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Cathepsin B inhibition attenuates cardiovascular pathology in mucopolysaccharidosis I mice

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ABSTRACT

Mucopolysaccharidosis type I (MPS I) is a lysosomal storage disorder with multisystemic features, including heart enlargement, heart valve dysfunction, and aortic stiffness and dilatation. Previous studies have shown that MPS I mice overexpress cathepsin B (CtsB) in multiple tissues, including those from the cardiovascular system. Here, we hypothesized that inhibition of CtsB could ameliorate cardiac function parameters, as well as aorta and valve abnormalities found in MPS I.

First, we found that total elastase activity in an MPS I aorta is elevated. Following that, we demonstrated that CtsB leaks from the lysosome in MPS I human fibroblasts, possibly acting as a degradative agent of extracellular matrix components from the aorta, cardiac muscle, and heart valves. We then used a CtsB inhibitor in vivo in the MPS I mouse model. After 4 months of treatment, partial inhibition of CtsB activity in treated mice reduced aortic dilatation, as well as heart valve thickening, and led to improvements in cardiac function parameters, although none of these were completely normalized. Based on these results, we conclude that lysosomal alterations in this disease promote leakage of CtsB to outside the organelle, where this protein can have multiple pathological roles. CtsB inhibition improved cardiovascular parameters in MPS I mice and can have a potential benefit in this disease.

1. Introduction

Mucopolysaccharidosis type I (MPS I) is an autosomal recessive disorder caused by deficiency of lysosomal enzyme alpha-L-iduronidase (IDUA), which is involved in the catabolism of glycosaminoglycans (GAG), heparan and dermatan sulfate. The disease is characterized by multiple organ dysfunction, which includes organomegaly, bone and joint deformities, short stature, abnormal facial features and, in severe cases, mental retardation. There is a spectrum of severity, ranging from the severe Hurler phenotype to the more attenuated Scheie phenotype. Death can occur from multiple causes in the first decades of life [1].

Heart and valve disease are present in all MPS types, and death from heart failure is common. MPS I patients suffer from heart enlargement [2], heart valve thickening and regurgitation, and aortic stiffness and dilatation [3,4]. Studies in animal models (both dogs and mice) have shown that MPS I animals have dilated hearts with reduced contractility, dilated ascending aortas with increased elastin breaks, and heart valve thickening and regurgitation [5–7].

The extracellular matrix (ECM) plays many roles in the cardiovascular wall and valve homeostasis [8]. Elastin and collagen are the main compounds of the cardiovascular ECM and play a vital role in biomechanical and functional properties in the myocardium, aorta and heart valves. Although the proportions of elastin and collagen vary considerably in these tissues, distribution and properties of these connective tissue proteins are important for structural integrity and function. An imbalance in the turnover of ECM proteins in the myocardium and vasculature can lead to cardiovascular disorders such as stenosis, left ventricular hypertrophy, heart failure and aortic aneurysm [9–11]. These changes have been attributed to the lack of control of proteolytic enzymes that degrade the major components of the ECM. The increase in protease activity (especially in Cathepsin B) subsequent to the primary defect has been described as a possible pathological mechanism present in different types of MPS [5,7,12–14].

Cathepsin B (CtsB) is a pH-dependent lysosomal protease

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responsible for normal proteolytic degradation that can degrade ECM components in a controlled remodeling processes. Several studies indicate imbalances in production and activity of this enzyme as responsible for many different diseases. Recently, it was demonstrated that this enzyme is overexpressed and maintains high activity in cardiovascular tissues of MPS I and MPS VII animal models [5,7,13,14]. Therefore, it is possible that abnormalities in the structure of the elastin and collagen related to cardiac dysfunction in MPS, are due to overexpression of CtsB.

Based on these studies, the present work was designed to verify the effects of using a pharmacological inhibitor of CtsB to evaluate the role of this enzyme in cardiac dysfunction of MPS I mice.

2. Materials and methods

2.1. In vitro assay

2.1.1. Immunofluorescence

Skin fibroblasts from human MPS I patients and normal (healthy) controls were obtained from patients referred to our institution after informed consent. The cells were grown in DMEM with 10% FCS, and 1×10^4 cells were cultured on a 6-well plate for 24 h. Subsequently, they were fixed and permeabilized in cold methanol for 10 min. Successive washes were performed with phosphate buffered saline (PBS) and samples were blocked with 2% bovine serum albumin (BSA) in PBS. Cells were incubated overnight (at 4 °C) with monoclonal anti-CtsB (ab58802; Abcam) and polyclonal anti-Lamp-1 (sc8098; Santa Cruz Biotechnology) antibodies diluted in BSA. After this, cells were washed 3 times with PBS and incubated for 1 h at room temperature with secondary antibodies as follows: goat anti-mouse IgG (H + L) FITC conjugated and rabbit anti-goat IgG (H + L) Alexa Fluor® 555 conjugated. After immunostaining, cells were washed in PBS, incubated in Fluoroshield with DAPI and mounted on glass slides. Samples were observed under a confocal laser-scanning microscope. Percentage of green signal (CtsB) in relation to total signal was quantified at the merge channel ($n = 5$ cells/group) to quantify co-localization of the enzyme with the lysosome.

2.2. In vivo experiments

2.2.1. Animals

All animal experiments were approved by the authors´ Institutional Review Board (#14-0417). Wild-type littermates and *Idua*$^{-/-}$ (MPS I) mice (kindly donated by Elizabeth Neufeld from UCLA) with a C57BL/6 background were used in this study [15]. Both male and female MPS I mice were included. Animals were assigned to three groups: Group I and II consisted of *Idua*$^{-/-}$ and wild-type mice without treatment ($n = 11$ each, males/females). Group III consisted of *Idua*$^{-/-}$ mice treated with the CtsB inhibitor (Ca-074 Me; $n = 6$ males). We additionally performed a group of *Idua*$^{-/-}$ mice ($n = 8$, males) treated only with the Ca-074 Me vehicle (DMSO 10%) to ensure that DMSO was not responsible for any potential benefits observed. These mice did not show any difference compared to the untreated MPS I mice and their results are shown as supplement (Fig. S1). All mice were sacrificed at 6 months of age.

2.2.2. Treatment

The inhibitor, Ca-074 Me (Apexbio, USA), was prepared in a stock solution at a concentration of 10 mg/ml in dimethyl sulfoxide (DMSO 10%). The solution was diluted 1:10 in saline and administered at a dosage of 10 mg/kg/day. Treatment by intraperitoneal injection began at 8 weeks of age and continued for 16 weeks. We also performed a pilot study treating mice with 5 mg/kg/day, but did not cause a significant inhibition in cathepsin B activity is tissues (data not shown), therefore we used the 10 mg/kg/day dose.

2.2.3. Elastase activity assay

For the elastase assay, samples were homogenized in buffer (100 mM Tris HCl, pH 8 to 25 °C) at 0.2 μg tissue/μl for heart samples or 50 μl of solution for aortic samples. Elastase activity was measured using substrate N-Succinyl-Ala-Ala-Ala-*p*-nitroanilide (Sigma-Aldrich, USA) in a continuous spectrophotometric rate determination. Substrate solution (4.4 mM $SucAla_3$-*p*NA solution) was prepared in Tris-HCl buffer. The amount of product was determined by absorbance using kinetic reading and comparison with 0.02 units of elastase solution from the porcine pancreas (Sigma-Aldrich, USA). Readings were performed for 2 h every 5 min at A_{410} nm. The results were expressed as U/mg protein. One unit of elastase hydrolyzes 1 μmol of N-Succinyl-Ala-Ala-Ala-*p*-nitroanilide per hour at pH 7.5 at 25 °C.

2.2.4. Collagenase activity assay

For the collagenase assay, samples were homogenized in buffer (50 mM Tricine with 10 mM calcium chloride and 400 nM sodium chloride, pH 7.5 to 25 °C) at a concentration of 0.2 μg/μl. Collagenase activity was measured using FALGPA substrate (1 mM N-(3-[2Furyl] acryloyl)-Leu-Gly-Pro-Ala solution in buffer) (Sigma-Aldrich, USA) in a continuous spectrophotometric rate determination. The amount of product was determined by absorbance using kinetic reading and comparison with 0.2 units of collagenase solution from *Clostridium histolyticum* (Sigma-Aldrich, USA). Readings were performed by recording the decrease in A345 nm for 2 h every 5 min. The results were expressed as U/mg protein. One unit of collagenase hydrolyzes 1 μmol of FALGPA per hour at 25 °C at pH 7.5 in the presence of calcium ions.

2.2.5. Cathepsin activity assay

Cathepsin B activity was measured in pH 7.4 using the specific fluorometric substrate, *Z*-Arg-Arg-AMC (Enzo Life Sciences, USA). Samples were homogenized in acetate buffer (100 mM sodium acetate, 0.1% Triton X, EDTA 2.5 mM, DTT 2.5 mM pH 7.4) and incubated with the substrate at a final concentration of 50 μM. Fluorescence was measured using Spectramax M3 every 5 min for 30 min at an excitation of 355 nm and an emission of 460 nm using the kinetic reading and comparison with 7-amino-4-methylcoumarin (AMC) standards. The results were expressed as nmol/h/mg protein. Protein content of each sample was measured by the Lowry technique for all assays.

Total cathepsin activity was measured using the *Z*-Phe-Arg substrate (Enzo Life Sciences, USA), 10 μM at pH 7.4, with the same buffer and parameters used for the CtsB assay. Inhibitors were from Calbiochem (San Diego, CA) and included CtsB inhibitor Ac-Leu-Val-Lysinal (#219385), CtsK inhibitor I [1,3-Bis (N-carbobenzoyloxy-L-leucyl) amino acetone; #219377] and CtsS *Z*-FL-COCHO (#219393). The inhibitors were added to some assays in final concentrations of 10, 100 or 1000 nM, as indicated. The results were expressed as nmol/h/mg protein.

2.2.6. Echocardiography

Transthoracic echocardiography was performed on anesthetized mice (2% isoflurane) and placed in the left lateral decubitus position to obtain cardiac images. An EnVisor HD System, Philips Medical (Andover, MA, USA), with a 12–4 MHz transducer was used, at 2 cm depth with fundamental and harmonic imaging. Images were captured by a trained user for the mouse echocardiography.

Left ventricular (LV) dimensions from diastolic and systolic transverse areas (cm2) were obtained by tracing the endocardial border at three levels: basal (at the tip of the mitral valve leaflets), middle (at the papillary muscle level) and apical (distal from the papillary muscle in the endocardium apex). The LV diameters (cm) at the end-diastolic (LVEDD) and end-systolic dimension (LVESD), as well as the diameters of posterior and anterior wall thickness, were obtained by M-MODE and were used as measures of hypertrophy and dilatation. The final value was obtained by taking the average of all three planes [16]. The assessment of LV systolic function was performed using the following