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SULFATED GLYCOSAMINOGLYCANS OF HUMAN AORTA: CHONDROITIN 6-SULFATE INCREASE WITH AGE

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SUMMARY: The proportions of chondroitin 4 and 6 sulfates of intima + media layers of normal human aortae vary with age. The two isomers are in approximately equal amounts in aortae of young individuals, while the 6-sulfate is more abundant in those of adult individuals. This increase of chondroitin 6-sulfate is even more pronounced for intima + media obtained from atherosclerotic aortae.

INTRODUCTION: Observations made during the past 20 years on the distribution of glycosaminoglycans (GAG) in aortic tissue have suggested a role of these compounds in the pathogenesis of atherosclerosis. However, studies specifically directed to the clarification of this role have given controversial results, both in man (1,2) and in animals (3,4,5).

More recent contributions have stressed the importance of studying the distribution of individual sulfated GAG. Thus, Murata et al. (6) have reported an increase in the proportion of dermatan and heparan sulfate of bovine aorta, progressing from the intima to the adventitia. Their results, however remain controversial in view of variations of GAG distribution in relation to age and degree of atherosclerosis (2, 7). Moreover, species variations have also been demonstrated (8).

As it was discovered that some lipoproteins could form, "in vitro" soluble and insoluble complexes with different sulfated glycans (9), the possibility that sulfated GAG could be involved in lipid deposition in vascular tissues became the subject of several investigations.

Abbreviations:- ΔDi-4S, 2-acetamino-2-deoxy-3-0-(β-D-gluco-4-enepyranosyluronic acid)- 4-0-sulfo-D-galactose; ΔDi-6S, 2-acetamino-2-deoxy-3-0- (β -D-gluco-4-enepyranosyluronic acid)-6-0-sulfo-D-galactose; GAG, glycosaminoglycan.

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The present communication reports the distribution of sulfated GAG in the different layers of human aortae in relation to age and degree of atherosclerosis.

MATERIAL AND METHODS:- Chondroitin 4-sulfate and 6-sulfate were purchased from Sigma Chem. Co. (St. Louis, Mo., USA). Dermatan sulfate, chondroitinase AC and ABC were purchased from Miles Lab. Co. (Elkhart, Ind., USA). One unit of chondroitinase is defined as the quantity that catalyzes formation of 1 µmole of product per minute. Heparan sulfate was prepared by a method previously described (10). Agarose purchased from Industrie Biologique Française (Gennevilliers, France); diaminopropane from Aldrich Chemical Co. (Milwaukee, Wis.,USA).

Extraction and identification of sulfated glycosaminoglycans: Fresh aortic tissues were obtained from autopsies and checked for normality by macroscopic and microscopic examination. The intima, media and adventitia were carefully separated by dissection under microscopic examination. In some experiments (Table II), the analysis are made on an inner portion of the aorta (intima + part of the media), since it is difficult to obtain pure intima from all ages to analyse the chondroitinase AC products. In the atherosclerotic aortae, the areas with lesions were cut and dissected for analyses while the apparently normal areas were excluded. The tissues were cut in small pieces and kept overnight at 4ºC in 10 volumes of acetone. The sulfated GAG were then extracted from the acetone powders essentially as previously described (11), using papain (10mg/100mg of dry tissue, preincubated for 30 min. at 60ºC in sodium acetate buffer 0.1 M pH 5.5, containing cysteine 5mM and EDTA 5mM) instead of trypsin.

The sulfated GAG were identified and quantitated by a combination of agarose gel electrophoresis and degradation with specific enzymes, as previously described (12, 13). GAG quantitation was performed by densitometry of the agarose slides after electrophoresis and toluidine blue staining, the error of the method being ±5%.

Quantitation of the chondroitin 4 and 6 sulfates: The amounts of chondroitin 4 and 6 sulfates in the mixtures were established by the amounts of unsaturated disaccharides formed from these compounds by the action of chondroitinase AC, as previously described (14). About 100 µg of sulfated GAG were incubated with 0.01 unit of chondroitinase AC in 0.05 M of ethylenediamine--acetate buffer pH 8.0 , for 12 hours, at 37ºC in a final volume of 20 µl. The mixtures were applied in Whatman nº 1 paper and subjected to descending chromatography in isobutyric acid, 1M NH_3 (5:3, v/v) for 24 hours. The products were visualized by silver nitrate staining and quantitated by densitometry as previously described (14).

RESULTS:- Sulfated glycosaminoglycans composition of the three layers human aorta: The agarose gel electrophoresis of the sulfated GAG obtained from the three layers of human aorta, before and after treatment with chondroitinases, is shown in figure 1. The sulfated GAG from the adventitia shows a single band identical with dermatan sulfate standard; those from the media show bands

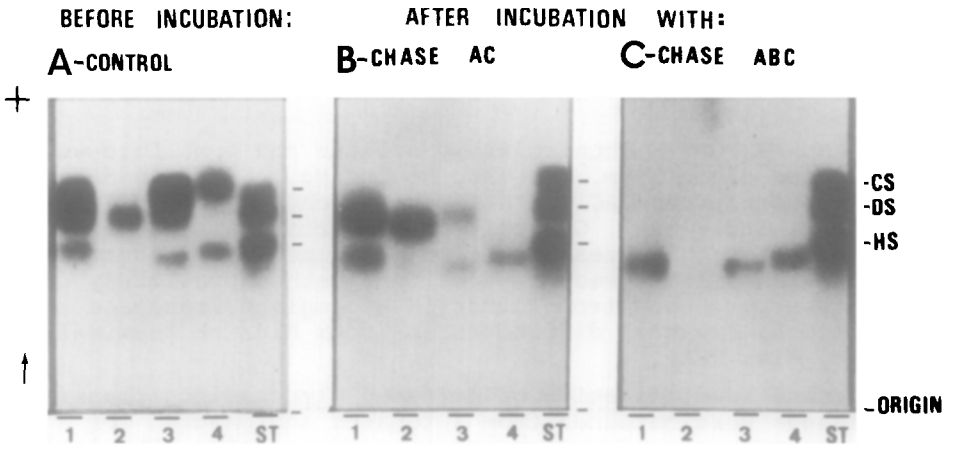

Fig. 1 - Agarose gel electrophoresis of glycosaminoglycans extracted from the three layers of human aorta before (A) and after (B,C) incubation with chondroitinase AC or ABC. About 100 µg of glycosaminoglycans from total aorta (1), adventitia (2), media (3) and intima (4) were incubated with 0.01 unit of chondroitinase AC (Chase AC) in 0.05M ethylenediamine-acetate buffer pH 8.0 at 37ºC for 8 hours and with 0.01 unit of chondroitinase ABC (Chase ABC) in 0.5 M TRIS-HCl buffer pH 8.0 at 37ºC for 8 hours, in a final volume of 20 µl . Appropriate controls were also incubated in absence of enzyme. After incubation 5 µl aliquots were submitted to agarose gel electrophoresis (0.05M 1,3-diaminopropane-acetate pH 9.0 for 1 hour at 120 V). The glycosaminoglycans in the gel were fixed with CETAVLON and stained with toluidine blue. St- standard mixture containing chondroitin 4/6 sulfates (CS) dermatan sulfate (DS) and heparan sulfate (HS).

corresponding to heparan sulfate, dermatan sulfate and chondroitin 4/6-sulfate, while those from intima show only two bands, with the electrophoretic migration of chondroitin 4/6--sulfate and heparan sulfate. Treatment with chondroitinase AC degraded the sulfated GAG of intima and media having the electrophoretic migration of chondroitin 4/6-sulfate standard. A small amount of dermatan sulfate in the media was still detectable after chondroitinase AC digestion. Treatment with chondroitinase ABC degraded all the sulfated GAG with migration similar to that of the standards of chondroitin 4/6-sulfate and dermatan sulfate, leaving those with migration similar to heparan sulfate standard. Crude extracts of *Flavobacterium heparinum*, which contain several mucopolysaccharidases, degraded all the sulfated GAG from aorta. The percent amounts and the types of sulfated GAG found in the

TABLE I

SULFATED GLYCOSAMINOGLYCANS COMPOSITION OF THE THREE LAYERS OF NORMAL HUMAN AORTA FROM ADULT INDIVIDUALS

Layer	Total sulfated glycosaminoglycans (mg/g dry tissue)	Sulfated glycosaminoglycans (%)		
		Chondroitin 4/6-sulfate	Dermatan sulfate	Heparan sulfate
Adventitia (16)*	0.7 (0.3 - 1.2)	5	90	5
Media (16)	7.8 (3.0 - 11.7)	80	12	8
Intima (6)	27.4 (17.0 - 44.0)	80	<2	20

* number of samples

different layers from aorta were calculated by densitometry and are given in Table I. Although a wide variation in the total amounts of sulfated GAG were observed, the relative amounts of chondroitin 4/6-sulfate, dermatan sulfate and heparan sulfate remained constant.

Relative amounts of isomeric chondroitin 4 and 6 sulfates in the combined intima and media layers of human aorta: Intima and media layers of normal aortae from individuals of various age and of aortae with atherosclerotic lesions were studied. The extracted sulfated GAG were degraded with chondroitinase AC. Two disaccharides, namely, ΔDi-4S derived from chondroitin 4-sulfate and ΔDi-6S derived from chondroitin 6-sulfate are formed. Only trace amounts of unsaturated non-sulfated disaccharides are concomitantly formed. The results reported in Table II indicate that aorta from normal young individuals (4 months to 5 years of age) have approximately equal amounts of the two isomers, whereas those from adults (16 to 78 years) have approximately 2/3 chondroitin 6-sulfate and 1/3 chondroitin 4-sulfate. This increase of chondroitin 6-sulfate is even more evident for the GAG extracted from atheromateous lesions.

DISCUSSION:- The results presented in figure 1 and Table I indicate that dermatan sulfate is the main GAG of human aortic adventitia, while chondroitin 4/6 sulfates are the most abundant GAG of the intima and media layers. However, Table II indicate that the proportion of the chondroitin 4/6 sulfates vary with age, from approximately equal in young individuals to prominently chondroitin 6-sulfate in adult individuals. This increase of

TABLE II

RELATIVE AMOUNTS OF ISOMERIC CHONDROITIN 4 AND 6 SULFATES IN THE INTIMA AND MEDIA LAYERS OF HUMAN AORTAE

Age	Sex	Total sulfated glycosaminoglycans* (mg/g dry tissue)	Chondroitinase AC products (%)		Ratio Δ Di-6S/ Δ Di-4S
			Δ Di-6S	Δ Di-4S	
4m	♂	3.7	51	49	1.04
1y	♂	3.0	50	50	1.00
2y	♂	7.3	48	52	0.92
3y	♀	6.6	53	47	1.13
5y	♀	6.7	50	50	1.00
16y	♂	6.3	66	34	1.94
18y	♂	7.0	69	31	2.23
23y	♀	5.2	65	35	2.60
36y	♂	5.3	68	32	2.13
46y	♀	6.7	75	25	3.00
55y	♂	9.0	70	30	2.33
58y	♂	10.8	70	30	2.33
60y	♀	10.7	71	29	2.45
67y	♂	8.6	70	30	2.33
69y	♀	9.5	75	25	3.00
73y	♀	11.7	73	27	2.70
Atheroma (66y)	♂	6.5	83	17	4.88
Atheroma (72y)	♂	7.8	70	30	2.33
Atheroma (72y)	♂	6.2	81	19	4.26
Atheroma (89y)	♀	5.4	86	14	6.14
Atheroma (89y)	♀	5.8	81	19	4.26

* No significance differences has been observed in the relative proportion of dermatan sulfate and heparan sulfate

chondroitin 6-sulfate was even more pronounced when the GAG were extracted from atherosclerotic aortae (see Table II).

Many experimental contributions have demonstrated that the GAG of the arterial wall may be involved in the transporting of plasma lipoproteins which eventually leads to lipid accumulation and formation of atherosclerotic lesions. Recent experiments (15) have also demonstrated that chondroitin 4-sulfate (but not chondroitin 6-sulfate) is capable of preventing the formation of complexes between low density lipoproteins and highly sulfated

glycosaminoglycans. In this connection, the relative increase of chondroitin 6-sulfate in aortic tissues of adult individuals and of individuals with atherosclerotic lesions may indicate structural and metabolic modifications which favor the deposition and accumulation of lipoproteins within the arterial wall.

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REFERENCES

1. Nakamura, M., Core, I. and Yabuta, N., (1965). Jap. Heart J., 6, 20-39.
2. Klynstra, F.B., Böttcher, C.J.F., Van Melsen, J.A. and VanDerlaan, E.J., . (1967) J. Atheroscler. Res., 7, 301-309.
3. Berenson, G.S. and Kumar, V. (1968). Acta cardiol., 23, 425-433.
4. Forman, D.T., Choi, S.S, and Taylor, C.B. (1968). Arch. Pathol., 85, 80-88.
5. Ho, K.J., Forestner, J.E. and Manalo-Estrella, P. (1971). Proc. Soc. Exp. Biol. Med. 137, 10-12.
6. Murata, K., Nakazawa, K. and Hamai, A., (1975). Atherosclerosis, . 21, 93-103.
7. Nakamura, T., Tokita, K. and Tateno, S. (1968). J.Atheroscler.Res. 8, 891-902.
8. Gardais, A., Picard, J. and Hermelin, B. (1973). Comp. Biochem. Physiol., 44B, 507-515.
9. Iverius, P.H. (1972). J. Biol. Chem. 247, 2607-2613.
10. Dietrich, C.P., Nader, H.B., Britto, L.R.C. and Silva, M.E. (1971). Biochim. Biophys. Acta, 237, 430-441.
11. Toledo, O.M.S. and Dietrich, C.P. (1977). Biochim.Biophys.Acta, 498, 114-122.
12. Dietrich, C.P. and Dietrich, S.M.C. (1972). Anal.Biochem., 46, 209-215.
13. Dietrich, C.P. and Dietrich, S.M.C. (1976). Anal. Biochem., 70, 645-647.
14. Mourão, P.A.S., Toledo, S.P.A., Nader, H.B. and Dietrich, C.P., (1973). Biochem. Med., 7, 415-423.
15. Nakashima, Y. Di Ferrante, N., Jackson, R.L. and Pownall, H.J. (1975). J.Biol. Chem., 250, 5386-5392.