Arterial smooth muscle cell proteoglycans synthesized in the presence of glucosamine demonstrate reduced binding to LDL[1]

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Abstract Atherosclerosis is the main cause of morbidity and mortality in diabetes, yet the underlying mechanisms remain unclear. Retention of atherogenic lipoproteins by vascular proteoglycans is thought to play a key role in the development of atherosclerotic lesions. High glucose levels cause a variety of diabetic complications by several mechanisms, including upregulation of the hexosamine pathway. Glucosamine, a component of the hexosamine pathway, is a precursor for the synthesis of glycosaminoglycan components of proteoglycans. This study evaluated whether high glucose or glucosamine supplementation of vascular smooth muscle cells would increase proteoglycan synthesis, leading to increased lipoprotein retention. Aortic smooth muscle cells were exposed to physiologic (5.6 mM) or high (25 mM) glucose levels, such as seen in diabetes, or to glucosamine (12 mM). Extracellular proteoglycans were characterized by sulfate incorporation, molecular sieve chromatography, and SDS-PAGE. LDL interactions were assessed by affinity chromatography and gel mobility shift assay. Proteoglycans synthesized in the presence of high glucose demonstrated no differences in size, sulfate incorporation, or LDL binding affinity compared with proteoglycans synthesized under physiological glucose conditions. However, proteoglycans synthesized in the presence of glucosamine had smaller glycosaminoglycan chains than control proteoglycans with a corresponding decrease in lipoprotein retention. Thus, glucose and glucosamine have different effects on proteoglycan biosynthesis and different effects on lipoprotein retention.—Tannock, L. R., P. J. Little, T. N. Wight, and A. Chait. **Arterial smooth muscle cell proteoglycans synthesized in the presence of glucosamine demonstrate reduced binding to LDL. *J. Lipid Res.* 2002.** 43: **149–157.**

Supplementary key words atherosclerosis • biglycan • decorin • glycosaminoglycan • hexosamine • lipoprotein • retention • versican • xyloside

Proteoglycans are a heterogeneous group of molecules that occur ubiquitously in the arterial wall. The major secreted extracellular vascular proteoglycans are the large chondroitin sulfate proteoglycan versican, and the small dermatan sulfate proteoglycans biglycan and decorin (1, 2). Alterations in proteoglycan concentrations and composition have been found focally in prelesional and lesional areas of atherosclerosis in diabetes (3, 4). The "response to retention" hypothesis of atherosclerosis states that proteoglycans bind and retain LDL in the subendothelial compartment. Retention of LDL by arterial proteoglycans has been suggested to increase the susceptibility of the LDL to modifications such as oxidation (5–8). Proteoglycans bind lipoproteins via interactions between negatively charged sulfate and carboxyl groups on their glycosaminoglycan side chains and positively charged amino acid residues on apolipoproteins B and E. Therefore, changes in the size or charge of proteoglycans or their glycosaminoglycan chains might have significant effects on their lipoprotein-binding properties (9, 10).

Glucosamine is a precursor in the synthesis of glycosaminoglycan chains. Glucosamine is gaining popularity as an over-the-counter supplement for arthritis and has been shown to improve joint pain and joint space narrowing in the knees (11). Glucosamine supplementation leads to increased glycosaminoglycan and proteoglycan synthesis in cartilage (12, 13), which has been proposed to account for improved symptoms.

Glucosamine also is a component of the hexosamine pathway, which has been demonstrated to be a mechanism by which glucose leads to diabetic complications (14). In the hexosamine pathway, glucose is metabolized to hexosamine via the transfer of an amide group from

Abbreviations: CPC, cetyl pyridinium chloride; GFAT, glutamine: fructose-6-phosphate amidotransferase; LDH, lactate dehydrogenase; MOPS, morpholinepropanesulfonic acid; UDP-GlcNAc, UDP-*N*-acetylglucosamine.

[1] Presented in part at the American Heart Association Scientific Sessions 2000, New Orleans, Louisiana, November 13, 2000.

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glutamine to fructose 6-phosphate to form glucosamine 6-phosphate. This subsequently is metabolized to hexosamines such as UDP-*N*-acetylglucosamine (UDP-GlcNAc) and UDP-*N*-acetylgalactosamine, which are the building blocks for glycosaminoglycan synthesis (15). The rate-limiting step in the hexosamine pathway is the initial transfer of the amide group from glutamine, which is catalyzed by the enzyme glutamine:fructose-6-phosphate amidotransferase (GFAT; EC 2.6.1.16) (14). GFAT activity is upregulated by high glucose and insulin levels (16), and is downregulated by UDP-GlcNAc (17). Under euglycemic conditions only 2–3% of glucose fluxes through the hexosamine pathway; however, the flux through this pathway is likely to be increased in the presence of hyperglycemia (18). Glucosamine enters the hexosamine pathway distal to the rate-limiting step, and therefore bypasses GFAT. Glucosamine, and the hexosamine pathway, have been found to be associated with increased synthesis of extracellular matrix proteoglycans in mesangial cells (19). The present study was performed to determine *a*) the effect of glucosamine on the synthesis of extracellular proteoglycans by vascular smooth muscle cells, and *b*) the ability of the secreted proteoglycans to bind lipoproteins. The study also evaluated whether the effects of glucosamine are mediated through the hexosamine pathway.

RESEARCH DESIGN AND METHODS

Chemicals and reagents were obtained from Sigma (St. Louis, MO) unless otherwise specified.

Cell culture techniques

Arterial smooth muscle cells isolated from monkey (*Macaca nemestrina*) thoracic aortas were plated at a density of 2.5×10^5 cells per 60-mm dish and grown to confluence in commercially available DMEM with 5.6 mM glucose, supplemented with pyruvate, nonessential amino acids, penicillin (10^5 U/l), streptomycin (10^5 μg/l), glutamine, and 5% calf serum. Cells were used between passages 6 and 18 and culture medium was changed every 48 to 72 h. Cells reaching visual confluence were made quiescent for 48 h by lowering the serum concentration to 0.1%. Quiescent cells were exposed to high (25 mM) or physiologic (5.6 mM) concentrations of glucose for 24 h in the presence of 5% serum. Cells were metabolically labeled with $^{35}SO_4$ (50–100 μCi/ml) or [^{35}S]methionine (10–20 μCi/ml) during this 24-h period. After removal of the radiolabeled culture medium the cell layer was washed once with PBS and then cell protein was determined by the method of Lowry et al (20). Parallel dishes were treated in an identical fashion except without $^{35}SO_4$ labeling, and cells were washed with PBS, trypsinized, and counted in a Coulter counter. Cell count and cell protein determination were performed on duplicate aliquots per dish, and in duplicate dishes per condition. Cytotoxicity was assessed by determining lactate dehydrogenase (LDH) activity of the medium [cytoxicity detection kit (LDH); Boehringer Mannheim, Indianapolis, IN]. In addition, the incorporation of [^{35}S]methionine into total cell protein was determined using total trichloroacetic acid (TCA)-precipitable counts (21).

Quiescent cells were supplemented with glucosamine (12 mM) added to DMEM containing 5.6 mM glucose. Glucosamine enters the cell through glucose transporters and is rapidly phosphorylated by hexokinase to glucosamine 6-phosphate. To test the role of the hexosamine pathway, quiescent cells were exposed to high glucose (25 mM) DMEM, or to high glucose medium in the presence of the GFAT inhibitor azaserine (5 μM) (14). Cells were exposed to these conditions for 24 h in the presence of 5% serum, and were metabolically labeled under these conditions, as described earlier. Time course studies were performed by exposing quiescent cells to physiologic glucose (5.6 mM) or various concentrations of glucosamine (0.12, 1.2, or 12 mM) for 8, 16, or 24 h in the presence of $^{35}SO_4$ for the last 8 h of incubation.

Proteoglycan isolation

Media from metabolically labeled cell cultures were collected and the protease inhibitors benzamidine-HCl (5 mM), 6-aminocaproic acid (100 mM), and phenylmethylsulfonyl fluoride (50 mM) were added immediately. Media from replicate dishes were pooled and frozen at −70°C for 1 to 3 days. The secreted proteoglycans synthesized under the different experimental conditions were concentrated on separate DEAE-Sephacel minicolumns equilibrated with 8 M urea buffer (8 M urea, 2 mM EDTA, 50 mM Tris-HCl, 0.5% Triton X-100, pH 7.5) with 0.25 M NaCl, and were eluted in 4 M guanidine-HCl buffer including 100 mM sodium sulfate, 100 mM Tris base, and 2.5 mM EDTA, pH 7.4.

Hydrodynamic size analysis

Molecular sieve chromatography was performed to determine the hydrodynamic size of proteoglycans. Fractions were applied to analytical Sepharose CL-2B columns (0.7×110 cm) equilibrated in 4 M guanidine-HCl buffer with 0.5% Triton X-100. Eluted fractions of 0.4 ml each were collected and assayed by liquid scintillation counting. The elution position of free $^{35}SO_4$ was used as a marker for the total volume (V_t) and the void volume (V_0) was determined by the elution position of [^{3}H]DNA (22).

Glycosaminoglycan analysis

Proteoglycans were treated with 1 M sodium borohydride in 50 mM NaOH for 24 h at 45°C to release the glycosaminoglycan chains. The reaction was terminated by neutralization with glacial acetic acid (23). The free glycosaminoglycan chains then were applied to a Sepharose CL-6B column (0.7×65 cm) in 0.2 M Tris, pH 7.0, with 0.2 M NaCl to determine chain length by size-exclusion chromatography (22). The elution position of free $^{35}SO_4$ was used as a marker for the total volume (V_t) and the void volume (V_0) was determined by the elution position of [^{3}H]DNA. Molecular weights were assessed as previously described (24).

Free glycosaminoglycan chains

To isolate free glycosaminoglycans, cells were cultured as described with the addition of 0.5 mM methyl β-D-xylopyranoside (xyloside), which serves as an artificial acceptor for the synthesis of glycosaminoglycan chains (25). Xyloside was added to the culture medium at the time of exposure to the various experimental conditions, and in the presence of $^{35}SO_4$. This results in the synthesis of free glycosaminoglycan chains that are not attached to core proteins. The free glycosaminoglycan chains generated were assessed by cetyl pyridinium chloride (CPC) precipitation (see below), and by size analysis on Sepharose CL-6B columns as described. These free glycosaminoglycan chains also were used in the gel mobility shift assay (see below).

Sulfate incorporation into proteoglycans and glycosaminoglycans

CPC precipitation was performed to measure the incorporation of $^{35}SO_4$ into glycosaminoglycan chains. Two 50-μl aliquots

of $^{35}SO_4$-labeled medium per tissue culture dish were spotted onto filter paper, air dried, and then washed five times for 1 h in 1% CPC with 0.05 M NaCl. The filter paper was dried and the ^{35}S content of the precipitate was determined by liquid scintillation counting (10, 23, 26).

SDS-PAGE chromatography

Proteoglycans were characterized by SDS-PAGE according to the procedure of Laemmli (22, 23, 27) on 4–12% gradient slab gels with a 3.5% stacking gel. To estimate apparent molecular sizes, proteoglycan bands were compared with prestained high molecular weight standards (Novex, San Diego, CA) that were run in separate lanes. Gels were dried and visualized on PhosphorImager screens (Molecular Dynamics, Sunnyvale, CA).

LDL preparation

LDL were isolated from the plasma of normal human volunteers as described previously (28). Briefly, LDL (d = 1.019–1.063 g/ml) was separated from normal human plasma by preparative ultracentrifugation in a Beckman (Palo Alto, CA) VTi 50 vertical rotor, and purified by sequential density gradient ultracentrifugation (28, 29). Lipoprotein content was determined by the method of Lowry et al. (20). LDL was stored in the dark at 4°C with 1 mM EDTA under nitrogen until used in the gel mobility shift assay.

Gel mobility shift assay

The interaction between proteoglycans or free glycosaminoglycan chains, synthesized under the various experimental conditions described, with native human LDL was assessed by a modified gel mobility shift assay (30). Native human LDL and proteoglycans eluted from DEAE columns were prepared for use in the gel mobility shift assay by extensive dialysis against morpholinepropanesulfonic acid (MOPS) sample buffer (20 mM MOPS, 140 mM NaCl, 5 mM $CaCl_2$, 2 mM $MgCl_2$, pH 7.4) at 4°C. Increasing concentrations of LDL were mixed with a fixed amount (approximately 1,500 dpm) of $^{35}SO_4$-labeled proteoglycans, or free glycosaminoglycan chains, in MOPS sample buffer for 1 h at 37°C. The samples then were applied to wells in 0.7% (w/v) NuSieve agarose (FMC, Rockland, ME) at 4°C in MOPS running buffer (20 mM MOPS, 2 mM $CaCl_2$, 4 mM $MgCl_2$, pH 7.2). In this assay proteoglycans or glycosaminoglycans that have bound to the LDL are retained at the origin and unbound proteoglycans or glycosaminoglycans migrate into the gel. The gels were fixed in CPC [0.1% (w/v), in 70% ethanol] for 1 h, air dried, and exposed to PhosphorImager screens. Analysis of the images was performed with Opti-Quant software (Packard, Downers Grove, IL). The amount of bound versus free proteoglycans or glycosaminoglycans in each lane was assessed and the percent bound was calculated as the proportion of radioactivity remaining at the gel origin relative to the total radioactivity per lane. Binding constants were calculated with the SAAM II modeling program (SAAM Institute, Seattle, WA), using the Michaelis-Menten equation, and statistical significance was determined by paired *t*-test.

LDL affinity chromatography

The binding of the total proteoglycan preparations to native human LDL also was assessed by affinity chromatography to obtain information regarding binding capacity. Metabolically labeled, protease inhibitor-containing culture medium from cells exposed to physiologic glucose (5.6 mM) or glucosamine (12 mM) was extensively dialyzed against buffer consisting of 10 mM HEPES, 20 mM NaCl, and 250 μM butylated hydroxytoluene, and then applied to separate columns containing native human

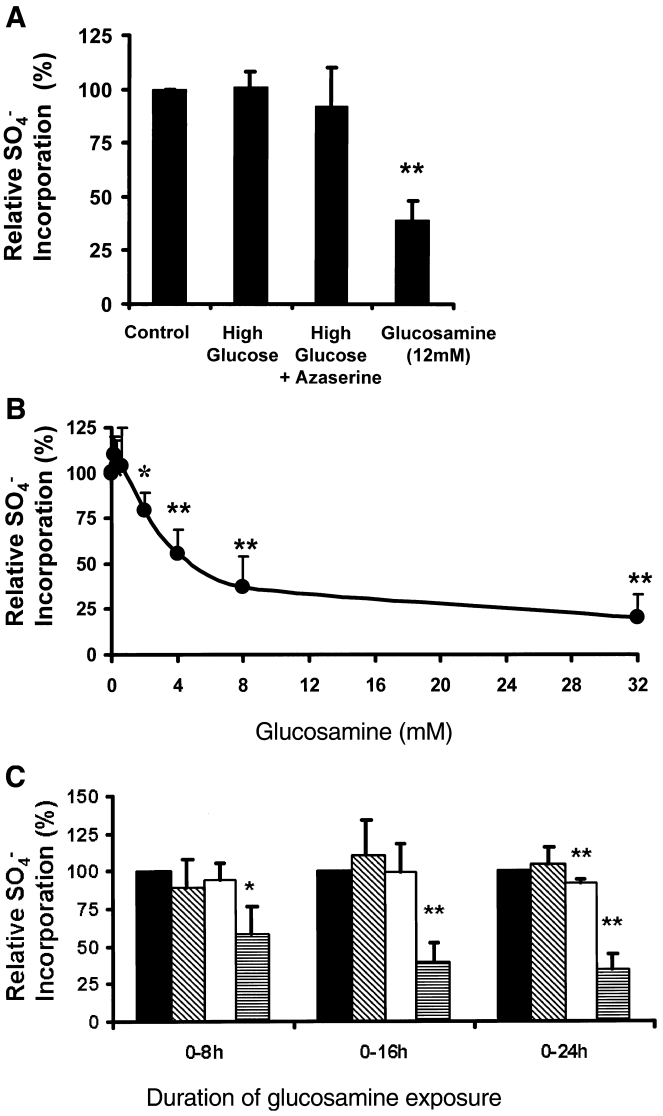

Fig. 1. Glucosamine inhibits proteoglycan sulfate incorporation. A: SMC exposed to physiological glucose (5.6 mM), high glucose (25 mM) with or without azaserine (5 μM) or glucosamine (12 mM) for 24 h were metabolically labeled with $^{35}SO_4$. The sulfate incorporation of proteoglycans secreted into the culture medium was analyzed by CPC precipitation as described in Research Design and Methods, and is expressed as incorporated sulfate counts per microgram of cell protein. Values are expressed as means ± SD relative to cells grown under physiological glucose conditions without additives, which is expressed as 100% (n = 7). B: SMC exposed to the indicated concentrations of glucosamine for 24 h were metabolically labeled with $^{35}SO_4$. The sulfate incorporation was analyzed by CPC precipitation, and is expressed as incorporated sulfate counts per microgram of cell protein. Values are expressed as means ± SD relative to cells grown under physiological glucose conditions without additives, which is expressed as 100% (n = 3). C: SMC were exposed to glucosamine for the indicated time periods, and were metabolically labeled with $^{35}SO_4$ for the last 8 h of glucosamine exposure. The sulfate incorporation was analyzed by CPC precipitation, and is expressed as incorporated sulfate counts per microgram of cell protein. Values are expressed as means ± SD relative to cells grown under low glucose conditions without additives, at each time point, which is expressed as 100% (n = 3). Solid columns, physiological glucose (5.6 mM); diagonally hatched columns, glucosamine at 0.12 mM; open columns, glucosamine at 1.2 mM; horizontally hatched columns, glucosamine at 12 mM. * $P = 0.01$, ** $P < 0.005$ versus control.

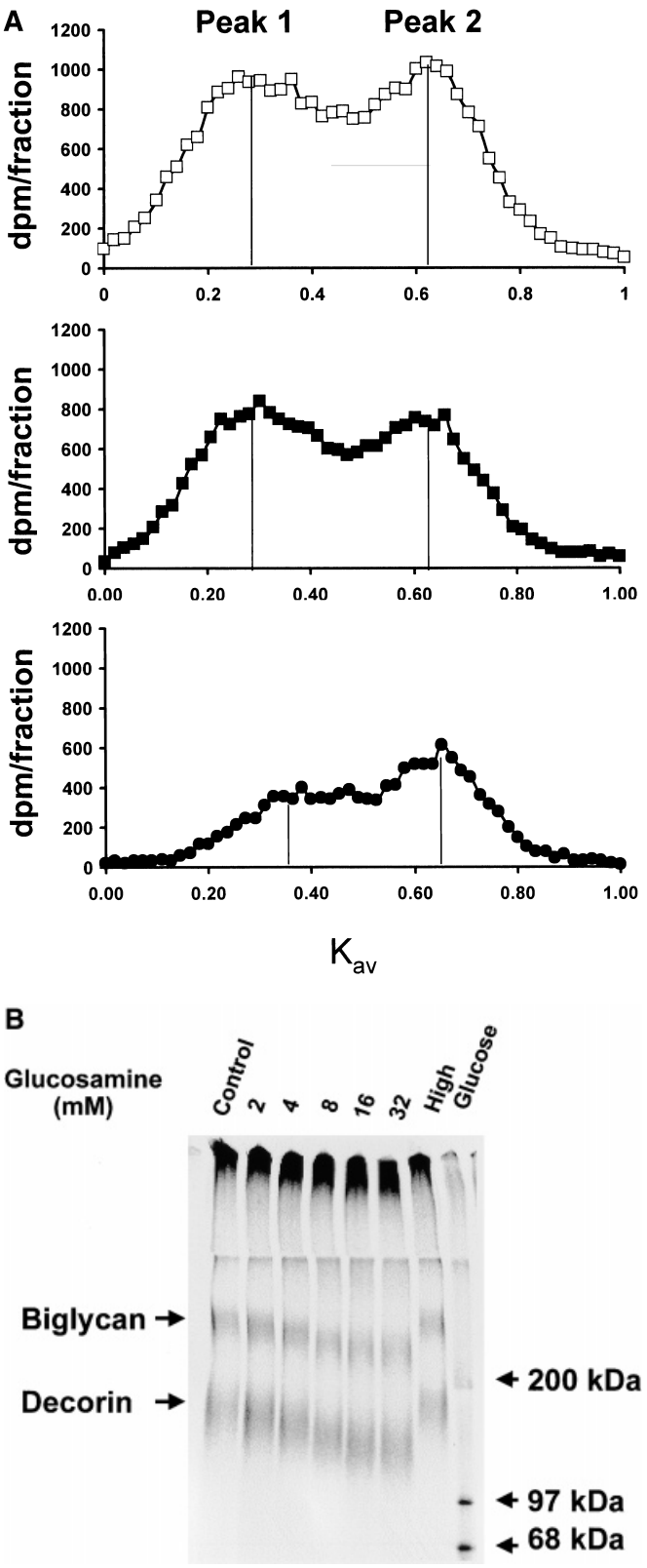

Fig. 2. Glucosamine decreases the size of proteoglycans. A: Size-exclusion chromatography was performed on proteoglycans synthesized in the presence of physiological glucose (control, 5.6 mM; open squares), high glucose (25 mM; solid squares), or physiological glucose plus glucosamine (12 mM; solid circles). Analytical Sepharose CL-2B columns were loaded with approximately 20,000 dpm. The dashed line indicates the K_{av} for peaks 1 (predominantly versican) and 2 (predominantly biglycan and decorin) for proteoglycans synthesized in the presence of physiological glucose levels. The graph shown is representative of three separate experiments. B: SDS-PAGE (3.5% stacking gel with 4–12% gradient resolving gel) analysis of radiolabeled secreted proteoglycans synthesized in the presence of the indicated concentrations of glucosamine for 24 h. Each lane was loaded with 25,000 dpm. The gel shown is representative of three separate experiments.

LDL bound to cyanogen bromide-activated Sepharose (31). The columns were washed extensively with the same buffer. Binding capacity was determined by calculating the radioactivity retained by the LDL column after extensive washing, and was expressed as a percentage of binding by control proteoglycans, which was set at 100%.

RESULTS

To test the effects of glucosamine on total secreted proteoglycans, the incorporation of radiolabeled sulfate into proteoglycans was assessed after exposure of cells for 24 h to glucosamine (12 mM). To assess the role of the hexosamine pathway, additional dishes were exposed to high glucose (25 mM) or high glucose with azaserine. Glucosamine supplementation (12 mM) resulted in a decrease in proteoglycan sulfate incorporation (**Fig. 1A**), which was the opposite of predicted. High glucose levels alone, or in the presence of azaserine, had no effect on proteoglycan sulfate incorporation (Fig. 1A). To further evaluate the role of glucosamine in glycosaminoglycan synthesis, vascular smooth muscle cells were metabolically labeled with $^{35}SO_4$ in the presence of concentrations of glucosamine ranging from 15 μM to 32 mM. A dose-dependent decrease in proteoglycan sulfate incorporation was seen with glucosamine concentrations greater than 2 mM ($P \leq 0.01$) (Fig. 1B). Time course studies performed by determining sulfate incorporation at 8, 16, and 24 h of glucosamine exposure showed that the decrease in sulfate incorporation occurred as early as 8 h in the presence of 12 mM glucosamine ($P = 0.01$) (Fig. 1C). Cytotoxicity of glucosamine was assessed by determination of cell culture medium LDH activity, and by determination of total protein synthesis by TCA precipitation of [^{35}S]methionine-labeled proteins. Cytotoxicity was found when cells were exposed to azaserine, or to 32 mM glucosamine, but cytotoxicity was not seen at lower concentrations of glucosamine (data not shown). Despite the finding of cytotoxicity with azaserine, proteoglycans synthesized in the presence of azaserine were not different from those synthesized under control conditions, suggesting that azaserine did not have a direct effect on proteoglycan synthesis.

The decrease in glycosaminoglycan chain sulfate incorporation found in response to glucosamine could be due to changes in the activity of sulfotransferases (15), or could be due to shorter glycosaminoglycan chain lengths with fewer sulfate-accepting groups. To assess glycosaminoglycan chain length, the hydrodynamic size of secreted proteoglycans was determined by molecular sieve chromatography. Proteoglycans synthesized under control conditions elute in two broad peaks: peak 1 (which contains

predominantly versican) with a K_{av} of 0.28, and peak 2 (which contains a mixture of biglycan and decorin), which has a K_{av} of 0.62 (**Fig. 2A**) (22, 23). There was no difference in the K_{av} of peak 1 or peak 2 between proteoglycans synthesized in the presence of physiologic or high glucose levels (Fig. 2A), or in the presence of high glucose with azaserine (data not shown). However, the proteoglycans synthesized in the presence of glucosamine (12 mM) were smaller (K_{av} of 0.36 and 0.65, respectively, for peaks 1 and 2) (Fig. 2A). To confirm these data, the apparent molecular size of glucosamine-modified proteoglycans was assessed by SDS-PAGE. A dose-dependent increase in mobility of two of the major proteoglycans produced by these cells, biglycan and decorin, was seen in the presence of glucosamine concentrations between 2 and 32 mM (Fig. 2B). No difference in proteoglycan size was seen between proteoglycans synthesized in the presence of control or high glucose levels (Fig. 2B), or in the presence of glucosamine concentrations less than 2 mM (data not shown). These experiments suggest that there is no change in proportion of chondroitin sulfate, dermatan sulfate, and heparan sulfate proteoglycans in the secreted proteoglycans synthesized in the presence of glucosamine. It previously has been reported that there is a change in the proportion of vascular proteoglycans in diabetes, with an increase in chondroitin sulfate and dermatan sulfate proteoglycans and a relative decrease in heparan sulfate proteoglycans (4).

To examine the effect of glucosamine supplementation on the glycosaminoglycan components of the proteoglycans, proteoglycans synthesized in the presence of glucosamine (12 mM) or synthesized under control conditions were treated with sodium borohydride to release their glycosaminoglycan chains. The size of these released chains was then determined by molecular sieve chromatography. The glycosaminoglycan chains cleaved from proteoglycans synthesized in the presence of 12 mM glucosamine were smaller [$K_{av} \approx 0.41$, corresponding to a molecular mass of 35 kDa (24)] than the chains cleaved from control proteoglycans [$K_{av} \approx 0.35$, corresponding to a molecular mass of 45kDa (24)] (**Fig. 3**). In addition, the effect of glucosamine supplementation on glycosaminoglycan synthesis was assessed by directly investigating free glycosaminoglycan chain synthesis. Free glycosaminoglycan chains were synthesized by adding exogenous xyloside to the culture medium to act as an artificial acceptor for glycosaminoglycan chain synthesis. This results in the synthesis of free glycosaminoglycan chains that are not attached to core protein molecules (25). The free glycosaminoglycan chains synthesized in the presence of glucosamine demonstrated a dose-dependent decrease in sulfate incorporation (**Fig. 4A**), and a decrease in chain size as determined by molec-

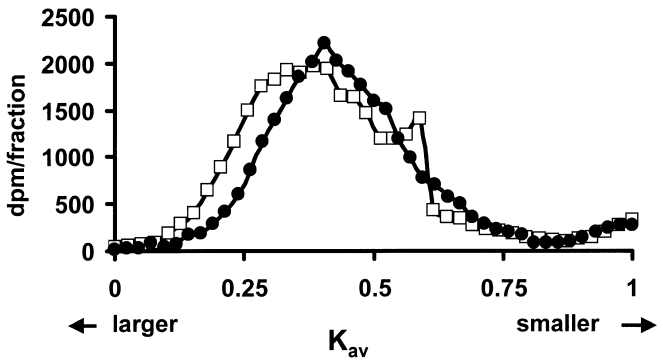

Fig. 3. Glucosamine decreases the size of glycosaminoglycans. Size-exclusion chromatography was performed on glycosaminoglycans cleaved from proteoglycans synthesized in the presence of physiological glucose (5.6 mM; open squares) or glucosamine (12 mM; solid circles). Analytical Sepharose CL-2B columns were loaded with approximately 20,000 dpm. The K_{av} for control glycosaminoglycans corresponds to a molecular mass of 45 kDa, and the K_{av} for glycosaminoglycans synthesized in the presence of glucosamine corresponds to a molecular mass of 35 kDa, using the standard curve of Wasteson (24). The graph shown is representative of three separate experiments.

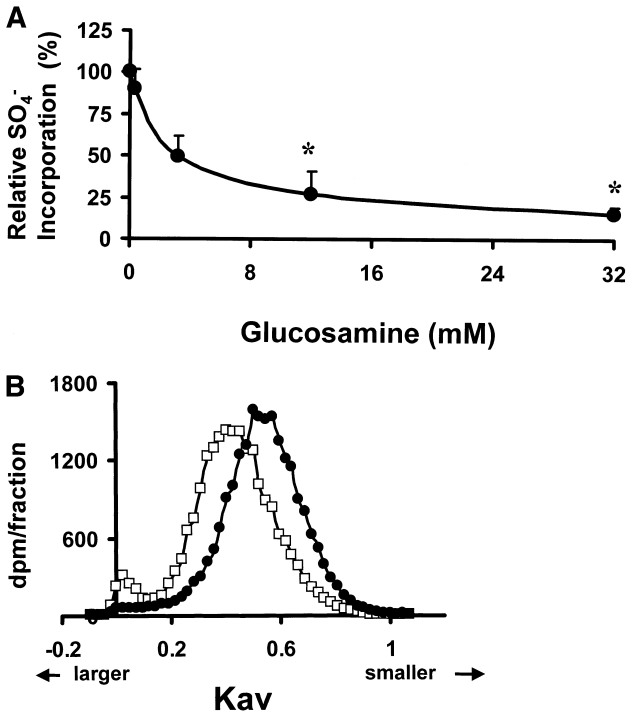

Fig. 4. Free glycosaminoglycans synthesized in the presence of glucosamine are smaller and less sulfated than control glycosaminoglycans. A: SMC were exposed to xyloside (0.5 mM) with the indicated concentrations of glucosamine for 24 h and were metabolically labeled with $^{35}SO_4$. The sulfate incorporation of the free glycosaminoglycans secreted into the culture medium was analyzed by CPC precipitation, and is expressed as a ratio of incorporated sulfate counts per microgram of cell protein. Values are expressed as means ± SD relative to free glycosaminoglycans synthesized in the presence of physiological glucose (control), which is expressed as 100%. * $P < 0.001$ versus control (n = 3). B: Size-exclusion chromatography was performed on free glycosaminoglycans synthesized in the presence of physiological glucose (5.6 mM; open squares) or glucosamine (12 mM; solid circles). Analytical Sepharose CL-2B columns were loaded with approximately 20,000 dpm. The K_{av} for control glycosaminoglycans corresponds to a molecular mass of 32 kDa, and the K_{av} for glycosaminoglycans synthesized in the presence of glucosamine corresponds to a molecular mass of 15 kDa, using the standard curve of Wasteson (24). The graph shown is representative of three separate experiments.

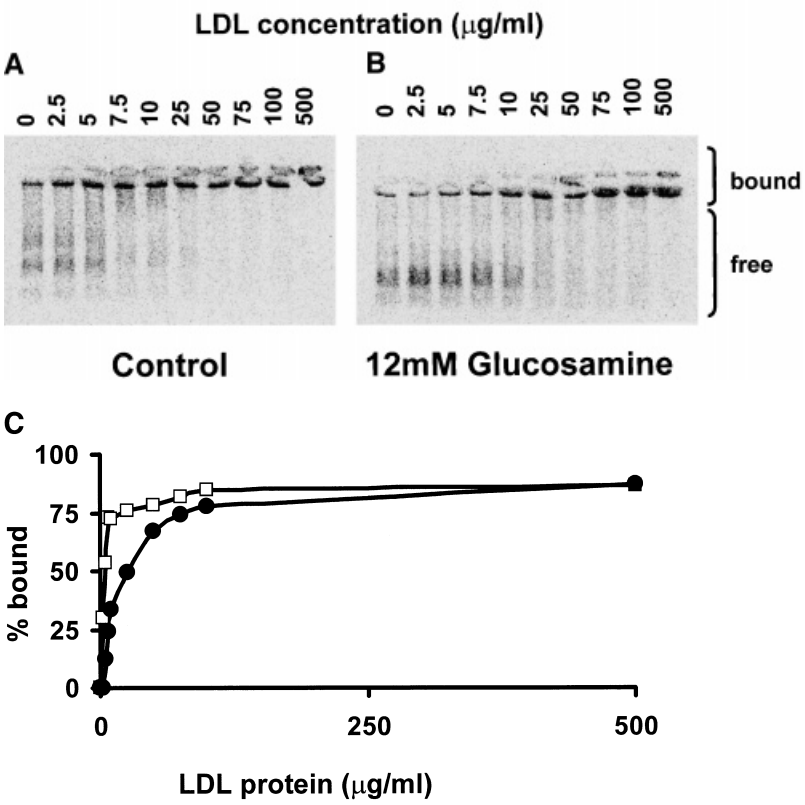

Fig. 5. Proteoglycans synthesized in the presence of glucosamine have reduced LDL-binding affinity compared with control proteoglycans. Equal amounts of total secreted proteoglycans (approximately 1,500 dpm) from control proteoglycans (A) or proteoglycans synthesized in the presence of 12 mM glucosamine (B) were incubated with the indicated concentrations of native human LDL under physiological conditions for 1 h. Samples were then applied to agarose gels and electrophoresed, as described in Research Design and Methods. Proteoglycans that have been bound by LDL are retained at or near the origin whereas free proteoglycans migrate into the gel. Binding curves (C) were calculated by expressing the percentage of proteoglycans bound as a ratio of total radioactivity in each lane. The lower K_d for control proteoglycans (open squares; K_d = LDL at 30 μg/ml or 5.45×10^{-8} M LDL) indicates higher binding affinity than for proteoglycans synthesized in the presence of glucosamine (solid circles; K_d = LDL at 79 μg/ml or 1.44×10^{-7} M LDL) ($P < 0.05$). Gels shown are representative of three separate experiments.

ular sieve chromatography (Fig. 4B). These data demonstrate that glucosamine supplementation directly affects glycosaminoglycan chain synthesis.

To determine whether the proteoglycans synthesized by cells exposed to glucosamine had a decrease in LDL-binding affinity consistent with their smaller size, a modified gel mobility shift assay was performed. In this assay, proteoglycans that have bound LDL are retained at or near the origin, whereas unbound proteoglycans migrate into the gel (30). Analysis and quantification of the binding pattern allow determination of binding affinity constants. Representative gel mobility shift assays for proteoglycans synthesized under control conditions (**Fig. 5A**) or with supplemental glucosamine (Fig. 5B), and the binding curves generated (Fig. 5C), show a significant decrease in LDL-binding affinity for proteoglycans synthesized in the presence of 12 mM glucosamine. Analysis of the gels shows that proteoglycan-lipoprotein complexes form at higher LDL concentrations when the proteoglycans are synthesized in the presence of glucosamine (Fig. 5B) compared with control proteoglycans (Fig. 5A). By using SAAM II modeling we determined that proteoglycans synthesized in the presence of 12 mM glucosamine had a lower binding affinity (K_d of 79 μg of LDL per ml, or 1.44×10^{-7} M LDL, assuming a molecular mass of 550,000 kDa for apoB) than control proteoglycans (K_d of 30 μg of LDL per ml or 5.45×10^{-8} M LDL; $P < 0.05$). No differences in LDL-binding affinities were seen for proteoglycans synthesized in the presence of high glucose levels (25 mM), or high glucose with azaserine, compared with control proteoglycans (data not shown).

To analyze the LDL-binding capacity of proteoglycans synthesized under control conditions or in the presence of glucosamine, LDL affinity chromatography was performed. Equal aliquots (approximately 500,000 dpm) of secreted proteoglycans synthesized in the presence of low glucose (5.6 mM), or in the presence of low glucose plus glucosamine (12 mM), were applied to columns containing native human LDL. The capacity of the proteoglycans to bind to the LDL was determined by calculating the relative retention of the radiolabeled proteoglycans. Proteoglycans synthesized in the presence of glucosamine (12 mM) had significantly lower binding capacity than control proteoglycans ($P < 0.001$) (**Fig. 6**). Thus, proteoglycans synthesized in the presence of glucosamine have reduced LDL-binding affinity and binding capacity compared with control proteoglycans.

To determine whether the effect of glucosamine supplementation on the LDL-binding affinity of proteoglycans was mediated through their shortened glycosaminoglycan chains, the binding affinity of free glycosaminoglycan chains initiated on xyloside (25) was determined by the modified gel mobility shift assay. Free glycosaminoglycans synthesized in the presence of glucosamine (12 mM) had a lower LDL-binding affinity (K_d of 772 μg LDL per ml or 1.4×10^{-6} M LDL) compared with modified free glycosaminoglycans synthesized under control conditions (K_d of 557 μg of LDL per ml or 1.0×10^{-6} M LDL) (**Fig. 7**). The lower binding affinity for free glycosaminoglycans compared with intact proteoglycans is likely due to their much smaller size. These data demonstrate that the effect of glucosamine supplementation on decreasing the LDL-binding affinity of pro-

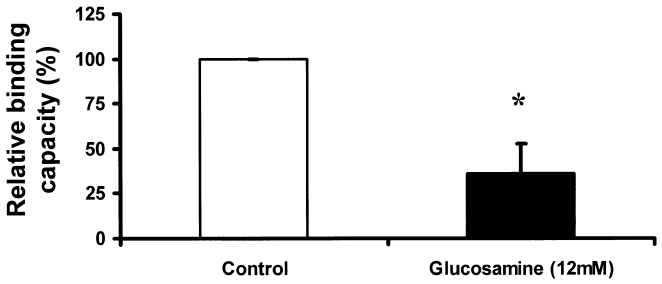

Fig. 6. Proteoglycans synthesized in the presence of glucosamine have reduced LDL-binding capacity. Proteoglycans (1 million dpm) synthesized in the presence of physiological glucose (5.6 mM; open column) or 12 mM glucosamine (solid column) were dialyzed against 10 mM HEPES with 20 mM NaCl and 250 μM butylated hydroxytoluene, and then were applied to separate LDL-Sepharose affinity columns. The binding capacity is determined by subtracting the disintegrations per minute eluted from the columns after extensive washing with 10 mM HEPES with 20 mM NaCl from the disintegrations per minute loaded. Values are expressed as means ± SD relative to the binding of proteoglycans synthesized under control conditions, which is expressed as 100%. * $P < 0.001$ versus control (n = 4).

teoglycans is mediated, at least in part, through the effects of glucosamine on the glycosaminoglycan chains.

DISCUSSION

The data presented demonstrate that under these experimental conditions extracellular proteoglycans synthesized in the presence of glucosamine are smaller, with shorter glycosaminoglycan chains. The proteoglycans synthesized in the presence of glucosamine have a lower binding affinity and capacity for LDL compared with proteoglycans synthesized in the presence of physiological glucose (5.6 mM, or control). However, proteoglycans synthesized in the presence of high glucose levels such as seen in diabetes, or in the presence of azaserine to inhibit glucose from entering the hexosamine pathway, do not differ from control proteoglycans in size, sulfate incorporation, or LDL-binding affinity. Azaserine is a glutamine analog that acts as a nonspecific inhibitor of GFAT. Although it is unstable in cell culture medium over long time periods after a single dose, azaserine is commonly used in this way to test the role of the hexosamine pathway (19). Because there were no differences between proteoglycans synthesized in the presence of high glucose, high glucose with azaserine, or control, this suggests that the flux of glucose through the hexosamine pathway is maximal at low glucose concentrations in this cell type. Therefore, inhibition of GFAT by azaserine does not have any effect on proteoglycan synthesis.

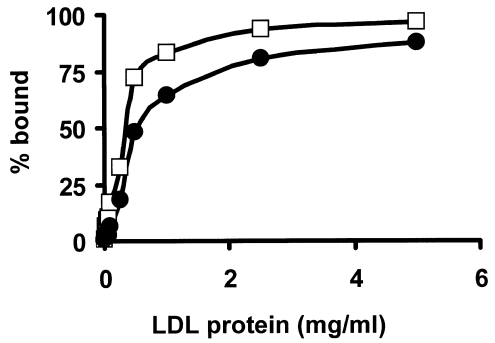

Fig. 7. Free glycosaminoglycans synthesized in the presence of glucosamine have lower LDL-binding affinity than control glycosaminoglycans. Equal amounts of labeled glycosaminoglycans (approximately 1,500 dpm) synthesized under control conditions (open squares) or in the presence of 12 mM glucosamine (solid circles) were incubated with indicated concentrations of native human LDL under physiological conditions for 1 h. Samples then were applied to agarose gels and electrophoresed. Glycosaminoglycans that have been bound by LDL are retained at or near the origin whereas free glycosaminoglycans migrate into the gel. The binding curves were calculated by expressing the percentage of glycosaminoglycans bound as a ratio of total radioactivity in each lane. The lower K_d for control glycosaminoglycans (K_d = 557 μg of LDL per ml or 1.0×10^{-6} M LDL) indicates a higher binding affinity than for glycosaminoglycans synthesized in the presence of glucosamine (K_d = 772 μg of LDL per ml or 1.4×10^{-6} M LDL). The graph shown is representative of two separate experiments.

Glycosaminoglycan chains cleaved from proteoglycans synthesized in the presence of glucosamine supplementation were shown to be smaller than glycosaminoglycan chains cleaved from proteoglycans synthesized under control conditions. Free glycosaminoglycan chains synthesized in the presence of glucosamine also were shorter, and bound LDL with lower binding affinity than free glycosaminoglycan chains synthesized under control conditions. This suggests that the glucosamine effect is being mediated on the carbohydrate moiety of proteoglycans, and is not due to a toxic effect on core protein synthesis; neither is it a limitation of core protein molecule availability to accept glycosaminoglycan chains during synthesis. The decreased sulfate incorporation seen in the presence of glucosamine is likely the result of shorter chain length with fewer sulfate-accepting groups, although a decrease in the sulfate-to-carbohydrate ratio cannot be ruled out by these experiments. These alterations in proteoglycans were seen rapidly: time course studies indicated that the inhibition of proteoglycan synthesis in the presence of glucosamine occurred within 8 h of exposure to glucosamine. This suggests that the effect of glucosamine is due to alterations in proteoglycan synthesis rather than to changes in proteoglycan degradation. The effect of glucosamine on proteoglycans is not due to cytotoxicity. Assays of cell culture medium LDH activity showed that there was no toxicity seen at glucosamine levels less than 12 mM. Further, total cell protein synthesis was decreased only in the presence of high levels of glucosamine supplementation (32 mM). Several groups have reported increased glycosaminoglycan synthesis with glucosamine supplementation in chondrocytes (12, 13), fibroblasts (32), and mesangial cells (19). However, the methods for detecting glycosaminoglycan synthesis vary widely between these reports and the present study, which could account for the differences seen between these present studies. Alternatively, the effect of glucosamine on glycosaminoglycan synthesis may be tissue specific, with in-

creased glycosaminoglycan synthesis in some tissues and decreased glycosaminoglycan synthesis in others. Further studies of the effect of glucosamine on glycosaminoglycan synthesis in various cell types are warranted.

One explanation for the lack of effect of high glucose on proteoglycan synthesis may be that vascular smooth muscle cells in vitro have glucose metabolism that differs from glucose metabolism of vascular smooth muscle cells in vivo. Several groups have previously reported that glucose metabolism by cultured cells varies depending on the availability of other oxidative substrates (33–35). Suzuki et al. (36) reported that >90% of glucose consumed by smooth muscle cells in vitro under both physiological and high glucose (25 mM) conditions is converted to lactate and secreted. They speculate that the high rate of anaerobic glycolysis by smooth muscle cells in vitro may be responsible for the relative insensitivity to high glucose levels that they observed. In addition, others have reported that high glucose levels can modify the ability of cytokines to affect proteoglycan synthesis (19, 37). It is conceivable that the effect of high glucose on proteoglycan synthesis by vascular smooth muscle cells in vivo may be different from that presented in this in vitro study.

Glucosamine is a nutritional supplement commonly claimed to alleviate joint pain in osteoarthritis. It has been available in Europe for many years, but is now gaining popularity in the United States as an over-the-counter remedy for arthritis. Despite the widespread and increasing use of glucosamine, little is known concerning its safety or efficacy. Glucosamine is a nutritional supplement and therefore the authority of the Food and Drug Administration to regulate it is now severely limited (38). There have been relatively few trials of glucosamine use in humans, and those that have been published are studies that examined the efficacy of glucosamine in the relief of joint pain (39). There have been no reported trials of long-term glucosamine use, and its long-term safety is unknown. Glucosamine consumption by the American public is on the rise (40), in part due to favorable, but scientifically inconclusive, reports of glucosamine therapy for arthritis symptoms (41). Glucosamine is available as an over-the-counter oral supplement, has few overt side effects, and is relatively inexpensive. Studies suggest that glucosamine supplementation may have significant benefits on osteoarthritis (11); however, the mechanisms underlying this effect are not yet known. The current recommended oral dose for humans is 1,500 mg of glucosamine daily, taken in divided doses. Setnikar and colleagues (42–44) have published several reports on the pharmacokinetics of glucosamine in rats, dogs, and humans. In both humans and rats, oral administration of radiolabeled glucosamine results in absorption of more than 90% of the ingested dose. Free glucosamine is not detectable in plasma, but after a lag time of 1–2 h the radioactivity appears in plasma proteins (42, 44). Glucosamine appears to undergo a significant first-pass effect in the liver, which metabolizes a significant proportion of the dose to CO_2, water, and urea (44). Olszewski, Szostak, and McCully (45) measured plasma glucosamine levels in survivors of recent myocardial infarctions and healthy control subjects by ion-exchange chromatography. They report levels of total glucosamine in the 4 mM range, and levels of free glucosamine (measured in deproteinized hydrolyzed plasma) in the range of 400 μM.

The majority of in vitro studies have used doses in the range of 1–12 mM glucosamine (16, 19, 46). Thus, the concentrations of glucosamine used in our study are compatible with the current literature. Plasma glucosamine levels are not detectable in humans after oral ingestion of supplements, but are likely to be in the micromolar range (44, 45). However, no evidence of cytotoxicity was found at this glucosamine concentration, and the concentration of glucosamine at the level of the artery wall is unknown. Our studies demonstrate that proteoglycans synthesized in the presence of various concentrations of glucosamine demonstrate a dose-dependent decrease in sulfate incorporation with glucosamine concentrations from 2 through 32 mM. The potential biological implications for these data is significant, if the same effects are confirmed in vivo. Given the vast clinical burden imposed by atherosclerotic disease, any potential atheroprotective interventions are of significant interest, and further studies into the role of glucosamine in vascular biology are needed.

This work was supported in part by grants DK02456 and DK35816-16, and by a grant from the Juvenile Diabetes Foundation. The authors thank P. Hugh Barrett for assistance with statistics, and thank Christina Tsoi and Mohamed Omer for expert technical assistance.

Manuscript received 21 June 2001 and in revised form 18 September 2001.

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