Endostatin binds biglycan and LDL and interferes with LDL retention to the subendothelial matrix during atherosclerosis[S]

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Abstract Retention of lipoproteins to proteoglycans in the subendothelial matrix (SEM) is an early event in atherosclerosis. We recently reported that collagen XVIII and its proteolytically released fragment endostatin (ES) are differentially depleted in blood vessels affected by atherosclerosis. Loss of collagen XVIII/ES in atherosclerosis-prone mice enhanced plaque neovascularization and increased the vascular permeability to lipids by distinct mechanisms. Impaired endothelial barrier function increased the influx of lipoproteins across the endothelium; however, we hypothesized that enhanced retention might be a second mechanism leading to the increased lipid content in atheromas lacking collagen XVIII. We now demonstrate a novel property of ES that binds both the matrix proteoglycan biglycan and LDL and interferes with LDL retention to biglycan and to SEM. A peptide encompassing the α coil in the ES crystal structure mediates the major blocking effect of ES on LDL retention. ES inhibits the macrophage uptake of biglycan-associated LDL indirectly by interfering with LDL retention to biglycan, but it has no direct effect on the macrophage uptake of native or modified lipoproteins. Thus, loss of ES in advanced atheromas enhances lipoprotein retention in SEM. Our data reveal a third protective role of this vascular basement membrane component during atherosclerosis.—Zeng, X., J. Chen, Y. I. Miller, K. Javaherian, and K. S. Moulton. **Endostatin binds biglycan and LDL and interferes with LDL retention to the subendothelial matrix during atherosclerosis. *J. Lipid Res.* 2005.** 46: **1849–1859.**

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Lipid accumulation and macrophage-derived foam cells are prominent features of atherosclerotic plaques. The response-to-retention hypothesis proposes that serum lipoproteins are retained by proteoglycans in the subendothelial matrix (SEM) and subsequently modified and taken up by macrophages to form foam cells that constitute the fatty streak type of atheroma (1–3). Biglycan, a dermatan sulfate proteoglycan in human and mouse atheromas, binds LDL by ionic interactions involving specific basic amino acids in apolipoprotein B-100 and sulfated sugar modifications of biglycan (4–6). LDL association or aggregation with arterial wall proteoglycans increases rates of LDL oxidation and macrophage uptake (7, 8). Mice expressing mutant apolipoprotein B-100 produce LDL particles with lower proteoglycan affinity and develop less atherosclerosis (9, 10). Dermatan sulfate and chondroitin sulfate proteoglycans such as biglycan and versican are increased, whereas heparan sulfate proteoglycans are reduced in atheromas compared with normal blood vessels (4, 11). Therefore, relative changes in the matrix composition of atheromas could enhance LDL retention of SEM during atherosclerosis.

The aorta is an abundant tissue source of the heparan sulfate proteoglycan collagen XVIII and its proteolytically released endostatin (ES) fragment, which has previously been shown to inhibit angiogenesis in cancer and atherosclerosis models (12–15). The ES portion of collagen XVIII has no attachment sites for glycosaminoglycans (GAGs) but has high affinity for heparin, which is necessary for its antiangiogenesis functions (16). Our recent data showed that collagen XVIII is differentially degraded in blood vessels affected by atherosclerosis. Loss of collagen XVIII/ES resulted in enhanced plaque neovascularization and vascular permeability to lipids in mice prone to develop atherosclerosis (17).

Abbreviations: B_{max}, maximum density of binding sites in matrix; ES, endostatin; GAG, glycosaminoglycan; HBS, HEPES-buffered saline; HUVEC, human umbilical vein endothelial cells; K_d, dissociation constant; OxLDL, oxidized low density lipoprotein; SEM, subendothelial matrix.

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[S] The online version of this article (available at http://www.jlr.org) contains an additional figure

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Impaired barrier function of the aorta would increase the influx of lipoproteins across the endothelium; however, we hypothesized that enhanced lipoprotein retention could be a second mechanism to increase lipid accumulation in atheromas lacking collagen XVIII and ES. The protease-susceptible N terminus of collagen XVIII proteins are depleted in early-stage atheromas compared with the ES fragment; therefore, we tested whether ES modifies lipoprotein retention to biglycan and to SEM (18, 19). We now show that ES interacts with biglycan and LDL and interferes with LDL retention to biglycan. ES binding to a more complex SEM also inhibited LDL retention. Interactions of ES with biglycan and LDL involve a common region that contains the α coil in the ES crystal structure. Finally, we show that ES inhibits the macrophage uptake of biglycan-associated LDL indirectly by interfering with LDL retention to biglycan. Thus, progressive loss of ES in atheromas may lead to enhanced subendothelial lipid retention.

MATERIALS AND METHODS

Expression and purification of recombinant proteins

Recombinant human ES containing the C-terminal residues (132–315) of collagen XVIII was expressed in *Pichia pastoria* (provided by EntreMed, Inc., Rockville, MD). ES amino acids are numbered based on residue 1 at the start of the NC1 domain (Brookhaven National Laboratory, Protein Data Bank code 1BNL). ES mutant protein was expressed as an Fc-fusion protein in mammalian myeloma cells that was affinity-purified and then separated from Fc after enterokinase cleavage (20). Two arginine-to-alanine substitutions generated an ES mutant (R158A/R270A) with impaired heparin affinity and antiangiogenesis activity (21). Rabbit polyclonal antibodies reactive to human and mouse ES were previously shown to lack reactivity for tissues from *Col18a1* null mice (20). Additional conformation-specific (12C1) and peptide-derived monoclonal (PDM, reactive to peptide 3) antibodies were tested for inhibition of ES-LDL binding (20).

Biglycan derived from bovine cartilage (Sigma) was used for LDL retention assays as described (9). Purity was assessed by the release of a major 45 kDa protein after chondroitin ABC lyase digestion and recognition by biglycan antibody (see Fig. 6 below). Rabbit polyclonal antibodies recognizing bovine biglycan were provided by Dr. Larry Fisher (National Institute of Dental Research) (22).

Synthetic ES peptides

We tested eight overlapping peptides spanning human ES to identify domains that interact with biglycan and LDL. ES peptides were synthesized (Synpep Corp., Dublin, CA) with the original intent to identify domains with antitumor activities (23). The N- and C-terminal amino acids for each peptide are indicated: peptide 1 (H132–G158), peptide 2 (M154–R178), peptide 3 (T176–V200), peptide 4 (A198–Q222), peptide 5 (G220–A244), peptide 6 (H242–Y265), peptide 7 (E266–Z290), and peptide 8 (L288–K315) (20, 24). The peptide 2 sequence is (MRGIRGADFQAFQQARAVGLAGTFR).

Protein binding assays

We performed solid-phase lipoprotein binding assays on the matrix components ES and biglycan (9). Maxisorp immunoplates (NUNC) were incubated overnight with 100 μl of HEPES-buffered saline (HBS, 20 mM HEPES and 150 nM NaCl, pH 7.4) containing ES (5 μg/ml) or biglycan (20 μg/ml dry weight, 30% protein content; Sigma). Wells were rinsed with HBS, blocked for 1 h with HBS containing 1% BSA (Sigma), and incubated with human LDL (range, 0–20 μg/ml; Biomedical Technologies, Inc., Stoughton, MA) in binding buffer (HBS + 0.1% BSA, 2 mM $CaCl_2$, and 2 mM $MgCl_2$) for 1.5 h at room temperature. After rinsing, bound LDL was detected with peroxidase-conjugated polyclonal antibodies reactive against human apolipoprotein B (diluted 1:750; The Binding Site) and absorbance at 450 nm of Turbo TMB-ELISA substrate (Pierce, Rockford, IL).

For solid-phase ES binding assays, we immobilized biglycan or LDL (10 μg/ml solution immobilized 0.462 μg of LDL per well), blocked the wells, and then incubated ES at various concentrations in HBS over the matrix or LDL-coated wells for 2 h. Bound ES was detected with rabbit polyclonal anti-human ES antibody (1:500), peroxidase-conjugated donkey anti-rabbit polyclonal antibody (1:1000; Amersham), and the substrate reaction described. For competition experiments, the indicated competitors were mixed with the solution-phase protein immediately before adding to the immobilized target protein.

Chondroitin ABC lyase treatment of biglycan

Biglycan-coated wells were digested with chondroitin ABC lyase (US Biological; 0.1 U/well, 37°C for 10 min) in digestion buffer (33 mM Tris, pH 8.0, 0.1% BSA, and 33 mM sodium acetate) containing protease inhibitors (1 mM PMSF, 5 mM benzamidine, 1 μM 1,10-diphenanthraline, 1 μM E64 cathepsin inhibitor, and Complete protease cocktail by Pierce). Control wells were incubated in the same digestion buffer lacking enzyme.

Immunoprecipitation of protein complexes

To detect ES-biglycan complexes formed in solution, we biotinylated biglycan using EZ-link sulfo-NHS-LC-biotin (Pierce), removed unbound biotin by dialysis, and confirmed that streptavidin resin pulled down biotin-biglycan. We incubated ES (20 μg/ml) and biotin-biglycan (40 μg/ml) overnight at 4°C in 200 μl of HBS, then added 20 μl of streptavidin resin, which had been blocked previously with HBS containing 1% BSA. The resin was collected 1 h later by centrifugation, rinsed four times with HBS containing 0.1% Nonidet P-40, extracted with SDS sample buffer, and separated by 12% SDS-PAGE. We detected ES in trapped biglycan complexes by Western blot analysis using rabbit polyclonal ES-IgG (1:1,000), horseradish peroxidase-conjugated donkey anti-rabbit IgG (1:5,000; Amersham International), and enhanced chemiluminescence.

To detect ES-LDL complexes that form in solution, we incubated increasing doses of ES with human LDL (10 μg/ml) in 200 μl of HBS containing 0.25% gelatin and 0.2% BSA for 3 h at 4°C, then added rabbit polyclonal apolipoprotein B antibody (1:750; The Binding Site) followed by 10 μl of protein A-Sepharose for 1 h each. We collected and rinsed the resin, resolved the trapped LDL complexes by gel analysis, and detected ES by Western blot analysis as described for biglycan pulldown methods.

Macrophage isolation

We collected resident mouse peritoneal macrophages from 4–6 week old C57BL6/J mice (Jackson Laboratory, Bar Harbor, ME) after peritoneal lavage and resuspended macrophages at 6×10^5 cells/ml density in RPMI 1640 medium (GIBCO/BRL Life Technologies).

Macrophage uptake and degradation assays

We tested whether ES directly altered cell binding, cell uptake, and degradation of native or modified lipoproteins when macrophages were exposed to soluble ES or seeded on ES ma-

trix. Native LDL or oxidized low density lipoprotein (OxLDL) was labeled with ^{125}I (Amersham International) in the presence of iodination beads (Pierce) and separated from unincorporated ^{125}I over a PD-10 column. The specific activity was typically 200 cpm/ng protein. Phenol chloroform extraction of labeled lipoproteins showed 90% of the radioactivity separated in the aqueous fraction.

For assays with ES added in solution, the medium over macrophages was replaced with RPMI medium containing ^{125}I-LDL (20 μg protein/well) plus ES (5 μg/ml), heparin binding mutant ES (5 μg/ml), biglycan (20 μg/ml), or laminin (20 μg/ml). Cell surface binding of lipoproteins was measured on plates incubated at 4°C for 4 h. Lipoprotein radioactivity associated with cells and with degradation products in the medium was measured on plates incubated at 37°C for the same period. The uptake of lipoprotein by macrophages was expressed as cell-associated labeled lipoprotein minus cell binding at 4°C (25). Radioactivity was converted to mass units based on the specific activity of LDL and normalized for cell number (^{125}I-LDL ng/mg cell protein).

For assays with immobilized ES, we coated 24-well plates with ES, heparin binding mutant ES, biglycan, laminin, or BSA, plated macrophages for 14 h, then added lipoproteins and measured cell binding, cell uptake, and degradation as described above. Binding of ^{125}I-labeled matrix and ELISA methods confirmed matrix absorbed to the coated tissue culture wells. In the third case, we tested whether retention of LDL to matrix before seeding macrophages would alter the cell association and degradation of LDL (8). Wells coated with ES and other matrices were incubated for 2 h with ^{125}I-LDL (20 μg protein/well) in a minimum volume of medium to retain LDL, 300,000 resident mouse peritoneal macrophages were added, and the cell-associated and degraded LDL products in the medium fractions were measured after 14 h at 37°C. In separate studies, unbound LDL not retained by the immobilized matrix was removed before adding macrophages.

Minimally OxLDL induced cytoskeletal rearrangements in macrophages

Minimally OxLDL was generated by an 18 h incubation of LDL with fibroblasts expressing 15-lipoxygenase. A bioassay measured the effects of minimally OxLDL on macrophage cell shape. J774 macrophages were incubated for 1 h with native or minimally modified LDL in the presence of ES or biglycan, and cell spreading was assessed microscopically and by levels of polymerized actin (26, 27).

Subendothelial basement membrane preparations

We prepared SEM from confluent monolayers of human umbilical vein endothelial cells (HUVEC; Cambria, La Jolla, CA) plated at near confluence on uncoated 96-well culture dishes for 2 days (11). Endothelial cells were detached after 5 min of incubation with 20 mM NH_4OH and 0.1% Triton X-100 and rinsed well with PBS followed by MEM and 3% BSA. SEM was incubated overnight with ES (range, 5 ng/ml to 5 μg/ml), biglycan, laminin, or control HBS buffer. Binding of ^{125}I-ES tracer and ELISA assays confirmed exogenous ES absorbed to the SEM. Saturation binding curves and 95% confidence intervals for LDL binding to SEM alone and to SEM incubated with ES were analyzed by GraphPad PRISM, version 4 (GraphPad Software, Inc., San Diego, CA). SEM with and without collagen XVIII was prepared from primary endothelial cells isolated from lungs of wild-type and *Col18a1*-null mice (28).

Aorta extractions

Aortas from C57BL6 and cholesterol-fed *Apolipoprotein E*$^{-/-}$ mice with increasing areas of atheromas were perfusion-rinsed, frozen in liquid nitrogen, pulverized, and homogenized in ice-cold RIPA buffer containing protease inhibitor cocktail (Pierce) and 10 mM EDTA to maximally extract ES-containing proteins (13). Aorta extract proteins (15 μg) were separated by 12% SDS-PAGE, and ES-containing proteins were detected by Western analysis relative to β-actin.

Statistical analysis

Data for protein binding assays, lipoprotein cell association, degradation, and cell binding were compared for statistical significance based on the ANOVA. Overall *P* values were calculated using GraphPad PRISM. In binding experiments, data points represent mean absorbance ± SD (error bars) from three samples. Experiments were performed in triplicate. Maximum density of binding sites in matrix (B_{max}) and dissociation constant (K_d) binding parameters were determined using the nonlinear regression function of GraphPad PRISM (version 4.0) and a one-binding-site model.

RESULTS

ES inhibits LDL retention to biglycan

Retention of LDL by arterial wall proteoglycans in the SEM is considered to be a key event in the development of atherosclerosis (1, 2). To determine whether ES alters LDL retention to biglycan, we compared LDL binding to immobilized biglycan and ES, separately and together, using previous assays for LDL retention to biglycan (9). We coated protein-absorbent plates with ES (5 μg/ml) and biglycan (20 μg/ml dry weight, 30% protein). The amount of immobilized protein was calculated from the percentage binding of ^{125}I-labeled ES and biglycan tracers. The indicated solutions absorbed 0.416 μg of ES and 1.04 μg of biglycan per well. Biglycan retained LDL, as shown previously, and ES retained LDL. Surprisingly, ES and biglycan in combination showed lower LDL binding than each component alone (**Fig. 1A**). ELISA showed that biglycan was reduced by 5% and ES was reduced by ~15% in combination wells relative to the respective biglycan- or ES-only wells; however, these differences in coating efficiencies could not account for the significant blocking effect on LDL retention. The negative interference of ES with LDL retention to biglycan suggested that ES and biglycan interacted with each other.

We performed solid-phase and solution-phase binding assays to determine whether ES bound biglycan. We immobilized biglycan on protein-absorbent plates, determined the average amount of biglycan bound per well, and performed saturation binding studies with ^{125}I-ES tracer. Statistical analysis of binding curves for ES binding to immobilized biglycan indicated that the B_{max} was 783 fmol ES/μg biglycan and the K_d was 110 nM (Fig. 1B).

To demonstrate that ES-biglycan complexes form in solution, we biotinylated biglycan and confirmed that it was trapped by streptavidin agarose. A Western blot of biotin-biglycan detected with avidin-HRP showed no biglycan core protein (data not shown). ES (20 μg/ml) and biglycan (40 μg/ml) were incubated overnight at 4°C. We pulled down biglycan-containing complexes with streptavidin agarose and separated the eluted complexes by 12% SDS-PAGE. ES was detected in targeted biglycan complexes but absent in control lanes lacking biglycan (Fig. 1C). These

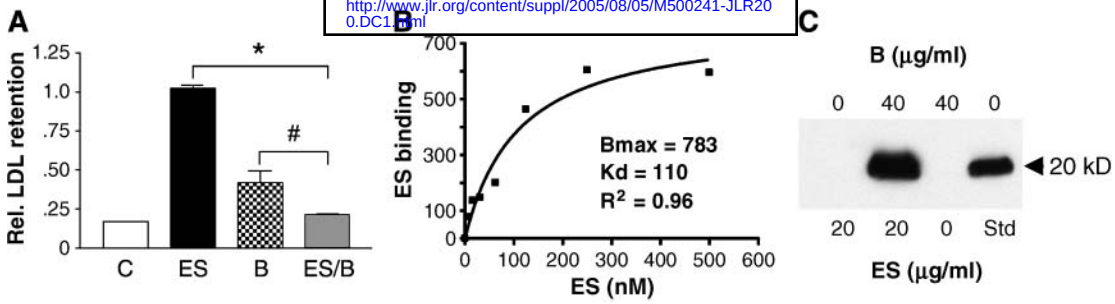

Fig. 1. Endostatin (ES) interferes with LDL retention to biglycan. A: Maxisorb plates were incubated overnight with ES (5 μg/ml) or biglycan (B; 20 μg/ml) separately and in combination. LDL (10 μg/ml) was incubated on matrix-coated wells, and retained LDL was measured relative to the absorbance of the ELISA substrate and normalized for the wells showing maximal retention (ES). ES and biglycan retained LDL, but LDL retention was reduced significantly in combination ES-biglycan wells compared with ES (* $P < 0.001$) and biglycan (# $P < 0.05$) alone. C, control BSA-coated wells. B: The saturation curve for binding of ^{125}I-ES to immobilized biglycan was determined over a range of ES concentrations (nM). Specific binding was calculated as fmol ES/μg biglycan (*y* axis). B_{max}, maximum density of binding sites in matrix; K_d, dissociation constant. C: Western blot of ES in complexes pulled down by streptavidin resin from solutions containing ES (20 μg/ml) and biotin-biglycan (40 μg/ml). The control lane verified that ES was pulled down only in trapped biglycan. Standard (Std) represents 50 ng of ES.

data confirmed that ES and biglycan interact with each other directly.

ES decreases LDL retention to SEM

To determine whether ES alters LDL retention to more complex SEM, we prepared SEM from confluent HUVEC monolayer cultures and determined the effect of added ES or other matrix molecules on LDL retention to SEM (11). LDL bound SEM at higher levels than BSA-coated wells (data not shown). SEM supplemented with ES inhibited LDL retention compared with control SEM or SEM incubated with biglycan or laminin (**Fig. 2A**). We compared saturation curves for LDL binding to SEM versus SEM absorbed with ES at 5 μg/ml (Fig. 2B). ES averaged

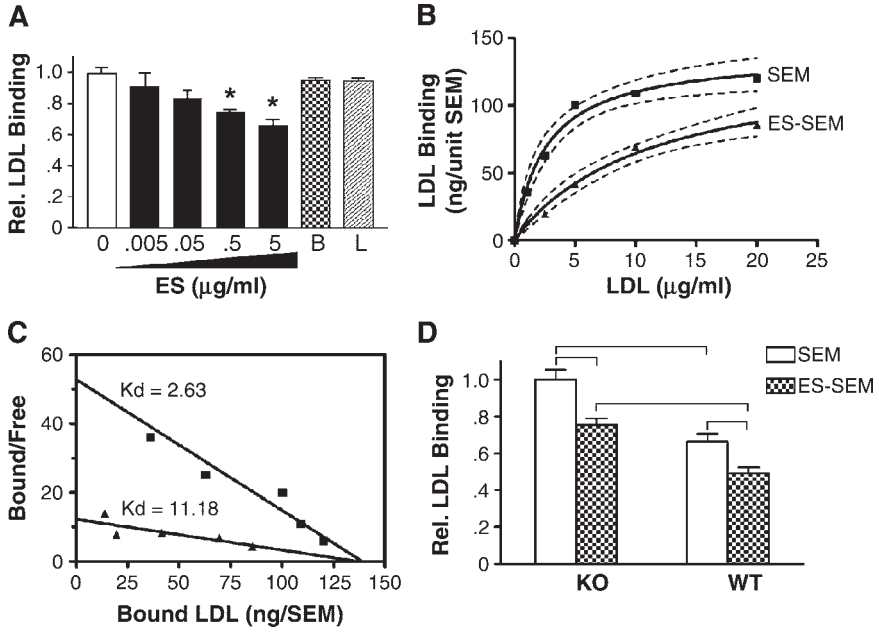

Fig. 2. ES reduces LDL binding to the subendothelial matrix (SEM). A: SEM was prepared from human umbilical vein endothelial cell monolayers, incubated overnight with ES (0.005–5 μg/ml), control buffer, biglycan (B; 20 μg/ml), or laminin (L; 20 μg/ml), then incubated with LDL (10 μg/ml) and assayed for LDL retention. LDL binding to SEM was inhibited by ES but not biglycan or laminin. Values shown are means ± SD. B: Saturation curves of ^{125}I-LDL binding to SEM (squares) or ES-treated SEM (triangles). The SEM and ES-SEM binding curves with their 95% confidence intervals (dashed lines) show no overlap and are therefore statistically different. C: Scatchard analysis of both retention curves shows similar B_{max} values (*x* intercept), but the affinity of LDL for ES-SEM is lower (K_d is 4.3-fold greater). D: SEM prepared from *Col18a1*-null (KO) endothelial cells showed greater LDL retention compared with SEM from wild-type (WT) endothelial cells (KO vs. WT, $P < 0.001$). LDL binding was normalized relative to KO-SEM (value = 1.0). ES supplementation of KO and WT- SEM reduced LDL retention (ES-SEM vs. SEM for KO endothelium, $P < 0.01$; ES-SEM vs. SEM for WT endothelium, $P < 0.05$). Values shown are means ± SD.

60 ± 5 ng in SEM wells without treatment and 82 ± 11 ng in ES-absorbed SEM wells, as determined by ELISA and an ES standard curve. Percentage binding of ^{125}I-ES determined that ~28 ng (range, 24–30 ng) of ES bound each SEM well. The saturation LDL binding curves with their 95% confidence intervals for SEM compared with ES-absorbed SEM did not overlap. Scatchard analysis of LDL binding showed similar B_{max} values (138.9 ± 7.2 vs. 136.6 ± 15.8) but a significant difference in K_d (2.63 ± 0.45 vs. 11.18 ± 2.5) for SEM and ES-SEM, respectively (Fig. 2C). Therefore, the difference in LDL retention is related to a lower affinity for ES-treated SEM. We further asked whether LDL retention to SEM lacking collagen XVIII and ES would be enhanced. We prepared SEM from monolayers of *Col18a1*-null and wild-type endothelial cells and compared each matrix for LDL retention. LDL retention was increased in *Col18a1*-null compared with wild-type SEM and was inhibited after absorption with ES (Fig. 2D). These data suggest that ES in SEM has a negative effect on LDL retention.

ES domain interactions with lipoproteins and biglycan

Our initial studies unexpectedly found that ES retained LDL, which was confirmed over a range of LDL doses (**Fig. 3A**). Saturation curves of ^{125}I-ES binding to LDL revealed a B_{max} of 2,054 fmol/μg LDL protein and a K_d of 78

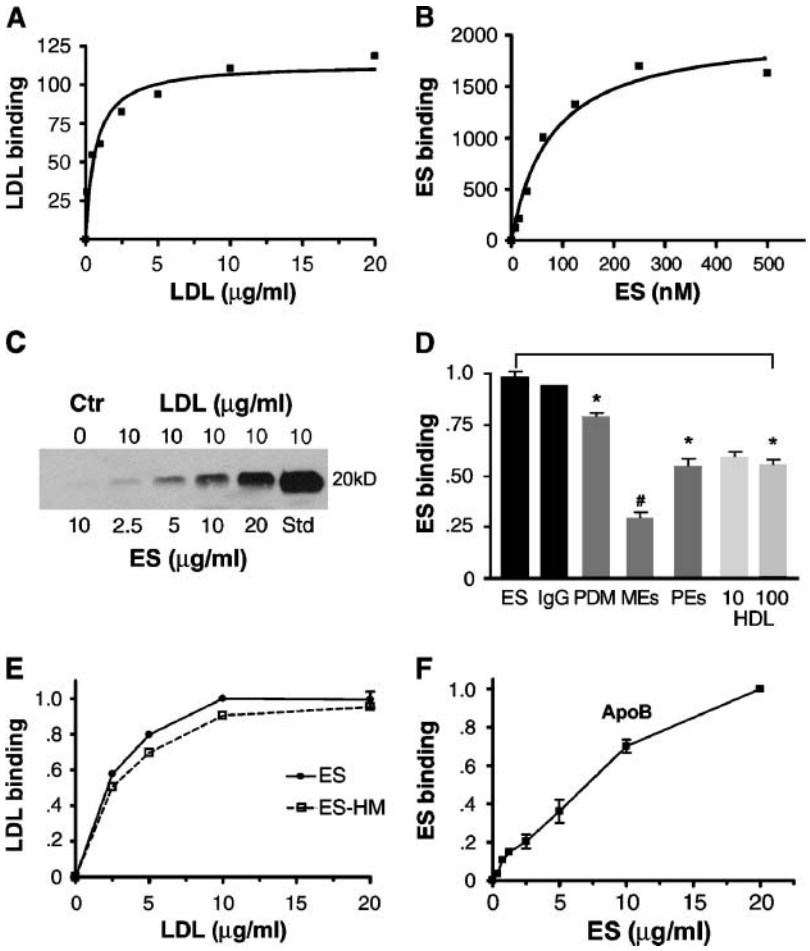

Fig. 3. ES has affinity for lipoproteins. A: ES (5 μg/ml) was absorbed on plates and incubated with human LDL (1–20 μg/ml). Retained LDL on ES was quantified by ELISA and converted to mass units by reference to a standard curve for LDL (*y* axis; ng LDL/μg ES matrix). B: Saturation curves of ^{125}I-ES binding to LDL-absorbed wells (0.462 μg/well) revealed a B_{max} of 2,054 fmol/μg LDL protein and a K_d of 78 nM. C: Western blot of ES in LDL complexes immunoprecipitated with apolipoprotein B antibody. ES-LDL complexes formed in solutions containing 10 μg/ml LDL and ES concentrations ⩾2.5 μg/ml. No ES was pulled down by the apolipoprotein B antibody in control samples lacking LDL (Ctr). The standard (Std) lane contained 100 ng of ES protein. D: ES-LDL binding was evaluated by competition with ES antibodies. ES-containing wells were incubated with control IgG, a peptide 3 monoclonal antibody (PDM), a conformation-specific monoclonal antibody (MEs), and rabbit polyclonal ES antibody (PEs), then rinsed and incubated with 10 μg/ml LDL. Bound LDL was measured based on the absorbance of the ELISA substrate and normalized relative to wells without competitors (value = 1). In separate experiments, LDL was mixed with HDL (10 or 100 μg/ml HDL), which partially competed for LDL binding to ES (* $P < 0.05$; # $P < 0.01$). E: Wells coated with 5 μg/ml wild-type (closed circles) or R158A/R270A heparin mutant (HM; open squares) ES were incubated with increasing LDL doses to measure relative LDL binding. Binding curves for LDL retention to ES or the heparin binding ES mutant were similar. F: ES bound immobilized apolipoprotein B protein (ApoB).

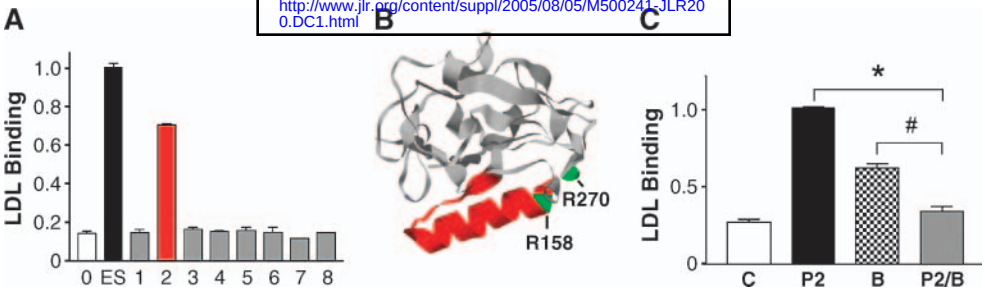

Fig. 4. The ES domain was involved in interference with LDL retention. A: Molar equivalent amounts of ES (5 μg/ml) and overlapping peptides (0.7 μg/ml) that span ES (residues 132–315) were immobilized on Maxisorb wells to identify ES domains that bound LDL. Peptide 2 accounted for ~70% of LDL binding activity compared with ES. B: The ES crystal structure displayed by the Ras Top program highlights peptide 2 (red), which includes the α coil and residue (R158). The R158A and R270A mutations (green) impair the heparin affinity but not the LDL affinity of ES. C: Peptide 2 (P2) corresponded to the major binding region for biglycan (B). LDL retained to peptide 2 and biglycan alone, but LDL retention was attenuated when peptide 2 and biglycan were coplated (combined vs. ES, * $P < 0.01$; combined vs. biglycan, # $P < 0.05$). C, control.

nM (Fig. 3B). To verify that ES interacts with LDL in solution, we used apolipoprotein B antibodies to immunoprecipitate LDL complexes that form in solutions containing ES. ES was detected in trapped LDL complexes that formed at ES concentrations ≥2.5 μg/ml (Fig. 3C).

We performed competition studies to determine the specificity and nature of ES-LDL interactions. Polyclonal and conformation-dependent (12C1) monoclonal antibodies reactive against ES significantly blocked ES-LDL binding, whereas a peptide-derived monoclonal antibody reactive to ES residues T176–V200 (peptide 3) showed partial inhibition of LDL binding compared with isotype-IgG (Fig. 3D). These neutralizing actions of ES antibodies demonstrated the specificity of the interaction for ES; however, ES binding to LDL was not specific because it also bound OxLDL at a similar affinity (data not shown). In competition studies, HDL at equal doses of LDL (10 μg/ml) competed for 45% of LDL binding; however, a 10-fold excess of HDL did not compete further for LDL binding sites in ES (Fig. 3D). Evidence to support the involvement of apolipoprotein B in LDL-ES interactions was provided by solid-phase assays of ES binding to immobilized apolipoprotein B (Fig. 3F).

A positively charged surface formed by a cluster of arginines in ES confers affinity for heparin or heparan sulfate proteoglycans and is necessary for its antiangiogenesis functions (24). To determine whether heparin affinity for ES is necessary for LDL binding, we compared curves for LDL binding to heparin binding mutant ES relative to immobilized ES. The R158A/R270A ES mutant has 90% reduced heparin affinity (21, 29), yet the LDL binding curve for the heparin binding mutant ES was nearly identical to that for ES (Fig. 3E). Furthermore, heparin at 5 and 10 μg/ml did not compete for LDL binding to ES (data not shown).

ES binding domains

We next screened overlapping ES peptides to identify domains that participate in binding with LDL and biglycan. Peptide 2 (M14–R178) spanned the α coil domain in the ES crystal structure and accounted for 70% of the binding activity for LDL compared with an equivalent molar amount of ES (**Fig. 4A**, **B**). Interestingly, this same peptide contained the major binding affinity for biglycan and contained no serine residues for potential sites of glycoaminoglycan modifications (30, 31). On wells coated with higher peptide concentrations (1 μg/ml), only peptide 3 showed a significant increase in LDL binding above baseline; no other peptides bound biglycan (data not shown).

Because peptide 2 sequences are involved in ES interactions with both LDL and biglycan, we tested whether peptide 2 would interfere with LDL retention to biglycan as we showed for the full-length ES. Wells coated with peptide 2 and biglycan alone showed increased LDL binding, yet LDL retention was reduced in combined peptide 2 and biglycan wells (Fig. 4C). Together, these data show that ES binds biglycan and interferes with LDL retention via sequences in peptide 2.

Competitive interactions between ES, biglycan, and lipoproteins

The previous studies showed that competitive interactions between ES and biglycan inhibited LDL retention to the immobilized matrix proteins; however, we wanted to directly compare the ability of ES and biglycan to compete with the other for binding to LDL. Wells coated with LDL (0.462 μg) were incubated for 1.5 h with 5 μg/ml ES with and without biglycan competitor, which was mixed at 0.5:1 and 1:1 molar ratios of competitor minutes before adding to the plates. At equal molar ratios, biglycan competed for 45% of ES binding to LDL (**Fig. 5A**). We incubated LDL-absorbed plates with 20 μg/ml biglycan mixed with and without ES competitor at the molar ratios given above. Bound biglycan was detected by ELISA using biglycan antibodies. ES mixed 1:1 with biglycan blocked 40% of biglycan binding to LDL (Fig. 5B). These data demonstrate that ES and biglycan compete with each other for LDL.

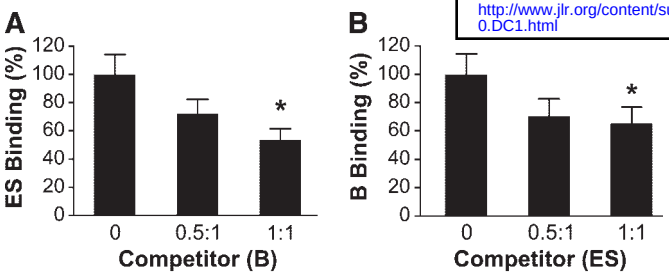

Fig. 5. Reciprocal competition between ES and biglycan for LDL. A: LDL-coated wells were incubated for 1.5 h with 5 μg/ml ES with and without biglycan (B) competitor mixed at 0.5:1 and 1:1 molar ratios. Bound ES was measured relative to maximal ES binding without competitor (100%). Biglycan competed for 45% of ES binding to LDL. B: LDL-coated wells were incubated for 1.5 h with 20 μg/ml biglycan with and without ES competitor mixed at the indicated molar ratios. Bound biglycan was measured by ELISA methods. At equal molar ratios, ES competed for 40% of biglycan binding to LDL. * $P < 0.05$, relative to ES or B binding without competitor.

Interactions with GAGs in biglycan

Other studies have suggested that LDL binding to biglycan involves interactions with GAG side chains on biglycan (32). We evaluated whether GAG modifications of biglycan are involved, or are essential, for ES binding. Gradient gel and Western blot analyses of biglycan showed that the majority of undigested biglycan migrated at high molecular mass and contained little core protein (**Fig. 6A**). Chondroitin ABC lyase digestion released a major 45 kDa protein that stained positive for biglycan antibodies on Western blot and produced no smaller proteolytic products of biglycan. To determine whether GAG modifications of biglycan are required for ES binding, we treated biglycan-absorbed wells with chondroitin ABC lyase or control digestion buffer lacking enzyme, rinsed the wells, then performed ES binding assays. ES binding to chondroitin-treated biglycan was increased compared with binding to undigested biglycan (Fig. 6B). We next tested whether dermatan sulfates or heparin would compete for ES binding to biglycan and whether competition would require the presence of GAG modifications on biglycan. Chondroitin-treated and control biglycan prepared as described above were incubated for 2 h with ES (20 μg/ml) alone or ES in the presence of dermatan sulfate (5 μg/ml) or heparin (5 μg/ml) competitors. Dermatan sulfates but not heparin significantly inhibited ES binding to biglycan, even after biglycan was digested with chondroitin ABC lyase (Fig. 6C). These data indicate that ES binding to biglycan is not solely dependent on its GAGs; however, they do not exclude the possibility that ES could interact with both core protein and GAG side groups of biglycan. We attempted to address whether ES interactions with GAG side groups on biglycan are necessary for ES interference with LDL retention; however, LDL retention to biglycan after chondroitin ABC lyase digestion was the same as background levels of LDL retention to BSA wells in Fig. 1, which obviated an evaluation for a secondary effect by ES.

ES exerts no direct effect on lipoprotein uptake and degradation by macrophages

Previous reports have shown that retention of LDL to proteoglycans enhances its susceptibility to oxidation and uptake by human monocyte-derived macrophages (7, 33). The specific receptors mediating enhanced macrophage uptake of retained LDL are not certain, but they may involve bridging or structural modifications of the lipid and apolipoproteins (8, 34–36). Therefore, we asked whether ES binding to lipoproteins alters its uptake or degradation rates by macrophages. Several variations were tested with macrophages exposed to soluble ES or seeded on ES ma-

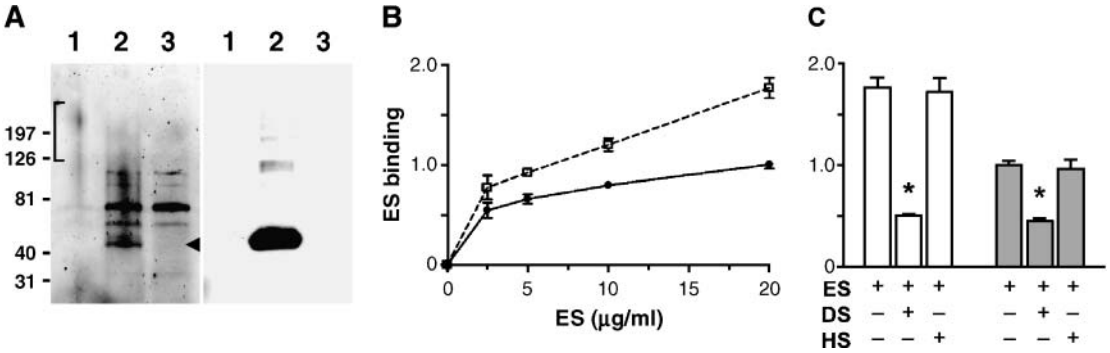

Fig. 6. Glycosaminoglycan (GAG)-dependent and -independent binding to biglycan. A: Tris-glycine gradient gel of biglycan (2 μg; lane 1), biglycan treated with chondroitin ABC lyase (lane 2), and digestion cocktail lacking biglycan (lane 3). The inverse image of a gel stained with Sypro-orange (left) shows a broad distribution of undigested proteins migrating at high molecular masses (bracket) and release of a unique protein at ~45 kDa (arrow) that reacts with biglycan antibodies after transfer (Western blot; right). Biglycan antibody was more reactive to biglycan after digestion. All other protein bands were found in the digestion cocktail. B: Biglycan-coated wells were treated with chondroitin ABC lyase or control digestion buffer under the same conditions used for samples in A, then incubated with ES to measure ES binding. ES retention to chondroitin-treated biglycan (open squares) was increased relative to control biglycan (closed circles) exposed to digestion buffer lacking enzyme. Retained ES was normalized relative to maximal ES binding to undigested biglycan (value = 1). C: Biglycan treated with (open bars) and without (closed bars) chondroitin ABC lyase was assayed for ES binding with and without dermatan sulfate (DS; 5μg/ml) or heparan sulfate (HS; 5 μg/ml) competitors. Dermatan sulfates competed for ES binding to biglycan regardless of chondroitin ABC lyase digestion. * $P < 0.05$, relative to ES binding to biglycan without DS.

trix, and the clearance rates for native and OxLDL were measured separately.

To evaluate the effects of soluble ES, we plated peritoneal macrophages overnight, then replaced the medium with medium containing ^{125}I-LDL (or OxLDL) plus ES, heparin binding mutant ES, biglycan, laminin, or BSA. We measured cell binding of lipoproteins in wells incubated for 4 h at 4°C. In wells incubated at 37°C, we measured radioactivity associated with cells and with degradation products released in the medium. Cell uptake of lipoproteins was determined from the cell-associated radioactivity minus cell binding at 4°C. We found that ES increased cell surface binding of LDL at 4°C; however, it did not increase cell uptake or degradation of LDL by macrophages (**Fig. 7A**). The ES heparin mutant had the same affinity for LDL as ES, but it did not increase cell binding of LDL.

In separate experiments with ^{125}I-OxLDL (data not shown), we observed the same relative effects by ES and other matrix components as shown for LDL. Lastly, we examined ES activity in a bioassay of the effects of minimally OxLDL on macrophage cell shape and spreading (26, 27). ES did not modify cytoskeletal changes in macrophages induced by modified LDL (data not shown).

Second, we plated macrophages overnight on wells coated with ES or other matrix molecules, provided medium with labeled lipoproteins, then repeated the cell binding, cell uptake, and degradation measurements normalized for cell protein in each well. Macrophages were 95% confluent and had similar cell morphology and plating efficiencies on the different matrices (i.e., cell protein/well). ES matrix did not alter uptake or degradation; however, plating of macrophages on biglycan increased the internalization and degradation of LDL (see supplementary data). In summary, we observed that ES had no direct effect on the clearance of lipoproteins by macrophages.

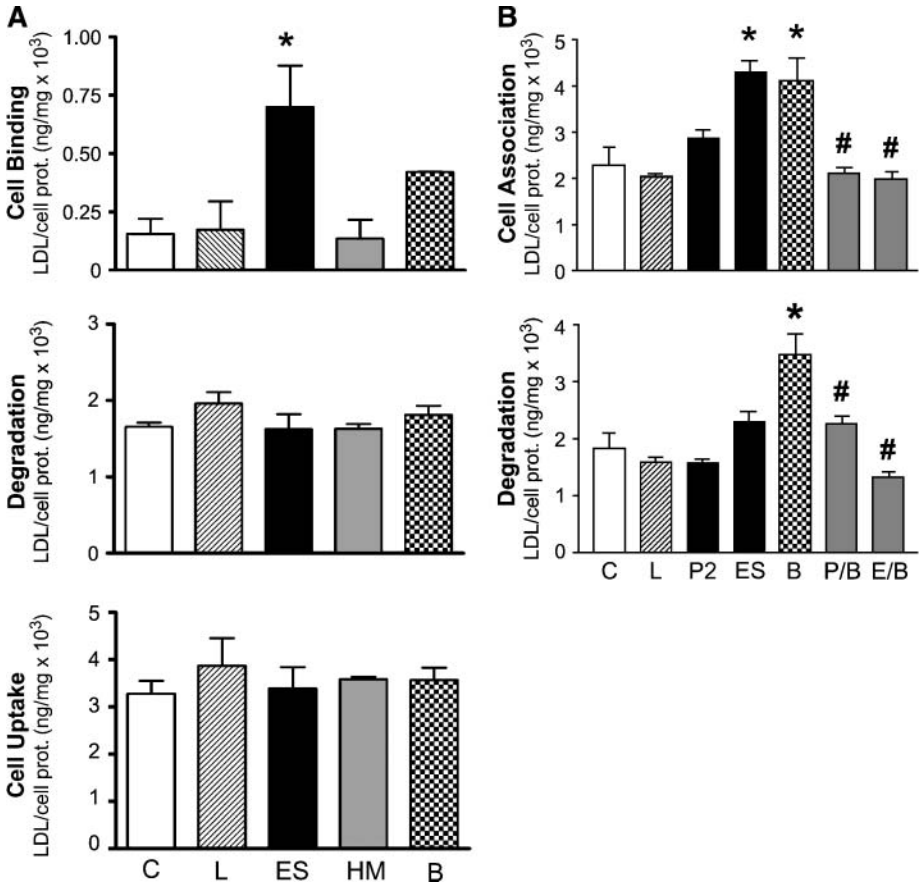

Fig. 7. ES interferes with the clearance of biglycan-retained LDL by macrophages. A: Cell binding, degradation, and cell uptake of ^{125}I-LDL by macrophages were measured with BSA (C, control), laminin (L), ES, heparin binding mutant ES (HM), or biglycan (B) added to the medium. Cell binding of LDL at 4°C was increased by ES but not by the heparin ES mutant. No matrix components altered cell uptake and degradation. B: LDL was retained to wells coated with control BSA (C), laminin (L; 20 μg/ml), peptide 2 (P2; 0.7 μg/ml), ES (5 μg/ml), or biglycan (B; 20 μg/ml) alone or in combination with ES or peptide 2 before plating resting peritoneal macrophages. Cell association and degradation of LDL retained by biglycan were increased. The positive effect of biglycan was inhibited by ES or peptide 2, consistent with their interference with LDL retention to biglycan. Data are means ± SD. * $P < 0.01$ (statistical significance of ES or biglycan lanes compared with BSA control); # $P < 0.01$ (statistical significance of combined biglycan and ES or peptide 2 wells compared with biglycan alone).

ES indirectly inhibits the uptake of biglycan-associated lipoproteins

Retention of LDL on proteoglycans facilitates its uptake and degradation by macrophages. We incubated ^{125}I-LDL with BSA, laminin, ES, peptide 2, biglycan, and combinations of ES-biglycan or peptide 2-biglycan under conditions that allowed LDL retention, then added resident mouse peritoneal macrophages and measured the rates of cell-associated and degraded LDL after 14 h. Biglycan increased the cell association and degradation of LDL relative to peptide 2, laminin, and control BSA wells, consistent with previous studies of proteoglycan-associated lipoproteins (8). The biglycan effect on macrophages was blocked in the combined ES-biglycan and peptide 2-biglycan wells (Fig. 7B). ES, but not peptide 2 or the heparin binding mutant, increased the cell association of LDL; however, in this experimental design, we could not adjust the cell-associated fraction for cell surface binding at 4°C to measure the internalization of retained LDL.

To determine whether the biglycan-mediated uptake of LDL by macrophages was mainly from the fraction of LDL retained to biglycan, we repeated the cell association and degradation experiments after rinsing away unbound LDL. We observed the same results as shown in Fig. 7B. Thus, ES has an indirect effect on the clearance of biglycan-retained LDL, attributable to its interference with LDL retention to biglycan.

Relative ES levels in normal and atherosclerotic aortas

Biglycan and other proteoglycans such as versican accumulate in murine and human atheromas (5). In contrast, we found that the relative levels of ES in aortas become reduced as the percentage area of atheromas in the aorta increases (**Fig. 8A**). Our present data show that ES interferes with LDL binding to SEM and biglycan; therefore, loss of ES in atherosclerotic arteries may be both a consequence of atherosclerosis and a pathogenic mechanism. Our data suggest a possible model for the progression of atherosclerosis, whereby the dual increase of biglycan and relative depletion of ES together promote lipid retention in diseased blood vessels (Fig. 8B).

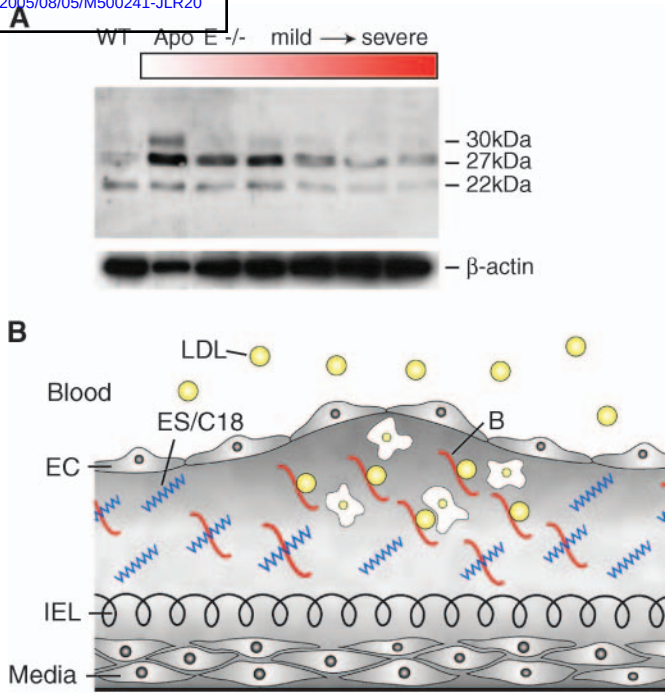

Fig. 8. Loss of ES in arteries during atherosclerosis. A: Western blot of aorta extracts from wild-type (WT) and *Apolipoprotein E*$^{-/-}$ (*Apo E*−/−) mice with increasing area of atheromas in the aorta. The abundance of ES-containing proteins relative to β-actin is reduced as the area of atheromas in the aorta is increased. B: Model for enhanced LDL retention in aortas associated with increased biglycan (B) and decreased abundance of ES and collagen XVIII (ES/C18). Interactions between ES (blue) and biglycan (red) inhibit LDL binding to either matrix component. The net effect of reduced ES and increased biglycan may increase the lipoprotein retention properties of SEM during atherosclerosis. EC, endothelial cells on the arterial endothelium; IEL, internal elastic lamina.

DISCUSSION

Our data reveal a novel property of ES that interferes with LDL retention to biglycan and SEM, an important step in the development of atherosclerosis. Depletion of collagen XVIII or its proteolytically released ES fragment in SEM enhances lipid retention as a second mechanism contributing to lipid accumulation in atherosclerosis-prone mice lacking collagen XVIII and ES (19). The mechanism for ES interference with LDL retention to biglycan involves direct binding between ES and biglycan. We further show that ES interference has indirect functional consequences on the uptake and degradation of biglycan-retained LDL by macrophages. Furthermore, ES interfered with LDL retention to a complex matrix produced by endothelial cells. Increasing the ES content of SEM significantly reduced its binding affinity for LDL (K_d increased) but not the B_{max}, which might have occurred if ES itself retained LDL or made high-affinity irreversible interactions with LDL binding sites in other matrix molecules. Consistent with a blocking effect of ES on LDL retention, SEM produced by *Col18a1*-null endothelial cells retained more LDL compared with wild-type SEM, and LDL retention was reduced after adding ES. The present study focused on the mechanisms for ES interference with retention; however, the potential contributions from other regions of collagen XVIII remain a subject for future investigations.

The specific mechanism for ES interference with LDL retention to biglycan involved sequences encompassing the α coil structure in ES that contained the major binding activities for both LDL and biglycan. This peptide domain was sufficient to interfere with LDL retention to biglycan. The LDL and biglycan binding regions of ES overlap but are not identical, because LDL binding activity extended into peptide 3, which did not bind biglycan.

The mechanisms for ES interference with LDL retention in SEM could include an effect on biglycan in SEM; however, SEM contains other proteoglycans, such as perlecan and versican, that are capable of retaining LDL (13, 30). Thus, ES interference with LDL retention to SEM

likely involves other matrix molecules besides biglycan. For instance, ES has been shown to bind nidogen, fibulin 1, fibulin 2, and perlecan, which are more abundant in atheromas; but the impact on LDL retention by ES interactions with these other matrix molecules is not yet understood (5).

Other studies have shown that LDL retention involves ionic interactions between certain basic amino acids in apolipoprotein B-100 and GAG side groups on biglycan (32). The competition of ES-biglycan binding by dermatan sulfates suggests that ES interacts with GAG side groups on biglycan that mediate LDL retention. Thus, a simple explanation for ES interference with LDL retention might arise from competition for GAG side groups on biglycan. ES-biglycan interactions are not exclusive to its GAG side groups, as revealed by the increased ES-biglycan binding observed after chondroitin ABC lyase digestion. It remains possible that ES interactions with residues in the biglycan core and/or apolipoprotein B in LDL particles could induce conformational changes in these proteins, which in turn could reduce the affinity of LDL for biglycan or SEM. Steric hindrance could be mediated by the close proximity of LDL and biglycan binding sites in ES. Understanding the complexity of three-component interactions between ES, LDL, and biglycan in concert may require a three-dimensional analysis of binding complexes. Mutational studies of the respective LDL and biglycan affinities of ES might determine whether its LDL affinity is required for its interference with retention to biglycan. For example, would an ES mutant with impaired LDL affinity but preserved biglycan affinity be able to interfere with LDL retention to biglycan? Mapping the ES binding domains in apolipoprotein B relative to sequences implicated in binding proteoglycans could determine whether ES binding to apolipoprotein B could alter its affinity for biglycan or other proteoglycans.

The physiologic correlate of ES binding to lipoproteins in vitro is not known at present. ES bound LDL in vitro, which suggests that it retains LDL and promotes atherosclerosis; however, it had the opposite effect in vivo and in vitro when other LDL-retaining matrix molecules were present. ES-LDL binding was detected at doses exceeding 1 μg/ml, which is 20-fold greater than ES levels in serum; therefore, the chance of forming significant ES-LDL complexes in the circulation is low. ES has no direct effect on macrophages; however, sequestration of OxLDL by ES might ameliorate toxic effects on other vascular cells (37). The antiretention functions of collagen XVIII or ES in vascular basement membranes could be necessary for the normal transport of lipoproteins from blood across the endothelium to peripheral tissues without trapping lipids in the extracellular matrix.

LDL retention studies performed in vitro have some limitations. SEM produced by cultured endothelial cells may differ from SEM in blood vessels. Basement membranes self-assemble into an organized structure, so ES-absorbed SEM may not represent the distribution of endogenous ES in the SEM of arteries. Exogenous ES has been shown to incorporate in remodeling tumor blood vessels (38). Near-infrared labeled ES injected in the peritoneal cavity of tumor-bearing mice shows selective uptake in subcutaneous tumors and in tumor blood vessels (39). Thus, the delivery of ES to SEM may have therapeutic benefits to attenuate LDL retention and lipid accumulation in atheromas. Our previous studies showed that ES inhibited atherosclerosis and plaque neovascularization as one mechanism for ES action; however, it is not yet known whether sufficient exogenous ES accumulates in SEM to alter LDL retention in vivo, or whether replacement doses can keep up with the rates of depletion that accompany the disease (15). The present findings demonstrate that the loss of ES and the increased abundance of atherogenic proteoglycans in atheromas together enhance LDL retention during atherosclerosis.

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