Critical role of TNF-alpha-TNFR1 signaling in intracranial aneurysm formation

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Abstract

Background: Intracranial aneurysm (IA) is a socially important disease due to its high incidence in the general public and the severity of resultant subarachnoid hemorrhage that follows rupture. Despite the social importance of IA as a cause of subarachnoid hemorrhage, there is no medical treatment to prevent rupture, except for surgical procedures, because the mechanisms regulating IA formation are poorly understood. Therefore, these mechanisms should be elucidated to identify a therapeutic target for IA treatment. In human IAs, the presence of inflammatory responses, such as an increase of tumor necrosis factor (TNF)-alpha, have been observed, suggesting a role for inflammation in IA formation. Recent investigations using rodent models of IAs have revealed the crucial role of inflammatory responses in IA formation, supporting the results of human studies. Thus, we identified nuclear factor (NF)-kappaB as a critical mediator of inflammation regulating IA formation, by inducing downstream pro-inflammatory genes such as MCP-1, a chemoattractant for macrophages, and COX-2. In this study, we focused on TNF-alpha signaling as a potential cascade that regulates NF-kappaB-mediated IA formation.

Results: We first confirmed an increase in TNF-alpha content in IA walls during IA formation, as expected based on human studies. Consistently, the activity of TNF-alpha converting enzyme (TACE), an enzyme responsible for TNF-alpha release, was induced in the arterial walls after aneurysm induction in a rat model. Next, we subjected tumor necrosis factor receptor superfamily member 1a (TNFR1)-deficient mice to the IA model to clarify the contribution of TNF-alpha-TNFR1 signaling to pathogenesis, and confirmed significant suppression of IA formation in TNFR1-deficient mice. Furthermore, in the IA walls of TNFR1-deficient mice, inflammatory responses, including NF-kappaB activation, subsequent expression of MCP-1 and COX-2, and infiltration of macrophages into the IA lesion, were greatly suppressed compared with those in wild-type mice.

Conclusions: In this study, using rodent models of IAs, we clarified the crucial role of TNF-alpha-TNFR1 signaling in the pathogenesis of IAs by inducing inflammatory responses, and propose this signaling as a potential therapeutic target for IA treatment.

Keywords: Inflammation, Intracranial aneurysm, Macrophage, MCP-1, NF-kappaB, TNF-alpha

Introduction

Intracranial aneurysm (IA) is a lesion with a regional bulging of intracranial arteries, usually located at bifurcation sites. IA is a common disease in the general public, with a prevalence of 1–5 percent [1], and is a major cause of subarachnoid hemorrhage [2]. Subarachnoid hemorrhage continues to be responsible for high mortality and morbidity rates, despite advances in medical technologies [3], and therefore, the prevention of pre-existing IA rupture is imperative. Furthermore, IAs have a remarkable negative impact on society, not only due to death or complications resulting from subarachnoid hemorrhage, but also due to the anxiety associated with potential rupture. Indeed, a recent report demonstrated that the quality of life of patients with IAs was significantly limited [4] even if IAs did not rupture. Importantly, treatment of IAs was also shown to restore the social activity of these patients. In this sense, the treatment of IAs is socially important. However, to date,
except for surgical procedures, there is no available medical treatment to prevent the rupture of IAs. Given the severity and negative social impact of a resulting subarachnoid hemorrhage after rupture, the mechanisms underlying IA formation and rupture should be investigated to develop a novel medical treatment for IAs.

Various studies using human IA specimens have revealed the involvement of active inflammatory responses in IA lesions, such as the expression of inflammatory cytokines, the infiltration of inflammatory cells into lesions, and the positive linkage of inflammatory-gene polymorphisms with IAs [5–9]. Furthermore, recent experiments using animal models of IAs support the interpretation of human studies that inflammatory responses in intracranial arterial walls regulate IA formation and progression. Among factors regulating inflammatory processes, we have revealed a critical role for nuclear factor (NF)-κB in the pathogenesis of IA formation. NF-κB mediates many physiological processes, and it is not a suitable therapeutic target. Indeed, despite the crucial role of NF-κB in various inflammatory diseases, no anti-NF-κB therapy has been established.

Tumor necrosis factor (TNF)-α is a cytokine that can strongly activate NF-κB, and TNF-α signaling has been implicated in the pathogenesis of human IAs. For example, increased TNF-α mRNA expression in lesions as determined by reverse transcription PCR (RT-PCR) [6], increased plasma TNF-α concentration in patients with IAs [12], and the possible linkage of TNF-α signaling with IA rupture in whole-genome expression profile analysis [9], have been reported. Furthermore, a positive correlation between single nucleotide polymorphisms in the TNF-α gene and increased risk of incidence and rupture, has been identified [13,14]. Based on these findings, in this study, we examined the contribution of TNF-α signaling to IA formation using tumor necrosis factor receptor superfamily member 1α (TNFR1)-deficient mice.

Materials and methods

Rodent IA models and histological analysis of induced IA

All of the following experiments, including animal care and use, complied with the National Institute of Health's Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Kyoto University Graduate School of Medicine.

Male Sprague–Dawley rats were purchased from Japan SLC (Shizuoka, Japan). TNFR1-deficient mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Animals were maintained on a light/dark cycle of 14 h/10 h, and had free access to chow and water. To induce IA, under general anesthesia by intraperitoneal injection of pentobarbital sodium (50 mg/kg), 7-week-old male rats or mice were subjected to ligation of the left carotid artery and systemic hypertension, induced by the combination of salt overloading and ligation of the left renal artery. This procedure is designed to increase hemodynamic stress, the trigger of IA formation [15–17], on the bifurcation site of intracranial arteries [18–20]. Immediately after the surgical procedure, animals were fed a special chow containing 8% sodium chloride and 0.12% 3-aminopropionitrile (Tokyo Chemical Industry, Tokyo, Japan), an inhibitor of lysyl oxidase that catalyzes the cross-linking of collagen and elastin. IA induction at the right anterior cerebral artery (ACA) and olfactory artery (OA) bifurcation, the contralateral side of the carotid ligation was assessed at times indicated in the corresponding Figure legends [18–20]. In histological analyses, after measurement of systemic blood pressure by the tail-cuff method, animals were deeply anesthetized by intraperitoneal injection with a lethal dose of pentobarbital sodium, and transcardially perfused with 4% paraformaldehyde. The right ACA-OA bifurcation including the IA lesion was then stripped, and serial frozen sections were made. The IA lesion was defined by the disruption of the internal elastic lamina, as visualized by Elastica van Gieson staining.

Immunohistochemistry

Immunohistochemical analyses were performed as previously described [19]. Briefly, at the indicated period after aneurysm induction, 5-um-thick frozen sections were made as described above. After blocking with 3% donkey or goat serum (Jackson ImmunoResearch, Baltimore, MD, USA), slices were incubated with primary antibodies followed by incubation with fluorescence-labeled secondary antibodies (Jackson ImmunoResearch). Finally, fluorescent images were acquired through a confocal fluorescence microscope system (CTR6500, Leica Microsystems, Tokyo, Japan). Representative images from at least 3 independent samples are shown in Figures 1 and 2. The relative intensity of positive staining in IA walls from each experiment was measured by imaging software and statistically analyzed.

The following primary antibodies were used: mouse monoclonal anti-smooth muscle alpha actin antibody
(Thermo Scientific, Waltham, MA, USA), rabbit monoclonal anti-phospho NF-kappaB p65 (ser536) antibody (Cell Signaling Technology, Danvers, MA, USA), rat monoclonal anti-TNF-alpha antibody (Lifespan Bioscience, Seattle, WA, USA), goat polyclonal anti-MCP-1 antibody (Santa Cruz Biotechnology, Dallas, TX, USA), rabbit polyclonal anti-COX-2 antibody (Cayman Chemicals, Ann Arbor, MI, USA), rat monoclonal anti-F4/80 antibody (Abcam, Cambridge, UK).

Macrophage count
Macrophages were defined as cells positive for F4/80 staining in immunohistochemistry. The number of infiltrated macrophages was calculated within 10000 um$^2$ around the dome of induced aneurysms (wild-type; n = 7, TNFR1-deficient mouse; n = 5).

Quantitative real-time PCR analysis
RNA purification from the ring of Willis of mice 5 months after aneurysm induction (n = 4 in wild-type and 3 in TNFR1-deficient mice) and subsequent reverse transcription were performed using a RNeasy Fibrous Tissue Mini Kit (QIAGEN, Hilden, Germany) and a High Capacity cDNA Reverse Transcription Kit (Life Technologies Corporation, Carlsbad, CA, USA), respectively, according to the manufacturers’ instructions. For the quantification of gene expression, PCR reactions were performed using the LightCycler 480 system with a SYBR Premix Ex Taq II (Takara Bio Inc., Shiga, Japan), using the expression of beta-actin as an internal control.

The following primers (forward and reverse, respectively) were used in experiments: 5′-tctgcagccatttccttctcc-3′ and 5′-aaaggcctccattgaccagagc-3′ for COX-2, 5′-atgtctggacccattccttcttg-3′ and 5′-tcccaatgagtaggctggaagc-3′ for MCP-1, 5′-gctcgttgccaatagtgatgaccg-3′ and 5′-cgtgctgtgacatcaaagagaagc-3′ for beta-actin.

Quantification of TNF-alpha protein content
The TNF-alpha protein content of intracranial arteries with aneurysm induction was examined using multiplex suspension array techniques, according to the manufacturer’s instructions. For the quantification of gene expression, PCR reactions were performed using the LightCycler 480 system with a SYBR Premix Ex Taq II (Takara Bio Inc., Shiga, Japan), using the expression of beta-actin as an internal control.

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Figure 1 Increased TNF-alpha content in the IA lesion. (A) Immunohistochemistry to detect TNF-alpha in IA walls located at right anterior cerebral artery-olfactory artery bifurcations in a rat model. Immunohistochemistry to detect TNF-alpha in control arterial and IA walls from rats, 3 months after aneurysm induction, is shown. The white arrow and arrow head indicate positive staining in endothelial cells and outer membranes, respectively. The star in the left panel indicates the media of arterial walls. Bar = 50 um. In the right panel, the relative intensities of positive signals in IA walls from the left panel are shown. n = 3 in each group, and * indicates p < 0.05. All bars indicate the mean ± SEM. (B) Increased TNF-alpha content in intracranial arteries after aneurysm induction. TNF-alpha content was analyzed by multi-suspension array using intracranial arteries from rats at indicated time points after aneurysm induction. n = 6 in each group. All bars indicate the mean ± SEM, and * indicates p < 0.05. M; month after aneurysm induction. (C) Enhanced tumor necrosis factor alpha converting enzyme (TACE) activity in intracranial arteries after aneurysm induction. TACE activity was measured as explained in the Methods section. n = 4 in both groups. All bars indicate the mean ± SEM, and * indicates p < 0.05. M; month after aneurysm induction.
DC Protein Assay (Bio-Rad), using the Luminex 2000 System (Luminex, Tokyo, Japan) for detection, and DNASIS Plex version 2.5 (HitachiSoft, Tokyo, Japan) for analysis, according to the manufacturer's instructions.

Measurement of TNF-alpha converting enzyme (TACE) activity
The total protein from intracranial arteries after aneurysm induction was purified by Complete M Lysis kit (Roche, Basel, Switzerland) according to the manufacturer’s instructions (n = 4 in each group). Each experiment used 25 μg of protein. Total protein and the fluorescence-labeled synthetic substrate for TACE (5-FAM-Ser-Pro-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-Arg-Lys; BioMol, Plymouth, PA, USA) were incubated in buffer containing 50 mM Tris–HCl (pH 7.4), 25 mM NaCl and 4% glycerol for 1 h at 37°C. Enzyme activity was evaluated based on the increase in fluorescence at 535 nm (excitation 485 nm).

Statistical analysis
Data are shown as the mean ± SEM, and 2 groups were statistically compared using the Mann–Whitney U test. Statistical comparisons among more than 2 groups were conducted using the Kruskal–Wallis test followed by the Steel–Dwass test. The incidence of IAs was analyzed by Fisher’s exact test. A p value smaller than 0.05 was defined as statistically significant.

Results
Increased TNF-alpha content in IA lesion in a rat model
In this study, we used the rodent model of IAs to examine the contribution of TNF-alpha signaling to IA formation. In this model, IA was induced through increased hemodynamic stress, a trigger of IAs in humans [15-17], loaded on the bifurcation sites of intracranial arteries. Three months after aneurysm induction, IA, defined as a lesion with disrupted internal elastic lamina, was induced at the right ACA-OA bifurcation in all rats.

The immunoreactivity of TNF-alpha was remarkably increased in IA lesions compared with control intracranial arteries, especially in endothelial cells and the outer membrane, and the difference in relative intensity of positive signals in IA walls was statistically significant (n = 3 in each group, p = 0.049) (Figure 1A). Consistent with these results, the content of TNF-alpha in the intracranial artery, examined by multi-suspension array, was significantly increased in rats with advanced stage IAs three months after aneurysm induction (0 months, 39.4 ± 7.2 pg/mg total protein; 0.5 months, 37.0 ± 4.8 pg/mg total protein; 1 month, 27.3 ± 3.0 pg/mg total protein; 3 months, 75.2 ± 6.1 pg/mg total protein; n = 6 in each group, 0 months compared to 3 months, p = 0.0497; 0.5 months compared to 3 months, p = 0.0497; 1 month compared to 3 months, p = 0.020) (Figure 1B). TNF-alpha is released from the cytoplasmic membrane through shedding, by the action of TACE. We therefore examined the activity of TACE in intracranial arteries including IA lesions, from a rat model using labeled synthetic substrate. As a result, in intracranial arteries from rats with aneurysm induction, the activity of TACE was significantly increased compared to control rats, suggesting up-regulation of TNF-alpha production in IA lesions, consistent with increased TNF-alpha content in IA lesions (0 months, 1.00 ± 0.36-fold, n = 4; 3 months, 2.78 ± 0.71-fold, n = 4; 0 months compared to 3 months, p = 0.0497; 0.5 months compared to 3 months, p = 0.0497; 1 month compared to 3 months, p = 0.020) (Figure 1C).

Suppression of IA formation due to TNFR1 deficiency
Next, to clarify the involvement of TNF-alpha signaling in IA formation, we used mice deficient in its receptor, TNFR1, based on the finding that TNFR1 was abundantly expressed in intracranial arteries (data not shown). As a result, 15 out of 22 wild-type mice, 1 out of 7 TNFR1-heterozygous, and 1 out of 8 TNFR1-deficient mice, had
IAs at right ACA-OA bifurcations. Statistically, the incidence of IA was significantly suppressed in TNFR1-heterozygous and TNFR1-deficient mice compared with wild-type mice (wild-type compared to TNFR1-heterozygous, \( p = 0.026 \), wild-type compared to TNFR1-deficient, \( p = 0.012 \)) (Figure 2A), suggesting a critical role forTNF-alpha-TNFR1 signaling in IA formation. Because IA formation in the mouse model was greatly influenced by systemic blood pressure, we confirmed the independence of genotypes from systemic blood pressure after aneurysm induction (systolic blood pressure: wild-type mice, 115.0 ± 10.3 mmHg, \( n = 8 \); TNFR1-heterozygous mice, 113.0 ± 9.0 mmHg, \( n = 7 \); TNFR1-deficient mice, 112.3 ± 8.6 mmHg, \( n = 8 \); mean blood pressure: wild-type mice, 77.3 ± 7.2 mmHg, \( n = 8 \); TNFR1-heterozygous mice, 78.1 ± 9.8 mmHg, \( n = 7 \); TNFR1-deficient mice, 74.8 ± 7.2 mmHg, \( n = 8 \); mean blood pressure: wild-type mice, 113.0 ± 9.0 mmHg, \( n = 7 \); TNFR1-deficient mice, 115.0 ± 10.3 mmHg, \( n = 8 \); TNFR1-heterozygous mice, 112.3 ± 8.6 mmHg, \( n = 8 \); TNFR1-deficient mice, \( p = 0.012 \) (Figure 2B), suggesting a critical role for TNF-alpha-TNFR1 signaling in IA formation. 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Conclusions

In this study, we used rodent models of IA through increased hemodynamic stress to investigate the contribution of TNF-alpha-TNFRI signaling to the pathogenesis of IA formation and progression. We confirmed an increase in TNF-alpha content in IA lesions in rat models during...
IA formation. Furthermore, TNFR1 deficiency suppressed IA formation by inhibiting inflammatory responses in IA walls such as NF-kappaB activation, MCP-1 induction, and macrophage infiltration. These results suggest a crucial role for TNF-alpha-TNFFR1 signaling in IA formation and progression.

Abbreviations
AIA: Anterior cerebral artery; IA: Intracranial aneurysm; MMP: Matrix metalloproteinase; OIF: Orbital artery; TACE: Tumor necrosis factor alpha converting enzyme; TNF-alpha: Tumor necrosis factor alpha; TNFR1: Tumor necrosis factor receptor superfamily, member 1a.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
TA conceived the study, carried out the molecular biological experiments, secured grants, and drafted the manuscript. MF carried out the molecular biological experiments. MN and KN participated in the design and coordination of the study. SN conceived the study, secured grants, and participated in the design and coordination of the study. SN also helped to draft the manuscript. All authors read and approved the final manuscript.

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