Alternative Splicing of Endothelial Fibronectin Is Induced by Disturbed Hemodynamics and Protects Against Hemorrhage of the Vessel Wall

Patrick A. Murphy, Richard O. Hynes

Objective—Abnormally low-flow conditions, sensed by the arterial endothelium, promote aneurysm rupture. Fibronectin (FN) is among the most abundant extracellular matrix proteins and is strongly upregulated in human aneurysms, suggesting a possible role in disease progression. Altered FN splicing can result in the inclusion of EIIIA and EIIIB exons, generally not expressed in adult tissues. We sought to explore the regulation of FN and its splicing and their possible roles in the vascular response to disturbed flow.

Approach and Results—We induced low and reversing flow in mice by partial carotid ligation and assayed FN splicing in an endothelium-enriched intimal preparation. Inclusion of EIIIA and EIIIB was increased as early as 48 hours, with negligible increases in total FN expression. To test the function of EIIIA and EIIIB inclusion, we induced disturbed flow in EIIIA−/− mice unable to include these exons and found that they developed focal lesions with hemorrhage and hypertrophy of the vessel wall. Acute deletion of floxed FN caused similar defects in response to disturbed flow, consistent with a requirement for the upregulation of the spliced isoforms, rather than a developmental defect. Recruited macrophages promote FN splicing because their depletion by clodronate liposomes blocked the increase in endothelial EIIIA and EIIIB inclusion in the carotid model.

Conclusions—These results uncover a protective mechanism in the inflamed intima that develops under disturbed flow, by showing that splicing of FN mRNA in the endothelium, induced by macrophages, inhibits hemorrhage of the vessel wall. (Arterioscler Thromb Vasc Biol. 2014;34:2042-2050.)

Key Words: alternative splicing • fibronectins • hemorrhage • macrophages

Risk of aneurysm is determined globally by genetic defects and locally by hemodynamic effects. Genetic risk is often traced to defects in extracellular matrix (ECM) proteins, such as fibrillin1, collagens, fibulin-4, and elastin, in the case of thoracic aneurysm, and collagens in intracranial aneurysm and hemorrhagic stroke. Other pathways, such as transforming growth-factor-β and lysyl oxidase, are indirectly linked to ECM protein deposition or organization. However, even given pre-existing genetic defects, not all blood vessels develop aneurysms; hemodynamics are a critical determinant of the location of aneurysms. For example, intracranial aneurysms are more likely to grow and rupture in regions of the vasculature with disturbed flow. The frictional force of flow, or shear stress, is sensed by the endothelium, which plays a critical role in regulating the vessels’ response to these flows. Thus, ECM composition and the endothelial response to disturbed flow are key determinants of aneurysm progression.

Fibronectin (FN) is a principal component of the vascular ECM and is highly upregulated in human aneurysm. The assembly of FN is considered a crucial step toward the assembly of new vascular ECM and essential for the matrix integration of many of the aforementioned aneurysm-linked proteins. FN production and deposition by the endothelium are increased in vitro by low and disturbed flow, and increased FN has been observed in areas of vasculature chronically exposed to disturbed flow. However, because FN deposition also seems to increase the proinflammatory signaling of overlying endothelial cells (ECs) potently and play a prominent role in leukocyte recruitment in vivo, the role of FN in vasculature exposed to low and disturbed flow is not yet clear. Although its structural function might suggest protection, it could also promote proinflammatory signaling thought to drive aneurysm progression. Thus, although the role of FN in aneurysm is not yet clear, it is poised to be a major player in regulating disease progression.

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FN is alternatively spliced, resulting in variable inclusion of the EIIIA (EDA) and EIIIB (EDB) domains and portions of the variable (V or IIICS) domain. Among vertebrates, EIIIA and EIIIB domains are as highly conserved at the amino acid level as other FN domains, suggesting strong evolutionary pressure. Their expression patterns are conserved among all vertebrates.
examined, including human, mouse, rat, cow, chicken, frog, and zebrafish, most prominently around the developing heart and blood vessels. Nearly complete inclusion during early development drops to <5% to 10% postnatally. Although adults express little EIIIA or EIIIB, inclusion is increased in a variety of vascular injuries resulting in damage to the endothelium, such as balloon or wire injury in animal models. Increased inclusion of EIIIA and EIIIB has also been observed in human aneurysms. But how expression of EIIIA and EIIIB is regulated in aneurysm progression remains unclear. Also unclear is how the production of EIIIA+EIIIB+FN affects vasculature damaged by exposure to low and disturbed flow.

We hypothesized that low and disturbed flow might promote the increased inclusion of alternative exons EIIIA and EIIIB in the arterial endothelium and, furthermore, that the upregulation of these alternative exons might play a role in preventing the destructive effects of low and disturbed flow on the arterial endothelium. Here, we describe experiments supporting these hypotheses.

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Results
Low and Disturbed Arterial Flow Promotes Increased Inclusion of FN Alternative Exons, EIIIA and EIIIB
To examine alternative splicing in response to low and disturbed arterial flow, we performed near-complete carotid ligation on C57BL/6J mice. This severely reduces mean blood velocity in the ligated carotid artery (by ≈75%; Figures 1 and 4) and results in the proinflammatory activation of the endothelium. We collected RNA from the intima of these mice by a Trizol flush following published methods, yielding a 3000-fold enrichment of endothelial mRNA Pecam1 (CD31) relative to smooth muscle mRNA acta2 (α-smooth muscle actin) in the intimal preparation relative to the remainder of the carotid artery (Figure IA in the online-only Data Supplement). Purified intimal RNA was pooled from 3 groups of 4 to 5 vessels for each flow regime: low-flow (ligated) or high-flow (contralateral to the ligated artery). The mRNA pool was sequenced, and splicing was assessed by MISO (mixture-of-isomorphs). We found that, at 48 hours, the levels of both EIIIA and EIIIB inclusion in the intima increased by ≈3-to-4-fold over the levels in contralateral carotid intima (Figure 1A and 1B). Baseline levels of EIIIA inclusion (17%) were higher than EIIIB inclusion (5%), as were the levels under disturbed flow (55% for EIIIA and 19% for EIIIB).

RNA-seq analysis provided an aggregate assessment of splicing changes in a large pool of mice. To determine splicing changes with greater resolution in the intima of individual carotid arteries, we established a quantitative polymerase chain reaction assay to measure fold changes in the levels of EIIIA+ and EIIIB+ mRNAs relative to total FN mRNA (Figure II in the online-only Data Supplement). We extrapolated the actual inclusion rates from the quantitative polymerase chain reaction data, using cDNA prepared from sequenced samples to calibrate.

Using these methods, we examined splicing in 2 more cohorts of mice, at 48 hours and at 7 days after nearly complete carotid ligation. We found that the mean changes in inclusion observed by quantitative polymerase chain reaction in these new cohorts replicated the results obtained by RNA-seq. Under low-flow conditions, EIIIA and EIIIB inclusion rates increased 2-to-3-fold (from 19% to 47% for EIIIA and from 5% to 11% for EIIIB) over the high-flow contralateral carotid at 48 hours (Figure 1C). The high inclusion levels were maintained at the 7-day time point (Figure 1D). We included sham-operated mice to control for splicing changes induced by the trauma of the surgery in the absence of flow changes. Although surgery alone induced a slight increase in EIIIA and EIIIB inclusion at 48 hours, this was less than the change induced by low and disturbed flow (compare ligated low [L] versus sham noΔ[L] at 48 hours), and the effect disappeared by 7 days. Interestingly, the change in splicing occurred without a significant change in the total FN expression at either 48 hours or 7 days (Figure 1E and 1F). Thus, low and disturbed flow acutely increases intimal EIIIA and EIIIB inclusion, as measured by 2 independent techniques, and this occurs without significant increases in total FN expression.

We then asked whether similar changes occurred in the media and adventitia, or whether they were confined to the intima. We used the same quantitative polymerase chain reaction approach, except that analysis was performed on the remainder of the carotid artery, after the intimal flush. We found that, in contrast to the intimal changes, there were no significant differences in splicing between ligated and sham-operated arteries at 48 hours (Figure IIIA in the online-only Data Supplement). After exposure to 7 days of low and disturbed flow, inclusion of both EIIIA and EIIIB was increased ≈2-fold in the media and adventitia relative to the sham-operated arteries at 48 hours (Figure IIIA in the online-only Data Supplement). After exposure to 7 days of low and disturbed flow, inclusion of both EIIIA and EIIIB was increased ≈2-fold in the media and adventitia relative to the sham-operated arteries at 48 hours (Figure IIIA in the online-only Data Supplement). After exposure to 7 days of low and disturbed flow, inclusion of both EIIIA and EIIIB was increased ≈2-fold in the media and adventitia relative to the sham-operated arteries at 48 hours (Figure IIIA in the online-only Data Supplement). After exposure to 7 days of low and disturbed flow, inclusion of both EIIIA and EIIIB was increased ≈2-fold in the media and adventitia relative to the sham-operated arteries at 48 hours (Figure IIIA in the online-only Data Supplement). After exposure to 7 days of low and disturbed flow, inclusion of both EIIIA and EIIIB was increased ≈2-fold in the media and adventitia relative to the sham-operated arteries at 48 hours (Figure IIIA in the online-only Data Supplement). After exposure to 7 days of low and disturbed flow, inclusion of both EIIIA and EIIIB was increased ≈2-fold in the media and adventitia relative to the sham-operated arteries at 48 hours (Figure IIIA in the online-only Data Supplement). After exposure to 7 days of low and disturbed flow, inclusion of both EIIIA and EIIIB was increased ≈2-fold in the media and adventitia relative to the sham-operated arteries at 48 hours (Figure IIIA in the online-only Data Supplement).
These results indicate that the endothelium is the primary source of alternatively spliced FN in the intimal response to altered shear.

Absence of EIIIA and EIIIB Inclusion Promotes Hemorrhage of the Vessel Wall in Response to Low and Disturbed Flow

To test the function of EIIIA and EIIIB inclusion in the vascular response to low flow, we performed carotid ligation in EIIIAB−/− mice unable to include EIIIA and EIIIB. After 7 days of low flow, carotid arteries of EIIIAB−/− mice showed localized hemorrhage of the vessel wall, typically associated with bulges in the artery (Figure 2A).

To quantify these differences further, excised vessels were scored for an injury phenotype based on the degree of hemorrhage and bulging of the wall (Figure IV in the online-only Data Supplement). To confirm the presence of blood beneath the endothelium, we performed immunofluorescence staining for the EC marker Pecam1 (CD31) and the erythrocyte marker Ter119. We found large areas of Ter119-labeled blood cells beneath the CD31-labeled endothelium, confirming that blood had leaked into the vessel wall and beneath the endothelium.

Figure 1. Low and disturbed flow promotes inclusion of alternative exons EIIIA and EIIIB. A, Relative read densities in alternative exon EIIIA (EDA) of fibronectin (EIIIA), alternative exon EIIIB (EDB) of fibronectin (EIIIB), and flanking exons from the sequenced intimal RNA of low-flow (ligated) and high-flow (contralateral) carotid arteries 48 hours after surgery. Numbered curves indicate exon-spanning reads. B, Inclusive and exclusive reads were used by MISO (mixture-of-isofoms) to calculate the percentage inclusion rate (Psi) of alternative fibronectin (FN) exons EIIIA and EIIIB. Average of 3 pools (4–5 arteries each) and the SD is shown. C and D, Exon inclusion in individual carotid arteries as measured by quantitative polymerase chain reaction at 48 hours (C) and 7 days (D) after nearly complete carotid ligation or sham operation. E and F, Fold-change in FN over sham-operated contralateral artery. Each dot (C–F) represents analysis of the intimal flush from a single carotid artery. Significance of the difference between (B) low-flow and high-flow pools by Student t test and (C–F) the ligated left carotid measurements and the other groups by ANOVA and Tukey multiple comparison test: **P<0.01, ***P<0.001, and ****P<0.0001.

Figure 2. Mice deficient in alternative Fibronectin exons EIIIA (EDA) and EIIIB (EDB) are prone to subendothelial hemorrhage in response to low and disturbed flow. A, Isolated carotid arteries from EIIIAB−/− (EIIIAB−/−) mice after 7 days of low flow and disturbed flow conditions. Note the visible extravascular blood in the EIIIAB−/− mouse after saline perfusion (arrowheads). B, Combined staining for CD31 and Ter119 shows blood cells beneath the endothelium. Percentage of mice with extravascular blood after saline perfusion is shown in the graph. P=0.04 by 2-tailed Fisher exact test. C, Mean ultrasound velocity from in vivo measurements at 7 days after nearly complete carotid ligation. Each dot is the measurement of a single carotid artery. Red dots indicate arteries with subendothelial hemorrhage. Scale bars, 100 and 25 μm (higher magnification).
endothelium (Figure 2B; Figure V in the online-only Data Supplement). Although hemorrhages also occurred in the wild-type littermate controls, they were much less frequent (14% versus 47%; P=0.02, Fisher exact test). Importantly, we observed no differences in the level of flow reduction induced by carotid ligation in EIIIAB−/− and EIIIAB+/+ mice, indicating that the inability to splice FN alternatively is the underlying cause of the hemorrhages, rather than a quantitative difference in the hemodynamic stimulus (Figure 2C).

Histological analysis of the arteries suggests that a local expansion of the adventitia may contribute to the appearance of the bulged regions because significant differences could be observed in thickness of the adventitia across multiple regions of EIIIAB−/− arteries versus EIIIAB+/+ or EIIIAB−/+ arteries (Figure VI in the online-only Data Supplement). In addition to the localized expansion of the adventitia, we also observed focal dilation of the artery lumen in vivo, by high-resolution ultrasound images collected at the 7-day end point. These focal dilations resulted in regions 30% to 70% larger than ultrasound images collected at the 7-day end point. These focal dilation of the artery lumen in vivo, by high-resolution ultrasound images collected at the 7-day end point. These focal dilations resulted in regions 30% to 70% larger than ultrasound images collected at the 7-day end point. These focal dilations resulted in regions 30% to 70% larger than ultrasound images collected at the 7-day end point. These focal dilations resulted in regions 30% to 70%

Because the hemorrhage and injury we observed resembled the development of vessel-wall hemorrhage in EIIIAB−/− mice led us to question whether the absence of EIIIA and EIIIB resulted in reduced FN expression or deposition. We examined constitutive FN mRNA in the intimal flush and in the remaining media and adventitia of EIIIAB−/− mice and littermate controls subjected to near complete carotid ligation. We found that there was no difference in the level of total FN mRNA in the intima at 7 days after nearly complete carotid ligation in EIIIAB−/− mice relative to their littermate controls (Figure 3A). So, the absence of EIIIA and EIIIB did not impinge on the levels of FN transcript in either the intima or the media and adventitia.

At the protein level, results were similar. Increased FN deposition could be observed in the intima of ligated carotid arteries, relative to sham-operated controls, but the level of staining was not significantly reduced in EIIIAB−/− mice (Figure 3B). Instead, there was increased deposition of FN in the intima of EIIIAB−/− mice, as determined by quantitative immunofluorescence (Figure 3C). Although all mice that developed vessel-wall hemorrhage had high levels of intimal FN, only in the EIIIAB−/− group was FN deposition increased in the absence of severe injury (score ≥2). It is not yet clear whether the increased FN deposition represents leak of plasma FN; extravasation of plasma albumin was not significantly increased by the same methods although it was increased relative to the contralateral artery (Figure X in the online-only Data Supplement).

Acute Deletion of FN Promotes Hemorrhage of the Vessel Wall in Response to Low and Disturbed Flow

Hemorrhage in response to low and disturbed flow could be a consequence of the absence of the EIIIA and EIIIB isoforms throughout vascular development, the inability to increase the expression of these isoforms under low and disturbed flow, or both. To tease apart these possibilities, we investigated whether there was an acute requirement for FN expression in the response to low and disturbed flow. We had previously observed that the acute deletion of floxed FN in the endothelium by Cdh5-CreER72 almost entirely ablated endothelial FN mRNA (Figure I in the online-only Data Supplement) but did not predispose to flow-induced hemorrhage (Figure 4; Figure
and Cdh5-CreERT2 to delete endothelial FN acutely <1 week before carotid ligation in the absence of smooth muscle FN.

As expected, Rosa26-CreERT2 effectively activated a Cre reporter throughout most, but not all, of the endothelial and smooth muscle cells (Figure 4) and ablated plasma FN within 2 days (data not shown), resulting in a nearly complete inhibition of FN protein deposition in the intima of vessels exposed to low and disturbed flow (Figure 4). Without the ability to increase FN production acutely, arteries were susceptible to hemorrhage (2/8 mice and none of the littermate controls) and almost all of the arteries demonstrated a failure in inward remodeling (Figure 4; Figures IV and VII in the online-only Data Supplement). Importantly, neither injury nor hemorrhage was observed in the contralateral arteries or sham-operated Rosa26-CreERT2; FNf/f arteries, indicating that enlargement of the artery and hemorrhage was specifically induced by low and disturbed flow conditions, rather than by the acute ablation of FN expression.

However, Rosa26-CreERT2 removes all sources of FN production, including EIIIA− EIIIB− plasma FN. To address more specifically the acute requirement for the EIIIA+ and EIIIB+FN variants produced by the endothelium and to delete FN more stringently in the vessel wall, we examined Cdh5-CreERT2; SM22-Cre; FNf/f mice after the acute deletion of endothelial FN (<1 week before nearly complete carotid ligation). SM22-Cre allowed stronger deletion of FN in the vessel wall than Rosa26-CreERT2, and Cdh5-CreERT2 was more effective in the deletion of endothelial FN than Rosa26-CreERT2. Despite abundant FN, presumably plasma-derived and EIIIA− EIIIB−, in the intima, arteries unable to produce EIIIA+ and EIIIB+FN variants in the endothelium-developed hemorrhage. Importantly, none of these defects were observed in SM22-Cre; FNf/f littermates, which were born in Mendelian ratios without noticeable vascular defects (Figure 4; Figure IV in the online-only Data Supplement) and resembled littermate FNf/f mice in carotid structure and response to low and disturbed flow, indicating that the defects observed were a result of the absence of endothelial FN.

Therefore, both approaches that acutely blocked the ability to produce new endothelial EIIIA+EIIIB+FN replicated the hemorrhages in the vessel wall observed in EIIAB−/− mice. Notably, of the 2 approaches, a stronger phenotype was observed using Cdh5-CreERT2; SM22-Cre; FNf/f mice after the acute deletion of endothelial FN (<1 week before nearly complete carotid ligation), Cdh5-CreERT2 was more effective in the deletion of endothelial FN than Rosa26-CreERT2. Despite abundant FN, presumably plasma-derived and EIIIA− EIIIB−, in the intima, arteries unable to produce EIIIA+ and EIIIB+FN variants in the endothelium-developed hemorrhage. Importantly, none of these defects were observed in SM22-Cre; FNf/f littermates, which were born in Mendelian ratios without noticeable vascular defects (Figure 4; Figure IV in the online-only Data Supplement) and resembled littermate FNf/f mice in carotid structure and response to low and disturbed flow, indicating that the defects observed were a result of the absence of endothelial FN.

Macrophages Are Required for the Induction of EIIIA and EIIIB in the Vessel Wall

Given the importance of the endothelial FN splicing switch in preventing the damaging effects of disturbed blood flow on the vessel wall, we investigated how the splicing switch is regulated. We found that macrophages, which are often recruited to sites of injury where EIIIA and EIIIB expression is increased, are recruited to the carotid arterial endothelium under disturbed flow conditions (Figure XI in the online-only Data Supplement). However, a more potent phenotype was observed using Cdh5-CreERT2; SM22-Cre; FNf/f mice after the acute deletion of endothelial FN (<1 week before nearly complete carotid ligation), Cdh5-CreERT2 was more effective in the deletion of endothelial FN than Rosa26-CreERT2. Despite abundant FN, presumably plasma-derived and EIIIA− EIIIB−, in the intima, arteries unable to produce EIIIA+ and EIIIB+FN variants in the endothelium-developed hemorrhage. Importantly, none of these defects were observed in SM22-Cre; FNf/f littermates, which were born in Mendelian ratios without noticeable vascular defects (Figure 4; Figure IV in the online-only Data Supplement) and resembled littermate FNf/f mice in carotid structure and response to low and disturbed flow, indicating that the defects observed were a result of the absence of endothelial FN.

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IV in the online-only Data Supplement). However, our analysis of FN splicing had revealed increased EIIIA+EIIIB+FN mRNA in the adjacent media after carotid ligation (Figure IIIC in the online-only Data Supplement), suggesting that smooth muscle cells may be compensating for endothelial FN production. Further supporting this line of reasoning, there was a trend toward increased expression of EIIIA+EIIIB+FN in the media and adventitia of EIIIAB+/+ and EIIIAB−/− mice 7 days after ligation or sham operation. Each dot represents analysis of the intimal flush from a single carotid artery. A, Immunofluorescence staining for total FN (red) and CD31 (green) in sections of carotid arteries, from a single carotid artery.

Figure 3. Deficiency in alternative fibronectin exons EIIIA (EDA) and EIIIB (EDB) does not inhibit fibronectin (FN) expression or deposition. A, Expression of FN mRNA in the intima and media/adventitia of EIIIAB+/+ and EIIIAB−/− mice 7 days after ligation or sham operation. Each dot represents analysis of the intimal flush from a single carotid artery. B, Immunofluorescence staining for total FN (red) and CD31 (green) in sections of carotid arteries, 7 days after ligation, with and without severe injury. C, Fluorescence intensity in sections of ligated carotid arteries in the intima or in the medial cell layers. Each dot represents a single carotid artery. **P<0.01 by 2-tailed Student t test. Scale bars, 100 μm.
Data Supplement). Therefore, we hypothesized that they might promote EIIIA and EIIIB inclusion.

To test this, we used clodronate liposomes to deplete macrophages in mice subjected to carotid ligation and examined EIIIA and EIIIB expression in the intimal flush. Analysis of the macrophage-specific mRNA Cd68 revealed that the 7-fold increase induced by low and disturbed flow conditions could be completely blocked by clodronate-liposome treatment (Figure 5A). Inhibiting the recruitment of macrophages resulted in a suppression of increased FN expression (Figure 5B) and a complete abrogation of the induction of EIIIA and EIIIB by low and disturbed flow (Figure 5C and 5D). Therefore, the switch in endothelial splicing under low and disturbed flow depends on macrophages, which are recruited in large numbers under these conditions.

Discussion

Low and disturbed arterial flow is known to promote aneurysm growth and rupture.5,6 The ECM is profoundly altered in late-stage aneurysms, but how disturbed flow regulates ECM composition in vivo remains poorly characterized. Furthermore, how acute changes in individual ECM components during aneurysm formation might contribute to or inhibit further progression is unclear. Here, we show that macrophages, recruited by disturbed flow,29 promote a rapid change in FN splicing resulting in increased expression of EIIIA+ and EIIIB+ variants as early as 48 hours after a change in flow. The acute upregulation of these isoforms inhibits hemorrhage of the vessel wall, revealing a protective splicing mechanism in the vascular endothelium of regions exposed to disturbed flow severely.

Upregulation of Alternative Splice Variants of FN Protects Against Hemorrhage of the Vessel Wall Under Low and Disturbed Flow

Our results suggest that alternative FN splicing in the vessel wall is an important protective mechanism against hemorrhagic rupture of the intima and, therefore, may play a role in the growth and rupture of areas of aneurysm exposed to disturbed flow. Increased EIIIA and EIIIB inclusion has been observed in late-stage human aortic aneurysms.22 Interestingly, EIIIA failed to increase in a subgroup of patients at higher risk of dissecting aneurysm.22 The same patient group (bicuspid aortic valve) is also at increased risk of intracranial aneurysm, although it is not clear whether this is because of altered FN splicing.30 Given the phenotypic complexity of human samples, these observations remained correlative. Here, we provide genetic evidence that deficient EIIIA and EIIIB inclusion directly increases the risk of damage to the vessel wall.

It is not clear why deficiency in EIIIA and EIIIB inclusion promotes hemorrhage in response to disturbed flow but not in response to Ang-II. Perhaps a clue lies in the cell types most affected. Low and disturbed flow acts primarily on the endothelium. In the carotid ligation model, macrophage accumulation and changes in FN splicing were strongest in the intima. In contrast, it has been proposed that macrophages recruited...
to the adventitia drive aortic dissection in the Ang-II model. Our data show that both macrophage recruitment and alternative FN splicing are reduced in the Ang-II intima, relative to the intima of vessels exposed to low and disturbed flow. Thus, we suggest that alternative FN splicing will be of greatest importance in preventing vascular damage in diseases with a strong component of endothelial activation and inflammation.

The mechanisms of protection conferred by EIIIA and EIIIB are of considerable interest. Several might be proposed: (1) EIIIA is known to bind and activate TLR4. This could skew macrophage phenotype directly or affect the presentation of autoantigens from the lesion to innate immune cells (EIIIA has been shown to be a potent antigen, and immunization of mice with FN decreases atherosclerotic vascular injury)\(^3\); (2) EIIIA inclusion in full-length recombinant FN has been shown to increase cell spreading in vitro, suggesting that it may affect proliferation and apoptosis pathways linked to spreading\(^3\); (3) suppressing EIIIA and EIIIB inclusion in ECs may affect fibrillogenesis,\(^3\) and thus both EC-cell junctions and permeability\(^3\); (4) Mice with increased EIIIA inclusion clot faster under arterial flow rates,\(^3\) suggesting that increasing EIIIA (and perhaps also EIIIB) inclusion may help quickly staunch intimal hemorrhage. A better understanding of how EIIIA and EIIIB prevent vessel damage should reveal new ways in which the vasculature locally resists flow-induced damage.

**Macrophages Promote the Increased EIIIA and EIIIB Inclusion in the Endothelium**

We show that macrophage recruitment is critical for the increased expression of EIIIA and EIIIB. In a variety of other fibrotic injury settings, such as organ transplant rejection, and skin, lung, kidney, and liver damage, and cancer, EIIIA and EIIIB inclusion is increased.\(^1\) Among isolated cell types from the liver, ECs showed the strongest increase in inclusion.\(^3\) This switch is thought to be one of the earliest steps in fibrosis, which is EIIIA dependent in both lung and liver injury models.\(^3\) Our data suggest that recruitment or activation of macrophages could provide signals to promote EIIIA inclusion. The specific mechanisms through which they induce FN splicing are not yet clear, but we have observed a good correlation between plasminogen activator inhibitor 1 (PAI1) and the inclusion of EIIIA and EIIIB, as well as total FN expression (Figure XII in the online-only Data Supplement), perhaps implicating transforming growth factor-β, known to regulate PAI1 and EIIIA inclusion in vitro.\(^4\)

Conditional deletion of macrophages has revealed a critical role in driving fibrosis in the liver and the lung.\(^4\) So, in a variety of fibrotic settings, macrophages may play a similar role in the induction of alternative FN splicing, with important implications for the composition and signaling of the fibrotic ECM.

Increased numbers of macrophages are found in ruptured intracranial aneurysms, and clodronate depletion experiments suggest macrophages drive the growth of intracranial aneurysms in elastase-induced animal models.\(^4\) We observed that the chronic depletion of macrophages in our carotid ligation model acutely suppresses the flow-mediated activation of the endothelium (indicated by reduced intimal PAI1 and α-smooth muscle actin expression; Figure XIII in the online-only Data Supplement) and arterial stiffness (indicated by pulse distension; Figure XIII in the online-only Data Supplement), markers of arterial injury. Our current model (Figure XIV in the online-only Data Supplement) is that the exposure of the arterial endothelium to reduced blood flow increases the expression of leukocyte adhesion receptors (eg, Icam1 [intercellular adhesion molecule 1] and Vcam1 [vascular cell adhesion protein 1]), resulting in the recruitment of blood-derived monocytes to the intima. Monocytes differentiate to macrophages and elicit changes in the endothelium, including the alternative splicing of FN, that are critical in preventing damage to the vessel wall. Because macrophages are also instigators of arterial injury, alternative FN splicing in the endothelium may be thought of as a mechanism induced by macrophages that protects against their own damaging effects on the vessel wall.

**Acute Flow-Induced Hemorrhage of the Vessel Wall**

Hemorrhage in the carotid artery under disturbed flow came as a surprise because other investigators used the same model and even injected FN assembly (using a small peptide inhibitor) but did not report vessel-wall hemorrhage.\(^4\) A likely explanation is that the penetrance of this phenotype on the wild-type background is low (4/27 AB\(^+/−\) littermate controls, 0/15 C57 mice\(^+/−\) clodronate liposomes, 1/9 EIIIA\(^+/−\) littermate controls, 0/22 FN\(^+/−\) or FN\(^+/−\) littermate controls), and may have escaped the notice of previous investigators. In a typical cohort of 5 to 10 mice, such defects would be rare. However, in the EIIIA\(^+/−\), ROSA-CreER; FN\(^+/−\), and Cdhr5-CreER; SM22-Cre; FN\(^+/−\) mice, hemorrhage within the vessel wall was seen in higher numbers (9/19, 2/8, and 2/8, respectively) and thus was obvious. It is also possible that hemorrhage
occurs acutely and is resolved by later, 2- to 3-week time points; we have not yet examined this in detail. Consistent with the idea that vessel-wall hemorrhage is a partially penetrant phenotype that can be exacerbated by genetic background, hemorrhage has been observed in the carotid plaques of ApoE-deficient mice subjected to nearly complete carotid ligation.

Similar to these ApoE−/− hemorrhages, we noted endothelial sprouting into the vessel wall76 (Movies I and II in the online-only Data Supplement). Interestingly, our en face imaging suggests that these sprouts originate from the lumen, rather than the vasa vasorum. Therefore, we suggest that the established carotid ligation model can be used as a platform to investigate the contributions of various genetic and epigenetic regulators of disturbed flow on intimal damage and hemorrhage of the vessel wall.

In conclusion, we report that the alternatively spliced EIIIA and EIIIB exons of FN, long-known to be conserved and highly regulated in all vertebrates but without clearly understood functions, play a significant role in protecting the vasculature from inflammation caused by disturbed flow, leading to rapid endothelial dysfunction and atherosclerosis. The subendothelial extracellular matrix modulates NF-κB activation by flow: a potential role in atherosclerosis. J Cell Biol. 2005;169:191–202.

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Aneurysms are fairly common in the brain, aorta, and elsewhere, but their catastrophic rupture is not. Disturbed flow within brain aneurysms is one of the best predictors of rupture, risk of which is strongly modified by extracellular matrix composition. Fibronectin (FN), an essential extracellular matrix protein, is abundant in human aneurysms where it is also alternatively spliced. However, neither the regulation of alternative FN isoform regulation in Mammalian tissues. (2012;338:1593–1599).

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Figure I. Measurement of Gene Splicing in the Endothelium.

A) Levels of CD31 and alpha-smooth muscle actin mRNAs were assayed by quantitative PCR in the intimal flush and in the remaining media/adventitia. Graph shows the relative levels of CD31 to alpha-smooth muscle actin in the intima, as fold increase over the media/adventitia. B) Images show the low flow carotid artery 7 days after ligation, the mT/mG reporter switches from red to green upon Cre excision. Similar results were observed in the contralateral and sham-operated arteries. C) Expression of total, EIIIA+ or EIIIB+ FN mRNA, in the intima and media/adventitia 7 days after nearly complete carotid ligation, as determined by quantitative PCR in mice with and without deletion of endothelial FN. Expression is shown as a percentage of similarly treated control arteries (littermate controls without Cre excision). Each dot represents analysis of the intimal flush from a single carotid artery.
Figure II. Validation of quantitative PCR primers for measurement of EIIIA and EIIIB inclusion.
A) Location of the probes used in quantitative PCR analysis of total FN, EIIIA or EIIIB marked on a schematic of the FN protein domains. B) Correlation between the change in threshold cycle (CT) and fold change of EIIIA- and EIIIB-inclusive cDNA, determined by serial dilutions of cDNA from AB+/+ aortic endothelial cells in cDNA from AB−/− aortic endothelial cells. Levels of constitutive FN did not vary between the cDNA pools. C) Registration of relative changes in EIIIA and EIIIB inclusion, determined by qPCR, to the absolute percent inclusion, determined by MISO analysis of RNA-seq data, from the same pools of intimal flush RNA. Each dot represents analysis of the intimal flush from a pool of 4-5 carotid arteries.
Figure III. Changes in FN expression and splicing in the media/adventitia of the carotid artery under low and disturbed flow.
Gene expression in carotid media/adventitia RNA as measured by quantitative PCR at 48hrs (A&B) and 7 days (C&D) after nearly complete carotid ligation or sham operation. (A&C) Exon inclusion in individual carotid arteries as measured by quantitative PCR. (B&D) Fold-changes are relative to sham-operated contralateral carotid. Each dot represents analysis of the intimal flush from a single carotid artery. Significance of the difference between the ligated left carotid measurements and the sham-operated measurements by ANOVA and Tukey’s multiple comparison test: *P<0.5, **P<0.01, ***P<0.001, ****P<0.0001.
Figure IV. Quantification of injury phenotype in carotid arteries.
A) Images showing a range of carotid injury phenotypes in EIIIA/B-/- carotid arteries 7 days after nearly complete carotid ligation along with the scoring system used. B) Scoring of carotid phenotype 7 days after nearly complete carotid ligation or sham operation. Each point represents a single carotid artery.
Figure V. Hemorrhage in the media between elastin layers
Combined staining for CD31 and Ter119 shows blood cells beneath the endothelium, and between the autofluorescent elastin layers of the vessel wall.
Figure VI. Local variation in adventitia thickness in EIIIAB-/− arteries exposed to low and disturbed flow

A) Focal expansion of the adventitia in damaged regions of the EIIIAB-/− artery. Artery was divided into thirds, each section represents a different region of the artery. Subendothelial hemorrhage was observed in the middle section (not shown here), corresponding to the bleed and bulge in the center of the artery. B) Graph quantifies the variation in media and adventitia thickness in a subset of arteries for which multiple regions were measured. Each point represents a single artery and red points indicate hemorrhaged arteries. **P<0.01 by two-tailed Student’s t-test.
Figure VII. *In vivo* lumen diameter 7 days after nearly complete carotid ligation

A) High-resolution ultrasound imaging of mouse carotid artery at 7 days after sham, or nearly complete carotid ligations. ECA=external carotid, ICA=internal carotid, CC=common carotid. Line indicated lumen measurement.

B) Frequency of local dilation (>30% increase in diameter relative to adjacent artery) in the indicated genotypes with and without visible intramural hemorrhage.

C&D) Summary of *in vivo* measurements of maximum carotid artery lumen diameter, as determined by high-resolution ultrasound. Each point indicates a single carotid artery. Differences between EIIIAB−/− and EIIIAB+/+ diameters were significant by Student’s t-test (P=0.04). Among all FN deleted arteries, only differences between FN ff and Rosa-CreERT2; FN ff were significant by ANOVA and Tukey’s post-hoc analysis (P<0.001).
Figure VIII. Hemorrhage of the vessel wall in EIIIA-/- mice.
Images of carotid arteries from mice of the indicated genotypes 7 days after nearly complete ligation. Cross sections of the arteries stained for CD31 and Ter119, demonstrating the presence of blood beneath the endothelium in the EIIIA-/- carotid.
Figure IX. No significant difference in susceptibility to dissection in Angiotensin-II driven model

A) Histogram summarizing the aneurysm score (according to Daugherty's classification scheme, with the addition of V to indicate death from rupture). B) Fold increase in EIIIA and EIIIB inclusion in RNA isolated from the aortic intima 14 days after AngII treatment, or in RNA isolated from the carotid intima 7 days after nearly complete carotid ligation. All samples are relative to aorta with no AngII treatment. C) Fold increase in CD11b RNA in the same samples, relative to aorta with no AngII treatment. Each point (B&C) indicates a single carotid artery.
Figure X. Extravasation by albumin Immunofluorescence
Immunofluorescence staining for albumin in carotid arteries of the indicated genotypes 7 days after nearly complete carotid ligation, showing increased albumin staining in the intima of ligated versus contralateral control arteries. Quantitation is shown below.
Figure XI. No significant difference in macrophage recruitment or localization

Left panels show immunofluorescence staining of F4/80+ macrophages in across several regions of the same EIIIAB+/+ and EIIIAB-/- ligated carotid arteries, as well as their contralateral arteries. Higher magnification of the boxed areas (a,b) is shown on the upper right, F4/80+ cells could be observed in the intima (a), just beneath the intima (b) as well as media and adventitia. Graphs show the increase in RNA markers of macrophages in the RNA isolated from the intima and the media/adventitia fractions, relative to the sham operated contralateral artery of EIIIAB+/+ mice. Each point in the graphs indicates a single carotid artery.
Figure XII. Correlation between PAI1 expression and Fn splicing
Graphs show the correlations between the increase in PAI1 expression and the indicated gene expression or splicing changes in mRNA harvested from the carotid intima 48hrs after nearly complete carotid ligation +/- clodronate-mediated macrophage depletion. Significance of the Pearson correlation is indicated in the upper left.
Figure XIII. Acute and chronic effects of macrophage depletion
A) Quantitative PCR results, showing the effects of acute depletion of macrophages on the levels of the indicated mRNA in the carotid intima. B) Measurements of pulse distension (as a percentage of minimal carotid diameter) and maximal carotid diameter obtained from high-resolution ultrasound measurements 7 days after nearly complete carotid ligation, with or without chronic clodronate liposome treatment (every 3 days). C) Increase in intimal immunofluorescence (over EIIIAB+/+ contralateral artery) in the ligated arteries of mice treated for 7 days with either clodronate or PBS liposomes. Each point in each graph indicates a single carotid artery. (D) Excised vessels and sections of carotid artery for the indicated treatments. Arrowhead indicates a mild bulge in the artery. Scale bars = 100µm.
Figure XIV. Model depicting the induction and role of FN splicing in preventing vessel dissection

Model shows the temporal progression (left to right) of the arterial response to low and disturbed flow. (1) By 48 hrs, ICAM and VCAM expression is increased and monocyte adhesion to the endothelium has begun. (2) At 48hrs, monocytes are necessary to increase inclusion of the alternative FN exons EIIIA and EIIIB in the endothelium through an unknown mechanism (dotted line). Increase in EIIIA and EIIIB inclusion continues to at least 7 days. Monocytes are recruited in equal numbers to the intima in mice unable to include EIIIA or EIIIB (EIIIAB-/-), but they cannot induce the expression of EIIIA+ and EIIIB+ FN in the vessel wall. (3) This results in increased damage and hemorrhage of the vessel wall.
Movie 1. Confocal imaging of carotid injury in wild-type mouse
En face staining of the injured carotid artery with VE-cadherin and DAPI. Imaging stack moves in Z from the vessel lumen into the vessel wall.

Movie 2. Confocal imaging of contralateral carotid artery in wild-type mouse
En face staining of carotid artery with VE-cadherin and DAPI. Imaging stack moves in Z from the vessel lumen into the vessel wall.
Materials and Methods:

Mice

EIIIAB/- mice on a C57BL/6J background, were previously reported 1. To obtain endothelial or global deletion of FN, FN f/f mice (obtained from R. Fassler 2) were crossed with Cdh5-CreER\textsuperscript{T2} (obtained from R. Adams 3) or ROSA-CreER\textsuperscript{T2} (obtained from T. Jacks 4) mice. To acutely delete endothelial FN in the absence of smooth muscle FN, SM22-Cre mice were crossed with Cdh5-CreER\textsuperscript{T2} mice 5. Cre-mediated excision efficiency was monitored with the fluorescent mT/mG Cre-reporter mice 6, crossed with the FN f/f mice. The reporter switches from red to green when activated by Cre.

In experiments with Cdh5-CreER\textsuperscript{T2} mice, excision was induced with 3 x 1mg Tamoxifen by intraperitoneal injection one week before surgery. Excision in ROSA-CreERT2 mice was induced with 5 x 1mg Tamoxifen by intraperitoneal injection one week before surgery.

Nearly complete carotid ligation was performed as previously described 7, except that anesthesia used was isoflurane, 9-0 Ethilon suture was used for ligation, and skin was closed with 7-0 nylon monofilament. Sham operations consisted of the same carotid dissection and encircling with suture, except that the vessels were not tied off. High-resolution ultrasound, using the VisualSonics Vevo 770, was performed at the experimental endpoint (2-7 days after partial carotid ligation) to confirm vessel patency.

Angiotensin II (AngII, Sigma 9525) infusion was performed as previously described 8. AngII was administered by subcutaneous osmotic pump (Alzet) at a dose of 2,500 ng/kg/min for a maximum of 14 days.

Macrophage depletion by chlodronate liposomes was performed as previously described 9. Briefly, mice were injected through the tail vein with 150 uL chlodronate-loaded liposomes (5 mg/mL) or PBS-loaded control liposomes several hours before partial carotid ligation, and then with another 150uL by intraperitoneal injection one day after partial carotid ligation. Clodronate liposomes or PBS liposomes were purchased from ClodronateLiposomes.com (Kruisweg 59, 2011 LB Haarlem, The Netherlands).

All mice were housed and handled in accordance with approved Massachusetts Institute of Technology Division of Comparative Medicine protocols.

RNA isolation and quantification

Carotid arteries were isolated for gross images following PBS perfusion from the left ventricle and out through the right atrium. RNA was isolated as described, except that 150uL of Sigma Trizol solution was used to flush the carotid, and
arteries were not washed in HBSS before being placed in Trizol for extraction of the medial and adventitial RNA. Flushes of the aorta were performed with a 26-gauge needle and 500uL of Trizol. RNA was extracted using the Qiagen RNAeasy mini-column kit, after combining the chloroform extract from Trizol 1:1 with 70% ethanol. A Promega kit was used to random prime a cDNA library from each carotid artery before expression analysis using the Bio-Rad iQ sybr mix.

**Primers for quantitative PCR**

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<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>Murine 18s</td>
<td>GTAACCCGTGATACCCATT</td>
<td>CCATCAAATCGGTAGTGCG</td>
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<tr>
<td>Murine FN</td>
<td>CTTTGGTAGCTGTCATGGGTCTC</td>
<td>AGCAAGTCAGGAATGTTTCA</td>
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<tr>
<td>Murine EllI</td>
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<td>GAGTAAGCTGGTGTTGTTG</td>
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<tr>
<td>Murine EllIB</td>
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<tr>
<td>Murine CD31</td>
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<tr>
<td>Murine alpha-smooth muscle actin</td>
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<td>Murine CD68</td>
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<td>Murine F4/80</td>
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<td>Murine Klf2</td>
<td>CCAACTGACGGCAAGACCTA</td>
<td>AGTGGGCCAGGCCAAGAGT</td>
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<tr>
<td>Murine PAI1</td>
<td>TCAGGATCGAGGTAAACGAGA</td>
<td>GCCGAACCACAAAGAGAAG</td>
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**Staining and fluorescence quantification**

For quantification of FN or albumin fluorescence, carotid or aorta were fixed in zinc-fix (BD), embedded in paraffin and sectioned. Sections blocked in 10% BSA, 0.1% TritonX-100 in PBS were stained for FN (lab generated rabbit polyclonal antibody, 1:200) or albumin (Abcam, ab19194) and CD31 (BD clone MEC13.3, 1:200) followed by fluorescently conjugated secondary antibodies. All samples were stained and imaged in parallel for experiments in which comparisons are made. Pixel intensity analysis of FN signal was performed using ImageJ software, on regions of the intima and the media defined by CD31 staining.

For CD31 and Ter119 co-staining, citrate retrieval was performed on zinc-fixed carotid arteries. Sections blocked in 10% BSA, 0.1% TritonX-100 in PBS were stained for CD31 (Abcam 28364 rabbit polyclonal antibody, 1:100) and Ter119 (BD 550565 rat monoclonal antibody, 1:100) followed by fluorescently conjugated secondary antibodies.

For co-staining of FN in mT/mG cre-reporter mice, carotid arteries were fixed in 1% PFA overnight at 4C, sucrose dehydrated, and then frozen in Optimum Cutting Temperature medium (O.C.T., Tissue-Tek). Sections were washed in PBS, blocked in 10% BSA, 0.1% TritonX-100 in PBS and then stained for FN (lab generated rabbit polyclonal antibody, 1:200) followed by Alexa647-conjugated secondary antibody. All samples were stained and imaged in parallel for
experiments in which comparisons are made. Channels showing mT/mG reporter expression were independently adjusted, since baseline fluorescence in the reporter was not equal between animals.

For whole-mount immunofluorescence, arteries were briefly fixed in 1% PFA on ice before being opened with fine vanna scissors and mounted on glass coverslip. Samples were blocked in 2% FN depleted goat serum, 0.1% TritonX-100 in PBS and then stained for VE-cadherin (BD, 1:200) followed by fluorescently conjugated secondary.

**Quantitation of vessel compartment areas**

Each zinc-fixed carotid artery was bisected or trisected and imbedded in paraffin. Thus, sections of the block provided 2 to 3 widely separated areas of the same artery. For quantitation, DAPI labeled images were taken, and the elastin autofluorescence was used to measure the perimeter of the lumen and the thickness of the media and the adventitia in ImageJ. For the media and adventitia thickness measurements, four independent measurements perpendicular to the intima were taken and averaged together for the section. The difference in these measurement gives the change in mean thickness (e.g. [AVG of 4 media measurements from portion 1 of the artery] minus [AVG of 4 media measurements from portion 2 of the artery]).

**Quantitation of ultrasound diameter**

Internal carotid diameter along the common carotid artery was measured in B-mode images of the right and left carotid artery of each mouse at 7 days (and 48hrs, when harvested at an earlier time-point). Since diameter changes with pulse, both maximal and minimal diameters were measured. The difference between these values is reported as the pulse distension (and given as a percentage of the minimal diameter). To quantify focal bulging of the carotid artery exposed to low and disturbed flow, we measured internal carotid diameter in the bulge as well as the adjacent artery. The percentage increase in diameter between the maximally distended region of the bulge and the adjacent artery is reported as local dilation.

**Statistics**

Statistical analyses were performed with the GraphPad Prism Software. Briefly, the Student's t test was used to compare means of two independent groups to each other, one-way ANOVA followed by Tukey's posttest was used to compare the means of more than two independent groups, and Fisher's exact test to measure contingency tables (association of hemorrhage with genotypes). Where indicated, a Pearson correlation was used to assess the correlation between gene expression changes.
References


