Role of estrogen deficiency in the formation and progression of cerebral aneurysms. Part I: experimental study of the effect of oophorectomy in rats

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Object. Estrogen has been shown to play a central role in vascular biology. Although it may exert beneficial vascular effects, its role in the pathogenesis of cerebral aneurysms remains to be determined. To elucidate the role of hormones further, the authors examined the effects of bilateral oophorectomy on the formation and progression of cerebral aneurysms in rats.

Methods. Forty-five female, 7-week-old Sprague–Dawley rats were divided into three equal groups. Group I consisted of intact rats (controls). To induce cerebral aneurysms, the animals in Groups II and III were subjected to ligature of the right common carotid and bilateral posterior renal arteries. One month later, the rats in Group II underwent bilateral oophorectomy. Three months after the experiment began all animals were killed and cerebral vascular corrosion casts were prepared and screened for cerebral aneurysms by using a scanning electron microscope. Plasma was used to determine the level of estradiol and the gelatinase activity.

Hypertension developed in all rats except those in the control group. The estradiol level was significantly lower in Group II than in the other groups (p < 0.01). The incidence of cerebral aneurysm formation in Group II (60%) was three times higher than that in Group III (20%), and the mean size of aneurysms in Group II (76 ± 27 μm, mean ± standard deviation) was larger than that in Group III (28 ± 4.6 μm) (p < 0.05). No aneurysm developed in control animals (Group I), and there was no significant difference in plasma gelatinase activity among the three groups.

Conclusions. The cerebral aneurysm model was highly reproducible in rats. Bilateral oophorectomy increased the susceptibility of rats to aneurysm formation, indicating that hormones play a role in the pathogenesis of cerebral aneurysms.

KEY WORDS • cerebral aneurysm • estrogen • vascular cast • electron microscopy • oophorectomy • rat

Intracranial aneurysms reportedly occur in 2 to 5% of adults and present with SAH in the majority of cases. A Despite current advances in microsurgical and endovascular techniques, ruptured cerebral aneurysms continue to be associated with high morbidity and mortality rates. Approximately 50% of patients die by the 30th postinjury day, and 40% are dead before they arrive at the hospital. A Half of the survivors manifest physical or psychosocial deficits 1 year after SAH. A As prevention of aneurysm formation and progression remains the most practical way to avoid the devastating effects of SAH, risk factors that contribute to the pathogenesis of cerebral aneurysms must be identified.

Hemodynamic shear stress appears to be the major aneurysmogenic factor. A The vascular endothelium was found to be the principal mediator in neutralizing hemodynamic stress and protecting the arterial vascular wall, mainly via the release of NO, which induces arterial dilation. A degenerative changes in the endothelium are considered to be the earliest event in the formation of cerebral aneurysms; these changes are followed by alterations in the underlying elastic lamina and, later, in the medial layer. A

Estrogen plays a central role in vascular biology; it enhances endothelial cell function and stimulates the release of NO. Estradiol also affects smooth muscle cell function and collagen degradation, and attenuates the formation of abdominal aortic aneurysms in mice. A Estradiol in the pathogenesis of cerebral aneurysms nonetheless remains to be determined. To elucidate the role of hormones, we examined the effects of oophorectomy on the formation and progression of cerebral aneurysms in rats.

Materials and Methods

All experiments and protocols were conducted in accordance with the Japanese standards for the care and use of laboratory animals and were approved by the Animal Care Committee at The University of Tokushima.

Seven-week-old female Sprague–Dawley rats were used. Before any procedure was performed, anesthesia was induced in the animals by means of isofluorane (2–4%) inhalation. The rats were divided into three groups of 15 animals each. Group I consisted of intact females (control). The 30 rats in Groups II and III underwent ligation.
of the right CCA and the bilateral posterior renal arteries. One week later, the animals were given a 1% NaCl solution as their drinking water. One month after the ligation procedure, the Group II rats underwent bilateral oophorectomy. Blood pressure measurements were obtained once a month in unanesthetized rats by using the tail-cuff auto-pickup method (Unicom, Chiba, Japan). At 3 months postligation, the animals were anesthetized, killed, blood was withdrawn to determine gelatinase and estradiol levels, and a vascular corrosion cast of the cerebral arteries was prepared.

Vascular Corrosion Casts

To obtain these casts, we adapted previously reported methods.18,19 The tip of a 19-gauge, 1.25-in-long plastic cannula was inserted into the left ventricle and secured in the ascending aorta. Heparinized (20 U/ml) phosphate-buffered saline (100 ml) was administered by a perfusion pump at a rate of 10 ml/minute; the right atrium was cut for drainage. Subsequently, we manually injected 10 ml of Batson No. 17 plastic (Polysciences, Inc., Warrington, PA). After polymerization (24 hours at room temperature), the entire brain was removed and digested for 24 to 72 hours in 20% KOH with intermittent water rinses. The resulting vascular cast was mounted on a scanning electron microscope stub with colloidal silver paste that was sputter-coated with gold, and the cast was screened for the presence of cerebral aneurysms by using the scanning electron microscope (S-100; Hitachi, Tokyo, Japan) at a beam voltage of 15 kV. Special care was taken in the examination of the arterial bifurcation of major arteries. Aneurysms were defined in this study as an outward bulging of the vascular cast by an arterial bifurcation of major arteries. Aneurysms were defined in this study as an outward bulging of the vascular cast at an arterial bifurcation, which was detected using the scanning electron microscope. To obtain the aneurysm size we measured the distance between the aneurysm neck and dome by using the Image Processing Toolkit version 3.0 in Adobe Photoshop (Adobe Systems, Inc., San Jose, CA). Assessments of the vascular casts were done by two examiners in a blinded manner.

Measurement of Estradiol Levels

Estradiol was measured using the Tosoh II E-test and an automated enzyme immunoassay system (Ala-600; Tosoh, Tokyo, Japan). The assay sensitivity (the detection limit) was 91.8 pmol.

Determination of MMP Gelatinase Activity

Plasma gelatinase activity was measured to assess the magnitude of enzymatic degradation of collagens and other structural proteins, which compromises the mechanical integrity of cerebral vessels and leads to conditions that favor aneurysm formation. The activities of Gelatinase A (MMP-2, a 72-kD Type IV collagenase) and Gelatinase B (MMP-9, a 92-kD Type IV collagenase), members of the family of MMPs able to degrade components of the extracellular matrix, were determined using an MMP gelatinase activity assay kit (Chemicon Int., Temecula, CA) according to the manufacturer’s instructions. Briefly, after incubating the plasma samples with biotinylated gelatinase substrate, the biotinylated gelatinase substrate cleaved by active MMP-2 and MMP-9 was added to a biotin-binding microplate and detected with the aid of streptavidin–enzyme complex. An addition of enzyme substrate resulted in a colored product that was detected by its optical density at 450 nm. Values are expressed as the percentage of the positive control.

Statistical Analysis

Sequentially obtained data, expressed as means ± SDs, were analyzed using the Mann–Whitney U-test for two-group comparisons, analysis of variance, and the Scheffé test for three-group comparisons. Statistical analyses were performed on a Macintosh computer running statistical software (StatView version 5; SAS Institute, Inc., Cary, NC). Differences were considered statistically significant at a probability level less than 0.05.

Results

Hypertension developed in all rats except those in the control group. The mean systolic blood pressure in the control animals (Group I) was 127 ± 14.3 mm Hg; in the animals in Groups II and III it was 185 ± 15.8 and 170 ± 23.1 mm Hg, respectively (p < 0.01, Group I compared with Groups II and III; Fig. 1A).

The estradiol level in the rats in Group II, which underwent oophorectomy, was significantly lower than that in animals in Groups I and III. The mean levels of estradiol in Groups I, II, and III were 257.4 ± 69.1, 115.2 ± 16.8, and 258.7 ± 43.0 pmol, respectively (p < 0.01, Groups I and III compared with Group II; Fig. 1B).

With the assistance of the scanning electronmicroscope,
we were able to obtain three-dimensional images of the aneurysms. Figure 2 displays the circle of Willis in a healthy rat as viewed at low magnification. Imprints of lining endothelial cells are clearly seen in Figs. 3 and 4. On the aneurysms, the endothelial markings were irregularly shaped and randomly distributed; portions of large aneurysms were devoid of endothelial imprints (Fig. 3B).

As shown in Table 1, nine (60%) of the 15 rats subjected to ligation and oophorectomy (Group II) were later found to have aneurysms; three of the animals had two aneurysms. In seven rats, the aneurysm was located at ACA–OA bifurcation. In the 15 rats that did not undergo oophorectomy (Group III), aneurysm changes manifested in three. No aneurysms developed in the control animals. Aneurysms identified in rats that had undergone oophorectomy tended to be larger than those in rats with intact ovaries (Figs. 1C and 4). The mean sizes of aneurysms in Groups II and III were 76 ± 27 and 28 ± 4.6 μm, respectively (p < 0.05). Although larger aneurysms developed in rats that underwent oophorectomy (Group III), aneurysm changes manifested in three. No aneurysms developed in the control animals. Aneurysms identified in rats that had undergone oophorectomy tended to be larger than those in rats with intact ovaries (Figs. 1C and 4). The mean sizes of aneurysms in Groups II and III were 76 ± 27 and 28 ± 4.6 μm, respectively (p < 0.05). Although larger aneurysms developed in rats that underwent oophorectomy, there was no evidence of aneurysm rupture in any rat in Group II or Group III.

All aneurysms arose on the side contralateral to the site of CCA occlusion. Fenestration of the ACA was apparent in approximately half of the rats in each group; there was no correlation between ACA fenestration and the incidence of cerebral aneurysms.

There was no significant difference in plasma MMP gelatinase activity among the different animal groups (p > 0.2) (Fig. 1D).

Discussion

In the rat aneurysm induction model described by Hashimoto and colleagues\textsuperscript{11,12} renal hypertension and unilateral ligation of the CCA are used to exacerbate hemodynamic forces on one side of the Willis circle. This activity produces cerebral aneurysms without direct manipulation of vessels in approximately 3 months. The rate of aneurysm formation in male and female rats when lathyrogen is used is approximately 30%. The addition of oophorectomy to this model greatly increased the rate of cerebral aneurysm formation: without lathyrogen treatment, cerebral aneurysms developed in approximately 60% of rats subjected to our combined procedure, indicating that hormones play a role in the pathogenesis of cerebral aneurysms. Specifically, estrogen exerts a protective role against the development and progression of cerebral aneurysms.

Different mechanisms may contribute to the protective effects of estrogen against aneurysms. Estradiol was found to stimulate NO production by endothelial cells through the phosphorylation and activation of an endothelial isoform of NO synthase (immediate effect)\textsuperscript{5,30} and also by upregulation of endothelial NO synthase expression (chronic or delayed effect).\textsuperscript{14} In addition, it accelerates reendothelialization in the mouse carotid artery and enhances vascular recovery after injury.\textsuperscript{2} Estrogen was also found to exert an antioxidant activity via the stimulation of antioxidant enzymes.\textsuperscript{5} These protective mechanisms of estradiol are receptor mediated and not limited to the endogenous form of estrogen.\textsuperscript{6,7,26,33}

Estrogen deficiency has been found to reduce endothelial functional capacity, to increase vascular free-radical production, and enhance angiotensin II–induced vasoconstriction.\textsuperscript{2,37} We posit that in rats without ovaries, these effects provide a good environment for the formation of cerebral aneurysms. When the effects of estrogen deficiency were
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Combined with the high hemodynamic stress produced by unilateral CCA ligation and renal hypertension, aneurysms developed at a higher rate and grew to a greater size than they did in rats exposed to increased hemodynamic stress alone.

Hypertension is a known risk factor for the development of cerebral aneurysms in humans. Oophorectomy has been associated with vasoconstriction and higher blood pressure, both of which are mediated by an increase in the Type 1 angiotensin receptor and can be reversed by addition of estradiol. We produced renal hypertension in rats by ligating the bilateral posterior renal arteries and found that oophorectomy did not lead to a further increase in blood pressure (Fig. 1A). Based on these observations, we posit that the higher incidence of cerebral aneurysms in rats subjected to oophorectomy was not attributable to the effects of this procedure on blood pressure.

Activation of MMP contributes to cerebral aneurysm formation and progression in humans; MMP-9 and MMP-2

**TABLE 1**

**Frequency and location of cerebral aneurysm in 45 rats***

<table>
<thead>
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<tr>
<td>no. of rats (%)</td>
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<tr>
<td>total</td>
<td>15</td>
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<tr>
<td>w/ cerebral aneurysms</td>
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<td>w/ multiple cerebral aneurysms</td>
<td>0 (0)</td>
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<td>w/ ACA fenestration</td>
<td>8 (53)</td>
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<td>no. of cerebral aneurysms (%)</td>
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*ICA = internal carotid artery; — = not applicable.
† Group I = control female rats; Group II = female rats that underwent CCA ligation and oophorectomy; Group III = female rats that underwent CCA ligation only.

**FIG. 3.** Scanning electron micrographs depicting a cerebral vascular cast in a rat that underwent oophorectomy. A: Note the bilateral ACA fenestration with a large fusiform ACoA aneurysm (arrow) and saccular ACA–OA aneurysms (arrowhead). B and C: Magnified views of the ACoA and ACA–OA aneurysms shown in A. Note the irregular endothelial cell imprints of the aneurysms and the absence of endothelial cell markings over parts of the fusiform ACoA aneurysm (B). Bar = 100 μm.
were markedly increased in the aneurysm wall compared with intact intracranial arteries. Estradiol was found to decrease pro-matrix MMP-2 and MMP-9, and to increase tissue inhibitors of metalloproteinases in cultured fibroblasts. The absence of estradiol following oophorectomy may have contributed to the higher incidence and larger size of cerebral aneurysms in the animals that underwent that procedure. When we assessed plasma gelatinase activity in the three groups of rats, we found no significant differences (Fig. 1D). These results suggest that the level of plasma gelatinase activity is not indicative of the incidence of cerebral aneurysms in rats. Further studies are needed to examine the correlation between cerebral aneurysms and plasma gelatinase activity.

Vascular casting greatly facilitates the study of arterial vascular systems because it avoids shrinkage artifacts and maintains arterial geometry. This technique yields material with which we can study the ultrastructural and three-dimensional morphological characteristics of large areas of the vascular tree. Using this technique we examined the major arterial bifurcations for the presence and size of cerebral aneurysms.

Epidemiological studies revealed that female sex is a significant independent risk factor for de novo aneurysm formation. In the entire aneurysm population, women in the 50 to 59-year age group predominated. In humans younger than 39 years of age, 44% of cerebral aneurysms were found in females; this rate increased to 55% in people 40 to 49 years of age and to 77% in those 50 to 59 years old. This suggests that sex-specific hormonal factors may play a role in the pathogenesis of cerebral aneurysms. A 36% decline in the odds ratio for the development of SAH was reportedly associated with hormone replacement therapy, although researchers in other studies found no clear association. The higher incidence of aneurysms in our rats that underwent oophorectomy supports the hypothesis that estrogen exerts a protective role against the development and progression of cerebral aneurysms.

Conclusions

We have demonstrated that oophorectomy increases the susceptibility for cerebral aneurysm formation in rats. This observation suggests that hormones play a role in the pathogenesis of cerebral aneurysms. Ours is a simple, highly reproducible model for aneurysm formation in rats and is of value in studies regarding the pathogenesis and progression of cerebral aneurysms. Based on the results reported here, we are pursuing additional studies on the effects of hormone replacement therapy on the pathogenesis of cerebral aneurysms.

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References

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