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Contribution of Matrix Metalloproteinase-9 to Cerebral Edema and Functional Outcome following Experimental Subarachnoid Hemorrhage

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Key Words
Subarachnoid hemorrhage · Cerebral edema · Outcome · Matrix metalloproteinases · Knockout mice

Abstract
Background: Cerebral edema is an important risk factor for death and poor outcome following subarachnoid hemorrhage (SAH). However, underlying mechanisms are still poorly understood. Matrix metalloproteinase (MMP)-9 is held responsible for the degradation of microvascular basal lamina proteins leading to blood-brain barrier dysfunction and, thus, formation of vasogenic cerebral edema. The current study was conducted to clarify the role of MMP-9 for the development of cerebral edema and for functional outcome after SAH. ***Methods:*** SAH was induced in FVB/N wild-type (WT) or MMP-9 knockout (MMP-9$^{-/-}$) mice by endovascular puncture. Intracranial pressure (ICP), regional cerebral blood flow (rCBF), and mean arterial blood pressure (MABP) were continuously monitored up to 30 min after SAH. Mortality was quantified for 7 days after SAH. In an additional series neurological function and body weight were assessed for 3 days after SAH. Subsequently, ICP and brain water content were quantified. ***Results:*** Acute ICP, rCBF, and MABP did not differ between WT and MMP-9$^{-/-}$ mice, while 7 days' mortality was lower in MMP-9$^{-/-}$ mice (p = 0.03; 20 vs. 60%). MMP-9$^{-/-}$ mice also exhibited better neurological recovery, less brain edema formation, and lower chronic ICP. ***Conclusions:*** The results of the current study suggest that MMP-9 contributes to the development of early brain damage after SAH by promoting cerebral edema formation. Hence, MMP-9 may represent a novel molecular target for the treatment of SAH.

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Introduction

Subarachnoid hemorrhage (SAH) is a subtype of hemorrhagic stroke with a particularly high mortality and morbidity [1]. Among others, brain edema has been identified as a major independent risk factor for mortality and poor outcome after SAH [2]. Posthemorrhagic brain edema is mostly vasogenic, i.e. it results from a dysfunction of the blood-brain barrier [3]. As previously shown, blood-brain barrier dysfunction starts 6 h after experi-

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mental SAH, peaks at 2 days, and correlates with the degradation of collagen IV [4], a major component of the microvascular basal lamina which serves as an important component of the blood-brain barrier [5].

Collagen IV, amongst other basal lamina proteins, can be degraded by matrix metalloproteinase-9 (MMP-9, type IV collagenase), a member of the MMP family of zinc-containing proteinases [6]. MMPs play a pivotal role for the development and normal function of the brain and other organs by modulating cell-matrix interactions [7]. Under pathological conditions, e.g. focal cerebral ischemia, MMP-9 expression is detectable in patients [8] and in experimental animals [9], and is held responsible for the development of vasogenic cerebral edema [10]. Recent publications have demonstrated that MMP-9 is upregulated and activated after SAH [11, 12]; however, since highly specific pharmacological inhibitors are missing, the pathophysiological role of MMP-9 after SAH has not been clarified yet.

The aim of the current study was therefore to clarify the role of MMP-9 for the development of cerebral edema and for functional outcome following SAH using MMP-9 knockout mice.

Methods

Thirty-five male and female MMP-9 knockout (MMP-9$^{-/-}$) mice (body weight 20–25 g; Jackson Laboratory, Bar Harbor, Me., USA) and 40 age- and weight-matched FVB/N wild-type (WT) mice (Charles River, Sulzfeld, Germany) were used for the current experiments. FVB/N mice were chosen as controls because MMP-9$^{-/-}$ mice, originally generated by Vu et al. [13], were bred and back-crossed for more than 10 generations on this background.

The genotype of MMP-9$^{-/-}$ mice was confirmed by polymerase chain reaction. The following primers were used to amplify a 172-bp mutant product (oIMP0158, 5′-CTG AAT GAA CTG CAG GAC GA-3′ and oIMR0159, 5′-ATA CTT TCT CGG CAG GAG CA-3′) or the 277-bp WT product (oIMR1760, 5′-GTG GGA CCA TCA TAA CAT CAC A-3′ and oIMR1761, 5′-CTC GCG GCA AGT CTT CAG AGT A-3′).

Experimental animals were cared for prior to and at all stages of the experiments in compliance with institutional guidelines and approved by the Animal Care Committee of the District Government of Upper Bavaria (protocol number 221-2531-117/05 and 66/08).

Anesthesia, Monitoring, and Induction of SAH
Anesthesia was induced by intraperitoneal injection of midazolam, fentanyl and medetomidin [14]. Subsequently, animals were intubated and mechanically ventilated, and end-tidal pCO_2 was measured by microcapnometry (CI240, Columbus Instruments, Columbus, USA). After medial skin incision, dissection of the temporal muscle and drilling of a ~1.5-mm burr hole, an intracranial pressure (ICP) probe (Johnson & Johnson Medical Limited, Berkshire, UK) was positioned in the epidural space above the frontal cortex of the right hemisphere to monitor ICP prior and after induction of SAH. Regional cerebral blood flow (rCBF) was measured continuously with a flexible laser Doppler probe (rCBF; Perimed 4001 Master, Perimed, Järfälla, Sweden) being glued onto the skull above the territory of the left middle cerebral artery. Mean arterial blood pressure and blood gases were measured through a catheter placed in the left femoral artery. SAH was induced using the endovascular perforation model as previously described [15]. Briefly, the common carotid artery was exposed and a 5-0 monofilament was introduced into the internal carotid artery via the left external carotid artery. The filament was advanced distally until a sudden sharp increase in ICP indicated SAH. Thereafter, the filament was immediately withdrawn and the external carotid artery was ligated. At the end of posthemorrhagic monitoring probes were removed, the burr hole was closed with dental cement, and anesthesia was terminated by intraperitoneal injection of atipamezol, naloxon, and flumazenil [14]. Mice were kept in an incubator at an ambient temperature of 33°C for 24 h.

Experimental Groups
One single investigator performed all surgeries (S.F.). All individuals involved in the current study were blinded towards the genotype of the animals until completion of data analysis. Animals were allocated to groups using block randomization.

In the first experimental series multimodal monitoring (ICP, rCBF, and mean arterial blood pressure) was carried out for 30 min after SAH in WT and MMP-9$^{-/-}$ mice (n = 7 in each group). Subsequently, arterial blood samples were analyzed and animals were sacrificed.

In the second experimental series multimodal monitoring was also carried out. Thereafter, WT and MMP-9$^{-/-}$ mice (n = 10 in each group) were observed for a postoperative period of 7 days to determine long-term mortality.

In the third experimental series – after multimodal monitoring – neurological deficits, body weight, and mortality was quantified during an observation period of 3 days in MMP-9$^{-/-}$ and WT mice. After 3 days, ICP was measured again and brains were removed for quantification of brain water content. Animals that died after the operation were replaced until n = 12 animals in the MMP-9$^{-/-}$ group and n = 11 animals in the WT group survived the 3 days' observation period. For this purpose surgery had to be performed in n = 14 MMP-9$^{-/-}$ mice and n = 19 WT mice. Due to technical reasons 3-day ICP and brain water content data are only available in n = 11 MMP-9$^{-/-}$ animals. Brain water content was also measured in n = 4 naive WT and MMP-9$^{-/-}$ mice (that did not undergo any invasive procedure) in order to obtain normal values.

Neurological Function and Body Weight
The neurological function was assessed as previously described using a global SAH score that particularly considers SAH-specific neurological deficits [16]. The best achievable total score was 1 point, the worst 19 points (table 1). Body weight was assessed prior to SAH and daily for the 3 days thereafter and was expressed in percent of the preoperative value.

To avoid bias caused by the occurring mortality, all functional data were corrected for mortality as previously described [16].

Table 1. Neuroscore (global SAH score)

Item	Points	Best	Worse
Consciousness	spontaneous movements/animal explores (1) spontaneous movements/animal walks in the cage (2) spontaneous movements/animal walks on the point (3) movements after mild tactile stimulus (4) animal movements after vertical movements of the cage (5) movements after intense tactile stimulus (6) coma (7)	1	7
Righting reflex	present (0) absent (1)	0	1
Corneal reflex	bilateral present (0) unilateral present (1) absent (2)	0	2
Ear twitch reflex	bilateral present (0) unilateral present (1) absent (2)	0	2
Sensory deficits	reaction to tail pinch present (0) reaction to tail pinch absent (1)	0	1
Whisker movements	present (0) absent (1)	0	1
Motor function [26]	no apparent deficit (0) contralateral forelimb flexion (1) lowered resistance to lateral push without circling (2) circling if pulled by tail (3) spontaneous circling (4) no spontaneous activity (5)	0	5
Total		1	19

Quantification of Chronic ICP and Brain Edema

Animals were re-anesthetized 3 days after SAH. The skin incision was reopened, the dental cement was removed, and the ICP microsensor was reinserted into the right frontal burr hole. ICP values were taken 5 min after readings stabilized. Subsequently, animals were sacrificed by cervical dislocation in deep anesthesia. Brains were removed from the skull and cerebellum, and olfactory bulbs were truncated. Hemispheres were separated and their wet weight (WW) was assessed using a precision balance. Thereafter, the hemispheres were dried for 24 h at 110°C and their dry weight (DW) was determined. Hemispheric water content (%) was calculated using the following formula: $[(WW - DW)/WW] \times 100$.

Statistical Analysis

Statistical analysis was performed using the SigmaStat 3.11 statistical software package (SPSS Science Inc., Chicago, Ill., USA) and STATISTICA 9.1 (mortality analysis; StatSoft, Hamburg, Germany). Data were analyzed with the Mann-Whitney rank sum test. For the comparison of repeated measurements the signed rank test was used. Mortality was compared with the Cox' F test. Statistical significance of results was assumed at $p < 0.05$. Data are presented as mean ± SEM if not indicated otherwise.

Results

Physiological Parameters

There were no significant differences in physiological parameters between MMP-9$^{-/-}$ and WT groups 30 min after SAH (table 2).

SAH resulted in an immediate increase of ICP from 7 to 60–70 mm Hg ($p < 0.001$ vs. pre-SAH baseline) followed by a decrease to a plateau of ~25 mm Hg within 10 min after SAH (fig. 1a). This plateau was maintained up to 30 min after SAH in all groups. There were no significant differences between groups indicating comparable degrees of SAH in all experimental animals.

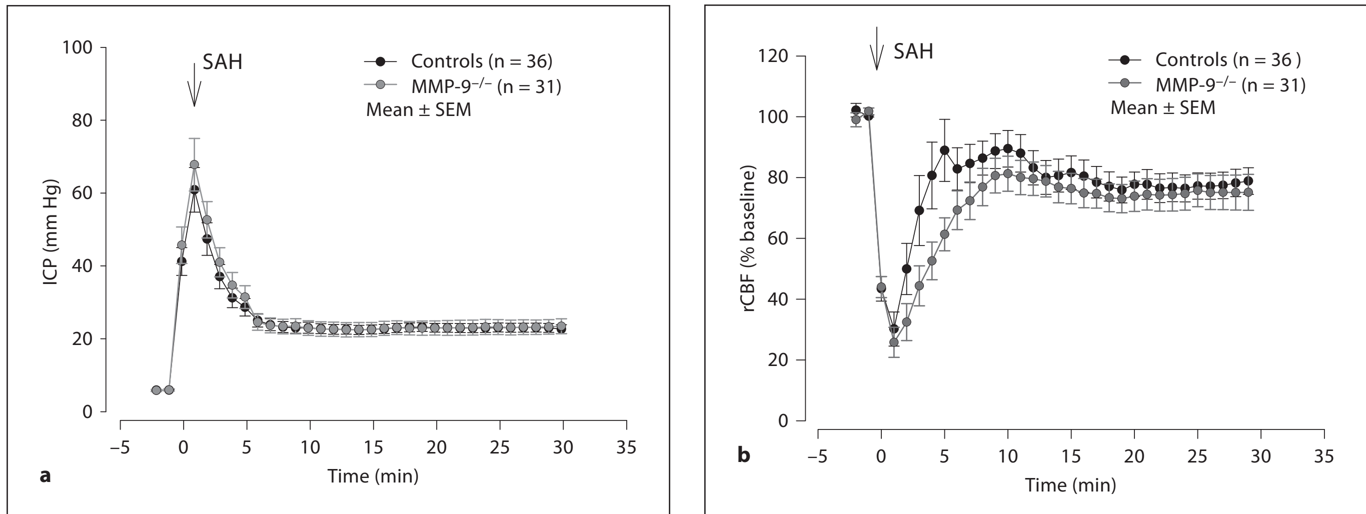

Fig. 1. Time course of ICP (**a**) and ipsilateral rCBF (**b**) up to 30 min after SAH in WT (controls) and MMP-9$^{-/-}$ mice. SAH resulted in an acute ICP elevation with a subsequent decline to a plateau phase in all groups. In addition, SAH resulted in an acute massive impairment of cerebral perfusion followed by a gradual rCBF recovery to ∼80% of baseline during the monitoring period. There were no differences between groups.

Table 2. Physiological parameters 30 min after SAH in WT and MMP-9$^{-/-}$ mice

	WT	MMP-9$^{-/-}$
pH	7.31 ± 0.09	7.35 ± 0.03
$PaCO_2$, mm Hg	43 ± 5	38 ± 6
Lactate, mg/dl	0.8 ± 0.2	0.9 ± 0.1
MABP, mm Hg	64.6 ± 11.2	76.8 ± 13.1

Data are expressed as mean ± SD (n = 7 in each group). pH = Arterial pH; $PaCO_2$ = arterial pCO_2; MABP = mean arterial blood pressure.

Induction of SAH additionally resulted in a sharp decline of ipsilateral rCBF ($p < 0.001$ vs. baseline) by over 70% to ∼25–30% of baseline values followed by recovery to a plateau of ∼80% of baseline values after 10–15 min (fig. 1b). There were no significant differences between groups.

Mortality

Mortality was assessed in all experimental groups, i.e. in animals sacrificed 3 or 7 days after SAH. Mortality within 24 h was 14% in MMP-9$^{-/-}$ and 37% in WT mice in the first series (3 days survival) and 10% in MMP-9$^{-/-}$ and 30% in WT mice in the second series (7 days' survival), i.e. very consistent between groups. The same consistency was observed after 3 days. In the 3 days' observation series mortality was significantly (p = 0.03) lower in the MMP-9$^{-/-}$ group (2/14; 14%) as compared to the WT group (8/19; 42%); in the 7 days' survival group mortality was 20% in MMP-9$^{-/-}$ and 40% in WT mice. This difference persisted and became even more evident at the end of the observation period 7 days after SAH. No additional MMP-9$^{-/-}$ mice died during this period of time (total 7-day mortality: 20%; p = 0.03 vs. WT; fig. 2a) while 2 more mice died in the WT group (total 7-day mortality: 40%; fig. 2a).

Neurological Function and Body Weight

Animals of both mouse strains exhibited no neurological deficits prior to SAH. During the 3 days' posthemorrhagic observation period, MMP-9$^{-/-}$ mice had significantly ($p < 0.05$) less neurological deficits as compared to WT mice (fig. 2b).

Body weight was 87.4 ± 1.3, 84.3 ± 1.5, and 84.4 ± 3.1% of preoperative values on days 1, 2, and 3, respectively, in the MMP-9$^{-/-}$ group, and 86.7 ± 1.0, 80.8 ± 2.1, and 80.6 ± 3.5% on days 1, 2, and 3 in the WT group. Although there was a tendency towards a less pronounced postoperative weight loss in MMP-9$^{-/-}$ mice this difference did not reach statistical significance.

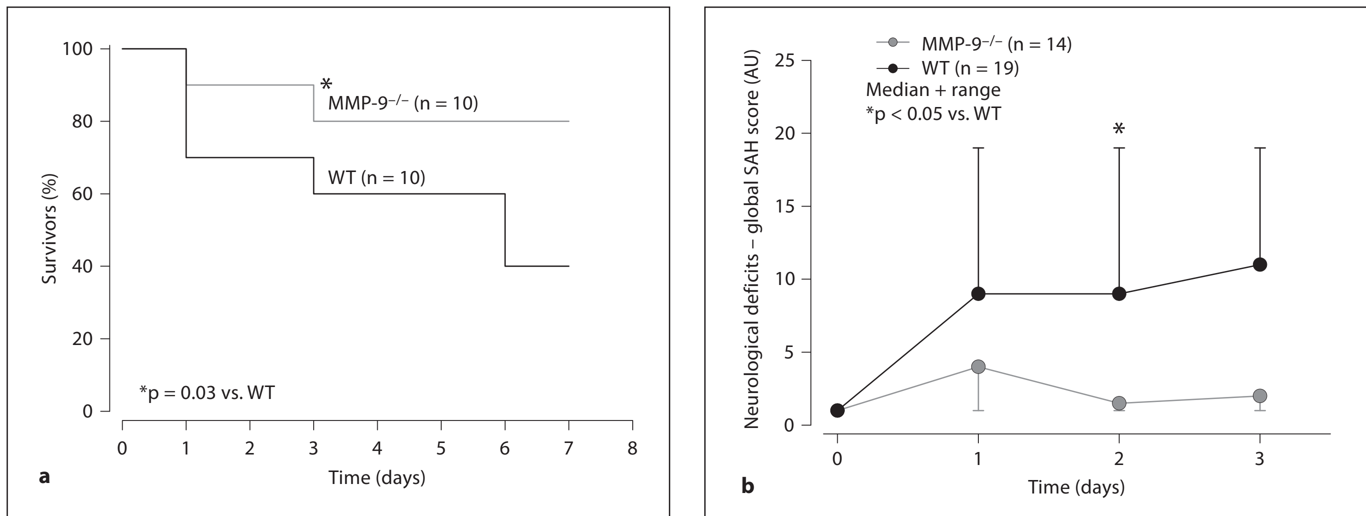

Fig. 2. Kaplan-Meier curve displaying posthemorrhagic mortality (**a**) and time course of neurological deficits (**b**). MMP-9$^{-/-}$ mice exhibited a significantly lower postoperative mortality (p = 0.03; Cox' F test) and lesser neurological deficits, reaching statistical significance ($p < 0.05$; rank sum test) on posthemorrhagic day 2 compared to WT mice.

ICP and Brain Edema 3 Days after SAH

MMP-9 null mice showed significantly ($p < 0.05$) lower ICP values (5.4 ± 2.4 mm Hg) than WT mice (11.2 ± 2.4 mm Hg) 3 days after SAH (fig. 3).

There was no difference in brain water content between naive MMP-9 and WT mice. SAH resulted in a significant increase ($p < 0.001$) in brain water content on posthemorrhagic day 3. The brain water content of the ipsilateral hemisphere was significantly ($p < 0.05$) lower in MMP-9$^{-/-}$ mice (78.7 ± 0.3%) compared to WT mice (79.6 ± 0.4%), whereas the brain water content of the contralateral hemisphere did not differ between groups (MMP-9$^{-/}$: 79.1 ± 0.2%; WT: 79.6 ± 0.4%; fig. 4).

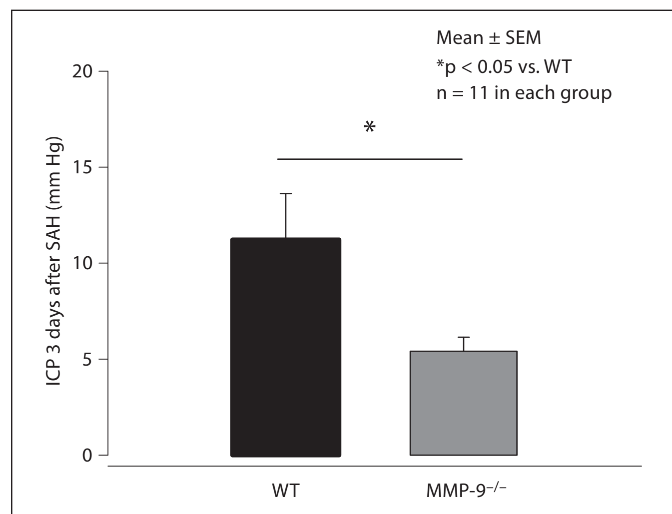

Fig. 3. Chronic ICP in animals subjected to SAH are displayed. MMP-9$^{-/-}$ mice exhibited lower ICP values ($p < 0.05$; rank sum test) compared to WT mice 3 days after SAH.

Discussion

In the current study we intended to evaluate the role of MMP-9 for the pathophysiology of SAH. Since highly specific pharmacological inhibitors for MMP-9 are not available, we used MMP-9$^{-/-}$ mice and showed that these animals had a significantly lower mortality, a better functional outcome as well as less brain edema formation and lower ICP 3 days after SAH as compared to WT mice. These findings suggest that MMP-9 is involved in the development of brain edema following SAH.

The endovascular perforation model in mice used in the current study resembles the high mortality observed in SAH patients [1] quite well, i.e. most animals that did not survive the observation period died within the first 7 days after SAH. An additional advantage of the mu-

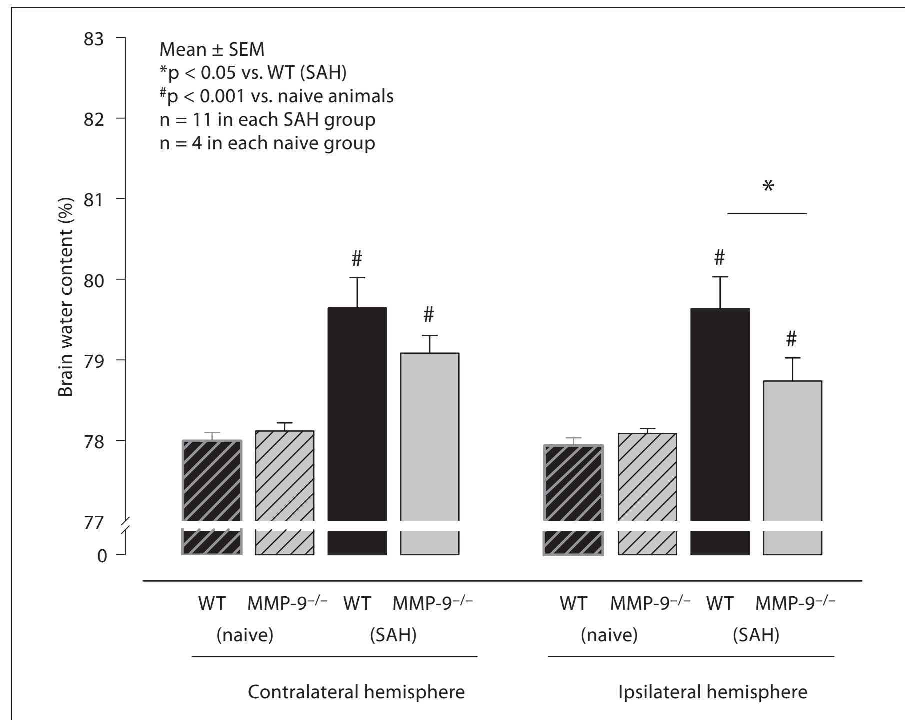

Fig. 4. Brain water content of the ipsilateral and the contralateral hemisphere in animals subjected to SAH and naive animals is displayed. MMP-9$^{-/-}$ mice exhibited a lesser increase ($p < 0.05$; rank sum test) of ipsilateral brain water content compared to WT mice 3 days after SAH.

rine SAH model is the possibility to use genetically engineered animals for the study of SAH. In the specific case of MMP-9 the use of knockout mice allowed us to investigate its pathophysiological role after SAH with high specificity and selectivity. The experimental setup that we use includes multimodal monitoring and is highly standardized [15, 16]. Epidural ICP measurement, in particular, verifies the extent of SAH [15] and was used in the current study in addition to rCBF measurement to prove comparable degrees of SAH in both study groups.

MMP-9 expression/activity has recently been characterized after SAH by Sehba et al. [11]. MMP-9 expression was increased by immunohistochemistry and staining was distributed segmentally along the microvasculature. Expression peaked at 6 h and recovered at 48 h after SAH. This is supported by results from Guo et al. [12], who described an early significant increase of MMP-9 activity from 12 to 72 h after SAH peaking at 24 h. Accordingly, this increase of MMP-9 activity seems to take place within the same 24- to 48-hour time frame as the degradation of microvascular collagen IV, a major MMP-9 target molecule, and the subsequent disruption of the blood-brain barrier [4]. These findings suggest that SAH-induced activation of MMP-9 contributes to the formation of posthemorrhagic cerebral edema by cleavage of microvascular protein components.

Cerebral edema was identified as one of the major independent risk factors for death and poor outcome after SAH [2] and, therefore, represents a key target for the development of novel therapeutic options. Despite the known fact that cerebral edema after SAH is, particularly in the first days, predominantly of vasogenic origin [3, 4, 17], the mechanisms of the formation of posthemorrhagic brain edema are still not well understood. MMP-9 that is regulated by a complex cascade involving plasmin, other MMPs (e.g. MMP-3, membrane type 1-MMP), the MMP inducer protein EMMPRIN and the tissue inhibitors of MMPs [18] is involved in brain edema formation after ischemic and hemorrhagic stroke [19, 20], as well as after traumatic brain injury [21]. The major mechanisms postulated are the cleavage of extracellular matrix proteins including collagen IV, laminin, and fibronectin [22, 23], and the disruption of tight junction proteins like occludin [24] leading to increased blood-brain barrier leakage and formation of vasogenic cerebral edema [9, 25]. However, MMP-9 might also be involved in the development of cytotoxic cerebral edema [12].

The role of MMP-9 for the formation of cerebral edema after SAH, however, had not been addressed so far.

Our current data provide for the first time clear evidence that MMP-9 is involved in the formation of cerebral edema after SAH. The parallel temporal pattern of MMP-9 activity shown by Guo et al. [12] and of blood-brain barrier damage/collagen IV reduction that we previously demonstrated [4] as well as similar findings by Sehba et al. [11] suggest that cleavage of collagen IV by MMP-9 contributes to the formation of posthemorrhagic cerebral edema in the rodent brain.

The potential effect of an MMP-9-targeted antiedema therapy can be guessed by the remarkably reduced mortality and functional outcome in MMP-9-deficient mice that we found. This neuroprotective effect is best explained by the reduced formation of cerebral edema leading to a reduction of intracranial hypertension.

In conclusion, our current results suggest that MMP-9 contributes to brain edema formation after experimental SAH and might represent a promising pharmacological target for the treatment of SAH.

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