NF-κB Is a Key Mediator of Cerebral Aneurysm Formation

Tomohiro Aoki, MD; Hiroharu Kataoka, MD, PhD; Munehisa Shimamura, MD, PhD; Hironori Nakagami, MD, PhD; Kouji Wakayama, MD; Takuya Moriwaki, MD, PhD; Ryota Ishibashi, MD; Kazuhiko Nozaki, MD, PhD; Ryuichi Morishita, MD, PhD; Nobuo Hashimoto, MD, PhD

Background—Subarachnoid hemorrhage caused by the rupture of cerebral aneurysm (CA) remains a life-threatening disease despite recent diagnostic and therapeutic advancements. Recent studies strongly suggest the active participation of macrophage-mediated chronic inflammatory response in the pathogenesis of CA. We examined the role of nuclear factor-κB (NF-κB) in the pathogenesis of CA formation in this study.

Methods and Results—In experimentally induced CAs in rats, NF-κB was activated in cerebral arterial walls in the early stage of aneurysm formation with upregulated expression of downstream genes. NF-κB p50 subunit-deficient mice showed a decreased incidence of CA formation with less macrophage infiltration into the arterial wall. NF-κB decoy oligodeoxynucleotide also prevented CA formation when it was administered at the early stage of aneurysm formation in rats. Macrophage infiltration and expression of downstream genes were dramatically inhibited by NF-κB decoy oligodeoxynucleotide. In human CA walls, NF-κB also was activated, especially in the intima.

Conclusions—Our data indicate that NF-κB plays a crucial role as a key regulator in the initiation of CA development by inducing some inflammatory genes related to macrophage recruitment and activation. NF-κB may represent a therapeutic target of a novel medical treatment for CA. (Circulation. 2007;116:2830-2840.)

Key Words: aneurysm ■ experimental animal models ■ endothelium ■ inflammation ■ NF-kappa B

Cerebral aneurysm (CA) is a common lesion, with a prevalence ranging from 1% to 5% in large autopsy studies.1 CA can cause a catastrophic subarachnoid hemorrhage, which has a 30-day mortality rate of 45%. An estimated 30% of survivors have moderate to severe disability.2 Despite its public importance, the mechanisms of the initiation, progression, and rupture of CAs remain to be elucidated.

Clinical Perspective p 2840

CA is characterized by the excessive degradation of extracellular matrix and accumulation of inflammatory cells in aneurysmal walls. Studies using human CA specimens revealed macrophage accumulation3 in aneurysmal walls. Macrophage accumulation also was prominent in experimentally induced CA in rats.4 By using the experimentally induced CA model, we have identified inducible nitric oxide synthase (iNOS),5 interleukin-1β (IL-1β),6 and matrix metalloproteinase (MMP)-2 and -97 as exacerbating factors of CA progression. These results suggest that the pathogenesis of CA may be linked to chronic inflammation mediated by macrophages in vascular walls. However, it is still unclear which inflammatory pathway is involved in CA formation and what triggers the inflammatory cascade.

Nuclear factor-κB (NF-κB) is a family of transcriptional factors regulating the expression of a variety of genes in response to inflammatory mediators,7 thus contributing to the initiation and progression of atherosclerosis8,9 and abdominal aortic aneurysm.10,11 NF-κB transactivates genes related to endothelial dysfunction, including vascular cell adhesion molecule-1 (VCAM-1)12,13 and monocyte chemoattracting protein-1 (MCP-1).14,15 NF-κB also regulates the transcription of some proinflammatory genes such as iNOS16 and MMPs17 which are of functional importance for the progression of CAs.

In the present study, we investigated the role of NF-κB in the initiation and progression of CAs by using an experimentally induced CA model in rats and mice. Here, we show for the first time that NF-κB participates in the initiation of CA formation by transactivating downstream genes in the early stage of aneurysm formation with upregulated expression of downstream genes. NF-κB p50 subunit-deficient mice showed a decreased incidence of CA formation with less macrophage infiltration into the arterial wall. NF-κB decoy oligodeoxynucleotide also prevented CA formation when it was administered at the early stage of aneurysm formation in rats. Macrophage infiltration and expression of downstream genes were dramatically inhibited by NF-κB decoy oligodeoxynucleotide. In human CA walls, NF-κB also was activated, especially in the intima.

Conclusions—Our data indicate that NF-κB plays a crucial role as a key regulator in the initiation of CA development by inducing some inflammatory genes related to macrophage recruitment and activation. NF-κB may represent a therapeutic target of a novel medical treatment for CA. (Circulation. 2007;116:2830-2840.)

Key Words: aneurysm ■ experimental animal models ■ endothelium ■ inflammation ■ NF-kappa B

Cerebral aneurysm (CA) is a common lesion, with a prevalence ranging from 1% to 5% in large autopsy studies.1 CA can cause a catastrophic subarachnoid hemorrhage, which has a 30-day mortality rate of 45%. An estimated 30% of survivors have moderate to severe disability.2 Despite its public importance, the mechanisms of the initiation, progression, and rupture of CAs remain to be elucidated.

Clinical Perspective p 2840

CA is characterized by the excessive degradation of extracellular matrix and accumulation of inflammatory cells in aneurysmal walls. Studies using human CA specimens revealed macrophage accumulation3 in aneurysmal walls. Macrophage accumulation also was prominent in experimentally induced CA in rats.4 By using the experimentally induced CA model, we have identified inducible nitric oxide synthase (iNOS),5 interleukin-1β (IL-1β),6 and matrix metalloproteinase (MMP)-2 and -97 as exacerbating factors of CA progression. These results suggest that the pathogenesis of CA may be linked to chronic inflammation mediated by macrophages in vascular walls. However, it is still unclear which inflammatory pathway is involved in CA formation and what triggers the inflammatory cascade.

Nuclear factor-κB (NF-κB) is a family of transcriptional factors regulating the expression of a variety of genes in response to inflammatory mediators,7 thus contributing to the initiation and progression of atherosclerosis8,9 and abdominal aortic aneurysm.10,11 NF-κB transactivates genes related to endothelial dysfunction, including vascular cell adhesion molecule-1 (VCAM-1)12,13 and monocyte chemoattracting protein-1 (MCP-1).14,15 NF-κB also regulates the transcription of some proinflammatory genes such as iNOS16 and MMPs17 which are of functional importance for the progression of CAs.

In the present study, we investigated the role of NF-κB in the initiation and progression of CAs by using an experimentally induced CA model in rats and mice. Here, we show for the first time that NF-κB participates in the initiation of CA formation by transactivating downstream genes in the early stage of aneurysm formation with upregulated expression of downstream genes. NF-κB p50 subunit-deficient mice showed a decreased incidence of CA formation with less macrophage infiltration into the arterial wall. NF-κB decoy oligodeoxynucleotide also prevented CA formation when it was administered at the early stage of aneurysm formation in rats. Macrophage infiltration and expression of downstream genes were dramatically inhibited by NF-κB decoy oligodeoxynucleotide. In human CA walls, NF-κB also was activated, especially in the intima.

Conclusions—Our data indicate that NF-κB plays a crucial role as a key regulator in the initiation of CA development by inducing some inflammatory genes related to macrophage recruitment and activation. NF-κB may represent a therapeutic target of a novel medical treatment for CA. (Circulation. 2007;116:2830-2840.)

Key Words: aneurysm ■ experimental animal models ■ endothelium ■ inflammation ■ NF-kappa B

Cerebral aneurysm (CA) is a common lesion, with a prevalence ranging from 1% to 5% in large autopsy studies.1 CA can cause a catastrophic subarachnoid hemorrhage, which has a 30-day mortality rate of 45%. An estimated 30% of survivors have moderate to severe disability.2 Despite its public importance, the mechanisms of the initiation, progression, and rupture of CAs remain to be elucidated.
genes related to macrophage recruitment and vascular inflammation.

Methods

Induction of Experimentally Induced CAs
In 7-week–old male Sprague-Dawley rats, CAs were induced as previously described by Nagata et al. We also induced CAs in NF-κB p50 subunit knockout mice, which have a background of C57/B6;129P2 (p50−/− mice; The Jackson Laboratory, Bar Harbor, Me), and their littersmates (p50+/+ mice), as previously described by Morimoto et al. Blood pressure was measured by tail-cuff method. At the indicated time point, animals were euthanized, and the anterior cerebral artery/olfactory artery (ACA/OA) bifurcation was stripped and observed under a light microscope after elastica van Gieson staining. Induced aneurysms were classified into 2 categories. Internal elastic lamina (IEL) disruption refers to a lesion with the discontinuity of IEL without apparent outward bulging of the arterial wall, which represents early change of CA formation. Aneurysm refers to an obvious outward bulging of the arterial wall with the fragmentation or disappearance of IEL. Three independent researchers assessed the histopathological changes. Aneurysm size was expressed as the maximum diameter of CAs. Animal care and experiments complied with Japanese community standards on the care and use of laboratory animals.

Immunohistochemistry and Cell Counting
After blocking, 5-μm frozen sections were incubated with primary antibodies for 1 hour at room temperature, followed by incubation with fluorescence-labeled secondary antibodies for 1 hour at room temperature. Then, the slides were observed under a fluorescence microscope system (BX51N-34-FL-1, Olympus, Japan). The primary antibodies used in the present study are listed in the online-only Data Supplement, expanded Methods section. To quantify macrophage accumulation, the number of CD68-positive cells was counted in a 100-μm² area in rats and in a 50-μm² area in mice.

Electrophoretic Mobility Shift Assay
Nuclear protein from the whole Willis ring was extracted with the Qproteome Nuclear Protein Kit (Qiagen, Hilden, Germany) according to the manufacturer’s directions. Electrophoretic mobility shift assay (EMSA) was performed by LightShift Chemiluminescent EMSA Kit (Pierce, Rockford, Ill). Nuclear extract (5 μg) was incubated with 20 fmol biotin 3′ end-labeled oligonucleotides containing the κB sequence (5′-GGGATTCCC-3′). After electrophoresis, transfer, and cross-linking, the signal was detected by a peroxidase/luminol system (Chemiluminescent Nucleic Acid Detection Module, Pierce). To confirm the specificity, a 200-fold excess amount of cold NF-κB oligonucleotides (cold probe) or mutated binding motif (5′-GGCCATTTC-3′) was added. Supershift assays using an anti-p50, anti-p52, or anti-p65 subunit antibody (Santa Cruz, Carpinteria, Calif) served as control. NF-κB binding site was served as control. NF-κB decoy ODN (5′-ttgccgtacctgacttagcc-3′ and 5′-ggagggaaatcccttcaagg-3′) served as control. NF-κB (40 μg) or scrambled decoy ODN in 60 μL PBS was injected into the cisterna magna every 2 weeks under general anesthesia.

To confirm the specificity of NF-κB decoy ODN, EMSA for the NF-κB binding site was performed as described above with a 200-fold excess amount of cold NF-κB or scrambled decoy ODN. In addition, the specificity of NF-κB decoy ODN was examined by luciferase assay using bovine aortic endothelial cells transfected with the luciferase gene driven by the NF-κB binding site (BD Bioscience Clontech, Palo Alto, Calif), PRT-TK plasmids (Promega, Madison, Wis), and 50 nmol/L NF-κB or scrambled decoy ODN. Transfected cells were incubated for 12 hours with or without human recombinant 10 ng/mL tumor necrosis factor-α (TNF-α; PeproTec, London, UK). NF-κB activity was measured with the Dual-Luciferase Assay System (Promega) according to the manufacturer’s instructions.

Quantitative PCR
In NF-κB- or scrambled decoy ODN–treated rats, quantitative PCR was performed using QuantiTect SYBR Green PCR Kit (Qiagen) and LightCycler quick system 330 (Roche, Basel, Switzerland). Constructs were produced by the TOPO TA Cloning Kit (Invitrogen, Carlsbad, Calif) from cDNA according to the manufacturer’s directions. β-Actin was used as an internal control. The second derivative maximum method was used for crossing point determination using LightCycler Software 3.3 (Roche). In addition, 3 independent samples were examined in 1 experiment. The primer sets and conditions for PCR are described in the online-only Data Supplement, expanded Methods section. Sham-operated rats were used as control.

Immunohistochemistry for Human Samples
Human CA samples were obtained from 7 patients who underwent neck clipping for unruptured aneurysms with informed consent. The middle cerebral artery (n=4) obtained at the superficial temporal artery–middle cerebral artery bypass surgery served as control. Paraffin sections (4 μm) were cut and mounted on slides. After deparaffination and blocking of endogenous peroxidase activity with 0.3% H₂O₂, a mouse monoclonal antibody against the rat DNA-binding form of the NF-κB p65 subunit, which cross-reacts with human p65, was incubated for 12 hours at 4°C, followed by incubation with biotin-labeled secondary antibodies for 30 minutes at room temperature. Then, the slides were incubated with streptavidin-conjugated peroxidase. Finally the signal was detected by 3,3′-diaminobenzidine system (Dako, Carpenteria, Calif).

Statistical Analysis
Data (mean±SD) were analyzed by use of the Mann–Whitney U test for a 2-group comparison and Kruskal-Wallis 1-way ANOVA on ranks, followed by the Turkey-Kramer test for multiple comparisons. The incidence of aneurysmal changes was analyzed by use of Fisher exact test. Differences were considered statistically significant at P<0.05.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

NF-κB Activation in an Experimentally Induced CA in Rats
Immunohistochemistry with an anti–NF-κB p65 subunit antibody that recognizes only the DNA-binding form was separated by the electrophoresis in 2% agarose gel. Densitometric analysis includes data of 6 samples per group.
revealed that NF-κB was highly activated in arterial walls at the ACA/OA bifurcation 2 weeks, 1 month, and 3 months after aneurysm induction whether they showed histological changes or not (Figure 1C through 1H). Although NF-κB also was activated at the contralateral ACA/OA bifurcation, which was not subjected to excessive hemodynamic stress as a result of CCA ligation, the extent of NF-κB activation was more abundant in the aneurysm side (Figure I of the online-only Data Supplement). In contrast, NF-κB activation occurred in only a few cells of the cerebral arterial walls before aneurysm induction (Figure 1A and 1B). The DNA-binding form of p65 was merged with endothelial NOS (Figure 2A) and CD68 (Figure 2B), showing that NF-κB was activated in both endothelial cells and macrophages. Activated NF-κB also was costained with MCP-1 (Figure 2C) and VCAM-1 (Figure 2D).

**DNA Binding of NF-κB in an Experimentally Induced CA in Rats**

In EMSA, 1 specific complex band could be detected in aneurysmal walls 1 and 3 months after aneurysm induction (Figure 3A). This band could be completely abolished by competition with the κB oligonucleotide but not with a mutated binding motif (Figure 3B), confirming that the band was specific for NF-κB. Supershift assays with specific antibodies against various NF-κB subunits demonstrated that the NF-κB complex band was abolished by the antibody
Figure 4. CA formation in p50−/− mice. A, The ratio of aneurysmal changes in p50−/− and p50+/+ mice 5 months after aneurysm induction. Data were analyzed by use of Fisher exact test (n=10 per group; P=0.020 for all aneurysmal changes). B, Aneurysm size in p50−/− and p50+/+ mice 5 months after aneurysm induction. Data were analyzed by the Mann–Whitney U test (n=10 per group; P<0.01). C, Systemic blood pressure 5 months after aneurysm induction. Data were analyzed by the Mann–Whitney U test (p50+/+, n=10; p50−/−, n=7). D and E, Anatomy of the Willis ring in p50−/− mice (D) and p50−/− mice (E) observed under a light microscope.

against p50 and p65 but not by the antibody against p52 (Figure 3C).

Aneurysm Formation in p50−/− Mice
After 5 months of aneurysm induction, only 1 of 9 p50−/− mice (10%) presented IEL disruption, whereas 7 of 10 p50+/+ mice (70%) developed aneurysmal changes, including IEL disruption, which were aneurysms in 3 of the mice (Figure 4A). The incidence of aneurysmal changes was significantly lower in p50−/− mice than in p50+/+ mice (P=0.020; n=10 per group). Aneurysms induced in p50−/− mice (0 µm; n=10) were significantly smaller than in the p50+/+ mice (39.0±31.9 µm; n=10; P<0.01; Figure 4B). Blood pressure after aneurysm induction was not different between p50−/− and p50+/+ mice (p50+/+; 124.9±19.0 mm Hg, n=7; p50−/−; 125.1±18.1 mm Hg, n=10; Figure 4C). No anatomic difference was present in the Willis ring between p50−/− and p50+/+ mice (Figure 4D and 4E).

The expression levels of MCP-1, VCAM-1, MMP-2, MMP-9, IL-1β, and iNOS mRNA were elevated with aneurysm progression in p50+/+ mice (MCP-1, VCAM-1, MMP-2, MMP-9, IL-1β; P<0.01; iNOS; P=0.012), whereas mRNA expression of these molecules was not upregulated in p50−/− mice (P<0.01, p50+/+ versus p50−/− after 5 months; Figure 5A through 5G). Macrophage accumulation assessed by the number of CD68-positive cells in p50−/− mice (1.3±0.87 cells/50 µm²; n=10) was observed much less than in p50+/+ mice (3.7±1.6 cells/50 µm²; n=10; P<0.01; Figure 5H). Immunohistochemistry also demonstrated the upregulation of MCP-1, VCAM-1, MMP-2, MMP-9, IL-1β, and iNOS in aneurysmal walls of p50−/− mice. Although these genes were also expressed in an upregulated fashion in the contralateral ACA/OA bifurcation, expression levels were higher in the aneurysm side than in the contralateral side (Figure II of the online-only Data Supplement).

Effect of NF-κB Decoy ODN on CA Formation
Two weeks after the first injection (40 μg in 60 μL PBS), fluorescein isothiocyanate (FITC)–conjugated NF-κB decoy ODN (FITC-decoy) was incorporated into the intima and media of the cerebral artery (Figure 6A and 6B). Double immunohistochemistry with the anti–endothelial NOS antibody revealed that FITC-decoy was transferred into endothelial cells (Figure 6C and 6D). The specificity of NF-κB decoy ODN was confirmed by EMSA and luciferase assay. The NF-κB complex band in rat CAs 1 month after aneurysm induction disappeared by preincubation with a 200-fold excess amount of cold NF-κB decoy ODN but not with a 200-fold excess amount of cold...
scrambled decoy ODN (Figure 6E). The TNF-α-induced increase in luciferase activity in bovine aortic endothelial cells was inhibited by NF-κB decoy ODN but not by scrambled decoy ODN (Figure 6F).

When injection of NF-κB decoy ODN started at the same time as aneurysm induction, the incidence of aneurysmal changes, including IEL disruption, was significantly lower in the NF-κB decoy group (40%, n=10) than in the scrambled decoy group (100%, n=10; P=0.011; Figure 6H). Aneurysms were significantly smaller in the NF-κB decoy group (5.0±6.7 μm; n=10) than in the scrambled decoy group (55.0±32.6 μm; n=10; P<0.01; Figure 6I). When injection of NF-κB decoy ODN was started 1 week after aneurysm induction, the incidence of aneurysmal changes was significantly lower in the NF-κB decoy group (P=0.011; n=10 per group; Figure 6J). Aneurysms also were significantly smaller in the NF-κB decoy group (6.5±10.5 μm; n=10) than in the scrambled decoy group (47.5±30.0 μm; n=10; P<0.01; Figure 6K). However, when the injection of NF-κB decoy ODN started 2 weeks after aneurysm induction, the incidence of CA formation did not differ significantly between the 2 groups (P=0.47; Figure 6L). No statistically significant difference in aneurysm size existed between the 2 groups (scrambled decoy: 55.3±36.0 μm, n=10; NF-κB decoy: 23.5±14.7 μm, n=10; P=0.17; Figure 6M). Systemic blood pressure was not different between the 2 groups in any time course experiment (Figure 6G).

**Figure 5.** Expression of downstream genes and macrophage infiltration into aneurysmal walls in p50+/+ and p50−/− mice before aneurysm induction (0M) and 5 months after aneurysm induction (5M). A, Reverse-transcription PCR analysis for MCP-1, VCAM-1, MMP-2, MMP-9, IL-1β, and iNOS in p50−/− and p50+/+ mice. B through G, Densitometric analysis of MCP-1 (B), VCAM-1 (C), MMP-2 (D), MMP-9 (E), IL-1β (F), and iNOS (G) mRNA expression. Data were analyzed by the Mann–Whitney U test (n=6 per group). H, The number of macrophages that infiltrate into aneurysmal walls. Data were analyzed by the Mann–Whitney U test (n=6 per group).
operated rats (MCP-1: P < 0.015; VCAM-1, MMP-2, MMP-9, IL-1β: P < 0.01, scrambled decoy group versus sham group; Figure 7A through 7F). In contrast, the mRNA expression level of these molecules was significantly lower in the NF-κB decoy group (P < 0.01, scrambled decoy group versus NF-κB decoy group; Figure 7A through 7F). In immunohistochemistry, MCP-1 and VCAM-1 were expressed mainly in the intima of aneurysmal walls 1 month after aneurysm induction in the scrambled decoy group (Figure 7G through 7I). However, only a few positive signals for MCP-1 and VCAM-1 could be detected in the NF-κB decoy group (Figure 7J through 7L). Macrophage infiltration into the arterial walls also was reduced in the NF-κB decoy group compared with the scrambled decoy group (NF-κB decoy group: 1.3 ± 1.1 cells/100 μm², n = 7; scrambled decoy group: 5.4 ± 1.5 cells/100 μm², n = 10; P < 0.01; Figure 7M). NF-κB Activation in Human CAs

Immunohistochemistry using an antibody against the DNA-binding form of NF-κB p65 subunit revealed that NF-κB was highly activated in aneurysmal walls, especially in the intima (Figure 8B). In contrast, NF-κB activation occurred in only a few cells in the control cerebral artery (Figure 8A).

Discussion

In the present study, we provide the first evidence that NF-κB plays a crucial role in the formation of CAs by transcriptionally activating the expression of some proinflammatory genes, leading to macrophage recruitment and extracellular matrix degradation. In unstimulated cells, NF-κB is located in the cytoplasm through association with inhibitory IκB proteins that mask their nuclear localization signal. After activating signals, IκB is phosphorylated and proteolytically degraded, resulting in NF-κB translocation to the nucleus. Immunohistochemistry with an antibody directed against the nuclear localization signal of the p65 subunit of NF-κB demonstrated increased NF-κB activation in arterial walls, especially in the early stage of CA formation in rats (Figure 1). NF-κB activation also was observed in the intima of

Figure 6. Effect of NF-κB decoy ODN on the initiation and progression of CAs. A and B, Uptake of FITC-decoy in cerebral arterial walls after 2 weeks of injection. A differential interference contrast image (A) and a fluorescence image (B) are shown. Bar=100 μm. C and D, Double staining of FITC-decoy (green) with an anti-endothelial NOS antibody (red). Hematoxylin and eosin staining (C) and a fluorescence image (D) of the serial section are shown. Bar=100 μm. E, A competition assay by use of EMSA. Nuclear extract from rat cerebral arteries 1 month after aneurysm induction was incubated with a 200-fold excess amount of cold NF-κB decoy ODN or cold scrambled decoy ODN. Representative data of 3 independent experiments are shown. F, Luciferase assay with NF-κB and scrambled decoy ODN in bovine aortic endothelial cells stimulated by TNF-α. Increased luciferase activity by TNF-α was inhibited by NF-κB decoy ODN but not by scrambled decoy ODN. Data were analyzed by use of the Kruskal-Wallis 1-way ANOVA on ranks, followed by the Turkey-Kramer test (n=4 per group). G, Systemic blood pressure 1 month after aneurysm induction in each group. Data were analyzed by the Mann–Whitney U test (n=10 per group). H through M, Incidence of aneurysmal changes (H, J, and L) and aneurysm size (I, K, and M) 1 month after aneurysm induction in rats. NF-κB or scrambled decoy ODN was injected into the cisterna magna every 2 weeks from the day of aneurysm induction (0W; H and I), from 1 week after aneurysm induction (1W; J and K), and from 2 weeks after aneurysm induction (2W; L and M). Data were analyzed by Fisher exact test (n=10 per group).
human CA samples (Figure 8). Enhanced DNA binding of NF-κB in aneurysmal walls was confirmed by EMSA (Figure 3A). NF-κB consists of homodimers or heterodimers of 5 known subunits in mammalian cells: p50, p52, RelA (p65), RelB, and RelC. Experiments with specific antibody to various NF-κB subunits demonstrated that the NF-κB complex activated in aneurysmal walls consisted of p50/p65 heterodimers (Figure 3C).

NF-κB activation occurred mainly in the endothelial cells and macrophages (Figure 2A and 2B). Furthermore, activated NF-κB was costained with MCP-1 and VCAM-1 (Figure 2C and 2D), both of which are prerequisites for macrophage accumulation in vascular walls, suggesting that activated NF-κB may mediate macrophage recruitment into aneurysmal walls through upregulated expression of MCP-1 and VCAM-1.

To determine the functional importance of NF-κB activation in CA formation, we used mice deficient in the p50 subunit of NF-κB (p50−/− mice). The p50−/− mice are viable and demonstrate specific defects only in immune responses.22 The anatomy of the Willis ring and systemic blood pressure are not different between p50−/− and p50+/+.
mice (Figure 4C through 4E). Nevertheless, the incidence of aneurysmal changes, including IEL disruption, and aneurysm size were significantly lower in p50/−/−/− mice than in p50+/−/+ mice (Figure 4A and 4B). As shown in Figure 5, mRNA expression of MCP-1 and VCAM-1 was upregulated in CAs of p50+/−/+ mice. In contrast, upregulation of these 2 genes did not occur even after aneurysm induction in p50−/− mice. Macrophage accumulation in cerebral arterial walls also was reduced in p50−/− mice (Figure 5H). These findings indicate that the NF-κB p50 subunit plays an important role in the initiation of CA formation through the transactivation of downstream genes causing macrophage accumulation.

We next used NF-κB decoy ODN to determine when NF-κB plays a role in the pathogenesis of CA. NF-κB decoy ODN was shown to bind NF-κB and to inhibit the binding of
NF-κB to DNA.\textsuperscript{11} NF-κB decoy ODN was injected into the cisterna magna every 2 weeks in rats. The dose of NF-κB decoy ODN was determined by a pilot study with FITC-decoy, and successful transfer to the cerebral arteries was confirmed (Figure 6A through 6D). The specificity of NF-κB decoy ODN was demonstrated in previous studies.\textsuperscript{23,24} We also confirmed its specificity in our model by EMSA, in which the addition of cold NF-κB decoy ODN inhibited the binding of NF-κB to DNA in CAs (Figure 6E). Luciferase assay revealed the inhibitory effect of NF-κB decoy ODN to TNF-α–induced NF-κB activation in vitro (Figure 6F). These data reinforce our hypothesis that NF-κB decoy ODN specifically inhibits DNA binding of NF-κB.

Incidence of aneurysmal changes, including IEL disruption, and aneurysm size were significantly reduced when injection of NF-κB decoy ODN was started within 1 week after aneurysm induction (Figure 6H through 6M), strongly suggesting that NF-κB may be involved in the early stage of CA formation. The expression of MCP-1 and VCAM-1 was inhibited by NF-κB decoy ODN (Figure 7A, 7B, and 7G through 7L), consequently reducing the number of macrophages accumulating in arterial walls (Figure 7M). These results indicate that macrophage recruitment into aneurysmal walls depends on the NF-κB pathway.

As we previously demonstrated,\textsuperscript{4–6} MMP-2, MMP-9, IL-1β, and iNOS were expressed in an upregulated fashion in aneurysmal walls with CA progression. In p50\textsuperscript{–/-} mice, mRNA expression of these molecules was not upregulated (Figure 5D through 5G). The upregulated expression of these genes also was inhibited by NF-κB decoy ODN (Figure 7C through 7F). MMP-2 and MMP-9 cause the loss of crucial extracellular matrix components, including collagen and...
elastin. IL-1β and iNOS induce apoptosis in medial smooth muscle cells (SMCs). Extracellular matrix degradation and apoptosis of SMCs result in thinning of the media, leading to progression and rupture of CAs. Our findings suggest that degenerative changes in aneurysmal walls also may be regulated by NF-κB. Because these genes are expressed mainly in macrophages and SMCs in aneurysmal walls, decreased expression of these genes in aneurysmal walls by the treatment with NF-κB decoy ODN or p50 deficiency may be derived in part from the decreased macrophage infiltration.

CAs tend to be formed at the arterial bifurcation suffering from high shear stress. Fluid shear stress promotes the translocation into the nucleus of NF-κB in cultured endothelial cells by activating IκB kinase. Therefore, NF-κB activation caused by excessive hemodynamic stress resulting from anatomic architecture and hypertension is considered the first step in CA formation. In fact, NF-κB was activated at the ACA/OA bifurcation before histological changes occurred (Figure 1C and 1D). Even in the contralateral side, which is influenced only by hemodynamic stress resulting from hypertension, NF-κB activation and upregulated expression of downstream genes were observed (Figures I and II of the online-only Data Supplement). However, the activation or upregulation was more prominent in the aneurysm side than in the contralateral side, suggesting that hemodynamic stress resulting from anatomic architecture may enhance the inflammatory reaction in an NF-κB–dependent manner. One major direct downstream pathway of NF-κB activation is upregulation of MCP-1 and VCAM-1 gene expression, resulting in macrophage recruitment into the aneurysmal walls. Macrophages secrete MMP-2 and MMP-9, causing extracellular matrix degradation in aneurysmal walls, and release nitric oxide via iNOS upregulation, which mediates apoptosis in SMCs. We previously found extensive apoptotic cell death of SMCs in aneurysmal walls of experimentally induced CA in rats. Although it is still controversial whether NF-κB has a proapoptotic or an antiapoptotic effect, another possible potential role of NF-κB in the pathogenesis of CA is NF-κB–mediated regulation of apoptosis in SMCs.

In the present study, we have identified for the first time that NF-κB is a key regulator of CA formation. Our results shed light on the pathogenesis of CA and support the notion that CA is a chronic inflammatory disease mediated mainly by macrophages. NF-κB–targeted therapy may provide preventive therapeutic options for CA, the major cause of a catastrophic subarachnoid hemorrhage.

Source of Funding
This work was supported by a Grant-in-Aid for Scientific Research (No. 17390399) from the Ministry of Education, Science, and Culture of Japan.

Disclosures
None.

References

**CLINICAL PERSPECTIVE**

Subarachnoid hemorrhage caused by the rupture of cerebral aneurysm (CA) is one of the most severe forms of stroke. Many people die of aneurysmal subarachnoid hemorrhage despite recent diagnostic and therapeutic advancements. At present, patients with unruptured CAs must undergo microsurgical clipping or endovascular coiling to prevent CA rupture because no effective medical treatment for it exists. In the present study, we have demonstrated for the first time that nuclear factor-κB (NF-κB) is a key mediator of CA formation by using an experimentally induced CA model in mice and rats. NF-κB was activated in cerebral arterial walls in the early stage of aneurysm formation with upregulated expression of downstream genes. NF-κB p50 subunit–deficient mice showed decreased incidence of CA formation with less macrophage infiltration into the arterial wall. NF-κB decoy oligodeoxynucleotide also prevented CA formation when it was administered at the early stage of aneurysm formation in rats. Macrophage infiltration and expression of downstream genes were dramatically inhibited by NF-κB decoy oligodeoxynucleotide. In human CA walls, NF-κB also was activated, especially in the intima. These data indicate that inflammation elicited by NF-κB activation plays a crucial role in the initiation and progression of CA formation. Anti-inflammatory agents, especially those with an inhibitory effect on NF-κB, may be a promising medical treatment for CA in the future.
NF-κB Is a Key Mediator of Cerebral Aneurysm Formation
Tomohiro Aoki, Hiroharu Kataoka, Munehisa Shimamura, Hironori Nakagami, Kouji Wakayama, Takuya Moriwaki, Ryota Ishibashi, Kazuhiko Nozaki, Ryuichi Morishita and Nobuo Hashimoto

Circulation. 2007;116:2830-2840; originally published online November 19, 2007;
doi: 10.1161/CIRCULATIONAHA.107.728303
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2007 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/116/24/2830

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2007/12/04/CIRCULATIONAHA.107.728303.DC1
http://circ.ahajournals.org/content/suppl/2007/12/04/CIRCULATIONAHA.107.728303.DC2

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org//subscriptions/
aneurysm contralateral

A

B

C

D

E

F

G

H

I

J

K

L
Table 1 The incidence of aneurysmal changes and blood pressure in the experimentally induced cerebral aneurysm model of rats (A) and mice (B).

<table>
<thead>
<tr>
<th>A. Rat</th>
<th>no change</th>
<th>IEL disruption</th>
<th>aneurysm</th>
<th>total</th>
<th>Blood Pressure</th>
</tr>
</thead>
<tbody>
<tr>
<td>0M</td>
<td>9 (82%)</td>
<td>2 (18%)</td>
<td>0</td>
<td>11</td>
<td>103.7±3.2</td>
</tr>
<tr>
<td>1M</td>
<td>2 (11%)</td>
<td>10 (53%)</td>
<td>7 (36%)</td>
<td>19</td>
<td>152.9±17.0</td>
</tr>
<tr>
<td>2M</td>
<td>0</td>
<td>5 (50%)</td>
<td>5 (50%)</td>
<td>10</td>
<td>160.0±20.7</td>
</tr>
<tr>
<td>3M</td>
<td>0</td>
<td>2 (10%)</td>
<td>19 (90%)</td>
<td>21</td>
<td>160.5±20.1</td>
</tr>
</tbody>
</table>

(=SD)

<table>
<thead>
<tr>
<th>B. Mouse</th>
<th>no change</th>
<th>IEL disruption</th>
<th>aneurysm</th>
<th>total</th>
<th>Blood Pressure</th>
</tr>
</thead>
<tbody>
<tr>
<td>0M</td>
<td>8 (80%)</td>
<td>2 (20%)</td>
<td>0</td>
<td>10</td>
<td>91.5±17.7</td>
</tr>
<tr>
<td>3M</td>
<td>5 (50%)</td>
<td>5 (50%)</td>
<td>0</td>
<td>10</td>
<td>132.6±12.7</td>
</tr>
<tr>
<td>5M</td>
<td>3 (30%)</td>
<td>4 (40%)</td>
<td>3 (30%)</td>
<td>10</td>
<td>137.0±15.5</td>
</tr>
</tbody>
</table>

(=SD)
Table 1 The incidence of aneurysmal changes and blood pressure in the experimentally induced cerebral aneurysm model of rats (A) and mice (B).

<table>
<thead>
<tr>
<th></th>
<th>Rat</th>
<th>no change</th>
<th>IEL disruption</th>
<th>aneurysm</th>
<th>total</th>
<th>Blood Pressure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0M</td>
<td>9 (82%)</td>
<td>2 (18%)</td>
<td>0</td>
<td>11</td>
<td>103.7±3.2</td>
</tr>
<tr>
<td></td>
<td>1M</td>
<td>2 (11%)</td>
<td>10 (53%)</td>
<td>7 (36%)</td>
<td>19</td>
<td>152.9±17.0</td>
</tr>
<tr>
<td></td>
<td>2M</td>
<td>0</td>
<td>5 (50%)</td>
<td>5 (50%)</td>
<td>10</td>
<td>160.0±20.7</td>
</tr>
<tr>
<td></td>
<td>3M</td>
<td>0</td>
<td>2 (10%)</td>
<td>19 (90%)</td>
<td>21</td>
<td>160.5±20.1</td>
</tr>
</tbody>
</table>

B. Mouse

<table>
<thead>
<tr>
<th></th>
<th>no change</th>
<th>IEL disruption</th>
<th>aneurysm</th>
<th>total</th>
<th>Blood Pressure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0M</td>
<td>8 (80%)</td>
<td>2 (20%)</td>
<td>0</td>
<td>10 91.5±17.7</td>
</tr>
<tr>
<td></td>
<td>3M</td>
<td>5 (50%)</td>
<td>5 (50%)</td>
<td>0</td>
<td>10 132.6±12.7</td>
</tr>
<tr>
<td></td>
<td>5M</td>
<td>3 (30%)</td>
<td>4 (40%)</td>
<td>3 (30%)</td>
<td>10 137.0±15.5</td>
</tr>
</tbody>
</table>

(mean±SD)