Relationship of Sulfated Glycosaminoglycans and Cholesterol Content in Normal and Arteriosclerotic Human Aorta

Jürgen Hollmann, Annette Schmidt, Dirk-Barthold von Bassewitz, and Eckhart Buddecke

Sulfated glycosaminoglycans were extracted from arteriosclerotic and adjacent nonarteriosclerotic areas of human aortas from persons ages 28 to 83 years; the glycosaminoglycans were compared with the cholesterol and triglyceride content of the tissues. Sulfated glycosaminoglycans were isolated after proteolytic digestion of defatted arterial tissue and were quantified after reductive labeling with NaB\(^{3}H_{4}\). The amount of glycosaminoglycans in the aorta increased with the age of the person and the cholesterol content (degree of arteriosclerosis) of the aorta. The proportion of chondroitin sulfate/dermatan sulfate increased significantly with age and cholesterol content, whereas the corresponding amounts of heparan sulfate decreased.

(Arteriosclerosis 9:154–158, March/April 1989)

Proliferation of arterial smooth muscle cells and accumulation of lipids are basic events in the pathogenesis of arteriosclerosis.\(^{1,2}\) Proliferating arterial smooth muscle cells synthesize and secrete increased amounts of proteoglycans,\(^{3}\) which are capable of interacting with lipoproteins.\(^{4-7}\) The complexes thus formed cause the accumulation of low density lipoprotein in the arterial wall and the subsequent development of arteriosclerosis.

Proteoglycans containing chondroitin sulfate, dermatan sulfate, and heparan sulfate glycosaminoglycans have been detected in human\(^{8}\) and in mammalian\(^{9-12}\) arteries. Such proteoglycans have been characterized as individual macromolecular species by chemical and physiochemical procedures.\(^{13}\) Extracellular proteochondroitin sulfate/dermatan sulfate may constitute a viscoelastic gel that regulates the flux of macromolecular plasma constituents into the vessel wall.\(^{14}\) Proteoheparan sulfate, on the other hand, is thought to be involved in the control of smooth muscle cell growth, because cell-associated heparan sulfate from confluent arterial smooth muscle cells\(^{15}\) and arterial endothelial cells\(^{16,17}\) specifically inhibits the proliferation of arterial smooth muscle cells.

It has been established that the content of sulfated glycosaminoglycans in the artery increases as arteriosclerosis progresses,\(^{18,19}\) but no information is available about corresponding changes in the content of heparan sulfate. In view of the antiproliferative activity of heparan sulfate and its potential role in the pathogenesis of arteriosclerosis,\(^{19}\) the quantitative evaluation of sulfated glycosaminoglycans is of special interest. The present study shows that increasing cholesterol content during the development of arteriosclerosis in the human aorta is accompanied by decreasing amounts of heparan sulfate and an increase in chondroitin sulfate/dermatan sulfate.

Methods

Materials

Chondroitin ABC lyase was purchased from Saikagaku, Tokyo, Japan and heparin lyase, from Miles Scientific, Munich, FRG. Sodium \(^{3}H\)-borohydride (specific radioactivity 500 mCi/mmol) was obtained from Amersham Buchler (Braunschweig, FRG). Whatman DE 52 cellulose ion exchanger was from Whatman Limited, United Kingdom, and Sephadex G 50 fine was from Pharmacia (Freiburg, FRG). All chemicals were purchased from Merck (Darmstadt, FRG) and Serva (Heidelberg, FRG).

Isolation and Fractionation of Sulfated Glycosaminoglycans

Thoracic aortas were obtained from the Institute of Pathology, University of Münster. Twenty-five human aortas were obtained at autopsy within 8 hours after death. Segments of the aortas ranging from the left arterial subclavia to the sixth arterial intercostal were used for the studies. Throughout the preparation of samples, all aortas were kept on ice. The specimens were freed from fat and adhering connective tissue and were rinsed with cold saline. After removal of the adventitia, macroscopically normal appearing and adjacent arteriosclerotic specimens (on the average 3 cm apart) ranging from 0.2 to 2.5 g were selected for analysis. Focal intimal thickening with the appearance of fatty streaks and/or fibrous plaques were regarded as arteriosclerotic. Plaques with hemorrhage, ulceration, or mineralization were not included. The samples were minced into 5×5 mm pieces, were delipidated with chloroform/methanol (2:1), and were dried under vacuum in the presence of paraffin. Total lipids
were recovered after solvent removal and were analyzed for cholesterol and triglyceride by standard methods. The defatted samples were subjected to proteolysis in 0.1 M acetate buffer (pH 5.8) containing 0.5% papain (200 U/g), 0.05% EDTA, and 0.005 M cysteine at 65°C for 24 hours. After papain digestion, which completely dissolved the arterial tissue, the glycosaminoglycans were precipitated with cetylpyridinium chloride at a final concentration of 1% (wt/vol). The cetylpyridinium-glycosaminoglycan precipitate was dissolved in 2 ml of 1 M MgCl₂ and was precipitated with 2.5 volumes of ethanol containing potassium acetate (final concentration 1% [wt/vol]). The precipitated potassium salts of the glycosaminoglycans were centrifuged and dissolved in distilled water. Glycosaminoglycans were precipitated by the addition of cetylpyridinium chloride in the presence of MgCl₂ to final concentrations of 1% [wt/vol]. and 0.125 M, respectively. Under these conditions, hyaluronate remained in solution. The insoluble cetylpyridinium salts of glycosaminoglycans were pelleted by centrifugation, were dissolved in 1 M MgCl₂, were precipitated with ethanol containing potassium acetate (final concentration, 1% [wt/vol]), were washed twice with ethanol and once with ether, and then were dried under a stream of air. Glycosaminoglycans were radiolabeled by reduction with NaBH₄, according to the procedure of Glaser and Conrad. After destruction of excess borohydride, glycosaminoglycans were recovered by gel filtration on a Sephadex G 50 column (0.8 x 50 cm) equilibrated with 1 M NaCl at ambient temperature. Material eluting with 1 M NaCl between Kav = 0 and Kav = 0.1 was pooled and, after adding 0.5 mg unlabeled chondroitin sulfate, was dialyzed against 6 M urea in 0.1 M Tris-HCl, pH 7.0. For separation of glycosaminoglycans by ion exchange chromatography, the dialyzed pools were applied to a 2 ml DE 52 column equilibrated with the above buffer at ambient temperature. After elution of unbound material with 5 ml of buffer, bound glycosaminoglycans were eluted with a linear gradient of NaCl (0 to 0.6 M, 10 g/10 g) in the above buffer. Characterization of the arterial glycosaminoglycans was accomplished by enzymatic analysis by using chondroitin ABC lyase (EC 4.2.2.4) and heparitin lyase (EC 4.2.2.8). Material eluting 0.2 M NaCl was chondroitin ABC lyse-resistant and heparitin lyase-sensitive and hence was heparan sulfate; material eluting with 0.36 M NaCl was chondroitin ABC lyase-sensitive and heparitin lyase-resistant and thus represented chondroitin sulfate/dermatan sulfate.

Further Procedures

Chondroitin sulfate and heparan sulfate were assayed enzymatically as described elsewhere. After enzyme digestions, samples were thermally inactivated and subjected to gel filtration on a Sephadex G 50 fine column (0.8 x 50 cm) equilibrated and eluted with 1 M NaCl. The appearance of ³H-labeled material in the total volume (V₀) was indicative of degradation. Radioactivity was measured with a liquid scintillation counter (Packard A 4430, Packard Instruments GmbH, Frankfurt, FRG) by using Instagel (Packard Instruments GmbH) as the scintillation medium.

Results

The total cholesterol and triglyceride content and the total sulfated glycosaminoglycans were analyzed in grossly normal appearing regions and in adjacent atherosclerotic areas of 25 human aortas from subjects 28 to 83 years old. The total cholesterol and triglyceride content was quantified by enzymatic analysis according to standard procedures. The total content of sulfated glycosaminoglycans was determined after quantitative release of chondroitin sulfate, dermatan sulfate, and heparan sulfate from the respective proteoglycans by proteolytic digestion and complete dissolution of the arterial wall followed by β-elimination and ³H-labeling of the monosaccharide residue (xylose) at the reducing end of the polysaccharide chain. ³H-radioactivity reflected the number of glycosaminoglycan molecules. All values were expressed as milligrams or ³H-cpm/g dry weight of tissue.

No effort was made to quantify native proteoglycans, because these are not quantitatively extractable from tissue with dissociative solvents.

Total Glycosaminoglycan Content Increases with Age and Degree of Arteriosclerosis

Plots of the radioactivity of ³H-glycosaminoglycans against age or cholesterol content of the aorta indicated that the glycosaminoglycan content increased proportionally (Figure 1). The age-dependent increase appeared more pronounced in atherosclerotic tissue than in normal areas, but the correlation coefficient was less than 0.7, so that the difference was not significant. No correlation between triglyceride and ³H-glycosaminoglycan contents was found (data not shown).

Amount of Heparan Sulfate Relative to Total Glycosaminoglycan Decreases, and Chondroitin Sulfate/Dermatan Sulfate Increases, with Increased Age and Cholesterol Content

Heparan sulfate and chondroitin sulfate/dermatan sulfate were separated on the basis of their different anionic charges (Figure 2). Heparan sulfate was distinguished by
its sensitivity to heparitinase, chondroitin sulfate/dermatan sulfate, by its resistance to heparitinase and its susceptibility to chondroitinase ABC.

When age and cholesterol-dependent changes in the glycosaminoglycan fraction were sought, it became clear that the relative proportion of heparan sulfate decreased linearly with increasing age and increasing cholesterol content in both normal and arteriosclerotic regions of the arteries. The correlation coefficients were $r = -0.91$ for normal, and $r = -0.95$ for arteriosclerotic segments (Figures 3A and 3B). From the data in Figure 3, it can be calculated that the amount of heparan sulfate relative to total glycosaminoglycans decreased from 41% to 20% with increasing age and from 41% to 23% with increasing cholesterol content.

In contrast, the chondroitin sulfate/dermatan sulfate fraction showed a marked linear increase with increasing cholesterol content and age, the correlation coefficients being $r = 0.92$ and $r = 0.95$, respectively, for normal and arteriosclerotic areas of the arteries (Figures 4A and 4B).

**Discussion**

The glycosaminoglycan content of arterial tissue and its alteration during arteriosclerosis has been the subject of several reports (See references 23 and 24 and the references cited therein). However, no information is available on changes in glycosaminoglycan concentration in relation to the lipid content of human arteries. Recently Yla-Herttua et al.16 studied the composition of glycosaminoglycans in human coronary arteries and found that with increasing age and in advanced arteriosclerotic lesions there were increases in the proportion of chondroitin sulfate/dermatan sulfate and decreases in heparan sulfate. However, the arteriosclerotic lesions were not characterized with respect to their lipid content.

In our research, a significant age- and cholesterol-dependent increase in the percentage composition of chondroitin sulfate and dermatan sulfate, and a corresponding decrease in heparan sulfate, was demonstrated. Macroscopically normal appearing and arteriosclerotic specimens were included in the analysis. However, since even ostensibly undiseased segments of vessels may contain early arteriosclerotic lesions, cholesterol content, which is the characteristic feature of arteriosclerotic lesions, was considered the definitive parameter for the degree of arteriosclerosis.

In our study, quantification of the individual glycosaminoglycans was based on selective $^3$H-labeling of the reducing terminus of each polysaccharide chain. Therefore, the radioactivity does not reflect the glycosaminoglycan concentration, but rather the number of glycosaminoglycan chains. Consequently, the relative increase in chondroitin sulfate/dermatan sulfate and the decrease in heparan sulfate with increasing age and increasing degree of arteriosclerosis indicate corresponding changes in the number of glycosaminoglycan chains. However, when the chain length of heparan sulfate isolated from areas with low cholesterol content was compared with that from areas with high cholesterol content, no significant differences were found, as judged from the elution profile of the heparan sulfate chains on Sephadex S 300 (Hollmann, unpublished observations). Likewise, the lengths of chondroitin sulfate and dermatan sulfate chains were not significantly different, although in some cases longer chondroitin sulfate/dermatan sulfate chains were isolated.
from atherosclerotic lesions than from normal areas. This confirms the observation of Wagner et al.\textsuperscript{29} who calculated that in atherosclerotic plaques there are fewer, but longer, chondroitin sulfate chains relative to core protein in the proteoglycan molecule.

The increase of chondroitin sulfate/dermatan sulfate with increasing cholesterol content (Figure 3A) is in accordance with the finding that cholesterol-rich, low density lipoproteins in atherosclerotic arteries accumulate concomitantly with glycosaminoglycans.\textsuperscript{7,25} This phenomenon is explained by our results. Proliferation of arterial smooth muscle cells is a characteristic feature in the development of atherosclerotic plaques. Since proliferating arterial smooth muscle cells have been shown to synthesize and secrete larger amounts of dermatan sulfate-rich proteoglycans than quiescent cells,\textsuperscript{3} the known low density lipoprotein binding capacity of dermatan sulfate-rich proteoglycans causes trapping of lipoprotein, preferentially in areas of cell proliferation. On the other hand, the decrease in heparan sulfate with increasing severity of atherosclerosis (Figure 4) is of special interest, because arterial smooth muscle cells produce a heparan sulfate species with antiproliferative activity.\textsuperscript{15} Thus, heparan sulfate is thought to be involved in controlling the growth of smooth muscle cells.\textsuperscript{15,16,17} However, it remains to be established whether loss of heparan sulfate can cause accelerated cell proliferation, which is known to be an early event in the development of atherosclerotic plaques.

Atherogenesis and the concomitant changing of the glycosaminoglycan pattern may be initiated by hyperlipoproteinemias. This assumption is supported by the finding that the accumulation of plasma low density lipoprotein in the arterial wall after hypercholesterolemia induces altered glycosaminoglycan synthesis in medial smooth muscle cells.\textsuperscript{20}

References

1. Rosse R, Glomset JA. Medical progress: The pathogenesis of atherosclerosis. N Engl J Med 1976;295:265–369
2. Rosse R. The pathogenesis of atherosclerosis—an update. N Engl J Med 1986;314:488–500
3. Schmidt A, Bunce A, Buddecke E. Proliferation-dependent changes of proteoglycan metabolism in arterial smooth muscle cells. Bioclin Chim Hoppe-Seyler 1987;358:277–284
4. Camejo G, Acquatella H, Lelegua F. The interaction of low density lipoproteins with arterial proteoglycans: an additional risk factor. Atherosclerosis 1980;36:55–65
5. Vijayagopai P, Srinivasan SR, Radhakrishnamurthy B, Berenson GS. Hemostatic properties and serum lipoprotein binding of a heparan sulfate proteoglycan from bovine aorta. Biochim Biophys Acta 1983;735:70–83
6. Srinivasan SR, Dolan P, Radhakrishnamurthy B, Berenson GS. Lipoprotein- and mucopolysaccharide complexes of human atherosclerosis lesions. Biochim Biophys Acta 1975;358:58–70
7. Srinivasan SR, Radhakrishnamurthy B, Dalleres ER Jr, Berenson GS. Collagenase-solubilized lipoprotein glycosaminoglycan complexes of human aortic fibrous plaque lesions. Atherosclerosis 1979;34:105–118
8. Sellasbury B, Wagner WD. Isolation and preliminary characterization of proteoglycans dissolubly extracted from human aorta. J Biol Chem 1981;256:8650–8657
9. Schmidt A, Prager M, Selmke P, Buddecke E. Isolation and properties of proteoglycans from bovine aorta. Eur J Biochem 1982;125:95–101
10. Vijayagopai P, Radhakrishnamurthy B, Srinivasan SR, Berenson GS. Studies of biological properties of proteoglycans from bovine aorta. Lab Invest 1980;42:190–196
11. Horn MC, Brelton M, Deudon E, Bernou E, Picard J. The structural characterization of proteoglycans of cultured aortic smooth muscle cells and arterial wall of the pig. Biochim Biophys Acta 1983;755:95–105
12. Oegeins TR Jr, Hascall VC, Eiselstein R. Characterization of bovine aorta proteoglycan extracted with guanidinium hydrochloride in the presence of protease inhibitors. J Biol Chem 1979;254:1312–1318
13. Schmidt A, Schäfer E, Buddecke E. Isolation and characterization of two proteoheparan sulfate species from calf arterial tissue. Eur J Biochem 1988;173:561–566
14. Wight TN. Vessel proteoglycans and thrombogenesis. In: Spier E, ed. Progress in hemostasis and thrombosis, vol 5. New York: Grune and Stratton, 1980:1–39
15. Fritz E LMS, Reilly CF, Rosenberg RD. An antiproliferative heparan sulfate species produced by postconfluent smooth muscle cells. J Cell Biol 1985;100:1041–1046
16. Oosta GM, Favreau LV, Benner DL, Rosenberg RD. Purification and properties of human platelet heparinase. J Biol Chem 1982;257:11249–11255
17. Casteljat JJ, Favreau LV, Karnovsky MJ, Rosenberg R. Inhibition of vascular smooth muscle cell growth by endothelial cell-derived heparin. J Biol Chem 1982;257:11256–11260
18. Stevens RL, Colombo M, Gonzales JJ, Hollandar W, Schmidt K. The glycosaminoglycans of the human artery and their changes in atherosclerosis. J Clin Invest 1976;58:470–477
19. Wagner WD. Proteoglycan structure and function as related to atherosclerosis. Ann NY Acad Sci 1985;454:52-68
20. Filipovic I, Buddecke E. Increased fatty acid synthesis of arterial tissue in hypoxia. Eur J Biochem 1971;20:587-592
21. Glaser JH, Conrad HE. Chick embryo liver $\beta$-glucuronidase. J Biol Chem 1979;254:6588-6597
22. Suzuki S. Chondroitinases from Proteus vulgaris and Flavobacterium heparinum. In: Ginsburg V, ed. Methods in enzymology, vol 28. New York: Academic Press, 1972: 911-917
23. Wagner WD, Salisbury BG, Rowe HA. A proposed structure of chondroitin 6-sulfate proteoglycan of human normal and adjacent atherosclerotic plaques. Arteriosclerosis 1986; 6:407-417
24. Yli-Herttuala S, Sumuvuori H, Karkola K, Möttönen M, Niikari T. Glycosaminoglycans in normal and atherosclerotic human coronary arteries. Lab Invest 1985;54: 402-407
25. Hervius P-H. The interaction between human plasma lipoproteins and connective tissue glycosaminoglycans. J Biol Chem 1972;247:2607-2613
26. Hoff HF. Plasma low density lipoprotein accumulation in aortas of hypercholesterolemic swine correlates with modifications in aortic glycosaminoglycan composition. Arteriosclerosis 1986;6:231-236

Index Terms: heparan sulfate • cholesterol • human aorta • atherosclerosis