Statin-exposed vascular smooth muscle cells secrete proteoglycans with decreased binding affinity for LDL

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Abstract Retention of LDL in the artery intima is mediated by extracellular matrix proteoglycans and plays an important role in the initiation of atherosclerosis. Compared with quiescent cells, proliferating smooth muscle cells secrete proteoglycans with elongated glycosaminoglycan side chains, which have an increased binding affinity to LDL. Because 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors (statins) decrease smooth muscle cell proliferation, we hypothesized that statin exposure would decrease both the size and LDL binding affinity of vascular proteoglycans. Monkey aortic smooth muscle cells grown in culture were exposed to simvastatin (10 and 100 µM) and cerivastatin (0.1 and 1 µM), and newly secreted proteoglycans were quantified and characterized. Both simvastatin and cerivastatin caused a concentration-dependent reduction in cell growth and reduced $^{35}SO_4$ incorporation into secreted proteoglycans, on both an absolute and a per cell basis. Interestingly, statin exposure increased the apparent molecular weight and hydrodynamic size of secreted proteoglycans. However, proteoglycans secreted from statin-exposed cells demonstrated a reduction in binding affinity to LDL. Thus, statins may induce atheroprotective changes in vascular proteoglycans and lower LDL retention in the vessel wall. ▰▰ These findings suggest a mechanism whereby statins may benefit atherosclerosis in a manner unrelated to serum LDL lowering.—Meyers, C. D., L. R. Tannock, T. N. Wight, and A. Chait. **Statin-exposed vascular smooth muscle cells secrete proteoglycans with decreased binding affinity for LDL.** *J. Lipid Res.* 2003. **44:** 2152–2160.

Supplementary key words glycosaminoglycan • atherosclerosis • extracellular matrix • mevalonate

Elevated LDL cholesterol is a well-known risk factor for coronary artery disease, and the deposition of LDL in the arterial wall plays a key role in the initiation and progression of atherosclerosis (1, 2). Over the past 20 years, inhibitors of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, or statins, have become the first-line agent for lowering serum LDL, due to their efficacy and tolerability. Recent large clinical trials have demonstrated that statins effectively reduce major cardiovascular events and death in patients with pre-existing cardiovascular disease (3, 4) and in subjects without underlying heart disease (5, 6). While most of the clinical benefit of statins is attributed to LDL lowering, these drugs have many noncholesterol effects (7, 8).

By inhibiting the enzyme HMG-CoA reductase, statins deplete intracellular mevalonate, which is a direct substrate for the synthesis of cholesterol (9). The resulting decrease in intracellular cholesterol leads to an upregulation of LDL receptors at the cell surface, and an increase in LDL clearance from the circulation (10). Mevalonate is also a substrate for the synthesis of isoprenoids such as farnesylpyrophosphate and geranylgeranyl pyrophosphate, which are important in regulating a variety of cell processes (11). For example, post-translational prenylation of the small GTP binding proteins *rho* and *ras* is essential for the initiation of cell proliferation (12).

Vascular smooth muscle cells play an important role in the development and progression of atherogenesis. Intimal hyperplasia, a hallmark finding of atherosclerosis, results from the migration and proliferation of smooth muscle cells originating in the vessel media (13). Statins inhibit vascular smooth muscle cell proliferation, primarily by inducing cell cycle arrest in G_1 (14). Cell cycle arrest can be prevented by the addition of geranylgeranyl pyrophosphate to statin-exposed cells, suggesting the importance of protein prenylation in smooth muscle cell proliferation (15). Statin-mediated inhibition of vascular smooth muscle cell proliferation might, in part, account for the beneficial effect of statins in reducing atherosclerotic events.

Vascular smooth muscle cells also are a major source of intimal extracellular proteoglycans, a diverse class of macromolecules consisting of glycosaminoglycan side chains

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attached to specific core proteins (16). Negatively charged glycosaminoglycan side chains on vascular proteoglycans interact with positively charged amino acid residues on apolipoprotein B and apolipoprotein E, leading to lipoprotein retention, a critical step in the initiation of atherosclerosis (17). Proteoglycans synthesized by actively proliferating smooth muscle cells have longer glycosaminoglycan side chains and bind to LDL more avidly than do proteoglycans secreted by quiescent cells (18). Thus, actively proliferating smooth muscle cells not only lead to intimal hyperplasia but also secrete potentially proatherogenic proteoglycans.

The specific effects of statins on the regulation of vascular smooth muscle cell proteoglycan secretion have not been extensively studied. Because statins inhibit smooth muscle cell proliferation, we hypothesized that statins would decrease the size of secreted proteoglycans and decrease the binding affinity of these proteoglycans to LDL. We demonstrate here that exposure of vascular smooth muscle cells to statins leads to the synthesis of proteoglycans with lower LDL binding affinity compared with proteoglycans secreted from control cells. These findings suggest that statins may reduce the retention of LDL in the artery wall by a mechanism unrelated to serum LDL lowering.

MATERIALS AND METHODS

Statins and isoprenoid intermediates

Cerivastatin was received as a generous gift from Bayer, AG, while simvastatin was prepared from Zocor® tablets. Cerivastatin stock solution was prepared by dissolving the statin in Dulbecco's modified Eagle's medium (DMEM, Bio Whittaker, Walkersville, MD), followed by sterile filtration. Simvastatin stock solution was prepared after conversion from the inactive lactone form to the active open ring form (19), suspended in DMEM, and sterile filtered. Mevalonolactone (Sigma, St. Louis, MO) was converted to the open-ring product mevalonate, as with simvastatin. All-*trans* farnesol (Sigma) and all-*trans* geranylgeraniol (American Radiolabeled Chemicals, Inc., St. Louis, MO) stock solutions were prepared in DMSO. Vehicle controls for each of the statins and the isoprenoid intermediates were prepared in a manner identical to stock solutions, except for the addition of the active chemical.

Cell culture, exposures, and proteoglycan labeling

Monkey (*Macaca nemestrina*) aortic smooth muscle cells isolated by the explant method were maintained in culture as described previously (20). The media were changed every 2–3 days until visual confluence was achieved, then cells were made quiescent for 48 h in media containing 0.1% calf serum. After this period of growth arrest, cells were exposed to cerivastatin (0.1 and 1 μM) and simvastatin (10 and 100 μM), as well as appropriate controls, for up to 72 h. All drug exposures took place in the presence of 5% calf serum to promote cell growth, as vascular smooth muscle cells do not demonstrate contact inhibition. Some cells were exposed concurrently to the statins and the isoprenoid intermediates mevalonate (100 μM), farnesol (10 μM), or geranylgeraniol (10 μM). Parallel dishes of cells were also exposed to the isoprenoid intermediates alone. Nascent proteoglycans were metabolically labeled with either $^{35}SO_4$ (100 μCi/ml) or [^{3}H]glucosamine (25 μCi/ml), which are incorporated into the proteoglycan glycosaminoglycan side chains (21), or [^{35}S]methionine (20 μCi/ml), which is incorporated into the proteoglycan core protein. The radioactive compounds were added directly to the exposure medium for the final 24 h of each experiment.

Measures of cell growth and cytotoxicity

At the time of media harvest, the cell layers were washed in sterile PBS and incubated at 37°C in 2% trypsin for 20 min. This cell suspension was then diluted, and the number of cells was quantified using a Coulter counter. Parallel dishes were washed in PBS, and cell protein was determined using the method of Lowry (22). Cell viability was determined by measuring the lactate dehydrogenase (LDH) activity of the medium [cytotoxicity detection kit (LDH); Boehringer Mannheim, Indianapolis, IN].

Proteoglycan quantification and purification

$^{35}SO_4$, [^{35}S]methionine, and [^{3}H]glucosamine incorporation into secreted proteoglycans was quantified using cetyl pyridinium chloride (CPC) precipitation (23). Fifty microliter aliquots of radiolabeled medium were spotted in duplicate on filter paper, allowed to dry, and then washed five times with 1% CPC in 0.05 M NaCl. Precipitated $^{35}SO_4$, [^{35}S]methionine, or [^{3}H]glucosamine was quantified using liquid scintillation counting (24). The remaining medium for each condition was combined with the protease inhibitors benzamidine-HCl (Sigma, 5 mM), 6-aminocaproic acid (Sigma, 100 mM), and PMSF (Sigma, 50 mM) prior to purification.

^{35}S-labeled proteoglycans were purified from the harvested media using ion-exchange chromatography (23). Media samples containing radiolabeled proteoglycans were applied to DEAE-Sephacel minicolumns equilibrated in urea buffer [8 M urea, 2 mM EDTA, 50 mM Tris-HCl, and 0.5% Triton X-100 (pH 7.5)]. The columns were then washed five times in urea buffer and twice in urea buffer without Triton X-100 to remove free radiolabel. Proteoglycans were eluted from the columns in 4 M guanidine-HCl buffer with 100 mM sodium sulfate, 100 mM Tris-HCl base, and 2.5 mM EDTA (pH 7.4). The radioactivity of eluted proteoglycans was determined by liquid scintillation counting.

Characterization of proteoglycans

SDS-PAGE (25) was used to evaluate the presence of different proteoglycans as well as to estimate the apparent molecular weight of each of the proteoglycans (26). Equal counts (20,000 dpm) of purified $^{35}SO_4$-labeled proteoglycans, along with prestained molecular weight markers, were run through a 4–12% gradient polyacrylamide gel with a 3.5% stacking gel. Dried gels were exposed to PhosphorImager screens (Molecular Dynamics, Sunnyvale, CA), and images from the screens were scanned and analyzed using Opti-Quant software (Packard, Downers Grove, IL).

Hydrodynamic sizing of intact proteoglycans was performed using size exclusion chromatography (27). Equal counts (25,000 dpm) of purified $^{35}SO_4$-labeled proteoglycans were applied to analytical Sepharose CL-2B columns (0.7–110 cm), which were equilibrated in a 4 M guanidine-HCl buffer with 0.5% Triton X-100. Fractions were eluted in 0.4 ml of the same buffer, and the radioactivity of each fraction was determined using liquid scintillation counting. The void volume (V_0) of the columns was determined by the elution profile of [^{3}H]DNA, while the total volume (V_t) was determined by the elution profile of free $^{35}SO_4$ (28).

The hydrodynamic size of free glycosaminoglycan chains also was determined, using size exclusion chromatography. Equal counts (20,000 dpm) of $^{35}SO_4$-labeled intact proteoglycans were treated with 1 M sodium borohydride in 50 mM NaOH for 24 h at 45°C to liberate the glycosaminoglycan chains, after which the reaction was terminated with glacial acetic acid. The free chains

then were applied to a Sepharose CL-6B column (0.7 × 65 cm) and equilibrated in 0.2 M Tris-HCl, pH 7.0, with 0.2 M NaCl. Fractions of radiolabeled glycosaminoglycan chains were eluted and collected in aliquots, which were analyzed for radioactivity. As with the intact proteoglycans, the elution profiles of free $^{35}SO_4$ and [^3H]DNA were used to determine the V_t and V_0, respectively (27).

Proteoglycan binding of LDL

Human LDL (d = 1.019–1.063 g/ml) was isolated from the pooled sera of healthy volunteers. LDL was isolated from plasma by preparative ultracentrifugation in a Beckman VT50 vertical rotor and then purified by sequential density gradient ultracentrifugation as described previously (29). Proteoglycan binding to native human LDL was characterized by a modified gel mobility shift assay (30). $^{35}SO_4$-labeled proteoglycans were extensively dialyzed against morpholinepropanesulfonic acid (MOPS) sample buffer [20 mM MOPS, 140 mM NaCl, 5 mM $CaCl_2$, and 2 mM $MgCl_2$ (pH 7.4)] at 4°C. Increasing concentrations of LDL (0–500 μg/ml) were incubated with a fixed amount (1,500 dpm) of purified proteoglycans in the MOPS sample buffer for 1 h at 37°C. The samples were loaded into wells of 0.7% (w/v) NuSieve agarose gels (FMC, Rockland, ME) in MOPS running buffer [20 mM MOPS, 4 mM $MgCl_2$, and 2 mM $CaCl_2$ (pH 7.2)]. Samples were electrophoresed for 2 h at 60 V and 4°C. The gels then were fixed in 0.1% CPC [0.1% (w/v), in 70% ethanol] for 1 h, and air dried overnight. PhosphorImager screens were exposed to dried gels, and the resulting images were analyzed using Opti-Quant software.

Statistical analysis

Data from experiments (expressed as means and standard errors of the mean) were analyzed using GraphPad Prism software (San Diego, CA). Differences in means were calculated using either a two-tailed Student's *t*-test (for comparisons of two conditions) or one-way ANOVA (for comparisons of three conditions). Statistically significant differences were defined by a P value of <0.05.

Size exclusion chromatographs were generated by plotting radioactivity in eluted fractions against K_{av} of the fractions. Calculations were done using $K_{av} = (V_f - V_0)/(V_t - V_0)$ where V_f is fraction volume, V_0 is the void volume, and V_t is the total volume. Proteoglycan:LDL binding curves were plotted as percent proteoglycan bound versus concentration of LDL in μg/ml, and dissociation constant (K_d) values were generated from these curves using nonlinear regression analysis.

RESULTS

Effect of statins on cell number and cytotoxicity

We confirmed previous reports that statin exposure decreases cell proliferation in a concentration-dependent manner. After 72 h at a concentration of 100 μM, simvastatin-exposed cells remained quiescent, with cell numbers equaling only 55.7 ± 6.5% of control cells ($P <$ 0.05; data not shown). Similarly, cells exposed to cerivastatin (1 μM) showed a decreased proliferation, with final cell numbers equaling only 67.4 ± 11.1% of control cells at the end of 72 h ($P <$ 0.05; data not shown). Cytotoxicity, as measured by the LDH assay, was performed in parallel experiments. No significant increases in cell toxicity were observed at the concentrations of simvastatin (10–100 μM) or cerivastatin (0.1–1 μM) used in the study (data not shown).

Effect of statins on $^{35}SO_4$, [^{35}S]methionine, and [^3H]glucosamine incorporation into secreted proteoglycans

After 72 h of exposure, simvastatin decreased the total $^{35}SO_4$ incorporation into secreted proteoglycans in a concentration-dependent manner (**Fig. 1A**). At 10 μM, simvastatin decreased $^{35}SO_4$ incorporation by 42.1 ± 15.2%, while at 100 μM, incorporation was reduced by 64.7 ± 15.2% ($P =$ 0.001 vs. control). Similar results were observed for cerivastatin, with 0.1 μM causing a 16.1 ± 8.6% decrease, and 1 μM causing a 49.6 ± 14.1% decrease in $^{35}SO_4$ incorporation (Fig. 1A) ($P <$ 0.05 for both 0.1 μM and 1 μM cerivastatin vs. control).

The statin-induced reduction of $^{35}SO_4$ incorporation could not be entirely explained by a decrease in cell number. When corrected for cell number, $^{35}SO_4$ incorporation was decreased by 26.2 ± 13.9% with simvastatin at 10 μM, and by 35.8 ± 14.8% with simvastatin at 100 μM (Fig. 1B) ($P <$ 0.05 for both 10 μM and 100 μM simvastatin vs. control). Similar results were seen with cerivastatin: at a concentration of 1 μM, cerivastatin decreased the relative $^{35}SO_4$ incorporation into secreted proteoglycans by 27.5 ± 20.2%, compared with controls ($P <$ 0.05; data not shown).

One possible explanation for the decrease in relative $^{35}SO_4$ incorporation is that statins inhibit the synthesis
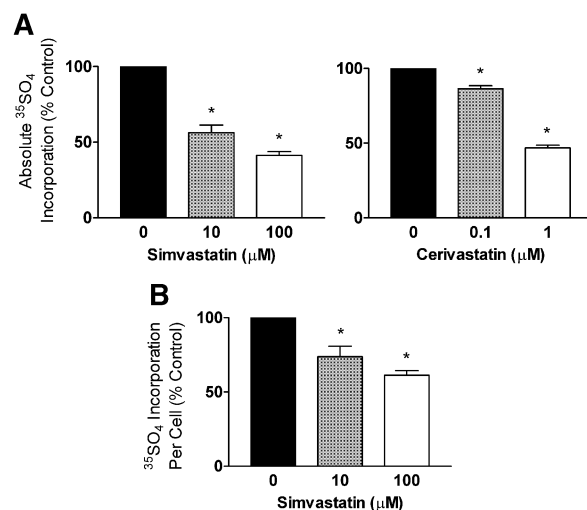

Fig. 1. Simvastatin and cerivastatin inhibit incorporation of $^{35}SO_4$ into nascent proteoglycans. A: Cells in culture were exposed to simvastatin (10 and 100 μM), cerivastatin (0.1 and 1 μM), and control conditions for 72 h. For the final 24 h of each experiment, newly synthesized proteoglycans were metabolically labeled with $^{35}SO_4$. Incorporation of $^{35}SO_4$ into proteoglycans was measured by cetyl pyridinium chloride (CPC) precipitation followed by liquid scintillation counting. B: Monkey vascular smooth muscle cells in culture were exposed to simvastatin (10 and 100 μM) as well as control conditions for 72 h. For the final 24 h of each experiment, newly synthesized proteoglycans were metabolically labeled with $^{35}SO_4$. Incorporation of $^{35}SO_4$ into CPC-precipitated proteoglycans was measured with liquid scintillation counting and then corrected for cell number. Values are expressed as means ± SEM relative to control conditions, and represent three separate experiments. * $P <$ 0.05 versus control.

and secretion of intact proteoglycans. To test this hypothesis, we metabolically labeled statin-exposed cells with [35S]methionine, which is incorporated into the core proteins of proteoglycans, and is a measure of intact proteoglycan secretion. After 72 h of exposure, simvastatin at 100 μM did not change the relative [35S]methionine incorporation into secreted proteoglycans, compared with controls (**Fig. 2A**). Similarly, cerivastatin at 1 μM did not change the relative [35S]methionine incorporation into proteoglycan core proteins (data not shown). This indicates that the decrease in $^{35}SO_4$ incorporation induced by statins cannot be explained by a reduction in the number of intact proteoglycan molecules secreted per cell.

Glycosaminoglycan side chains are synthesized with a variable degree of sulfation but with a relatively constant pattern of glucosamine residues. Because of this, the ratio of $^{35}SO_4$ incorporation relative to [3H]glucosamine incorporation gives a measure of the degree of sulfation of proteoglycans. Simvastatin treatment (100 μM) significantly reduced the ratio of $^{35}SO_4$/[3H]glucosamine incorporation into nascent proteoglycans (Fig. 2B). In control cells, the ratio of $^{35}SO_4$/[3H]glucosamine incorporation was 5.90 ± 0.49, whereas in simvastatin-treated cells, the ratio was 1.20 ± 0.49 ($P = 0.001$). A similar reduction in the ratio of $^{35}SO_4$/[3H]glucosamine incorporation was observed with cerivastatin treatment (1 μM; data not shown).
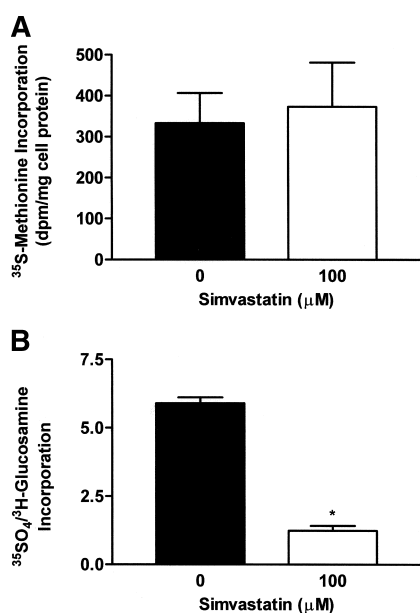
Statin effects prevented by mevalonate and geranylgeraniol

We next assessed whether the inhibitory effects of statins on proteoglycan secretion were due to an interruption of the HMG-CoA pathway. Mevalonate (100 μM) (**Fig. 3A**) completely prevented the inhibitory effects of simvastatin (100 μM) on $^{35}SO_4$ incorporation into proteoglycans ($P < 0.05$). Mevalonate also prevented the effects of cerivastatin (1 μM) on $^{35}SO_4$ incorporation into proteoglycans (data not shown). This concentration of mevalonate also prevented the inhibitory effects of simvastatin and cerivastatin on cell number and cell protein (data not shown). Mevalonate alone had no effect on $^{35}SO_4$ incorporation (Fig. 3A), cell number, or protein content (data not shown), compared with controls.

Other isoprenoid molecules downstream from mevalonate in the cholesterol cascade were also added to statin-exposed cells. The concurrent addition of all-*trans* geranylgeraniol (10 μM) (Fig. 3B) prevented simvastatin (100 μM) from inhibiting $^{35}SO_4$ incorporation into secreted proteoglycans ($P < 0.05$). Likewise, all-*trans* geranylgeraniol (10 μM) prevented the inhibitory effects of

Fig. 2. Simvastatin effects on [35S]methionine incorporation, and the ratio of $^{35}SO_4$/[3H]glucosamine incorporation into proteoglycans secreted by vascular smooth muscle cells. A: Cells were exposed to simvastatin (100 μM) as well as control conditions for 72 h and were metabolically labeled with [35S]methionine for the final 24 h. Incorporation of [35S]methionine into CPC-precipitated proteoglycans was measured with liquid scintillation counting and then corrected for cell number. B: Cells exposed to simvastatin (100 μM) or control conditions were colabeled with $^{35}SO_4$ and [3H]glucosamine. Relative incorporation of the radionuclides was measured as described. Values presented are means ± SEM relative to control conditions and represent three separate experiments. * $P <$ 0.05 versus control.
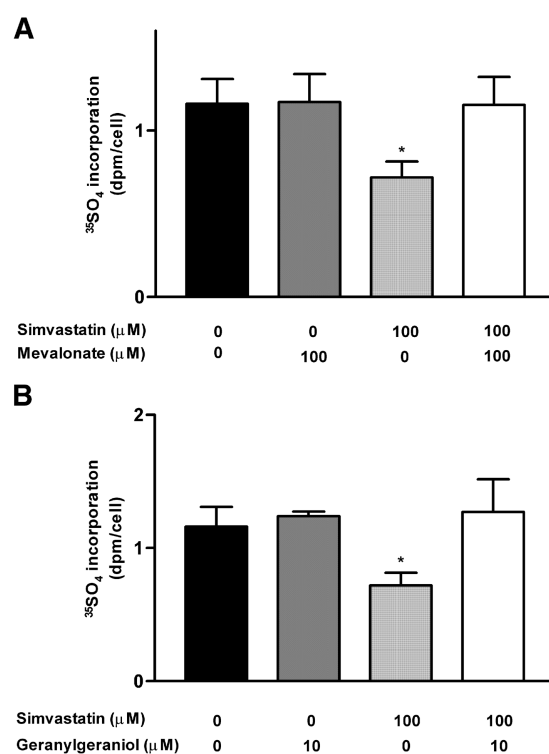

Fig. 3. Statin effects can be prevented by isoprenoid intermediates. A: Monkey vascular smooth muscle cells in culture were exposed to control conditions, mevalonate (100 μM), simvastatin (100 μM), or both for 72 h. For the final 24 h of each experiment, newly synthesized proteoglycans were metabolically labeled with $^{35}SO_4$. Incorporation of $^{35}SO_4$ into precipitated proteoglycans was measured and corrected for cell number. B: Cells also were exposed to all-*trans* geranylgeraniol (10 μM) alone or in combination with simvastatin (100 μM). Values presented are means ± SEM relative to control conditions, and represent three separate experiments. * $P < 0.05$ versus control.

cerivastatin (1 μM) on $^{35}SO_4$ incorporation (data not shown). Geranylgeraniol also prevented the inhibitory effects of the statins on cell number and cell protein (data not shown). All-*trans* farnesol (10 μM) did not prevent any of the measured effects of the statins, and neither geranylgeraniol nor farnesol alone had any significant effects on $^{35}SO_4$ incorporation, compared with controls (data not shown).

Statin exposure increases the apparent molecular weight and size of secreted proteoglycans

The effect of statins on the apparent molecular weight of proteoglycans secreted from vascular smooth muscle cells was evaluated using SDS-PAGE. Four major bands of secreted proteoglycans are visible in the gels (**Fig. 4A**), previously identified as: *1*) versican, *2*) heparan sulfate/chondroitin sulfate proteoglycan (HS/CSPG), *3*) biglycan, and *4*) decorin (23, 31). After 72 h of exposure, simvastatin (100 μM) induced a shift (toward the origin of the gel) to a larger apparent molecular weight of both biglycan and decorin. Cerivastatin at 1 μM had effects similar to those of simvastatin in shifting bands 3 and 4 to larger apparent molecular weights (data not shown). Because of the large size of versican and HS/CSPG, SDS-PAGE is insensitive in identifying changes in the apparent molecular weights of these proteoglycans.

We next performed size exclusion chromatography on the proteoglycans, because of a better sensitivity than SDS-PAGE in measuring size changes in the large proteoglycan

species versican and HS/CSPG. Size exclusion chromatographs of secreted proteoglycans from smooth muscle cells contained two peaks (Fig. 4B). Peak 1 contained primarily versican and HS/CSPG, whereas peak 2 contained primarily biglycan and decorin (23, 27). After 72 h of exposure to simvastatin (100 μM), smooth muscle cells secreted proteoglycans with an increased hydrodynamic size compared with controls(Fig. 4B, data from one representative experiment). Both of the major proteoglycan peaks seen on the chromatograph were shifted to the left, representing an increase in K_{av} and therefore in the size of proteoglycans. Using data from five separate experiments, we found that the peak 1 K_{av} shifted from 0.24 ± 0.012 in controls to 0.20 ± 0.023 in simvastatin-treated cells ($P <$ 0.01), whereas the K_{av} of peak 2 shifted from 0.59 ± 0.053 in controls to 0.57 ± 0.024 in simvastatin-exposed proteoglycans ($P =$ ns). The shift of peak 1 to a higher molecular size confirms that simvastatin increases the size of the larger proteoglycan species, not just biglycan and decorin, as seen in SDS-PAGE (Fig. 4A). Cerivastatin at 1 μM had similar effects on the hydrodynamic size of secreted proteoglycans (data not shown).

Glycosaminoglycan chains were liberated from whole proteoglycans and subjected to size exclusion chromatography (Fig. 4C, data from one representative experiment) to assess whether an increase in size of the intact proteoglycans was mediated through an increase in size of the glycosaminoglycan side chains. The chromatograph of glycosaminoglycan side chains from cells exposed to sim-
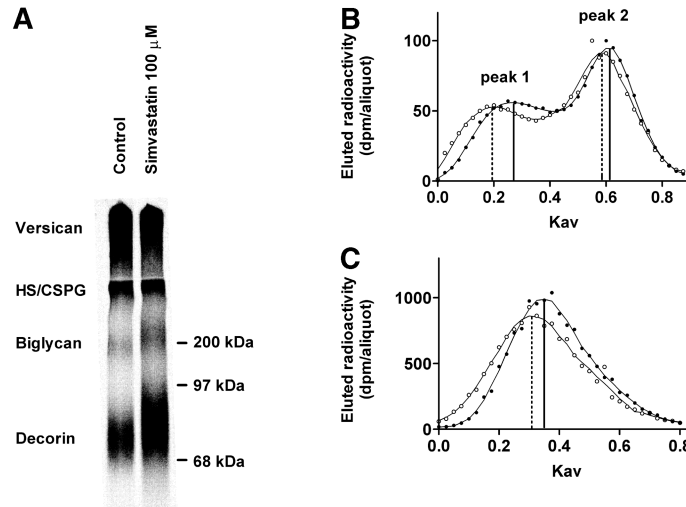

Fig. 4. Simvastatin increases the apparent molecular weight and hydrodynamic size of secreted proteoglycans from vascular smooth muscle cells. A: Monkey smooth muscle cells were exposed to control conditions or simvastatin (100 μM) for 72 h, and nascent proteoglycans were collected in the media and purified by ion exchange chromatography. Equal counts (20,000 dpm) of purified proteoglycans were exposed to conditions of SDS-PAGE. This gel is representative of three separate experiments. B: Purified $^{35}SO_4$-labeled proteoglycans from simvastatin-exposed cells (100 μM) were analyzed using size exclusion chromatography. Analytical sepharose CL-2B columns were loaded with 25,000 dpm per sample, and $^{35}SO_4$ activity in eluted aliquots was measured using liquid scintillation counting. Eluted $^{35}SO_4$ activity (dpm/aliquot) is plotted here against K_{av}. The data shown here are from one experiment representative of five separate experiments. C: Free $^{35}SO_4$-labeled glycosaminoglycan chains (20,000 dpm) liberated from intact proteoglycans were run on analytical sepharose CL-6B size exclusion columns. Aliquots were collected, and radioactive counts (dpm/aliquot) are plotted here against K_{av}. Data presented here are from one experiment representative of five separate experiments. Closed circles, control; open circles, simvastatin.

vastatin (100 μM; 72 h) was shifted to the left, indicating a reduction in K_{av}, or an increase in hydrodynamic size. Using data from five separate experiments, we found that the K_{av} of glycosaminoglycan chains from control cells was 0.36 ± 0.010 compared with 0.31 ± 0.026 for glycosaminoglycans from simvastatin-exposed cells (100 μM; $P <$ 0.05). Similar results were seen with cerivastatin exposure (1 μM; data not shown).

The concurrent addition of mevalonate (100 μM) or all-*trans* geranylgeraniol (10 μM) to simvastatin-exposed cells entirely prevented the statin-induced changes seen in SDS-PAGE and size exclusion chromatography. Mevalonate and all-*trans* geranylgeraniol alone had no effects on either the types of proteoglycans synthesized or the apparent molecular weights and hydrodynamic sizes of the secreted proteoglycans. All-*trans* farnesol did not prevent the statin effects on molecular weight or hydrodynamic size (data not shown).

Statin-altered proteoglycans have decreased binding affinity to native LDL

The binding interaction between proteoglycans from statin-exposed cells and native human LDL was measured using a modified gel mobility shift assay. In this assay, free proteoglycans migrate into the gel, whereas proteoglycans bound to LDL remain at or near the origin of the gel. Proteoglycans isolated from simvastatin-exposed cells bound to LDL with a lower affinity than did proteoglycans isolated from control cells (**Fig. 5A**). At an LDL protein concentration of 10 μg/ml, control proteoglycans were nearly all bound to LDL, but at the same concentration, most proteoglycans secreted from statin-exposed cells were free. Similar results were seen with cerivastatin (1 μM, data not shown).

Figure 5B shows binding curves generated from analysis of three binding experiments. The binding curves demonstrate decreased binding of LDL to proteoglycans from simvastatin-exposed cells, compared with proteoglycans from control cells. The K_d, or concentration of LDL at which half of the proteoglycans are bound, was higher in proteoglycans from simvastatin-exposed cells than in controls. The K_d from control cells was 11.8 ± 2.2 μg/ml, whereas the K_d from simvastatin-exposed cells was 29.5 ± 4.4 μg/ml ($P <$ 0.05). Thus, statin-exposed cells secrete proteoglycans that bind less avidly to LDL.

DISCUSSION

Summary of findings

In the present study, we demonstrate that simvastatin and cerivastatin decrease the incorporation of $^{35}SO_4$ into proteoglycans secreted by vascular smooth muscle cells. The decrease in $^{35}SO_4$ incorporation is, in part, explained by decreased cellular proliferation, a finding previously described for statins (32). However, $^{35}SO_4$ incorporation is also reduced on a per-cell basis. Our finding that statins do not decrease the relative [^{35}S]methionine incorporation into proteoglycan core proteins suggests that there is
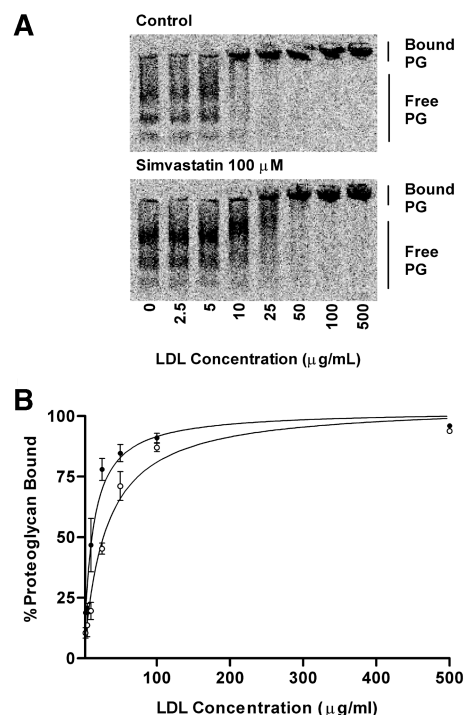

Fig. 5. Simvastatin-exposed cells secrete proteoglycans that have a reduced binding affinity for LDL. A: Equal counts (1,500 dpm) of purified $^{35}SO_4$-labeled proteoglycans from simvastatin-exposed cells (100 μM) and control conditions were coincubated with increasing concentrations of LDL (μg/ml) then electrophoresed in an agarose gel. Proteoglycans bound to LDL (Bound PG) remain at the gel's origin, while free proteoglycans (Free PG) migrate into the gel. These gels are representative of three separate experiments. B: Binding curves were generated by plotting percent proteoglycans bound to LDL versus concentration of LDL. The curve was generated using data from three separate experiments. Closed circles, control; open circles, simvastatin. Error bars indicate ± SEM.

no change in the molar concentration of proteoglycans secreted per cell. Therefore, statin exposure appears to decrease the degree of sulfation of glycosaminoglycan side chains. This is an important finding, because glycosaminoglycan sulfation largely determines the binding affinity of proteoglycans for LDL (33).

In addition to being undersulfated, proteoglycans secreted by statin-exposed cells have slightly larger apparent molecular weights and hydrodynamic sizes, mediated by an elongation of glycosaminoglycan side chains. This is a novel finding, because quiescent cells previously have been shown to have smaller glycosaminoglycan side chains compared with actively growing cells (18). Thus, statins appear to have the specific effect of promoting glycosaminoglycan chain elongation while cells are quiescent.

Our most significant finding is that proteoglycans secreted by statin-exposed cells have a decreased binding affinity for LDL, a finding that may explain part of the beneficial effects of statins on atherogenesis (34). The binding coefficient for LDL-proteoglycan interaction is physiologically significant, because it is within the range of LDL concentrations present in interstitial fluid (35). Be-

cause we used equal counts of $^{35}SO_4$-labeled proteoglycans in our binding assay, we likely underestimated the differences in LDL binding between fully sulfated proteoglycans from control cells and undersulfated proteoglycans from statin-exposed cells. If loaded on an equal molar basis, the differences between the binding of LDL with control proteoglycans and the binding of LDL with statin-exposed proteoglycans might have been more pronounced.

Potential mechanism of statin-induced proteoglycan changes

The observed changes in secreted proteoglycans appeared to have been mediated by a blockade of isoprenoid synthesis, because the addition of either mevalonate or geranylgeraniol prevented the effects of the statins. Prenylation of intracellular proteins allows a close interaction with lipid membranes (36), and statins alter a variety of cell functions that are dependent on protein prenylation (37). Protein prenylation may play a significant role in the post-translational modification of proteoglycans, a process that requires that the core protein closely associate with the Golgi membrane (16). Specific inhibition of farnesylation has been shown to reduce proteoglycan deposition in the intima of balloon-injured porcine coronary arteries, although this effect may also be due to an inhibition of smooth muscle cell proliferation (38).

The effects of statins on proteoglycan production appear to be class effects, inasmuch as we saw the same findings with both cerivastatin and simvastatin. Cerivastatin, before it was removed from the market in 2001 for muscle toxicity, was prescribed at doses between 0.4 mg and 0.8 mg a day, whereas simvastatin is prescribed at doses ranging from 20 mg to 80 mg (39). The peak serum concentration after a single dose of cerivastatin (0.8 mg) is ~0.03 μM (40), whereas the peak concentration after a single dose of simvastatin (80 mg) is ~0.2 μM (41). The difference in equipotent concentrations of cerivastatin versus simvastatin (~100-fold; see Fig. 1) mirrors the difference in clinical doses prescribed to patients.

The concentrations of cerivastatin and simvastatin used in our study are higher than those observed in human serum. However, the portal blood flow to the liver contains a much higher concentration of statins than does the peripheral blood. Evidence suggests that inhibition of hepatic HMG-CoA reductase, and the subsequent decrease in circulating isoprenoid levels, may mediate pleiotropic effects in the periphery (11). As with many of the "pleiotropic" effects of statins, the physiological relevance of our in vitro findings can only be established after extension of the findings into animal models of atherosclerosis.

Vascular proteoglycans, atherosclerosis, and statins

An increase in the retention of LDL by vascular proteoglycans is likely to be atherogenic, because this retention directly leads to the uptake of LDL by macrophages (42). Exposure of vascular smooth muscle cells to oxi-

dized LDL causes cells to secrete larger and more highly sulfated proteoglycans, which interact more avidly with LDL (43). Proteoglycans secreted in the presence of nonesterified fatty acids contain glycosaminoglycan side chains that are longer and that bind LDL more avidly (44). Likewise, transforming growth factor β_1 exposure to smooth muscle cells causes an increase in the size and charge of glycosaminoglycans, and leads to an increased binding of LDL (45).

On the other hand, any intervention that results in a decreased interaction of proteoglycans with LDL may be atheroprotective. Recently, glucosamine was shown to reduce the size of glycosaminoglycan side chains of proteoglycans (20). This decrease in proteoglycan size led to a reduced binding interaction with LDL. Quiescent cells secrete proteoglycans that are relatively undersulfated compared with those of actively proliferating cells, and these may be atheroprotective (18). Our current findings suggest that some of the beneficial effects of statins may be due to alterations in vascular proteoglycans. The beneficial effects of statin-altered vascular proteoglycans may be amplified in vivo, inasmuch as each molecule of undersulfated proteoglycan would bind fewer bridging molecules, such as lipoprotein lipase or aggregated forms of LDL.

Little has been published about the effects of statins on proteoglycan regulation. Using cultured human smooth cells, one group has shown by Northern analysis that 72 h of lovastatin exposure decreases biglycan mRNA expression (46). This lovastatin effect was completely prevented with the coaddition of mevalonate. In a more recent study (47), human vascular smooth muscle cells exposed to cerivastatin also exhibited a significant decrease in biglycan mRNA expression. A specific analysis of the effects of statins on the glycosaminoglycan makeup of individual species of vascular proteoglycans and their ability to bind LDL is beyond the scope of our current work, but deserves attention. The potential physiologic relevance of our in vitro findings can better be established by evaluating the effects of statins on vascular proteoglycans with in vivo models of atherosclerosis.

Conclusions

Reduction in LDL retention by the vessel wall slows atherosclerosis (1). Current clinical prevention of atherosclerosis focuses on lowering serum LDL cholesterol; however, an additional site of intervention may be the vessel extracellular matrix. Specifically, alterations in the LDL binding capacity of vascular proteoglycans may be of clinical benefit. We have shown that statin exposure decreases the sulfation of glycosaminoglycans, leading to less-negatively charged proteoglycans. Importantly, undersulfated proteoglycans from statin-exposed cells bind less LDL than do proteoglycans secreted from control cells. By decreasing the binding interaction of LDL with vascular proteoglycans, and the subsequent retention of LDL in the arterial intima, statins may benefit atherosclerosis in a manner unrelated to serum cholesterol lowering.

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