REPORT

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Novel Role of ADAMTS-5 Protein in Proteoglycan Turnover and Lipoprotein Retention in Atherosclerosis*[S]

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Background: In atherosclerosis, proteoglycan accumulation results in increased lipoprotein retention.
Results: ADAMTS-5 is reduced in aortas of apolipoprotein E-null mice. ADAMTS-5 deficiency impairs processing of vascular proteoglycans, and ADAMTS-5 activity affects proteoglycan-mediated lipoprotein retention.
Conclusion: ADAMTS-5 regulates vascular proteoglycan catabolism and alters lipoprotein retention.
Significance: This is the first study implicating ADAMTS-5 proteolytic activity in atherosclerosis.

Atherosclerosis is initiated by the retention of lipoproteins on proteoglycans in the arterial intima. However, the mechanisms leading to proteoglycan accumulation and lipoprotein retention are poorly understood. In this study, we set out to investigate the role of ADAMTS-5 (a disintegrin and metalloprotease with thrombospondin motifs-5) in the vasculature. ADAMTS-5 was markedly reduced in atherosclerotic aortas of apolipoprotein E-null (apoE$^{-/-}$) mice. The reduction of ADAMTS-5 was accompanied by accumulation of biglycan and versican, the major lipoprotein-binding proteoglycans, in atherosclerosis. ADAMTS-5 activity induced the release of ADAMTS-specific versican (DPEAAE^{441}) and aggrecan (^{374}ALGS) fragments as well as biglycan and link protein from the aortic wall. Fibroblast growth factor 2 (FGF-2) inhibited ADAMTS-5 expression in isolated aortic smooth muscle cells and blocked the spontaneous release of ADAMTS-generated versican and aggrecan fragments from aortic explants. In aortas of ADAMTS-5-deficient mice, DPEAAE^{441} versican neoepitopes were not detectable. Instead, biglycan levels were increased, highlighting the role of ADAMTS-5 in the catabolism of vascular proteoglycans. Importantly, ADAMTS-5 proteolytic activity reduced the LDL binding ability of biglycan and released LDL from human aortic lesions. This study provides the first evidence implicating ADAMTS-5 in the regulation of proteoglycan turnover and lipoprotein retention in atherosclerosis.

According to the response-to-retention hypothesis (1), apolipoprotein B (apoB)[2]-containing lipoproteins are deposited in the arterial intima during the early stages of atherosclerosis. These proatherogenic molecules, in particular low-density lipoprotein (LDL), are trapped by proteoglycans in atherosclerosis-prone areas and become modified, commonly by aggregation and oxidation (2). The accumulation of modified lipoproteins elicits an inflammatory response, alterations in proliferative and matrix-producing properties of smooth muscle cells (SMCs), and remodeling of extracellular matrix (ECM).

The interaction of lipoproteins with proteoglycans is ionic. Positively charged residues on apoB (and other apolipoproteins) interact with sulfated, negatively charged glycosaminoglycan (GAG) side chains of proteoglycans (3). Other, more complex interactions involve the lipid moieties of lipoproteins and the proteoglycan core proteins (4). Proteoglycans with chondroitin sulfate GAGs, such as biglycan and versican, play the major role in LDL retention (5). Both proteoglycans accumulate in atherosclerosis-prone arteries, and their GAG composition is affected by proatherogenic stimuli (6). Biglycan is associated with apoB-containing lipoproteins at all stages of atherosclerosis (7), even in the lipid-rich necrotic core of advanced human plaques (8). It is also known to accumulate in murine lesions (9). Apart from their role in lipoprotein retention, proteoglycans are important determinants of plaque stability (10, 11). Although the role of proteoglycans in the vasculature is well characterized, little is known about mechanisms that control their remodeling and accumulation in atherosclerosis.

In this study, we characterized the catabolic properties of a major proteoglycan-degrading enzyme, ADAMTS-5 (a disintegrin and metalloprotease with thrombospondin motifs-5), on vascular proteoglycans and revealed its ability to alter proteoglycan-mediated lipoprotein retention. For the first time, we implicate reduction of ADAMTS-5 activity in proteoglycan accumulation and lipoprotein binding in atherosclerosis.

EXPERIMENTAL PROCEDURES

Human Tissue—Aortic specimens were collected from three patients (44-year-old female, 47- and 55-year-old males) upon aortotomy performed during routine aortic valve replacement. All patients gave written informed consent. The study was approved by the Research Ethics Committees of St. George's Hospital and King's College London.

Murine Tissue—Abdominal aortas were dissected from 10- and 48-week-old wild-type (WT), apoE$^{-/-}$ (The Jackson Laboratory) or adamts5-ΔCat mice (12) (a kind gift of Prof. Amanda Fosang) from the diaphragm to the iliac bifurcation. Periaortic fat and lymph nodes were removed under a dissecting microscope. Cleaned aortas were kept frozen (−80 °C).

Extraction of Aortic ECM Proteins—Murine aortas were extracted using an adaptation of a published extraction methodology (13). Briefly, 2–3 aortas were pooled for each experi-

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[S] This article contains supplemental Figs. 1–3.

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[2] The abbreviations used are: apoB, apolipoprotein B; ADAMTS-5, a disintegrin and metalloprotease with thrombospondin motifs-5; SMC, smooth muscle cell; ECM, extracellular matrix; GAG, glycosaminoglycan; MMP, matrix metalloproteinase; ANOVA, analysis of variance.

mental condition. ECM proteins were solubilized in 4 M guanidine-HCl, 50 mM sodium acetate, pH 5.8 buffer (5:1 buffer volume to tissue weight), supplemented with proteinase inhibitor mixture (Sigma-Aldrich) and 25 mM EDTA, for 48 h at room temperature. Then, the guanidine extracts were mixed with 100% ethanol to remove guanidine and precipitate the proteins. Protein pellets were redissolved in deglycosylation buffer to remove glycosaminoglycan side chains (11).

Western Blotting—Experiments were performed as described previously (11). The following primary antibodies were used: ADAMTS-1 (sc-5468), ADAMTS-5 (sc-83186), versican (full-length) (sc-25831), FGF-2 (sc-7911), and link protein 1 (sc-46826), all from Santa Cruz Biotechnology; DPEAAE versican fragments (ab19345), biglycan (ab54855), cytoplasmic β-actin (ab8226), apoA1 (ab7613), and apoE (ab7620) from Abcam; and ALGS-aggrecan fragments (AB8135) from Chemicon.

Diaminobenzidine Chemical Immunostaining—Murine sections were probed with antibodies to apoB (ab20737) and $DPEAAE^{441}$ fragments and stained as described in Ref. 11.

In Vitro Metalloprotease Digestion Assay—Murine or human aortic specimens were dissected into smaller pieces and washed five times in ice-cold PBS. The tissue blocks were mixed with 10 volumes of metalloprotease reaction buffer (10 mM $CaCl_2$, 120 mM NaCl, and 50 mM Trizma (Tris base), pH 7.5), supplemented with protease inhibitor mixture (Sigma-Aldrich), which was either used plain (control) or supplemented with different concentrations (50–100 pM) of recombinant enzymes; murine (Chinese hamster ovary cell line) and human (murine myeloma cell line) ADAMTS-1; and murine (Chinese hamster ovary cell line; discontinued) and human ADAMTS-5 (murine myeloma cell line), all purchased from R&D systems. The samples were incubated for 24 h at 37 °C. Tissue extracts were deglycosylated and immunoblotted as indicated.

Murine SMCs—Aortic SMCs were isolated from 4-week-old, wild-type or $apoE^{-/-}$ mice (both C57BL6 background) (14) and used between the 10th and 15th culture passage. SMCs were cultured in DMEM and 10% fetal calf serum (FCS). Confluent SMCs were FCS-starved for 2 h and then either stimulated with 50 ng/ml murine recombinant fibroblast growth factor 2 (FGF-2; PeproTech) or left untreated for 24 h in FCS-free DMEM.

Aortic Explants—Two abdominal aortas from 10-week-old wild-type mice were pooled for each experimental condition. The tissue was directly incubated in FCS-free DMEM and either stimulated with 100 ng/ml murine recombinant FGF-2 (PeproTech) or left untreated for 24 h at 37 °C and 5% CO_2. Alternatively, aortas were incubated with or without 1.2 μM of an aryl thioxothiazolidinone ADAMTS-5 inhibitor (Calbiochem, IC_{50}, 1.1 μM) (15), dissolved in dimethyl sulfoxide (DMSO).

Conditioned Medium—Conditioned medium derived either from SMCs or from aortic explants (typically 300 μl) was collected and deglycosylated as above. Samples were then acetone-precipitated, and the air-dried protein pellets were dissolved in 30 μl of 1× PBS. Prior to electrophoresis, protein concentration was estimated using Bradford reagent (Bio-Rad).

Gelatinolytic Zymography—MMP-2 in the SMC and aortic explant conditioned medium was detected as described previously (13).

Quantitative PCR—Total RNA was extracted from murine abdominal aortas (two aortas were pooled for each experimental condition) or isolated aortic SMCs (2×10^6) using the TRIzol reagent (Invitrogen) and purified using a spin column kit (Qiagen) (16). 1 μg of RNA from SMCs or 700 ng of RNA from abdominal aortas was converted to cDNA using the high capacity RNA-to-cDNA kit (Applied Biosystems). 10 ng of RNA/reaction was quantified with the TaqMan real-time PCR system (Applied Biosystems), using their prevalidated primers/probe mixes, at a concentration of 900 nM for each primer and 200 nM for the probe. TaqMan Probes: biglycan, Mm00455918_m1; versican, Mm01283063_m1; and gapdh, Mm99999915_g1. Real-time PCR was performed using an ABI Prism thermocycler (Applied Biosystems). Data analysis was carried out with the SDS software (version 2.2) from the same company. All samples were measured in duplicate and compared with the expression levels of gapdh.

Solid-phase Biglycan-LDL Binding Assay—The assay was performed as described (6). Following the binding of human LDL on biglycan immobilized on MaxiSorp 96-well immunoplates (NUNC), the wells were covered either with plain MMP buffer or MMP buffer supplemented with 100 pM ADAMTS-5 for 5 h at 37 °C. The immunoplates were blocked with 5% bovine serum albumin for 1 h followed by 1:50 lipoprotein-deficient serum (Sigma-Aldrich) for 1 h. Finally, the wells were developed with horseradish peroxidase-conjugated polyclonal antibodies against human apoB-100 using the 1-Step Turbo TMB-ELISA substrate (Thermo Scientific).

ApoB-100 Release from Aortic Specimens—Following treatment with ADAMTS-5, aortic supernatants (10 μg/ml) were used to coat immunoplates overnight at room temperature and apoB-100 was quantified as above.

RESULTS AND DISCUSSION

Reduction of ADAMTS-5 in Aortas of $apoE^{-/-}$ Mice—Levels of ADAMTS-5 were compared in aortas of young (10-week) and old (48-week) WT and $apoE^{-/-}$ mice. Representative aortic root sections were stained with Oil red O to visualize atherosclerotic lesions (17) (Fig. 1*A*). 10-week-old $apoE^{-/-}$ mice had mild lesions (8.5% ± 2.4 S.D.) as compared with 48-week-old $apoE^{-/-}$ animals (45.75% ± 7.5 S.D.). No lesions were observed in aortic roots of WT mice (Fig. 1*A*). Immunoblotting revealed a reduction of the active form of ADAMTS-5 (~75 kDa) in atherosclerotic aortas (Fig. 1, *B* and *C*), which was accompanied by accumulation of biglycan and versican (Fig. 1, *B* and *C*). Because transcript levels were unchanged (Fig. 1*D*), accumulation of these proteoglycans could be, at least in part, due to reduced catabolism. The pro-form of ADAMTS-1 (~110 kDa), another versican-cleaving protease (18) previously found in SMCs of human atherosclerotic lesions (19), was also reduced in the 48-week-old $apoE^{-/-}$ aortas (Fig. 1*B*). The loss of ADAMTS-5 was associated with reduction in ADAMTS-generated $DPEAAE^{441}$ versican fragments in aortas of 10- and 48-week-old $apoE^{-/-}$ mice (Fig. 1*D*). The ability of ADAMTS-5 to cleave versican at the Glu^{441}-Ala^{442} bond has been recently demonstrated in a study of interdigital ECM web regression (20). It is analogous to its well studied activity in articular cartilage, where the cleavage of the aggrecan Glu^{373}-Ala^{374} bond results in the generation of $NITEGE^{373}$-^{374}ALGS

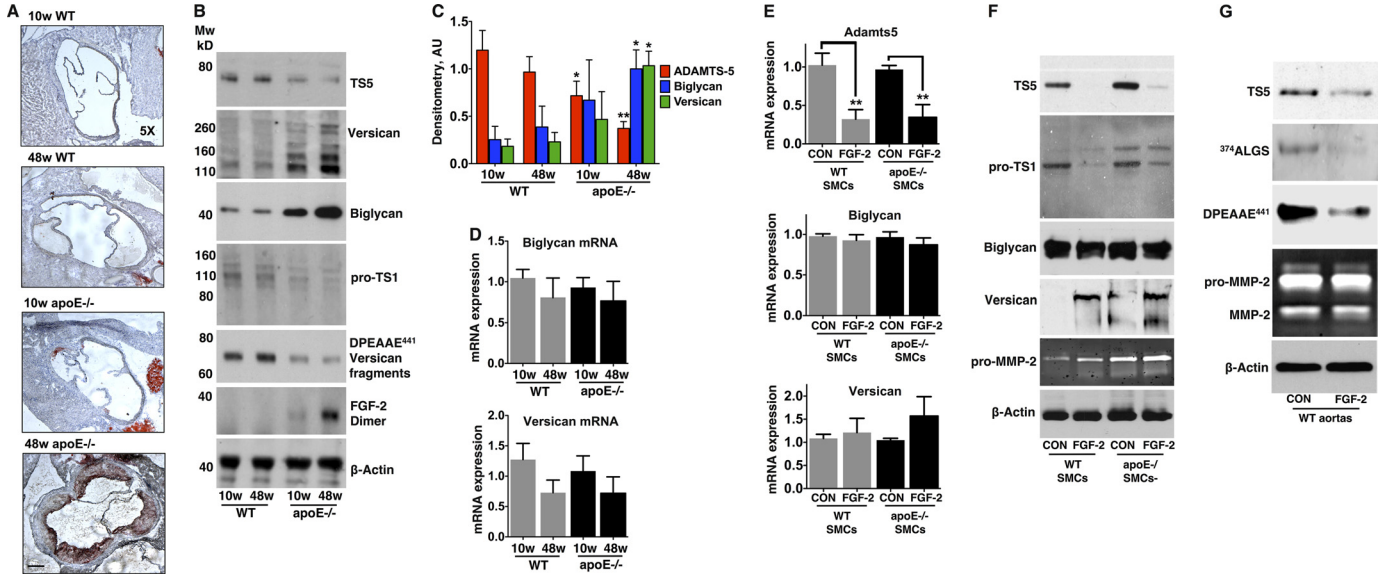

FIGURE 1. **Loss of ADAMTS-5 and proteoglycan accumulation in atherosclerosis.** *A*, Oil red O-stained cross sections of WT and apoE$^{-/-}$ aortic roots. Results are representative of three aortic roots. *Scale bar* 100 μm. *B*, immunoblotting of aortic extracts for ADAMTS-5 (*TS5*), versican, biglycan, ADAMTS-1 (*TS1*), DPEAAE^{441} versican neoepitopes, and FGF-2. β-Actin served as loading control. Three aortas were pooled for each experimental condition. *MW*, molecular weight. *C*, densitometry measurements of immunoblots for ADAMTS-5 (*red bars*), biglycan (*blue bars*), and versican (*green bars*). Values were normalized to β-actin. Results are mean $\pm$ S.D. from $n = 3$ independent experiments. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's post hoc comparisons with WT controls (*, $p \leq 0.05$, **, $p \leq 0.01$). *AU*, absorbance units. *D*, mRNA expression of biglycan and versican as measured by quantitative PCR. Values were normalized to gapdh. Results are mean $\pm$ S.D. from $n = 4$ experiments. *E*, aortic SMCs derived from WT and apoE$^{-/-}$ mice were either stimulated with 50 ng/ml FGF-2 or left untreated (*CON*) for 24 h. Quantitative PCR of adamts5, biglycan, and versican with values normalized to gapdh was performed. Results are mean $\pm$ S.D. from $n = 3$ independent experiments. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's post hoc test (**, $p \leq 0.01$). *F*, immunoblotting for ADAMTS-5, ADAMTS-1, versican, and biglycan in the conditioned medium of aortic SMCs, incubated with or without FGF-2 (50 ng/ml) for 24 h. MMP-2 was detected by zymography. Cellular lysates were immunoblotted for β-actin. Results are representative of at least 3 independent experiments. *G*, immunoblotting for ADAMTS-5, ^{374}ALGS aggrecan fragments, and DPEAAE^{441} versican fragments in the conditioned medium of *ex vivo* cultured abdominal aortas incubated with or without FGF-2 (100 ng/ml) for 24 h. Aortic lysates were immunoblotted for β-actin.

fragments (^{374}ARGS in human aggrecan), the hallmark of ADAMTS-5 activity (21). Interestingly, dimeric FGF-2 (~35 kDa) accumulated in 48-week-old apoE$^{-/-}$ aortas (Fig. 1*D*). Although FGF-2 is a key regulator of ADAMTS-5 expression and activity in cartilage (21, 22) and is up-regulated in atherosclerosis (23–25), its effect on ADAMTS-5 levels in vascular cells has not been demonstrated thus far.

FGF-2-mediated Inhibition of ADAMTS-5 Expression in Aortic SMCs—Aortic SMCs were isolated from WT or apoE$^{-/-}$ mice (14). Both expressed (Fig. 1*E*) and secreted ADAMTS-5 (Fig. 1*F*). Stimulation with FGF-2 reduced ADAMTS-5 expression (Fig. 1, *E* and *F*) but increased levels of versican in the culture medium. Biglycan was unchanged. FGF-2 also inhibited the secretion of the pro-form of ADAMTS-1, whereas MMP-2 (a metalloprotease unrelated to ADAMTSs) was not affected (Fig. 1*F*), indicating that FGF-2 is not a global inhibitor of metalloprotease expression in SMCs. When aortic explants of 10-week-old WT mice were cultured *ex vivo* for 24 h, stimulation with FGF-2 reduced ADAMTS-5 levels in the conditioned medium and inhibited the spontaneous release of ^{374}ALGS aggrecan and DPEAAE^{441} versican fragments (Fig. 1*G*), indicating reduction of ADAMTS catabolic activity. Although little is known about the regulation of ADAMTS-5 expression and activity in tissues, our findings are in line with recent studies reporting increased ADAMTS-5 activity and aggrecan breakdown in fgf2$^{-/-}$ mice (22). In another study, stimulation of cartilage explants and chondrocytes with FGF-2 inhibited ADAMTS-5 mRNA expression and blocked the release of aggrecan fragments following stimulation with IL-1 (21). In the vasculature, the suppression of ADAMTS-5 by FGF-2 could be related to the ability of the growth factor to increase neointima formation following arterial injury (25).

ADAMTS-5 Cleaves Vascular Proteoglycans—To assess the proteolytic properties of ADAMTS-5 on vascular proteoglycans, murine aortas were incubated with recombinant ADAMTS-5. ADAMTS-1 was used for comparison. ADAMTS-5 caused the release of ^{374}ALGS aggrecan and DPEAAE^{441} versican fragments as well as full-length biglycan and link protein (HPLN1) (Fig. 2*A*). Biglycan is a known target of ADAMTS-5, *in vitro* (26) and in cartilage (27). The release of link protein indicates that ADAMTS-5 catabolic activity could compromise the binding of proteoglycans with hyaluronan (28). When proteoglycans from ADAMTS-treated aortas were extracted using 4 M guanidine, ADAMTS-1 was also able to generate DPEAAE^{441} fragments (Fig. 2*B*), but unlike ADAMTS-5, it did not induce their release into the supernatant (Fig. 2*A*). Next, incubation of human aortic tissue with either ADAMTS-1 or ADAMTS-5 resulted in the release of DPEAAE^{441} fragments (Fig. 2*C*). The additional DPEAAE-immunoreactive band at ~150 kDa most likely represents the less abundant versican V0 isoform cleaved at the DPEAAE^{1428} bond (18). Instead, HPLN1 and biglycan were only released following treatment with ADAMTS-5. ^{374}ARGS (human aggrecan neoepitope) was not detected in the aortic supernatant. The activity of the proteases was also examined using purified bovine substrates; both ADAMTS-1 and ADAMTS-5 digested biglycan (supplemental Fig. 1*A*), but only ADAMTS-5 generated aggrecan fragments (supplemental Fig. 1*B*). To further investi-

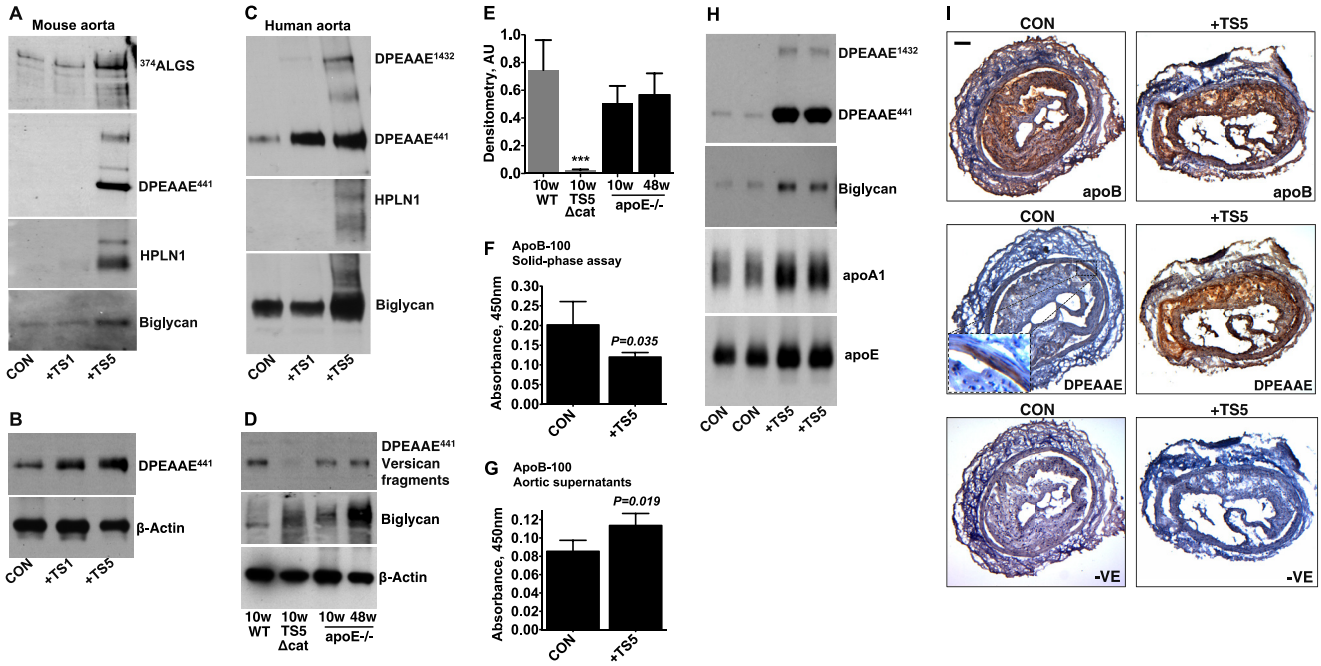

FIGURE 2. **ADAMTS-5-mediated cleavage of vascular proteoglycans affects lipoprotein retention.** *A*, aortas from 10-week-old WT mice were incubated either in plain MMP reaction buffer (*CON*) or in MMP reaction buffer supplemented with either 50 pM murine recombinant ADAMTS-1 (+*TS1*) or 50 pM ADAMTS-5 (+*TS5*) for 24 h at 37 °C. The supernatants were analyzed by immunoblotting for ^{374}ALGS and DPEAAE^{441} neoepitopes, HPLN1, and biglycan. Each lane had 20 μg of protein. Results are representative of at least 3 independent experiments. *B*, guanidine extracts from ADAMTS-treated aortas were immunoblotted for versican neoepitopes and β-actin. *C*, a human aortic specimen was dissected into three equal blocks incubated either in plain MMP reaction buffer or in MMP reaction buffer supplemented with either 100 pM human recombinant ADAMTS-1 or 100 pM ADAMTS-5 for 24 h at 37 °C. The supernatants were immunoblotted for DPEAAE^{441}, HPLN1, and biglycan. *D*, immunoblotting of WT, TS5 Δcat, and apoE$^{-/-}$ aortic extracts for DPEAAE^{441} neoepitopes, biglycan, and β-actin. *E*, densitometry measurements of DPEAAE^{441} neoepitopes normalized to β-actin. Results are mean ± S.D. from $n = 3$ independent experiments. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's post hoc comparison with the 10-week-old WT control (***, $p \leq 0.001$). *AU*, absorbance units. *F*, amount of LDL (measured as apoB-100) bound to biglycan after 5 h of digestion with 100 pM ADAMTS-5. Values are mean ± S.D. from $n = 4$ independent experiments, each performed with six replicates/condition. Statistical analysis was performed using two-tailed, unpaired *t* test. *G*, three human aortic specimens with subendothelial lipid accumulation were treated either with plain MMP reaction buffer or with 100 pM ADAMTS-5 for 24 h at 37 °C. The aortic supernatants were used to coat 96-well immunoplates, and the amount of LDL was quantified using HRP-conjugated antibodies against apoB-100. Values are mean ± S.D. from $n = 3$ independent experiments and six replicates/condition. Statistical analysis was performed using two-tailed, unpaired *t* test. *H*, aortic supernatants from two human specimens used in *G* were immunoblotted for DPEAAE^{441}, biglycan, apoA1, and apoE. *I*, consecutive, parallel sections from 48-week-old apoE$^{-/-}$ abdominal aortas were probed with antibodies to apoB or DPEAAE^{441} neoepitope. Note the difference in staining for DPEAAE^{441} following incubation with 50 pM ADAMTS-5 for 24 h at 37 °C (*right panels*) as compared with controls incubated in plain MMP reaction buffer (*left panels*). Negative controls were probed with secondary antibodies only (−*VE*). Nuclei were counterstained with Mayer's hematoxylin. *Scale bar*, 25 μm.

gate the proteolytic role of the enzyme in the vasculature, aortas from mice lacking ADAMTS-5 catalytic activity (TS5 Δcat) (12) were compared with aortas of WT and apoE$^{-/-}$ mice (Fig. 2*D*). DPEAAE^{441} versican neoepitopes were barely detectable in TS5 Δcat aortas (Fig. 2, *D* and *E*). In contrast, biglycan was increased, reaffirming the role of ADAMTS-5 in the processing of vascular proteoglycans *in vivo*. Moreover, 10-week-old WT murine aortas were cultured *ex vivo* in the presence of a specific ADAMTS-5 inhibitor (IC_{50} 1.1 μM) (15) (supplemental Fig. 2). At 1.2 μM, the inhibitor completely blocked the generation of DPEAAE^{441} in aortic explants. Taken together, our findings are consistent with recent studies reporting that ADAMTS-5 deletion attenuates versicanolysis in the skin pericellular matrix, evidenced by the reduction of DPEAAE^{441} fragments (30).

ADAMTS-5 Activity Affects Retention of LDL on Proteoglycans—Given the key role of chondroitin sulfate proteoglycans in lipoprotein retention, we investigated whether ADAMTS-induced proteoglycan fragmentation affects LDL binding. 96-well immunoplates coated with biglycan were used as binding substrates for purified, human LDL (6). Digestion of biglycan with ADAMTS-5 caused loss of apoB-100 from the biglycan-coated surface (Fig. 2*F*). Moreover, treatment of human aortic specimens (supplemental Fig. 3*A*) with ADAMTS-5 resulted in the release of LDL from the vascular matrix (Fig. 2*G*), accompanied by DPEAAE^{441} fragments and biglycan as well as apoA1 and apoE (Fig. 2*H*), also known to interact with biglycan (29). Coomassie Blue staining revealed a consistent protein pattern in the supernatants (supplemental Fig. 3*B*). Our data suggest that proteoglycan catabolism by ADAMTS-5 alters lipoprotein binding to the vascular matrix, and this could have important implications in atherosclerotic lesion formation. Next, we examined the relationship between apoB and versican neoepitopes in advanced murine plaques. Strong apoB staining was seen on the entire lesion surface. The signal for DPEAAE^{441} fragments was weak (Fig. 2*I*, *left panels*), confirming low ADAMTS proteolytic activity. Following incubation of the aorta with ADAMTS-5 (+*TS5*), the newly generated DPEAAE^{441} fragments colocalized with apoB (Fig. 2*I*, *right panels*), demonstrating that processing by ADAMTS-5 may affect the interaction of versican with lipoproteins. Although other metalloproteases could also induce loss of lipoproteins from the vascular extracellular space by degrading the ECM, the specific and potent activity of ADAMTS-5 on the LDL-binding, proteoglycan fraction of the matrix is intriguing.

Study Limitations—Our study does not exclude the role of other metalloproteases in the processing of proatherogenic proteoglycans. For instance, we failed to detect the mature form of ADAMTS-1 (~80 kDa), but other, potentially active forms may be present. Nonetheless, aortas of ADAMTS-5-deficient mice showed a lack of ADAMTS-induced versicanolysis (Fig. 2, *D* and *E*). At present, we have no *in vivo* evidence for the role of ADAMTS-5 in atherosclerosis, but its function in vascular proteoglycan remodeling and lipoprotein retention warrants further investigation.

Conclusions—In this study, we showed for the first time that ADAMTS-5 is reduced in a murine model of atherosclerosis and that this reduction coincides with the accumulation of biglycan and versican, the major LDL-binding species.

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