Suppression of cerebral aneurysm formation in rats by a tumor necrosis factor–α inhibitor

Laboratory investigation

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Object. Although cerebral aneurysmal subarachnoid hemorrhage is a devastating disease for humans, effective medical treatments have not yet been established. Recent reports have shown that regression of some inflammatory-related mediators has protective effects in experimental cerebral aneurysm models. This study corroborated the effectiveness of tumor necrosis factor–α (TNF-α) inhibitor for experimentally induced cerebral aneurysms in rats.

Methods. Five-week-old male rats were prepared for induction of cerebral aneurysms and divided into 3 groups, 2 groups administered different concentrations of a TNF-α inhibitor (etanercept), and 1 control group. One month after aneurysm induction, 7-T MRI was performed. The TNF-α inhibitor groups received subcutaneous injection of 25 μg or 2.5 μg of etanercept, and the control group received subcutaneous injection of normal saline every week. The TNF-α inhibitor administrations were started at 1 month after aneurysm induction to evaluate its suppressive effects on preexisting cerebral aneurysms. Arterial circles of Willis were obtained and evaluated 3 months after aneurysm induction.

Results. Rats administered a TNF-α inhibitor experienced significant increases in media thickness and reductions in aneurysmal size compared with the control group. Immunohistochemical staining showed that treatment with a TNF-α inhibitor suppressed matrix metalloproteinase (MMP)–9 and inducible nitric oxide synthase (iNOS) expression through the luminal surface of the endothelial cell layer, the media and the adventitia at the site of aneurysmal formation, and the anterior cerebral artery–olfactory artery bifurcation. Quantitative polymerase chain reaction also showed suppression of MMP-9 and iNOS by TNF-α inhibitor administration.

Conclusions. Therapeutic administration of a TNF-α inhibitor significantly reduced the formation of aneurysms in rats. These data also suggest that TNF-α suppression reduced some inflammatory-related mediators that are in the downstream pathway of nuclear factor-κB.

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Key Words • tumor necrosis factor–α inhibitor • cerebral aneurysm • nuclear factor kappa B • rat • vascular disorders

Cerebral aneurysmal subarachnoid hemorrhage is a catastrophic disease for humans, but effective medical treatments have not yet been established. The pathogenesis of cerebral saccular aneurysms has been investigated using several aneurysm models.5,15,28,32,33 Hashimoto and colleagues established an experimentally induced cerebral aneurysm model in rats, and hemodynamic stress has been emphasized for aneurysmal formation. In the early stage after aneurysm induction, disruption of the elastic laminae occurs,22,24 which is followed by infiltration of the inflammatory-related mediator to the arterial wall, and proteolytic damage to the arterial wall caused by inflammation-related mediators appears to play a crucial role. Several kinds of mediators have been reported to be decisive causes of the formation of abdominal aortic aneurysms,13,23 and Chyatte et al. demonstrated the expression of immunoglobulin, macrophages, and T-lymphocytes in humans in the walls of unruptured cerebral aneurysms.11 Macrophages can induce programmed cell death through a tumor necrosis factor (TNF)–α dependent pathway.8 Jayaraman et al. have demonstrated TNF-α mRNA and protein in ruptured cerebral aneurysms in humans.19 Tumor necrosis factor–α activation induces apoptosis via activation of caspase-8,1 which leads to the activation of inducible nitric oxide synthase (iNOS). Aoki et al. showed important roles of nuclear factor-κB (NF-κB) in the induc-
tion and formation of cerebral aneurysms in rat aneurysmal models. Nuclear factor-κB also regulates the transcription of some proinflammatory genes such as iNOS and matrix metalloproteinases (MMPs), which are of functional importance for the progression of cerebral aneurysms. Recent reports have shown that regression of some inflammatory-related molecules have protective effects in experimental cerebral aneurysm models. One of the main activators of NF-κB is TNF-α, which is one of the main proinflammatory cytokines and plays a fundamental role in initiating and regulating the cascade of events leading to inflammatory reactions. The main aim of this study was to elucidate the role of TNF-α in the formation and augmentation of cerebral aneurysms, to seek possible medical therapeutic approaches for the suppression and prevention of aneurysmal formation and subarachnoid hemorrhage.

Methods

Experimentally Induced Cerebral Aneurysms in Rats

Animal care and experiments complied with Japanese community standards on the care and use of laboratory animals, and all protocols were permitted by the Ethical Committee of the Research Center of Animal Life Science at Shiga University of Medical Science. Cerebral aneurysms were induced using a method previously described. While anesthetized with 50 mg/kg of intraperitoneal pentobarbital, the left common carotid artery and posterior branches of the bilateral renal arteries were ligated at the same time with 3-0 nylon thread in 5-week-old male Sprague-Dawley rats (Oriental Bioservice). A total of 36 rats were operated on. All of the aneurysm-induced rats (n = 36) were divided into 3 groups in random order: 1) a high-dose TNF-α inhibitor group (n = 12); 2) a low-dose TNF-α inhibitor group (n = 12); and 3) a vehicle group (control group, n = 12). After aneurysm induction, animals in all groups were fed with a high-salt diet containing 8% sodium chloride (Oriental Bioservice) for 3 months. At 1 and 3 months after aneurysm induction, systemic blood pressure was measured by the tail-cuff method (Softron) with rats kept in a small container without any anesthesia.

Magnetic Resonance Imaging

We conducted 7-T MRI at 1 month after aneurysm induction while the rats were anesthetized using an intraperitoneal injection of 30 mg/kg of pentobarbital. The main purpose of this MRI evaluation was confirmation of aneurysm formation when the etanercept administration was started. We intended to emphasize that the effectiveness of this drug was reduction of or cure from the preexisting aneurysm, not prevention of aneurysm formation. The MRI was performed on a Unity INOVA 7T MR machine (Varian) equipped with a Magnex gradient system capable of producing pulse gradients of 400 mT/m in each of the 3 orthogonal axes, and with the JMTB-7.0/400/SS conducting magnet (Jastec; bore size 400 mm, central field magnetic force 7 T). We used a 38-mm volume coil for the brain scan procedure. The MRI parameters were as follows: TR 40 msec, TE 3.24 msec, flip angle 45°, thickness 1.5 mm, scanning length 40 mm, 32 slices, with no gap. We conducted MRI 3 times on each rat, with the slice offset shifted to the same direction by 0.5° every time. In all 3 groups, 1 of the rats was chosen by random sampling at 1 month after aneurysm induction. The 7-T MR angiography revealed a saccular-type aneurysm at the right anterior cerebral artery (ACA)–olfactory artery bifurcation in all 3 groups (Fig. 1).

Administration of TNF-α Inhibitor

To examine the effect of a TNF-α inhibitor on preexisting aneurysms, TNF-α inhibitor administration started 1 month after aneurysm induction. We used etanercept as a TNF-α inhibitor. Etanercept was provided by Takeda Pharmaceutical Co. Ltd. and Pfizer Co., Inc. Animals in

Fig. 1. Magnetic resonance angiograms obtained 1 month after aneurysm induction in each of the 3 groups. Arrowheads indicate saccular aneurysms at the bifurcation of the ACA and olfactory artery. A: Control group. B: Low-dose etanercept group. C: High-dose etanercept group.
the high-dose TNF-α inhibitor group received subcutaneous injection of 25 μg of etanercept with 0.5 ml of normal saline every week for 2 months. Those in the low-dose TNF-α inhibitor group received 2.5 μg of etanercept with 0.5 ml of normal saline in the same fashion. The control group received subcutaneous injection of 0.5 ml of normal saline every week for 2 months. The doses were selected based on previous studies that demonstrated the effectiveness of etanercept in other disease models.21,27,31

Evaluation of Induced Cerebral Aneurysms in Rats

Arterial circles of Willis were evaluated 3 months after aneurysm induction. After systemic blood pressure was measured by the tail-cuff method, rats were deeply anesthetized and perfused transcardially with 4% paraformaldehyde by perfusion pump (Atto) at 100–150 ml/m. We conducted this immobilization and euthanasia of 7 rats in each group by random sampling. The ACA and olfactory artery bifurcation was stripped and embedded in optimal cutting temperature compound (Sakura). Five-μm sections were cut in the sagittal plane, mounted on silane-coated slides, and observed under a light microscope after H & E and Masson trichrome staining. To evaluate the pathological changes occurring in aneurysmal walls, we analyzed the thinning of the medial smooth-muscle cell layer and aneurysmal size. The thickness of media was evaluated by the ratio of minimal thickness in aneurysmal walls to thickness in surrounding normal arterial walls. Aneurysmal size was calculated as the mean of the maximal longitudinal diameter and the maximal transverse diameter.

Quantitative Polymerase Chain Reaction

For the analysis of gene expression of cerebral aneurysms in rats, total RNA was extracted from the frozen arterial circles of Willis by the acid guanidium thiocyanate–chloroform method as previously reported.10 Real-time polymerase chain reaction (PCR) was performed with a LightCycler 480 SYBR MasterMix and LightCycler 480 System II (Roche Diagnostics) by 45 cycles of denaturation (95°C, 15 sec), annealing (60°C, 15 sec), and extension (72°C, 15 sec). Gene expression was normalized with the β-actin gene. The rat-specific primer sequences are listed in Table 1. We conducted this quantitative PCR of 3 rats in each group by random sampling. All quantification analysis was performed in triplicate.

Immunohistochemical Analysis

The ACA and olfactory artery bifurcation was stripped and embedded in optimal cutting temperature compound. Five-μm sections were cut and mounted on silane-coated slides in the sagittal plane. After blocking with 5% donkey serum (Jackson Immune Research), primary antibodies were incubated for 1 hour at room temperature followed by incubation peroxidase-labeled secondary antibodies (simply stain MAX-PO; Nichirei) for 1 hour at room temperature. The primary antibodies and titer of antibodies used in the present study were goat polyclonal MMP-9 antibody (Santa Cruz Biotechnology, Inc.) and rabbit polyclonal anti–iNOS antibody (Santa Cruz Biotechnology, Inc.). We conducted this immunohistochemical analysis of 2 rats in each group by random sampling.

Statistical Analysis

Data (means ± SDs) were analyzed using the Mann-Whitney U-test for 2-group comparisons. Differences were considered statistically significant at p < 0.05.

Results

Effect of Etanercept on Aneurysmal Formation

In each group, systolic blood pressure was significantly elevated at 3 months after aneurysm induction as compared with the systolic blood pressure at 1 month after aneurysm induction. There was no significant difference at 1 month or 3 months after aneurysm induction between the control group (74.8 ± 2.4 mm Hg after 1 month, 118.8 ± 2.8 mm Hg after 3 months), low-dose etanercept group (75.8 ± 2.1 mm Hg after 1 month, 115.7 ± 21.2 mm Hg after 3 months) and high-dose etanercept group (76.1 ± 1.9 mm Hg after 1 month, 121.1 ± 1.9 mm Hg after 3 months; Fig. 2A and B). The maximum size of the aneurysm was significantly smaller in the low-dose and high-dose etanercept groups than in the control group, and the maximum size of the aneurysm in the high-dose etanercept group was significantly smaller than in the low-dose etanercept group (Fig. 2C). The thickness of media was significantly larger in the low-dose and high-dose etanercept groups than in the control group (Fig. 2D). Aneurysm formation was markedly suppressed in the etanercept groups, and the internal elastic membrane was also thicker in the etanercept-treated groups as compared with the control group (Fig. 3). We

<table>
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<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tr>
<td>iKKα</td>
<td>TCCAAAGTCCAAAAACAGAGAAGCC</td>
<td>CCATTGCTAGAAGGGCACATC</td>
</tr>
<tr>
<td>iKKβ</td>
<td>TCACAAACAGCCTCCAGATGG</td>
<td>GCCATCATCCCGTTACAGG</td>
</tr>
<tr>
<td>NFκB</td>
<td>CTGGCCATGCTGAGGGGTTG</td>
<td>GCTCATAGAAGGCTTCAAGTTC</td>
</tr>
<tr>
<td>IL-1β</td>
<td>CCTGGACGCTGAGGCTGTC</td>
<td>GGTGCCATGGTAGGCAAC</td>
</tr>
<tr>
<td>nO2</td>
<td>AGGGGTCACTGCCACCCCAAC</td>
<td>CAGGGATTGCTGCAATCTG</td>
</tr>
<tr>
<td>MMP-9</td>
<td>TTTCAGGCACAGCTGGATG</td>
<td>CCTTGGAGACTGCGGATCTC</td>
</tr>
<tr>
<td>β-actin</td>
<td>AAGCTTGGCTAGATTCCT</td>
<td>AAGCAATGTAGTGTCCTC</td>
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TABLE 1: Oligonucleotides used for quantitative real-time PCR
created these definitions to measure each length objectively. In this study, the evaluators were not blinded; however, the methodology of each evaluation was standardized.

**Immunohistochemical Staining**

The discontinuity of the internal elastic lamina at the ACA–olfactory artery bifurcation occurred in all 3 groups, but apparent outward bulging of the arterial wall was distinct in the control group. There was some elastin, which formed from tropoelastin. There was some inflammation-related reaction just outside of the internal elastic laminae. The cells in the photomicrographs were infiltrated monocytes from the internal vessels, which differentiate into macrophages and dendritic cells. According to the immunohistochemical analysis, iNOS and MMP-9 were expressed mainly in the area with fragmentation or disappearance of the internal elastic lamina (Fig. 4). This tendency was obvious in the control group and low-dose TNF-α inhibitor group. The expressions of iNOS and MMP-9 proteins in the media were reduced at the aneurysm formation site in the etanercept groups, but we could not find a significant reduction in the expression of these molecules in the adventitia due to edge artifact.

**Quantitative PCR Analysis**

We conducted a significance test between rats in the control group and those in the high-dose etanercept group. In the etanercept group, mRNA expression levels of MMP-9, iNOS, and interleukin (IL)-1β were significantly lower than those in the control group at 3 months after aneurysm induction (Fig. 5A). In the etanercept group, mRNA expression levels of NF-κB, phospho-IKKα, and phospho-IKKβ were lower than those in the control group at 3 months after aneurysm induction (Fig. 5B).

**Discussion**

Recent studies revealed the involvement of inflammation in cerebral aneurysm formation.14,17 Arterial walls are continuously loaded by circulating blood flow, which generates hemodynamic force. Because hemodynamic force with subsequent mechanical injury gives rise to infiltration of macrophages into the arterial wall, iNOS is constitutively expressed mainly in smooth muscle cells. Histopathological studies on clipped human aneurysms have identified macrophages and lymphocytes in the aneurysmal wall.11,14 Chyatte et al.11 revealed that the number of macrophages accumulated in the luminal surface of the endothelium and in the adventitia increase with progression of an aneurysm. Hashimoto et al. investigated the total and phosphorylated levels of different signaling proteins or cytokines produced by inflammatory cells known to participate in vascular remodeling or inflammation of cerebral aneurysms such as...
MMP. Investigations in several animal models of aortic aneurysms have demonstrated that increased macrophage expression of MMP-9 plays a critical role in the development and progression of abdominal aortic aneurysms. In addition to the mechanisms above, the functional role of NF-κB that activates MMP-9 expression has been demonstrated in a variety of animal models of abdominal aortic aneurysms. A recent paper indicated that activation of NF-κB also appeared at the site of cerebral aneurysm formation. Cerebral aneurysms tend to be formed at the arterial bifurcation by hemodynamic shear stress, and fluid shear stress has been shown to promote the translocation into the nucleus of NF-κB in cultured endothelial cells by activating IκB kinase. One of the main activators of NF-κB is TNF-α, which is one of the main proinflammatory cytokines and plays a key role in initiating and regulating the cascade of events leading to inflammatory reactions.

Tumor necrosis factor–α is encoded on chromosome 6 and exists in its biologically active form as a homotrimer of a 17-kD subunit. Tumor necrosis factor–α can locate upstream of the NK-κB pathway, and TNF-α–induced NF-κB activation results from activation of the inhibitor of the κB kinase (IKK) complex, which phosphorylates

**Fig. 3.** Photomicrographs of H & E (A–C) and Masson trichrome staining (D–F) of the ACA–olfactory artery bifurcation in a rat 3 months after aneurysm induction. * Outer lumen of the vessels. ** Internal lumen of the vessels. Bar = 50 μm.

**Fig. 4.** Immunohistochemical staining of iNOS (A–C) and MMP-9 (D–F) 3 months after aneurysm induction. Arrowheads indicate iNOS-positive cells. Arrows indicate MMP-9–positive cells. * Outer lumen of the vessels. ** Internal lumen of the vessels. Bar = 50 μm.
the inhibitor of κB family members that are normally bound to NF-κB and thereby retains it in the cytoplasm. Tumor necrosis factor-α has a broad range of biological activities associated with inflammatory-related disorders. The significant increase in mRNA expression of TNF-α was also observed in human cerebral aneurysms. The increased expression of TNF-α in cerebral aneurysms may lead to activation of its downstream signaling components to initiate vessel weakening and remodeling via inflammation and apoptosis. Thus, activation of the proinflammatory cytokine TNF-α may contribute to the development of abnormal vasculature during aneurysmal formation. We are planning to determine the relationship between an immune reaction and TNF-α upregulation by using immunohistochemical double staining of CD-68 and the secondary antibody of anti-rat TNF-α.

After we discontinued the administration of the TNF-α inhibitor in the aneurysmal model, we did not observe the arterial circles of Willis. We have no data about the change in aneurysms after we ceased admin-

![Bar graphs showing quantitative PCR-measured mRNA expression.](image)
A TNF-α inhibitor in cerebral aneurysm suppression

istration of the TNF-α inhibitor. We are currently working on observing the changes in aneurysms after ceasing the administration of TNF-α inhibitor in rats. We assume that hemodynamic stress will cause aneurysmal formation in these animals as well, using sodium loading and lasting renal hypertension. To reduce the matured aneurysms would require suppression of inflammatory cells infiltrated into the aneurysmal wall.

In this paper, we examined the distribution of TNF-α inhibitor in cerebral aneurysm development using an experimental model in rats. We showed that aneurysmal formation was significantly reduced by 2-month treatment with etanercept 1 month after aneurysm induction in rats, which suggests that TNF-α may be involved in the enlargement of cerebral aneurysms. These data suggest that inhibition of TNF-α and its downstream signaling components contributes to the pathological mechanisms of cerebral aneurysms via the suppressive effect for pro-inflammatory status. The suppression of not only NF-κB, phospho-IKKα, and phospho-IKKβ mRNAs but also iNOS and MMP-9 mRNA (one of the downstream molecules of the NF-κB pathway) by a TNF-α inhibitor might indicate that the inhibition of aneurysm formation by the TNF-α inhibitor occurs through an NF-κB pathway. One of the major target molecules of TNF-α is NF-κB, and its activation leads to macrophage recruitment into the aneurysmal walls. Macrophages secrete MMP-9, which causes extracellular matrix degradation in aneurysmal walls, and release of nitric oxide via iNOS upregulation. A genetic analysis also revealed that a TNF-α-linked locus significantly influences the risk of aneurysm formation.

The results of this study suggest that TNF-α inhibitors have therapeutic potential in the prevention of cerebral aneurysm formation. Additional investigations in higher mammals will be needed to determine the possible clinical application of TNF-α inhibitors to human cerebral aneurysms. We are currently studying the effectiveness and side effects of these inhibitors using mammal models.

Conclusions

Therapeutic administration of a TNF-α inhibitor significantly reduced aneurysm formation in rats. These data suggest that the inhibition of TNF-α might be a novel tool in the treatment of cerebral aneurysms by reducing some inflammatory-related mediators.

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Disclosure

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

Author contributions to the study and manuscript preparation include the following. Conception and design: Yokoi. Acquisition of data: Yokoi, Isono, Saitoh, Yoshimura. Analysis and interpretation of data: Yokoi, Isono. Drafting the article: Yokoi, Isono. Critically revising the article: all authors. Reviewed submitted version of manuscript: all authors. Approved the final version of the manuscript on behalf of all authors: Yokoi. Statistical analysis: Yokoi. Administrative/technical/material support: Yokoi. Study supervision: Nozaki.

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