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Effects of Tenascin-C Knockout on Cerebral Vasospasm After Experimental Subarachnoid Hemorrhage in Mice

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Abstract A matricellular protein tenascin-C (TNC) has been suggested to play a role in the pathogenesis of cerebral vasospasm after subarachnoid hemorrhage (SAH), but the direct evidence remains lacking. In this study, we examined effects of TNC knockout (TNKO) on cerebral vasospasm after experimental SAH in mice. C57BL/6 wild-type (WT) or TNKO mice were subjected to SAH by endovascular puncture. Ten WT and ten TNKO mice were randomized to WT sham (n = 4), TNKO sham (n = 4), WT SAH (n = 6), and TNKO SAH (n = 6) groups. In addition to neurobehavioral impairments and severity of SAH, cerebral vasospasm was assessed by morphometric measurements of the left internal carotid artery (ICA). Infiltration of inflammatory cells in the subarachnoid periarterial space was also assessed, and expressions of TNC and mitogen-activated protein kinases (MAPKs) in the ICA were immunohistochemically evaluated at 24 h post-surgery. TNC was induced in the smooth muscle cell layers and the adventitia in the spastic ICAs as well as the periarterial inflammatory cells in WT SAH mice. Compared with WT SAH mice, TNKO SAH mice showed better neurological scores and less severe cerebral vasospasm, as well as fewer inflammatory cell infiltration in the periarterial space. Post-SAH activation of MAPKs in the smooth muscle cell layers of the ICAs was also prevented in TNKO SAH mice. The findings in the present study suggest that TNC causes the development of cerebral vasospasm via pro-inflammatory effects and activation of MAPKs.

Keywords Cerebral vasospasm · Mitogen-activated protein kinase · Subarachnoid hemorrhage · Tenascin-C

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Introduction

Cerebral vasospasm is one of the important treatable causes of delayed cerebral ischemia and poor outcome after aneurysmal subarachnoid hemorrhage (SAH) [1, 2]. Despite numerous preclinical and clinical studies, however, the pathogenesis of cerebral vasospasm remains poorly understood.

Recently, we reported that tenascin-C (TNC), a matricellular protein, was induced in cerebrospinal fluid and serum after aneurysmal SAH associated with cerebral vasospasm [3, 4]. Furthermore, our experimental studies showed that TNC was induced in the spastic cerebral arteries [3, 5] and that imatinib mesylate, a tyrosine kinase inhibitor of platelet-derived growth factor receptor, prevented cerebral vasospasm in rat SAH models associated with downregulation of TNC and inactivation of mitogen-activated protein kinases (MAPKs) [5]. In other experimental studies, an intracisternal injection of TNC activated MAPKs and caused prolonged cerebral arterial constriction via Toll-like receptor 4 and epidermal growth factor receptor in healthy rats [6–8]. TNC is thus considered to play an important role in the pathogenesis of cerebral vasospasm after SAH, but the direct evidence has not been provided thus far. In the present study, in order to obtain the direct evidence linking TNC with cerebral vasospasm, we evaluated effects of TNC knockout (TNKO) on cerebral vasospasm in an established endovascular perforation model of SAH in mice.

Materials and Methods

Animals

All procedures were approved by the Animal Ethics Review Committee of Mie University and were carried out in accordance with the institution's Guidelines for Animal Experiments. The original TNKO mouse was backcrossed with C57BL/6 inbred mice for more than ten generations [9]. C57BL/6 wild-type (WT) littermates were used as controls. Mice were maintained on a constant 12-h light/12-h dark cycle in a temperature- and humidity-controlled room and were given ad libitum access to food and water.

Study Protocol

To study effects of TNKO on cerebral vasospasm after SAH, ten WT and ten TNKO mice (female; weight, 20–25 g) were randomly divided into WT sham ($n = 4$), TNKO sham ($n = 4$), WT SAH ($n = 6$), and TNKO SAH ($n = 6$) groups. Mice underwent endovascular perforation SAH or sham operation. After evaluating neuroscore at 24 h post-surgery, mice were sacrificed, and high-resolution pictures of the base of the brain depicting the circle of Willis and basilar arteries were taken for assessing the severity of SAH. Then, cerebral vasospasm and immunohistochemical findings were evaluated at 24 h post-surgery (Fig. 1a).

Mouse SAH Model

The endovascular perforation model of SAH was produced by a modification of the method previously described [10–12]. Each animal was anesthetized with an intraperitoneal injection of tribromoethanol (250 μg/g body weight). A sharpened 5-0 monofilament nylon suture was advanced rostrally into the left internal carotid artery (ICA) from the external carotid artery stump to perforate the bifurcation of the left anterior and middle cerebral arteries. In the sham surgery, the filament was advanced 5 mm through the ICA without perforating the artery. Blood pressure and heart rate were measured noninvasively from the tail.

Neurobehavioral Test

Neurological impairments were blindly evaluated using two methods as previously described [10–12]. Neurological scores

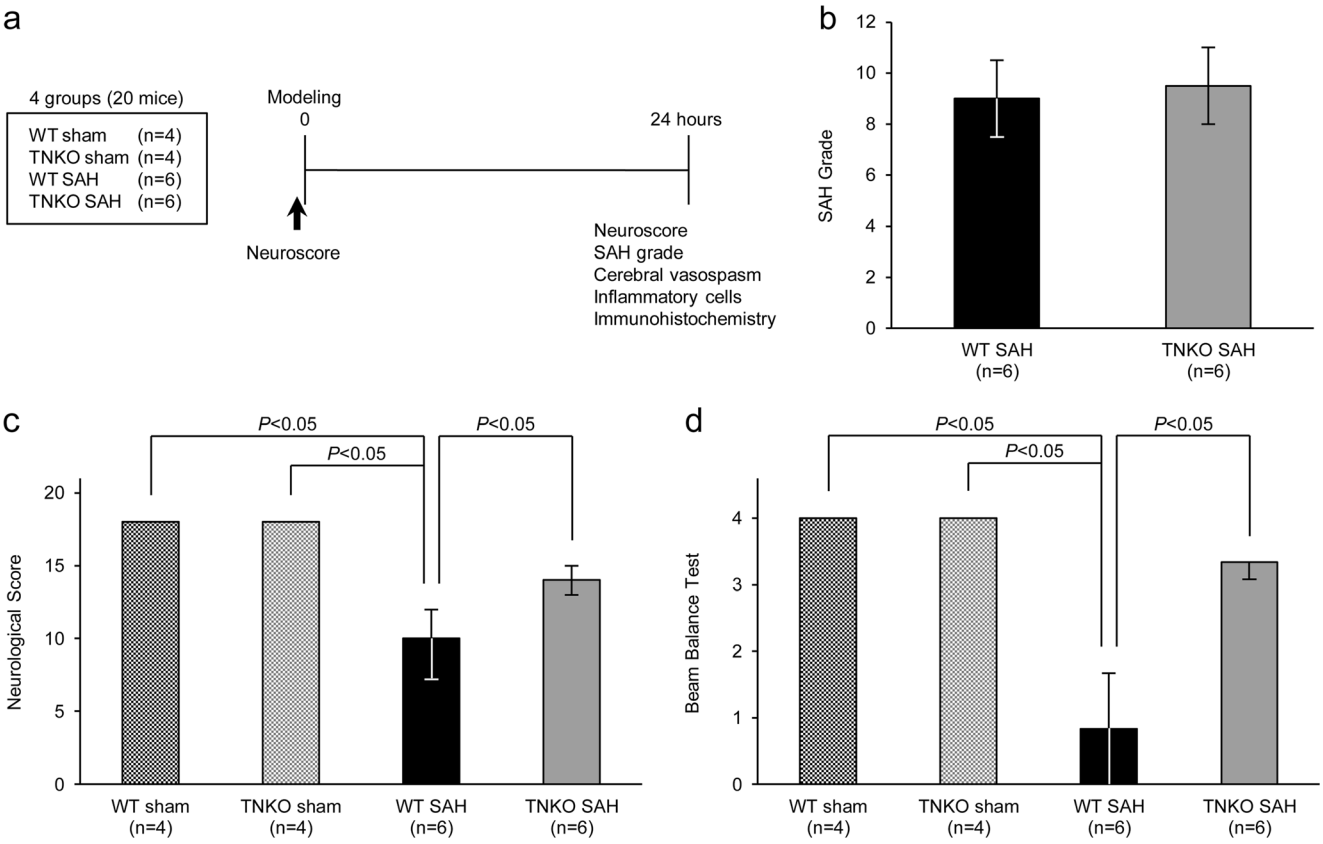

Fig. 1 Experimental designs (**a**). Effects of tenascin-C knockout (*TNKO*) on the severity of subarachnoid hemorrhage (*SAH*) (**b**), neurological score (**c**), and beam balance test (**d**) at 24 h after SAH. Experiment is designed to examine effects of TNKO on cerebral vasospasm following SAH. Data are expressed as median ± 25th–75th percentiles. Kruskal-Wallis tests. *WT* wild type

(3–18) were assessed by summing six test scores (spontaneous activity, spontaneous movement of four limbs, forepaw outstretching, climbing, body proprioception, and response to whisker stimulation). A beam balance test investigated the animal's ability to walk on a narrow wooden beam for 60 s: four points, walking ≥20 cm; three points, walking ≥10 cm but <20 cm; two points, walking ≥10 cm but falling; one point, walking <10 cm; and zero points, falling with walking <10 cm. The mean score of three consecutive trials in a 5-min interval was calculated.

Severity of SAH

The grading system for evaluating SAH severity in endovascular perforation rodent models showed a strong correlation with neurological status and degree of cerebral vasospasm [13]. The severity of SAH was blindly assessed using the high-resolution photographs as previously described [10–12]. The basal cistern was divided into six segments, and each segment was allotted a grade from 0 to 3 depending on the amount of subarachnoid blood clot in the segment: grade 0, no subarachnoid blood; grade 1, minimal subarachnoid blood; grade 2, moderate blood clot with recognizable arteries; and grade 3, blood clot obliterating all arteries within the segment. The animals received a total score ranging from 0 to 18 after adding the scores from all six segments.

Histological Preparation

At 24 h post-surgery, mice were anesthetized with an intraperitoneal injection of tribromoethanol (250 μg/g body weight) and euthanized in preparation for perfusion and fixation. The ascending aorta was cannulated with a blunted 18-gauge needle attached to flexible plastic tubing, which was connected to a pressure transducer (Nihon Kohden Co., Tokyo, Japan) and a syringe on an automatic infusion pump (KD Scientific Inc., Holliston, MA). After an incision was made in the right atrium to allow for the outflow of perfusion solutions, 50 mL of phosphate-buffered saline (PBS) and 15 min of 10% neutral buffered formalin were infused through the closed circuit at 60–80 mmHg. The brain was harvested and high-resolution pictures of the base of the brain depicting the circle of Willis and basilar arteries were taken for assessing the severity of SAH. Then, the brain was stored in 10% neutral buffered formalin for approximately 12 h at 4 °C for fixation. Cerebrum with the ICA was removed and then embedded in paraffin. The tissue was coronally cut at 1 mm dorsal from bifurcation of the left anterior and middle cerebral arteries, and transverse sections (4 μm thick) of the left intracranial ICA were obtained with a microtome. Tissue slices were mounted on glass slides for hematoxylin and eosin (HE) or immunohistochemical stainings [6–8].

HE Staining: Vasospasm Measurement and Counting of Inflammatory Cells in the Subarachnoid Periarterial Space

The sections for HE staining were stained with hematoxylin for 10 min and eosin for 10 min. Cross sections of the left intracranial ICA were digitized using a video-assisted microscope (×400; Olympus Co., Tokyo, Japan). The circumferences and the thickness of the left ICA were measured using ImageJ software (National Institutes of Health, Bethesda, MD). The circumference of the left ICA ($C = 2\pi r$) was used to calculate the cross-sectional area (area = $C^2/4\pi$) of the artery [14]. Artery wall thickness was measured at four equally spaced points along the artery circumference and averaged to obtain artery thickness [15].

Inflammatory cells in the subarachnoid periarterial space per high-powered field were detected and the number was counted. All measurements were performed by an experienced researcher who was unaware of the treatment groups.

Immunohistochemical Staining

Immunohistochemical staining on formalin-fixed paraffin-embedded sections was performed as described previously [6–8]. After dewaxing and rehydration, the sections were treated with 3% hydrogen peroxide for 10 min to block endogenous peroxidase activity, placed in 1 mmol ethylenediaminetetraacetic acid (pH 8.0), and heated in an autoclave at 80 °C for 20 min. The sections were then blocked with 5% goat or horse serum and incubated overnight at 4 °C with the rabbit polyclonal anti-TNC antibody (1 μg/mL), rabbit polyclonal anti-phosphorylated extracellular signal-regulated kinase (ERK) 1/2, mouse monoclonal anti-phosphorylated c-Jun N-terminal kinase (JNK), and mouse monoclonal anti-phosphorylated p38 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA) antibodies. They were subsequently incubated with biotinylated anti-rabbit or anti-mouse immunoglobulin (Vector Laboratories, Burlingame, CA) for 30 min and then with an avidin-biotin complex for 30 min at room temperature. Color reactions were developed in diaminobenzidine/hydrogen peroxide solution, and the sections were counterstained with hematoxylin solution for light microscopic examination. Negative controls consisted of serial sections incubated with buffer alone instead of the primary antibodies.

Statistics

Neurological, beam balance scores, and SAH grade were expressed as median ± 25th–75th percentiles and were analyzed with Mann-Whitney *U* tests or Kruskal-Wallis tests, followed by Steel-Dwass multiple comparisons. Other values were expressed as mean ± standard deviation, and one-way analysis of variance (ANOVA) with Tukey-Kramer post hoc tests were used. $P < 0.05$ was considered significant.

Results

TNKO Prevents Cerebral Vasospasm After SAH

Comparisons of physiological parameters revealed no significant differences among the groups (data not shown). The severity of SAH grading score was similar between the WT and TNKO groups at 24 h post-surgery (Fig. 1b). All mice exhibited no neurological deficits before surgery. In the sham group, neurological findings, and the lumen cross-sectional area and the wall thickness of the left intracranial ICA showed no difference between the WT and TNKO mice. In the WT mice, SAH caused significant neurological impairments, significantly reduced the lumen cross-sectional area, and increased the wall thickness of the left ICA associated with an increase in inflammatory cells in the subarachnoid periarterial space (Figs. 1c, d, 2, and 3). On the other hand, TNKO SAH mice showed significantly better neurological findings and

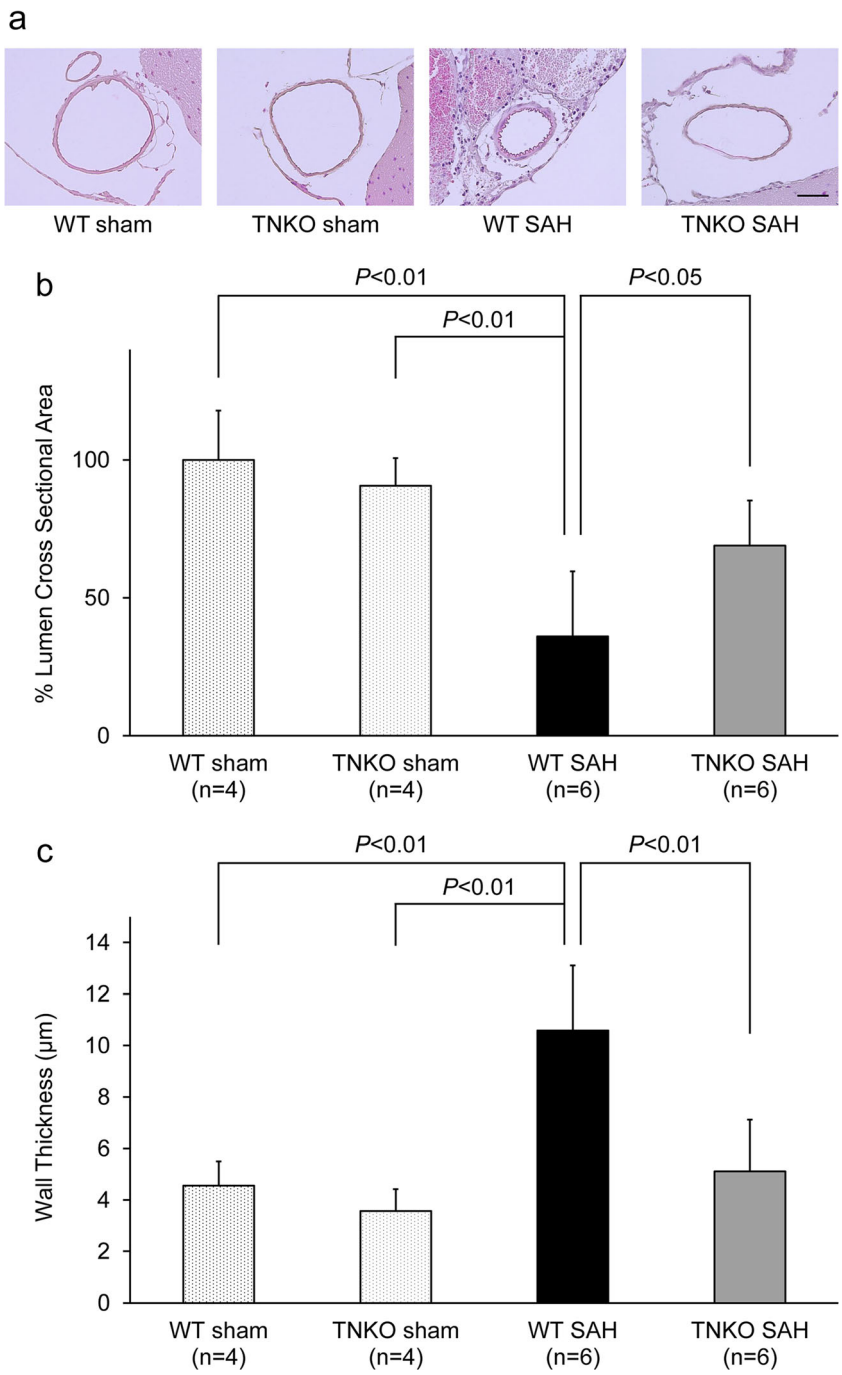

Fig. 2 Effects of tenascin-C knockout (*TNKO*) on vasospasm of the left internal carotid artery at 24 h after subarachnoid hemorrhage (*SAH*). Representative hematoxylin-eosin staining (**a**), % lumen cross-sectional area (**b**) and wall thickness (**c**) of the left internal carotid artery versus that of the wild-type (*WT*) sham mice. *Scale bar* = 50 μm. Data are expressed as mean ± standard deviation. ANOVA

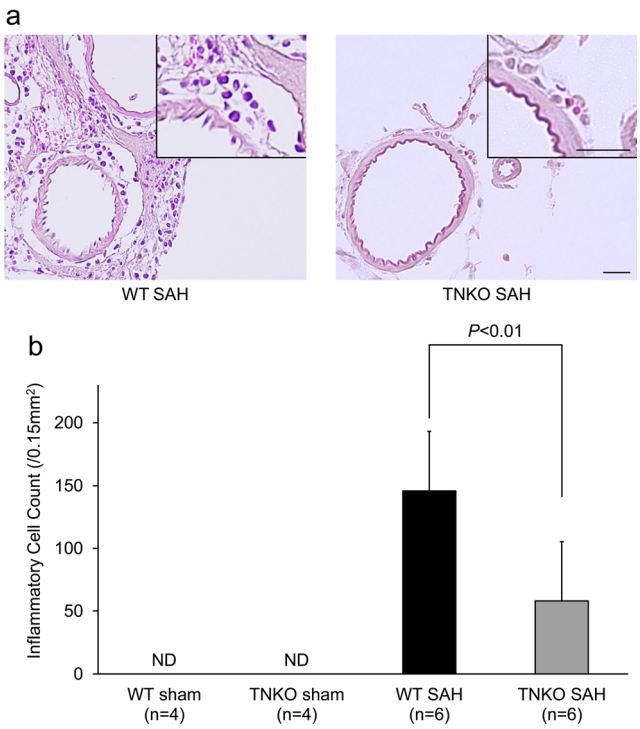

Fig. 3 Effects of tenascin-C knockout (*TNKO*) on inflammatory cell infiltration in the subarachnoid space around the left internal carotid artery at 24 h after subarachnoid hemorrhage (*SAH*). Representative hematoxylin-eosin staining (**a**). Inflammatory cell counting (**b**). *Scale bar* = 20 μm. Data are expressed as mean ± standard deviation. ANOVA. *ND* no detection, *WT* wild type

less severe cerebral vasospasm associated with fewer inflammatory cells in the periarterial space compared to the WT SAH mice (Figs. 1c, d, 2, and 3).

TNKO Blocks Activation of MAPKs in the Left ICA after SAH

Immunohistochemical stainings showed that TNC was mainly induced in the adventitia in the spastic ICA in the WT SAH mice in addition to the smooth muscle cell layers (Fig. 4). TNC was also intensively expressed in the periarterial inflammatory cells in the WT SAH mice (Fig. 5). Immunostainings of phosphorylated ERK1/2, JNK, and p38 were very limited in the sham group. In the WT SAH mice, strong immunolabeling of phosphorylated ERK1/2, JNK, and p38 was observed in the smooth muscle cells in the spastic ICA, and these post-SAH immunoreactivities were blocked by TNKO (Fig. 4).

Discussion

In this study, we investigated the role of TNC in cerebral vasospasm after SAH using TNKO mice. The findings indicated that deficiency of TNC resulted in better neurological function, less severe cerebral vasospasm, and less infiltration of inflammatory cells into the periarterial space after SAH.

TNC is an inducible, nonstructural, and secreted extracellular matrix protein categorized as a matricellular protein [16–18]. TNC exerts diverse functions through direct binding to cell surface receptors, other matrix proteins, and soluble extracellular factors such as growth factors and cytokines [17]. As with other matricellular protein knockout mice, TNKO mice were initially reported to undergo normal development and have a normal life span and fertility without distinct phenotypes [9, 19, 20]. However, many studies using TNKO mice have shown differences in specific cell behavior and responses in various diseases and reported that TNC may play an important role in the development of the diseases [10, 21–24].

Our previous studies suggested the possible link between TNC and cerebral vasospasm [3–5, 25–27]. In clinical researches, TNC levels in serum and cerebrospinal fluid in patients with cerebral vasospasm were significantly higher than those without vasospasm after aneurysmal SAH [3, 4, 28]. In experimental SAH produced by a single blood injection into

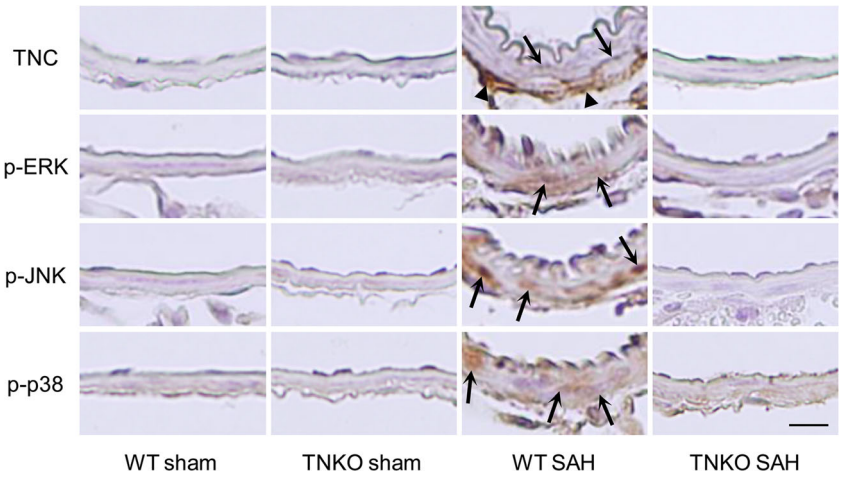

Fig. 4 Effects of tenascin-C (*TNC*) knockout (*TNKO*) on immunohistochemical stainings of TNC, phosphorylated extracellular signal-regulated kinase1/2 (*p-ERK*), c-Jun N-terminal kinase (*p-JNK*), and p38 (*p-p38*) in the left internal carotid artery at 24 h after subarachnoid hemorrhage (*SAH*). *Scale bar* = 10 μm. *WT* wild type. Immunoreactive smooth muscle cell (*arrow*). Immunoreactive adventitial cell (*arrowhead*)

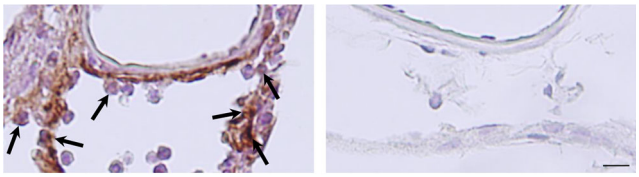

Fig. 5 Effects of tenascin-C (TNC) knockout (TNKO) on immunohistochemical stainings of TNC in inflammatory cells around the left internal carotid artery at 24 h after subarachnoid hemorrhage (SAH). *Scale bar* = 10 μm. *WT* wild type. Immunoreactive inflammatory cell (*arrow*)

the cisterna magna and a filament perforation to the bifurcation of the anterior and middle cerebral arteries in rats, TNC immunoreactivity was induced in cerebral artery walls with the development of vasospasm and decreased as vasospasm improved [3, 5]. Furthermore, imatinib mesylate, a tyrosine kinase inhibitor of platelet-derived growth factor receptor, prevented cerebral vasospasm in a rat SAH model associated with the reduction of TNC and inactivation of MAPKs [5]. In other experimental studies, an intracisternal injection of TNC caused prolonged cerebral arterial constriction via Toll-like receptor 4 and epidermal growth factor receptor, and associated with activation of MAPKs in healthy rats [6–8]. However, this study first provided the direct evidence showing that TNC causes the development of cerebral vasospasm via activation of MAPKs as well as inflammatory cell infiltration into the periarterial space.

Recent investigations suggest that other factors such as early brain injury in addition to cerebral vasospasm also cause delayed cerebral ischemia [29], although it is well-known that delayed cerebral ischemia more frequently occurs associated with severe vasospasm [30]. As with this study, previous studies showed that blockage of TNC had no effects on SAH severity in the endovascular puncture model of rats or mice [5, 10, 12]. However, TNKO [10] as well as pharmacological blockage of TNC [12] prevented early brain injury in terms of blood-brain barrier disruption. Thus, neurological improvement in this study may have been brought about by TNKO's preventive effects on cerebral vasospasm and/or early brain injury.

Findings from both clinical and animal studies have indicated that inflammatory reactions may contribute to the development of cerebral vasospasm after SAH [31–33]. Human studies have repeatedly shown elevated inflammatory mediators such as endothelin-1 [34], tumor necrosis factor-alpha [35], and interleukins-1 and -6 [36] in cerebrospinal fluid after SAH. In experiment studies, several pro-inflammatory agents such as talc (crystallized hydrous magnesium sulfate) [37], polystyrene latex beads [38], and lipopolysaccharide [39] were administered intracisternally to show that prolonged vasoconstriction resembling cerebral vasospasm occurred in the absence of blood but associated with inflammation. Under the state of SAH, blood clots in the subarachnoid space can activate inflammatory responses through a complex series of cellular and molecular events as follows: (1) leukocyte recruitment, infiltration, and activation; (2) cytokine production; (3) immunoglobulin and complement activation; and (4) transcription factor activation [40]. TNC is highly expressed during embryonic development, but the distribution of TNC is typically limited in adult tissues. However, TNC expression is induced rapidly at sites of inflammation, apparently regardless of the location or type of causative insult, by various pro- and anti-inflammatory cytokines, hypoxia, reactive oxygen species, and mechanical stress [17]. Depending on the pathological conditions, TNC modulates activation, adhesion, rolling, and infiltration of inflammatory cells via various signaling pathways, resulting in promotion or inhibition of inflammatory reaction [17]. This study first revealed that TNC promoted inflammation at least in terms of inflammatory cell infiltration in the periarterial space after SAH, which might contribute to the development of vasospasm as above. On the other hand, SAH may induce matrix metalloproteinases and serine proteases, which can cleave TNC [10, 17]. Cleavage of TNC may release cryptic sites that create adhesive sites for cell surface receptors, activating different signaling and exerting diverse cell responses via the receptors [17]. Although the full extent of the functions is not currently clear, TNC may activate MAPKs and cause cerebral vasospasm in the text of SAH [5]. As TNC may have the positive feedback mechanisms on upregulation of TNC itself in an acute phase of SAH [27], the vicious cycle may lead to more activation of inflammatory reactions and MAPKs, both of which cause the development or aggravation of cerebral vasospasm. Thus, this study showed that TNC is a promising therapeutic target against cerebral vasospasm and warrants further studies.

Conclusions

We demonstrated that TNKO prevented post-SAH cerebral vasospasm development via suppressing inflammation and inactivating MAPKs. Targeted suppression of TNC expression may provide a novel therapeutic approach against post-SAH vasospasm.

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Compliance with Ethical Standards

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Conflict of Interest The authors declare that they have no conflict of interest.

Research Involving Animals All procedures were approved by the Animal Ethics Review Committee of Mie University and were carried out according to the institution's Guidelines for Animal Experiments.

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