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ORIGINAL ARTICLE

Effect of genetic depletion of MMP-9 on neurological manifestations of hypertension-induced intracerebral hemorrhages in aged mice

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Abstract Clinical and experimental studies show that hypertension induces intracerebral hemorrhages (ICH), including cerebral microhemorrhages in the aged brain, which contribute to the pathogenesis of vascular cognitive impairment (VCI). Previous studies showed that aging increased oxidative stress-mediated activation of matrix metalloproteinases (MMPs) that importantly contributes to the pathogenesis of ICHs. In particular, oxidative stress has been implicated in activation of MMP-9, which is known to be involved in the degradation of the extracellular matrix and cleavage of collagen IV, a key constituent of the basal membrane of cerebral vessels. To determine the role of MMP-9 activation in the genesis of ICHs, we induced hypertension in 20-month-old MMP-9 null and age-matched control mice by angiotensin II and L-NAME treatment. Contrary to our hypothesis, MMP-9 deficiency did not delay the onset or incidence of neurological consequences of hypertension-induced ICHs. Our results indicate that MMP-9

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activation does not play a role in the age-related exacerbation of hypertension-induced ICH.

Keywords Microbleed · Artery · Arteriole · Cerebral microhemorrhage · Stroke · Oxidative stress · Aging

Introduction

Stroke is the second most common cause of death in the European Union, the fourth leading cause of death in the USA, and one of the leading causes of long-term disability in both continents [1, 2]. Intracerebral hemorrhages (ICH) account for approximately 10–15% of all strokes, and mortality rates range from 35 to 52% [3, 4]. Significant advances in magnetic resonance imaging (MRI) techniques (including T2* gradient-recall echo, susceptibility-weighted imaging MRI sequences) have allowed the detection of previously undetectable small ICH, termed cerebral microhemorrhages, in elderly patients [5]. Cerebral microhemorrhages are small (< 5 mm) vascular lesions associated with rupture of small intracerebral vessels, which contribute to cognitive decline [6, 7].

Advanced age and hypertension are the primary risk factors for the development of both larger ICH [8–10] and cerebral microhemorrhages [5, 11]. Incidence of ICH increases in persons older than 55 years and doubles with each decade until age 80 years. The prevalence of cerebral microhemorrhages also significantly increases with advanced age, from ~ 6.5% in persons aged 45 to 50 years to ~ 35 to 50% or more in older adults [11]. Recent data from rodent models extend the clinical observations, showing that aging and high blood pressure synergistically interact to exacerbate the genesis of ICHs [12]. Preclinical studies suggest that in addition to stiffening of the conduit arteries [13–17] and increased penetration of high pressure waves into the vulnerable distal portion of the cerebral microcirculation [18–29], aging likely promotes the development of ICHs and cerebral microhemorrhages by exacerbating vascular oxidative stress and activation of matrix metalloproteinases (MMPs), which compromise the structural integrity of the cerebral vasculature [12]. Yet, the role of specific MMPs in increased susceptibility of the aged cerebral vasculature to rupture remains elusive.

MMP-9, also known as type IV collagenase or gelatinase B, is a collagenase enzyme involved in the degradation of the extracellular matrix [30, 31]. Aging associates with increased MMP-9 expression in many tissues, including the heart [32, 33] and the human aorta [34]. Increased MMP-9 activation has been causally linked to the genesis of ICH in various experimental murine models, including ICH associated with chronic hypertension and cerebral amyloid angiopathy [35–38]. Importantly, MMP-9 deletion and inhibition have been shown to confer protective effects in a range of animal models of cardiovascular disease [30].

The present study was designed to test the hypothesis that MMP-9 contributes to the development of hypertension-induced ICH in aging mice. To test this hypothesis, we induced hypertension in aged mice with genetic depletion of MMP-9 and respective controls (by treatment with angiotensin II [Ang-II] and the NO synthesis inhibitor L-NAME) and compared the incidence of neurological manifestations of ICH. Our previous studies demonstrate that the approach used in the present study, longitudinal analysis of hypertension-induced changes in the mouse neuroscore, closely predict the incidence of histologically verified ICH in the mouse brain [12, 39, 40]. In aging, the activity of the vascular renin-angiotensin system is elevated. Moreover, hypertension in older adults can be successfully treated with angiotensin converting enzyme inhibitors and angiotensin II receptor blockers. Thus, aged mice with Ang-II-induced hypertension is a clinically highly relevant animal model to investigate hypertension-related cerebrovascular alterations in the context of aging [21]. Previous studies by the Heistad laboratory [35, 41] and subsequent investigations by our investigative team [12, 39] showed that co-administration of L-NAME and Ang-II results in an ~ 15-mmHg additional increase in blood pressure, which associates with a significant increase in the incidence of ICH in the presence of underlying microvascular fragility.

Methods

Experimental animals

Male MMP-9 null mice were used from a breeding colony that originated with mice generated by Zena

Werb's laboratory and backcrossed by Lynn Matrisian's laboratory [42, 43]. Male C57BL/6 J mice purchased from Jackson Laboratories were used as the control group (n = 17).

Animals were identically housed in the Rodent Barrier Facility at OUHSC under specific pathogen-free barrier conditions, on a 12-h light/12-h dark cycle, with access to standard rodent chow (Purina Mills, Richmond, IN) and water ad libitum. Animals were randomized to groups, and investigators were blinded to group throughout the protocol.

Induction of spontaneous ICH

To study the effects of MMP-9 on hypertension-induced intracerebral hemorrhages, we used a previously well-characterized mouse model [12, 35, 41]. Briefly, in 20-month-old male MMP-9 deficient mice ($n = 22$) and respective age-matched control mice (n = 17), hypertension was induced by a combination treatment with ω-nitro-L-arginine-methyl ether (L-NAME, 100 mg/kg/day, in drinking water) and administration of angiotensin II (Ang-II; s.c. via osmotic mini-pumps [Alzet Model 2006, 0.15 µl/h, 42 days; Durect Co, Cupertino, CA]). Pumps were filled either with saline vehicle or solutions of angiotensin II (Sigma Chemical Co., St. Louis, Missouri, USA) delivered subcutaneously at 1 µg/min/kg of angiotensin II, thus generating two experimental groups: (1) wild type control + Ang-II + L-NAME and (2) $MMP9^{-/-}$ + Ang-II + L-NAME. Pumps were placed into the subcutaneous space of isoflurane anesthetized mice through a small incision in the back of the neck that was closed with surgical sutures. All incision sites healed rapidly without the need for additional medication. Since aging is associated with increased activity of the vascular renin-angiotensin system and Ang-II-dependent hypertension is common among older individuals [44], Ang-II-dependent hypertension is a clinically highly relevant model to study aging-related cerebrovascular alterations [21].

Blood pressure of the animals was recorded before the treatment and every second day during the treatment period using a tail-cuff blood pressure machine (CODA Non-Invasive Blood Pressure System, Kent Scientific Co., Torrington, CT), as described [12, 19, 21]. Each experimental group was closely monitored, and mice were sacrificed upon the occurrence of clinical signs of intracerebral hemorrhages.

All procedures were approved by the Institutional Animal Use and Care Committees of the University of Oklahoma Health Sciences Center.

Standardized neurological examination of mice

To assess the occurrence of clinical features of hemorrhages, daily neurological examination was performed by assessing each animal's spontaneous activity, symmetry in the movement of the four limbs, forelimb outstretching, climbing ability, body proprioception, response to vibrissae touch, and gait coordination. Each examined animal was provided with a daily score calculated by the summation of all individual test scores. When a consistent decline in the neurological score was observed or on day 11 of the study, mice were euthanized by CO_2 asphyxiation.

Statistical analysis

Cumulative incidence of neurological signs of ICH was evaluated using a Kaplan–Meier test, and the difference among groups was analyzed by log-rank test (Mantel-Cox). A p value less than 0.05 was considered statistically significant.

Results

Incidence of neurological signs of hypertension-induced ICHs in MMP-9 null mice

Treatment with Ang-II plus L-NAME resulted in comparable increases in blood pressure both in MMP-9 null (149 ± 6 mmHg) and age-matched control wild-type mice (150 ± 5 mmHg).

We found that during the experimental period, 35% of control mice and 77% of MMP-9 null mice developed clinically manifest signs of hypertension-induced ICH, as assessed by neurological examination. The cumulative distribution curves for time-to-event in the two groups were statistically different (log rank [Mantel-Cox] test; $\chi^2 = 5.701$; P = 0.017).

Discussion

This is the first study to demonstrate that genetic MMP-9 deficiency does not ameliorate the incidence of neurological manifestations of hypertension-induced ICHs in aged mice (Fig. 1).

Several lines of evidence suggest that both aging and hypertension upregulate MMP-9 in the vasculature. First, increased expression of MMP-9 was reported in aorta samples derived from older adults [34]. Older patients with small vessel vascular dementia also present with elevated levels of MMP-9 in the cerebrospinal fluid [45]. Interestingly, no increase in MMP-9 expression was found in smooth muscle cells derived from aged mice [46]. Second, Ang-II-induced hypertension in mice also increases MMP-9 activity in the cerebral vasculature due to increased levels of oxidative stress, which could potentially be linked to the genesis of ICHs [35, 41]. Finally, MMP-9 is upregulated in the brain after development of ICH, which has been linked to disruption of the blood brain barrier [47–51].

Contrary to the prediction based on our hypothesis, our findings show that genetic depletion of MMP-9 does not prevent/delay neurological manifestations of hypertension-induced ICHs in aged mice.

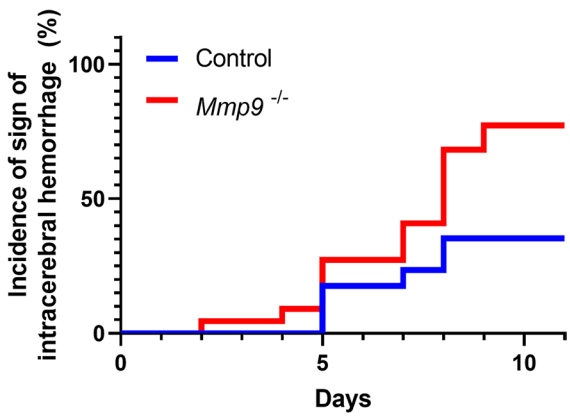

Fig. 1 Genetic MMP-9 deficiency does not ameliorate, rather increases, the incidence of hypertension-induced intracerebral hemorrhages in aged mice. Shown are cumulative incidence curves for neurological signs of hypertension-induced intracerebral hemorrhages in 20-month-old control (n = 17) and age-matched MMP-9 null mice (n = 22). In MMP-9 deficient mice, the incidence of ICHs was higher to that in control mice after induction of hypertension (log-rank test; Mantel-Cox; p = 0.017)

These results are consistent with the concept that the cellular and molecular mechanisms responsible for increased susceptibility of aged cerebral microvessels to rupture are not dependent on the presence of MMP-9 and likely involve other MMPs. Genetic MMP-9 deficiency was also reported to enhance, rather than attenuate, collagenase-induced ICHs, brain injury, and mortality in mice [52]. This observation is also in line with our findings and refutes the idea that increased MMP-9 is a cause for increased incidence and mortality of ICH in aging.

In addition to MMP-9, a wide range of other MMPs are expressed in the cerebral arteries and the brain, which likely play complex roles in the pathogenesis of ICHs. They are known to degrade collagen and elastin and other components of the basal lamina and extracellular matrix, compromising the structural integrity of the cerebral vasculature. Cerebral arteries express MMP-1 (collagens, types I, II, and III), MMP-2 (collagens I, II, III, IV, VII, X), MMP-3 (collagens II, IV, IX, X, X), MMP-8 (collagens I, II, III, VII, VIII, X), MMP-12 (elastin, fibronectin, collagen IV), and MMP-13 (collagens I, II, III, IV, IX, X, XIV). Previous studies demonstrate that aging is associated with MMP-2 expression in the human aorta [34, 53], mammary artery [54], and aortas of non-human primates [55] and rodent models [56–58]. MMP-3 and MMP-12 are also upregulated in the cardiovascular system [59] and the brain [60] of aged mice. MMP-3 has also been linked to aging-induced vascular remodeling in humans [61]. Aging is also associated with the accumulation of senescent cells in the cerebral circulation [62]. Senescent cells can affect the surrounding tissue microenvironment, one of these effects is the senescence-associated secretory phenotype (SASP), characterized by up-regulation and local the release of elastase and various MMPs (including MMP-1, MMP-3 and MMP-13), whose proteolytic activity can lead to focal weakening of the vascular wall, potentially creating loci of least resistance, promoting the genesis of ICHs and cerebral microhemorrhages. Further, in response to ICH, several MMPs were reported to be upregulated in the brain, which likely plays an important role in blood–brain barrier dysfunction, and thereby affect the extent of the neuronal damage and survival [52, 63, 64]. At present, it is unclear how age-related changes in MMP expression alter susceptibility of cerebral vessels to pressure-induced rupture. There is

strong evidence that in MMP-9 null mice, the expression of other MMPs is dysregulated [52], which may overcompensate the loss of MMP-9, altering microvascular fragility and influencing bleeding and brain injury. In MMP-9 null mice, increased expression of MMP-2, MMP-3, MMP-8, and/or MMP-13 as well as TIMP-1 has been documented in various tissues, including cardiomyocyte s [65, 66].

Previously, advanced aging was found to exacerbate hypertension-induced generation of reactive oxygen species (ROS) in cerebral vessels, suggesting that redox-sensitive MMP activation [35, 67] may potentially contribute to the observed phenotype. This concept is supported by the observations that increased hypertension-related MMP activation in the aged cerebral vasculature can be attenuated by antioxidative treatments [12]. Increased hypertension-induced oxidative stress in aged arteries in murine models has been attributed to increased activation/expression of NAD(P)H oxidases (NOX enzymes), increased ROS production by mitochondrial sources, and age-related impairment of Nrf2-dependent cellular antioxidant defense pathways [12, 68–70]. Growing evidence suggests that inhibition of ROS generation by these sources can prevent/delay development of ICHs in models of aging, hypertension, [12, 35] and even Alzheimer's disease [71].

The present study has important limitations. Several animal models of hypertension have been used to investigate the effects of high blood pressure on the brain [72–79]. Although none of the aforementioned models can fully recapitulate the hemodynamic alterations, vascular pathologies, and long-tern neurological consequences associated with chronic presence of "essential" hypertension in humans, these experimental models have proved to be valuable tools to elucidate the potential mechanisms underlying the susceptibility of the brain to hypertension-induced injury. The mouse model used in the present study (Ang-II plus L-NAME to induce hypertension), along with similar murine models, has been specifically developed to study the pathogenesis of hypertension-induced ICHs [35, 41, 80]. It is an advantage of the model that due to the "aggressive" regimen of hypertension induction, the development of ICHs can be studied in a short time window. Although in the present study histological evaluation of ICHs has not been performed, previous studies demonstrated that the incidence of the neurological manifestations of ICHs closely correlates with the susceptibility of the cerebral microvasculature to rupture and the actual ICH burden 12, 40, 39].

In conclusion, our results do not support a key role for MMP-9 in the pathogenesis of hypertension-induced ICHs in the aged mouse brain. Future studies should determine the role of specific MMPs in the genesis of ICHs using selective pharmacological inhibitors and inducible knockout mouse models. The causal link between increased oxidative stress, MMP activation and genesis of ICHs should also be determined.

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Declarations

Disclaimer The funding sources had no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; and in the decision to submit the article for publication.

Competing interests Dr. Anna Csiszar serves as Associate Editor for *The Journal of Gerontology, Series A: Biological Sciences and Medical Sciences and GeroScience*. Dr. Andriy Yabluchanskiy serves as Guest Editor for *The American Journal of Physiology-Heart and Circulatory Physiology*. Dr. Zoltan Ungvari serves as Editor-in-Chief for GeroScience and as Consulting Editor for *The American Journal of Physiology-Heart and Circulatory Physiology*.

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