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Short communication

Plasmatic dermatan sulfate and chondroitin sulfate determination in mucopolysaccharidoses

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ABSTRACT

The evaluation of plasmatic galactosaminoglycans, dermatan sulfate (DS) and chondroitin sulfate (CS) can be helpful in the early identification of MPS patients, also considering that primary storage of one type of GAG can lead to secondary accumulation of other lysosomal substrates. We explore the possibility to determine plasmatic DS and CS in numerous healthy pediatric (and sometimes adult) subjects depending on age and in patients affected by various forms of MPS. A highly sensitive HPLC separation and fluorescence detection was applied for plasma/serum DS and CS determination after a specific enzymatic treatment able to release their constituent disaccharides. DS and CS content decrease significantly with age in controls having high values in the first year (~8 μg/mL). A highly significant decrease was observed for 1–5-year-old (~−33%) and 5–10-year-old (~−65%) healthy subgroups. No further decrease was determined showing a stabilization after 5 years of age. MPS I Scheie and Hurler patients showed rather similar DS and CS content significantly higher than controls matched for age. Similarly, MPS II, III and IV subjects all presented significantly higher plasmatic DS and CS content compared to healthy subjects matched for age. The same trend was determined for the only patient affected by MPS VI. Plasmatic DS and CS analyzed by the present procedure may be a useful diagnostic and screening marker for various forms of MPS.

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1. Introduction

Mucopolysaccharidoses (MPS), belonging to the group of the lysosomal storage diseases, are progressive and multisystem disorders caused by genetic defects in the catabolism of macromolecules known as glycosaminoglycans (GAGs) [1,2]. Each MPS disorder results from a deficiency in the activity of a specific lysosomal enzyme required for GAG catabolism. MPS types I through IX involve deficiencies of one of the key 11 enzymes needed for the stepwise degradation of various GAGs, i.e. dermatan sulfate (DS), heparan sulfate (HS), keratan sulfate (KS), chondroitin sulfate (CS), very rarely hyaluronan, or in combination.

In all MPS subtypes, partially degraded GAG(s) accumulate in the lysosomes of affected cells and/or are eliminated in the blood and excreted in the urine. As a consequence, these diseases are biochemically characterized by an accumulation of intact and partially degraded polysaccharides within lysosomes and in biological fluids [1,3]. The GAG accumulation results in progressive cellular damage affecting multiple organ systems and leading to organ failure, cognitive impairment, and reduced life expectancy.

Due to the elevated structural heterogeneity of GAGs as well as to their variable content in cells, tissues and organs [1–3], the clinical heterogeneity seen in each of the MPSs presents a challenge for diagnosis and management. Variability in the age of the onset of symptoms as well as in the rate of disease progression in each organ system involved has been observed in all of the MPSs. Each of the MPSs demonstrates this extensive clinical heterogeneity, and thus severe or attenuated classification is used for all of the MPSs [2].

Early diagnosis and treatment improves outcomes in MPS [4–6], particularly in those disorders that can be treated with enzyme-replacement therapy (ERT) or hematopoietic stem cell

Abbreviations: CS, chondroitin sulfate; DBS, dried blood spots; DS, dermatan sulfate; ERT, enzyme-replacement therapy; GAG(s), glycosaminoglycan(s); HS, heparan sulfate; HSCT, hematopoietic stem cell transplantation; KS, cheratan sulfate; MPS, mucopolysaccharidoses; 0s, ΔUA-(1→3)-GalNAc; 4s, ΔUA-(1→3)-GalNAc-4s; 6s, ΔUA-(1→3)-GalNAc-6s; 2,6dis, ΔUA-2s-(1→3)-GalNAc-6s; 4,6dis, ΔUA-(1→3)-GalNAc-4,6dis; 2,4dis, ΔUA-2s-(1→3)-GalNAc-4s; 2,4,6tris, ΔUA-2s-(1→3)-GalNAc-4,6dis.

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transplantation (HSCT). Furthermore, because the MPS disorders produce a wide variety of clinical manifestations, diagnosis is often delayed, particularly in those patients without cognitive impairment [4]. This is highly important considering that the earlier ERT and HSCT are initiated, the better will be the potential outcome because of the irreversible nature of some of the abnormalities associated with MPS disorders [4,6].

Several established procedures have been adopted in clinical laboratories to diagnose MPS. Apart from enzyme-activity assays that are the golden standard procedures to confirm diagnosis, biochemical and clinical markers are useful procedures to diagnose MPS [2,7]. Primary biomarkers are represented by the primary storage material, i.e. GAGs and related fragments accumulated in tissues and biological fluids, the latter being particularly useful in early determination of MPS depending on the severity [8–10] and in the evaluation of the metabolic fate of GAGs [3,11–13] during therapies. Serum/plasma GAGs have been proposed as a possible biomarker in MPS. Several studies have been performed for GAGs level determination in MPS patients' serum/plasma [7,10,14,15]. Furthermore, in previous studies, we determined plasmatic GAGs in patients affected by MPS I [3] and MPS II [11] and subjected to ERT with specific evaluation of the metabolic fate of GAGs after infusion of enzymes. Moreover, the dried blood spot (DBS) method has evident advantages over other laboratory methods thanks to its easy sampling, shipping and to the stability of the samples, and can be particularly helpful in the early identification of affected MPS patients through neonatal screening, high-risk population screening or family screening [7]. Finally, recent studies strongly support the concept that the primary storage of one type of GAG can lead to secondary storage of other lysosomal substrates [14,16]. In fact, we detected a high concentration of DS and CS also in MPS patients affected by Sanfilippo (subtype with HS as primary storage) syndrome [8,17]. On the basis of these considerations, in this study we explore the possibility to use a specific test for plasma/serum DS and CS (named galactosaminoglycans due to the presence of *N*-acetyl-galactosamine in the structural disaccharides) determination after treatment with a specific enzyme able to produce constituent disaccharides separated by highly sensitive HPLC and fluorescence detection. The total content of galactosaminoglycans was tested in numerous healthy pediatric (and sometimes adult) subjects depending on age and patients affected by various forms of MPS.

2. Methods

2.1. Materials

0.45 μm and Microcon YM-3 filters with an MM cut-off of 3000 Da were from Millipore (Billerica, MA, USA). Chondroitinase ABC from *Proteus vulgaris* [E.C. 4.2.2.4], proteinase K [3.4.21.64] and unsaturated DS/CS disaccharides (used for quantitative evaluation) were purchased from Sigma–Aldrich (St. Louis, MO, USA). All the other reagents were analytical grade generally supplied by Sigma–Aldrich.

2.2. Control subjects and MPS patients

Normal subjects and patients affected by MPS type I (Hurler and Scheie), MPS II (severe form), MPS III, MPS IV and MPS VI (Table 1) were registered in the Pediatric Division, Department of Clinical Sciences, Polytechnic University of the Marche, Presidio Salesi, Ancona, Italy. The diagnosis of MPS was performed on the basis of the pathological pattern of urinary GAGs and enzymatic deficiency. The different forms of MPS I (Hurler and Scheie) and MPS II (severe form) were established on the basis of the peculiar clinical signs and of the presence or absence of mental retardation. In all subjects the parents gave informed consent for the collection of plasma samples. This study was approved by an institutional review board of the Department of Life Sciences, University of Modena and Reggio Emilia, Italy.

Table 1
Characteristics of the healthy subjects and various MPS-affected patients.

	Subjects (number)	Female/Male	Mean age (years)
Healthy group	71	28/43	8.8 ± 12.9 (0.04–66)
MPS I Scheie	3	3/0	19.8 ± 27.9 (2.5–52)
MPS I Hurler	3	2/1	5.5 ± 6.1 (1.5–12.5)
MPS II Severe	3	0/3	2.4 ± 0.5 (2–3)
MPS III	4	2/2	8.1 ± 3.3 (5–12)
MPS IV	3	2/1	2.88 ± 1.6 (1–4)
MPS VI	1	0/1	13

Data are reported as mean ± Standard deviation. Minimum and maximum values are illustrated in brackets.

Blood samples collected from human subjects were immediately collected in tubes containing citrate as an anticoagulant and the plasma obtained was stored at −80 °C for analytical investigation.

2.3. Extraction of plasma GAGs

100 μl of human plasma were lyophilized and reconstituted with 500 μl of TRIS-Cl 10 mM pH 7.5. After adding 20 μl of proteinase K, the samples were incubated at 60 °C for 12 h. After boiling for 5 min, the samples were centrifuged at 5000 RPM for 5 min and the surnatants were sequentially filtered on 0.45 μm and on YM-3 filters at 10,000 g for 60 min and lyophilized. The crude retained plasma GAG fraction was dissolved in 80 μl of 50 mM ammonium acetate pH 8.0 and treated with 20 μl of chondroitinase ABC at 37 °C for 6 h. After boiling for 5 min, 20 μl of samples were injected in HPLC.

2.4. Strong-anion exchange (SAX)-HPLC with postcolumn derivatization

The HPLC equipment was from Jasco (pump mod. PU-1580, pump for postcolumn reagent mod. PU-2080 Plus, fluorescence detector mod. FP-1520, Rheodyne injector equipped with a 20 μl loop, software Jasco-Borwin rel. 1.5). The column thermocontroller and the dry reaction bath were from Eldex, Mod. CH-150. The unsaturated disaccharides generated from DS and CS treated with chondroitinase ABC were separated by SAX-HPLC using an isocratic separation with 50 mM NaCl pH 4.00 for 5 min and a gradient separation from 50 mM NaCl pH 4.00 at 5 min to 1.2 M NaCl pH 4.00 at 60 min at a flow rate of 1 mL/min [18]. Aqueous 1% 2-cyanoacetamide in 100 mM sodium tetraborate, at the flow-rate of 0.4 mL/min by using the postcolumn pump, was added to the effluent. The mixture was made to pass through a reaction coil (0.8 mm inner dimension × 10 m) set in a dry reaction bath thermostated at 136 °C and a following cooling coil (0.25 mm inner dimension × 2 m). The effluent was monitored fluorometrically (Excitation 331 nm, Emission 383 nm).

2.5. Statistics

Micrograms of DS and CS were calculated by means of specific calibration curves (not shown) of pure unsaturated disaccharide standards from Sigma–Aldrich (and reported as μg/mL plasma). Data are expressed as means ± S.D. Statistical analysis was performed by analysis of variance (ANOVA), the Student–Newman–Keuls test and Mann–Whitney *U* test as

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