Cerebral aneurysms can cause a devastating SAH, with quite high mortality and morbidity rates. Despite its high prevalence and the severity of subsequent SAH, we have no option for medical treatment of unruptured cerebral aneurysms because detailed mechanisms of cerebral aneurysm formation and rupture remain to be elucidated. Our recent studies have revealed that chronic inflammatory response in arterial walls to hemodynamic stress is an active participant in cerebral aneurysm formation. The transcriptional factor NF-κB is a key mediator of this process; NF-κB is activated in the endothelial cell layer of the arterial bifurcation and transcriptionally regulates various inflammation-related genes. Macrophages infiltrate arterial walls by MCP-1 and VCAM-1 expression, transcriptionally regulated by NF-κB activation. However, detailed mechanisms of NF-κB activation in arterial walls are unclear.

Toll-like receptors are key regulators of innate immune responses. These receptors recognize various microbial-related molecules such as lipopolysaccharide and double-stranded RNA, and they contribute to an innate immunity. The TLRs consist of 13 family members (TLR1–13) in humans and mice. In rats, 11 members (TLR1–11) are registered in a worldwide database. The TLRs are expressed in various tissues and play a critical role in various immune responses. In addition to immune responses, several members of the TLRs are active participants in various inflammatory diseases. Among them, TLR2 and TLR4 are involved, and play a crucial role in the pathogenesis of inflammatory diseases like atherosclerosis.

Toll-like receptor 4 expression during cerebral aneurysm formation

Laboratory investigation

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Object. The pathophysiological origin of cerebral aneurysms is closely associated with chronic inflammation in arterial walls. Recently, the authors identified nuclear factor–kappa B (NF-κB) as a key mediator of cerebral aneurysm formation and progression. Because Toll-like receptor 4 (TLR4) stimulates NF-κB activation in arterial walls in atherosclerosis, the authors hypothesize that TLR4 expresses in cerebral aneurysms and contributes to the activation of NF-κB in cerebral aneurysm walls.

Methods. Cerebral aneurysms were induced in male Sprague-Dawley rats. Expression of TLRs in cerebral aneurysm walls was assessed using reverse transcriptase polymerase chain reaction (RT-PCR). The expression of TLR4 was examined using RT-PCR, immunohistochemical studies, and Western blotting. To assess TLR4 dependency on NF-κB activation, double immunostaining and a study using NF-κB–deficient mice were done. Finally, TLR4 expression in human cerebral aneurysm walls was assessed using immunohistochemical studies.

Results. In cerebral aneurysm walls, TLR1, -4, -5, -6, -10, and -11 were expressed. Among them, TLR4 and TLR10 expression changed during cerebral aneurysm formation. Expression of TLR4 was predominantly in the endothelial cell layer of cerebral aneurysm walls, and was transitionally upregulated at the early stage of cerebral aneurysm formation. The TLR4 expression coincided well with NF-κB activation. In human cerebral aneurysms, TLR4 was also expressed in the endothelial cell layer, as it was in rats.

Conclusions. Toll-like receptor 4 was expressed in cerebral aneurysm walls both in rats and humans. This receptor may play a crucial role in cerebral aneurysm formation through NF-κB activation in endothelial cells. The results of the present study will shed new light on the pathogenesis of cerebral aneurysm formation.

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Key Words • cerebral aneurysm • toll-like receptor • nuclear factor–kappa B • inflammation • rat

Abbreviations used in this paper: ACA-OA = anterior cerebral artery–olfactory artery; eNOS = endothelial nitric oxide synthase; MCA = middle cerebral artery; NF-κB = nuclear factor–kappa B; PCR = polymerase chain reaction; RT = reverse transcriptase; SAH = subarachnoid hemorrhage; TLR4 = Toll-like receptor 4.

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ation and contributes to inflammation in various inflammatory diseases. In cerebral aneurysms, similar mechanisms and a contribution from TLR4 may be present, but there is no experimental evidence indicating the role of TLRs in cerebral aneurysm formation.

In the present study, we clearly show the expression of TLRs in cerebral aneurysm walls, and provide a detailed evaluation of expression of TLR4 in a model of rat and human cerebral aneurysms.

Methods

Induction of Experimentally Induced Cerebral Aneurysms in Rats

Cerebral aneurysms were induced in rats as previously described by Nagata et al. After the induction of pentobarbital anesthesia (50 mg/kg intraperitoneally), the left common carotid artery and posterior branches of the bilateral renal arteries were ligated at the same time with 10-0 nylon in 7-week-old male Sprague-Dawley rats (Oriental Bioservice). Animals were fed a high-salt diet containing 8% sodium chloride and 0.12% β-aminopropionitrile (Tokyo Chemical), an inhibitor of lysyl oxidase that catalyzed the cross-linking of collagen and elastin. After 2 weeks, 1 month, or 3 months of aneurysm induction, the rats were killed as described above. Total RNA from the ring of Willis was isolated using the RNasey Fibrous Tissue Mini Kit (QIAGEN). Extraction was performed according to the manufacturer’s instructions. Total RNA was converted into cDNA by Sensiscript RT (QIAGEN). The PCR was performed using FastCycling Taq polymerase (QIAGEN); β-actin was used as an internal control.

The primer sets used were the following: 5′-GCT TTCGTGTAGTGAGTTGG-3′ and 5′-GGATGACAAA GATCCCATGC-3′ for TLR1; 5′-CAGGCTCTATTGT TACCCG-3′ and 5′-GCTGTGCGTTGCTGATCTTGGG-3′ for TLR2; 5′-GCTGTAGCTTGTGCTAACC-3′ and 5′-GGGTTCTTGGATCTGGAGC-3′ for TLR3; 5′-TG TCCACTGCGGCTTACG-3′ and 5′-AACCTCAAC GACTTCAAGG-3′ for TLR4; 5′-GATGGTGTTGGC TGAAGTTC-3′ and 5′-AATCAACAGCCTGTTTCAG C-3′ for TLR5; 5′-ATGTGAAAGTCTCTGAGTTG-3′ and 5′-GATGCACTGAGTGAAGCTGG-3′ for TLR6; 5′-TACCTCAAGGCTCAGAAGC-3′ and 5′-CAATG TGCAACAAAGG-3′ for TLR7; 5′-CCTCTTATTGGG CTAAGACC-3′ and 5′-GGGAAATTTAGTGGCTCTG TG-3′ for TLR8; 5′-TGATGGCTATTTGCCAGA G-3′ and 5′-TCAACAAGACCGCTGACTGGG-3′ for TLR9; 5′-CAGTTCCAGATCTGGAAGC-3′ and 5′-TC CGAAGACTTTCTTGGG-3′ for TLR10; 5′-GCACC TCTAGAAAGGAGGCTCC-3′ and 5′-ACTTTGCAAC CACTTTCAGG-3′ for TLR11; and 5′-AAGTCTCCAT CCTCAGAAAAG-3′ and 5′-AAGGAATGCTGTGAC CCTCCCAGG-3′ for β-actin. The condition necessary for PCR was as follows: 95°C for 5 minutes, followed by 35 cycles of 95°C for 5 seconds, 57°C for 5 seconds, and 68°C for 6 seconds. The PCR products were separated by electrophoresis in 2% agarose gels. Expression of each gene was calculated as a ratio to β-actin expression. Densitometric analysis includes data from 6 samples per group.

Western Blotting

Lysates from the rat cerebral artery adjacent to the ACA-OA bifurcation were extracted using Complete Lysis-M (Roche). After electrophoresis, transfer to a polyvinylidene difluoride membrane and blocking using enhanced chemiluminescence plus blocking agent (Amersham), the membrane was incubated with horseradish peroxidase–conjugated anti–mouse IgG antibody (Chemicon International, Inc.), rabbit monoclonal anti–NF-κB p65 antibody, or rabbit monoclonal antiphosphorylated NF-κB p65 (Ser536) antibody (Cell Signaling Technology) for 1 hour at room temperature, followed by incubation with horseradish peroxidase–conjugated anti–rabbit IgG antibody (GE Healthcare). The signal was detected by chemiluminescent reagent (enhanced chemiluminescence

Isolation of RNA and Performance of RT-PCR

Two weeks, 1 month, or 3 months after aneurysm induction, the rats were killed as described above. After decapitation, the brain was removed and the ACA-OA bifurcation was stripped, embedded, and frozen. Sections of 5-μm thickness were cut for histochemical studies. We have used this same image of elastic van Gieson-stained tissue in immunohistochemical studies of other molecules.

Immunohistochemical Studies

Two weeks, 1 month, or 3 months after aneurysm induction, all rats were deeply anesthetized and perfused transcardially with 4% paraformaldehyde. As a control, age-matched male Sprague-Dawley rats were killed as described above. The ACA-OA bifurcation was stripped, embedded, and frozen. Sections of 5-μm thickness were cut and mounted on silane-coated slides. After blocking with 5% donkey serum, the slides were incubated with primary antibodies for 1 hour at room temperature, followed by incubation with fluorescence-labeled secondary antibodies (fluorescein isothiocyanate–conjugated donkey anti–goat IgG antibody and Cy3–conjugated donkey anti–mouse IgG antibody [Jackson ImmunoResearch]) for 1 hour at room temperature. Finally, the slides were covered with PermaFluor mounting medium (ImmunoTech) and excited for fluorescence by illumination through a fluorescence microscope system (BX51N-34-FL-1, Olympus).

The primary antibodies used in the present study are the following: goat polyclonal anti-TLR4 antibody (Santa Cruz Biotechnology, Inc.); mouse monoclonal anti–NF-κB p65-subunit antibody (Chemicon International, Inc.), which recognized only the DNA binding form; and mouse monoclonal anti-eNOS antibody (Biomol Research Laboratories).

Several tissue slices were obtained from one experimental animal. One of these slices was treated with elastic van Gieson staining for a reference image of immunohistochemical studies. We have used this same image of elastic van Gieson-stained tissue in immunohistochemical studies of other molecules.
Toll-like receptor 4 expression and cerebral aneurysms

plus Western Blotting Detection System; GE Healthcare). The α-tubulin served as an internal control. Densitometric analyses included 5 independent experiments. We examined some molecules using the same membrane by stripping a previously used antibody. We then used the same blotting image of the internal control, α-tubulin, for Western blot analyses of other molecules.

Expression of TLR4 in NF-κB p50 Knockout Mice

We induced cerebral aneurysms in NF-κB p50 subunit knockout mice (C57/B6;129P2-Nfkb1tm1Bal/J, Jackson Laboratory) (p50−/− mice) and their littermates (p50+/+ mice) as described previously. The cerebral aneurysm specimens were subjected to immunohistochemical studies in the same protocol as described above for evaluation of the efficacy of NF-κB p50 deletion on TLR4 expression. The primary antibodies used were the same as in the rat studies.

Immunohistochemical Studies for Human Samples

Human cerebral aneurysm samples were obtained in 6 patients who underwent aneurysm neck clip occlusion for unruptured lesions, with informed consent. As a control, we used 3 specimens of the MCA obtained at autopsy. After deparaffinization and blocking of endogenous peroxidase activity with 0.3% H2O2, goat polyclonal anti-TLR4 antibody (the same as used in the rat study) was incubated for 30 minutes at room temperature, followed by incubation with biotin-labeled secondary antibody for 30 minutes at room temperature. Slides were incubated with streptavidin-conjugated peroxidase. Finally, the signal was detected using the 3,3′-diaminobenzidine system (DAKO). Nuclear staining was performed using a hematoxylin solution. As a negative control study, immunostaining without a primary antibody was done. For double staining, slides were incubated with primary antibodies for smooth-muscle α-actin or von Willebrand factor (both from DAKO) for 30 minutes at room temperature, followed by incubation with alkaline phosphatase–labeled secondary antibodies and fast red solution (Sigma Chemical Co.).

Statistical Analysis

Data (mean ± SD) were analyzed using the Mann-Whitney U-test for a 2-group comparison and the Kruskal-Wallis 1-way ANOVA on ranks, followed by the Tukey-Kramer test for a multiple comparison. Differences were considered statistically significant at p < 0.05.

Results

Activation of NF-κB in Cerebral Aneurysm Walls in Rats

Activation of NF-κB, assessed by the phosphorylation of NF-κB p65 subunit in Western blotting, had already occurred 2 weeks after aneurysm induction (Fig. 1A).

Expression of TLR mRNA in Cerebral Aneurysm Walls in Rats

In cerebral aneurysm walls of the rats 2 weeks after aneurysm induction, TLR1, -4, -5, -6, -10, and -11 mRNA was detectable in PCR (Fig. 1B). However, TLR11 mRNA expression was only weak. Expressions of TLR1, -5, and -6 mRNA did not change during cerebral aneurysm formation and progression. Expression of TLR10 mRNA gradually increased with cerebral aneurysm progression (Fig. 1C).

Expression of TLR4 During Cerebral Aneurysm Formation

The TLR4 was expressed in the endothelial cell layer of arterial walls at the ACA-OA bifurcation (Fig. 2F). Two weeks or 1 month after aneurysm induction, TLR4 expression was apparently upregulated in the endothelial cell layer and adventitia of aneurysm walls (Fig. 2G and H). Then, at 3 months after aneurysm induction, expression of TLR4 decreased to almost the same as or less than that of normal arterial walls (Fig. 2I). In the contralateral ACA-OA bifurcation, TLR4 expression was apparently weaker than that in aneurysm walls (Fig. 2J), suggesting

Fig. 1. Activation of NF-κB and expression of TLRs in cerebral aneurysm walls in rats. A: Western blotting of NF-κB p65 subunit and phosphorylated NF-κB p65 subunit; α-tubulin was used as an internal control. Representative data from 5 independent experiments are shown. B and C: Findings on RT-PCR studies of TLRs in cerebral aneurysm walls. Representative data from 6 independent experiments are shown. The β-actin was used as an internal control. 0M = before aneurysm induction; 0.5M = 2 weeks after aneurysm induction; 1M = 1 month after aneurysm induction.
that the upregulation of TLR4 was predominantly present at the cerebral aneurysm site. Expression of TLR4 was certainly present in the endothelial cells of cerebral aneurysm walls prepared with double immunostaining with eNOS (Fig. 2K). Expression of TLR4 coincided well with NF-κB activation (Fig. 2L). In NF-κB p50-deficient mice, TLR4 expression was the same as in wild-type mice (Fig. 2M and N).

In Western blotting, expression of TLR4 was significantly upregulated 1 month after aneurysm induction (5 data sets, p < 0.01). Three months after aneurysm induction, TLR4 expression decreased to the level before aneurysm induction (5 data sets, 1 month compared with 3 months; p = 0.021) (Fig. 3A and B). The mRNA expression of TLR4 transitionally increased 2 weeks after aneurysm induction followed by the decrease 1 month after aneurysm induction in RT-PCR (before compared with 2 weeks after induction, 1M:contra = contralateral ACA-OA bifurcation at 1 month after aneurysm induction. Panel A is reproduced with permission from Aoki et al: Arterioscler Thromb Vasc Biol 29:1080–1086, 2009. Panel C is reproduced with permission from Aoki et al: Stroke 38:162–169, 2007.
Expression of TLR4 in Cerebral Aneurysm Walls of Humans

The TLR4 was abundantly expressed in the endothelial cell layer and adventitia both in control arterial walls and cerebral aneurysm walls of human tissue samples (Fig. 4A, B, and E). It was expressed only slightly in medial smooth-muscle cells (Fig. 4F). Expression of TLR4 was present in the endothelial cells both in rat and human cerebral aneurysms. However, in control MCA walls of humans, TLR4 expression was also present in the adventitia, unlike in rats (Fig. 4).

Discussion

Cerebral aneurysm is a common clinical entity in the general population, with a prevalence ranging from 1 to 5%,37 and causes a devastating SAH. Approximately 50% of patients die of SAH, and 30% of patients have a mild to severe disability.24 Furthermore, a considerable number of survivors of SAH have a cognitive dysfunction and cannot return to the same activities of daily living as before the illness, even if they reach a good outcome.56 However, there is no medical treatment of unruptured cerebral aneurysms except for invasive surgical procedures. This is because the detailed mechanisms of cerebral aneurysm formation and rupture remain to be elucidated.

To date, we have clarified some of the important...
mechanisms of cerebral aneurysm formation by using a previously established animal model of cerebral aneurysms. TLR are identified. In rats, 11 family members are identified and registered in the database. For example, skeletal muscle expresses TLR2, -4, -5, and -9.4 Cardiomyocyte expresses TLR2, -3, -4, -5, -7, and -9.5 There are some previous studies about the role of TLRs in intracranial lesions, but there are no experimental data about the expression pattern of TLRs in intracranial arteries. In cerebral aneurysm walls, expressions of TLR1, -4, -5, -6, -10, and -11 mRNA were detected on PCR studies. Among them, the expression of TLR1, -5, and -6 mRNA was constitutive during cerebral aneurysm progression (Fig. 1). Expression of TLR4 and TLR10 mRNA changed during cerebral aneurysm progression, suggesting that TLR4 and TLR10 played a role in cerebral aneurysm progression (Figs. 1 and 3). Expression of TLR10 is restricted in B cells, dendritic cells, and regulatory T cells in previous reports.11,13,20 Previously we revealed that, in cerebral aneurysm walls, the main inflammatory cells were macrophages, whereas T cells and B cells were a minor population of inflammatory cells in cerebral aneurysm walls. However, increased expression of TLR10 mRNA may imply the important role of T cells or B cells in cerebral aneurysm formation. This point should be examined in future studies.

Expression of TLR4 significantly and temporarily increased at the early stage of cerebral aneurysm formation, both in mRNA and protein, but increased expression of TLR4 was temporary, only at the early stage of cerebral aneurysm formation. Expression of TLR4 was predominantly in the endothelial cell layer of arterial walls, where hemodynamic stress was loaded (Fig. 2). Certainly, in previous reports, shear stress has been found to induce TLR4 activation by shear stress. Uregulated TLR4 expression was coincided well with NF-κB activation. Previously, we showed that NF-κB activation occurred mainly in endothelial cells, suggesting that TLR4 expression and NF-κB activation had a correlation with each other. In NF-κB-deficient mice (p50 knockout mice), TLR4 expression was the same as in wild-type mice, suggesting that TLR4 regulates NF-κB activation (Fig. 2).

In many previous studies, TLRs certainly contribute to NF-κB activation. In human cerebral aneurysms, TLR4 expression was predominantly in the endothelial cell layer, as in the cerebral aneurysms of rats. However, TLR4 was also abundantly expressed in the adventitia of both human cerebral aneurysm and control MCA. In rats, TLR4 expression was definitely detected in the adventitia of cerebral aneurysm walls 2 weeks after aneurysm induction. A longer term of hemodynamic stress compared with rats may influence TLR4 expression in cerebral aneurysm walls. However, increased expression of TLR10 mRNA may imply the important role of T cells or B cells in cerebral aneurysm formation. This point should be examined in future studies.

A cerebral aneurysm is formed as a result of chronic inflammation in arterial walls by hemodynamic stress. Nuclear factor–kappa B is a key molecule of cerebral aneurysm formation, and is activated in endothelial cells at the arterial bifurcation by hemodynamic stress. This molecule transcriptionally upregulates inflammation-related genes such as MCP-1. Macrophages, which are a major type of inflammatory cell in cerebral aneurysm walls, accumulate at the arterial bifurcation, recruited by upregulated MCP-1 expression. Macrophages express tissue-destructive proteinases such as matrix metalloproteinase-2 and decompose extracellular matrix of arterial walls, resulting in thinning of the media. Chronic inflammation also causes apoptosis in medial smooth muscle cells and further promotes the degradation of arterial walls. Inhibition of NF-κB activation by p50 deletion, decoy oligonucleotides, or simvastatin, markedly prevents cerebral aneurysm formation, suggesting that NF-κB activation is a key step in cerebral aneurysm formation. Nuclear factor–kappa B is activated by various stimuli, including hemodynamic stress. In cerebral aneurysm walls, NF-κB is already activated 2 weeks after aneurysm induction, when cerebral aneurysm formation is at an early stage and gross pathological changes have not developed yet. However, the molecular basis of NF-κB activation by hemodynamic stress in cerebral aneurysm walls is not yet clear. The TLR is one of the candidates of these molecules.

The TLR is a mammalian homolog of Drosophila Toll, which regulates early morphogenetic patterning of Drosophila, known as dorsoventralization. In mammals, TLRs have a crucial role in innate and adaptive immunity. Toll-like receptors recognize microbially derived molecules such as lipopolysaccharide, DNA, and so on. Recent studies reveal that TLRs have another important role in various inflammatory diseases added to their contribution in immunity. The TLRs are originally receptors for outward stimuli; TLRs respond to changes of the outward environment and cause both immune and inflammatory responses. The TLRs regulate inflammatory cascades, especially NF-κB activation, and are involved in the pathogenesis of various inflammatory diseases, including atherosclerosis. Toll-like receptors have large numbers of family members and express in a different manner in different tissues. In humans and mice, 13 family members of NF-κB–regulated genes such as MCP-1 were macrophages, whereas T cells and B cells were a minor population of inflammatory cells in cerebral aneurysm walls. However, increased expression of TLR10 mRNA may imply the important role of T cells or B cells in cerebral aneurysm formation. This point should be examined in future studies.
stage of aneurysm formation, as we can in rats. So the results of immunohistochemical studies in human cerebral aneurysms do not disagree with the results in rats. For further evaluation of the role of TLR4 in cerebral aneurysm formation, experiments using TLR4-deficient mice are desirable. However, in our preliminary experiment, TLR4-deficient mice showed significantly lower systemic blood pressure compared with wild-type mice after aneurysm induction (data not shown). Because our cerebral aneurysm model is produced by induced hypertension, we cannot assess cerebral aneurysm formation in TLR4-deficient mice.

Toll-like receptor 4 is actively involved in inflammatory vascular diseases such as atherosclerosis.\textsuperscript{12,23,34,42,45,46,50,52} In the early stage of atherogenesis, endothelial cells expressing TLR4 regulate chronic inflammation, especially NF-κB activation, in arterial walls.\textsuperscript{12,23,34} suggesting that TLR4 plays a critical role in the initiation of atherosclerosis. Chronic inflammation by hemodynamic stress is a common pathogenesis of both atherosclerosis and cerebral aneurysm, and similar results about the expression of TLR4 were also present in our study, further suggesting that TLR4 played the critical role in the initiation of cerebral aneurysm.

Conclusions

In this study, we identified TLR4 as a factor related to NF-κB activation in endothelial cells of cerebral aneurysms walls. At the early stage of cerebral aneurysm formation, TLR4 expression is upregulated by increased hemodynamic stress and, as a result of TLR4 upregulation, NF-κB is activated. As a result, chronic inflammation in arterial walls is induced and a cerebral aneurysm is formed. The TLR4 is a first identified factor associated with NF-κB activation in cerebral aneurysms. Modulation of TLR4 activity may be a target of therapy for cerebral aneurysms. The results of the present study will shed new light on cerebral aneurysm formation.

Disclosure

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