

High matrix metalloproteinase-9 expression induces angiogenesis and basement membrane degradation in stroke-prone spontaneously hypertensive rats after cerebral infarction

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Abstract

Basement membrane degradation and blood-brain barrier damage appear after cerebral infarction, severely impacting neuronal and brain functioning; however, the underlying pathogenetic mechanisms remain poorly understood. In this study, we induced cerebral infarction in stroke-prone spontaneously hypertensive rats by intragastric administration of high-sodium water (1.3% NaCl) for 7 consecutive weeks. Immunohistochemical and immunofluorescence assays demonstrated that, compared with the non-infarcted contralateral hemisphere, stroke-prone spontaneously hypertensive rats on normal sodium intake and Wistar-Kyoto rats, matrix metalloproteinase-9 expression, the number of blood vessels with discontinuous collagen IV expression and microvessel density were significantly higher, and the number of continuous collagen IV-positive blood vessels was lower in the infarct border zones of stroke-prone spontaneously hypertensive rats given high-sodium water. Linear correlation analysis showed matrix metalloproteinase-9 expression was positively correlated with the number of discontinuously collagen IV-labeled blood vessels and microvessel density in cerebral infarcts of stroke-prone spontaneously hypertensive rats. These results suggest that matrix metalloproteinase-9 upregulation is associated with increased regional angiogenesis and degradation of collagen IV, the major component of the basal lamina, in stroke-prone spontaneously hypertensive rats with high-sodium water-induced focal cerebral infarction.

Key Words: nerve regeneration; cerebral infarction; matrix metalloproteinase-9; collagen IV; microvessel density; angiogenesis; basement membrane degradation; high sodium; stroke-prone spontaneously hypertensive; China Medical Board Project; neural regeneration

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Introduction

Cerebral infarction is a common complication of hypertension (Qureshi, 2008; Sun et al., 2011). The major underlying initiating cause of infarction is small vessel structural changes (*i.e.*, vessel wall thickening, disruption and eventual breakdown), leading to damage to the blood-brain barrier, consequently resulting in stroke (Fukuda et al., 2004; Liu et al., 2006; Bailey et al., 2009; Bernas et al., 2010; Goldstein et al., 2011; Iadecola and Anrather, 2011; Liu et al., 2011; Dirnagl, 2012; Hossmann, 2012; Yenari and Han, 2012). Angiogenesis is a major factor associated with improved neurologic recovery after stroke (Chopp et al., 2007; Zhao et al., 2007; Bosomtwi et al., 2008; Teng et al., 2008; Li et al., 2010; Potente et al., 2011; Reitmeir et al., 2012; Espinera et al., 2013; Xiong et al., 2013; Zechariah et al., 2013; Zhang et al., 2013; Kono et al., 2014; Omote et al., 2014).

Studies have demonstrated that matrix metalloproteinase-9, a member of the matrix metalloproteinase family of

zinc- and calcium-dependent enzymes, is thought to play key roles in the pathogenesis of blood-brain barrier breakdown following stroke (Asahi et al., 2001; Aoki et al., 2002; Maier et al., 2004; Gidday et al., 2005; Kelly et al., 2006; Rosell et al., 2006; Kamada et al., 2007; Rosell et al., 2008; Feiler et al., 2011; Kumari et al., 2011; Shichi et al., 2011; Wang et al., 2011; Graham et al., 2012; Lee et al., 2012; Liu et al., 2012; Ralay Ranaivo et al., 2012; Suofu et al., 2012; Wu et al., 2012; Xiang et al., 2012; Alam and Shuaib, 2013; Morancho et al., 2013; Russell et al., 2014; Wu et al., 2014), and it appears to be involved in ischemic stroke. Matrix metalloproteinase-9 is able to degrade the major components of the basement membrane around cerebral blood vessels, such as collagen IV (Rosell et al., 2008; Luo et al., 2011), which increases the risk of cerebral hemorrhage (Alam et al., 2011). Recent studies (Lee et al., 2004; Mira et al., 2004; Yang et al., 2013; You-suf et al., 2014) indicate that matrix metalloproteinase-9, in particular, plays a central role during angiogenesis. Mira et

al. (2004) confirmed a relationship between matrix metalloproteinase-9 and angiogenesis in the invasion and metastasis of malignant tumors. Others have shown that matrix metalloproteinase-9 expression is upregulated by growth factors, including vascular endothelial growth factor, and that the enzyme plays an important role in vasculogenesis and vascular remodeling in response to injury (Lee et al., 2004), such as stroke (Yang et al., 2013). Matrix metalloproteinase-9 may also mediate neurovascular remodeling *via* lipoprotein receptor signaling in the peri-infarct cortical region, and could be a useful biomarker (Xiong et al., 2013). However, it remains poorly understood whether matrix metalloproteinase-9 is involved in basement membrane degradation following infarction and angiogenesis induced by hypertension in the stroke-prone spontaneously hypertensive rat model. In this study, we investigate the relationship between matrix metalloproteinase-9, collagen IV expression and microvessel density in stroke-prone spontaneously hypertensive rats.

Materials and Methods

Experimental animals and establishment of cerebral infarction model

Experiments were performed in 20 male stroke-prone spontaneously hypertensive rats, weighing 230–280 g, and 20 male non-hypertensive Wistar-Kyoto rats weighing 300–350 g. All rats, aged 9 weeks, were obtained from the Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). The rats were maintained at the Laboratory Animal Center, Medical School of Xi'an Jiaotong University, China (license No. SYXK (Shaan) 2007-003). The rats were housed in a room at $22 \pm 1^\circ\text{C}$ with a 12-hour light/dark cycle. All protocols were performed in accordance with the European Communities Council Directive of 24 November, 1986 (86/609/EEC), or with the Guidelines laid down by the NIH in the US regarding the care and use of animals for experimental procedures.

Stroke-prone spontaneously hypertensive rats and Wistar-Kyoto rats received either high sodium intake (10 stroke-prone spontaneously hypertensive rats, 10 Wistar-Kyoto rats) or normal sodium intake (10 stroke-prone spontaneously hypertensive rats, 10 Wistar-Kyoto rats) starting at 9 weeks of age to accelerate stroke onset. Rats receiving normal sodium intake were given 0.9% NaCl, and rats receiving high sodium intake were given 1.3% NaCl to drink, with daily weigh-ins. All rats were fed with standard rat chow. Systolic blood pressure of conscious rats was measured over 5-second intervals every 10 minutes by tail-cuff plethysmography (Kvetnansky et al., 1977). We calculated the mean weekly systolic blood pressure values for each animal. We began the experiments at 3 weeks after the rats showed major stroke-associated signs, such as hyperirritability, paroxysm, palsy or hemiplegia.

Expression of collagen IV, matrix metalloproteinase-9 and factor VIII in rat brain as detected by immunohistochemical staining

Five stroke-prone spontaneously hypertensive rats with brain infarction, five stroke-prone spontaneously hypertensive rats

without brain infarction, five Wistar-Kyoto rats given high sodium intake and five Wistar-Kyoto rats given normal sodium intake were given an intraperitoneal injection of 10% chloral hydrate (400 mg/kg) and then intracardially perfused with 100 mL of PBS, followed by 60 mL of fixative (4% paraformaldehyde, 2% sucrose in PBS; pH 7.5). Dissected brains were stored in the same fixative at 4°C overnight, followed by 10% sucrose for 12 hours, 20% sucrose for 12 hours, and 30% sucrose for 12 hours. Fixed brains were sectioned coronally 3 mm anterior and 3 mm posterior to the mid-coronal plane. Serial transverse sections of frozen brain (4- μm -thick) were made using a cryostat and treated with 3-aminopropyl-triethoxysilane. One section from each experimental animal was stained with hematoxylin and eosin, and the other sections were stored at -80°C for further use.

Immunohistochemical staining using the streptavidin-peroxidase method was performed after sectioning. Sections (4- μm -thick) containing the frontoparietal cortex from infarcted stroke-prone spontaneously hypertensive rats and Wistar-Kyoto rats were incubated with rabbit anti-rat collagen IV monoclonal antibody (1:50; Dako, Carpinteria, CA, USA), rabbit anti-rat matrix metalloproteinase-9 monoclonal antibody (1:200; Dako) or rabbit anti-rat factor VIII monoclonal antibody (1:300; Dako) at 4°C overnight, followed by goat anti-rabbit IgG (1:100; Dako) at 37°C for 30 minutes. After three additional washes, sections were visualized using 3,3'-diaminobenzidine solution. PBS was used instead of 3,3'-diaminobenzidine for negative controls. Larynx squamous cell carcinoma was used as a positive control. We evaluated matrix metalloproteinase-9 expression by counting the number of matrix metalloproteinase-9-positive cells and matrix metalloproteinase-9-positive blood vessels in different areas of rat brain from 10 high-power fields ($\times 400$), and the values were averaged for each parameter. All collagen IV-positive staining was found in the basal lamina of cerebral blood vessels. Two patterns of labeling were found: continuous or discontinuous around microvessels. The evaluation of collagen IV immunostaining was similar to that for matrix metalloproteinase-9. Microvessel density was measured by counting the number of single or clustered factor VIII-positive endothelial cells in different areas of the brain. The mean microvessel density was obtained from five high-power fields ($\times 400$) by light microscopy (Leica, Solms, Germany) (Ritz et al., 2009).

Expression of collagen IV, matrix metalloproteinase-9 and factor VIII in rat brain as determined by immunofluorescence labeling

All rats were intraperitoneally injected with 10% chloral hydrate (400 mg/kg). FITC-dextran (2×10^6 molecular weight; Sigma, St. Louis, MO, USA; 50 mg/mL, 1 mL) was injected into rats (five stroke-prone spontaneously hypertensive rats with brain infarction, five stroke-prone spontaneously hypertensive rats without brain infarction, five high sodium intake Wistar-Kyoto rats and five normal sodium intake Wistar-Kyoto rats) *via* the left femoral vein. One minute later, the rats were sacrificed by decapitation. The brains

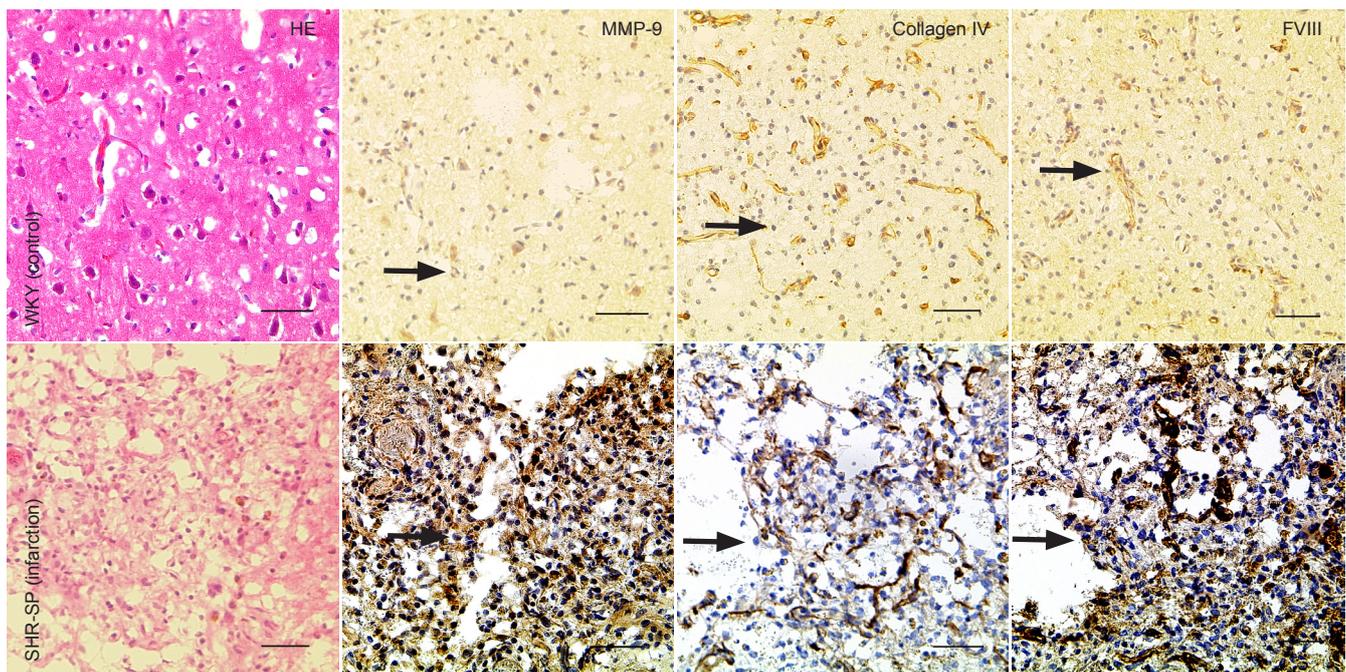


Figure 1 Histological changes in brain tissues of stroke-prone spontaneously hypertensive rats with cerebral infarction. Proliferative changes were visible in tissue. Cytoplasmic staining for matrix metalloproteinase-9 (MMP-9) is visible in vascular endothelial cells, astrocytes, neurons, gitter cells and inflammatory cells. Moderate disruption of the microvessel basal lamina was revealed by staining for collagen IV. There was strong cytoplasmic staining for factor VIII (FVIII) in vascular endothelial cells in stroke-prone spontaneously hypertensive rats. Arrows show positive expression. Scale bars: 50 μm. HE: Hematoxylin and eosin staining; WKY: Wistar-Kyoto; SHR-SP: stroke-prone spontaneously hypertensive.

Table 1 Expression of MMP-9 ($n/400 \times$ field of view), collagen IV ($n/400 \times$ field of view) and microvessel density ($n/400 \times$ field of view) in stroke-prone spontaneously hypertensive rats with or without cerebral infarction and in WKY rats

Group	MMP-9			Collagen IV ⁺ blood vessels	
	Cells	Blood vessels	Microvessel density	Continuous	Discontinuous
Normal sodium intake					
WKY	3.16±1.84	0.8±1.00	55.84±2.94	15.00±1.96	23.44±2.18
SHR-SP	51.60±6.20	3.84±1.82	54.60±6.83	17.08±2.94	23.16±2.23
High sodium intake*					
WKY	3.2±1.68	0.8±0.99	55.80±3.51	15.04±1.88	23.32±2.29
SHR-SP (NICH)	51.72±6.79	4.08±1.89	55.56±7.58	16.96±2.62	23.28±2.78
SHR-SP (IIH)	138.40±10.30	14.12±2.35	82.36±8.85	6.24±2.03	40.68±2.25

The data are presented as mean ± SD. Comparisons between two groups were analyzed by independent samples *t*-test. Compared to the contralateral hemisphere of stroke-prone spontaneously hypertensive rats or WKY rats on normal sodium intake, the number of MMP-9-positive cells and blood vessels, and the number of discontinuously collagen IV-labeled blood vessels and microvessel density were significantly higher, and the number of continuously collagen IV-labeled blood vessels was lower, in the infarct border zone of stroke-prone spontaneously hypertensive rats on high-sodium intake ($P < 0.01$). The numbers of MMP-9-positive cells and blood vessels were higher in the stroke-prone spontaneously hypertensive rats on normal sodium intake compared to WKY rats on normal sodium intake ($P < 0.01$). No significant difference in collagen IV-positive discontinuously or continuously-labeled blood vessels or microvessel density was detected between stroke-prone spontaneously hypertensive rats on normal sodium and WKY rats on normal sodium ($P > 0.05$). *The multicomparison analysis between normal sodium intake and high sodium intake was done by univariate analysis of variance ($F = 725.187$, $df = 16$, $P = 0.000 < 0.01$). SHR-SP: Stroke-prone spontaneously hypertensive; WKY: Wistar-Kyoto; NICH: non-infarcted contralateral hemisphere; IIH: infarcted ipsilateral hemisphere; MMP-9: matrix metalloproteinase-9.

were rapidly removed and fixed in 4% paraformaldehyde at 4°C for 24 hours. Serial transverse sections of frozen brain (30-μm-thick) were cut with a cryostat (JungCM 1900, Leica Instruments, Nussloch, Germany). Sections were treated with 3-aminopropyl-triethoxysilane and stored at -80°C for further use.

Sections were dried for an hour at room temperature and

immersed in 3% hydrogen peroxide for 10 minutes to suppress endogenous peroxidase activity. After three 5-minute rinses in PBS, sections were incubated at 4°C overnight with rabbit anti-rat collagen IV monoclonal antibody (1:50) or rabbit anti-rat matrix metalloproteinase-9 monoclonal antibody (1:100). After three 5-minute washes in PBS, sections were incubated with streptavidin-fluorescein (or Texas

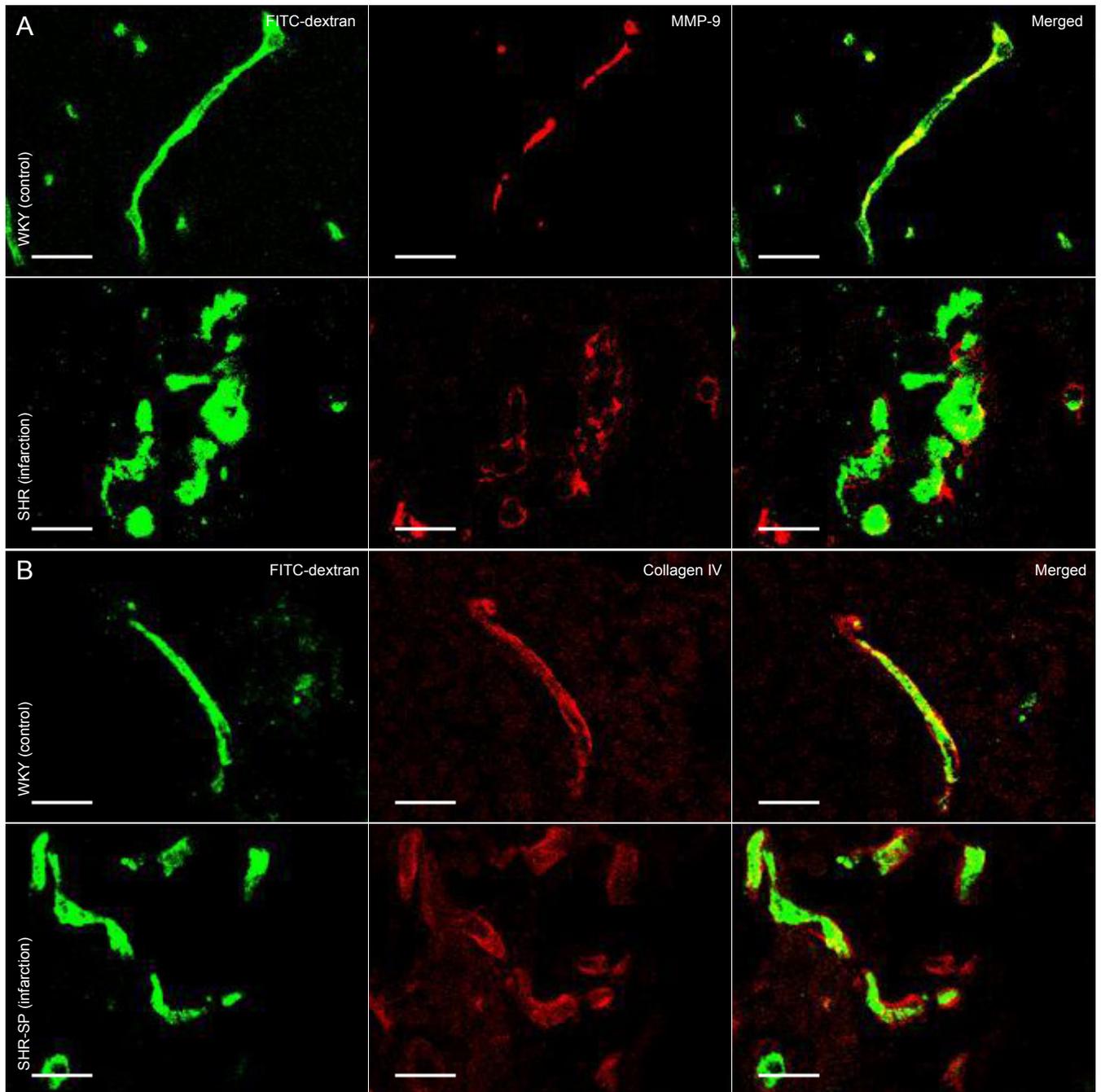


Figure 2 Expression of MMP-9 (A) and collagen IV (B) in the brain of stroke-prone spontaneously hypertensive rats with cerebral infarction (immunofluorescence staining).

FITC-dextran was injected into rats *via* the left femoral vein before being sacrificed. The architecture of microvessels is displayed with FITC dextran (green), and with MMP-9 and collagen IV immunofluorescence labeling (red). MMP-9 expression was increased in microvessels and endothelial cells in the infarct border zone in stroke-prone spontaneously hypertensive rats, and collagen IV was discontinuous in basal lamina of microvessels, compared to the corresponding areas of WKY rats. Scale bars: 20 μm. MMP-9: Matrix metalloproteinase-9; SHR-SP: stroke-prone spontaneously hypertensive; WKY: Wistar-Kyoto.

red)-labeled goat anti-rabbit IgG (1:800; Dako) in the dark at room temperature for 30 minutes. PBS was substituted for the negative control. Larynx squamous cell carcinoma was used as a positive control. Matrix metalloproteinase-9-positive blood vessels and collagen IV-positive blood vessels (red fluorescence) were visualized by confocal laser microscopy (Leica) (excitation, 488 nm). Imaging was performed under the same conditions, and laser excitation lasted for an hour.

Statistical analysis

All data were presented as mean \pm SD. Statistical analysis was performed with the Social Sciences Statistical package 13.0 (SPSS, Chicago, IL, USA). Data with two-group variables were analyzed by independent *t*-tests. Differences among multiple groups were assessed using univariate analysis of variance. The relationships between markers were evaluated with linear correlation analysis (Pearson correlation).

Differences between means were considered statistically significant at $P < 0.05$.

Results

Morphological changes in brain tissues of stroke-prone spontaneously hypertensive rats with cerebral infarction

Stroke-prone spontaneously hypertensive rats maintained on high-sodium water (1.3% NaCl) had a mean arterial blood pressure of 201 ± 8 mmHg. Decreased weight with a depressed state was apparent at 7 weeks. In comparison, stroke-prone spontaneously hypertensive rats given normal water had a mean arterial systolic blood pressure of 151 ± 4 mmHg, without a reduction in weight or depressive signs. Wistar-Kyoto rats on high-sodium water or normal water did not show abnormal symptoms and had a mean arterial systolic blood pressure of 115 ± 7 mmHg and 110 ± 5 mmHg, respectively.

There were no hemorrhages or infarcts in the brains of any control Wistar-Kyoto or stroke-prone spontaneously hypertensive rats with normal sodium intake (**Figure 1A**). Gross and histopathologic examination of the brain showed that they were normal. However, in all stroke-prone spontaneously hypertensive rats that ingested high-sodium water, gross pathologic examination showed softening in the frontoparietal or temporal cortex and surface bleeding, and the incidence rate of brain infarction was 100%. Hematoxylin-eosin-stained sections showed that the center of the softened area contained a cavity, and the boundary zone contained a small, softened area speckled with hemorrhages in stroke-prone spontaneously hypertensive rats with cerebral infarction. In the boundary zone, we observed proliferative changes in inflammatory cells, and degenerating astrocytes, neurons, gitter cells and capillary vessels (**Figure 1E**). No hemorrhages or infarction were observed in the non-infarcted hemisphere or in any control Wistar-Kyoto or stroke-prone spontaneously hypertensive rats with normal sodium intake.

Expression of matrix metalloproteinase-9 and collagen IV in the brain of stroke-prone spontaneously hypertensive rats with cerebral infarction

Immunohistochemical staining showed that matrix metalloproteinase-9 was mainly expressed in the cell membrane and cytoplasm of vascular endothelial cells, astrocytes, neurons, gitter cells and inflammatory cells (**Figure 1B and F**). The numbers of matrix metalloproteinase-9-positive cells and blood vessels were significantly higher in infarct border zones compared to the non-infarcted contralateral hemisphere, stroke-prone spontaneously hypertensive rats in normal water or Wistar-Kyoto rats ($P < 0.01$; **Table 1**). Immunofluorescence staining for matrix metalloproteinase-9 was especially robust and easily discerned in microvessels in experimental rats after injecting FITC (**Figure 2A**).

To assess basal lamina integrity in blood vessels, we stained brain sections for collagen IV. Two distinct patterns of collagen IV immunofluorescence were visible around microvessels-continuous and discontinuous (**Figure 2B**). Numerous blood vessels with continuous collagen IV labeling were

present in the non-infarcted contralateral hemisphere and in stroke-prone spontaneously hypertensive rats on normal sodium intake and in Wistar-Kyoto rats (**Figure 1C**). A lower number was observed in infarct border zones (**Figure 1G**). This difference was statistically significant ($P < 0.01$). The profile of discontinuous collagen IV labeling in blood vessels was essentially opposite to that of continuous collagen IV labeling ($P < 0.01$; **Table 1**).

Microvessel density in the brain of stroke-prone spontaneously hypertensive rats with cerebral infarction

Factor VIII expression was observed in the cytoplasm of cerebral vascular endothelial cells in all groups (**Figure 1D and H**). The number of factor VIII-positive microvessels and microvessel density were significantly higher in infarct border zones compared with the contralateral hemisphere, stroke-prone spontaneously hypertensive rats on normal sodium or Wistar-Kyoto rats ($P < 0.01$; **Table 1**).

Correlation of matrix metalloproteinase-9 with collagen IV and microvessel density

Linear correlation analysis showed that the number of matrix metalloproteinase-9-positive cells and blood vessels positively correlated with microvessel density and the number of blood vessels with discontinuous collagen IV labeling (matrix metalloproteinase-9-positive cells *versus* microvessel density: $r = 0.754$, $P < 0.01$, **Figure 3A**; matrix metalloproteinase-9-positive cells *versus* blood vessels discontinuously labeled for collagen IV: $r = 0.845$, $P < 0.01$, **Figure 3B**; matrix metalloproteinase-9-positive microvessels *versus* microvessel density: $r = 0.767$, $P < 0.01$, **Figure 3C**; matrix metalloproteinase-9-positive microvessels *versus* blood vessels discontinuously labeled for collagen IV: $r = 0.871$, $P < 0.01$, **Figure 3D**). In addition, the number of blood vessels with continuous collagen IV labeling was negatively correlated with the number of matrix metalloproteinase-9-positive cells ($r = -0.672$, $P < 0.01$; **Figure 3E**) and with the number of matrix metalloproteinase-9-positive blood vessels ($r = -0.719$, $P < 0.01$; **Figure 3F**). These results demonstrate that matrix metalloproteinase-9 expression is positively correlated with collagen IV degradation and angiogenesis in the cerebral infarct area of stroke-prone spontaneously hypertensive rats administered high-sodium drinking water.

Discussion

Stroke-prone spontaneously hypertensive rats are used as a stroke model because they exhibit cerebral hemorrhage and infarction similar to hypertensive humans (Bailey et al., 2009). However, they have a relatively low incidence rate of brain infarction, which is a concern in a model system (Yenari and Han, 2012). Studies reported a high proportion of strokes in stroke-prone spontaneously hypertensive rats on a high-salt diet, and they have severe hypertension and rapid stroke onset (Cho et al., 2007; Thoene-Reineke et al., 2011). Our observations of neurological changes in stroke-prone spontaneously hypertensive rats given high sodium (1.3%) drinking water showed mainly brain infarctions with

macroscopic and microscopic pathology, and the incidence rate of brain infarction was 100%. Thus, we suggest that stroke-prone spontaneously hypertensive rats on a high-salt regimen typically have hypertension and neocortical strokes, and may be a satisfactory experimental model of stroke.

Hitherto, the exact molecular mechanism responsible for clearing damaged tissue following stroke remained unknown. Studies demonstrated that matrix metalloproteinase-9 is upregulated early in injured tissue, suggesting a detrimental role, and that it is involved in brain damage in animal models of cerebral ischemia (Feiler et al., 2011; Kumari et al., 2011; Wang et al., 2011; Lee et al., 2012; Wu et al., 2012). We performed immunohistochemical staining for matrix metalloproteinase-9 expression in stroke-prone spontaneously hypertensive rats given high-sodium drinking water, and found that matrix metalloproteinase-9 expression was increased in the cell membranes and cytoplasm of all positive cell types, including astrocytes, neurons, glial cells, vascular endothelial cells and inflammatory cells in different regions of interest. We also found that the number of matrix metalloproteinase-9-positive cells and matrix metalloproteinase-9-positive blood vessels was high in infarct border zones. These findings are similar to those of a previous investigation by Shichi et al. (2011), which showed that protein levels of matrix metalloproteinase-9 were significantly increased 1 and 3 days after middle cerebral artery occlusion in a mouse model of cerebral infarction. Their results also demonstrated that matrix metalloproteinase-9 plays a detrimental role in brain damage in animal models of stroke.

Interestingly, matrix metalloproteinase-9 is associated with collagen IV degradation in cerebral stroke. The loss of basal lamina integrity has been postulated to be the primary cause of stroke, because matrix metalloproteinase-9 can degrade the main components of basal lamina, such as laminin, fibronectin and collagen IV (Rosell et al., 2008). Hence, matrix metalloproteinase-9 upregulation leads to a breakdown of the blood-brain barrier, and this ultimately results in brain hemorrhage and infarction. Studies have implicated matrix metalloproteinase-9 in cerebral stroke events and basal lamina destruction in rat models (Alam et al., 2011). Fukuda et al. (2004) showed that matrix metalloproteinase-9 significantly reduces levels of microvessel-associated collagen IV, laminin and heparan sulfate proteoglycans, and is acutely responsible for vascular matrix degradation in infarcted cerebral tissues after middle cerebral artery occlusion in adolescent male baboons. We found that the number of discontinuously collagen IV-labeled blood vessels mirrored the pattern of matrix metalloproteinase-9 expression. However, the number of continuously collagen IV-labeled blood vessels was low in infarct border zones. Linear correlation analysis showed that the number of matrix metalloproteinase-9-positive cells and matrix metalloproteinase-9-positive blood vessels were positively correlated with discontinuously collagen IV-labeled blood vessels, and it was negatively correlated with the number of continuously collagen IV-labeled blood vessels. Therefore, our findings confirm the associ-

ation between matrix metalloproteinase-9 overexpression and collagen IV degradation in infarct areas in stroke-prone spontaneously hypertensive rats, suggesting that matrix metalloproteinase-9 may be a key contributor to collagen IV degradation in brain after stroke.

Although many studies have reported matrix metalloproteinase-9 expression in vascular endothelial cells (Asahi et al., 2001; Aoki et al., 2002; Lee et al., 2004; Maier et al., 2004; Yang et al., 2013), the association between matrix metalloproteinase-9 expression and angiogenesis in infarcted brain tissue is still unclear. Zhao et al. (2007) showed that, in the peri-infarct area of the cortex, matrix metalloproteinases might mediate neurovascular remodeling *via* lipoprotein receptor signaling, and suggested its use as a biomarker. Therefore, to gain more insight into the mechanisms of the microvascular proliferative effects of matrix metalloproteinase-9 in stroke-prone spontaneously hypertensive brain infarcts (Kvetnansky et al., 1977; Bosomtwi et al., 2008), we measured markers such as microvessel density. Our results show that microvessel density mirrored the pattern of matrix metalloproteinase-9-positive blood vessels; *i.e.*, microvessel density was significantly higher in infarct border zones compared with the non-infarcted contralateral hemisphere and corresponding areas of stroke-prone spontaneously hypertensive rats on normal sodium or Wistar-Kyoto rats. Furthermore, microvessel density was positively correlated with the number of matrix metalloproteinase-9-positive cells and matrix metalloproteinase-9-positive blood vessels using linear correlation analysis, which suggests that matrix metalloproteinase-9 upregulation is associated with regional angiogenesis in the stroke-prone spontaneously hypertensive rat model of brain infarction. This is supported by the results of Li et al. (2010), which showed that vessel density and matrix metalloproteinase-9 protein levels were augmented in the Goto-Kakizaki rat model of type 2 diabetes, and further demonstrated that matrix metalloproteinase-9 augments angiogenesis at the capillary level in the stroke-prone spontaneously hypertensive rat model of brain infarction.

Taken together, our findings demonstrate a relationship between matrix metalloproteinase-9 upregulation, a reduction in continuous collagen IV labeling of microvessels and increased microvessel density in cerebral infarcts in stroke-prone spontaneously hypertensive rats given high-sodium (1.3%) drinking water. Furthermore, this study suggests that high-sodium water induces focal brain infarction in stroke-prone spontaneously hypertensive rats. Our findings suggest that matrix metalloproteinase-9 may play a key role in accelerating regional angiogenesis and degrading collagen IV.

Author contributions: Hou HL designed and performed the study, analyzed experimental data and wrote the manuscript. Zhang GJ, Wang HY, Gong HL, Wang CB and Zhang XB wrote the manuscript and provided critical revision of the manuscript for intellectual content. All authors approved the final version of the paper.

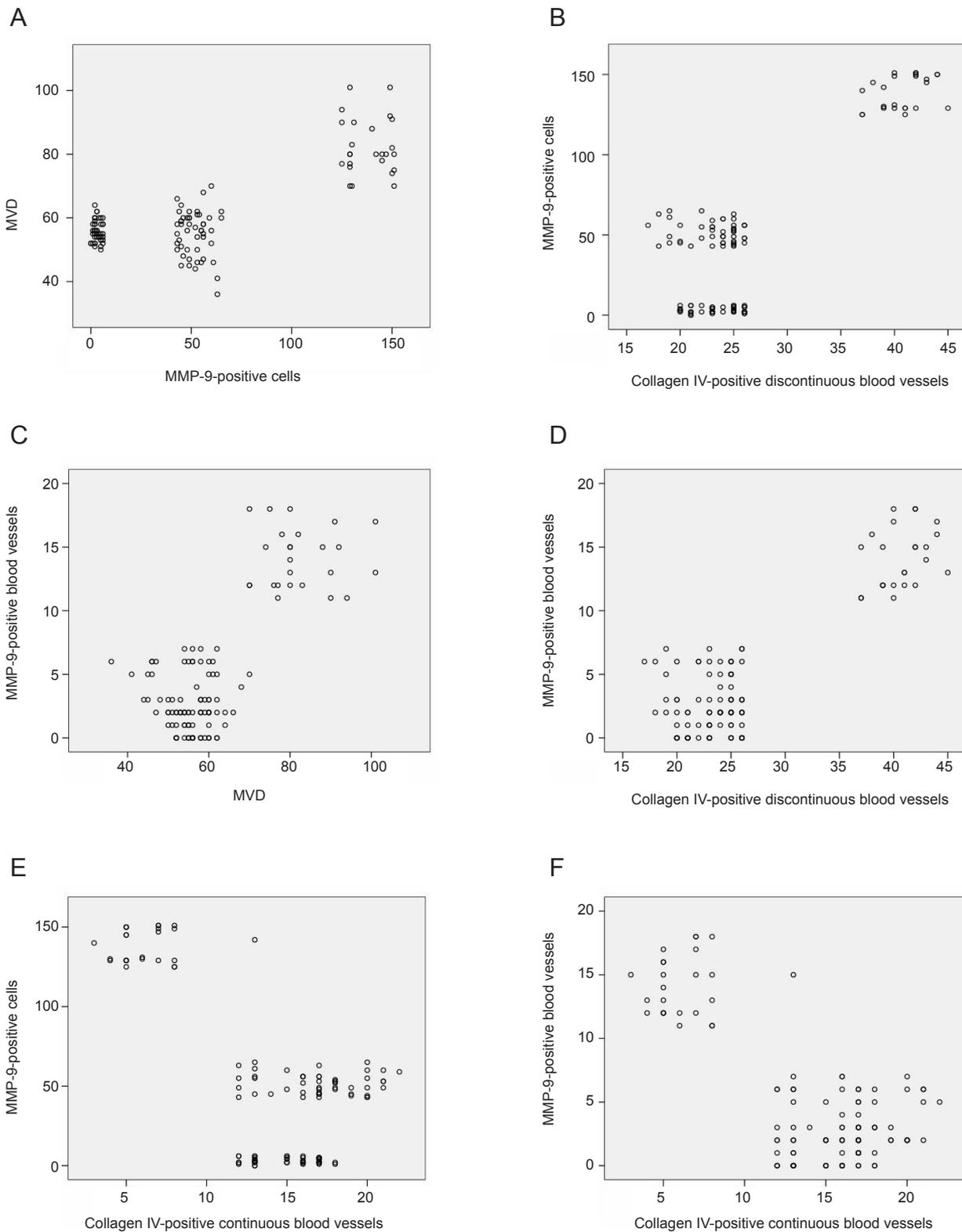


Figure 3 Correlation of MMP-9 with collagen IV and microvessel density in the brain of stroke-prone spontaneously hypertensive rats with cerebral infarction.

Linear correlation analysis showed that the number of MMP-9-positive cells and blood vessels positively correlated with microvessel density and the number of collagen IV-positive discontinuously-labeled blood vessels. The correlations were positive for MMP-9-positive cells *versus* microvessel density ($r = 0.754$; A) and *versus* collagen IV-positive discontinuously-labeled blood vessels ($r = 0.845$; B); and for MMP-9-positive microvessels *versus* microvessel density ($r = 0.767$; C) and *versus* collagen IV-positive discontinuously-labeled blood vessels ($r = 0.871$; D). The correlations were negative for the number of collagen IV-positive continuously-labeled blood vessels *versus* MMP-9-positive cells ($r = -0.672$; E) and *versus* MMP-9-positive blood vessels ($r = -0.719$; F). MVD: Microvessel density; MMP-9: matrix metalloproteinase-9.

Conflicts of interest: None declared.

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