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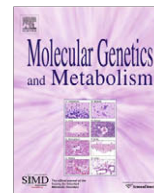

Evaluation of heparin cofactor II–thrombin complex as a biomarker on blood spots from mucopolysaccharidosis I, IIIA and IIIB mice

Kia Langford-Smith [a], Malani Arasaradnam [a], J. Ed Wraith [b], Rob Wynn [c], Brian W. Bigger [a,c,*]

[a] MPS Stem Cell Research Group, Faculty of Medical and Human Sciences, University of Manchester, 3.721 Stopford Building, Manchester M13 9PT, UK
[b] Genetic Medicine, St. Mary's Hospital, Manchester, UK
[c] Bone Marrow Transplant Unit, Royal Manchester Children's Hospital, Manchester, UK

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A B S T R A C T

Mucopolysaccharide (MPS) diseases are lysosomal storage disorders caused by deficiencies of enzymes catabolising glycosaminoglycans (GAGs). Abnormal GAG accumulation leads to symptoms including severe progressive neurological decline, skeletal deformities, organomegally, respiratory compromise and premature death. Treatment is available for some MPS diseases; enzyme replacement therapy for MPS I, II and VI, and haematopoietic stem cell transplantation for MPS I, VI and VII. These treatments are reliant on early diagnosis of the disease and accurate monitoring of treatment outcomes.

Blood enzyme levels and total urinary GAGs are commonly used biomarkers in diagnosis of MPS but are not good measures of treatment outcome. Serum heparin cofactor II–thrombin complex (HCII-T), which is a GAG regulated serpin–protease complex, has recently been identified as a promising biomarker for MPS diseases.

Here we present an assessment of the HCII-T biomarker in mouse models of MPS I, IIIA and IIIB, which suggests that HCII-T is a reliable marker for MPS I when measured in serum or dried blood spots stored for over a year at 4 °C, but that murine MPS IIIA and IIIB cannot be reliably detected using this biomarker. We also show that HCII-T formation *in vivo* is dependent on the presence of excess intravenous dermatan sulphate (DS), whilst intravenous heparan sulphate (HS), does not promote complex formation effectively. This suggests that HCII-T will prove effective as a biomarker for MPS I, II, VI and VII diseases, storing dermatan sulphate but may not be as appropriate for MPS III, storing heparan sulphate. With careful sample preparation, HCII-T ELISA could prove to be a useful biomarker for both newborn screening and measurement of treatment outcomes in selected MPS diseases.

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Introduction

The mucopolysaccharidoses (MPS) are lysosomal storage disorders caused by specific deficiencies in lysosomal enzymes that catabolise glycosaminoglycans (GAGs). Excess GAG accumulation leads to a variety of symptoms, which are dependent on the specific GAGs stored. Symptoms can include severe progressive neurological decline, skeletal and facial deformities, respiratory difficulties, cardiomyopathy, corneal clouding and organomegally [1].

Enzyme replacement therapy (ERT) is currently available for MPS IS, IHS, II and VI [2–4] and is moderately successful in patients with non-neuronal attenuated disease, whilst treatment with haematopoietic stem cell transplantation (HSCT) has been successful in MPS IH, VI and VII [5–8]. These treatments are most effective when the disease is diagnosed early and treatment can begin before irreversible damage occurs in affected tissues. Therefore biomarkers which can be used for early diagnosis of MPS are an essential part of providing a successful treatment. It is also important to be able to monitor treatment outcomes in these patients using a cheap and reliable method.

Currently in the clinic, MPS is diagnosed by a combination of urinary GAG analysis and measurement of blood enzyme levels of the deficient enzyme. Urinary GAGs vary with the hydration state of the patient and also with age, and despite normalisation against creatinine, often give a variable prediction of disease severity and treatment outcomes. In clinical trials urinary GAG analysis has often been combined with clinical disease predictors but it is not clear how well it correlates with these, given its variability. A recent trial of ERT for MPS VI for example, measured urinary GAG analysis, alongside measurement of the patients walking ability, joint flexibility and grip strength [4].

One alternative that has been used extensively in Manchester for monitoring treatment outcomes in MPS I and II patients is to

* Corresponding author. Address: MPS Stem Cell Research Group, Faculty of Medical and Human Sciences, University of Manchester, 3.721 Stopford Building, Manchester M13 9PT, UK. Fax: +44 (0) 161 275 1781.
E-mail address: Brian.Bigger@manchester.ac.uk (B.W. Bigger).

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normalise the level of a stored GAG, dermatan sulphate (DS), against a GAG that is not stored, such as chondroitin sulphate (CS) (DS/CS ratio) [9]. This is a good predictor of metabolic outcome; where for example, a lower DS/CS ratio correlates very well with a higher blood enzyme level following transplant in patients with MPS I, and can clearly distinguish patients on ERT from those receiving related heterozygote transplants and those receiving unrelated homozygote transplants [10]. However, it is laborious and time consuming to perform and it cannot be easily scaled down to accommodate the smaller volumes encountered when sampling mice.

An alternative method for diagnosis of MPS diseases is measurement of urinary GAG oligosaccharides that can be detected using electrospray ionisation tandem mass spectroscopy. Levels of these oligosaccharides are increased in MPS patients, and different profiles of urinary oligosaccharides are associated with each type of MPS, allowing all types of MPS to be distinguished from one another except MPS IIIB and MPS IIIC [11]. This is a promising technique but is very difficult to optimise and perform for the majority of laboratories. More recently, heparin cofactor II–thrombin complex (HCII-T) was identified as a promising biomarker for MPS. Initially identified as a marker for MPS I, HCII-T is now reported to be elevated in serum samples from patients with MPS I, II, III, IV and VI [12,13]. Levels of HCII-T appear to be responsive to treatment and disease progression, but are not yet in clinical diagnostic use.

Here we present an assessment of the HCII-T biomarker in mouse models of MPS I, IIIA and IIIB, and test which stored glycosaminoglycans are important for HCII-T complex formation in the blood.

Materials and methods

Mouse colony maintenance and blood sample collection

Animal maintenance and all procedures were ethically approved and carried out in accordance with UK Home Office regulations. The MPS I [14], IIIA [15] and IIIB [16] mouse models were maintained as heterozygote lines on an inbred C57BL/6 background at the University of Manchester. Animals were kept in a 12/12 h light/dark cycle and constant temperature with food and water supplied *ad libitum*.

Blood samples were collected from anaesthetised mice by incision of the tail vein. In some cases 160 μg dermatan sulphate (DS) or 160 μg heparan sulphate (HS) were delivered to mice by tail vein injection 30 min before blood sample collection. Blood (40 μl) was added to a blood spot card and allowed to dry before being stored at 4 °C. Serum was collected from blood without anticoagulant that was allowed to clot for 30 min at room temperature, before centrifugation at 3000*g* for 15 min at room temperature. Plasma was collected by immediately adding blood to sodium citrate in a ratio of 3:1 blood to sodium citrate and storing at 4 °C before centrifugation at 1500*g* for 15 min at 4 °C. Serum and plasma were stored at −20 °C.

Measurement of heparin cofactor II–thrombin (HCII-T) complex

A polyclonal sheep anti-thrombin capture antibody and a peroxidise-conjugated polyclonal goat anti-HCII detection antibody (Affinity Biologicals) were used to quantify the HCII-T complex in mouse serum, plasma and dried blood spots. A 96 well EIA/RIA plate (Corning) was coated with capture antibody diluted in coating buffer (50 mM carbonate pH 9.6) overnight at 4 °C. Wells were blocked with blocking buffer (PBS 10% BSA, pH 7.4) for 90 min at room temperature. Standards, serum and plasma samples were diluted in General Purpose Serum Diluent (Serotec) up to 25,000-fold. Samples from dried blood spot cards were prepared by punching a sample from the blood spot using a Wallac DBS Puncher (DELFIA) and eluting with 200 μl General Purpose Serum Diluent for 80 min at room temperature. Sample diluent (HBS, 1% BSA, 0.1% TWEEN-20, pH 7.2) was added to all standards and samples. Following three washes with wash buffer (PBS 0.1% TWEEN-20 pH 7.4), 100 μl of standards and samples were added to the wells for 2 h at room temperature. After three washes with wash buffer the detecting antibody, diluted in sample diluent, was added for 60 min at room temperature. Following three washes with wash buffer *O*-phenylenediamine (OPD) substrate dissolved in citrate–phosphate buffer (pH 5.0) was added for exactly 10 min and the reaction quenched using 2.5 M H_2SO_4. The plate was read at 490 nm within 10 min of quenching on a Synergy HT Microplate Reader. All standards and samples were assayed in duplicate, with serum samples tested at three different dilutions each and blood spot samples tested at one or two dilutions each.

Formation of heparin cofactor II–thrombin (HCII-T) complex

A 1 μM HCII-T standard was made by reaction of 1 μM human thrombin with 5 μM human heparin cofactor II (Enzyme Research Laboratories) for 30 min at 37 °C in standard reaction buffer (Tris buffered saline (TBS) with 1 mM EDTA, 0.05 U/ml heparin). This was used to make a series of standards from 5 to 100 pM. To test the effect of heparan sulphate and dermatan sulphate on HCII-T complex formation 1 μM human thrombin was reacted with 2.5 μM human heparin cofactor II for 5 min at 37 °C in standard reaction buffer with varying concentrations of HS and DS. Each reaction mixture was diluted in General Purpose Serum Diluent up to 50,000-fold and tested using the HCII-T ELISA.

Limitations of the HCII-T ELISA

Samples were tested at several dilutions to establish the linear portion of the standard curve. Samples with standard deviations greater than 10% of the mean absorbance value or those falling outside this region of the standard curve were excluded and retested. Analytical sensitivity, which is defined as the lowest concentration of HCII-T in a sample that can be accurately distinguished from zero, was determined to be 5.8 pM. Intra-assay imprecision was determined by running replicates across a range of different HCII-T concentrations, with the coefficient of variance demonstrating that the intra-assay imprecision was <6.5%. Inter-assay imprecision was calculated to be <22%. Functional sensitivity is defined as the lowest concentration of HCII-T that can be measured based on an inter-assay CV of 10% and was estimated to be 7.5 pM. Linearity of dilution was calculated to show that it is accurate to consider the standard curve is linear between 5 and 100 pM HCII-T.

Statistical analysis

Data were analysed using one way ANOVA, applying Tukey's multiple comparisons test as a post hoc comparator. Where appropriate, data were log transformed before statistical analysis. Significance was assumed where p values were less than 0.05 of a studentised range of Q. Error bars represent standard deviations about the mean.

Results

HCII-T complex was measured in samples from the murine models of MPS I, IIIA and IIIB to determine the best methods of sample collection and storage. The reliability and reproducibility

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