000), endothelial cells (von Willebrand factor [vWF], rabbit polyclonal, Abcam, 19.5 μg/mL), basal lamina (collagen IV, rabbit polyclonal, Abcam, 0.63 μg/mL) and MMP-2 (rabbit polyclonal, Millipore, 5 μg/mL, periodate-lysin-parafomaldehyde-fixation). Alexafluor secondary antibodies were used (Invitrogen). Tetramethyl rhodamine isothiocyanate conjugated egg-white avidin (Sigma-Aldrich, 1:2000) stains MCs.\textsuperscript{11} Acetone fixation for 5 minutes and 30 minutes blocking with 0.1 mol/L TrisHCl, 3% bovine serum albumin, 20% fetal bovine serum were used with immunohistochemistry and avidin staining. Mouse IgG1 (Dako) and rabbit IgG (Vector Labs) were used in equivalent concentrations as negative controls and no specific immunostaining was detected.

For microvessel analysis, 5 regions of interest were acquired from vWF+ ISZ-double-stained sections (Figure 1A) from predefined sites (3 cortical, 2 subcortical) from both hemispheres at 20× magnification with an epifluorescent Axioplan2 microscope (Carl

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**Table. Drug Protocols in Rats Undergoing MCAo**

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ICV injection, 0.9 mm posterior and 1.6 mm lateral from the bregma, 3.4 mm depth from the skull surface. Modified with permission from Strbian et al.\textsuperscript{8} MCAo indicates middle cerebral artery occlusion.

*10 μL 
†100 μL.
‡Cromoglycate ICV
§C 48/80 intravenous compound 48/80 0.25 mg/mL in saline (100 μL).
Confocal microscopy was performed with a Zeiss LSM510 Meta microscope. Image Analysis

For quantification of microvascular gelatinase activity (Figure 1A), we developed an automated high-throughput image analysis workflow using the Anduril workflow framework (http://csbi.ltdk.helsinki.fi/anduril/). The program automatically segments microvessels from the endothelial cell marker channel (vWF, Figure 1B). Using this selection, the relative ISZ-brightness of each object is calculated as a margination value by dividing the object’s brightness by the brightness of a 20-pixel-wide ring surrounding the object (Figure 1C). When the margination value is >1, the object is brighter than its immediate local background, indicating elevated activity. The mean margination value of each animal represents the ISZ activation level of the whole analyzed microvasculature, whereas the percentage of microvessels with high ISZ-positivity refers to the portion of highly activated vessels (Figure 1A; http://stroke.ahajournals.org). Microvessels with high ISZ-activity were defined with a threshold value 1 SD higher than the mean margination value of the contralateral hemisphere of the saline/WT group in each experiment (Figure 1A).

To quantify gelatinase activity area within the ischemic lesion, montage images of coronal brain sections were analyzed (ImageJ, National Institutes of Health). Briefly, an intensity subtraction removed background noise. The infarcted brain area was outlined, and the total area of ISZ-positive pixels was calculated as percentage of total infarcted area. All image analyses were performed in a blinded fashion.

Statistics

Data are presented as mean±SEM. Statistical analyses were performed with unpaired t-test for comparisons of 2 groups, and continuous variables of multiple groups with 1-way ANOVA followed by the Holm-Sidak posthoc test. Kruskal-Wallis ANOVA was used for nonparametric variables. Spearman’s rank correlation was used. Significance was considered at \( P<0.05 \).

Results

Physiological Variables, Cerebral Blood Flow, and Corrected Infarct Volume

No significant intergroup differences existed in physiological parameters (body weight, mean arterial blood pressure, body temperature, pH, \( \text{paCO}_2 \), \( \text{paO}_2 \), blood glucose), cerebral blood flow, or corrected infarct volumes.8

Cellular Localization of Gelatinase Activity

We observed activated MCs surrounded by a halo of gelatinase-positive granules in the ischemic hemisphere (Figure 2A). Partly degranulated perivascular MCs showed strong gelatinase activation in the adjacent vessel segment (Figure 2B, Supplemental Figure II). Three hours after reperfusion, gelatinase activity in the ischemic hemisphere colocalized with a portion of vWF-positive microvessels (Figure 1A). In the contralateral hemisphere, this activity was weaker and...
Gene manipulation | Pharmacological modulation

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Figure 3. ISZ-activity area within the ischemic hemisphere. A Genetic MC deficiency led to a reduced area of gelatinase activity within the ischemic tissue compared with WT littermates \( (P<0.001) \). B There was a trend toward an intergroup difference in groups with pharmacological MC modulation \( (P=0.07; \text{ANOVA}) \). Separate t-tests showed a significant difference in groups with pharmacological MC modulation \( (P<0.001) \). Separate t-tests showed a significant difference between both saline and cromoglycate-treated groups compared with compound 48/80 (both \( P<0.001 \)).

sparse. NeuN-positive neurons within the ischemic lesion showed strong gelatinase activity (Figure 2C), which was localized in both the neuronal nucleus and cytosol. Again, gelatinase activity was weaker and more scattered in the contralateral hemisphere. Glial fibrillary acidic protein-labeled astrocytes only rarely colocalized with gelatinase activity (Figure 2D), mostly in the vicinity of ISZ-active microvessels (Supplemental Figure III). MMP-2 immunoreactivity colocalized with ISZ-active neurons and with a portion of ISZ-active microvessels, and showed staining of astrocytic endfeet that were not ISZ-active (Supplemental Figure IV).

Global Gelatinase Activity in the Ischemic Lesion

MC-deficient animals showed a significantly lower percentage of gelatinase-active area within the ischemic lesion than did WT littermates \((-69\%; P<0.001; \text{Figure 3A})\). A trend toward an intergroup difference in gelatinase-active area was seen in the pharmacological treatment groups \( (P=0.07; \text{ANOVA}) \). T-tests showed a significant increase in the compound 48/80-treated group (compared separately with cromoglycate \([+62\%]\) and saline \([+55\%]\), both \( P<0.001 \) Figure 3B). Pharmacologically treated, sham-operated animals showed equally low percentages of ISZ-active area: cromoglycate-sham \(2.43\%\pm0.30\), saline-sham \(2.96\%\pm1.00\), and compound-sham \(48/80\) \(4.08\%\pm1.00\) \( (P=0.43; \text{ANOVA}) \). Gel zymography from brain homogenate further confirmed gelatinase activity, detecting both 72kDa MMP-2 and 92kDa MMP-9 bands 3 hours after reperfusion (Supplemental Figure V).

Quantification of Microvascular Gelatinase Activity

MC deficiency was associated with significantly lower mean gelatinase activity of microvessels in the ischemic hemisphere as compared with WT \((-57\%; P=0.002; \text{Figure 4A, left})\). Similarly, the percentage of microvessels with high activity was also significantly lower in the ischemic hemisphere \((-64\%; P=0.003; \text{Figure 4B, left})\). Strikingly, in the MC-deficient groups, microvascular gelatinase activity of the ischemic hemisphere remained at a level equal to that of the contralateral hemisphere in both analyses. The pharmacological interventions also impacted the percentage of microvessels with high gelatinase activity in the ischemic hemisphere \( (P=0.02; \text{ANOVA}; \text{Figure 4B, right}) \), with a significant difference between cromoglycate-treated and saline groups \((-36\%; P<0.05)\). The pharmacological interventions showed a borderline significance for lower levels of mean microvascular gelatinase activity in the ischemic hemisphere \( (-21\%; P=0.054; \text{ANOVA}; \text{Figure 4A, right}) \). In the pharmacologically treated, sham-operated groups, the mean microvascular gelatinase activity of both hemispheres was comparable with contralateral levels of corresponding MCA-operated groups: cromoglycate-sham \(1.34\pm0.05\), saline-sham \(1.35\pm0.04\), and compound 48/80-sham \(1.26\pm0.01\) \( (P=0.147; \text{ANOVA}) \).

Correlations Between Brain Swelling, EBA Extravasation, and Microvascular Gelatinase Activity

At 3 hours after reperfusion, the groups showed significant brain swelling and EBA extravasation. As reported earlier, genetic MC deficiency and pharmacological MC stabilization led to significant reductions in these parameters, and enhanced activation of MCs with compound 48/80 increased them.

The genetically manipulated groups showed a strong positive correlation between measurements of microvascular gelatinase activity and brain swelling \( (r=0.84, P<0.001) \) in mean microvessel activity; \( r=0.80, P<0.001 \) in percentage of microvessels with high activity; \( \text{Figure 4C, Supplemental Figure VI} \). In the pharmacologically manipulated cromoglycate and saline groups, the percentage of microvessels with high activity had a significant correlation with brain swelling \( (r=0.61; P=0.02; \text{Figure 4C} \). There was a trend toward correlation between microvascular gelatinase activity and EBA leakage in all groups (Supplemental Figure VI). Although compound 48/80 was shown to promote severe brain swelling and EBA extravasation, we could not confirm this to be associated with increased microvascular gelatinase activity in the present experiments.

Discussion

We and others have previously presented data suggesting the involvement of MCs in the pathophysiology of cerebral ischemia–reperfusion injury, namely BBB disruption and edema formation. The molecular mechanisms behind such action, however, have remained elusive. Here, we provide mechanistic insight into the processes leading to BBB disruption following reperfusion-induced MC activation. Our
present results show that pharmacological modulation of MC-activation influences posts ischemic activity of gelatinase enzymes within the neurovascular unit. The link between biological effects of MCs and gelatinase activity was strongly supported by additional experiments with MC-deficient rats.

In line with an earlier report, ISZ revealed striking posts ischemic gelatinase activity in several cellular elements such as neurons, astrocytes, and microvascular endothelium. A halo of ISZ-positive granules was seen around degranulated MCs, fitting with that also MCs themselves can release gelatinases. Because MCs are situated in perivascular positions ready to influence the function of a neurovascular unit, we focused this report on the microvasculature, where even short-lived gelatinase activation could trigger rapid BBB degradation and vasogenic edema. High topographical accuracy is needed in evaluation of innumerable, variably oriented, and differentially activated microvessels in multiple regions of interest, and we developed a high-throughput computerized image analysis workflow for quantitative study of double-stained sections (Figure 1).

To study MC-specific responses in our experimental setting, MCAo was induced in genetically MC-deficient rats and their WT littermates. Genetic MC deficiency led to a striking decrease in total gelatinase-active area (Figure 3), and decreased microvascular gelatinase activity to levels corresponding to the intact hemisphere (Figure 4). Fittingly, microvascular gelatinase activity correlated with the degree of brain swelling, and showed a strong trend toward correlation with EBA leakage (Supplemental Figure VI). We therefore believe that MCs could trigger localized MMP activation, leading to acute posts ischemic BBB degradation and vasogenic edema.

We have previously shown that pharmacological MC stabilization with cromoglycate can ameliorate posts ischemic brain edema, BBB damage, and hemorrhages after transient experimental MCAo with and without thrombolysis. The present data suggest that these effects granted by cromoglycate could be mediated through inhibition of MC-dependent microvascular gelatinase activation (Figure 4). Again, microvascular gelatinase activity correlated with brain swelling (Figure 4C), but total ISZ-positive brain area was not reduced (Figure 3), a result that may reflect relative weakness of pharmacological MC-stabilization compared with genetic absence of MCs. In addition, using a fixed dose of cromoglycate may have added variability of the results to some extent. The effects of compound 48/80 on gelatinase activity were not entirely congruent. Microvascular gelatinase activity was not increased (Figure 4), but the percentage of gelatinase-active area was significantly elevated in montage analysis, which also captures activity of nonvascular cell types (Figure 3B).

MCs contain a broad mediator assortment, which can affect gelatinase activity after ischemia-reperfusion through parallel pathways. MCs can directly mediate proteolytic activation of progelatinases and degrade tissue inhibitor of metalloproteinase-1 through released chymase and tryptase. MCs may potentiate the secretion of gelatinases from neighboring cells by releasing preformed or newly synthesized tumor necrosis factor-α. In addition, MCs are capable of secreting tissue-type plasminogen activator, which can enhance MMP-9 activation through subsequent plasmin and MMP-3 activation. Finally, MCs increase post-
ischemic neutrophil infiltration, which may contribute to microvascular MMP-9 accumulation. The relative significance and convergence of these pathways remain to be elucidated.

Conclusions
The present observations support our earlier findings of MC involvement in early ischemic BBB disruption and edema formation, and suggest a mechanistic link via MC-dependent regulation of gelatinase activity within the neurovascular unit.

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Disclosures
None.

References
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Figure I. Distribution of gelatinase activity in the microvasculature of the ischemic hemisphere in two representative animals.

Genetic MC deficiency reduced perimicrovascular gelatinase activity following experimental MCAo. (A) Histograms illustrating the distribution of gelatinase activity in the analyzed microvessels of a representative wildtype (top) and a MC-deficient WsRc<sup>Ws</sup>/Ws (bottom) rat. *Mean gelatinase activity* refers to the mean relative gelatinase activity of all the vessels per each animal (represented by diamond). The *percentage of microvessels with high gelatinase activity* was counted according to a calculated cut-off point (represented by vertical line). The relative gelatinase activity of 1.0 represents an object with ISZ brightness equal to the surrounding background. In the case of WsRc<sup>Ws</sup>/Ws rats and their littermates, the cut-off point for high gelatinase activity was set at 1.339, and in the pharmacological groups (not shown here) it was 1.486 (see Methods for details). (B) The width of the selected microvascular objects fell within known diameters of microvessels. Microvascular width was measured as the minor axis of an ellipse fitted around the selected object.
**Supplemental videos a-c. MCs release active gelatinases during degranulation**

Three-dimensional visualization of a confocal image stack showing a degranulating MC in the ischemic hemisphere. (A) ISZ-channel, (B) Avidin-TRITC-channel and (C) overlay. A halo of gelatinase positive granules is seen around the cell.
Figure II. Mast cell mediated microvascular gelatinase activation 3 hours after reperfusion in the ischemic hemisphere. (A&B) Fluorescent avidin shows perivascular MCs (arrow), surrounded by significant gelatinase activation (ISZ) in the adjacent vessel segment. Scale Bar = 20µm
Figure III. Astrocytes co-localize with gelatinase activity near active microvessels. GFAP-positive astrocyte endfeet surrounding a typical gelatinase positive microvessel (scale bar=50µm).
Figure IV. Immunohistochemistry for MMP-2
Immunohistochemistry for MMP-2 co-localized with ISZ-active neurons and with a portion of ISZ-active microvessels. It also showed staining of astrocytic end-feet which were not ISZ-active, indicating latent MMP-2 in these cells (scale bar=50µm).
Figure V. Gel zymography
Gelatinase activity in gel zymography from exemplary comoglycate-treated and saline-treated animals 3 hours after reperfusion.

For gel zymography, the ischemic territory and a corresponding area from the contralateral hemisphere were homogenized from 1mm thick coronal brain sections. MMP-2 and MMP-9 in homogenates were concentrated from 100μg of total protein using gelatin-sepharose beads with established methodology (Zhang and Gottschall, J Neuroscience Methods 1997, PMID: 9334934). Equal amounts of extracted samples were analyzed on a 8% gelatin SDS-PAGE gel. After electrophoresis the gel was incubated for 40 hours in a renaturing buffer at 37°C. The gel was stained with standard coomassie blue staining as described before (Kleiner and Stetler-Stevenson, Analytical Biochemistry 1993, PMID: 8074288). Recombinant rat proMMP-2 (0.5ng) and proMMP-9 (0.25ng, both from RnD systems), were used as positive controls.
Figure VI. Supplemental correlation graphs