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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 have developed a method to induce experimental cerebral aneurysms in rats, monkeys, and mice. Interleukin-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 five week-old male C57BL/6 mice, IL-1β gene deficient mice (IL-1β-/-) 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 early stage of anerysmal 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 to wild-type animals. Additionally, the number of apoptotic cells assessed by ssDNA immunoreactivity and TUNEL was significantly reduced in IL-1β deficient mice compared to 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.

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Cerebral aneurysm rupture with subsequent subarachnoid hemorrhage remains a life-threatening medical emergency despite recent diagnostic and therapeutic advances. The mechanisms of aneurysm initiation, progression, and rupture remain unclear, however (1). Most studies examining the pathophysiology of cerebral aneurysm have relied upon 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 (2-6), quite resemble to human cerebral aneurysms in their anatomic location and histological structure (3,5), 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 in the aneurysmal walls using this model system (7). Additionally, using inducible nitric oxide synthase (iNOS) inhibitor and iNOS deficient mice, we demonstrated that iNOS is important in cerebral aneurysm progression (8,9). Interleukin-1 (IL-1) is a proimflammatory cytokine highly produced by mononuclear phagocytes, and to a lesser extent by other cell types, in response to injury and infection (10,11). IL-1β is produced as an inactive precursor protein that is proteolytically cleaved by an endoproteinase originally termed IL-1β-converting enzyme (ICE), which was subsequently found to be caspase-1 (12). 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. (10,11). Additionally, IL-1β is a potent iNOS activator (13,14).

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

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Materials and Methods

Induction of Experimental Cerebral Aneurysms

Seven week-old male C57BL/6 mice were purchased from Shimizu-jikken-doubutsu (Kyoto Japan), and 5-6 week-old IL-1β gene deficient mice (IL-1β-/-) (15) and age-matched control B10 mice (wild-type) were purchased from Taconic (Petersburgh, NY). Cerebral aneurysms were induced using the procedure reported by Morimoto et al (6). 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% 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

2 weeks and 3 months after the operation of the C57BL/6 mice and 5 months after the operation of the IL-1β gene deficient mice (IL-1β-/-) and age-matched control B10 mice, total RNA was isolated using the RNeasy Fibrous Tissue Mini Kit from Qiagen (Hilden, Germany). Extraction was performed according to the manufacturer's directions. 10ng of total RNA were converted to cDNA using Sensiscript reverse transcriptase (Qiagen). The conditions for the cDNA synthesis were: 60 min at 37°C followed by heating at 93°C for 5 min. 2µl of the samples were used in PCR reaction using HotStarTaq polymerase (Qiagen). PCR was started at 94°C for 15 min, followed by 45 cycles (94 °C for 1min , 53 °C for 1 min, and 72 °C for 1 min), ended with a 10-min incubation at 72 °C for Caspase-1. In case of GAPDH and β-actin, cycles were 40, and IL-1β were 45cycles (94 °C for 20sec, 55 °C for 45 sec, and 72 °C for 40sec). Primers were as follows: caspase-1, forward

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5'-GAGAGGAGAGTGCTGATTCAGG-3' and reverse 5'-CAAGACGTGTACGAGTGGTTGT-3', product size 400 bp; GAPDH, forward 5'-ACCACAGTCCATGCCATCAC-3' and reverse 5'-TCCACCACCCTGTTGCTGTA-3', product size 452 bp; IL-1β, forward 5'-GGTGTGTGACGTTCCATTAGA-3' and reverse 5'-CATGGAGAATATCACTTGTTGGTTGA-3', product size 143 bp; β-actin, forward 5'-GTATGCCTCGGTGGTACCA-3' and reverse 5'-CTTCTGCATCCTGTCAGCAA-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 approximately 0.7 mL/h using a peristaltic pump (RP-2000, EYELA, Tokyo). 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., Tokyo, Japan). 4 µm semithin sections were cut and mounted on silane-coated slides. We could make about 10 slides per one lesion.

Definitions

Aneurysm, as defined here, refers to an outward bulging of the arterial wall detected by light microscopy (9). *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 (Fig1 g and h). *Advanced cerebral aneurysm* refers to an obvious outward bulging of the arterial wall with fragmentation and disappearance of the internal elastic lamina (Fig1 i). Three independent researchers assessed the histopathological changes and selected the section at the maximum diameter of each aneurysm in a blinded manner.

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Orcein staining and size measurement

Specimens were washed and incubated in orcein solution (orcein 0.1g, 70% Ethanol 100 ml, 35% HCl 2 ml) for 24 h. 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, Eugene, OR) for 1 h at room temperature. Mouse staining was performed using rabbit polyclonal anti single-stranded DNA antibody (1:500; DAKO, Glostrup, Denmark) or rabbit polyclonal anti Caspase-1 antibody (1:200; Santa-Cruz, Santa Cruz, CA) and Cy3-conjugated mouse monoclonal anti-α-smooth muscle actin antibody (Sigma, St. Louis, MO) 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, Burlingame, CA) for 1 h at room temperature (RT). After three more washes with PBS-T, Alexa Fluor Fluoronanogold streptavidin 488 (Molecular Probes) was applied for 30 min at RT. Slides were washed 3 more times with PBS-T and covered with PERMAFLUOR (Immunotech, Marseille, France) and excited for fluorescence by illumination through a fluorescence microscope system (BX51N-34-FL-1, Olympus, Tokyo, Japan). The anti-IL-1β antibody does not cross-react with IL-1α isoforms.

TUNEL

Cell death was detected in situ by enzymic labelling of DNA strand breaks using an In Situ Cell

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Death Detection Kit, Fluorescein (Roche Diagnostics, Indianapolis, IN) according to the

manufacturer's instructions. And co-stained with Cy3-conjugated mouse monoclonal anti-α-smooth

muscle actin antibody (Sigma, St. Louis, MO) as previously discribed.

Cell Counting

The observer captured the image under microscope from total sections including aneurysm from one

animal (three to five). Image-Pro plus (Media Cybernetics; San Diego, CA) was used for photo

imaging and cell counting. Mean number of positive cells per section was recorded.

Statistical Analysis

The values were expressed as means ± SD. Statistical analysis was performed using χ^2 for

independence test for aneurysm formation rate, Mann-Whitney's U test for advanced aneurysm

change and Welch's t-test for others. Differences were considered statistically significant at $P < 0.05$.

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 (Fig. 1 red, b and c) and interleukin-1β indicated that IL-1β was

expressed in medial smooth muscle cells (green b and d). 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 (Fig 1 e and f) revealed that 2 weeks

groups (n=3) had significantly higher expression of IL-1β mRNA than controls (n=4, P=0.003) and

3 months groups (n=5, P=0.004).

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Characteristics of experimental cerebral aneurysms in IL-1β deficient mice

Five months after surgery, we analyzed cerebral aneurysmal changes in IL-1β deficient mice (n=11) and wild-type controls (n=13). Examining the wild-type mice, we identified four animals with advanced aneurysmal changes (AA Fig1 i), four animals with early aneurysmal changes (EAC Fig1 g and h), and five mice with no changes (NC). In contrast, when the IL-1β-/- animals were examined, no AA lesions were found, six mice had EAC, and five animals NC animals. As shown in Figure 2-a, the rate of the aneurysmal change is not different between the wild-type and IL-1β deficient mice (p=0.527). However, the rate of advanced aneurysm per total aneurysmal change (AA + EAC) was significantly larger in wild-type controls than in IL-1β deficient mice (p=0.048). Mean blood pressure of the two 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 single-stranded DNA in IL-1β deficient 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, single-stranded DNA (ssDNA) and TUNEL, which are as markers of apoptosis. We observed increased caspase-1 immunoreactivity in IL-1β deficient mice compared to wild-type mice and were most pronounced in the media (Fig. 3). We next examined the apoptosis in the aneurismal walls. The cells expressing ssDNA and TUNEL were quantified with Image-Pro plus for comparison of IL-1β-/- and wild-type animals. ssDNA immunopositive and TUNEL-positive cells were primarily found in the media, and reduced number of positive cells was seen in IL-1β-/- mice compared to wild-type animals (Fig. 4). The total number of ssDNA positive cell was 27±9.24 (n=5) and TUNEL-positive cells was 30.2±10.6 in the wild-type group and 10±4.32 (n=4), and 3.6±3.78

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(n=5) in the IL-1β-/- group. These differences were significant (p=0.027, p=0.0033, respectively). Furthermore, significantly more smooth muscle cells contained ssDNA and TUNEL in the wild-type group (14.7±7.70 cells and 14.6±3.65 cells, respectively) compared to the IL-1β knock-out mice (2.75±2.63, p=0.049 and 0.6±0.55, p= 0.0011, respectively). No differences were seen between the number of ssDNA positive endothelial cells (EC) between wild-type (4.7±3.27) and knock-out 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 to wild-type mice. An inability to produce IL-1β also led to increased caspase-1 expression but decreased ssDNA immunoreactivity. Taken together, these data indicate that IL-1β expression in smooth muscle cell promotes SMC apoptosis and this may enhance aneurysm formation.

Since Hashimoto et al. originally described the experimental induction of cerebral aneurysms in rats (2), we have 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 al. (6) developed a cerebral aneurysm model in mice, we showed that both cerebral aneurysm size and the number of apoptotic vascular smooth muscle cells in iNOS deficient mice were significantly reduced compared to wild-type littermates (9). Activation of iNOS depends on cellular exposure to immunologic or inflammatory stimuli such as bacterial endotoxins,

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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 (ROI), 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 inflammatory mediators can lead to cellular damage (18). In

particular, Cox-2 generates ROI and contributes to tissue damage (10-12). IL-1 can also induce the

expression of macrophage inflammatory proteins (MIPs), focal adhesion kinase (FAK), Mac-1 and

CD4. Expression of cytokines, chemokines and inflammation-associated factors including IL-6,

TNFa, MCP-1, MIPs, Cox-2 and iNOS are reduced in IL-1 receptor1 null mice (10-12). 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. (19) reported that intracerebroventricular injection of neutralizing anti- IL-1β

antibody to rats reduced ischemic brain damage. Mice lacking the IL-1 receptor1 gene experience

50% less damage after cerebral hypoxia/ischemia (20).

There are two different IL-1 isoforms, IL-1α and IL-1β (10,11). 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-kD caspase-1 precursor requires two internal

cleavages before becoming enzymatically active; the active form is a heterodimer comprised of a 10-

and a 20-kD chain (12). Two caspase-1 heterodimers form a tetramer with two molecules of pro-

IL-1β during the cleavage reaction. Macrophages from caspase-1-deficient mice do not release

mature IL-1β upon stimulation with endotoxin (12). Mice lacking the gene for caspase-1 or

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expressing a dominant negative form of caspase-1 also exhibit reduced ischemic brain damage (21,22). Interestingly, in the present study, increased caspase-1 expression was seen in the cerebral aneurysms of IL-1β deficient 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 (9). From the ssDNA staining and TUNEL results, the apoptosis was mainly found in SMC.

We assessed the ssDNA expression and TUNEL in the study. We don't present the data about cytochrome-c release or caspase-9 expression. As for the pathway of cell death in aneurismal walls, additional experiments are necessary. As for cleaved casapase-3, we detected more obvious expression of that in wild-type mice than in IL-1β deficient mice, but this tendency didn't 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 backcrossed for 3 generations to a B10. Thus it was the closest control that we could use. In our model, BAPN 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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Figure legend

Figure 1. IL-1β expression in experimentally induced aneurysm in mice (a-f). a. hematoxilin and eosin staining of the arterial bifurcation of ACA(Anterior cerebral artery) and OA(Olfactory artery). b-d. Double staining for α-actin (red, b and c), and interleukin-1β (green, b and d) in ACA-OA bifurcation of this model. Merged cells in yellow signals (b) indicate that smooth muscle cells express interleukin-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. Densitometric analysis indicates that the expression of IL-1β mRNA is higher in 2 weeks group than control and 3 months group. These differences are statistically significant (P < 0.05). Classification of this study in Orcein staining (g-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) ACA; Anterior cerebral artery, OA; Olfactory artery, Lu; Lumen side, * indicate P< 0.05 to the other groups, Bars; 50μm

Figure 2. Characterization of experimentally induced aneurysms in IL-1β deficient and wild-type

mice. a. Incidence of experimental cerebral aneurysmal changes in IL-1β -/- and wild-type mice. No

significant differences are seen in the incidence of aneurysm development. b. Incidence of advanced

aneurysmal changes among total aneurysmal changes. In IL-1β-/- mice, the incidence of advanced

aneurysmal changes is significantly smaller than in wild-type mice (P=0.048).* indicate P<0.05.

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Figure3. Caspase-1 expression in experimentally induced aneurysms. a-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-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; 50μm. 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.

Figure 4. Single-stranded DNA expression (a-f) and TUNEL staining (g-l) in experimentally induced aneurysms in wild-type and IL-1β deficient mice. a-c. In wild-type mice, more ssDNA immunoreactive cells were seen compared to 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-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-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-l. In IL-1β-/- mice, little TUNEL-positive cells was 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; 50μm.