Disruption of Gene for Inducible Nitric Oxide Synthase Reduces Progression of Cerebral Aneurysms

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Background and Purpose—The rupture of a cerebral aneurysm is a major cause of subarachnoid hemorrhage, but the mechanism of its development remains unclear. Inducible nitric oxide synthase (iNOS) is expressed in human and rat cerebral aneurysms, and aminoguanidine, a relatively selective inhibitor of iNOS, can decrease the number of the aneurysms in rats. In this study we applied our new mouse model of cerebral aneurysms to the iNOS gene knockout mice and observed experimental cerebral aneurysms in these animals to elucidate the role of iNOS in the process of cerebral aneurysm formation.

Methods—Eight C57/Bl6 mice and 16 iNOS knockout mice received a cerebral aneurysm induction procedure. Four months after the operation, the mice were killed, their cerebral arteries were dissected, and the region of the bifurcation of the anterior cerebral artery/olfactory artery was examined histologically and immunohistochemically.

Results—No significant difference was seen in the incidence of cerebral aneurysms between iNOS+/+ and iNOS−/− mice. However, the size of advanced cerebral aneurysms and the number of apoptotic smooth muscle cells were significantly greater in iNOS+/+ mice than in iNOS−/− mice.

Conclusions—Inducible NOS is not necessary for the initiation of cerebral aneurysm. However, the results of this study suggest that regulation of iNOS may have therapeutic potential in the prevention of the progression of cerebral aneurysms. (Stroke. 2003;34:2980-2984.)

Key Words: animal models | cerebral aneurysm | genetics | nitric oxide synthase

Subarachnoid hemorrhage is still a life-threatening disease despite the recent advancement of diagnostic and therapeutic strategies. The rupture of a cerebral aneurysm is a major cause of subarachnoid hemorrhage, and the size of the aneurysm correlates with the likelihood of its rupture.1 However, the mechanisms of its initiation, progression, and rupture remain unclear.

Many studies have been performed with the use of cerebral aneurysm specimens obtained during surgery or autopsy. However, these studies could not identify the key molecule for the development of cerebral aneurysm because the cerebral aneurysms used in the studies were always mature ones. We have developed a method to induce experimental cerebral aneurysms in rats,2 monkeys,3 and mice,4 without performing any direct manipulations of the cerebral artery itself. Our experimental cerebral aneurysms in rats and monkeys resemble human cerebral aneurysms in their anatomic location and histological structure.3 This model enables us to explore the process of cerebral aneurysmal development.

Nitric oxide (NO), which plays diverse roles in the cardiovascular system, is produced by nitric oxide synthase (NOS). Three isoforms of NOS are known: endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS). In general, eNOS and nNOS are constitutively expressed, and NO production is regulated predominantly by intracellular Ca2+ fluxes that permit the calmodulin binding that activates the enzymes.6 In contrast, iNOS is transcriptionally regulated and is not normally produced by most cells.6,7 Typically, iNOS is expressed in the case of cellular immunity or vascular injury and generates 100- to 1000-fold more NO than its constitutive counterparts that are involved in physiological regulation.8

We previously reported that iNOS was expressed in human and rat cerebral aneurysms and that aminoguanidine, a relatively selective inhibitor of iNOS, decreased the number of early aneurysmal changes in rats.9 This drug, however, exerts various other effects, particularly inhibitory effects on eNOS and nNOS. Therefore, we cannot exclude the possibility that factors other than iNOS may contribute to the formation of cerebral aneurysms.

In this study we applied our cerebral aneurysm induction procedure to iNOS gene knockout mice10 and observed the experimental cerebral aneurysms with the goal of clarifying the role of iNOS in the process of cerebral aneurysm formation.

Materials and Methods

Induction of Experimental Cerebral Aneurysms

Nine-week-old male C57/Bl6Tac mice (iNOS+/+) and iNOS gene knockout mice (iNOS−/−) were purchased from Taconic (Seattle,
Wash). We used the procedure for induction of experimental cerebral aneurysms reported by Morimoto et al.4 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, the posterior branch of the right renal artery was ligated to induce renal hypertension. The mice were fed food containing 8% sodium chloride and 0.12% β-aminopropionitrile (BAPN). Sodium chloride was used to enhance the degree of hypertension. BAPN is an inhibitor of lysyl oxidase that catalyzes cross-linking between 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. Four months after the operation, the blood pressure of each mouse was measured by the tail-cuff method.

**Polymerase Chain Reaction**

To confirm the iNOS genotype of the mice, we extracted genomic DNA from their tails by using a DNeasy mini kit (Qiagen). Tails were cut into pieces of 0.4 to 0.6 cm and incubated with proteinase K solution, and the genomic DNA was then eluted according to the manufacturer’s instructions. The polymerase chain reaction was performed as described4 with synthetic gene-specific primers for wild-type iNOS (upstream primer, 5’-ATCACCCCTCTGCTGTCCTC-3’; downstream primer, 5’-GGCTTTCTGCTTCCTCCTC-3’; product length, 413 bp) and knockout iNOS (upstream primer, 5’-GAGCAATGTGACAAAGCTTCCAGATCCTTTCTCTGTCTCC-3’; downstream primer, 5’-GGCTGAAGAAATCCTTCAGACTAG-3’) primer, 5'-GCCTTTCTGCTGTTCTCTC-3’; product length, 1288 bp) by using HotstarTaq polymerase (Qiagen) for 30 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and elongation at 72°C for 1 minute. One hundred nanograms of DNA were used per reaction. The PCR products were then separated on 1% agarose gel, and the products were visualized with ethidium bromide. The mean maximal arterial blood pressure of iNOS+/− group and the iNOS−/− group was 131.6±9.2 mm Hg, whereas that of iNOS+/+ mouse was 134.5±8.3 mm Hg. No significant difference was observed between these 2 groups (P=0.45; Figure 2A).

**Tissue Preparation**

Four months after the induction procedure, the mice were deeply anesthetized with ether and perfused transcardially with 0.1 mol/L sodium chloride and 0.12% sodium carbonate, followed by 50% paraformaldehyde at a pressure of approximately 80 mm H2O. Cerebral arteries were stripped from their brains under a surgical microscope. The anterior cerebral artery/olfactory artery (ACA/OA) bifurcation of each sample was embedded in OCT compound (Tissue-Tek), and 6-μm semithin sections were cut with a Leica CM 1850 and mounted on silane-coated slides. We could make 10 to 15 slides per 1 sample containing the aneurysm.

**Definitions**

Aneurysm, as defined here, refers to an outward bulging of the arterial wall detected by light microscopy.4 We defined the terms to describe the development of cerebral aneurysms as follows: Early aneurysmal change consists of discontinuity of the internal elastic lamina, which can be visualized by orcein stain, and no apparent outward bulging of the vascular wall. Advanced cerebral aneurysm refers to an obvious outward bulging of the arterial wall with complete disappearance of the internal elastic lamina. Initiation indicates the process that leads cerebral arteries to early aneurysmal changes, and progression refers to the process that leads early aneurysmal changes to advanced aneurysms.

Three independent researchers assessed the histopathological changes and selected the section at the maximal diameter of each aneurysm in a blinded manner. The size of cerebral aneurysms was measured from the proximal to the distal end of the aneurysmal orifice.

**Inducible NOS Immunohistochemistry**

The sections were washed 3 times with 0.1 mol/L PBS for 5 minutes each time. After a section had been blocked with 3% normal goat serum, rabbit anti-mouse iNOS antibody (1:500 dilution, Upstate Biotechnology) was applied overnight at 4°C. Then the slides were washed 3 times with PBS and incubated with secondary antibody (anti-rabbit Alexa-Fluor 488 antibody, Molecular Probes) for 1 hour at room temperature. After they were washed 3 final times with PBS, the sections were covered and excited for fluorescence by illumination through a confocal laser scanning microscope system (FV300, Olympus). The iNOS antibody does not cross-react with other NOS isoforms. As negative controls for all primary antibodies, we performed the immunohistochemical staining in the same manner without the primary antibodies and confirmed that no signal was identified. We used mouse lung, palatine tonsil, and aorta for positive controls of iNOS, single-stranded DNA, and α-smooth muscle actin, respectively.

**Double Staining**

Sections were first washed 3 times with PBS, and then diluted normal goat serum was applied to them for blocking. The double staining was performed by using rabbit polyclonal anti–single-stranded DNA antibody (DAKO) and Cy3-conjugated mouse monoclonal anti–α-smooth muscle actin antibody (Sigma) as primary antibodies. The sections were incubated with the solution of primary antibodies overnight at 4°C. The slides were then washed 3 times with PBS and subsequently incubated with secondary antibody (anti-rabbit Alexa-Fluor 488 antibody, Molecular Probes) solution for 1 hour at room temperature. After they were washed 3 times with PBS, the sections were examined as described above. On each slide containing an advanced cerebral aneurysm, 3 independent researchers counted apoptotic vascular smooth muscle cells in the ACA/OA bifurcation in a blinded manner, and the mean score from the 3 examiners was recorded.

**Statistical Analysis**

The values of blood pressure and aneurysm size were expressed as mean±SD. Statistical analysis was performed by using unpaired t and χ² tests. Differences were considered statistically significant at P<0.05.

**Results**

**Immunohistochemistry of iNOS**

Experimental cerebral aneurysms induced in wild-type mice and observed at 4 months after the operation revealed strong immunoreactivity indicating NOS, which was mainly observed in the media and adventitia (Figure 1). We also performed iNOS immunohistochemistry for all samples from iNOS−/− mice and confirmed that no iNOS immunoreactivity was observed (data not shown).

**Blood Pressure Measurement**

Maximal arterial blood pressure of the mice just before they were killed was measured by utilizing the tail-cuff method. The mean maximal arterial blood pressure of iNOS+/+ mouse was 131.6±9.2 mm Hg, whereas that of iNOS−/− mice was 134.5±8.3 mm Hg. No significant difference was observed between these 2 groups (P=0.45; Figure 2A).

**Incidence of Experimental Cerebral Aneurysms at 4 Months After Operation**

We examined the incidence and the progression of experimental cerebral aneurysms. In the iNOS+/+ group (n=8), the ACA/OA bifurcation showed no aneurysmal change in 2 mice, an early aneurysmal change in 3 mice, and an advanced aneurysm in 3 mice. In the iNOS−/− group (n=16), the bifurcation showed no aneurysmal change in 6 mice, an early aneurysmal change in 4 mice, and an advanced aneurysm in...
6 mice. No significant difference was found between the 2 groups concerning each category of experimental cerebral aneurysms ($P = 0.77$; Figure 2B).

Size of Experimental Cerebral Aneurysm
The size of each experimental cerebral aneurysm was measured. Representative data for the 2 groups are shown graphically in Figure 3. The mean aneurysm size in \textit{iNOS}+/+ mice (32.7$\pm$4.6 m) was significantly larger than that in \textit{iNOS}−/− mice (12.7$\pm$2.1 m; $P = 0.0002$).

Apoptotic Vascular Smooth Muscle Cell Count
The spatial relationship between vascular smooth muscle cell and apoptosis was examined by double staining. Colocalization of $\alpha$-smooth muscle actin and single-stranded DNA within the same spot was detected as yellow fluorescence as a result of direct superimposition of red and green colors. A typical image is shown in Figure 4. The number of apoptotic vascular smooth muscle cells was 72.7$\pm$9.2 in \textit{iNOS}+/+ mice and 28.7$\pm$9.2 in \textit{iNOS}−/− mice, and this difference was significant ($P = 0.0003$; Figure 5).

Discussion
\textit{iNOS} is expressed in macrophages and vascular smooth muscle cells in the case of cellular immunity, atherosclerosis, or vascular injury and is thought to play a central role in chronic inflammation and connective tissue degradation within the vessel wall.$^{12}$ Shear stress is one of the major stimulants of the vascular wall and induces \textit{iNOS} expression in medial smooth muscle cells in vitro.$^{13}$ Inflammation, connective tissue degradation, and shear stress are all related to cerebral aneurysm formation,$^{5,14,15}$ and we have postulated that \textit{iNOS} plays a potential role in the formation of cerebral aneurysms.

Earlier our group reported that \textit{iNOS} was markedly expressed in human and rat cerebral aneurysms and that aminoguanidine, a relatively selective inhibitor of \textit{iNOS}, suppressed the incidence of experimental cerebral aneurysms in rats.$^{9}$ Because there was a possibility that aminoguanidine has some inhibitory effect on e\textit{NOS} and n\textit{NOS} or has some effect on other molecules, we considered it important to use \textit{iNOS} gene knockout mice to elucidate the relationship between \textit{iNOS} and cerebral aneurysms. Our recently developed mouse model$^{4}$ of cerebral aneurysm enabled us to use such genetically modified mice to conduct this experiment.
Unlike in our previous study using aminoguanidine, we could not detect any significant difference in the initiation of experimental cerebral aneurysms between the normal and knockout groups. Neither the incidence of cerebral aneurysm nor that of early aneurysmal change was significantly different between them. These findings may be interpreted in 2 ways. One is that the differences observed by using aminoguanidine are attributable to the other effects of this agent and that iNOS is not a major inducing factor of cerebral aneurysms. Aminoguanidine has been reported to have the inhibitory effects on eNOS, nNOS, and advanced glycation end products.11 The other is that the iNOS condition of the mice in this study before the induction was simply different from that in the previous study. In this study iNOS was deleted during development, whereas in the previous study the rats received aminoguanidine after normal vascular development. Targeted gene disruption after the induction of the aneurysm is now technically difficult for us. Further establishment of experimental technique, for example, long-term administration of RNA interference after the induction, may clarify the result of this experiment.

Like the results of the present study, some results obtained from animal disease models in iNOS knockout mice11,12 are not in accordance with the results found using aminoguanidine.16,17 For example, Johanning et al16 reported that the expression of iNOS was induced and plasma nitrite/nitrate levels were increased in experimental abdominal aneurysm and that inhibition of iNOS by aminoguanidine limited NO production and iNOS expression, resulting in aneurysms of smaller size. On the other hand, Lee et al12 used iNOS knockout mice and reported that iNOS was not required for the development of elastase-induced experimental abdominal aortic aneurysms, although extensive protein nitration and increased expression of iNOS accompany the development. Together with this study, we deduced that iNOS is not a necessary factor in the induction of experimental cerebral and abdominal aneurysms.

The size of experimental cerebral aneurysms and the number of apoptotic vascular smooth muscle cells were significantly greater in the iNOS+/+ group than in the iNOS−/− group. Vascular smooth muscle cells are known to synthesize and secrete connective tissue components, including elastic fibers.18 Therefore, the disappearance of viable vascular smooth muscle cells through apoptosis may cause a decline in elastic fiber synthesis. Hara et al19 and Kondo et al20 proved by using the terminal deoxynucleotidyl transferase–mediated dUTP–biotin nick end-labeling (TUNEL) method that apoptotic cells exist in the human or rat cerebral aneurysmal wall and that the number of such apoptotic cells directly correlates with the probability of the rupture of a cerebral aneurysm.19 We have hypothesized that after the initiation of a cerebral aneurysm, the vascular smooth muscle cells die gradually, resulting in a decreased content of elastin that leads to the enlargement and rupture of the cerebral aneurysm. Disruption of the collagen/elastin synthesis is one of the possible mechanisms underlying cerebral aneurysm formation.21

We examined the mice at only 1 time point, ie, 4 months after the induction, and there still remains a possibility that the cerebral aneurysm in iNOS−/− mice might have become greater in size if the animals had been examined for a longer period. However, we propose from the present results that ablation of the iNOS gene may delay the progression of a cerebral aneurysm.

In the present study we used an antibody against single-stranded DNA, which is produced during the process of nuclear fragmentation in apoptosis. The simple immunohistochemical method using this antibody can detect apoptotic cells more specifically than the TUNEL method,22,23 which also detects fragmented double-stranded DNA that forms during necrosis. We tested the other apoptotic markers, cleaved caspase-3 and Bax, and also performed the TUNEL method. All apoptotic markers and the TUNEL method indicated results similar to that of single-stranded DNA (data not shown), but the staining methods of these markers and TUNEL required a more complicated process than that of single-stranded DNA. The vessels prepared for this study.
were very fragile, and most of them were corrupted during the staining of the other apoptotic markers except for single-stranded DNA. That is why we used the single-stranded DNA antibody for double staining instead of the other markers.

The mechanism of NO-induced vascular smooth muscle cell apoptosis is not fully understood. Several mechanisms have been suggested for NO-induced cytotoxicity, including disruption of mitochondrial function, activation of p53, up-regulation of Fas, activation of c-Jun N-terminal kinase, and induction of ceramide synthesis. Recently, Idel et al. showed that phytic acid, a branch-chain fatty acid, induced apoptosis in vascular smooth muscle cells and that this acid exerted its effect via tumor necrosis factor-α secretion and iNOS upregulation. Additionally, our group reported that the level of phosphorylated c-Jun N-terminal kinase was increased in the vascular wall of human cerebral aneurysms. One or more of these mechanisms may be involved in the development of the cerebral aneurysm.

Although genetic ablation of inducible NOS did not influence the incidence of cerebral aneurysms, both the cerebral aneurysm size and the number of apoptotic vascular smooth muscle cells in iNOS−/− mice were significantly smaller than those in iNOS+/+ mice. Although this study had the limitation that resulted from small sample size, we deduced that iNOS may participate in the progression, but not in the initiation, of experimental cerebral aneurysms. These observations suggest that the regulation of iNOS and NO-induced apoptosis of vascular smooth muscle cells may pave the way to prophylactic therapy for the cerebral aneurysm.

Acknowledgments

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References

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