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SPONDYLOEPIPHYSEAL DYSPLASIA, CHONDROITIN SULFATE TYPE: A POSSIBLE DEFECT OF PAPS - CHONDROITIN SULFATE SULFOTRANSFERASE IN HUMANS.

Paulo A.S.Mourão[+*], Setuko Kato[+] and Patricia V. Donnelly[≠]

+ Laboratório de Investigação em Reumatologia e Unidade de Genética, Faculdade de Medicina da Universidade de São Paulo, São Paulo, SP - Brasil and ≠ Department of Biochemistry, Baylor College of Medicine, Houston, TX - USA.

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SUMMARY: Four patients with an unusual form of spondyloepiphyseal dysplasia excreted in the urine undersulfated chondroitin 6-sulfate (Biochem. Med. 7, 415-423, 1973). The sera of these patients show a low activity of PAPS - chondroitin sulfate sulfotransferase, while the undersulfated chondroitin sulfate present in their urine is a much better acceptor of $^{35}SO_4$ than standard chondroitin sulfate when they are incubated with [^{35}S]PAPS and normal sulfotransferases. These results suggest that in these patients the skeletal lesions are secondary to a defect in the synthesis of chondroitin sulfate involving specifically the sulfotransferase activity.

INTRODUCTION: In the past years several heritable disorders involving lysosomal enzymes responsible for the degradation of sulfated glycosaminoglycans (GAG) have been described (1-5). However, only recently some indications of possible genetic disorders involving the biosynthetic pathways of the sulfated GAG have emerged.

In 1973 we reported an abnormal urinary excretion of GAG in four patients with an unusual form of spondyloepiphyseal dysplasia. The patients excreted a normal amount of hexuronic acid-containing GAG. The isolated urinary GAG, however, when degraded with chondroitinase AC gave a high percentage of unsaturated nonsulfated

* All correspondence should be address to Paulo A. S. Mourão, Departamento de Bioquímica, Centro de Ciência da Saúde, Universidade Federal do Rio de Janeiro, 21 910, Rio de Janeiro, RJ - Brasil.

Abbreviations:- GAG, glycosaminoglycans; PAPS, 3'- phosphoadenosine 5'-phosphosulfate; PPO, 2,5-diphenyloxazole; ΔGlcUA-GalNAc4S, 2-acetamido-2-deoxy-3-0-(β-D-glyco-4-enepyranosyluronic acid)-4-0-sulfo-D--galactose; ΔGlcUA-GalNAc6S, 2-acetamido-2-deoxy-3-0-(β-D-glyco-4-enepyranosyluronic acid)-6-0-sulfo-0-galactose; ΔGlcUA-GalNAc, 2-acetamido-2-deoxy-3-0-(β-D-glyco-4-enepyranosyluronic acid)-D-galactose; ΔGlcUA-GluNAc, 2-acetamido-2-deoxy-3-0-(β-D-glyco-4-enepyranosyloronic acid)-D-glucose.

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disaccharide (ΔGlcUA-GalNAc) and a low percentage of unsaturated 6-sulfated disaccharide (ΔGlcUA-GalNAc6S), as compared to GAG derived from normal urine (6,7). These results were interpreted as an indication that the patients excreted a chondroitin 6-sulfate of low sulfate content and it was thought that the specific metabolic defect in this syndrome could be ascribed to a decrease in the activity of PAPS-chondroitin 6-sulfate sulfotransferase.

Recent studies of Orkin et al.(8) on the homozygous brachymorphic mice have shown a considerable increase in the amounts of nonsulfated disaccharides (ΔGlcUA-GalNAc) obtained enzymically from the chondroitin sulfate extracted from the abnormal cartilage. This, however, contains normal amounts of total GAG. Additional studies of Sugahara et al.(9) on these mice suggest a possible defect in the synthesis of phosphoadenosine 5'-phosphosulfate (PAPS) from ATP and $SO_4^=$, the availability of PAPS possibly being the rate limiting factor in the sulfation of the GAG.

The present communication reports further studies of the patients affected by the spondyloepiphyseal dysplasia, chondroitim sulfate type and discusses some similarities with the brachymorphic mice.

MATERIAL AND METHODS: Materials - Chondroitin 4-sulfate, Chondroitin 6-sulfate and chondroitinase AC were purchased from Miles Laboratories (Elkhart, IN, USA). Partially desulfated chondroitin 4- and 6-sulfate were prepared by the method of Kantor and Schubert (10). The extent of desulfation achieved is shown in Table I. Urinary GAG were precipitated from urine with cetyltrimethylammonium bromide, after dilution by addition of 0.5 volumes of distilled water, by the method of Meyer et al. (11). [^{35}S]PAPS was prepared as described by Robbins (12) and purified by paper electrophoresis in 0.1 M Tris:acetate buffer pH 6.5 (13). [1,6-^{3}H]glucosamine hydrochloride was purchased from New England Nuclear (Boston, MA, USA); [^{35}S]H_2SO_4 from Instituto de Energia Atômica (São Paulo, SP, Brasil); glucosamine:HCl and galactosamine:HCl from British Drug House Ltd. (England).

Sulfation of urinary chondroitin sulfate by sulfotransferases from chicken embryo epiphyseal cartilages - Sulfotransferases from 13 day chicken embryo epiphyseal cartilages were prepared as described by Meezan and Davidson (14). The endogenous acceptors were removed by incubation with 1% protamine:hydrochloride at 4°C for 30 min, followed by centrifugation at 35,000 x g for 1 hr. 30 µl of the supernatant were incubated with 100 µg of different chondroitin sulfates and 150,000 cpm of [^{35}S]PAPS in the following buffer: 0.05 M Tris:HCl pH 8.0, containing 0.125 M KCL, 0.010 M $MgCl_2 \cdot 6H_2O$, 0.01 M EDTA and 0.006 M L-cysteine HCl·H_2O. After 3 hr the mixture was applied to Whatman No. 1 chromatography paper and developed in isobutyric acid:1 N NH_4OH (5:3, v/v) for 24 hr. The GAG were eluted from the origin of the chromatograms and incubated with 0.01 units of chondroitinase AC in 0.05 M ethylenediamine:acetate buffer, pH 8.0 for 8 hr (15). The ^{35}S-labeled degradation products were separated by paper chromatography as previously described (16) and the amount of ^{35}S incorporated in the unsaturated 4- and 6-sulfated disaccharides (ΔGlcUA-GalNAc4S or 6S) was measured.

TABLE I

Sulfation of different preparations of chondroitin sulfate by sulfotransferases present in extracts of chicken embryo epiphyseal cartilages.

Acceptor Chondroitin sulfate from:	ΔGlcUA-GalNAc formed by chondroitinase AC (% of total disaccharides)	$^{35}SO_4$ incorporated (cpm/100 µg of CS)	Ratio $^{35}SO_4$ incorporated by different acceptors/$^{35}SO_4$ incorporated by Ch-6-SO_4
Normal urine	<5	10,461	1.8
Patient 1 (L.M.)*	24	22,472	4.0
Patient 2 (M.E.M.)	23	30,015	5.3
Patient 3 (A.S.M.)	50	45,618	8.1
Ch-4-SO_4 (Miles)	<5	8,764	1.5
Partially desulfated Ch-4-SO_4	83	56,711	10.1
Ch-6-SO_4 (Miles)	<5	5,615	1.0
Partially desulfated Ch-6-SO_4	70	45,481	8.1

* See reference 6.

Measurement of chondroitin sulfate-sulfotransferases in human serum - The presence of chondroitin sulfate sulfotransferases in human serum has been previously reported by Adams(17). In this work, a modified method has been employed to assay these enzymes. 40 µl of serum were incubated with 300,000 cpm [^{35}S]PAPS, 0.05 M NaF and different amounts of desulfated chondroitin 4- or 6-sulfate as exogenous acceptors. After incubation at 37°C for 3 hr, the GAG were purified by phenol extraction, applied to Whatman No. 3 MM chromatography paper and developed in isobutyric acid:1 N NH_4OH (5:3, v/v) for 24 hr. The origin of the chromatograms were cut and the radioactivity quantitated in 10 ml 0.5% PPO/toluene in a L-S 100 Beckman spectrometer.

Glycosaminoglycans from cultured fibroblasts - Fibroblasts cultures derived from punch skin biopsies of normal individuals and from patients with spondyloepiphyseal dysplasia were established and maintained as previously described (18). When the cultures were at the required cell density (approximately 3 x 10^6cells/flask), 40 µCi carrier-free [^{35}S]H_2SO_4 or 200 µCi [1,6-^{3}H]glucosamine were added to the culture. The cells were then incubated at 37°C for 72 hr. The pericellular and intracellular GAG from the cultured fibroblasts were prepared as previously described(16). GAG from the medium were purified by ECTEOLA column (19).

The ^{35}S-labeled GAG (about 5000 cpm) were incubated with 0.01 units of chondroitinase AC in 0.05 M ethylenediamine:acetate buffer pH 8.0 for 8 hr (16). The unsaturated disaccharides were separated by paper chromatography in isobutyric acid:1 M NH_4OH (5:3, v/v) for 24 hr. The ^{35}S-labeled degradation products were detected by radioautography of the chromatograms and the bands with identical chromatographic migration of the standard sulfated disaccharides (ΔGlcUA-GalNAc4S and ΔGlcUA-GalNAc6S) were cut and quantitated in a L-S 100 Beckman spectrometer.

The ^{3}H-labeled GAG were degraded by chrondroitinase AC and the products were separated by paper chromatography, as described for the ^{35}S-labeled GAG. The ^{3}H-labeled degradation products with identical chromatographic migration to the standard sulfated disaccharides (ΔGlcUA-GalNAc4S and 6S) and standard nonsulfated disaccharides (ΔGlcUA-GalNAc and ΔGlcUA-GluNAc) were cut and quantitated in a L-S Beckman spectrometer. After that, the nonsulfated disaccharides were eluted from the paper, hydrolyzed with 6.0 M HCl at 100°C for 6 hr and chromatographed on Whatman No. 1 paper for 48 hr with butanol:pyridine:water (4:3:1, v/v/v) as descending solvent (6) to determine the amounts of [^{3}H]-glucosamine and [^{3}H]galactosamine. With this technique, the percentage of glucosamine and galactosamine - containing nonsulfated disaccharides was calculated.

RESULTS: Sulfation of urinary chondroitin sulfate by sulfotransferases from chicken embryo cartilages - The results reported in Table I indicate that the urinary chondroitin sulfate from patients with spondyloepiphyseal dysplasia, when incubated with [^{35}S]PAPS and sulfotransferases from chicken embryo cartilages, is 4-8 times a better acceptor of $^{35}SO_4$ than standard chondroitin 6-sulfate. Chemically desulfated chondroitin 4- and 6-sulfate are both as good acceptors as the urinary chondroitin sulfate from patients with spondyloepiphyseal dysplasia, while chondroitin sulfate from normal urine is only 1.8 times better acceptor than

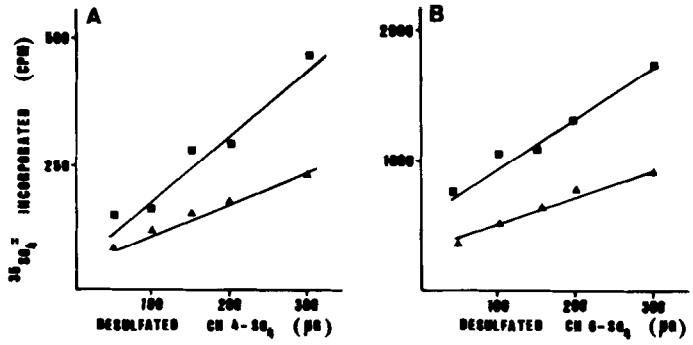

Figure 1. Chondroitin sulfate-sulfotransferases in human serum. 40 µl of serum from normals (■ - ■) and from patients with spondyloepiphyseal dysplasia (▲ - ▲) were incubated with 300,000 cpm [^{35}S]PAPS, 0.05 M NaF and increasing amounts of desulfated chondroitin 4-sulfate (A) or desulfated chondroitin 6-sulfate (B) as exogenous acceptor. After incubation at 37°C for 3 hr, the GAG were purified by phenol extraction and paper chromatography. The origin of the chromatograms were cut and the radioactivity quantitated in 10 ml 0.5% PPO/toluene in a L-S 100 Beckman spectrometer.

standard chondroitin 6-sulfate. Degradation of the [$^{35}SO_4$]GAG by chondroitinase AC indicates that the sulfate was incorporated only in the 6 position of the N-acetylgalactosamine in all substrates indicated in Table I.

Chondroitin sulfate-sulfotransferases in human serum - Chondroitin sulfate-sulfotransferases in human serum were measured with desulfated chondroitin 4- and 6-sulfate as exogenous acceptor and [^{35}S]PAPS as the sulfate donor. Figure 1 indicates a deficiency of sulfotransferases in the serum of patients with spondyloepiphyseal dysplasia, detectable with both exogenous acceptors. The parents have a normal level of sulfotransferases (not shown). Chondroitin sulfate could not be sulfated with $^{35}SO_4$, ATP and human serum, suggesting that the latter lacks the PAPS-synthesizing enzymes.

Glycosaminoglycans from cultured fibroblasts - The study of chondroitin 4- and 6-sulfate in the medium, pericellular and intracellular pools of cultured skin fibroblasts using $^{35}SO_4$ and

[^{3}H]glucosamine showed no differences between normal fibroblasts and fibroblasts from a patient with spondyloepiphyseal dysplasia. As shown in Table II the relative proportions of [^{35}S]Δ GlcUA-GalNAc4S/[$^{35}S_4$]ΔGlcUA-GalNAc6S are normal, there is no detectable amount of [^{3}H]ΔGlcUA-GalNAc and no decrease in the amount of [^{3}H]ΔGlcUA-GalNAc4S + 6S. Also, several lysosomal enzymes involved in the degradation of complex carbohydrates (β-hexosaminidase, β-glucuronidase, β-galactosidase, α-mannosidase, arylsulfatase A, α-fucosidade and α-L-iduronidase) were gently measured by Dr. L. J. Shapiro and the activities are within normal values.

DISCUSSION: Chondroitin 6-sulfate with low sulfate content was previously reported to be excreted in the urine of patients with an unusual form of spondyloepiphyseal dysplasia, having an autosomal recessive inheritance (6,7). This undersulfated chondroitin sulfate could be the result either of a defective enzymic step in the sulfation process, or of defective synthesis of glycan backbone which could not be sulfated by normal sulfotransferases.

The results reported in this communication indicate that the urinary chondroitin sulfate isolated from the urine of these patients can be readily sulfated by chick embryo cartilage sulfotransferases, at the same rate as chemically desulfated chondroitin sulfate. However, when sera of normal individuals and of the patients were used as a source of sulfotransferases, deficient incorporation of sulfate from [^{35}S]PAPS into desulfated chondroitin sulfate was observed with the serum of the patients.

Surprisingly, no abnormalities were found in the degree of sulfation of the chondroitin sulfates synthesized by cultured skin fibroblasts of these patients. This finding, however, is in line with those of Orkin *et al.*(8) who reported that in the homozygous brachymorphic mice the defect is limited to the cartilage GAG, while those prepared from the skin are normal. Also, Sugahara *et al.*(20) studying the tissue distribution of the defective sulfate pathway in homozygous brachymorphic mice showed normal PAPS synthesis by skin extracts and skin cultured fibroblasts. The normal synthesis of chondroitin sulfate by cultured fibroblasts from patients with spondyloepiphyseal dysplasia, chondroitin sulfate type strongly

TABLE II

Chondroitin sulfate and hyaluronic acid from fibroblasts of normals and of patients with spondyloepiphyseal dysplasia.

Fraction	Fibroblasts	Products formed by chondroitinase AC (cpm/10^3 cells)					
		[^{3}H]ΔGlcUA-GalNAc4S + [^{3}H]ΔGlcUA-GalNAc6S**		[^{3}H]ΔGlcUA-GluNAc		[^{3}H]ΔGlcUA-GalNAc	
Intracellular	Normal	1051	(16)*	5426	(84)	not detected	(<5)
	Brachyolmia	1798	(17)	8623	(83)	not detected	(<5)
Pericellular	Normal	825	(15)	4643	(85)	not detected	(<5)
	Brachyolmia	788	(19)	3370	(81)	not detected	(<5)
Medium	Normal	162	(2)	7444	(98)	not detected	(<5)
	Brachyolmia	107	(1)	8253	(99)	not detected	(<5)
		[^{35}S]ΔGlcUA-GalNAc4S		[^{35}S]ΔGlcUA-GalNAc6S			
Intracellular	Normal 1	2810	(59)	1941	(41)		
	Normal 2	1224	(71)	509	(29)		
	Normal 3	1124	(72)	442	(28)		
	Average	1719	(67)	962	(33)		
	Brachyolmia	1053	(64)	603	(36)		
Pericellular	Normal 1	1832	(44)	2328	(56)		
	Normal 2	1196	(48)	1275	(52)		
	Normal 3	1315	(68)	623	(32)		
	Average	1447	(53)	1408	(47)		
	Brachyolmia	2640	(55)	2181	(45)		

* Percent values.
** When the GAG were labelled with |^{3}H|glucosamine it was difficult to distingue both sulfated disaccharides.

reinforces the evidence that differences may exist in the distribution of enzymes involved in the sulfation of chondroitin sulfates. These results may have considerable implications in the future studies of biosynthetic metabolic defects of the sulfated GAG.

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