Impaired Progression of Cerebral Aneurysms in Interleukin-1β–Deficient Mice

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Background and Purpose—Subarachnoid hemorrhage caused by cerebral aneurysm rupture remains a life-threatening emergency despite advances in treatment. However, the mechanisms underlying aneurysm initiation, progression, and rupture remain unclear. We developed a method to induce experimental cerebral aneurysms in rats, monkeys, and mice. Interleukin-1β (IL-1β) is a key inflammatory mediator, and it is thought to be a promising target for the treatment of inflammatory diseases. In the present study, we examined the role of IL-1β in cerebral aneurysm development.

Methods—Cerebral aneurysms were experimentally induced in 5-week-old male C57BL/6 mice, IL-1β gene–deficient (IL-1β−/−) mice, and age-matched control B10 mice (wild-type). Their cerebral arteries were dissected and examined histologically and immunohistochemically.

Results—IL-1β was expressed in vascular media in mice at an early stage of aneurysmal models’ cerebral arteries. No differences were seen in the rate of aneurysm development between IL-1β−/− and wild-type mice, but the percentage of advanced aneurysm change was significantly larger in wild-type animals. Furthermore, in IL-1β−/− mice, increased caspase-1 expression was seen compared with wild-type animals. Additionally, the number of apoptotic cells assessed by single-stranded DNA immunoreactivity and TUNEL was significantly reduced in IL-1β−/− mice compared with wild-type animals.

Conclusions—IL-1β is important for the progression of cerebral aneurysms in a mouse model. Disruption of the IL-1β gene results in the reduced incidence of mature experimental cerebral aneurysms. (Stroke. 2006;37:000-000.)

Key Words: animal models • apoptosis • cerebral aneurysm • cerebrovascular disorders • interleukins

Cerebral aneurysm rupture with subsequent subarachnoid hemorrhage remains a life-threatening medical emergency despite recent diagnostic and therapeutic advances. However, the mechanisms of aneurysm initiation, progression, and rupture remain unclear. Most studies examining the pathophysiology of cerebral aneurysm have relied on specimens obtained during autopsy or surgery. These samples are not suitable for examining the molecular factors leading to aneurysm initiation and progression.

We developed a novel experimental model of cerebral aneurysms in rats, monkeys, and mice that quite resembles human cerebral aneurysms in their anatomic location and histological structure, and it allowed us to examine the early stages of cerebral aneurysm development in greater detail.

We previously found extensive apoptotic cell death of smooth muscle cells (SMCs) in the aneurysmal walls using this model system. Additionally, using inducible NO synthase (iNOS) inhibitor and iNOS-deficient mice, we demonstrated that iNOS is important in cerebral aneurysm progression. Interleukin-1 (IL-1) is a proinflammatory cytokine highly produced by mononuclear phagocytes, and to a lesser extent by other cell types, in response to infection and injury. IL-1β is produced as an inactive precursor protein that is proteolytically cleaved by an endoprotease originally termed IL-1β–converting enzyme (ICE), which was subsequently found to be caspase-1. IL-1 functions in a broad array of normal and pathological inflammatory, hematopoietic, and immunologic situations. IL-1 is thought to be a key inflammatory mediator, and it is a potential target for therapy in inflammatory diseases. Additionally, IL-1β is a potent iNOS activator.

In the present study, we examined the distribution of IL-1 in cerebral aneurysm development using an experimental model in mice.

Materials and Methods

Induction of Experimental Cerebral Aneurysms

Seven-week-old male C57BL/6 mice were purchased from Shimizu-jikken-doubutsu (Kyoto Japan), and 5- to 6-week-old IL-1β gene–deficient (IL-1β−/−) mice and age-matched control B10 mice (wild-type) were purchased from Taconic (Hudson, NY). Cerebral aneurysms were induced using the procedure reported by Morimoto et al. The left common carotid artery and posterior branch of the left renal artery were ligated with 10-0 nylon under general anesthesia with 1% to 2% halothane. One week later, another side was operated. The mice were fed 8%...
sodium chloride and 0.12% β-aminopropionitrile (BAPN) containing food. Sodium chloride was used to enhance the degree of hypertension. BAPN is an inhibitor of lysyl oxidase, the enzyme that catalyzes the cross-linking of collagen and elastin, and was used to increase vessel fragility. Animal care and experiments complied with Japanese community standards on the care and use of laboratory animals.

RNA Isolation and Reverse Transcription

Two weeks and 3 months after the operation of the C57BL/6 mice and 5 months after the operation of the IL-1β/−/− and age-matched control B10 mice, total RNA was isolated using the RNeasy Fibrous Tissue Mini Kit from Qiagen. Extraction was performed according to manufacturer directions. A total of 10 ng of total RNA was converted to cDNA using Sensiscript reverse transcriptase (Qiagen). The conditions for the cDNA synthesis were: 60 minutes at 37°C followed by heating at 93°C for 5 minutes. A total of 2 μL of the samples was used in polymerase chain reaction (PCR) using HotStarTaq polymerase (Qiagen). PCR was started at 94°C for 15 minutes, followed by 45 cycles (94°C for 1 minute, 53°C for 1 minute, and 72°C for 1 minute), ended with a 10-minute incubation at 72°C for caspase-1. In case of GAPDH and β-actin, cycles were 40, and IL-1β were 45 cycles (94°C for 20 s, 55°C for 45 s, and 72°C for 40 s). Primers were as follows: caspase-1, forward 5'-GAGAGGAGAGTGCTGATTCAGG-3' and reverse 5'-CAAGACGTGTACGAGTGGTTGT-3', product size 400 bp; GAPDH, forward 5'-ACCACAGTCCATGCCATCAC-3' and reverse 5'-TCCACCACC-CTGTGCTGTA-3', product size 452 bp; IL-1β, forward 5'-GGTGAGGAGTGTGAGTGCTGATTAGA-3' and reverse 5'-CATGGAGAATATCACTTGTTGGTTGA-3', product size 499 bp. GAPDH and β-actin were used as a control, and the PCR products were separated by electrophoresis in 2% agarose gels.

Tissue Preparation

Five months after aneurysm induction, animals were deeply anesthetized and perfused transcardially with physiological saline followed by 4% paraformaldehyde at a speed of ~0.7 mL per hour using a peristaltic pump (RP-2000; EYELA). The anterior cerebral artery/olfactory artery (ACA/OA) bifurcation and another aneurysm susceptible portion were stripped and embedded in OCT compound (Tissue-Tek; Sakura Fine Technical Co.). Semithin 4-μm sections were cut and mounted on silane-coated slides. We could make ~10 slides per 1 lesion.

Definitions

Aneurysm, as defined here, refers to an outward bulging of the arterial wall detected by light microscopy. Early aneurysmal change refers to a lesion with discontinuity of the internal elastic lamina visualized by orcein stain without apparent outward bulging of the vascular wall (Figure 1g and 1h). Advanced cerebral aneurysm refers to an obvious

![Figure 1. IL-1β expression in experimentally induced aneurysm in mice (a through f).](http://stroke.ahajournals.org/)

- **a**: Hematoxylin and eosin staining of the arterial bifurcation of ACA and OA.
- **b** through **d**: Double staining for α-smooth muscle actin (red; **b** and **c**) and IL-1β (green; **b** and **d**) in ACA/OA bifurcation of this model. Merged cells in yellow signals (**b**) indicate that SMCs express IL-1β in induced mice aneurysms.
- **e**: RT-PCR analysis of IL-1β mRNA in control (n=4), 2 weeks after the operation (n=3), and 3 months after the operation (n=5). **f**: Denitometric analysis indicates that the expression of IL-1β mRNA is higher in the 2-week group than control and the 3-month group. These differences are statistically significant (P<0.05). Classification of this study in orcein staining (**g** through **i**). **g** and **h**: Early aneurysmal change sample. Fragmentation of internal elastic laminae is seen. **g**: Lower magnification; **h**: higher magnification.
- **i**: Advanced aneurysm sample. Vascular wall is protruded laterally at ACA just distal of juxta-apical groove (lower magnification). Lu indicates lumen side; Bars=50 μm.
outward bulging of the arterial wall with fragmentation and disappearance of the internal elastic lamina (Figure 1i). Three independent researchers assessed the histopathological changes and selected the section at the maximum diameter of each aneurysm in a blinded manner.

**Orcein Staining and Size Measurement**
Specimens were washed and incubated in orcein solution (0.1 g orcein, 100 mL 70% ethanol, 2 mL 35% HCl) for 24 hours. After dehydration, delipidation, and enclosure, all samples were classified into 3 groups: no change group (NC), early aneurysmal change group (EAC), and advanced cerebral aneurysm group (AA).

**Immunohistochemistry**
Slides were then washed 3 times with PBS containing 0.1% Tween 20 (PBS-T) and incubated with secondary antibody (anti-goat Alexa-Fluor 488 antibody and anti-mouse Alexa-Fluor 546 antibody; Molecular Probes) for 1 hour at room temperature (RT). Mouse staining was performed using rabbit polyclonal anti single-stranded DNA (ssDNA) antibody (1:500; DAKO) or rabbit polyclonal anti–caspase-1 antibody (1:200; Santa Cruz Biotechnology) and Cy3-conjugated mouse monoclonal anti–α-smooth muscle actin antibody (Sigma) as primary antibodies. The sections were incubated with primary antibody solutions overnight at 4°C. The slides were then washed 3 times with PBS-T and subsequently incubated with biotinylated anti-rabbit IgG (1:250; Vector Laboratories) for 1 hour at RT. After 3 more washes with PBS-T, Alexa Fluor Fluoromonganold streptavidin 488 (Molecular Probes) was applied for 30 minutes at RT. Slides were washed 3 more times with PBS-T and covered with PERMAFLUOR (Immunotech) and excited for fluorescence by illumination through a fluorescence microscope system (BX51N-34-FL-1; Olympus). The anti–IL-1β antibody does not cross-react with IL-1β isoforms.

**Terminal Deoxynucleotidyltransferase-Mediated dUTP Nick End Labeling**
Cell death was detected in situ by enzymic labeling of DNA strand breaks using an In Situ Cell Death Detection Kit, Fluorescein (Roche)

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**Figure 2.** Characterization of experimentally induced aneurysms in IL-1β−/− and wild-type mice. a, Incidence of experimental cerebral aneurysmal changes [(AA+EAC)/(AA+EAC+NC)] in IL-1β−/− and wild-type mice. No significant differences are seen in the incidence of aneurysm development (IL-1β−/−, 6 of 11; wild-type, 8 of 13). b, Incidence of advanced aneurysmal changes among total aneurysmal changes [(AA)/(AA+EAC)]. In IL-1β−/− mice, the incidence of advanced aneurysmal changes is significantly smaller than in wild-type mice (IL-1β−/−, 0 of 6; wild-type, 4 of 8; P=0.048. All statistical analysis was performed by Mann–Whitney U test.

**Figure 3.** Caspase-1 expression in experimentally induced aneurysms. a through c, In wild-type mice, little caspase-1 immunoreactivity was seen in vascular walls. a, Microscopic view; b, double staining for α-actin and caspase-1 (red, α-actin; green, caspase-1); c, hematoxylin and eosin staining. d through f, In IL-1β−/− mice, more caspase-1 immunoreactive cells were seen compared with wild-type mice. d, Microscopic view. e, double staining for α-actin and caspase-1 (red, α-actin; green, caspase-1); f, hematoxylin and eosin staining. Bars=50 μm. g, RT-PCR analysis of caspase-1 mRNA in wild-type and IL-1β−/− mice. h, Densitometric analysis showed that increased expression of caspase-1 mRNA in IL-1β−/− mice.
Results

IL-1β Expression in Aneurysmal Walls of Mouse Cerebral Aneurysm

We examined IL-1β expression in the aneurysmal vessel walls by immunohistochemical method. Double staining for α-actin (Figure 1b and 1c, red) and IL-1β indicated that IL-1β was expressed in medial SMCs (Figure 1b and 1d, green). Next we analyzed the expression of IL-1β at 2 weeks and 3 months after the surgery. In our model, the typical EAC and AA histology was observed at 2 weeks and 3 months, respectively. RT-PCR (Figure 1e and 1f) revealed that 2-week groups (n=3) had significantly higher expression of IL-1β mRNA than controls (n=4; P=0.003) and 3-month groups (n=5; P=0.004).

Characteristics of Experimental Cerebral Aneurysms in IL-1β−/− Mice

Five months after surgery, we analyzed cerebral aneurysmal changes in IL-1β−/− mice (n=11) and wild-type controls (n=13). Examining the wild-type mice, we identified 4 animals with advanced aneurysmal changes (AA; Figure 1g and 1h), and 5 mice with no changes (NC). In contrast, when the IL-1β−/− animals were examined, no AA lesions were found, 6 mice had EAC, and 5 were NC animals. As shown in Figure 2a, the rate of the aneurysmal change [(AA+EAC)/(AA+EAC+NC)] assessed by Mann–Whitney U test was significantly larger in wild-type controls than in IL-1β−/− mice (P=0.527). However, the rate of advanced aneurysm per total aneurysmal change [(AA)/(AA+EAC)] assessed by the same test was not different between the wild-type and IL-1β−/− mice (P=0.048). Mean blood pressure of the 2 groups did not show significant difference before the surgery (wild-type 97.03±4.27 to IL-1β−/− 92.16±6.65; P=0.20) and after the surgery (wild-type 129.22±8.76 to IL-1β−/− 130.11±10.16; P=0.89).

Expression of Caspase-1 and ssDNA in IL-1β−/− Mice

Given the differences in aneurysm progression in wild-type and IL-1β−/− animals, we wished to examine other pathways downstream of IL-1β. We analyzed the expression of caspase-1, which activates pro–IL-1β, ssDNA, and TUNEL.

Figure 4. ssDNA expression (a through l) and TUNEL staining (g through l) in experimentally induced aneurysms in wild-type and IL-1β−/− mice. a through c, In wild-type mice, more ssDNA immunoreactive cells were seen compared with IL-1β−/− mice. a, Microscopic view (green, ssDNA); b, double staining for α-actin and ssDNA (red, α-actin; green, ssDNA); c, hematoxylin and eosin staining. d through f, In IL-1β−/− mice, little ssDNA immunoreactivity was seen in the media. d, Microscopic view (green, ssDNA); e, double staining for α-actin and ssDNA (red, α-actin; green, ssDNA); f, hematoxylin and eosin staining. g through i, More TUNEL-positive cells were seen in wild-type specimen. g, Microscopic view (green, TUNEL); h, double staining for α-actin and TUNEL (red, α-actin; green, TUNEL); i, hematoxylin and eosin staining. j through l, In IL-1β−/− mice, few TUNEL-positive cells were seen in the media. j, Microscopic view (green, TUNEL); k, double staining for α-actin and TUNEL (red, α-actin; green, TUNEL); l, hematoxylin and eosin staining. Bars=50 μm.
which are as markers of apoptosis. We observed increased caspase-1 immunoreactivity in IL-1β/H9252/H11002/H11002 mice compared with wild-type mice and were most pronounced in the media (Figure 3). We next examined the apoptosis in the aneurysmal walls. The cells expressing ssDNA and TUNEL were quantified with Image-Pro plus for comparison of IL-1β/H9252/H11002/H11002 and wild-type animals. ssDNA immunopositive and TUNEL-positive cells were primarily found in the media, and a reduced number of positive cells was seen in IL-1β/H9252/H11002/H11002 mice compared with wild-type animals (Figure 4). The total number of ssDNA-positive cells was 27.924 (n=5) and for TUNEL-positive cells 30.2 ± 10.6 in the wild-type group, and 10.4.32 (n=4) and 3.6 ± 3.78 (n=5) in the IL-1β/H11002/H11002 group. These differences were significant (P=0.027 and P=0.0033, respectively). Furthermore, significantly more SMCs contained ssDNA and TUNEL in the wild-type group (14.7 ± 7.70 cells and 14.6 ± 3.65 cells, respectively) compared with the IL-1β knockout mice (2.75 ± 2.63; P=0.049 and 0.6 ± 0.55, P=0.0011, respectively; Figure 5). No differences were seen between the number of ssDNA-positive endothelial cells (ECs) between wild-type (4.7 ± 3.27) and knockout mice (2.75 ± 2.63; P=0.079).

Discussion
Using an experimental model of cerebral aneurysms that closely mimics the histopathology seen in human lesions, we have clearly shown that IL-1β is induced in the aneurysmal walls during the early stages of aneurysm development. Moreover, animals deficient in IL-1β exhibit delayed aneurysm progression compared with wild-type mice. An inability to produce IL-1β also led to increased caspase-1 expression but decreased ssDNA immunoreactivity. Together, these data indicate that IL-1β expression in SMCs promotes SMC apoptosis, and this may enhance aneurysm formation.

Because Hashimoto et al originally described the experimental induction of cerebral aneurysms in rats,2 we analyzed the mechanisms of cerebral aneurysm formation using rats and monkeys.2–6 We confirmed that SMCs in the arterial walls of aneurysms die via apoptosis.7,16 In addition, we reported that iNOS was markedly induced in human and rat cerebral aneurysms, and aminoguanidine, a relatively selective iNOS inhibitor, suppressed the incidence of experimental cerebral aneurysms in rats.8 Recently, Morimoto et al6 developed a cerebral aneurysm model in mice, and we showed that both cerebral aneurysm size and the number of apoptotic vascular SMCs in iNOS-deficient mice were significantly reduced compared with wild-type littermates.9 Activation of iNOS depends on cellular exposure to immunologic or inflammatory stimuli such as bacterial endotoxins, tumor necrosis factor, and IL-1.13,14 IL-1 is normally expressed at low levels, but it is rapidly induced in response to local or peripheral insult.10,11 During inflammation, IL-1, a potent proinflammatory cytokine, is released with other cytokines, chemokines, prostaglandins, reactive oxygen species (ROS), NO, and proteases.17 In addition to inducing genes in the iNOS system, IL-1 also leads to the expression of phospholipase A2 and cyclooxygenase-2 (Cox-2). Phospholipase A2 increases cellular levels of arachidonic acid, and Cox-2 catalyzes the first steps of prostaglandin synthesis.10–12 These lipid-derived inflam-
Inflammatory mediators can lead to cellular damage. In particular, Cox-2 generates ROS and contributes to tissue damage. IL-1 can also induce the expression of macrophage inflammatory proteins, focal adhesion kinase, Mac-1 (CD11b/CD18), and CD4. Expression of cytokines, chemokines, and inflammation-associated factors including IL-6, tumor necrosis factor-α, monocyte chemotactic protein-1, macrophage inflammatory proteins, Cox-2, and iNOS are reduced in IL-1 receptor-1 null mice. These findings indicate that IL-1 is a key upstream regulator of the inflammatory and immune responses. Defective IL-1 signaling also appears to protect cells from inflammation-associated cell death. Yamasaki et al reported that intracerebroventricular injection of neutralizing anti–IL-1 antibody to rats reduced ischemic brain damage. Mice lacking the IL-1 receptor-1 gene experience 50% less damage after cerebral hypoxia/ischemia.

There are 2 different IL-1 isoforms: IL-1α and IL-1β. Both are synthesized as precursor molecules that are then processed to their mature forms. However, pro–IL-1α is fully functional, whereas pro–IL-1β is biologically inactive. ICE, also known as caspase-1, is an intracellular cysteine protease that cleaves and activates pro–IL-1β. The 45-kDa caspase-1 precursor requires 2 internal cleavages before becoming enzymatically active; the active form is a heterodimer comprised of a 10- and a 20-kDa chain. Two caspase-1 heterodimers form a tetramer with 2 molecules of pro–IL-1β during the cleavage reaction. Macrophages from caspase-1-deficient mice do not release mature IL-1β on stimulation with endotoxin. Mice lacking the gene for caspase-1 or expressing a dominant-negative form of caspase-1 also exhibit reduced ischemic brain damage. 

Interestingly, in the present study, increased caspase-1 expression was seen in the cerebral aneurysms of IL-1β−/− mice. It is possible that the absence of IL-1β perturbs the normal feedback regulation of caspase-1. Such a feedback system has not been described. 

During apoptosis, nuclear fragmentation and the generation of ssDNA occur. From the ssDNA staining and TUNEL results, the apoptosis was mainly found in SMCs. We assessed the ssDNA expression and TUNEL in the study. We do not present the data about cytochrome-c release or caspase-9 expression. As for the pathway of cell death in aneurysmal walls, additional experiments are necessary. As for cleaved caspase-3, we detected more obvious expression of that in wild-type mice than in IL-1β−/− mice, but this tendency did not reach significance (P = 0.058; n = 5 per each group). As for wild-type mice, we used B10 mice. B10 mice are not complete wild-type. But IL-1β−/− mice was back-crossed for 3 generations to B10. Thus, it was the closest control that we could use. In our model, BAPNs were used to induce aneurysms. It is possible that BAPN plays a role in apoptosis of vascular walls. The present data indicate that IL-1β plays an important role in the development and progression of cerebral aneurysms. Disruption of the IL-1β gene resulted in the reduced incidence of mature cerebral aneurysms.

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