

Immunohistochemical Alterations of Fibronectin During the Formation and Proliferative Repair of Experimental Cerebral Aneurysms in Rats

[Original Contribution]

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Abstract

Background and Purpose: To determine whether distributional changes of fibronectin, a factor promoting wound healing, occur during the formation and repair of cerebral saccular aneurysms, we performed immunohistochemical analyses in experimental aneurysms.

Methods: Cerebral aneurysms were induced in rats by both the ligation of the unilateral common carotid artery and induced hypertension. Intimal proliferation in aneurysmal walls was induced by the ligation of the preserved common carotid artery 3 months after the first operation. The distribution of fibronectin was examined by immunohistochemistry in anterior cerebral artery-olfactory artery bifurcations under the following three conditions: normal bifurcations in control rats, early aneurysmal lesions during the aneurysm induction, and aneurysmal lesions with intimal proliferation. Furthermore, the immunohistochemical distributions of type I and IV collagens were examined to evaluate the specificity of fibronectin immunoreactivity.

Results: In the normal bifurcations, fibronectin was positive in the subintimal space, the surrounding area of the medial smooth muscle cells, and the adventitial fibrous tissue. In early aneurysmal lesions, linear staining of fibronectin and type I and IV collagens in the subendothelial space disappeared with the loss of the internal elastic lamina. In the intimal proliferation of early aneurysmal lesions, fibronectin was strongly immunostained in the subendothelial space and diffusely immunostained in the widened extracellular space surrounding proliferated cells. In contrast, the stainings of type I and IV collagens were sparse or negative.

Conclusions: Although the present findings regarding dynamic changes of fibronectin distribution do not prove any causality in the process of aneurysm formation and repair, these immunohistochemical changes may constitute the crucial sequela of intimal endothelial damage and its subsequent recovery in cerebral aneurysms.

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Key Words: rats, cerebral aneurysm, collagen, fibronectin, immunohistochemistry

The pathogenesis of cerebral saccular aneurysms still remains obscure. However, the insufficiency of the wound-healing mechanism in an arterial wall that generally follows vascular injury may be involved in the formation of cerebral aneurysms. [1,2] An initiating episode in cerebral aneurysm formation is considered to be degeneration of the endothelial cells, [3-5] in accordance with that in atherosclerosis. [6] However, during their development, experimental cerebral aneurysms lack a proliferative response of smooth muscle cells, [2] which is indispensable in atherosclerosis. [6] Some factors that contribute to wound healing may be inhibited in the course of cerebral aneurysm formation. Accordingly, it may be essential to evaluate the alterations in such factors during the development of cerebral aneurysms to elucidate their pathogenesis.

In previous reports, [1,2,7] proliferative repair in the wall of experimental aneurysmal lesions was induced by various methods, such as decrease of hemodynamic stress, [1] administration of blood coagulation factor XIII, [1,2] or administration of basic fibroblast growth factor. [7] In the most advanced cases, the aneurysmal lumens were completely filled with proliferated cells and matrix substance. [1,2,7] These findings indicate the therapeutic possibility of preventing the development of cerebral aneurysms or inducing thickening of the aneurysmal wall with the use of certain factors that facilitate wound healing. Furthermore, they indicate the importance of elucidating the mechanism of intimal proliferation in the cerebral aneurysmal wall to investigate such factors.

Fibronectin is a noncollagenous extracellular matrix glycoprotein and one of the factors that promotes wound healing through its facilitating effects of cell adhesion or migration. [8-11] In arterial walls, fibronectin can be produced by endothelial cells, [12] smooth muscle cells, [13] and fibroblasts [14] and exists in all layers of the walls. [15] It has been shown to be strongly immunostained in the early stage of atherosclerosis [15,16] and the proliferative response after vascular injury. [17] In the present study we investigated changes in fibronectin immunoreactivity in early aneurysmal lesions during both the formation and the proliferative repair of aneurysmal walls experimentally induced in rats. Additionally, the immunohistochemical distribution of type I and IV collagens was examined to evaluate the specificity of fibronectin distribution.

Materials and Methods

Animal Preparation

Experimental cerebral aneurysms were induced in 25 male Sprague-Dawley rats (age range, 6 to 7 weeks) according to the method of Hashimoto et al. [18,19] Ligation of the

left common carotid artery and the posterior branches of both renal arteries was performed under anesthesia with the use of an intraperitoneal injection of chloral hydrate (3%, 0.01 mL/g body wt). One week after the operation, 1% saline was substituted for drinking water. The previous literature reported this method of preferentially induced experimental cerebral aneurysms at the right anterior cerebral artery (ACA)-olfactory artery (OA) bifurcations, where hemodynamic stress was assumed to increase by the ligation of the opposite common carotid artery. [5] At 3 months, these rats were divided into two groups. In group 1, 13 rats were cannulated into the ascending aorta through the left cardiac ventricle under general anesthesia and perfused at a pressure of 80 mm Hg with 4% paraformaldehyde in PBS. In the remaining 12 rats (group 2), proliferative repair was induced by the ligation of the common carotid artery ipsilateral to the aneurysm formation, [1] which decreased the hemodynamic stress to the aneurysms. These rats underwent ligation of the right common carotid artery and were supplied with tap water for drinking. Two months after the second operation, rats in group 2 were perfused in the same manner as those in group 1. An additional 5 age-matched rats served as controls.

After the perfusion fixations, the major arteries at the base of the brain were carefully dissected under a surgical microscope. The specimens were further immersed in 4% paraformaldehyde in PBS for 24 hours. After dehydration in graded alcohol, the specimens were embedded in paraffin, and sections 4 micro meter thick were cut. Using the specimens stained by elastica-van Gieson stain, we performed a microscopic examination of the right ACA-OA bifurcations.

Definition of Aneurysmal Changes

Aneurysmal changes were defined as lesions representing the outward dilatation of the wall that were accompanied by discontinuity of the internal elastic lamina in more than half the length of the dilated wall. The lesions were classified into two stages: (1) a stage of early aneurysmal lesion preserving the smooth muscle cell layer in the whole length of the dilated wall and (2) a stage of saccular aneurysm lacking the smooth muscle cell layer in even a part of the entire length of the lesion. The lesions accompanied by intimal proliferation were defined as those with a cellular component existing between endothelial cells and the residual internal elastic lamina. Using the serial sections of normal ACA-OA bifurcations in control rats, the early aneurysmal lesions in group 1, and the early aneurysmal lesions accompanied by intimal proliferation in group 2, we performed the following immunohistochemical analysis.

Immunohistochemical Analysis

An immunohistochemical analysis was performed with the use of the avidin-biotin-peroxidase technique. We used rabbit polyclonal antibodies: A-245 against human fibronectin at a dilution of 1/100, LB-1104 against rat type I collagen at a dilution of 1/400, and LB-1407 against bovine type IV collagen at a dilution of 1/100. After the inactivation of intrinsic peroxidase with H₂ O₂ in methanol and blocking of nonspecific binding with normal horse serum at a dilution of 1/50, the antibodies were applied for 24

hours at 4 degrees C to the serial sections. The sections were then incubated with biotin-labeled horse IgG against rabbit IgG at a dilution of 1/100 for 1 hour at 37 degrees C, followed by the avidin-biotin procedure, and counterstained with hematoxylin. The specificity of the immunostaining was confirmed by replacing the primary antibody with nonimmune rabbit serum. To control for potential interanimal variation in the overall degree of immunoreactivity, immunohistochemical staining was evaluated semiquantitatively by comparison with the staining intensity of the media in the normal arterial wall: (minus) when negative, (plus) when weaker than in the media, (plus plus) when the same as in the media, and (plus plus plus) when stronger than in the media. When more than two of the three blinded investigators (O.T., S.H., and T.Y.) made the same evaluation, it was conclusive.

Results

ACA-OA Junction Under elastica-van Gieson Staining

In the specimens stained by elastica-van Gieson stain, the wall of the ACA-OA junctions in control rats consisted of a monolayer of endothelial cells covering the inner surface of the arterial wall, the internal elastic lamina continuous along the curvature of the arterial lumen, a medial smooth muscle cell layer, and an adventitial fibrous connective tissue [Figure 1A](#). At the distal side of the ACA adjacent to the apex, there was an intimal pad representing a focal intimal protrusion. At the margin of the intimal pad, the internal elastic lamina was splitted into several layers and fragmented beneath the intimal pad. In the ACA just distal to the intimal pad, the arterial wall was occasionally depressed outward in a mild degree, and the medial smooth muscle cell layer was slightly thinner than the other portions of the artery.

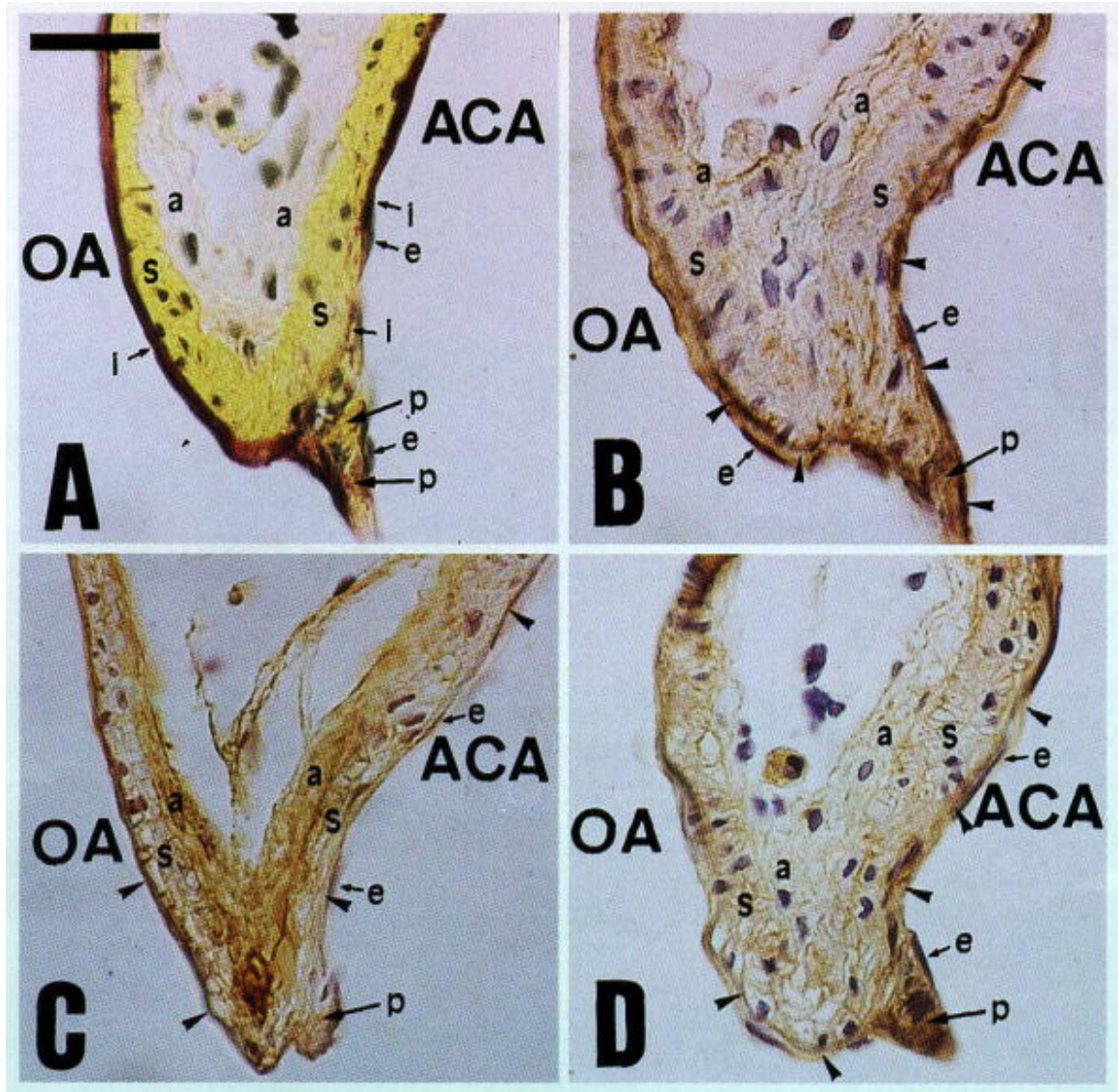
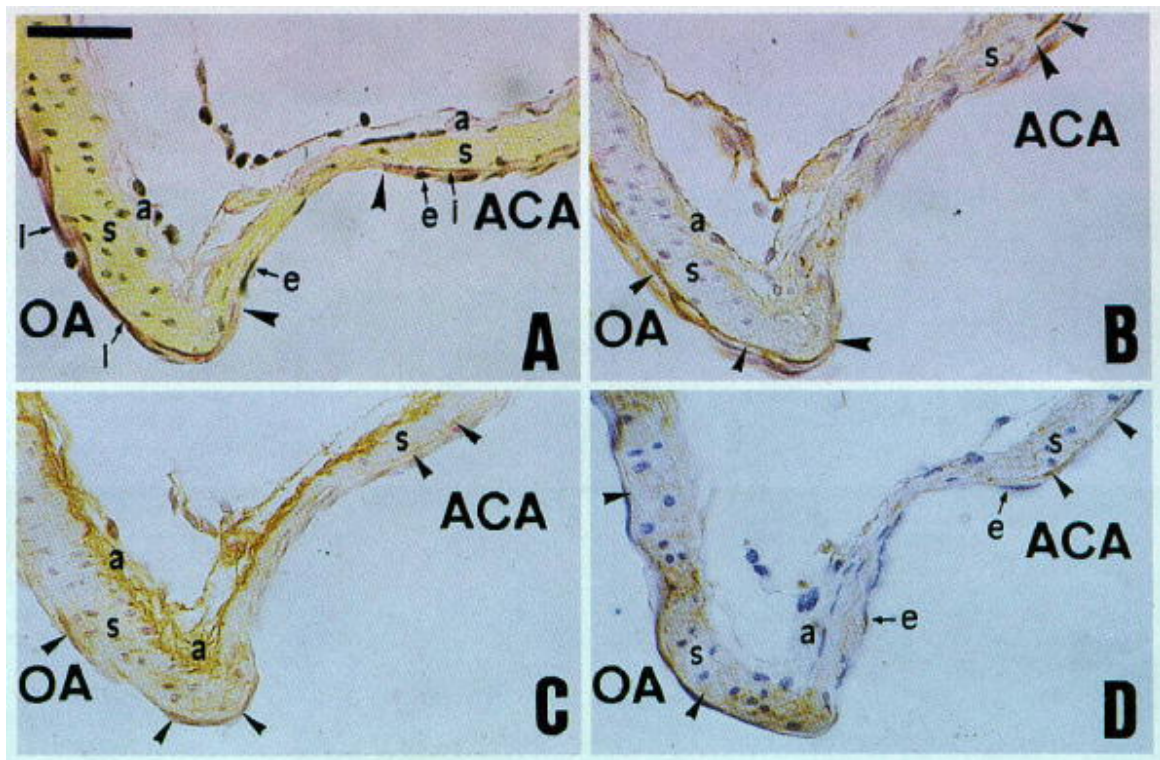


Figure 1. Photomicrographs of normal bifurcations of the anterior cerebral artery (ACA) and the olfactory artery (OA) on the nonligated side in control rats. A, Elastica-van Gieson staining. The internal elastic lamina continues along the curvature of the arterial lumen except for the portion beneath an intimal pad. B, C, and D, Immunohistochemical staining using anti-fibronectin (B), anti-type I collagen (C), and anti-type IV collagen (D) antibodies. Fibronectin and both types of collagens are present in the subintimal space as a linear staining (arrow-heads), the surrounding area of the medial smooth muscle cells, and the adventitial fibrous tissue. a indicates adventitia; i, internal elastic lamina; e, endothelial cells; s, smooth muscle cells; and p, intimal pad. Bar equals 10 micro meter (A, B, C, D).

Early aneurysmal lesions were observed in none of the control rats, whereas they were observed in 6 of 13 rats in group 1 [Figure 2A](#) and 5 of 12 rats in group 2 [Figure 3A](#). These early aneurysmal lesions were always located in accordance with the depression shown in normal bifurcations. The wall of the lesions dilated in various degrees toward the outside

of the artery. Medial smooth muscle cells of the lesions were frequently thinned and elongated but remained in the entire course of the wall of the lesions. In these early aneurysmal lesions, none of 6 in group 1 showed intimal proliferation. In contrast, 3 of 5 in group 2 showed variable degrees of cellular components between the residual and fragmented internal elastic lamina and a monolayer of endothelial cells [Figure 3A](#). These proliferating cells were composed of two or three layers of cells that had oval nuclei with spindle-shaped cytoplasm. In the most advanced cases, proliferated cells completely filled the original lumen of the lesion and extended into the subendothelial space along the internal elastic lamina.



[Figure 2](#). Photomicrographs of early aneurysmal lesions in group 1 rats, in which the unilateral common carotid artery was ligated and hypertension was induced by renal infarction and salt solution. A, Elastica-van Gieson staining. The internal elastic lamina abruptly ends at the entrance of the lesion (arrowheads). B, C, and D, Immunohistochemical staining using anti-fibronectin (B), anti-type I collagen (C), and anti-type IV collagen (D) antibodies. The subendothelial stainings of extracellular matrices (arrowheads) are abolished in the wall of the lesions. See [Figure 1](#) legend for abbreviations. Bar equals 20 micro meter (A, B, C, D).

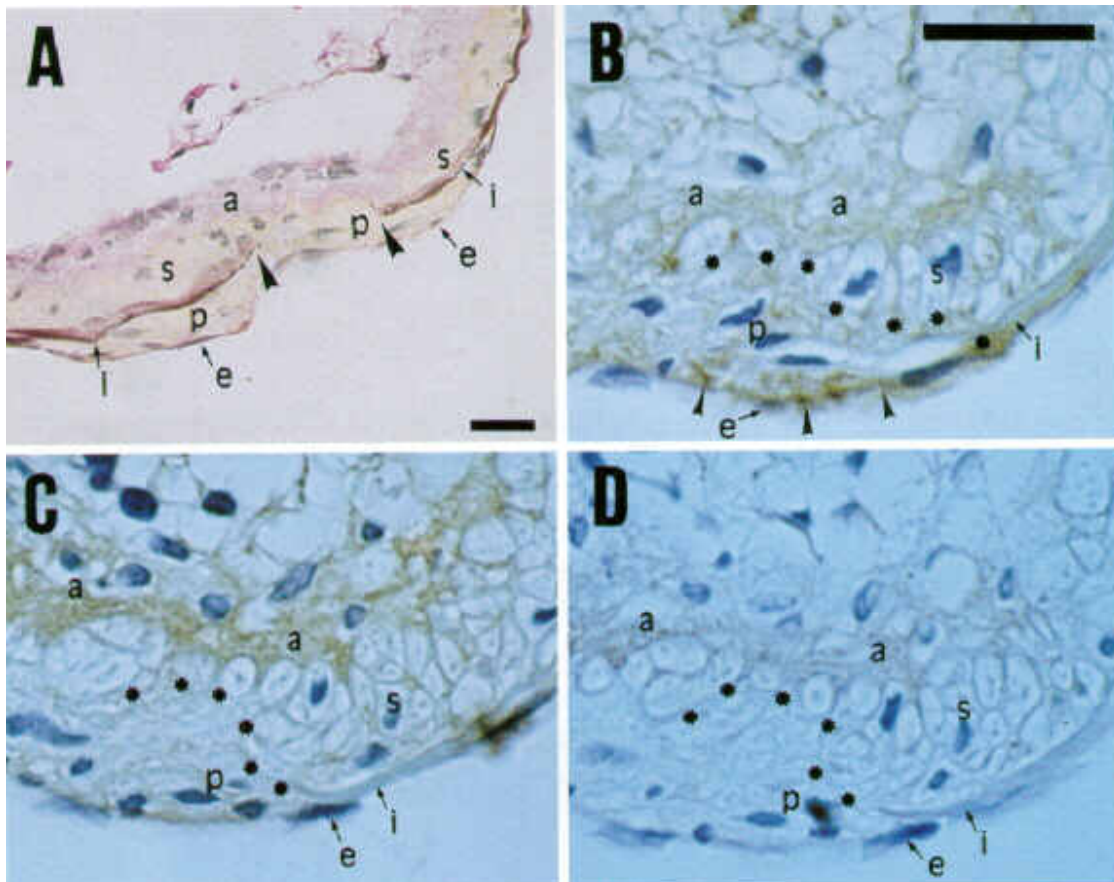


Figure 3. Photomicrographs of an early aneurysmal lesion accompanied by prominent intimal proliferation in a group 2 rat. A, Elasticin-van Gieson staining. The lesion was induced by ligation of the nonligated side of the common carotid artery at 3 months after surgery to induce the aneurysm. The internal elastic lamina abruptly ends at the entrance of the original lesion (arrowheads). Proliferated cells fill the original lumen of the lesion, protrude inward, and prominently extend along the internal elastic lamina. B, C, and D, Immunohistochemical staining of the bordering area of the original arterial wall (the right sides of B, C, and D) and the intimal proliferation (the left sides of B, C, and D) in the serial sections of the same lesion, using anti-fibronectin (B), anti-type I collagen (C), and anti-type IV collagen (D) antibodies. Asterisks indicate the borderline. The subendothelial linear staining of fibronectin shows an irregular configuration (arrowheads in B). Throughout the entire area of intimal proliferation, fibronectin is diffusely and abundantly stained. On the other hand, stainings of type I and IV collagens in the area of intimal proliferation are sparse or indistinct compared with those in other portions of the artery. Bar equals 6 micro meter (A) and 10 micro meter (B-D). See [Figure 1](#) legend for abbreviations, except p indicates proliferated cells.

Saccular aneurysms were absent in all of the control rats. However, 3 of 13 rats in group 1 and 3 of 12 rats in group 2 showed distinct saccular aneurysms (not shown). Medial smooth muscle cells abruptly ended at the orifice of the lesions or were attenuated in the wall of the lesions. The wall of the lesions was mainly composed of endothelial cells and fibrous connective tissue of the adventitia and was prominently dilated outward. Except for the remaining intimal pads, there were no apparent cellular components protruding into the lumen in the subendothelial space, indicating proliferative cells, in the saccular aneurysms in group 1 or 2.

Immunohistochemistry in Normal Control Rats

In the normal ACA-OA junctions of the control rats, fibronectin was stained as a linear structure in the subendothelial basal layer, as a fibrillary structure in the spaces surrounding the medial smooth muscle cells, and throughout the adventitia [Figure 1B](#). In the intimal pads, fibronectin was stained more strongly than in the media. The subendothelial linear staining was continuous along the curvature of the arterial lumen. Similar to the distribution of fibronectin, type I and IV collagens were present in the subendothelial basal layer as a linear staining, in the area surrounding the medial smooth muscle cells as a fine reticular pattern, and diffusely in the fibrous adventitia [Figure 1C](#) and [Figure 1D](#)). However, the subendothelial linear staining was the most obvious in fibronectin within these three extracellular matrices. In contrast, in the media fibronectin represented the faintest fibrillary staining compared with the other proteins. On the other hand, in the diffuse staining in the adventitia, the staining of type I collagen was apparently more prominent compared with the others.

Immunohistochemistry of Developing Early Aneurysmal Lesions

In the early aneurysmal lesions induced in group 1, the subendothelial linear staining of fibronectin ended at the entrance of the lesions and completely disappeared in the wall of the lesions except for occasionally existing segmental fragments of residual staining [Figure 2B](#). The ends of the subendothelial staining were nearly consistent with those in the internal elastic lamina under elastica-van Gieson stain. The staining of fibronectin in the media and the adventitia of the lesions was not significantly different from that in the normal ACA-OA bifurcations or that in the other portions of the artery. Similar to fibronectin staining, the subendothelial linear staining of type I and IV collagens also disappeared in the wall of the lesions [Figure 2C](#) and [Figure 2D](#)).

Immunohistochemistry of Early Aneurysmal Lesions With Intimal Proliferation

In the wall of the early aneurysmal lesions with intimal proliferation in group 2, subendothelial linear staining of fibronectin was observed, although without well-demarcated configurations [Figure 3B](#). Throughout the entire area of intimal proliferation, fibronectin was diffusely and abundantly stained, except for scattered proliferated cells. On the other hand, the stainings of type I and IV collagens in the area of intimal proliferation were evidently sparse or indistinct compared with those in the other portions of the artery [Figure 3C](#) and [Figure 3D](#)).

The [Table 1](#) summarizes the immunoreactivity of the various layers constituting the wall of normal ACA-OA bifurcations, early aneurysmal lesions during development, and early aneurysmal lesions accompanied by intimal proliferation, against anti-fibronectin, anti-type I collagen, and anti-type IV collagen antibodies.

Layer of the Wall	Fibronectin	Type I Collagen	Type IV Collagen
Normal arterial bifurcation			
Subendothelial layer	+++	+++	++
Media	++	++	++
Adventitia	+	+++	+
Early aneurysmal lesion			
Subendothelial layer	-	-	-
Media	++	++	++
Adventitia	+	+++	+
Early aneurysmal lesion with intimal proliferation			
Subendothelial layer	+++	+	-
Area surrounding proliferated cells	+++	+	-
Media	++	++	++
Adventitia	+	+++	+

Immunoreactivity is indicated as follows in each extracellular matrix: -, negative; +, weaker than in the media; ++, same as in the media; and +++, stronger than in the media.

[Table 1.](#) Intensity of Immunoreactivity in Various Layers of Normal Arterial Bifurcations, Early Aneurysmal Lesions, and Early Aneurysmal Lesions With Intimal Proliferation

Discussion [↑](#)

In 1978 Hashimoto et al [\[18\]](#) induced saccular cerebral aneurysms in rats by ligating one common carotid artery, making the animals hypertensive, and feeding them beta -aminopropionitrile, one of the lathyrogens, to inhibit cross-linkage of collagen and elastin. To induce cerebral aneurysms, beta -aminopropionitrile was not always needed, but this lathyrogen apparently increased the incidence of cerebral aneurysms. [\[19\]](#) Patients with Ehlers-Danlos syndrome type IV associated with a relative deficiency of type III collagen occasionally develop cerebral aneurysms. [\[20,21\]](#) Moreover, a deficiency of type III collagen was often demonstrated in patients with cerebral aneurysms. [\[22,23\]](#) These findings indicate the role of extracellular matrices in the formation of cerebral aneurysms.

Fibronectin is not only one of the major mechanical properties of arterial walls but also a multifunctional glycoprotein. [\[8\]](#) Fibronectin has been implicated in a variety of cellular properties, particularly those involving the interactions of cells with extracellular

matrices. [8] In arterial walls, fibronectin is produced by endothelial cells, [12] smooth muscle cells, [13] and fibroblasts [14] and is found in connective tissue matrix and associated with basement membranes [24] that underlie endothelial cells and envelop smooth muscle cells. Meanwhile, of 13 distinct subtypes of collagens, [25-27] six types (I, III, IV, V, VI, and VIII) of collagens are present in arterial walls. [28] All of these collagen types except type VIII are known to be synthesized by smooth muscle cells, and all except type VI are synthesized by endothelial cells. [28] Types I and III are fibril-forming interstitial collagens that constitute 80% to 90% of total arterial collagen. [28] They colocalize in extracellular matrix of the intima, media, and adventitia of normal arteries. Type IV collagen is a constituent of the basement membrane. [25]

In the present study, considering that fibronectin and type IV collagen concomitantly constitute the basement membrane and that fibronectin and type I collagen exist in the subendothelial connective tissue, the immunohistochemical distributions of type I and IV collagens were examined to evaluate the specificity of fibronectin in the distributional changes. During the development of aneurysmal lesions, fibronectin in the subendothelial space was immunohistochemically demonstrated to disappear together with type I and IV collagens. This finding may represent the degeneration of the endothelial basement membrane and the subendothelial connective tissue. The precise mechanism of these alterations in these extracellular matrices remains unclear. However, it is compatible with the degeneration of endothelial cells in aneurysmal walls demonstrated by scanning electron microscopy. [3-5] Degenerated endothelial cells may decrease the production of fibronectin or type I and IV collagens. Accordingly, the alterations of the subendothelial extracellular matrices may represent one of the earliest manifestations of endothelial dysfunction.

Fibronectin plays an important role in tissue repair, [9,10] showing molecular affinities for collagen, [31] hyaluronic acid, [32] and fibrin, [33] which are three major components of early wounds. [10,34] Fibronectin in clots cross-links to the alpha -chain of fibrin by plasma transglutaminase, activated blood coagulation factor XIII, during blood coagulation [33] and forms the adhesive sites on the fibrin molecule to which cells can bind. [11] Epidermal cells contact the fibrin filaments during their migration toward the wound center. [9] Fibroblasts are also chemoattracted by fibronectin. [35] Thus, fibronectin with collagen and hyaluronic acid in the developing matrix of granulation tissue and with fibrin in fibrin clots can provide a substratum for migrating cells that functions to unite the opposed tissues. [9] In arterial walls, fibronectin is strongly immunostained in the early stage of atherosclerosis [15,16] and proliferative response after vascular injury. [17] Type I collagen also promotes the migration of fibroblasts. [36] Accordingly, the lack of fibronectin and type I and IV collagens in the subendothelial space of aneurysmal lesions may not only increase the mechanical vulnerability of the wall but also impair wound healing in aneurysmal walls. [1,2]

Using rats subjected to ligation of the unilateral common carotid artery and renal hypertension, Kang et al [1,2] produced intimal proliferation in aneurysmal lesions by three different methods: decreasing hemodynamic stress by the ligation of the nonligated common carotid artery, [1] administration of an antihypertensive drug, [1] and

administration of blood coagulation factor XIII. [1,2] We induced a similar intimal proliferation by administration of basic fibroblast growth factor. [7] In these experiments, smooth muscle cells were consistently demonstrated to proliferate in the subendothelial space. [1,2,7]

In the present study we induced intimal proliferation by the ligation of the common carotid artery ipsilateral to the aneurysm formation. [1] The incidences of intimal proliferation in the ACA-OA junctions and in aneurysmal changes were 25% and 37.5%, respectively. In immunohistochemistry, we found diffuse staining of fibronectin in the area of the intimal proliferation induced in early aneurysmal lesions. It is likely that fibronectin is an important factor in the repair of aneurysmal lesions. In such cases, fibronectin may provide a substratum for smooth muscle cells to migrate and proliferate in the subendothelial space of the wall. Furthermore, the distinct subendothelial staining of fibronectin in the intimal proliferation may indicate the subsequent recovery of endothelial cells as a result of decreasing hemodynamic stress to aneurysmal lesions.

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