Endothelial injury and inflammatory response induced by hemodynamic changes preceding intracranial aneurysm formation: experimental study in rats

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Object. Intracranial aneurysms are the leading cause of subarachnoid hemorrhage, which is associated with high morbidity and mortality rates. Despite advances in the microsurgical and endovascular treatment of intracranial aneurysms, little is known about the mechanisms by which they originate, grow, and rupture. To clarify the series of early events leading to formation of intracranial aneurysms, the authors compared aneurysmal morphological changes on vascular corrosion casts with parallel pathological changes in the cerebral arteries of rats.

Methods. The authors induced cerebral aneurysms by renal hypertension and right common carotid artery ligation in 40 male Sprague–Dawley rats; 10 intact rats served as the controls. The anterior cerebral artery–olfactory artery bifurcation was assessed morphologically by using vascular corrosion casts of Batson plastic reagent and immunohistochemically by using antibodies against endothelial nitric oxide synthase, α–smooth muscle actin, macrophages, and matrix metalloproteinase–9.

Results. Surgically treated rats manifested different degrees of aneurysmal changes. Based on these staged changes, the authors propose that the formation of intracranial aneurysms starts with endothelial injury at the apical intimal pad (Stage I); this leads to the formation of an inflammatory zone (Stage II), followed by a partial tear or defect in the inflammatory zone. Expansion of this defect forms the nidus of the intracranial aneurysm (Stage III).

Conclusions. This is the first study to demonstrate the in vivo mechanisms of intracranial aneurysm formation. The inflammatory response that follows endothelial injury is the basic step in the pathogenesis of these lesions. In this study the investigators have expanded the understanding of the origin of intracranial aneurysms and have contributed to the further development of measures to prevent and treat aneurysms. (DOI: 10.3171/JNS-07/08/0405)

KEY WORDS • endothelial cell • inflammatory zone • intracranial aneurysm • macrophage • matrix metalloproteinase • rat • smooth-muscle cell migration • vascular corrosion cast

INTRACRANIAL aneurysms are a major public health problem; it is estimated that approximately 5% of the population harbors an unruptured intracranial aneurysm.14 The consequences of rupture are catastrophic: approximately 50% of patients die during the first postrupture month, and 60% of deaths occur within 2 days of the onset of SAH.14 Half of the survivors manifest physical or psychosocial deficits 1 year after SAH.9 Clip placement and coil occlusion to treat ruptured intracranial aneurysms, aimed at avoiding recurrent bleeding, have no direct effect on the recovery from the initial hemorrhage. Therefore, despite advances in microsurgical and endovascular instrumentation and techniques, a patient’s clinical status at the time of SAH onset remains the major predictive factor of the clinical outcome. The mechanisms underlying the formation, growth, and rupture of intracranial aneurysms remain poorly understood. Given the poor prognosis of ruptured intracranial aneurysms, clarification of the pathological changes that lead to their formation is of paramount importance.

There is considerable controversy regarding the arterial layer critical for the formation of intracranial aneurysms, the factors that damage this layer, and the formation of the an-
eurysm wall. We previously used the aneurysm induction model of Nagata et al.,23 in which renal hypertension and unilateral ligation of the CCA were used to induce lesions, and first reported the early in vivo morphological changes that led to the formation of intracranial aneurysms. Using vascular corrosion casts we demonstrated that changes in the morphological characteristics of endothelial cell imprints were the earliest changes in the process of aneurysm formation; these changes were predominant at the ACA–OlfA bifurcation.15 Here we compared the aneurysms' morphological changes observed with the aid of vascular corrosion casts and the pathological changes found on immunostained serial sections of the arterial bifurcation to gain a better understanding of the series of events leading to the lesions' formation. As did our vascular corrosion casts, serial sections of the ACA–OlfA bifurcation demonstrated that endothelial cell injury, evidenced by the loss of eNOS expression at the apical intimal pad, was the earliest pathological change in the process of aneurysm formation. We also found that the inflammatory reaction that follows endothelial injury represents the basic step in the pathogenesis of intracranial aneurysms.

Materials and Methods

We induced intracranial aneurysms in 40 male 7-week-old Sprague–Dawley rats, which had been anesthetized by 2 to 4% isofluorane inhalation, by ligating the right CCA and bilateral posterior renal arteries. One week later, 1% NaCl solution was substituted for the rats’ drinking water. The controls were 10 7-week-old Sprague–Dawley rats.

The blood pressure of unanesthetized rats was periodically measured using the tail-cuff, auto-pickup method (Unicom); blood flow in the left CCA was determined using a laser Doppler flowmeter (Advance). Two months after the aneurysm induction procedure, the rats were deeply anesthetized and perfused with 100 ml heparinized (20 U/ml) PBS. Vascular corrosion casts of the cerebral arteries were prepared from 20 of the animals after they were killed. In the other 20, the bilateral ACA–OlfA bifurcation was carefully dissected and immersed in 4% paraformaldehyde. The arteries were rinsed with PBS, embedded in OCT compound (Tissue-Tek, Inc.) and 7-μm-thick serial sections from the ACA–OlfA bifurcation were cut with a cryotome (CM 1850; Advance). Two months after the aneurysm induction procedure, the rats were deeply anesthetized and perfused with 100 ml heparinized (20 U/ml) PBS by using a perfusion pump. This was followed by the major injection of 10 ml of plastic solution (Batson No. 17 Plastic Replica and Corrosion Kit; Polysciences). After 24-hour polymerization at room temperature, the whole brain was removed and digested in 20% KOH for 24 to 72 hours with intermittent water rinses. The remaining vascular cast was mounted on a scanning electron microscope stub (S-100 SEM; Hitachi Corp.) with colloidal silver paste, sputter-coated with gold, and screened for the presence of aneurysmal changes. The beam voltage was 15 kV and special care was taken in examining the arterial bifurcations of major arteries, particularly the ACA–OlfA bifurcation.

Statistical Analysis

Sequentially obtained data, expressed as the mean ± standard deviation, were analyzed using the Mann–Whitney U-test. Statistical analyses were performed on a Macintosh computer running statistical software (StatView 5). Differences were considered statistically significant at probability values of less than 0.05.

Results

Systolic Blood Pressure and CCA Blood Flow

As shown in Fig. 1, all rats with induced intracranial aneurysms manifested systolic hypertension; their mean systolic blood pressure (182.0 ± 15.9 mm Hg) was significantly higher than in the controls (117.2 ± 14.3 mm Hg; p < 0.05). Left CCA blood flow in the experimental rats was significantly higher (32.5 ± 4.3 ml/minute) than in the controls (18.1 ± 5.6 ml/minute; p < 0.05).

Sequential Morphological and Pathological Changes at the ACA–OlfA Bifurcation

The animals with artery ligation manifested different degrees of morphological and pathological aneurysmal changes. Based on the serial changes we observed, we propose the sequence of pathological and morphological aneurysm changes shown in Table 1.

![Fig. 1. Bar graphs showing the mean systolic blood pressure (left) and CCA blood flow (right) in the control and experimental (operated) rats. Data represent means ± standard deviations (*p < 0.05).](image-url)
Inflammatory zone and cerebral aneurysms

<table>
<thead>
<tr>
<th>Stage</th>
<th>Morphology†</th>
<th>Immunohistochemistry‡</th>
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<tr>
<td>I</td>
<td>endothelial changes</td>
<td>partial loss of endothelial cells at AIP, normal SMC</td>
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<tr>
<td>II</td>
<td>endothelial changes + intimal pad elevation</td>
<td>partial loss of endothelial cells at AIP, macrophage infiltration, SMC migration, intimal swelling, inflammatory zone formation</td>
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<tr>
<td>IIA</td>
<td>early inflammatory stage: no vessel wall protrusion</td>
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<tr>
<td>IIB</td>
<td>late inflammatory stage: destruction &amp; protrusion of vessel wall</td>
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<tr>
<td>III</td>
<td>saccular aneurysm</td>
<td>partial loss of endothelial cells at AIP, macrophage infiltration, SMC migration, intimal swelling, defect of inflammatory zone, destruction &amp; protrusion of vessel wall, aneurysm formation</td>
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† Evaluated on vascular corrosion casts.
‡ Performed using eNOS, α-SMA, macrophage, and MMP-9 antibodies.

Normal Arterial Bifurcation. The vascular corrosion cast of a normal rat arterial bifurcation exhibited no bulges or depressions, and the endothelial cell imprints were regular in shape and arranged in the direction of the blood flow (Fig. 2A). Immunostaining showed the expression, in a continuous layer and with no defect or discontinuity, of eNOS and α-SMA along the inner and outer surfaces of the vessel wall, respectively (Fig. 2F). There was no expression of macrophage-specific antigen in the vessel wall of normal arterial bifurcations.

Endothelial Changes (Stage I). These were observed just distal to the apical intimal pad. Compared with normal bifurcations, vascular corrosion casts revealed that the lining surface was rough; irregular cell imprints were evident (Fig. 2B). There was no arterial wall dilatation or depression. These changes reflected the partial loss of eNOS expression at the apical intimal pad. There was no change in the expression of α-SMA (Fig. 2G); macrophage-specific antigen was not expressed.

Formation and Progression of the Inflammatory Zone (Stage II). On serial sections of ACA–OlfA bifurcations, the formation of an inflammatory zone was evidenced by swelling of the luminal side of the vessel wall (Fig. 3A and B). This swollen area was positive for macrophage-specific antigen (CD68) and showed subendothelial expression of α-SMA; it also manifested a loss of eNOS (Figs. 2H, 3C, and 3D). There was no protrusion on the adventitial side of the vessel wall at this early inflammatory stage (Stage IIA). The progression of inflammation resulted in arterial wall destruction and the development of a defect in the inflammatory zone presenting as a narrow slit; this was associated with protrusion of the vessel wall (Stage IIB). The inflammatory zone defect did not extend beyond the area of SMC migration. The defect was continuous with the lumen of the parent artery, lacked eNOS expression, contained a thin layer of α-SMA–positive cells, and was positive for macrophage expression (Figs. 2I and 4).

Vascular corrosion casts demonstrated slight elevation of the vessel wall at the apical intimal pad; the elevated area was surrounded by a depression in the cast (Fig. 2C). The elevated area mirrored the defect in the inflammatory zone; the surrounding depression mirrored the swollen vessel wall. The degree of elevation varied from moderate (Stage IIA; Fig. 2C) to a pronounced pyramid shape (Stage IIB; Fig. 2D) and depended on the size of the inflammatory zone defect.

Saccular Dilation (Stage III). Expansion of the inflammatory zone defect resulted in the formation of a saccular dilation at the arterial bifurcation and within the migrated SMC layer. The walls of this cavity continued to lack eNOS expression, contained a thin layer of α-SMA–positive cells, and were positive for MMP-9 expression (Figs. 2J and 5). Vascular corrosion casts demonstrated swelling of the saccular shape; it was covered by abnormal cellular imprints (Fig. 2E).

Of the 40 rats, 20 manifested endothelial changes, 12 had inflammatory changes, and four had changes consistent with saccular aneurysm formation. All aneurysms were located on the left side; that is, contralateral to the side of CCA occlusion. None of the rats in the aneurysm induction group manifested evidence of aneurysm rupture, and none of the control rats had aneurysmal changes.

Discussion

We used vascular corrosion casts and immunohistochemical staining of serial sections of the ACA–OlfA bifurcation to examine which arterial layer is critically involved in the formation of saccular aneurysms, and how the aneurysm wall is formed. Our findings clearly demonstrated the aneurysmal morphological changes that occur in vascular corrosion casts and the parallel pathological changes on ACA–OlfA bifurcation. As summarized in Figs. 1 and 6, our findings indicate that endothelial injury triggers the infiltration by macrophages and the proliferation and migration of SMCs, and that this results in the formation of an inflammatory zone at the apical intimal pad. Progression of the inflammatory reaction leads to proteolytic destruction of the vessel wall and creates a defect in the inflammatory zone. This defect, restricted to the area of migrated SMCs, represents the nidus of the intracranial aneurysm.

Increased shear stress is considered a major factor in the origin of intracranial saccular aneurysms. We used the aneurysm induction model of Nagata et al.,23 in which renal hypertension and unilateral ligation of the CCA are used to induce hemodynamic changes within the Willis circle. All rats in the experimental group had high blood pressure and elevated left CCA blood flow levels (Fig. 1). Although this model requires a relatively long time (3 months) to produce aneurysmal changes and is reported to have low reproducibility, lesions formed with this model simulate the pathological features and distribution of human aneurysms.8,11,12,23

Vascular casting is a powerful tool for studying arterial vascular systems; it avoids shrinkage artifacts and maintains a 3D geometry.18,20 In our study, the shape of the vascular corrosion cast represents the shape of the arterial lumen and simulates images obtained by 3D cerebral angiography. Using a scanning electron microscope, we were able to observe ultrastructural changes in the vascular casts, including the imprints made by endothelial cells. We prepared ACA–OlfA sections in the sagittal plane and perpendicular to the bifurcation. The functional integrity of the endothelial layers of the vessel wall was assessed by the expression of
eNOS, and the integrity of the smooth-muscle layer by the expression of α-SMA. We also used the expression of macrophage-specific antigen (CD68) and MMP-9 to demonstrate the basic role of the inflammatory reaction that follows endothelial cell injury in the pathogenesis of cerebral aneurysms.

The tendency to develop cerebral aneurysms differs from one rat to another; whereas in some rats aneurysmal changes developed early after the aneurysm induction procedure, in others additional time was required to show such changes. Because of these variations, we proposed that these aneurysmal changes, although observed at a single time, represent sequential changes.

**Endothelial Injury**

The endothelium can be seen as a biosensor; it reacts to a large variety of stimuli and serves as an important autocrine and paracrine organ that regulates vascular wall functions. Under basal conditions, eNOS produces low concentrations of nitric oxide, a powerful vasodilator and relaxant of vascular smooth muscles. Nitric oxide is necessary for endothelial cell function and integrity, and it is considered to be the major defense molecule against oxidative stress. Chronic exposure of endothelial cells to supraphysiological high shear-stress levels results in their dysfunction, with possible progression to endothelial cell degeneration.

Based on our findings, we suggest that the endothelial layer is the critical structure in the process of intracranial aneurysm formation. This hypothesis is supported by the early development of irregularly shaped endothelial cell imprints at the apical intimal pad of the arterial bifurcation that precedes arterial wall dilation (Stage I; Fig. 2B). At this early stage, immunostaining of ACA–OlfA sections showed partial loss of eNOS expression at the apical intimal pad (arrowhead). H: Swelling of the vessel wall at the apical intimal pad; part of this swollen area lacks eNOS expression and shows subendothelial expression of α-SMA–positive cells (arrow). D and I: Stage III B. D: Pyramid-shaped elevation of the apical intimal pad; note that the surface of this elevation is covered by abnormal imprints. E: Thinning and degradation of the smooth-muscle layer creates a defect in the inflammatory zone (arrow) and produces vessel wall protrusion. E and J: Stage III. E: Saccular aneurysm covered with abnormal imprints. J: Expansion of the inflammatory zone defect, and destruction and protrusion of the vessel wall representing the nidus of the cerebral aneurysm. OA = OlfA. Bars = 50 μm.

**Role of Macrophage Infiltration and Proliferation and Migration of SMCs**

Exposure of the subendothelial matrix initiates platelet activation and adhesion. Activated platelets release proinflammatory mediators and secrete growth factors that contribute to the recruitment of inflammatory cells and promote the proliferation and migration of vascular SMCs. These processes result in the formation of an inflammatory zone at the apical intima. We clearly demonstrate this localized in-
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Fig. 3. Early inflammatory changes characterized by SMC migration and macrophage infiltration (Stage IIA). A and B: Sagittal cut of the left ACA–OlfA bifurcation viewed at low (A) and high magnification (B) showing swelling of the apical intimal pad. C: Double immunostaining of an ACA–OlfA section with eNOS antibodies (green) and α-SMA (red) shows swelling of the vessel wall at the apical intimal pad; part of this swollen area lacks eNOS expression and shows migration of α-SMA–positive cells into the neointima (arrow). D: Triple immunostaining of an ACA–OlfA section with antibodies against eNOS (green), α-SMA (red), and macrophages (blue). Macrophage expression confirms the presence of an inflammatory zone. Bars = 50 μm.

Fig. 4. Late inflammatory changes characterized by progression of inflammation, resulting in a defect within the inflammatory zone. A: Double immunostaining of an ACA–OlfA section. The eNOS antibodies (green) and α-SMA (red) demonstrate the thinning and destruction of the smooth-muscle layer. This creates a defect (arrow) in the inflammatory zone that appears to lack eNOS expression and has a thin layer of α-SMA–positive cells. B: Magnified view of panel A demonstrating the inflammatory zone defect (arrow). This defect lacks eNOS expression. C: Positive expression of macrophages (blue) around the inflammatory defect. D: Merged images. Lu = lumen. Bars = 10 μm.

The early inflammatory zone is mirrored on the vascular cast by a slight elevation at the apical intimal pad (Stage IIA; Fig. 3A and B). Immunostaining of the localized swelling demonstrated the expression of macrophages, loss of eNOS expression, and the subendothelial expression of α-SMA (Fig. 3C and D). The adventitial side of the vessel wall appeared to be normal and without protrusion at this early inflammatory stage (Stage IIA). We propose that at this stage the intrinsic strength of the vessel wall is preserved and inflammation is limited to the luminal side of the vessel wall.

The early inflammatory zone is mirrored on the vascular cast by a slight elevation at the apical intimal pad; this elevation is surrounded by a depressed area that mirrors the swollen intimal pad. Both the elevated and depressed parts of the apical intimal pad were covered by abnormally shaped cellular imprints (Fig. 2C, Stage IIA). These abnormal imprints may derive from abnormal endothelial cells or hyperplastic subendothelial SMCs that develop secondary to endothelial injury and congregate in the inflamed area of the vessel wall.

Although the proliferation and subintimal migration of vascular SMCs are recognized as the main factors involved in arterial narrowing in proliferative disorders such as atherosclerosis, their involvement in the pathogenesis of intracranial aneurysm formation has not been described elsewhere in the literature. The significance of these SMC changes that are triggered by macrophage infiltration in the formation of intracranial aneurysms is unknown. We propose that SMC proliferation and migration may produce SMCs of fragile phenotypes, and may also mechanically interfere with the healing of the endothelial layer.

We postulate that the swollen inflammatory zone at the apical intimal pad simulates the intimal thickening that occurs in atherosclerosis, in which SMCs play a predominant role. Polymorphonuclear leukocyte invasion of the media through the endothelium triggers the proliferation of SMCs, which then migrate from the media through the lamina interna into the intima. This supports our hypothesis that in the development of intracranial aneurysm and atherosclerosis, similar early pathological changes take place; their presence at opposite sides of the arterial bifurcation may be the main determinant of the difference in their presentation.

Inflammation leads to the release of MMPs and other proteolytic enzymes that degrade and remodel the vascular extracellular matrix. The combined effects of hemodynamic changes and the direct or indirect destructive effects of macrophages through their release of proteolytic enzymes may lead to development of a defect within the inflammatory zone. The spread of the inflammatory reaction into different levels of the smooth-muscle layer and the development of a defect in the inflammatory zone appear to compromise the intrinsic strength of the vessel wall and result in its outward protrusion (Stage IIB; Fig. 2I).

This defect is clearly identified by direct examination of
serial sections of the ACA–OlfA bifurcation. The walls of this defect lack eNOS expression, contain a thin layer of \( \alpha \)-SMA–positive cells, and are positive for macrophage expression (Fig. 4). We found that although the degree of extension of this defect in the vessel wall varied among rats, it did not penetrate beyond the area of the migrated SMCs. This observation supports our proposal that SMC proliferation and migration produce fragile phenotypes of SMCs compared with naïve cells. The extension of the defect along the whole thickness of the arterial wall may be responsible for the clinical entity termed SAH of unknown origin. Scanning electron microscopy of the ACA–OlfA bifurcation showed a small pyramidal protrusion at the apical intimal pad (Fig. 2D) covered by irregularly shaped imprints. This tear becomes the nidus for the subsequent intracranial aneurysm (Figs. 2E, 2J, and 5).

Proteolytic damage to the vessel wall caused by inflammation plays a crucial role in the development of atherosclerosis\(^{10,13}\) and abdominal aortic aneurysms;\(^{6,17}\) however, its role in the pathogenesis of intracranial aneurysms is not well understood. Consistent with our findings, Chyatte et al.\(^2\) demonstrated the expression of immunoglobulins, macrophages, and T lymphocytes in the wall of unruptured intracranial aneurysms in humans. Kataoka et al.\(^{16}\) showed that ruptured intracranial aneurysms manifest significant inflammatory cell invasion compared with unruptured ones. Although their findings demonstrated the presence of inflammatory reaction in the wall of mature (particularly ruptured) aneurysms, its origin and role in the formation of the aneurysm wall remained to be elucidated. Our findings clearly demonstrate the mechanisms underlying intracranial aneurysm formation and confirm that inflammation subsequent to endothelial injury plays a basic role in the pathogenesis of cerebral aneurysms.

The early aneurysmal changes, that is, endothelial injury, inflammation of the apical intimal pad, and the tear in the inflammatory zone (Fig. 6), cannot be identified by methods previously used to investigate the pathological features of intracranial aneurysm in clinical and experimental studies. The detection of aneurysmal changes by angiography and dissecting microscopy relies on the appearance of arterial wall dilation. By examining serial sections of the ACA–

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**Fig. 5.** Expansion of the inflammatory zone resulting in saccular aneurysm formation. A: Sagittal cut of the left ACA–OlfA bifurcation showing the nidus of a cerebral aneurysm (arrow). B: Double immunofluorescence labeling with antibodies against eNOS (green) and \( \alpha \)-SMA (red) shows the lack of eNOS expression (arrows) in the aneurysm wall. Note the subendothelial migration of SMCs (arrowheads). The aneurysm cavity is limited to the area of SMC migration. C: Positive expression of MMP-9 (blue) resulting in vessel wall destruction. D: Merged images. Bars = 10 \( \mu \)m.

**Fig. 6.** Schema outlining our hypothesis for cerebral aneurysm formation.

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**Hemodynamic changes**

- Injury of endothelial cells at the apical intimal pad (AIP) characterized by loss of eNOS expression and abnormal endothelial cell imprints

- Local inflammation of the vessel wall at the AIP characterized by macrophage infiltration and the proliferation and subintimal migration of smooth muscle cells

- Defect of the inflammatory zone produced by destruction of the vessel wall components by macrophages and the release of proteolytic enzymes

- Saccular aneurysm formation (expansion of the defect)
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OlfA bifurcation in all rats, even those without apparent arterial dilation on examination with the dissecting microscope, and by using scanning electron microscopy to screen vascular corrosion casts, we were able to identify early changes that precede the appearance of a well-developed saccular aneurysm. Nevertheless, because it is technically impossible to prepare vascular corrosion casts and pathological sections in the same animal, we identified and compared these early morphological and pathological changes in different rats, which is the main limitation of our study.

Conclusions

Ours is the first demonstration of the sequence of ultrastructural morphological and pathological changes leading to the formation of saccular intracranial aneurysms in vivo. Detailed knowledge regarding these changes is crucial for an understanding of the pathogenesis of intracranial aneurysms and for the development of preventive and therapeutic strategies that interrupt this cascade of aneurysmal changes.

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