Prevention of Rat Cerebral Aneurysm Formation by Inhibition of Nitric Oxide Synthase

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Background—Cerebral saccular aneurysm is a major cause of subarachnoid hemorrhage, one of the cerebrovascular diseases with the highest mortality. The mechanisms underlying the development of aneurysms, however, still remain unclear. We have made a series of reports on an animal model of experimentally induced cerebral aneurysms that resemble human cerebral aneurysms in their location and morphology, suggesting that the arterial wall degeneration associated with aneurysm formation develops near the apex of arterial bifurcation as a result of an increase in wall shear stress. Using the animal model and human specimens, we examined the role of nitric oxide (NO) in the degenerative changes and cerebral aneurysm formation.

Methods and Results—Inducible NO synthase (iNOS) was immunohistochemically located at the orifice of human and rat aneurysms. Nitrotyrosine distribution was also seen in the human aneurysm. Although no iNOS immunostaining was found in normal arteries, iNOS immunoreactivity was observed in parallel with the development of early aneurysmal changes in rats. In contrast, during the early development of aneurysm, endothelial NOS immunostaining in the endothelium was weakened compared with that in the control arteries. An NO inhibitor, aminoguanidine, attenuated both early aneurysmal changes and the incidence of induced aneurysms. A defibrinogenic agent, batroxobin, which may diminish shear stress by reduction of blood viscosity, prevented iNOS induction as well as early aneurysmal changes.

Conclusions—The evidence suggests that NO, particularly that derived from iNOS, is a key requirement for the development of cerebral aneurysm. The iNOS induction may be caused by an increase in shear stress near the apex. (Circulation. 2000;101:2532-2538.)

Key Words: aneurysm • nitric oxide • cerebrovascular disorders • hemodynamics

The pathogenesis of cerebral saccular aneurysms still remains unresolved. Our experimentally induced aneurysms in rats and monkeys resemble human cerebral aneurysms in their location and microscopic structure. They have been accepted as good models for studying the pathogenesis of human aneurysms. In our microscopic studies, the initial changes associated with aneurysm formation were observed mainly at arterial bifurcations, particularly at the distal side of the major branch adjacent to the apex (Figure 1A). The degenerative changes were localized almost exclusively at the intimal pad and its neighboring distal portion, the so-called juxta-apical groove (JAG).

The mechanism of the arterial wall degeneration at the JAG, however, is still unclear. Nitric oxide (NO) is one of the possible contributors to the arterial wall degeneration. We show here that NO synthesized by inducible NO synthase (iNOS) may serve to damage the arterial wall and lead to aneurysm formation. Distributions of iNOS and nitrotyrosine in human and rat cerebral aneurysms were studied immunohistochemically. The association between tissue damage and induction of iNOS and endothelial NOS (eNOS) during the early development of aneurysm was also examined immunohistochemically. The NOS inhibitor aminoguanidine was used to confirm whether NOS inhibition prevents both the early aneurysmal changes and the formation of aneurysms in rats.

We also examined the mechanism of iNOS induction in the development of aneurysms. Clinical and epidemiological evidence suggests that the heightened hemodynamic stress due to an increase in blood flow may be an important factor underlying aneurysm development. Aneurysms in our model develop at several sites along the circle of Willis, where blood flow is increased in compensation for unilateral common carotid artery ligation and experimental hyperten-
sion. The data suggest that the hemodynamic stress and hypertension are of primary importance. Our hemorheological studies in rat aneurysms showed that in early aneurysm development, the wall shear stress was increased and highest at the distal end of the JAG, the distal end of the aneurysmal orifice. Moreover, the initial dilatation and degeneration of the arterial wall invariably developed in the same area. Other investigators have also indicated that the wall shear stress is the highest at the aneurysmal end. The data suggest that the increase in shear stress is associated with the formation of aneurysm. An increase in shear stress is known to induce the production of various mediators, including NO. Thus, we hypothesized that iNOS may be induced by an increase in shear stress. A defibrinogen agent, batroxobin (BX), was used to decrease wall shear stress.

Methods

Cerebral Aneurysm–Inducing Surgery in Rats

For aneurysm induction, 147 male Sprague-Dawley rats 6 to 8 weeks old were subjected to ligations of the right common carotid artery and the posterior branches of both renal arteries after pentobarbital anesthesia (50 mg/kg IP). One week after the operation, 0.5% saline was substituted for drinking water.

Treatment of Rat Induced Aneurysms With BX and AG

To clarify the effect of BX and AG on early aneurysmal changes, from the day after the surgery, the animals received an injection of 100 mg·kg⁻¹·d⁻¹ AG IP (Sigma) (n=6), 200 mg·kg⁻¹·d⁻¹ AG (n=6), 100 mg·kg⁻¹·d⁻¹ AG plus 300 mg·kg⁻¹·d⁻¹ L-arginine (Sigma) (n=10), 60 U·kg⁻¹·d⁻¹ BX (Tobishi Pharmaceutical Co and Nippon Chemipharm Co) (n=16), or an equivalent volume of physiological saline (n=27) for 14 consecutive days. To examine the effect of AG on mature aneurysm formation, the animals received 100 mg·kg⁻¹·d⁻¹ AG (n=13) or physiological saline (n=8) for 90 consecutive days. The animals were then anesthetized with 1.5 mL/min halothane. The MABP was recorded. The CBF was monitored at the left parietofrontal cortex with a laser Doppler flowmeter.

Electron Microscopy for Early Aneurysmal Changes and Light Microscopy for Aneurysm Formation in Rats

The animals were deeply anesthetized and perfused with 0.5% glutaraldehyde in 0.1 mol/L cacodylate buffer for electron microscopy or 4% paraformaldehyde in 0.1 mol/L PBS for light microscopy. The brains were immersion-fixed with 3% paraformaldehyde, 0.5% glutaraldehyde, and 0.01% tannic acid for electron microscopy or 4% paraformaldehyde for light microscopy. Arteries of the circle of Willis were embedded in epoxy resin. For early aneurysmal changes, ultrathin sections of bifurcations of the left anterior cerebral artery and olfactory artery were examined with a Hitachi H-600 electron microscope. Light microscopy was used for mature aneurysm formation after serial sections 1 μm thick were cut and stained with toluidine blue. “Aneurysm,” as defined here, refers to an outward bulging of the arterial wall detected by light microscopy. The assessments were done by 3 examiners in a blinded manner.

Preparation of Rat Tissue for Immunohistochemistry

Tissues were fixed with Zamboni’s fixative by the same methods as described above. The tissues at the apex areas obtained 2 weeks after the surgery were cut off from the bifurcations as shown in Figure 1B and were put in 0.4% Triton-X (Sigma) in PBS. The tissues were immunostained without embedding, and the inner wall of the tissues was opened and placed on a glass slide (Figure 1B). The mature aneurysmal tissues obtained 3 months after the surgery were embedded in paraffin, and sections 4 μm thick were cut.

Preparation of Human Tissue for Immunohistochemistry

A human cerebral aneurysm was obtained from a 69-year-old woman during brain surgery. The large aneurysm was resected out from the right middle cerebral artery, followed by arterial bypass surgery. The specimens were immediately fixed in formalin and embedded in paraffin, and sections 4 μm thick were cut.

Immunohistochemistry in Human and Rat Specimens

After the inactivation of intrinsic peroxidase with 0.3% H₂O₂, in methanol and blocking with 15% normal goat serum, either a rabbit antibody against mouse iNOS (Upstate Biotechnology) (1:500), a monoclonal antibody against bovine eNOS (Calbiochem-Novabiochem Corp) (1:500), or a monoclonal anti-nitrotyrosine antibody (1:50) was applied overnight (NOS antibodies) or for 18 hours (nitrotyrosine antibody) at 4°C, followed by application of the immunoperoxidase technique using ABC kits (Vector Laboratories). The specificity of the immunostaining was confirmed by replacing the primary antibody with either nonimmune rabbit IgG, nonimmune mouse IgG, or an anti-nitrotyrosine antibody in the presence of 10 mmol/L nitrotyrosine. The anti-nitrotyrosine antibody recognizes nitrotyrosine in the human tissue. The anti-iNOS and anti-eNOS antibodies were also used.
antibodies recognize human and rat iNOS and rat eNOS, respectively, and do not cross-react with other NOS isoforms.\textsuperscript{18,19}

Effect of BX on Fibrinogen Concentration
The animals received 60 U \textsuperscript{z} kg \textsuperscript{2} d \textsuperscript{1} BX (n = 5) or physiological saline (n = 5). Their blood was collected just before and 1, 3, 6, 10, and 24 hours after the injection, and the fibrinogen concentration was measured by the modified weight method.\textsuperscript{15}

Results

iNOS and Nitrotyrosine Immunoreactivities in Cerebral Aneurysms

iNOS immunoreactivity could be demonstrated in smooth muscle cells (SMCs) at the distal end of the neck of the rat cerebral aneurysm (Figure 2A), whereas no such immunoreactivity was found at the control bifurcations (Figure 2B). No nitrotyrosine immunoreactivity was seen in rat specimens.

In the human aneurysm, iNOS and nitrotyrosine distributions were observed in SMCs of the aneurysmal orifice (Figure 3A and 3B).

Effects of BX and AG on Early Aneurysmal Changes in Rats

Damage to endothelial cells (ECs) and SMCs at the JAG was classified into 5 grades according to electron microscopic pathological changes as follows: grade 1 (Figure 4A), no EC or SMC damage; grade 2 (Figure 4B), mild EC damage, such as a wavy rippling of plasma membrane, in the majority of ECs without SMC damage; grade 3 (Figure 4C), moderate EC damage, such as some vacuoles in the cytoplasm without SMC damage; grade 4 (Figure 4D), severe EC damage, such as cell deformation and/or many vacuoles in the cytoplasm and nucleus without SMC damage; and grade 5 (Figure 4E), severe EC damage in association with SMC damage.

Although no damage was seen in the nonsurgical group, severe damage to ECs and SMCs was observed in the saline group (Table 1). BX ameliorated EC and SMC damage (Table 1). AG at 100 and 200 mg \textsuperscript{z} kg \textsuperscript{2} d \textsuperscript{1} diminished EC and SMC damage. AG plus l-arginine, however, did not decrease the damage. The grade of damage in the AG plus l-arginine group was higher than that in the 100-mg \textsuperscript{z} kg \textsuperscript{2} d \textsuperscript{1} AG group. There was no significant difference in cerebral blood flow (CBF) or mean arterial blood pressure (MABP) in any group compared with the saline group. MABP in the AG
plus L-arginine group was significantly lower than that in the 100-mg/kg AG group. SMC damage without severe EC damage was not seen in any group.

### iNOS and eNOS Immunoreactivities in Early Aneurysmal Changes in Rats

In the nonsurgical group, eNOS immunoreactivity was seen in all arterial endothelium examined (Figure 5A). In particular, eNOS staining was stronger at the area around the JAG than any other area (Figure 5A). In the saline group, eNOS immunoreactivity at the area around the JAG, in which mild tissue damage could be seen, was weakened compared with that in the nonsurgical group, leading to homogeneous eNOS distribution in the arterial wall (Figure 5B). eNOS staining was no longer observed at the JAG where severe damage was observed.

In contrast with eNOS, neither iNOS immunoreactivity nor tissue damage was seen in the nonsurgical group. In 6 of 8 animals of the saline group, iNOS immunostaining was seen in the arterial wall of the JAG and the intimal pad near the apex, with severe tissue damage (Figure 5D). The tissue damage was severe not at the apex, but rather in the areas surrounding the apex, especially at the JAG, in parallel with the iNOS immunoreactivity. There was no apparent iNOS immunoreactivity with mild tissue damage in 2 animals of the saline group.

To clarify whether reduction of shear stress with BX results in prevention of iNOS induction, we also examined the correlation between iNOS immunoreactivity and tissue damage in the BX group. iNOS immunoreactivity was not seen in any animal of the BX group with any tissue damage in light microscopy (4 animals) (Figure 5C) or with very mild tissue damage (4 animals). There was no significant difference in MABP or CBF among the groups.

### Effects of AG on Incidence of Induced Aneurysm Formation in Rats

In the saline group, aneurysm formation was common (Table 2). In the AG group, the frequency of aneurysm formation was significantly below that in the saline group. MABP in the apex, with severe tissue damage (Figure 5D). The tissue damage was severe not at the apex, but rather in the areas surrounding the apex, especially at the JAG, in parallel with the iNOS immunoreactivity. There was no apparent iNOS immunoreactivity with mild tissue damage in 2 animals of the saline group.

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**TABLE 1. Effect of BX and AG on Early Aneurysmal Changes in Rats**

<table>
<thead>
<tr>
<th>No. of Animals</th>
<th>Grade of Damage</th>
<th>Amelioration of Damage</th>
<th>MABP, mm Hg</th>
<th>CBF (ratio)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1   2   3   4   5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>9   0   1   3   1   4</td>
<td></td>
<td>110±15</td>
<td>100±10</td>
</tr>
<tr>
<td>BX</td>
<td>8   1   2   4   1   0</td>
<td>*</td>
<td>122±23</td>
<td>109±14</td>
</tr>
<tr>
<td>AG 100 mg/kg</td>
<td>6   1   3   2   0   0</td>
<td>*</td>
<td>121±18</td>
<td>91±17</td>
</tr>
<tr>
<td>AG 200 mg/kg</td>
<td>6   3   1   2   0   0</td>
<td>*</td>
<td>124±13</td>
<td>87±15</td>
</tr>
<tr>
<td>AG+L-arginine</td>
<td>10  2   0   1   5   2</td>
<td>†</td>
<td>96±12</td>
<td>93±9</td>
</tr>
</tbody>
</table>

The average value of CBF in the saline group was set to 100 U. *P<0.05 vs saline group; †P<0.05 vs AG 100-mg/kg group (Mann-Whitney U test for the grade of damage, Scheffé-type multiple comparison test for CBF and MABP).
AG group was significantly higher than that in the saline group. CBF remained the same in the 2 groups.

**Effect of BX on Fibrinogen Concentration**
The fibrinogen concentration in the BX group was significantly lower than that in the saline group 1, 3, 6, 10, and 24 hours after the BX treatment (Figure 6).

**Discussion**
We have demonstrated that aneurysmal changes are initiated by endothelial degenerative changes and that aneurysmal alterations progress from the luminal toward the abluminal side of the arterial wall.3,20 In our rat model, ≈3 months was usually needed for mature aneurysms to develop. Two weeks after the aneurysm-inducing surgery, severe damage first to ECs and in time to SMCs at the JAG was observed, whereas no damage was seen in the nonsurgical group (Table 1). The EC and SMC damage thus reflects the early changes of aneurysm development, which are compatible with the findings of the early phases of development in previous studies.20

**TABLE 2. Effect of AG on Incidence of Induced Aneurysm Formation in Rats**

<table>
<thead>
<tr>
<th></th>
<th>No. of Animals</th>
<th>No. of Aneurysms</th>
<th>MABP, mm Hg</th>
<th>CBF (ratio)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>8</td>
<td>9</td>
<td>141±16</td>
<td>100±28</td>
</tr>
<tr>
<td>AG 100 mg/kg</td>
<td>13</td>
<td>2*</td>
<td>184±35*</td>
<td>109±16</td>
</tr>
</tbody>
</table>

The average value of CBF in the saline group was set to 100 U.

*P<0.01 vs saline group (Fisher’s protected least significant difference).

*Figure 5. Immunohistochemical distributions of eNOS (A and B) and iNOS (C and D) in rat arterial walls. In nonsurgical group, eNOS immunoreactivity is seen in all endothelium; eNOS staining in particular is stronger at area around apex (A). eNOS staining is also strong at area around apex of a small branch (arrow). In saline group, eNOS immunoreactivity at area around apex is weakened, with mild tissue damage (B). No iNOS immunoreactivity is seen with any apparent tissue damage in BX group (C), whereas iNOS immunoreactivity is seen with severe tissue damage at JAG and intimal pad near apex in saline group (D). Intimal pad near apex is severely damaged and has partly disappeared. Arrows indicate JAG; AP, apex; and IP, intimal pad. Bars=25 μm.

*Figure 6. Effect of BX on fibrinogen concentration. *P<0.05 vs control (Fisher’s protected least significant difference).
iNOS was immunohistochemically demonstrated in the aneurysmal orifice, whereas there was no iNOS immunoreactivity in control arteries (Figures 2 and 3). iNOS distribution was also observed at the JAG during the development of early aneurysmal changes in parallel with the tissue damage (Figure 5). iNOS, which does not ordinarily exist in the vascular wall, is induced by various stimuli in both ECs and SMCs and synthesizes a large amount of NO, which appears to be involved in various types of EC and SMC injury and degenerative changes.8,9,17 Thus, iNOS induction in the arterial wall may contribute to tissue damage and the development of aneurysm. This hypothesis is further supported by experiments that showed that the NOS inhibitor AG suppressed both the early aneurysmal changes and the incidence of aneurysm formation in rats (Tables 1 and 2). Neither MABP nor CBF in the AG groups was lower than those in the saline groups. Although AG has other pharmacological actions,18 the suppression of aneurysm development by AG may not be attributable to these other actions, because this ameliorating effect could be antagonized by L-arginine (Table 1). The effect may be mediated by L-arginine–NO pathways. AG is a relatively selective inhibitor for iNOS.6 MABP in the AG group 3 months after the surgery, however, was significantly higher than that in the saline group (Table 2), suggesting that AG also inhibited eNOS at least partially. To evaluate whether eNOS is also involved in aneurysm formation, we observed eNOS immunoreactivity during the early development of aneurysm. In contrast with iNOS, eNOS immunostaining at the JAG was weakened as the aneurysmal changes developed (Figure 5). Therefore, eNOS downregulation and iNOS induction were observed in parallel with the development of early aneurysmal changes, suggesting that eNOS has little effect on aneurysm development. Moreover, the amount of NO derived from eNOS is much less than that of NO synthesized by iNOS, too little to damage ECs and SMCs.23 Thus, NO derived from iNOS, but not from eNOS, may be a key requirement for tissue damage and degenerative changes in the arterial wall and the formation of cerebral aneurysms from the early phases of development. Although the ideal experiment to more clearly define the role of iNOS in the formation of aneurysm may be one that shows that iNOS is not induced with an increase in hemodynamic stress in mice lacking iNOS, an experimental induction of aneurysms in mice has never been successful, probably because of their small size. This may need further investigation in the future.

A question may be raised here as to the mechanism of iNOS induction in the development of aneurysm. One likely explanation is that iNOS is induced in response to an excessive increase in wall shear stress. We have shown that the wall shear stress at the distal end of the JAG is highest during the early development of aneurysms10,11 and that the initial dilatation of the arterial wall develops at this area.3 BX had a significant amelioration of EC and SMC damage (Table 1). Although whole blood is a non-Newtonian liquid, at high shear stresses it behaves as a Newtonian liquid,24 in which the wall shear stress is the product of shear rate and Newtonian viscosity. The shear rate depends on the flow rate and the arterial diameter. A major factor determining whole blood viscosity is the hematocrit, with a secondary determinant being fibrinogen concentration.24 BX is used clinically as a defibrinogenic agent for humans.15,16 BX at a dose of 60 U · kg⁻¹ · d⁻¹ in rats decreases fibrinogen concentration and lowers the whole-blood viscosity without any influences on inflammatory events.16,25 A dose of 60 U/kg BX significantly reduced the fibrinogen concentration in rats for 24 hours (Figure 6). BX did not reduce CBF or MABP (Table 1). In the cerebral circulation, when both CBF and MABP are the same, cerebral arterial tonus may be kept the same by means of cerebral autoregulation. The arterial diameter, therefore, is also likely to be the same. Thus, BX probably reduced the overall blood viscosity without a corresponding change in the shear rate. The tissue damage at the JAG was probably ameliorated by a decrease in the wall shear stress resulting from the reduction of whole-blood viscosity by BX, although it still remains possible that some other effect of BX caused the reduction of tissue damage, because we did not directly measure the wall shear stress in the BX-treated animals. The data suggest that the increase in wall shear stress may be involved in the mechanisms of early aneurysm development. Moreover, iNOS immunoreactivity was attenuated in parallel with the reduction of tissue damage by BX treatment, suggesting that a decrease in the level of shear stress by BX treatment may prevent iNOS induction during the period of aneurysm development. The increase in wall shear stress may play an important role in iNOS induction.

The mechanism of iNOS induction in response to high shear stress is not clear. The promoter of human iNOS contains a shear-stress–responsive element,26 and nuclear factor-κB, which is required for iNOS gene expression, is activated by shear stress.27 An increase in shear stress may directly promote iNOS induction, or high shear stress may induce functional tissue damage and then the functional damage may lead to iNOS induction. In addition to ECs directly exposed to blood flow, SMCs are also exposed and can respond to shear stress due to interstitial flow derived from transmural pressure gradients even in intact arteries.28 Endothelial denudation caused by endothelial injury may lead to an exposure of SMCs to higher levels of fluid shear stress. iNOS is not induced in SMCs in response to shear stress on the order of 1.1 to 25 dyn/cm² in a short time, up to 24 hours.29 Maximum shear stresses on the wall of human cerebral aneurysms are estimated to be ≈50 dyn/cm².13 iNOS may be induced by exposure to higher magnitudes of shear stress in a longer time.

NO-related cell injury was evaluated with an anti-nitrotyrosine antibody as a marker of peroxynitrite (Figure 3). Peroxynitrite, which is a reaction product of NO and superoxide, is a potentially cytotoxic agent.4,17 Both iNOS and nitrotyrosine immunoreactivities were found in the medial layer of the human aneurysm (Figure 3), suggesting that the NO synthesized by iNOS may damage the arterial wall at least partly by producing peroxynitrite. We tested the anti-nitrotyrosine antibody at dilutions from 1:5 to 1:100 and could get the best contrast immunohistochemical images using the antibody at a dilution of 1:50. Some investigators reported good results using a higher dilution of the antibody.17 The difference may be a result of differences in procedure and/or specimens.
The results all suggest the importance of enhanced wall shear stress, iNOS induction, and the subsequent NO synthesis as the mechanism for aneurysm development. Although the present experiments were performed mainly on animals with experimental aneurysms, the results may also shed light on the mechanisms of human cerebral aneurysms, because the iNOS immunoreactivity was also demonstrated in the human cerebral aneurysm.

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