Demonstration of a Keratan Sulfate-Containing Proteoglycan in Atherosclerotic Aorta

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Proteoglycans were isolated from either grossly normal or atherosclerotic pigeon aortas after extraction with 4 M guanidine hydrochloride and purification by ion-exchange and size-exclusion chromatography. The small-size proteoglycans (Km, 0.4, on Sepharose CL-4B) from both normal and atherosclerotic tissue contained primarily a dermatan sulfate proteoglycan with an intact molecular size of 220–330 kd and a 45-kd core protein. In addition to the dermatan sulfate proteoglycan, the preparation contained a proteoglycan recognized by monoclonal antibody (MAb) 5-D-4, indicating the presence of sulfated poly-N-acetyllactosamine sequences common to corneal and cartilage keratan sulfate. Electrophoresis on sodium dodecyl sulfate-polyacrylamide gel revealed a polydisperse proteoglycan of 60–150 kd that was recognized by MAB 5-D-4. Significantly greater immunoreactivity with MAB 5-D-4 was observed for atherosclerotic compared with normal artery. After endo-β-D-galactosidase treatment of the proteoglycan from atherosclerotic aorta, diminished MAB 5-D-4 reactivity observed by both Western blot analysis and enzyme-linked immunosorbent assay demonstrated that the material was keratan sulfate. Endo-β-D-galactosidase treatment of the intact proteoglycan generated core proteins of 28 and 38 kd. These studies suggest the presence of one or more keratan sulfate proteoglycans in grossly normal and atherosclerotic arteries. Immunochemical data suggest that sulfation of the keratan sulfate proteoglycan may be greater in atherosclerotic aorta. (Arteriosclerosis and Thrombosis 1992;12:83–91)

Proteoglycans (PGs) have been implicated in the development of atherosclerosis through their ionic binding and retention of low density lipoproteins.1-3 The most consistently reported change in arterial PGs during atherosclerosis progression has been an increase in the aortic content of sulfated glycosaminoglycans (GAGs), primarily dermatan sulfate (DS)-PG.4-6 Recently, using immunohistochemistry, we have demonstrated the presence of chondroitin 4-sulfate (C4S)/DS in spontaneous and diet-induced atherosclerotic lesions of the White Carneau pigeon.7 In these studies we also demonstrated the presence of keratan sulfate (KS) glycoconjugates that were exclusively localized to the atherosclerotic lesions.

Several reports exist regarding the presence of KS in arterial tissue. Initially in studies by Buddecke,8 it was suggested that KS was a normal component of arterial tissue. Later, Stuhlsatz et al9 analyzed the carbohydrate composition of GAG fractions isolated from normal human aorta, and, based on ratios of galactose to glucosamine, they reported that the concentration of KS chains increased from proximal to distal aortic sites and increased with age. Oegema et al10 and Kapoor et al11 described KS-like oligosaccharides present on the large CS-PGs isolated from bovine aorta, and later Akiyama et al12 demonstrated immunochemically the presence of KS chains on the large CS-PGs from normal human aortas.

KS-PGs are a major extracellular matrix component of cartilage and cornea. Corneal KS-PG contains one or two KS chains of M r ~7,000 covalently linked through N-acetylgalcosamine to an asparagine residue of a 30–40-kd core protein.13 In cartilage, 10–15 KS chains of M r ~8,000 are covalently linked by O-glycosidic bonds between N-acetylgalactosamine and the hydroxyl groups of serine or threonine residues of the large CS-PGs and are concentrated in a region of core...
protein adjacent to the hyaluronic acid–binding region. The KS chains consist of repeating N-acetyllactosamine (Galβ1-4GlcNAc) disaccharides, which may be sulfated at the C-6 position of either galactose or N-acetylglucosamine.

Recently, anti-KS antibodies, which recognize highly sulfated regions of the KS chain, have been used to localize the PG in chondrocyte cultures, in normal and pathological muscle, in human serum, in human synovial fluid, and in other tissues from several different species including humans, chickens, cows, monkeys, rats, fish, and pigeons. Although these reports have demonstrated KS-containing PGs in a variety of tissues other than cornea and cartilage, few studies have described the isolation and structural properties of these molecules. In the present study, we present the immunohistochemical and biochemical characterization of KS-PGs isolated from grossly normal and atherosclerotic aortas of the White Carneau pigeon. The findings suggest that the KS-PG is discrete from the large CS-PG containing KS chains in artery and that the sulfation of the KS-PG is enhanced in atherosclerotic tissue.

Methods

Materials
Ultrapure guanidine hydrochloride (GdnHCl) and urea were purchased from Schwartz-Mann (Cleveland, Ohio); Coomassie blue RR 250, 6-aminohexanoic acid, Alcian blue, x-ray film (X-omat AR), and cellulose-coated plastic thin-layer chromatography plates from Eastman Kodak Co. (Rochester, N.Y.); and N,N-methylene-bis-acrylamide, sodium dodecyl sulfate (SDS), tris(hydroxymethyl)aminomethane (Tris), glycine, 4-chloro-l-naphthol, and acrylamide from Bio-Rad (Richmond, Calif.). Chondroitinase ABC (from Proteus vulgaris) and keratanase (from Pseudomonas sp.) were purchased from Seikagaku America, Inc. (St. Petersburg, Fla.). Trypsin (diphenylcarbamyl chloride-treated, type XI), phenylmethylsulfonyl fluoride (PMSF), and peptatin were obtained from Sigma Chemical Co. (St. Louis, Mo.). Endo-β-D-galactosidase (from Escherichia freundii) was from V-Labs, Inc. (Covington, La.). Horseradish peroxidase (HRP)—conjugated antirabbit immunoglobulin G (IgG) was obtained from Cappel Laboratories (Cochransville, Pa.). HRP-conjugated Ig (IgG plus IgM) fraction was obtained from Southern Biotechnology (Birmingham, Ala.). Nitrocellulose membranes (0.45 μm) were obtained from Schleicher and Schuell (Keene, N.H.). High-performance liquid-chromatography reagents were obtained from Waters Division of Millipore (Milford, Mass.). All other chemicals were reagent grade and were purchased from Fisher Scientific (Pittsburgh, Pa.).

Monoclonal Antibodies
The production and characterization of the monoclonal antibody (MAb) used in this study have been thoroughly described. In brief, the MAb 5-D-4 directed against KS recognizes a pentasulfated hexa-saccharide common to both corneal and skeletal KS and can bind KS in its native form. The MAb 2-B-6 directed against C4S or DS recognizes the terminal unsaturated disaccharide, sulfated in the C-4 position of the N-acetylexosamine residue present on the carbohydrate stub of the core protein subsequent to chondroitinase ABC treatment.

Source of Animals

Pigeons were obtained from the production colonies of the Bowman Gray School of Medicine. Pigeons were randomly bred White Carneau of either sex and ranged in age from 1 to 5 years. Of the 222 pigeons used, 138 were fed a cholesterol-free diet throughout their lives and 84 were fed a cholesterol-containing diet for 6 months. The cholesterol-containing diet consisted of pigeon pellets containing 0.5% cholesterol and 10% lard by weight. White Carneau pigeons develop naturally occurring atherosclerosis at several arterial sites, most consistently near the bifurcation of the celiac artery in the distal part of the thoracic aorta. The atherosclerotic lesions are histologically and biochemically similar to human atherosclerotic lesions. Feeding of cholesterol-containing diets has been shown to accelerate atherosclerosis in this species.

Extraction and Purification of Proteoglycans

Only the thoracic aorta extending from the inferior border of the right brachiocephalic artery to 2 mm distal to the inferior border of the branch point of the celiac artery was used in the study. The atherosclerotic lesions consisted of mixed lesions with variable amounts of smooth muscle cells, macrophages, and connective tissue. Grossly normal aortic tissue (41.9 g) and 10.2 g atherosclerotic tissue were obtained from aortas of 222 pigeons. After removal of periadventitial tissue, aortic intima–medias were finely minced (1–2 mm³) and extracted with 16 volumes of 4 M GdnHCl and 0.05 M sodium acetate (pH 5.8) containing 100 mM 6-aminohexanoic acid, 11.1 mM EDTA, 5 mM benzamidine hydrochloride, 2.5 mM 1,10-phenanthroline, 5.1 mM tryptamine hydrochloride, and 0.5% 3-(3-cholamidopropyl)dimethylammonium]-1-propanesulfonate (CHAPS) for 48 hours at 4°C. The filtered extracts were concentrated on Amicon YM 30 membranes and dialyzed against 7 M urea in 0.05 M Tris, pH 6.8, containing 0.5% CHAPS. PGs (including in vitro 35S-labeled PG from pigeon aorta as an internal marker) were fractionated on DEAE–Sephacel by an initial elution with 60 ml 7.0 M urea in 0.05 M Tris HCl (pH 6.8), followed by a continuous 100-m1 NaCl gradient of 0–1 M NaCl.

Fractions containing the PG, which eluted with 0.38–0.47 M NaCl, were pooled, concentrated, and dialyzed against 4 M GdnHCl containing protease inhibitors. The PG types were separated by size-exclusion chromatography on a column of Sepharose CL-4B, with 4.0 M GdnHCl, 0.05 M sodium acetate (pH 5.8) containing 0.5% CHAPS, and protease inhibitors as the eluant. The PG eluted as two peaks of...
material, a large PG, eluting at the \( V_v \) and a small population of PG, included with a \( K_w \) of \(-0.4\). Fractions corresponding to PG-I or PG-II were pooled, dialyzed against double-distilled water, and lyophilized. In previous studies of human aorta\(^9\) and pigeon aorta,\(^40\) C6S-PG was found to be the major component of large PG material and DS-PG was the major component of the small PG material. Four preparations of PG were obtained: 1) small PG from normal aorta, 2) small PG from atherosclerotic aorta, 3) large PG from normal aorta, and 4) large PG from atherosclerotic tissue. In our previous study,\(^7\) dot-blot immunoblot indicated that KS was present mainly in the small PG of atherosclerotic tissue. Therefore, preparations of small PG were used in the present study.

**Enzyme Digestions**

PG preparations (1 mg/ml) were digested with chondroitinase ABC, 0.025 units/ml, in 0.1 M Tris, 0.03 M sodium acetate, and 10 mM EDTA (pH 8.0) containing 0.036 mM pepstatin, 0.5 mM PMSF, and 1.0 mM N-ethylmaleimide for 16 hours at 37°C; or with protease-free chondroitinase ABC under the same conditions excluding protease inhibitors. Digestions with endo-\(\beta\)-d-galactosidase (from E. freundii, 0.025 units/ml) were carried out in 50 mM sodium acetate, pH 5.5, for 16 hours at 37°C in the presence of the protease inhibitors mentioned previously. Digestion with keratanase (Pseudomonas sp., 0.025 units/ml in 0.1 M Tris acetate, pH 8.0) was carried out for 16 hours at 37°C in the presence of the protease inhibitors.

**Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (PAGE)**

Analytical polyacrylamide gradient (4–20%) SDS slab gels (160×140×1.5 mm) were formed with a 3.6% stacking gel by the method of Laemmli and Favre.\(^41\) SDS-PAGE electrophoresis was carried out at 200 V for 4 hours. Protein bands were detected by Coomassie blue staining, and acidic polysaccharides were detected by Alcian blue staining. The staining procedure included a first staining with Coomassie blue followed by destaining. Gels were then stained with Alcian blue followed by a repeat of the Coomassie blue staining. In some cases, samples were directly blotted to nitrocellulose after electrophoresis.

**Western Blotting**

Electrotransfer of proteins from SDS gels to nitrocellulose sheets was carried out at 4°C at 30 V overnight followed by 200 V for 3 hours in a Bio-Rad Transblot apparatus according to the method of the manufacturer. After transfer of intact PG to nitrocellulose, the epitopes recognized by MAb 2-B-6 were generated by treatment of the nitrocellulose sheet with 0.5 units chondroitinase ABC in 20 ml 0.1 M Tris acetate buffer containing 0.1% bovine serum albumin (BSA) for 1 hour at 37°C.

**Immunocharacterization**

After electrotransfer of proteins to nitrocellulose, the membrane was blocked with Tris-buffered saline (TBS), pH 7.4, containing 5% BSA and 0.5% nonfat dry milk for 1 hour at 23°C. After washing with TBS, the membranes were incubated for 2 hours at 23°C with the appropriate MAb (MAb 2-B-6 diluted 1:2,000 or MAb 5-D-4 diluted 1:2,000) in TBS containing 0.5% BSA and 0.5% nonfat dry milk. The membrane was washed three times for 15 minutes each with TBS and incubated with a secondary goat anti-mouse antibody (diluted 1:1,000 in antibody buffer) conjugated to HRP for 1 hour at 20°C. After washing six times for 10 minutes each with TBS, substrate solution (12.4 mg 4-chloro-l-naphthol in 4.15 ml methanol, 20.75 ml TBS, and 8.3 \( \mu l \) 30% \( \text{H}_2\text{O}_2 \)) was added. The reaction was stopped with phosphate-buffered saline (PBS) containing 0.03% NaOAc.

**Enzyme-Linked Immunosorbent Assay (ELISA)**

ELISA plates were irradiated for 18 hours under ultraviolet light. Each well was filled with 0.2 \( \mu g \) protein in 200 \( \mu l \) PBS–NaOAc, incubated for 18 hours at 4°C, washed three times with PBS–NaOAc, and blocked by treatment with PBS–NaOAc containing 0.5% BSA and 0.5% nonfat dry milk for 1 hour at 37°C. Samples were washed three times with PBS–NaOAc, and incubated with a 1:2,000 dilution of MAb 5-D-4 for 2 hours at 37°C. The wells were washed six times with TBS and incubated with a 1:1,000 dilution of an HRP-conjugated goat anti-mouse IgG for 1 hour at 37°C. Then wells were washed four times with TBS and incubated for 30 minutes at 37°C with \( \alpha\)-phenylenediamine substrate (20 ml \( \alpha\)-phenylenediamine buffer containing 10 \( \mu l \) 30% \( \text{H}_2\text{O}_2 \), 150 \( \mu l \)well). The color reaction was stopped by adding 50 \( \mu l \) 2 M \( \text{H}_2\text{SO}_4 \) to each well, and the plates were read at 492 nm with a Titertek Multiskan, Baxter Diagnostics, Chicago, Ill. Bovine nasal cartilage core protein prepared as a chondroitinase ABC-treated A,D, fraction\(^31\) was used as a positive control for the enzymatic action of endo-\(\beta\)-d-galactosidase and keratanase.

**Analytical Methods**

The hexuronic acid content of the PG preparations was determined by the method of Blumenkrantz and Asboe-Hansen,\(^42\) with glucuronic acid as the standard. Protein was measured according to the method of Lowry et al,\(^43\) with BSA as the standard.

**Results**

Preparations of small arterial PGs were enzymatically and electrophoretically characterized on a 4–20% gradient SDS-polyacrylamide gel. The reduced intact PG molecules stained with Alcian blue/Coomassie blue were identified as a polydisperse band. Intense staining was seen for both normal artery and atherosclerotic artery preparations at \( M_s \), \(-220\)–330, but staining of material was also apparent from \( M_s \), \(-60\)–230 (Figure 1, lanes 1 and 2). Core
Figure 1. Electrophoretic separation of intact small proteoglycan (PG) and chondroitinase ABC-generated core proteins on a 4–20% gradient sodium dodecyl sulfate polyacrylamide gel stained with Alcian blue/Coomassie blue. Lane 1, intact PG from grossly normal aorta (5 µg protein); lane 2, intact PG from atherosclerotic aorta (5 µg protein); lane 3, chondroitinase ABC enzyme; lane 4, chondroitinase ABC-generated core proteins of PG from grossly normal aorta (5 µg protein); lane 5, chondroitinase ABC-generated core proteins of PG from atherosclerotic aorta (5 µg protein); lane 6, protein molecular weight standards. Electrophoresis was carried out at 200 V for 4 hours at 12°C.

Proteins of the PG obtained after chondroitinase ABC treatment are shown in lanes 4 and 5. A major Coomassie blue-stained protein corresponding to 45 kd was generated, apparently from 220–330-kd material in both PG preparations. The 45-kd protein was not present in the enzyme mixture alone (lane 3). The 45-kd protein corresponds to the core protein of arterial DS-PG and is similar to preparations of pigeon and human artery DS-PG core proteins and the core protein of DS-PG in other tissues. Recent studies have demonstrated two DS-PG gene products, biglycan (DS-PG I) and decorin (DS-PG II), differing in overall molecular size but having similar-size core protein molecular weights. Further work is needed to demonstrate if the intact DS-PG material with Mr 220–330 kd represents both decorin and biglycan that may not have separated effectively in this system. In addition, material migrating at 94 kd was apparent in both preparations. This material appears to have been present in lanes 1 and 2 but in combination with polydisperse PG was not easily discernable as a discrete band.

To determine the location of the KS and/or CS chains, intact PGs were electrophoresed on a 4–20% gradient gel, transferred to nitrocellulose, then treated with chondroitinase ABC, and probed with MAbs 2-B-6 and MAb 5-D-4. The majority of MAb 2-B-6 (CS4/DS) reactivity was associated with the 220–330-kd PG in both normal and atherosclerotic aorta samples (Figure 2, lanes 2 and 3). The 60–150-kd PG contained KS chains as observed by MAb 5-D-4 reactivity (Figure 2, lanes 5 and 6); no MAb 5-D-4 reactivity was associated with the 220–330-kd PG. Significantly greater immunoreactivity was observed in the intact PG preparation from atherosclerotic aorta (Figure 2, lane 6), in comparison to PG from normal artery, from which only trace immunoreactive material is presented (Figure 2, lane 5). To further examine the GAGs and N-linked oligosaccharides associated with the two major proteins, chondroitinase ABC-generated core proteins were electrophoresed on 4–20% gradient SDS-PAGE gels, transferred to nitrocellulose, and probed with MAbs 2-B-6 and MAb 5-D-4.

Figure 2. Western blot of intact small proteoglycan (PG), with monoclonal antibody (MAb) 2-B-6 or MAb 5-D-4 to detect chondroitin 4-sulfate–dermatan sulfate and keratan sulfate. A portion of the intact PG (2.5 µg as protein) was electrophoresed on a 4–20% gradient sodium dodecyl sulfate polyacrylamide gel, electroblotted to nitrocellulose, treated with chondroitinase ABC, and detected with MAb 2-B-6 (lanes 2 and 3) or MAb 5-D-4 (lanes 5 and 6), followed by a peroxidase-conjugated second antibody. Lanes 1, 4, and 7, prestained protein molecular weight standards; lanes 2 and 5, PG from grossly normal aorta; lanes 3 and 6, PG from atherosclerotic aorta.

Figure 3. Western blot of proteoglycan (PG) cores generated by chondroitinase ABC treatment, alone or in combination with endo-β-D-galactosidase or N-glycanase, and probed with monoclonal antibody (MAb) 2-B-6 that recognizes chondroitin 4-sulfate–dermatan sulfate. Core proteins (2.5 µg) generated after chondroitinase ABC treatment were electrophoresed on a 4–20% gradient sodium dodecyl sulfate polyacrylamide gel, electroblotted to nitrocellulose, and detected with MAb 2-B-6 (chondroitin 4-sulfate–dermatan sulfate). Lanes 1, 5, and 9, prestained protein molecular weight standards; lanes 2–4, PG core preparations from grossly normal aorta; lanes 6–8, PG core preparations from atherosclerotic aorta; lanes 2 and 6, chondroitinase ABC treatment alone; lanes 3 and 7, chondroitinase ABC treatment followed by endo-β-D-galactosidase treatment; lanes 4 and 8, chondroitinase ABC treatment followed by N-glycanase treatment.
and there was minimal loss of MAb 5-D-4
nase treatment, no shifts in molecular weights were
KS chains (Figure 4, lanes 3 and 7). After /V-glyca-
ished immunoreactivity confirmed the presence of
some residual recognition of material by MAb 5-D-4
the 45-kd DS-PG core proteins. Although there is
extended from 60 to 150 kd and was comparable in
observed for PG prepared from atherosclerotic
C4S/DS chains. Minimal MAb 5-D-4 reactivity was
associated with the PG from normal aorta (Figure 4,
lanes 3 and 7), indicating that KS was not a compo-
ment of either the DS-PG core protein (45-kd band)
or the 94-kd protein. After N-glycanase treatment, a
significant shift in molecular weight was observed for
the 45- and 94-kd proteins in both the normal and
the atherosclerotic aorta preparations (Figure 3,
lanes 4 and 8), indicating that both proteins con-
tained /V-linked oligosaccharides.

A strong positive reaction with MAb 5-D-4 was
observed for PG prepared from atherosclerotic
plaque (Figure 4, lane 6). The reaction product
extended from 60 to 150 kd and was comparable in
to the nonenzyme-treated sample (Figure 2),
suggesting that the KS material did not contain any
C4S/DS chains. Minimal MAb 5-D-4 reactivity was
associated with the PG from normal aorta (Figure 4,
lane 2). No MAb 5-D-4 reactivity was associated with
the 45-kd DS-PG core proteins. Although there is
some residual recognition of material by MAb 5-D-4
after endo- /B-galactosidase treatment, the dimin-
ished immunoreactivity confirmed the presence of
KS chains (Figure 4, lanes 3 and 7). After N-glyca-
nase treatment, no shifts in molecular weights were
apparent, and there was minimal loss of MAb 5-D-4
reactivity, suggesting that the KS chains were not
N-linked to a core protein (Figure 4, lanes 4 and 8).
The degree of susceptibility of the MAb 5-D-4–
positive material to endo- /B-galactosidase and kera-
tanase treatment was estimated by ELISA. Endo- /B-
galactosidase treatment of the PG preparation
from atherosclerotic plaque resulted in a 73% reduc-
tion in immunoreactivity compared with a 61% re-
duction in recognition of bovine nasal cartilage core
protein used as a control. Keratanase treatment of
the PG resulted in a 13% reduction in immunoreac-
tivity. These results indicated that the immunoreac-
tive material recognized by MAb 5-D-4 was largely a
KS with variable regions of sulfation.

Because further examination of the protein asso-
ciated with KS was limited by low levels in the
sample, the PG preparations were radioiodinated,
subjected to selective enzymatic treatments, and
electrophoresed on SDS gels. The results (Figure 5)
confirm the previous findings (Figure 3) that the
intact 220–330-kd DS-PG from either normal artery
or atherosclerotic plaque (Figure 5, lanes 1 and 2)
was not affected by treatment with endo- /B-galacto-
sidase (Figure 5, lanes 5 and 6) but was susceptible
to chondroitinase ABC treatment, generating a
45-kd core protein (Figure 5, lanes 3 and 4). After
endo- /B-galactosidase treatment of intact PG, two
major proteins with approximate molecular weights
of 38 and 28 kd were observed in the sample from
atherosclerotic artery (Figure 5, lane 6) and appear
to have been generated from intact molecules of
70–150 kd. Both proteins were also present in normal
aorta (Figure 5, lane 5). The results suggest that the
core proteins of the KS-PG have molecular weights
of 38 and 28 kd.

Discussion

In the present study, we have reported the pres-
ence in artery of a previously undescribed small PG
containing KS chains. Although the KS-PG was coisolated with DS-PG and an unidentified 94-kd protein, the KS-PG was shown to be distinct from these materials. There are three criteria that define the arterial PG as a KS-PG: recognition by MAb 5-D-4 and sensitivity to two different enzymes, endo-β-d-galactosidase and keratanase. Recognition by the specific MAb 5-D-4 indicated the presence of epitopes characteristic of both skeletal and nonskeletal KS. Sensitivity of the molecule to endo-β-d-galactosidase provided confirmation of the poly-N-acetyllactosamine carbohydrate composition of the molecule. Endo-β-d-galactosidase is a highly specific enzyme that requires a minimum structure of GlcNAcβ(1-3)Galβ(1-4)GlcNAc. Degradation of the molecule with endo-β-d-galactosidase indicated that the molecule was authentic KS. The observation that treatment with keratanase produced a significantly decreased binding with MAb 5-D-4 indicated that the epitope resided in a KS chain.

Based on results of the immunoblots, the intact KS-PG migrated as polydisperse 60-150-kd material on SDS-PAGE gels, while the DS-PG had a range of 220-330 kd. After chondroitinase ABC treatment of the PG, no change was seen in the size of the KS-PG (compare Figure 2 with Figure 4). Decreases in immunoreactivity by both immunoblot and ELISA after endo-β-d-galactosidase treatment confirmed the presence of KS chains associated with the 60-150-kd PG.

Both the 45-kd core protein and the 94-kd protein possessed oligosaccharides containing 4-sulfated disaccharides because subsequent to chondroitinase ABC digestion, the proteins were recognized by MAb 2-B-6. The sizes of the 45-kd DS-PG core protein and 94-kd protein material were unaffected by endo-β-d-galactosidase treatment, demonstrating that these proteins did not contain KS chains. In atherosclerotic plaque, based on the appearance of 38- and 28-kd proteins and the loss of immunoreactivity to MAb 5-D-4 after endo-β-d-galactosidase treatment, it appears as if the PG molecules containing highly sulfated KS chains had core proteins of 38 and 28 kd. Both core proteins were also present in normal artery. The two different core proteins may represent differential processing of one KS-PG. Alternatively, based on the wide molecular weight range of material that was positive with the antibody for KS, there may be more than one type of KS-PG in the preparation, and the two proteins may represent two different KS-PGs. Funderburgh and Conrad recently described two KS-PGs isolated from bovine cornea that are comparable to the arterial KS-PG in the present study. The two corneal KS-PGs yielded core proteins of 37 kd and 25 kd after endo-β-d-galactosidase treatment. Each protein had a unique N-terminal amino acid sequence and distinct tryptic peptide map.

Several reports suggest the presence of KS-like oligosaccharides or KS chains as a component of the large, hyaluronic acid-binding CS-PG in normal human aorta and in bovine aorta. Therefore, it appears that the CS-PG of artery may be similar in structure to the large articular cartilage CS-PG in that it contains KS as well as CS chains. Recently from studies of immunoblot, we reported the presence of KS chains on pigeon arterial CS-PG. Although these studies demonstrated that KS was a component of the CS-PG, the immunolocalization of the KS in atherosclerotic lesions of the White Carneau pigeon suggested that the majority of the KS chains were localized in the intimal lesions and not codistributed with the 6-sulfated disaccharide-containing CS-PG that predominates in the normal media. Therefore, it appears that the KS-PG described in the present study is distinct from the KS on CS-PG.

A significant difference between the PG isolated from normal and atherosclerotic arteries was the increased recognition of KS-PG from atherosclerotic lesions by MAb 5-D-4. Mehmet et al showed that the smallest oligosaccharide fragment of the KS chain recognized by MAb 5-D-4 was a linear pentasulfated hexasaccharide. The antibody has been observed to be highly specific for sulfated poly-N-acetyllactosamine sequences, with sulfation being a major factor in recognition. Recent studies of corneal KS with endo-β-d-galactosidase-generated fractions of varying sulfation suggest that the KS chain contains domains of different charge and structure at specific positions of the polysaccharide chain. The failure to completely remove the MAb 5-D-4 reactivity by endo-β-d-galactosidase results from the inability of the enzyme to cleave sulfated residues. Keratanase alone also failed to abolish recognition with MAb 5-D-4. However, KS is not hydrolyzed by keratanase when galactose is sulfated at the C-6 position.

The increased MAb 5-D-4 reactivity observed in the sample from atherosclerotic plaque indicated either increased content and/or increased sulfation of the chain. Although the results of endo-β-d-galactosidase treatment, in which a 73% loss of reactivity to MAb 5-D-4 was observed with the atherosclerotic plaque material, might seem to argue against a high degree of sulfation of the chain, we do not know the oligosaccharide sequence and sulfation pattern of the polymer.

There are a number of possibilities that may account for the presence of a highly sulfated KS-PG in atherosclerotic lesions. Alterations in the glycosylation and/or sulfation of the PG in atherosclerotic lesions may represent a modification in smooth muscle cell synthesis of PG in the changing environment of the lesion. An obvious source of factors capable of modifying smooth muscle cell metabolism are macrophages, which are known to infiltrate the artery wall during the initial stages of atherogenesis. Macrophage products such as prostaglandins, platelet-derived growth factor, and interleukin-1 have been shown to modulate PG synthesis by aortic smooth muscle cells. Thus, macrophage-derived factors may stimulate arterial smooth muscle cells to synthe-
size a highly sulfated KS-containing PG. In studies by Plaas (personal communication), mature, non-dividing chondrocytes have been reported to produce fibromodulin without KS chains, whereas proliferating chondrocytes as well as chondrocytes from fetal calf cartilage produce fibromodulin with KS chains recognized by MAb 5-D-5. These studies further suggest a role for proliferating smooth muscle cells in the accumulation of KS-PG in the developing atherosclerotic lesion.

An alternative explanation for the appearance of MAb 5-D-4 reactivity in the PG from atherosclerotic artery can be drawn from observations by Scott and Haigh.59 They suggest that KS may substitute for CS in conditions of hypoxia. Aw and Jones60 have demonstrated that during hypoxia, hepatocytes reduce the production of CS precursor ([UDP]-GlcA from UDP-Glc and NAD), which requires oxygen and which may lead to an increase in the amount of UDP-Glc available for KS synthesis. KS biosynthesis does not require oxidation of the glucose precursor.61 Their hypothesis was based on studies of cartilage and cornea that demonstrated that KS was present in greater amounts in regions of the tissue further removed from a source of oxygen.69 Additionally, Balduini et al61 demonstrated that there was an inverse relation in the biosynthesis rates of KS and CS in bovine cornea.61 After inhibition of CS biosynthesis by UDP xylene, an increase in KS synthesis was observed.60 In addition to these findings, several reports suggest that hypoxic conditions may contribute to atherosclerotic lesion development.62,63 Low oxygen tension has been shown to stimulate arterial GAG synthesis in vivo64 and in cultured aortic smooth muscle cells.65 It may be possible that focal hypoxia in the atherosclerotic lesion may lead to a preferential synthesis of KS chains.

Another possibility for the increase of KS-containing PG is synthesis by macrophages, which are the major cell type present in developing atherosclerotic lesions. Human monocyte-derived macrophages and peritoeal macrophages from pigeon and guinea pig have been shown to synthesize PG in culture, primarily CS-PG,66-68 as well as oversulfated PG.66 Although the synthesis of KS-PG by macrophages has not been demonstrated to date, the environment of the atherosclerotic lesion may stimulate these cells to produce a KS-containing PG recognized by MAb 5-D-4.

As indicated, KS chains have been reported to be components of the large aortic CS-PG,10-12 and we have demonstrated MAb 5-D-4 reactivity associated with the CS-PG isolated from the atherosclerotic aorta of pigeons.7 It is possible that the 60-150-kd KS-PG results from partial degradation of the CS-PG. If the amino terminus of arterial CS-PG is rich in KS chains, it is conceivable that the KS-rich region may remain in the extracellular space after partial proteolysis of the CS-PG molecule. Plaas et al69 examined the origin of a 40-60-kd KS-PG in rabbit articular cartilage, and from pulse-chase experiments, they demonstrated that the KS-PG was not derived from proteolytic processing of the large KS-containing CS-PG.

Finally, KS-PG may be derived from the plasma and become sequestered in the atherosclerotic lesion. Thonar et al71 and Sweet et al18 have shown that KS chains, recognized by MAb 5-D-4 and presumably resulting from the turnover of cartilage CS-PG, are present in human plasma samples. Plasma KS is thought to exist as free GAG not associated with a core protein.17 In contrast, KS chains described for the KS-PG in the present study were associated with a core protein. In addition, the sample was isolated with detergents included in the extraction solvent and then concentrated by filtration on a 30,000-molecular weight cutoff membrane, a procedure that would result in the loss of small KS chains not having protein. Therefore, it appears unlikely that the source of the arterial KS-PG is plasma KS. Although there are several potential mechanisms to account for the presence of KS-PG in artery, additional studies are needed to determine the specific origin of the KS-PG.

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**KEY WORDS** • artery • atherosclerosis • proteoglycans • keratan sulfate • dermatan sulfate
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