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Composition and structure of glycosaminoglycans in DBS from 2-3-day-old newborns for the diagnosis of mucopolysaccharidosis

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ABSTRACT

Dried blood spot (DBS) technology is a cheap and easy method largely applied in newborn screening. Mucopolysaccharidoses (MPS) are characterized by the deficit of enzymes that degrade glycosaminoglycans (GAGs) characterized by progressive worsening of the conditions. For a possible early diagnosis of MPS, we developed a method of uronic acid (UA)-GAGs determination in DBS of 600 healthy newborns and from a small group of MPS subjects matched for age. Spotted blood UA-GAGs of the normal newborns are composed of 67.2% chondroitin sulfate (CS), 28.6% heparan sulfate (HS) and 4.4% hyaluronic acid with a CS/HS ratio of 2.35 and a total GAGs content of 0.43 μg/DBS. A chemical evaluation of CS and HS structure was performed by measuring their disaccharide composition, sulfation and the overall charge density. The DBS of four different MPS types presented an increase of total or single UA-GAGs content and/or modifications of the CS and HS disaccharide composition as well as chemical signature also related to the MPS enzymatic defect. The modifications of the UA-GAGs composition, parameters and structure of healthy newborns determined in DBS would be useful for a possible early diagnosis of various MPS types.

Introduction

Lysosomal storage disorders (LSDs) are a group of about 50 genetic diseases characterized by an accumulation of waste products in lysosomes caused by deficits in lysosomal enzymes. A wide range of manifestations can occur from early in life to adulthood such as neuronal disfunction, hepatomegaly and splenomegaly, two of the most common symptoms [1–3]. All LSDs are rare, causing diagnostic delays and missed cases [4]. In the late 1960's and early 1970's, the discovery that the enzymatic deficit is expressed in cultured fibroblasts opened the way for the diagnosis of these diseases [5].

Mucopolysaccharidoses (MPS) represent the largest group of LSD characterized by a deficit in one of the lysosomal enzymes required to degrade glycosaminoglycans (GAGs). To date, seven types of MPS have been identified caused by deficiency or absence of eleven enzymes [6]. Several therapeutic treatments can be adopted to treat the various forms of LSD [7] such as Haemopoietic Stem Cell Transplantation (HSCT) which has been carried out in early diagnosed babies [8]. However, except for MPS I, no clear benefits have been found for the nervous and/or skeletal system of these patients [8,9]. In the 1990's, several pharmaceutical companies started to develop Enzyme Replacement Therapy (ERT) as a potential therapy for the LSD, consisting of a weekly/biweekly IV administration of the functional protein [10]. However, ERT is unable to cross the blood-brain barrier and, therefore, in the present licensed form it is not a viable option for over half the MPS and related diseases associated with neuro-degeneration [11]. Finally, new therapeutic options are emerging as valid alternatives to classic LSD and MPS treatments [12,13].

MPS are often characterized by a progressive course of the disease and can therefore be controlled or slowed down if diagnosed early

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enough, even in the absence of obvious symptoms [14]. For this reason, an early diagnosis is essential for the correct growth of the child [15]. Therefore, in the field of preventive medicine, neonatal screening procedures play a vital role with the aim of identifying those individuals predisposed to develop a disease even before evident clinical signs and symptoms have appeared. Wilson and Jungner in 1968 [16] defined the criteria useful to justify the beginning of a screening program in a population. According to these criteria, the disease to be monitored must be relevant and constitute a major problem for the individual, the community and for the costs they face. The tests performed on newborn babies will help early diagnosis of any disease and increase the chances of treatment. Therefore, early detection, diagnosis and intervention are now considered a responsibility of the public health system and crucial for improving the health of affected children [16]. In recent years, newborn screening has changed thanks to progress in science. In fact, the state of the research has improved during the last decade and in particular it has been well exemplified by the introduction and development of mass spectrometry (MS) and tandem mass technologies [17,18] as well as the development of the spot diagnostic technology [19]. The technological progress has made newborn screening for a large group of metabolic diseases possible, offering the possibility of diagnosis and treatment before the onset of symptoms [20,21].

In general, the macromolecules accumulating in LSD, and in MPS, are also accumulated in biological fluids and, consequently, they can be detected in a patient's blood, urine and/or cerebrospinal fluid by laboratory investigations. The diagnosis is initially based on the dosage of specific metabolites and, subsequently, on the measurement of a presumed lack in or reduced enzyme activities and on the genetic studies [2,6]. Dried blood spot (DBS) technology is a cheap and easy method introduced during the last five decades to collect a small dried blood drop on a cellulose paper card (Guthrie cards) [22–25]. The usage of DBS has gained increasing importance since this method shows strong advantages compared to venipuncture [22–25]. Typical fields of application are newborn screening, where only a very small amount of blood (approximately 30–100 μL per spot) is available from heel prick in infants, soon after birth.

Uronic acid-bearing GAGs (UA-GAGs) are biomacromolecules formed of sequences of disaccharides, each of them composed of a uronic acid unit, glucuronic acid (GlcA) or iduronic acid (IdoA), and an amino sugar residue, *N*-acetyl-galactosamine (GalNAc) or *N*-acetyl-glucosamine (GlcNAc) [26,27]. The unique non-sulfated GAG is hyaluronic acid (HA) while chondroitin sulfate (CS), dermatan sulfate (DS), heparan sulfate (HS) and heparin are sulfated to a different extent and in various positions resulting very heterogeneous for charge density and physico-chemical properties. In MPS patients, these heteropolysaccharides are accumulated in tissues and organs [28] and excreted partially degraded in blood and urine depending on the type of MPS and on the absence of the specific enzyme. As a consequence, the accumulation of total or specific GAG in biological fluids is measured by a large variety of analytical methods with the aim of obtaining an early screening of the pathology.

Following a previous study performed on urine of 2-3-day-old newborns [27], here we developed an efficient method of UA-GAGs determination in DBS of healthy newborns for a possible diagnosis of MPS. Data of UA-GAGs from newborn DBS were compared with those obtained from a small group of MPS affected subjects matched for 2–3 days of age. The present procedure is able to measure abnormal amounts of UA-GAGs in DBS (and as a consequence in the blood) of newborn babies for a possible MPS diagnosis and treatment.

Material and methods

Newborns and MPS subjects

DBS samples of 600 healthy term 2-3-day-old newborns were collected by simple heel blood sampling. All newborns were term infants with no evident associated pathologies. Informed consent was administered to newborn parents after explaining the research purposes. At the same time, the ethic Committees' approval was obtained by the various research units involved in the patient recruitment.

DBS of one 2-3-day-old newborn affected by MPS I, two from MPS II (Hunter), one affected by MPS IIIA (Sanfilippo A) and two by MPS IVA (Morquio A) were also analyzed for UA-GAGs. The MPS I subject showed two nucleotide substitutions in heterozygosis c.979 G > C (p.Ala327Pro) c.1045 G > T (p.Asp349Tyr) (exon 8), a α-*L*-iduronidase activity of 0.6 nM/mg/h (normal values > 40) and a GAG urinary level of 1813 μg/mg creatinine (normal range 30–200). The first MPS II subject was diagnosed based on the small deletion c.589_592 CCTG (exon 5), while the second patient showed the p. Arg493Pro mutation and undetectable iduronate 2-sulfatase activity. The Sanfilippo A subject showed a heparan-*N*-sulphate sulfatase activity of 0.8 nmol/mg/17 h and a urinary level of 359.3 μg GAGs/mg creatinine (normal range of 22–86). The first Morquio A patient was found to have a homozygous nucleotide substitution c.29 G > A (p.trp10) with a galactose-6-sulphate sulfatase activity of 0.6 nmol/mg/17 h and the second MPS IVA subject was homozygous for the nucleotide substitution c.1043C > A (p.Thr348Asn) of the GALNS gene.

DBS cards with circular areas of 12 mm for a total blood volume of ~40 μL were used. DBS were prepared by the clinical Centers according to the standard National program of newborn screening by collecting a small amount of blood by means of infant heel prick soon after birth. DBS were sent to the analytical Center of Department of Life Sciences, University of Modena and Reggio Emilia, for UA-GAGs analysis.

Extraction of UA-GAGs from DBS and analysis

One single circular area of 12 mm, ~40 μL of blood, of each DBS sample was reconstituted with 100 μL of Tris-Cl 20 mM pH 7.4 and treated with 10 μL of proteinase K (from Sigma-Aldrich) at 60 °C overnight. After boiling for 10 min and centrifugation at 5000 × *g* for 15 min, the supernatant was filtered on YM-3 filters (from Millipore) possessing a cutoff of 3 kDa and lyophilised. After reconstitution in 100 μL water, an aliquot of 50 μL was added with 140 μL of 50 mM ammonium acetate pH 8.0 and treated with 10 μL of chondroitin ABC lyase (20 mU, [EC 4.2.2.4] from Sigma-Aldrich) at 37 °C for 4 h. The other aliquot of 50 μL was added with 135 μL of 0.1 M sodium acetate and 0.1 M calcium acetate pH 7.0 and treated with 15 μL of three different heparinases according to previous published procedure [27–30].

The first aliquot from each DBS having the HA and CS disaccharides and the second one from the same DBS with HS disaccharides were lyophilised and fluorotagged with AMAC [see 27–29]. Finally, derivatized disaccharides were separated and quantified by capillary electrophoresis (CE) interfaced to laser induced fluorescence (LIF) [see 27, 29 and 30 for CE-LIF separation].

10 DBS from healthy newborns were also treated as illustrated above, but the first extracted aliquot of 50 μL was submitted to chondroitinase B (10 μL, 20 mU, [no EC number] from Sigma-Aldrich) degradation instead of chondroitinase ABC. The produced disaccharides were derivatized with AMAC and analyzed by CE-LIF as above described.

Results

Six hundred DBS of healthy term 2-3-day-old newborns were collected from the heel of each newborn according to the standardized procedure applied for determining classic inborn metabolic diseases [20]. DBS were stored at room temperature and sent to the research unit laboratory thanks to the stability of spotted blood GAGs [19].

Blood from each circular area of 12 mm of DBS, ~40 μL, was recovered by extraction in Tris-Cl 20 mM pH 7.4 buffer and treated with a protease. After centrifugation, filtration and lyophilization, one aliquot