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Molecular Alterations Associated with Aneurysmal Remodeling are Localized in the High Hemodynamic Stress Region of a Created Carotid Bifurcation

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Abstract

Objective—Although elevated hemodynamics has been speculated to play a key role in intracranial aneurysm (IA) initiation, little is known about the specific hemodynamic microenvironment that triggers aneurysmal vascular degradation. We previously demonstrated maladaptive remodeling characteristic of IA initiation occurring in hemodynamic regions of combined high wall shear stress (WSS) and high WSS gradient near the apex of an experimentally created carotid bifurcation. This study examines whether this remodeling recapitulates the molecular changes found in IAs and whether molecular changes also correspond to specific hemodynamic environments.

Methods—De novo bifurcations were surgically created using both native common carotid arteries in each of 6 dogs. Bifurcations were imaged 2 weeks or 2 months after surgery by high-resolution 3-dimensional angiography, from which flow fields were obtained by computational fluid dynamics simulations. Subsequently, harvested tissues, demonstrating early aneurysmal changes near the apex, were immunostained for interleukin-1 β , endothelial and inducible nitric oxide synthases, nitrotyrosine, and matrix metalloproteinase-2 and -9. Spatial distributions of these molecules were compared with computational fluid dynamics results.

Results—The aneurysmal wall showed decreased endothelial nitric oxide synthase expression compared with surrounding segments, the feeding artery, and native controls, whereas all other markers increased. Anti-CD68 staining indicated the absence of inflammatory cells in the aneurysmal wall. Comapping molecular marker distributions with flow fields revealed confinement of these molecular changes within the hemodynamic region of high WSS and high, positive WSS gradient.

Conclusion—Aneurysm-initiating remodeling induced by combined high WSS and high, positive WSS gradient is associated with molecular changes implicated in IAs.

Keywords

Aneurysm; Initiation; Matrix metalloproteinase; Nitric oxide; Pathogenesis; Wall shear stress

Approximately 80% to 90% of intracranial aneurysms (IA) are saccular or berry pouches formed at or near bifurcation apices or on the outer curvature of curved vessels in regions of complex hemodynamics (42). IA formation is proposed to result from maladaptive vascular responses to elevated hemodynamic stress at these locations (24,26,37). However, the specific causative hemodynamic microenvironments responsible for the biological changes that underlie aneurysm-initiating remodeling have not been elucidated.

Recently, we surgically created a Y-shaped bifurcation from 2 native common carotid arteries (CCAs) in dogs (28) and examined how a naïve vessel responded to impinging flow at a bifurcation apex from time zero. We found that the periapical wall underwent destructive remodeling characteristic of IA initiation, including degradation of the internal elastic lamina, loss of smooth muscle cells (SMCs) and endothelial cells (ECs), and reduced cell proliferation (29). Using high-resolution in vivo image-based computational fluid dynamics (CFD) to quantify the impinging flow environment and its spatial association with the observed histological changes, we demonstrated that morphological and cellular aneurysmal changes were localized in a flow acceleration region characterized by high wall shear stress (WSS) and high WSS gradient (WSSG) (28,29). These observations suggest that the aneurysm-initiating

remodeling results from maladaptation of the periapical arterial wall to high WSS and high WSSG hemodynamic insult and that this hemodynamic microenvironment constitutes a “danger zone” prone to aneurysm initiation.

The current study examines whether the maladaptive remodeling in response to high WSS and high WSSG hemodynamics induces significant molecular up- or down-regulation of specific markers associated with IAs, such as endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS), nitrotyrosine, and matrix metalloproteinase (MMP)-2 and -9 (1,3,13,19,23,39). Additionally, we sought to localize these findings to their specific hemodynamic environments.

Materials and Methods

Bifurcation Creation

All procedures conformed to current regulations and guidelines for animal experiments and were approved by the Institutional Animal Care and Use Committee. Anastomosed CCA bifurcations were surgically created (28) (Fig. 1A) in each of 6 mongrel female dogs. In an attempt to induce hypertension, all dogs were fed a high-salt diet (0.4 g NaCl/kg per day) starting 4 to 14 weeks before surgery and continuing until euthanasia. In 3 of the 6 dogs, the right renal artery was ligated at least 2 weeks before creating the bifurcation to further elevate blood pressure. Systemic and diastolic blood pressures were measured noninvasively through a blood pressure cuff (SurgiVet; Smiths Medical, Waukesha, WI) on the dog's fore- or hindlimbs while the animal was resting. The measurements were done before surgery or the high-salt diet (122 ± 8 mm Hg) and at the end of the experiment (high-salt treatment alone: 106 ± 47 mm Hg; renal ligation plus a high-salt diet: 130 ± 48 mm Hg). However, no hypertension was detected in the animals throughout the duration of the study. Failure to detect hypertension may have been attributable to the excitability of the animals, making it difficult to obtain reliable blood pressure values. Alternatively, the effects of renal ligation might have been negated by the development of collateral renal arteries (29).

Histology and Immunohistochemistry

Either 2 weeks ($n = 2$) or 2 months ($n = 4$) after bifurcation creation, the animals underwent imaging with 3-dimensional (3D) rotational angiography under general anesthesia, followed by humane euthanasia via the intravenous administration of 100 mg/kg sodium pentobarbital. Bifurcations were immediately perfused with phosphate-buffered saline and pressure fixed in situ at 150 mm Hg with 10% buffered neutral formalin for 30 minutes. The tissue was then excised, embedded in paraffin, and sectioned longitudinally. Adjacent, 4- μ m-thick sections from the bifurcation median plane were stained with hematoxylin and eosin, van Gieson, or trichrome. For comparison, a segment of unmanipulated CCA was excised from 3 experimental animals before bifurcation creation and then processed and stained using the same methods.

For immunohistochemical analysis, sections were deparaffinized, rehydrated, and incubated with 3% H₂O₂ for 10 minutes to inactivate endogenous peroxidase. Antigen was unmasked by treating sections with 20 μ g/mL proteinase K (Roche Applied Biosciences, Indianapolis, IN) for 10 minutes at 37°C or by boiling sections in 10 mmol/L citric buffer (pH 6.0). Sections were then stained with rabbit polyclonal anti-interleukin (IL)-1 β (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti-eNOS or anti-iNOS (Lab Vision, Fremont, CA), rabbit polyclonal anti-nitrotyrosine (Millipore, Billerica, MA), mouse monoclonal anti-MMP-2 (Chemicon, Temecula, CA), goat polyclonal anti-MMP-9 (Santa Cruz Biotechnology), mouse monoclonal anti-macrophage (Abcam, Cambridge, MA), or mouse anti-rat CD68 (AbD Serotec, Raleigh, NC), and visualized with LSAB+ System-HRP kits (Dako, Carpinteria, CA). For MMP-2 and -9 staining, slides were counterstained with hematoxylin.

Flow Simulation and Comapping with Histology

Before euthanasia, the in vivo bifurcation geometry was imaged with high-resolution, digital subtraction rotational angiography, while flow rates in the feeding artery and bifurcation branches (under general anesthesia) were recorded using a perivascular flow probe (Transonic Systems, Inc., Ithaca, NY). Because we do not have the tools to measure flow in conscious dogs, flow measurements were taken during surgical procedures by wrapping the vessel with a perivascular flow probe. CFD analysis was conducted for each bifurcation, and the detailed in vivo 3D flow field was comapped with the histological sections. Details of the CFD method and mapping procedure have been described previously (28). Briefly, 3D CFD solutions under both steady-state (average peak Re, 590 ± 252) and pulsatile-flow conditions were obtained under assumptions of a Newtonian fluid (μ , 0.0035 Pa·s) and incompressible flow in a rigid-wall model. Both simplifications were justified, as reported previously (28). The WSS, which represents the fluid frictional force on the wall sensed by the endothelium, was calculated from the 3D flow velocity field and given as a distribution on the luminal surface. The WSSG, or spatial gradient of WSS, was calculated along the flow streamline, whereas the gradient normal to the streamline was negligible in comparison. Next, the 3D flow field of the bifurcation at peak systole was sliced, superimposed, and morphed onto the photomicrograph of the corresponding histological section. The detailed hemodynamics (including WSS and WSSG) were then comapped with immunohistochemical staining results to identify the specific hemodynamic environment in which these molecular changes occurred.

Quantitation of Immunohistochemistry

To minimize measurement errors, immunostaining of each molecule was performed at the same time for all tissue samples to avoid technical variance, all images were captured under the same illumination, and the measured area was normalized by subtracting the blank background intensity. In detail, protein expression was quantitated by densitometry of the immunohistochemistry images using ImageJ software (National Institutes of Health, Bethesda, MD). Digitized color images of stained sections were split into red-green-blue components, and the blue image was used for quantitation to obtain the best contrast for the brown color produced by immunohistochemistry. Average intensities were measured in manually outlined regions of interest and in a blank area in the lumen from the same section, with the latter providing the background intensity. The average intensity of the regions of interest, minus the background, was used as a measure of protein expression in that region. Regions with altered protein expressions relative to adjacent tissues were identified from the photomicrographs using both high- and low-magnification views. To position these regions on hemodynamic profiles, the distances from the flow impingement point to the boundaries of the region were measured and used to position bars indicating marker expression.

As an internal reference, the expression level in the feeding carotid artery of the bifurcation was determined by averaging 6 regions from the feeding segment distributed over the proximal, middle, and distal parts from each side of the vessel. Additionally, the expression level in unmanipulated native CCAs was measured by averaging 3 randomly selected regions.

Statistical Analysis

Measurements are presented as mean $\pm$ standard deviation. One-way analysis of variance with Bonferroni post hoc correction (Systat Software, Inc., Richmond, CA) was used to test for significant differences between the regions of interest and reference tissues (feeding arteries or normal CCAs). Differences were considered statistically significant at a *P* value of less than 0.01. The unpaired *t* test was used to compare the accumulated length of positive immunostaining results found in the positive WSSG and the negative WSSG regions. Differences were considered statistically significant at a *P* value of less than 0.05.

Results

Hemodynamic Insult in De Novo Bifurcations

Flow rates in the feeding artery of the de novo bifurcation increased by approximately 50% because of the combined effects of the ligation of the contralateral CCA and the anastomosis to an additional downstream outlet. Consequently, the feeding artery wall experienced an increase in WSS, whereas the periapical regions of the bifurcation were exposed to a more complex hemodynamic environment involving both elevated WSS and large magnitudes of WSSG, as shown by the example of a 2-month created bifurcation in Figure 2. Figure 2A shows the WSS distribution on the luminal surface; Figure 2B highlights differences between hemodynamic stress levels at the periapical bifurcation wall and at the feeding artery (a straight arterial segment). In the feeding artery, the WSS (blue dashed line) is relatively uniform and, thus, the WSSG (red dashed line) is close to zero (<5 Pa/mm). In contrast, WSS in the periapical region (blue solid line) increased drastically as the flow accelerated away from the impingement point to a maximum and then decreased further downstream to a level similar to that in the feeding artery. This acceleration and deceleration created a WSSG (red solid line) distribution consisting of a positive region (before maximum WSS) and a negative region (after maximum WSS).

According to our previous definition (28), the hemodynamic microenvironment along the apical wall of a bifurcation can be theoretically divided into 3 subregions in each branch: an impingement region (region I) at the apex, an acceleration region (region II) that starts at WSS of 2 Pa and ends at maximum WSS, and a recovery region (region III). In the example shown in Figure 2, we demonstrate that the apex itself can be exposed to high WSS (>2 Pa) such that it practically belongs to region II. This happens when the flow does not impinge perfectly perpendicular to the apex and so has a large component parallel to the wall, resulting in a non-zero WSS. Notice that region II is characterized by high WSS and high, positive WSSG, whereas region III is characterized by negative WSSG.

Molecular Changes near the Apex of Created Bifurcations

The created bifurcations exhibited early aneurysmal morphological changes near the apex as reported previously (29). An example of an early aneurysm in the right CCA 2 months after surgical bifurcation creation is shown in Figure 1B. To probe underlying molecular changes, these bifurcation tissues were first examined for MMP-2 and -9, which degrade extracellular matrix and are elevated in human and rodent IAs (1,23,39). We observed local up-regulation of both MMPs in the periapical region that exhibited early aneurysmal morphology (Fig. 3A). The increased expression of these molecules in the aneurysmal region is significant compared with that in the corresponding feeding arteries (44.11 ± 6.62 versus 28.78 ± 10.91 for MMP-2; 30.56 ± 6.98 versus 6.46 ± 2.40 for MMP-9; $P < 0.01$) (Fig. 3B). This up-regulation was similar in bifurcations harvested 2 weeks or 2 months after creation, and no significant differences could be detected between the 2 groups. Although both MMPs were increased in the periapical region of aneurysm remodeling (by comparison with feeding artery and unmanipulated CCA segment values), MMP-9 expression was substantially more elevated than MMP-2 (Fig. 3B).

Next, we examined the formation of peroxynitrite, a product of nitric oxide (NO) reacting with superoxide (O_2) that can augment MMP production and activation (5,32,33) and which is prominently increased in IAs (13). Peroxynitrite production was assessed by staining for the nitrotyrosine product formed by the reaction of the highly reactive peroxynitrite moiety with protein tyrosine residues (2). In a pattern similar to that for MMP expression, nitrotyrosine was found in a greater amount in the aneurysmal wall (Fig. 3A) than in the feeding artery (17.67 ± 8.70 versus 6.33 ± 6.78 ; $P < 0.01$) (Fig. 3B). Because such elevation is likely associated with increased production of NO, we examined eNOS and iNOS expression to explore whether

these NO synthases were up-regulated. We found that eNOS was absent from the intima along the aneurysmal wall, whereas the feeding artery and segments downstream (data not shown) showed continuous eNOS expression in the intima. In contrast, the expression of iNOS was markedly increased in the tunica media of the aneurysmal wall compared with the feeding artery (19.00 ± 6.12 versus 6.59 ± 3.00 ; $P < 0.01$) (Fig. 3B).

The distribution of IL-1 β , a known regulator of iNOS induction (38), was also examined to further probe the molecular events underlying aneurysmal remodeling. As was observed for iNOS and MMP-2 and -9, IL-1 β expression was elevated in the aneurysmal remodeling region, compared with the feeding artery (18.89 ± 5.16 versus 5.74 ± 2.63 ; $P < 0.01$) (Fig. 3B). The IL-1 β elevation and all other molecular up- or down-regulations were observed in both 2-week and 2-month bifurcations. There were no significant differences between these 2 groups.

To investigate whether inflammatory responses were involved in the periapical aneurysmal remodeling process, we stained for inflammatory cells with anti-CD68 because CD68 is a unique macrophage/monocyte marker. We found no macrophages or monocytes in the bifurcation tissues (Fig. 3C).

Quantitation of Molecular Changes in the Aneurysmal Remodeling Region

Quantitation of molecular changes showed that the levels of MMP-2 and -9, nitrotyrosine, iNOS, and IL-1 β in the feeding artery were similar to their levels in unmanipulated CCAs. However, in the aneurysmal remodeling regions, all 5 molecules were significantly higher than in either feeding arteries or unmanipulated CCAs ($P < 0.01$, one-way analysis of variance with Bonferroni correction) (Fig. 3B).

Correlation of Molecular Changes with High WSS and High, Positive WSSG

By comapping immunohistochemistry results with the local hemodynamics determined by CFD, we identified the specific hemodynamic environment in which molecular changes occurred. The comaps of molecular expression and hemodynamics are shown in Figure 4, A–D, for 4 branches in 3 dogs. Despite variations in bifurcation geometry and flow among individuals, there was extensive overlap among the regions of elevated expression for all 5 of the up-regulated molecules (MMP-2 and -9, nitrotyrosine, iNOS, and IL-1 β). The loss of eNOS expression also coincided with the regions in which the other markers were up-regulated. Furthermore, the molecular changes were largely confined to areas experiencing high WSS and high, positive WSSG, whereas significantly less molecular change was found in regions of negative WSSG, even in the presence of high WSS.

To test this relationship statistically, the total length of vessel wall that displayed each molecular change was uniformly sampled and measured separately in regions experiencing either positive or negative WSSG. The length of vessel segments exhibiting molecular markers of aneurysmal remodeling was significantly higher (longer) in positive WSSG regions than in the negative WSSG regions (unpaired t test, $P < 0.05$) (Fig. 4E).

Discussion

Molecular Alterations Associated with Aneurysmal Remodeling

Flow-induced IA formation has been demonstrated in a rodent model by Kondo et al. (24) and in a rabbit model by our group (14). We have previously reported aneurysmal destructive remodeling near the apex of a newly created carotid bifurcation after exposure to high impinging flow (29). This destructive remodeling was morphologically similar to that observed in early IA walls in humans and animals—associated with internal elastic lamina disruption, loss of ECs and SMCs, reduced proliferation of SMCs, loss of fibronectin, and media thinning.

To determine whether these morphological and cellular changes are also accompanied by molecular changes similar to those found in IAs, we examined key molecules implicated in aneurysm development. Our observations of increased MMP expression (1,3,23), peroxynitrite formation (13), IL-1 β expression (31), and iNOS induction, as well as decreased eNOS expression (13,19), further demonstrate that the destructive remodeling induced by impinging flow in created bifurcations is, at least in part, a recapitulation of IA initiation.

The aneurysmal remodeling and altered molecular expressions were induced by the increased hemodynamics at the bifurcation and not by the surgical procedure as these remodeling processes were both behaviorally and spatially distinct from the remodeling observed around the surgical arteriotomy sites (29). In addition, although hypertension induction was attempted in these animals, no significant difference in blood pressure was seen. Hence, the role of hypertension remains to be explored in flow-induced aneurysmal-initiating remodeling.

Implications of the Molecular Changes

Not only did we observe extensive EC loss, but we also found reduction or absence of eNOS expression in the aneurysmal remodeling region, even when ECs remained present. This is suggestive of injured or dysfunctional endothelium (6,12), which may be a critical early step in the initiation of aneurysmal remodeling. We also observed increased iNOS expression and peroxynitrite formation in the media of the aneurysmal remodeling region.

Decreased eNOS expression undermines NO bioavailability, potentially resulting in further endothelial dysfunction and consequent iNOS induction in SMCs (12,15,16). Compared with eNOS, iNOS has a longer-lived activity and produces at least 10-fold more NO per second (4). Under physiological conditions, low levels of NO are produced constitutively to maintain the anticoagulation and antiinflammation properties of the vessel wall (27). However, large amounts of NO have toxic effects on many cell types and can inhibit SMC proliferation and growth (20,41). With increased iNOS expression in the aneurysmal remodeling region, it seems likely that excessive NO production is, at least in part, responsible for the reduced SMC proliferation observed during the destructive remodeling (29). In addition, NO toxicity can be exacerbated by NO reacting with superoxide to form peroxynitrite. Increased peroxynitrite has been demonstrated in human IAs (13) and causes cell apoptosis and MMP activation (2,32). The latter is particularly noteworthy in view of the observed increase in the expression of MMP-2 and -9, along with nitrotyrosine elevation, in the aneurysmal remodeling region. Increased MMP expression in combination with activation by the elevated peroxynitrite would be expected to drive excessive vessel degradation, potentially contributing to aneurysm development.

We also found IL-1 β up-regulation in the media of the aneurysmal remodeling regions. In the mouse IA model, increased IL-1 β expression in the aneurysm wall has been reported 5 months after aneurysm induction (31). Reverse transcriptase–polymerase chain reaction technique in aneurysms in mice revealed up-regulation of IL-1 β messenger ribonucleic acid as early as 2 weeks after aneurysm induction, indicating that the signal for IL-1 β is triggered in the initial stage of aneurysm development (31). In addition, high flow can increase IL-1 β gene expression (21), making it not entirely surprising to find up-regulated IL-1 β in the flow-induced aneurysmal-initiating remodeling region.

IL-1 β is a proinflammatory cytokine, and inflammatory cells have been found in a rat aneurysm model (1,19). However, we did not detect inflammatory cell infiltration in the aneurysmal remodeling region of our created bifurcations. Although contrary to our original expectation, it is possible that inflammatory contribution to flow-induced aneurysmal remodeling is insignificant at this early stage of aneurysmal development. In fact, the absence of inflammatory cells in our aneurysmal remodeling region is consistent with Stehbens' (36)

observation that no inflammatory change or fibrinoid necrosis was detectable in the early human aneurysms. However, a significant correlation between macrophage infiltration and structural changes of the vessel wall in ruptured human IAs suggests a prominent role for inflammation in later stages of IA development and rupture (22).

Hemodynamic Insult Associated with Aneurysmal Remodeling

The localization of early aneurysmal cellular and molecular changes within the region of accelerating flow, where the hemodynamic forces experienced by the vessel wall are characterized by combined high WSS and high, positive WSSG, is crucial to understanding the potential contribution of hemodynamics to aneurysm initiation. The created bifurcation apex wall had possessed relatively uniform biological properties indistinguishable from the feeding artery before surgery. After the bifurcation creation, destructive vascular changes were localized to specific hemodynamic environments, which strongly suggests that such a response is uniquely associated with the local hemodynamic stimuli. Despite the variability in bifurcation geometry and differences in flow among animals, all 6 dogs shared certain important features. In contrast to the relatively uniform presurgical baselines of WSS (1.5–2 Pa) and negligible WSSG, the flow acceleration region of the created bifurcation had significantly elevated WSS and positive WSSG and was linked to aneurysmal remodeling, whereas the flow recovery region had negative WSSG and little aneurysmal remodeling was observed. Furthermore, the feeding artery, which also experienced elevated WSS after the surgery, showed no aneurysmal changes. Therefore, the combination of 2 hemodynamic factors—high WSS and high, positive WSSG—seems to promote aneurysmal-initiating destructive remodeling.

Such observation raises new questions about the role of high WSS and high WSSG in vascular pathophysiology. It has been described that WSS is sensed by the EC through a cascade of intracellular events including activation of ion channels, realignment of cytoskeleton fibers, and changes in gene expressions (6). Chronic high WSS is known to induce adaptive outward remodeling of arteries, resulting in increased lumen diameter and wall thickness (40). This physiological response is through an increase in eNOS expression and subsequent NO production (10,40), followed by cell proliferation (30,35). However, aneurysm initiation is associated with local decrease in eNOS expression (19), endothelial denudation (18), and cell apoptosis (25). Such pathological response to high WSS and high WSSG might suggest that the coexistence of WSSG compromises the normal, physiological response to high WSS. Limited in vitro studies suggest that WSSG might have detrimental effects on ECs when occurring in conjunction with low WSS (7,8,11), a hemodynamic environment that has a propensity for atherogenesis in vivo (9).

The preferential localization of aneurysmal molecular changes in the high, positive WSSG region is intriguing. It could be speculated that under a net stretching force exerted by the positive shear stress gradient, endothelial cell-cell junctions experience a large “pulling” force that weakens intercellular contact. A loss of intercellular contact has been linked to reduced eNOS expression and activity (6,17), and this could lead to EC loss and onset of destructive remodeling.

Admittedly, this study is limited by the inherent quantitation difficulties with the immunohistochemistry method, but methods such as Western blotting and quantitative polymerase chain reaction do not permit spatial localization of molecules. However, the novelty of the study is not quantification of altered molecules but correlation of the molecular changes with certain hemodynamic regions. Another limitation lies in the fact that the bifurcation is not a human vessel wall and it uses extracranial vessels. Intracranial vessels differ structurally from their extracranial counterparts in that they have a prominent internal elastic lamina, a relatively thin media, and an insignificant adventitia, surrounded by cerebrospinal

fluid in the subarachnoid space (34). However, it is important to note that this model only mimics the very upstream events associated with aneurysm initiation: the elevated hemodynamic insult in a complex geometry (flow impingement and acceleration) and the inside-out early response from endothelium to the media. Precisely because of its large size, the canine carotid bifurcation model is uniquely powerful, permitting accurate angiographic imaging and CFD calculation to quantitatively pinpoint the shear stress and gradient zones and colocalize with the altered molecular expressions. Our future studies may involve using intracranial vasculature in a canine model; however, the early information is important. These results underscore the significance of hemodynamics in aneurysm initiation through a direct linkage between hemodynamic factors and the secondary molecular changes that are likely causal in aneurysm formation.

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Abbreviations

3D	3-dimensional
CCA	common carotid artery
CFD	computational fluid dynamics
EC	endothelial cell
eNOS	endothelial nitric oxide synthase
IA	intracranial aneurysm
IL	interleukin
iNOS	inducible nitric oxide synthase
MMP	matrix metalloproteinase

NO	nitric oxide
O₂	superoxide
SMC	smooth muscle cell
WSS	wall shear stress
WSSG	wall shear stress gradient

FIGURE 1.

De novo bifurcation model. **A**, schematic of bifurcation creation. The left common carotid artery (LCCA) is transected proximally. L2 is mobilized under the trachea to the right side. The right common carotid artery (RCCA) is then proximally and distally closed with atraumatic clips and divided. R2 is anastomosed end to side to L2. An arteriotomy is performed on that connected segment and R1 is anastomosed, resulting in a Y-shaped bifurcation. (Adapted from Meng H, Swartz DD, Wang Z, Hoi Y, Kolega J, Metaxa EM, Szymanski MP, Yamamoto J, Sauvageau E, Levy EI: A model system for mapping vascular responses to complex hemodynamics at arterial bifurcations in vivo. *Neurosurgery* 59:1094–1101, 2006 [28].) **B**, histological sections illustrating an example of an aneurysmal bulge with extensive internal

elastic lamina loss (between the arrows) near the bifurcation apex (van Gieson staining) 2 months after bifurcation creation.

FIGURE 2.

Hemodynamic stress experienced by a de novo bifurcation 2 months after bifurcation creation. **A**, surface plot of wall shear stress (WSS). **B**, distributions of WSS (blue, left y-axis) and WSS gradient (WSSG) (red, right y-axis) along the wall in the periapical right branch (solid lines) compared with the feeding artery (dashed lines) in the respective boxes indicated in panel **A**. The blue band at the bottom represents the average baseline WSS (<2 Pa) in a native CCA. II, acceleration region; III, recovery region.

FIGURE 3.

Molecular expressions in the aneurysmal-initiating remodeling region. **A**, exemplar immunostaining for matrix metalloproteinase (MMP)-2, MMP-9, nitrotyrosine (NT), endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS), and interleukin (IL)-1 β —in the aneurysmal remodeling region compared with the feeding artery in the same animal (each bar = 100 μ m). **B**, quantitation of staining intensity in aneurysmal remodeling region compared with feeding arteries and unmanipulated common carotid arteries (CCAs) for all samples. The expressions in the aneurysmal region are significantly higher than those in the 2 control tissues (one-way analysis of variance [ANOVA] and Bonferroni post hoc correction, $P < 0.01$). **C**, absence of macrophages in the aneurysmal remodeling. Left, negative

anti-CD68 staining in the aneurysmal remodeling region in a 2-month created bifurcation. Right, positive anti-CD68 staining of macrophages (brown) in canine spleen. Scale bar = 100 μm .

FIGURE 4.

Molecular changes confined within specific hemodynamic regions in the branches from 3 of the dogs 2 weeks (A) or 2 months (B–D) after bifurcation creation. A–D, colocalization of elevated (matrix metalloproteinases [MMP], inducible nitric oxide synthase [iNOS], interleukin [IL]-1 β , and nitrotyrosine [NT]) or reduced (endothelial nitric oxide synthase [eNOS]) protein expressions with the acceleration region (II). The origin (zero) denotes the flow impingement point at the bifurcation apex. Shaded areas indicate the positive wall shear stress gradient (WSSG) regions. Bifurcation geometry is presented in the right upper corner with arrows showing the branch with the molecular mapping. E, total lengths of aneurysmal

changes by various molecular markers in positive WSSG regions are significantly greater than in negative WSSG regions (unpaired t test, $P < 0.05$).