Fibromodulin Deficiency Reduces Low-Density Lipoprotein Accumulation in Atherosclerotic Plaques in Apolipoprotein E–Null Mice

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Objective—The aim of this study was to analyze how an altered collagen structure affects development of atherosclerotic plaques.

Methods and Results—Fibromodulin-null mice develop an abnormal collagen fibril structure. In apolipoprotein E (ApoE)-null and ApoE/fibromodulin-null mice, a shear stress-modifying carotid artery cast induced formation of atherosclerotic plaques of different phenotypes; inflammatory in low-shear stress regions and fibrous in oscillatory shear stress regions. Electron microscopy showed that collagen fibrils were thicker and more heterogeneous in oscillatory shear stress lesions from ApoE/fibromodulin-null mice. Low-shear stress lesions were smaller in ApoE/fibromodulin-null mice and contained less lipids. Total plaque burden in aortas stained en face with Oil Red O, as well as lipid accumulation in aortic root lesions, was also decreased in ApoE/fibromodulin-null mice. In addition, lipid accumulation in RAW264.7 macrophages cultured on fibromodulin-deficient extracellular matrix was decreased, whereas levels of interleukin-6 and -10 were increased. Our results show that an abnormal plaque collagen fibril structure can influence atherosclerotic plaque development.

Conclusion—The present findings suggest a more complex role for collagen in plaque stability than previously anticipated, in that it may promote lipid-accumulation and inflammation at the same time as it provides mechanical stability. (Arterioscler Thromb Vasc Biol. 2013;33:354-361.)

Key Words: atherosclerosis ■ carotid arteries ■ collagen ■ fibromodulin ■ LDL

Assembly of extracellular matrix (ECM) directs the conditions for both growth and stability of an atherosclerotic plaque. Vascular smooth muscle cells (SMCs) in the normal media are ensased by a basement membrane embedded in collagens, adhesive proteins, and proteoglycans. At an early stage of the atherosclerotic process, the SMCs in the media transfer from a contractile to a synthetic phenotype and migrate to the intima, where they proliferate and produce a collagen-rich matrix. During development of an atherosclerotic plaque, there are marked changes in the structure of the ECM. About 60% of the total protein content in the plaque represents fibril-forming collagens type I and III, and the roles of collagens in atherogenesis are multifaceted where excess collagen production can promote plaque growth and thereby contribute to vascular stenosis, whereas defective fiber assembly or degradation can cause plaque rupture and subsequent thrombosis.

The collagen fibrillar structure is an important parameter in this aspect, and it has been shown that SMCs seeded on monomeric type I collagen exhibit increased proliferation, as well as migration, when compared with cells plated on fibrillar type I collagen. An in vivo injury experiment in pig coronary arteries supports this hypothesis by identifying a further connection between fibrillar collagen and inhibition of SMC proliferation through regulation of the cyclin-dependent kinase inhibitors, p21Cip1 and p27Kip1.

Macrophage function also depends on the monomeric/polymeric state of type I collagen. Both spreading and matrix metalloproteinase-9 production is inhibited in human macrophages when seeded on fibrillar type I collagen and increased when macrophages were seeded on monomeric type I collagen. Furthermore, type I collagen can influence both macrophage phagocytic activity, as well as their uptake of low-density lipoprotein (LDL). Therefore, it is clear that the physical state of type I collagen can have a great impact on both SMCs and macrophages, and thus also on the developing atherosclerotic plaque.

Small leucine-rich repeat proteoglycans are a group of structurally related proteins present in the ECM. Many of the small leucine-rich repeat proteoglycans interact with collagens and regulate the assembly of fibrillar collagen matrices. Fibromodulin is a 59-kDa small leucine-rich repeat proteoglycan primarily expressed in cartilage and tissues exposed to tensile stress, such as tendons, and is a close homolog to lumican. It regulates collagen fibril formation by binding collagen type I, and thereby influences the collagen scaffold formation by a still unknown molecular mechanism. In fibromodulin-null mice, abnormal...
collagen fibrils are found in tendons, skin, dermal wound healing, liver cirrhosis, and tumor stroma of subcutaneous experimental tumors. In the present study, we tested the hypothesis that fibromodulin, by regulating the collagen structure, affects the development of atherosclerotic plaque tissue.

Materials and Methods

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

Animals and In Vivo Alteration of Shear Stress

Animal tests were approved by the Malmö/Lund regional ethical committee (Sweden). Apolipoprotein E null mice, in a Bl6 background, and fibromodulin null mice were crossed, and ApoE/fibromodulin null mice were used in experiments with ApoE null mice as controls. As described previously by Cheng et al., standardized changes in shear stress were induced by a periadventitial cast placed around the right carotid artery of ApoE- and ApoE/fibromodulin-null female mice maintained on a cholesterolic diet, starting 2 weeks before surgery. Cast placement was performed on 18-week-old mice, and mice were euthanized at 30 weeks of age.

Immunohistochemistry and Histology

Carotid artery sections were stained using a Mac-2 antibody (Cedarlane; Burlington, ON, Canada), a smooth muscle α-actin antibody (Sigma-Aldrich), a fibromodulin antibody (gift from Prof. Dick Heinegård), the PCNA staining kit (Invitrogen), and a PCNA antibody (AbCam), and the TUNEL kit, TACS XLDAB In Situ Apoptosis Detection Kit ( Treviglen). Appropriate biotin-conjugated secondary antibodies were used, and to differentiate staining patterns during sequential double staining, 3,3′-diaminobenzidine and streptavidin alkaline phosphatase with StayRed/AP (AbCam) were used with the different antibodies. For histology, Oil Red O (0.3%, Sigma-Aldrich) and the Accustain trichrome stain (Masson; Sigma-Aldrich) were used. All stained sections were visualized and digitalized using an Aperio ScanScope digital slide scanner (ScanScope Console v8.2.0.1263, Aperio Technologies, Inc. Vista, CA).

Lesion size is expressed as area and intima-media ratio, and represents the mean value of 4 sections 15 µm apart, where the lesions were at their largest. Positively stained areas of Oil Red O–stained descending aortas mounted en face were quantified and expressed as the percentage of the inner arterial lining covered by lesions. Aortic root sections were collected between first appearance and disappearance of the aortic valves, and 1 section every 56 µm was stained. Lipid content in plaques from the carotid artery and aortic root was expressed as the mean percentage of lesion area that stained positive for lipids.

Transmission Electron Microscopy

Tissues were fixed in 0.15 mol/L sodium cacodylate-buffered 2% glutaraldehyde, postfixed in 0.1 mol/L collidine-buffered 2% osmium tetroxide, and embedded in epoxy resin.

RNA Extraction and Quantitative Real-Time PCR

Because of small sample size, low and oscillatory shear stress plaques from 3 ApoE- and ApoE/fibromodulin-null mice were pooled, yielding 2 groups per genotype, and uninjured carotid arteries from 2 mice were pooled, yielding 3 groups per genotype. Total RNA was extracted with RNAeasy Micro Kit (Qiagen). Fifty ng total RNA was subjected to first-strand cDNA synthesis with Superscript VILO cDNA Synthesis Kit (Invitrogen), and gene expression was analyzed using LightCycler software Version 3 (Roche Applied Science).

In Vitro Assays With RAW264.7 Macrophages

For ECM preparation, aortic SMCs were isolated from wild-type and fibromodulin-null C57BL/6 mice, and cultured cells were extracted with 0.5% Triton X-100 in PBS and 25 mmol/L NH4OH in PBS. LDL-uptake was assessed by growing RAW264.7 cells on wild-type and fibromodulin-null ECM, with the addition of 50 µL/mL native or Cu2+-oxidized LDL in Dulbecco’s Modified Eagle Medium GlutaMax supplemented with 10% lipoprotein-deficient human serum, 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco). After 4 days, cells were fixed and stained with Oil Red O, and the amount of lipids that had been taken up was quantified as Oil Red O–positive area relative to total cell area (values added from 20 micrographs per cover slip). Expression of fibromodulin by wild-type SMCs was confirmed through Western blot (Figure I in the online-only Data Supplement). Cytokine concentrations in cell lysates were determined by using Mouse ProInflammatory 7-Plex Tissue Culture Kit (Meso Scale Discovery).

Software and Statistical Methods

Sample size is expressed as n, and error bars represent standard deviation. Immunohistochemical and histological stains were quantified using BioPix iQ software (BioPix AB, Gothenburg, Sweden). Two-tailed t test (electron microscopy image analysis) was performed using Microsoft Excel. Mann–Whitney t test (image analysis), and Student t test and 1-way ANOVA analysis.

Figure 1. Decreased size of low–shear stress lesions in apolipoprotein E (ApoE)/fibromodulin-null (ApoE–/–FM–/–) mice. Representative sections of Masson trichrome–stained carotid lesions (A and B, low shear stress; C and D, oscillatory [Osc.] shear stress). Intima/media ratio is shown in E and total plaque area in F. Scale bars=100 µm.
with Bonferroni posttest (quantitative real-time-PCR) were performed using GraphPad Prism, version 4.03 (GraphPad Software, San Diego, CA).

Results

Fibromodulin Deficiency Reduces Plaque Size

To test our hypothesis that an altered collagen structure affects the development of atherosclerotic plaque tissue, we generated ApoE/fibromodulin-null double knockout mice and studied the development of carotid atherosclerotic lesions of stable and vulnerable phenotypes induced by a perivascular cast. In this technique, vulnerable lesions develop under conditions of low-shear stress proximal to the cast, whereas stable lesions develop distal to the cast because of oscillatory shear stress. Low-shear stress plaques were smaller in ApoE/fibromodulin-null mice than in ApoE-null mice (Figure 1A and 1B); the lesion/media ratio ($P=0.005$; Figure 1E), total plaque area ($P=0.006$; Figure 1F), and the length of the low-shear stress plaques (484.4±346.6 versus 256.9±232.3, $P=0.013$) were reduced in the ApoE/fibromodulin-null lesions. Note that there was no difference in the size of the oscillatory shear stress lesions between the 2 genotypes (Figure 1C and 1D).

Low-shear stress carotid lesions contained less collagen than the oscillatory shear stress lesions in both ApoE-deficient ($P=0.0011$) and ApoE/fibromodulin-null mice ($P=0.0010$), but there was no difference in collagen (Figure II in the online-only Data Supplement), SMC, or T-cell content (data not shown) between the genotypes. Additionally, we found no difference in the size of aortic root lesions (Figure III in the online-only Data Supplement).

Increased Collagen Fibril Thickness in Fibromodulin-Deficient Low–Shear Stress Plaques

Fibromodulin expression has previously been demonstrated in murine aortic atherosclerotic lesions. In addition, we found positive fibromodulin immunoreactivity in carotid lesions and, to some extent, in the medial layer associated with the lesions (Figure IV A and IVC in the online-only Data Supplement).

Figure 2. Thicker and more heterogeneous collagen fibrils in apolipoprotein E (ApoE)/fibromodulin-null lesions. Electron micrographs of atherosclerotic carotid lesions (n=3) of ApoE- (A) and ApoE/fibromodulin-null mice (B). Image analysis shown in C.

Figure 3. Decreased lipid and macrophage content in low-shear stress lesions in apolipoprotein E (ApoE)/fibromodulin-null mice. Image analysis of Oil Red O-stained low and oscillatory shear stress carotid plaques (A, representative low-shear stress lesions shown) and sections from aortic root lesions (B). Image analysis of Mac2-stained low-shear stress and oscillatory shear stress ApoE- and ApoE/fibromodulin-null carotid plaques (C–G). Scale bars=100 μm.
No positive fibromodulin staining was found in the ApoE/fibromodulin-null lesions, or in uninjured control arteries (Figure IVB and IVD–IVF in the online-only Data Supplement). These results are in agreement with the presence of fibromodulin transcripts in carotid lesions in ApoE-null mice, whereas no transcripts could be detected in ApoE/fibromodulin-null carotid lesions (Figure IVG in the online-only Data Supplement).

Electron microscopy of atherosclerotic oscillatory shear stress lesions from ApoE/fibromodulin-null mice revealed an altered structure of the collagenous matrix manifested by the presence of more heterogeneous collagen fibrils (Figure 2A and 2B). The average fibril diameter thickness was increased from 25 to 34 nm \( (P<0.0001; \text{Figure 3C}) \) in ApoE/fibromodulin-null compared with ApoE-null lesions. Collagen fibrils in low-shear stress lesions were disorganized to an extent that they were not quantifiable; however, the surrounding adventitia contained proper collagen fibrils (data not shown). We found no difference between collagen fibril diameters in healthy, uninjured carotid arteries or in descending aortas from ApoE- and ApoE/fibromodulin-null mice (Figure V in the online-only Data Supplement).

**Fibromodulin Deficiency Reduces Lipid Accumulation in Plaques**

Lipid retention is an essential process in atherosclerosis development,2 and ECM components are known to affect LDL-retention.25 We analyzed the lipid content of low-shear stress and oscillatory shear stress plaques in ApoE- and ApoE/fibromodulin-null by Oil Red O staining. Reduced lipid accumulation was observed in low (Figure 3A), but not in oscillatory (Figure VIB in the online-only Data Supplement), shear stress carotid plaques from ApoE/fibromodulin-null mice \( (P=0.028) \). A similar decrease in lipid accumulation was observed in the media surrounding these plaques \( (P=0.0019; \text{Figure VIA in the online-only Data Supplement}) \). We also analyzed lipid accumulation in aortic root lesions that were stained with Oil Red O and found that the lipid content was lower in lesions from ApoE/fibromodulin-null mice \( (P=0.0056; \text{Figure 3B}) \). In an atherosclerotic plaque, lipids primarily accumulate within macrophages and foam cells.27 Consequently, when comparing macrophage accumulation in lesions from ApoE- and ApoE/fibromodulin-null mice, we also found a significant decrease in total macrophage content in low-shear stress ApoE/fibromodulin-null plaques \( (P=0.0214; \text{Figure 3C–3G}) \).

In addition, to compare the general severity of atherosclerosis between mice with or without fibromodulin expression, we performed en face Oil Red O staining of the descending aorta and found a 30% decrease \( (P=0.0144) \) in total plaque burden in aortic lesions from ApoE/fibromodulin-null mice (Figure 4).

**A Fibromodulin-Deficient ECM Reduces Lipoprotein Uptake by Macrophages In Vitro**

To further explore the mechanisms involved in the reduced lipid accumulation in low–shear stress lesions from fibromodulin-deficient mice, we analyzed lipid uptake in macrophages cultured on fibromodulin-null ECM synthesized by SMCs. RAW264.7 macrophages were seeded on wild-type or fibromodulin-null ECM, and native or oxidized LDL was added to the culture medium. After 4 days, cells had grown to confluence (approximately 6550 cells/mm\(^2\)) and were stained with Oil Red O. Our results demonstrate a decreased accumulation of oxidized LDL in cells growing on a fibromodulin-deficient ECM compared with cells growing on a wild-type ECM \( (P=0.018; \text{Figure 5A}) \). There was no difference in the uptake of native LDL between RAW264.7 macrophages cultured on wild-type or fibromodulin-deficient ECM.

We also analyzed the migratory capacity of RAW264.7 macrophages and found no difference in migration between cells seeded on wild-type and fibromodulin-deficient collagen (data not shown).
A Fibromodulin-Deficient ECM Alters Cytokine Production In Vitro

Cytokines and chemokines produced by leukocytes in plaques play an important part in atherosclerosis development, including regulation of uptake and efflux of cholesterol. Accordingly, we analyzed whether an altered ECM also can have an impact on macrophage cytokine production. RAW264.7 cells were cultured on wild-type and fibromodulin-deficient ECM for 2 days in presence of native or oxidized LDL. The levels of interleukin (IL)-10 and IL-6 were found to be increased in cells cultured on fibromodulin-deficient ECM in presence of oxidized LDL (P=0.0028 and 0.04, respectively; Figure 5B and 5C). However, we found no differences in levels of tumor necrosis factor-α, IL-1, or IL-12, between cells growing on wild-type and fibromodulin-deficient ECM (data not shown).

In addition, we found no difference in plasma levels of inflammatory markers, including tumor necrosis factor-α, interferon-γ, IL-1β, -2, -4, -5, -10, -12, or keratinocyte-derived chemokine, at the time animals were euthanized (Figure VII in the online-only Data Supplement). There was no difference in plasma cholesterol levels (Figure VIII in the online-only Data Supplement).

Fibromodulin Deficiency Increases Cell Proliferation and Collagen Turnover

Proliferation of SMCs is another important mechanism regulating atherosclerotic plaque growth. The fibrillar state of collagen matrix can influence this process; the monomeric or degraded form stimulates, and the mature fibrillar state can inhibit cell proliferation. Thus, it is likely that the abnormal fibril formation in ApoE/fibromodulin-null mice affects cell proliferation in the atherosclerotic plaque. Low and oscillatory shear stress carotid lesions isolated from ApoE/fibromodulin-null mice were stained for proliferating cell nuclear antigen, and the proportions of positively stained nuclei, that is, proliferating cells, was determined (Figure XI in the online-only Data Supplement). The mRNA expression of lysyl oxidase, the main collagen cross-linking enzyme, was also enhanced in low–shear stress lesions from ApoE/fibromodulin-null mice (Figure XI in the online-only Data Supplement). This increase was significant also in comparison with oscillatory shear stress plaques, as well as with control arteries within the same genotype. A similar tendency was found in the expression of procollagen chain 1α1 (Col1α1; Figure XI in the online-only Data Supplement).

The mRNA expression of lysyl oxidase, the main collagen cross-linking enzyme, was also enhanced in low–shear stress lesions from ApoE/fibromodulin-null mice (Figure XI in the online-only Data Supplement). In addition, the basal expression level of lysyl oxidase was significantly higher in control arteries derived from ApoE/fibromodulin-null mice, when compared with ApoE-null mice, indicating a general increase in collagen turnover in mice lacking fibromodulin expression.

As tissue turnover also includes degradation, we analyzed the expression of urokinase plasminogen activator receptor-associated protein, an internalizing receptor that binds degraded collagen fragments for further uptake and degradation. Urokinase plasminogen activator receptor-associated protein expression was significantly increased in oscillatory shear stress plaques from ApoE/fibromodulin-null mice compared with ApoE-null mice (Figure XI in the online-only Data Supplement) and showed a tendency of higher expression in low-shear stress plaques. Urokinase plasminogen activator receptor-associated protein expression was also significantly increased in low–shear stress lesions compared with oscillatory shear stress lesions in both genotypes.

Discussion

The aim of the present study was to analyze how an altered collagen structure affects the development of atherosclerotic plaques in ApoE-null mice. Fibromodulin is a collagen-binding protein, with an important role in collagen fiber assembly. We crossed fibromodulin- and ApoE-null mice to generate atherosclerotic plaques with a defective collagen structure. In line with the previously published data on collagen structure in fibromodulin-null tendons, cornea, cartilage, and wound healing, the ApoE/fibromodulin-null mice developed atherosclerotic lesions with more heterogeneous and significantly larger fibrils, resulting in a disorganized collagen matrix. However, lack of fibromodulin did not alter the collagen content in the lesions.

However, we found plaque size, lipid accumulation, and macrophage content to be significantly decreased in lesions from ApoE/fibromodulin-null mice. It is possible that the decreased lipid content reduces the local inflammatory response, and thus also affects the size of the plaque. Our finding that the total amount of macrophages is decreased supports this hypothesis. Furthermore, collagen type I fibrils appear to be involved in lipid-retention and macrophage accumulation. In vitro experiments have previously shown that collagen type I stimulated monocyte differentiation, as well as uptake of lipids by macrophages. Thus, the abnormal collagen fibrils in ApoE/fibromodulin-null lesions may have reduced ability to stimulate lipid accumulation in macrophages, resulting in
reduced lipid content in these lesions. Indeed, our finding that RAW264.7 macrophages cultured on fibromodulin-null ECM has a decreased capacity to accumulate oxidized LDL supports this hypothesis.

Furthermore, the decrease in lipid accumulation in RAW264.7 macrophages grown on fibromodulin-deficient ECM was associated with an increased production of IL-6 and -10. In line with this, Frisdal et al reported that treatment with IL-6 promoted an anti-inflammatory cytokine profile, including increased secretion of the antiatherogenic cytokine, IL-10, as well as efflux of excess cholesterol from macrophages in vitro through ATP-binding cassette A1.

Collagen structure has general effects on cellular functions, but may also specifically affect the binding of LDL or oxidized LDL to the ECM. The tyrosine sulfate-rich N-terminal domain of fibromodulin has heparin-like properties, binds basic heparin-binding proteins, and may also bind LDL. Kaplan and Aviv suggested that the larger ECM GAG content in old mice resulted in a greater binding and uptake of oxidized LDL compared with young mice, and Tran-Lundmark et al performed a study, where LDL in an ApoE-null mouse with ECM containing heparan sulfate-deficient perlecan was shown to diffuse more rapidly into the vessel wall, but exhibited a reduced retention rate. Similarly, ECM synthesized in the absence of fibromodulin may be altered in a manner that LDL retention is reduced.

Although fibromodulin deficiency reduces lipid content in plaques both at the aortic root and at the low-shear stress region of the cast, it only attenuates the size of the latter type of lesion. The reason for this discrepancy remains to be fully elucidated, but may involve differences in shear stress patterns; the shear stress on the vessel wall is different in the aortic root, compared with the low-shear stress region proximal to the cast in the carotid artery. Van Doormaal et al demonstrated complex flows and a high oscillatory shear stress in the aortic root, whereas an oscillatory shear stress to the vessel wall is induced in the oscillatory shear stress region distally, but not proximally, to the cast in our model. Interestingly, similar to the aortic root lesions, there was no difference in the size of the low-shear stress lesions, when comparing the 2 genotypes. This should be compared with the low-shear stress on the vessel wall in the proximal region of the cast, where we could detect differences in lesion size.

In a study by Ranjzad et al, both the neointima and the neointimal collagen content was found to be decreased in human veins, after overexpression of fibromodulin through adenovirus-mediated gene transfer ex vivo. The effects were found to be mediated, at least in part, by antagonism of transforming growth factor-β1 and collagen homeostasis. There are several possible explanations to these apparently conflicting results. The present study focused on atherosclerotic plaque development, whereas the study by Ranjzad investigated neointimal hyperplasia; 2 processes that develops through different mechanisms. Thus, in our study, the effects of fibromodulin deficiency on plaque size are found to be principally because of alterations in lipid accumulation and macrophage lipid uptake, whereas, in contrast, overexpression of fibromodulin during neointimal hyperplasia was found to affect SMC and collagen accumulation. In addition, the discrepancies may be explained by differences in the experimental models used. The present study was performed on atherosclerotic lesions developed over 20 weeks in ApoE- and ApoE/fibromodulin-deficient mice, whereas the study of Ranjzad et al was performed using an ex vivo model with human veins maintained for 14 days in culture. However, it is interesting to note that results on cell density from the 2 studies are in agreement, as Ranjzad et al found neointimal cell density to be lowered after fibromodulin overexpression, whereas in the present study, cell density in atherosclerotic lesions was increased by fibromodulin deficiency.

We found that cell proliferation was increased in both low and oscillatory shear stress lesions in mice lacking fibromodulin. Because there is no correlation between fibromodulin expression and proliferation rate in individual plaques (Shami, unpublished data, 2011), we speculate that the increased cell...
proliferation and decreased lipid retention is instead directly affected by an altered surrounding collagen structure and that the increase in cell proliferation reflect an increased matrix turnover. To test this hypothesis, we quantified RNA transcripts for procollagens Col1a1 and Col1a2, and the collagen turn-over-related lysyl oxidase and urokinase plasminogen activator-receptor-associated protein in the plaques and in control arteries. The results indicated a higher collagen turnover in carotid lesions from ApoE/fibromodulin-null mice compared with ApoE-null mice. An increased collagen turnover resulting in a higher proportion of degraded or monomeric, nonfibrillar collagen may further enhance the disordered collagen scaffold in plaques lacking fibromodulin expression. This milieu could, in turn, stress the cells in the plaque to an increased rate of proliferation. In support of this conclusion, Koyama et al demonstrated that intact fibrillar collagen has an inhibitory effect on proliferation. In support of this conclusion, Koyama et al demonstrated that intact fibrillar collagen has an inhibitory effect on proliferation, whereas degraded or monomeric collagen promotes proliferation. Collagen type VIII, produced and deposited by SMCs, is suggested to cover native collagen type I, and thereby allow cells to overcome the inhibition of proliferation; a process that cell proliferation in fibromodulin-null lesions may not be dependent on. Thus, we speculate that abnormal collagen type I fibrils in fibromodulin-deficient ECM may lead to decreased inhibitory effects, as well as increased stimulatory effects, on proliferation compared with normal collagen fibrils of a fibromodulin-competent matrix. The increase in proliferation rate in cells embedded in a fibromodulin-deficient matrix may also be explained by altered downstream signaling caused by a different expression of cell surface collagen receptors, such as integrins and discoidin domain receptors.

It is likely that SMCs can adjust to an altered collagen matrix already during early vessel development. This adjustment can include different expression patterns of cell surface receptors, such as discoidin domain receptors (collagen-activated tyrosine kinases), and subsequently affect the response, and thus behavior, of the cells. Hou et al has shown that deletion of discoidin domain receptor-1 attenuates plaque development and shifts plaque composition toward reduced inflammation, as well as an early increase in ECM content and SMC proliferation.

In this study, we used low and oscillatory shear stress to induce the formation of vulnerable and stable atherosclerotic lesions, respectively, in ApoE-null mice. We demonstrate that a defective formation of collagen fibers because of fibromodulin-deficiency reduces the formation of lipid-rich vulnerable lesions, but does not affect the formation of more stable lesions induced by oscillatory shear stress. The present findings suggest a more complex role for collagen in plaque stability than previously anticipated, in that it may promote lipid-accumulation and inflammation at the same time as it provides mechanical stability.

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References
Fibromodulin Deficiency Reduces LDL Accumulation


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SUPPLEMENTAL MATERIAL

Animals
All animal tests were approved by the Malmoe/Lund regional ethical committee (Sweden). ApoE-null mice in a Bl6 background (B6.129P2-ApoEtm1UncN11) purchased from Taconic (Lille Skensved, Denmark) and fibromodulin-null mice generated by targeted disruption were crossed and the resulting ApoE/fibromodulin-null mice were used in experiments. ApoE-null mice were used as controls.

In vivo alteration of shear stress
As described previously by Cheng et al. standardized changes in shear stress were induced by a periadventitial cast placed around the right carotid artery of ApoE- and ApoE/fibromodulin-null female mice. Mice were kept on a cholesterol-rich diet throughout the experiment, starting two weeks before surgery. Cast placement was performed on 18-week-old mice anaesthetized with isoflurane carried by oxygen (initiated at 4% isoflurane and kept at 2-3% during surgery). Mice were given buprenorphine (0.1 mg/kg) subcutaneously once before surgery and once five to seven hours post-surgery. Mice were sacrificed at 30 weeks of age.

Tissue preparation
Mice were sacrificed through administration of an overdose of ketamine, xylazin and acepromazine and blood was collected from the right chamber of the heart. The mice were then either perfused with HistoChoice tissue fixative (Amresco Inc) before carotid arteries, aortas and hearts were removed and fixed for (a minimum of) several days with Histochoice; or perfused with PBS before carotid arteries were embedded in optimal cutting temperature compound (OTC, Sakura Finetek, Japan), frozen directly (isopropanol on dry ice) and sectioned (7 µm). Carotid arteries fixed with HistoChoice were rinsed in PBS, dehydrated in series of rising ethanol concentrations, two changes of xylene, paraffin overnight, then embedded in paraffin and sectioned (5 µm). Hearts were incubated in 30% sucrose overnight and in OTC with 15% sucrose for 30 min. They were then embedded in OTC, frozen and sectioned (7 µm). Descending aortas were cleaned and mounted en face on slides with ovalbumin/glycerine (Sigma-Aldrich).

Immunohistochemistry and histology
Carotid artery sections were stained for macrophages and foam cells with a Mac-2 antibody (Cedarlane; Burlington, Ontario, Canada), for SMCs with an alpha smooth muscle actin (α-SMA) antibody (clone 1A4, Sigma-Aldrich) and for fibromodulin (antibody kindly provided by Prof. Dick Heinegård). Endogenous peroxidase activity was quenched by incubation in 3% H2O2 (Sigma-Aldrich) and heat induced antigen epitope retrieval, pH 6.0, was performed for 11 minutes (Mac-2 and α-SMA staining). The Vectastain Elite Kit (goat anti-rabbit) and the MOM-kit (both from Vector laboratories; Burlingame, California) were used according to the manufacturer’s instructions, though the former with a goat anti-rat biotin-conjugated secondary antibody (Abcam; Cambridge, UK) during Mac-2 staining. Positive fibromodulin stains were visualized by incubation with ImmPACT NovaRed (Vector laboratories; Burlingame, California, USA) and counterstained with Harris’ hematoxylin (Sigma-Aldrich). Positive Mac2-stains were visualized by 3,3’-Diaminobenzidine and counterstained with Vector Methyl Green nuclear counterstain (Vector laboratories; Burlingame, California, USA). Apoptotic cells were stained using the TUNEL kit TACS XL DAB In Situ Apoptosis Detection Kit from Trevigen according to the manufacturer’s instructions.

PCNA stainings were performed using a PCNA antibody from AbCam and the “PCNA staining kit” from Invitrogen according to the manufacturer’s instruction with the addition of quenching of endogenous peroxidase activity as described previously as well as heat induced
antigen epitope retrieval pH 6.0 for 20 minutes. Sequential double staining was performed using the Mac-2 or α-SMA antibody together with the PCNA antibody with positive stains visualized by 3,3’-Diaminobenzidine and streptavidin alkaline phosphatase with StayRed/AP (AbCam) and counterstained with Harris’ hematoxylin.

Masson’s trichrome staining of carotid artery sections was performed using the “Accustain trichrome stain (Masson)” (Sigma-Aldrich) according to the manufacturer’s instructions. Frozen carotid artery and aortic root sections were incubated in 60% isopropanol, rinsed with dH2O, stained with 0.3% Oil Red O (Sigma-Aldrich) for 20 minutes and then mounted with GVA mount (Zymed, San Francisco, California, USA). Flat preparations of aortas were stained with Oil red O for 50 minutes and mounted with Mountquick (Daido Sangyo Co. LTD, Tokyo, Japan).

**Morphometric measurements**
Lesion size was expressed as area and intima-media ratio and represents the mean value of four sections 15 µm apart where the lesions were at their largest. Positively stained areas of Oil red O-stained descending aortas mounted en face were quantified and expressed as the percentage of the inner arterial lining covered by lesions. Aortic root sections were collected between first appearance and disappearance of the aortic valves. One section every 56 µm was stained with Oil red O. Lipid content was expressed as the mean percentage of lesion area which stained positive for lipids.

**Transmission electron microscopy**
Low and oscillatory shear stress carotid plaques as well as control common carotid arteries and descending aortas (segments cut immediately below the aortic arch; n=3) from ApoE- and ApoE/fibromodulin-null mice were fixed in 0.15 M sodium cacodylate-buffered 2% glutaraldehyde, postfixed in 0.1 M-collidine-buffered 2% osmium tetroxide, and embedded in epoxy resin 1. Ultrathin sections were analyzed in Philips CM-10 electron microscope (Philips, Amsterdam, The Netherlands). Collagen fibril thickness was measured with ImageJ (NIH).

**RNA extraction and quantitative real time PCR (qRT-PCR)**
Uninjured carotid arteries (as control) and low and oscillatory shear stress carotid plaques from ApoE- and ApoE/fibromodulin-null (n=6 per genotype) were isolated after saline perfusion of the mice. Due to the small sample size, low and oscillatory shear stress plaques from 3 mice were pooled yielding 2 groups per genotype. In addition, uninjured carotid arteries from 2 mice were pooled, yielding 3 groups per genotype. Samples were homogenized with TissueLyser (Qiagen, Valencia, CA) in 75 µl extraction buffer and total RNA was extracted with RNeasy Micro Kit (Qiagen) according to the manufacturers’ instructions. Fifty ng total RNA was subjected to first strand cDNA synthesis with Superscript VILO cDNA Synthesis Kit (Invitrogen). QRT-PCR were performed on LightCycler (Roche Applied Science) in duplicates by mixing cDNA with Maxima™SYBR Green qPCR Master Mix (Fermentas, St. Leon-Rot, Germany) and gene specific primer pairs (available upon request). Amplification results were analyzed using LightCycler software Version 3. The calculated threshold cycle values for each gene were normalized to the threshold cycle value of the internal standard hypoxanthine phosphoribosyltransferase 1 (Hprt1).

**Analysis of plasma cholesterol and cytokines**
The colorimetric assay Infinity Total Cholesterol (Thermo Scientific, Liverpool, U.K.) was used to quantify total plasma cholesterol and the mouse Th1/Th2 9-Plex (IFN-γ, IL-1β, TNF-α, IL-2, IL-12, IL-4, IL-5, IL-10, and KC) Ultra-Sensitive Kit (Meso Scale Discovery) was used to quantify plasma cytokine concentrations. Both analyses were performed according to instructions from the manufacturer.
In vitro assays with RAW264.7 cells
Aortic SMCs were isolated from fibromodulin-null and wild type C57BL/6 mice. The aortas were digested in 0.3% collagenase (type II, Gibco) and then seeded in plastic cell culture flasks and kept for up to six passages in Ham’s F-12 medium (Gibco) supplemented with 50 µg/ml gentamicin (Sigma-Aldrich), 5 mg/ml ascorbic acid (Sigma-Aldrich) and 10% NCS (Gibco).

Mouse RAW264.7 cells (gift from Prof. Fredrik Ivars) were grown in DMEM GlutaMax (Gibco) supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco) and 10% FBS Gibco).

Native human LDL (NatLDL; provided by the Experimental Cardiovascular Research Unit at the Clinical Research Center, Lund University) at a concentration of 1 mg/ml was oxidized with 5 µM CuCl$_2$ for 18h at 37°C. Oxidation was stopped by 1 mM EDTA and protein concentration was determined with the Pierce BCA protein assay kit (Thermo Scientific).

For ECM preparation wild type and fibromodulin-null SMCs were seeded on cover slips in 24 well-plates and cultured seven to nine days. Cells were then extracted with 0.5% Triton X-100 in PBS and 25 mmol/litre NH$_4$OH in PBS leaving cover slips covered with cell-free ECM. The effect of fibromodulin-null ECM on LDL-uptake was assessed through seeding RAW264.7 cells on the ECM-covered cover slips and adding 50 µl/ml native or oxidized LDL (in DMEM GlutaMax supplemented with 10% lipoprotein-deficient human serum, 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco)) to each well. RAW264.7 cells were then incubated for four days – fresh LDL was added after two days – and then fixed in 4% formaldehyde and stained with 0.3% Oil Red O for 10 minutes. Cells were counterstained with Methyl Green (Trevigen) and the intracellular lipid content was quantified as Oil Red O-positive area relative to total cell area (values added from 20 micrographs per cover slip).

For immunoblotting, the cell extract from the above-mentioned cultures was precipitated with 50% ethanol, and the pellet was dissolved in SDS-PAGE reducing loading buffer. The samples were run on 4-20% Bis-Tris SDS-PAGE gels and the proteins were transferred onto a nitrocellulose membrane. The membrane was blocked with 4% milk in PBS, and incubated with home-made anti-fibromodulin, or anti-calreticulin (Abcam ab14234) in 0.5% milk and 0.1% Tween-20 in PBS, and detected with HRP-conjugated secondary antibodies followed by ECL (SuperSignal West Pico Substrate, Thermo Scientific). All membrane washes were done in PBS with 0.1% Tween-20.

The effect of wild type and fibromodulin-null ECM on cytokine production in RAW264.7 cells was assessed using the Mouse ProInflammatory 7-Plex Tissue Culture Kit (IFN-γ, IL-1β, IL-10, IL-12 p70, IL-6, KC/GRO/CINC (CXCL1), TNF-α; Meso Scale Discovery). Cells were seeded on wild type and fibromodulin-null ECM-covered cover slips and native or oxidized LDL was added as described for LDL-uptake studies. After two days 30 ng/ml PMA and 1µg/ml ionomycin was added to the wells for 24 hours and cells were then lysed using the CellLytic MT reagent according to the manufacturer’s instructions (Sigma-Aldrich).

Software and statistical methods
Sample size is expressed as n and error bars represent standard deviation (S.D.). Stained sections were scanned and digitalized using an Aperio ScanScope digital slide scanner (Scanscope Console v8.2.0.1263, AperioTechnologies, Inc., Vista, California, USA ) and positively stained areas (fibromodulin, Mac2, Oil Red O and Masson’s Trichrome stain) were quantified using BioPix iQ software (BioPixAB, Gothenburg Sweden). Two-tailed t-test (electron microscopy image analysis) was performed using Microsoft Excel. Mann-Whitney t-test (image analysis) and Student’s t-test and one-way ANOVA analysis with Bonferroni post-test (qRT-PCR) were performed using GraphPad Prism version 4.03 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.
References


Supplemental figure I: Fibromodulin expression in SMCs \textit{in vitro}.
Supplemental Figure III: Size of aortic root lesions from ApoE- and ApoE/fibromodulin-null mice.
Supplemental Figure IV: Fibromodulin immunoreactivity in carotid lesions. Representative sections of fibromodulin-stained carotid lesions and control arteries from ApoE- (A, C and E) and ApoE/fibromodulin-null (B, D and F) mice. Quantitative PCR results showing mean expression levels of fibromodulin mRNA in carotid low and oscillatory shear stress lesions and control arteries isolated from ApoE-/- and ApoE/fibromodulin-/- mice (G). Scale bars = 100 µm.
Supplemental Figure V: Collagen fibril diameter similar in healthy arteries from ApoE- and ApoE/fibromodulin-null mice. Mean fibril diameter was calculated for each carotid artery or aorta from at least three electron micrographs per sample. Average fibril diameter in carotid artery vessel walls were 33 ± 1.8 nm from ApoE-null mice and 29 ± 0.4 nm from ApoE/fibromodulin-null mice (A; n = 3 for each genotype; p>0.09). Average fibril diameter in vessel walls from aorta were 37.5 ± 2.4 nm in ApoE-null mice and 39 ± 6.8 nm in ApoE/fibromodulin-null mice (B; n = 3 for each genotype; p>0.85). Scale bars = 200 nm in A and 100 nm in B, error bars represent standard error of the mean.
Supplemental Figure VI: Decreased lipid content in the media in ApoE/fibromodulin-null mice. Image analysis (A) and representative Oil Red O-stained sections from oscillatory shear stress carotid lesions from ApoE- and ApoE/fibromodulin-null mice. Scale bars = 100 µm.
Supplemental Figure VII: Plasma levels of interleukin-1β (A), -2 (B), -4 (C), -5 (D), -10 (E), -12 (F), interferon-γ (G), KC (H) and tumor necrosis factor-α (I). N=14 and 13 for ApoE-null and ApoE/fibromodulin-null mice, respectively.
Supplemental Figure VIII: Cholesterol content in plasma from ApoE- and ApoE/fibromodulin-null mice. N=14 and 10 for ApoE-null and ApoE/fibromodulin-null mice, respectively.
Supplemental Figure IX: Fibromodulin deficiency does not affect the rate of apoptosis in lesions from low and oscillatory shear stress regions. Representative TUNEL-stained carotid artery plaques from ApoE- and ApoE/fibromodulin-null mice. Scale bars = 100 μm.
Supplemental Figure X: SMC and macrophage proliferation in low and oscillatory shear stress carotid artery lesions from ApoE- and ApoE/fibromodulin-null mice. Representative sections stained for PCNA only (first, third column; positive nuclei are red) and the same sections double stained (second, fourth column) for PCNA and α-SMA or Mac-2 (positive stains are brown). Black squares represent insets. Scale bars = 100 µm.
Supplemental Figure XI: QRT-PCR results showing mean expression levels of Col1α1 (A), Col1α2 (B), Lox (C) and uParap (D) in carotid lesions (n=6). Relative transcript levels in control arteries and lesions were normalized to the reference gene Hprt1.