Abstract

Mucopolysaccharidosis type I (MPS I) is an autosomal recessive lysosomal storage disorder caused by deficiency of α-L-iduronidase, which leads to a wide range of clinical manifestations. Different secondary pathogenic cascades may be involved in the tissue specificity and heterogeneity of phenotypes, as the accumulation of glycosaminoglycans itself is not enough to explain the multisystemic alterations observed in MPS I. Autophagy is a self-digesting mechanism responsible for the degradation of long-lived proteins and cellular organelles, which alterations have been observed in many lysosomal storage disorders. The aim of this study was to evaluate some proteins involved in the autophagic process in the brain, liver and spleen of 3 months-old MPS I mice by Western blotting. Beclin-1 levels were higher in MPS I mice brains compared to controls ($p = 0.01$), but this protein was not detected in other tissues. Two isoforms of p62/SQSTM1 were identified in all tissues analyzed, with ~60kDa and ~50kDa, respectively. No differences between the levels of both isoforms were observed among the tissues from MPS I and control mice. However, the ratio between 50kDa and 60kDa isoforms was higher in the spleens of MPS I mice ($p = 0.04$). In addition, the ratio of these isoforms was shown to vary among tissues. Interestingly, levels of β-tubulin in the liver were different between MPS I and control mice ($p = 0.02$). These alterations in protein markers suggest that autophagy may be deregulated in MPS I mice in a tissue specific manner and that the cytoskeleton and vesicle trafficking in hepatocytes may be compromised.
Introduction

Mucopolysaccharidosis type I (MPS I) is an autosomal recessive lysosomal storage disorder (LSD) caused by deficiency of α-L-iduronidase, an enzyme responsible for the degradation of two glycosaminoglycans (GAGs): dermatan and heparan sulphates. As observed in many other LSDs, patients with MPS I present a wide range of clinical manifestations, including hepatosplenomegaly, developmental delay and cognitive decline, characteristic coarse facial features, respiratory insufficiency, valvular heart disease, corneal clouding, dysostosis multiplex and joint stiffness (1). The severity of phenotypes vary among MPS I patients, and it has recently been recommended to classify them into two broader groups – severe MPS I (corresponding to Hurler syndrome), in which patients usually die until the first decade of life, mainly as a result of cardiorespiratory failure and progressive neuropathology, and attenuated MPS I (corresponding to Hurler-Scheie and Scheie syndromes), in which many patients survive until adulthood with normal intelligence, presenting a variable range of other symptoms (2, 3).

The mechanisms by which the enzyme deficiency leads to all these multisystemic alterations are still not fully understood. Accumulation of undegraded or partially degraded GAGs in the lysosomes was thought to be the main cause of MPS I clinical manifestations; however, recent studies have demonstrated that secondary biochemical and cellular pathways play an important role in the physiopathology of many LSDs (4). The involvement of these different pathogenic cascades may explain the tissue specificity and heterogeneity of clinical manifestations observed in this group of diseases.
Secondary biochemical and cellular alterations as oxidative stress (5, 6), inflammation (7, 8), upregulation of elastases (9) and deficiency of cathepsin K (10) have already been associated with the physiopathology of MPS I. In addition, in a previous study by our group, alterations in $\text{Ca}^{2+}$ and $\text{H}^+$ homeostasis in MPS I mice splenocytes, in addition to lysosomal membrane permeabilization and increased apoptosis were observed (11), which could lead to impairment of the endocytic/autophagic pathway.

Macroautophagy, which will be herein referred to as autophagy, is a self-digesting mechanism responsible for the degradation of long-lived proteins and cellular organelles, which is important to maintain cellular homeostasis (12). During autophagy, double-membrane vesicles (autophagosomes) are formed to sequester the cytosolic material and then fuse with endosomes and lysosomes to complete the degradation process, which requires the action of many lysosomal hydrolases. Finally, the degradation products are transported back to the cytosol where they can be recycled for the synthesis of other molecules or metabolism (13-16).

There are many steps in the autophagic process and therefore several proteins are involved. Beclin-1 (homologue of yeast Atg6) is considered the main inductor of autophagy, as its conjugation with Vps34 is essential to induce the autophagosome membrane formation (17). To complete the process, the microtubule-associated protein 1 light chain 3 (LC3-I, homologue of yeast Atg8) is cleaved at its carboxy-terminal, and further modified to the lipid-conjugated LC3-II, which is integrated to autophagosome membranes. To select the proteins for degradation, the autophagosome membrane interacts with polyubiquinated
proteins aggregates, which is mediated by p62/SQSTM1 (p62/sequestosome1) which will be referred to as p62. Block of autophagy leads to an accumulation of p62, which has been used as an important marker of this alteration (18, 19).

Alterations in autophagy have already been observed in many LSDs, including other types of mucopolysaccharidoses (20-30). These studies were performed using different cell types and tissues, which was facilitated by the availability of animal models of some LSDs. The aim of this study was to evaluate some proteins involved in the autophagic process in the brain, liver and spleen of MPS I mice.

**Material and methods**

**Mice**

C57BL/6 *Idua* +/+ and *Idua* -/- mice were bred by heterozygous mating, which precursors were kindly provided by Dr. Elizabeth Neufeld (UCLA, U.S.A.) and Dr. Nance B. Nardi (UFRGS, Brazil). This MPS I mouse model has been briefly described by Ohmi *et al.* (8), and is similar to that described by Clarke *et al.* (31). Animals were maintained on a 12-h light/dark cycle with food and water available *ad libitum*. Genotyping was performed by polymerase chain reaction at the 30th day of life and all the experiments were performed with 3 months-old males and females. This study was approved by the Ethical Research Committee from Universidade Federal de São Paulo in 2007 (CEP # 1165/07).
**Protein extraction and quantification**

After euthanasia procedures, portions of brain, liver and spleen were collected from mice and homogenized with 1.5mL lysis buffer (PBS with 2% Triton and 10% proteases inhibitors cocktail) for protein extraction. Samples were centrifuged twice at 13000rpm/10min and the supernatants were collected. Protein quantification was performed by a commercial kit (RC DC™ Protein Assay - BIORAD) based on Lowry’s method.

**Antibodies**

Primary antibodies (all produced in rabbit) were: anti-Beclin-1 and anti-p62/SQSTM1 (Sigma-Aldrich), anti-β-tubulin (Novus Biologicals) and anti-GAPDH (Santa Cruz). Secondary antibody was Alexa Fluor 680 anti-rabbit IgG (Invitrogen). Antibodies were diluted in 5% non-fat milk.

**Western Blotting**

Total proteins (20µg) were loaded onto 13% polyacrilamide gels for electrophoresis at 170V/80min. Nitrocellulose membranes were used for transfer, which was performed at 400mA/60min. Membrane blocking was performed with 5% non-fat milk for 1h. Primary antibodies concentration and incubation conditions were: anti-Beclin-1 (1.5µg/mL, 1h at room temperature), anti-p62/SQSTM1 (0.5µg/mL, overnight at 4°C), anti-β-tubulin (0.1µg/mL, overnight at 4°C) and anti-GAPDH (0.5µg/mL, overnight at 4°C).

Membranes were visualized in Odyssey® infrared imaging system and target proteins were quantified relatively to β-tubulin or GAPDH.
Statistical analysis

Unpaired Student’s t test was used to compare data from both groups through STATISTICA 6.0 software, and the level of significance was set at p<0.05.

Results

Protein markers of different autophagy steps were analyzed in this study. To evaluate autophagy induction, Beclin-1 was quantified in the brain, liver and spleen of Idua +/+ and Idua -/- mice. Beclin-1 levels were higher in Idua -/- mice brain when compared to controls (p = 0.01, Figure 1), but this protein was not identified in the liver and spleen. Increased levels of Beclin-1 suggest that autophagy is up regulated in the brain of MPS I mice.

Levels of p62 were quantified by Western blot to investigate the accumulation of polyubiquinated protein aggregates due to an impairment of autophagy. Two isoforms of p62 were identified in all tissues analyzed, with approximately 60kDa and 50kDa. These two isoforms have been previously identified and described by Waguri and Komatsu (32), who speculate that the 50kDa protein may represent a partially degraded isoform. No differences between the levels of both isoforms were observed among the tissues from MPS I and control mice. However, the ratio between 50kDa and 60kDa isoforms was higher in the spleens of MPS I mice (p = 0.04, Figure 2). In addition, the ratio of these isoforms was shown to vary among tissues (Figure 2). This difference between the ratio of p62 isoforms suggest that the degradation of proteins via p62 by autophagy occurs at different levels among the tissues analyzed in this study, and it might be up regulated in the spleen of MPS I mice.
Levels of β-tubulin in the liver were different between *Idua* +/+ and *Idua* -/- mice (*p* = 0.02, Figure 3). As shown in Figure 3B, MPS I mice have very low levels of this protein in the liver, which probably compromises the cytoskeleton structure, vesicle sorting and trafficking in the cytosol. This difference between β-tubulin levels made it necessary to use GAPDH as a normalizing protein for liver samples.

**Discussion**

In a previous study by our group, we observed alterations in Ca\(^{2+}\) and H\(^+\) homeostasis in MPS I mice splenocytes, in addition to lysosomal membrane permeabilization (11). Ca\(^{2+}\) release from the lumen, low pH and membrane integrity are required for an efficient fusion between lysosomes and other vesicles (33). Therefore, we hypothesized that the endocytic/autophagic pathway might be also impaired in these cells, which would contribute to disease pathology.

Accordingly, many studies have demonstrated that a deregulation of autophagy may play an important role in the physiopathology of LSDs, supported by the evidences of autophagosome accumulation in Danon disease, Neuronal ceroid-lipofuscinoses, Pompe disease, Mucolipidosis type II and IV, Niemann-Pick type C, Multiple Sulphatase Deficiency, Mucopolysaccharidosis IIIA, GM1-Gangliosidosis and Fabry disease (20-30), probably caused by an impairment of autophagosome-lysosome fusion (33). In this present study, some protein markers from different steps of autophagy were evaluated to verify the involvement of this process in the physiopathology of MPS I in the brain, liver and spleen.

Increased levels of Beclin-1 were found in the brain of MPS I mice, suggesting that autophagy may be up regulated in this tissue, as the induction of
autophagy is physiologically dependent on this protein (17). There was no difference between p62 levels in the brain, which indicates that, despite the upregulation, there is still no autophagic build up in this tissue. High levels of Beclin-1 and p62 have been observed in Mucolipidosis IV and MPS VI fibroblasts (28, 34) and in both studies the authors suggest that the increase of Beclin-1 occurs due to the impaired degradation of protein aggregates, to stimulate cellular clearance. However, our results suggest that the increase of Beclin-1 levels occurs regardless of the accumulation of p62 in the brain of MPS I mice.

Besides, we have shown that there was no difference between the levels of both isoforms of p62 between groups in the other tissues analyzed, which suggests that there is also no accumulation of these protein aggregates in liver and spleen. However, the ratio between 50kDa and 60kDa isoforms was higher in the spleens of MPS I mice and it was shown to vary among tissues. This difference between the ratio of p62 isoforms suggest that the degradation of proteins via p62 by autophagy may occur at different levels among the tissues analyzed in this study, and it might be even up regulated in the spleen of MPS I mice.

Notably, the presence of this p62 isoform with ~50kDa is not discussed or shown in most articles regarding analysis of this protein. It has been previously identified and described by Waguri and Komatsu (32), who speculate that this isoform may represent a partially degraded p62. The tissue specific ratio between both isoforms found in this present study indicates that a better characterization of the protein is necessary to confirm if it indeed represents a partially degraded p62, a chemically modified protein or a homologue one.
Interestingly, levels of β-tubulin in the liver were different between Idua +/+ and Idua -/- mice, the later showing very low levels of this protein. Microtubules are composed by β-tubulin and α-tubulin heterodimers and are involved in many cellular processes, including vesicular transport and fusion (35-38). It has been shown that alteration in microtubules leads to the accumulation of autophagosomes and inefficient delivery of hydrolytic proteins to the lysosomes (35, 37, 39).

Alterations of α-tubulin have been demonstrated in autophagic areas from muscle cells with Pompe disease, which clearly showed disorganization of microtubular structure and network (21). Therefore, it is likely that the alterations of β-tubulin in MPS I also compromise the cytoskeleton structure, vesicle sorting and trafficking in the cytosol of hepatocytes. In the study with Pompe disease, the authors suggest that impairment of autophagy occurs by deficient fusion of vesicles, which in turn leads to microtubular alterations (21). However, reduced levels of β-tubulin and the absence of p62 accumulation found in MPS I liver cells indicate that the disorganization of microtubular network may precede the autophagic build up.

Identifying the time course of these pathogenic alterations is of great importance to elucidate the underlying mechanisms of LSDs, mainly because they are all characterized as progressive diseases. In this sense, it is important to emphasize that this present study was performed with 3 months-old mice and the results found may represent only initial steps of different cellular and biochemical alterations.
Our results suggest that autophagy may be deregulated in MPS I mice, in a tissue specific manner and that the cytoskeleton and vesicle trafficking in hepatocytes may be compromised. However, further analyses on the quantity of autophagosomes will be helpful for understanding the alterations in the autophagic flux observed in this study.

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Figure 1. Western blot analysis of Beclin-1 levels from lysates of brain from *Idua* +/+ and *Idua* -/- mice. A: Mean ± standard deviation of Beclin-1/β-tubulin ratio from *Idua* +/+ (n = 4 mice) and *Idua* -/- (n = 4 mice); *p* = 0.01. B: Western blot results of Beclin-1 and β-tubulin from samples representing both groups.
Figure 2. Ratio between both isoforms of p62 (~50kDa and ~60kDa) from lysates of liver, brain and spleen of Idua +/+ and Idua -/- mice, analyzed by Western blotting. A: Mean ± standard deviation of p62 50kDa and 60kDa ratio from Idua +/+ (n = 4 mice) and Idua -/- (n = 5 mice) tissues; *p = 0.04. B: Western blot results of p62, GAPDH and β-tubulin from samples representing both groups. a: GAPDH was used to normalize proteins from liver; b: β-tubulin was used to normalize results from brain and spleen.
Figure 3. Western blot analysis of β-tubulin from lysates of liver from *ldua* +/- and *ldua* -/- mice. A: Mean ± standard deviation of β-tubulin/GAPDH ratio from *ldua* +/- (*n* = 4 mice) and *ldua* -/- (*n* = 5 mice); *p* = 0.02. B: Western blot results of β-tubulin and GAPDH from samples representing both groups.
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3. DISCUSSÃO GERAL
3. Discussão geral

A ampla diversidade de manifestações clínicas observadas nas DDL e o acúmulo de metabólitos não diretamente relacionados à deficiência enzimática específica sugerem que várias vias bioquímicas e/ou celulares secundárias estejam envolvidas na fisiopatologia destas doenças (Futerman e van Meer, 2004; Walkley, 2009; Walkley e Vanier, 2009). Alterações em diversos processos celulares como vias de sinalização, homeostase de Ca\(^{2+}\) intracelular, biossíntese de lipídeos, tráfego intracelular, permeabilização da membrana lisossômica e autofagia já foram identificadas em diferentes DDL (Futerman e van Meer, 2004; Ballabio e Gieselmann, 2009).

Neste presente estudo, foram avaliados os processos de permeabilização da membrana lisossômica (PML), homeostase de Ca\(^{2+}\) intracelular e autofagia em diferentes tecidos de um modelo animal de MPS I. Os resultados obtidos foram apresentados separadamente em dois artigos, e nesta sessão serão discutidos de maneira conjunta.

Foram observadas alterações na homeostase de Ca\(^{2+}\) intracelular em esplenócitos de camundongos com MPS I, que apresentaram quantidades aumentadas do íon no retículo endoplasmático (RE) e nos lisossomos (Artigo 1, Figura 1, p.44). Similarmente, um aumento na liberação de Ca\(^{2+}\) do RE do tecido cerebral de pacientes com Doença de Gaucher já havia sido demonstrado, indicando uma maior quantidade do íon no RE (Pelled et al., 2003). Um estudo publicado em 2010 demonstrou que o aumento induzido de Ca\(^{2+}\) no RE é capaz de melhorar o dobramento da enzima β-glicosidase em fibroblastos de pacientes com a Doença de Gaucher. Em conseqüência desta melhora no dobramento da
β-glicosidase, uma quantidade maior da enzima foi transportada corretamente aos lisossomos, e foi observado um aumento na atividade enzimática (Ong et al., 2010).

A maior quantidade de Ca$^{2+}$ e a menor quantidade de H$^+$ nos lisossomos dos esplenócitos ldua -/- indicam uma alteração na homeostase iônica, que analisada em conjunto com os resultados do vazamento de proteases para o citosol evidencia o processo de PML (Artigo 1, Figuras 1, 3 e 4, respectivamente, p.44, 47 e 48). Além disso, o acúmulo de material não degradado dentro do lisossomo pode afetar a homeostase do Ca$^{2+}$ diretamente pelo seqüestro do íon, ou pela alteração de seus transportadores (Kiselyov et al., 2010). O tipo de alteração na homeostase do Ca$^{2+}$ parece ser diferente entre as DDL, pois em células de Niemman-Pick tipo C foi observada uma quantidade reduzida de Ca$^{2+}$ nos lisossomos (Lloyd-Evans et al., 2008), enquanto na Doença de Gaucher (Pelled et al., 2005) e neste presente estudo com MPS I foram observadas quantidades aumentadas de Ca$^{2+}$ dentro da organela.

O NAADP é o principal responsável pela liberação de Ca$^{2+}$ de organelas ácidas, e sua ação é mediada pela ligação aos canais TPC, que foram identificados nas organelas do sistema endossomo/lisossomo (Brailoiu et al., 2009; Calcraft et al., 2009; Pitt et al., 2010). Assim, outra hipótese provável para explicar o acúmulo de Ca$^{2+}$ encontrado nos lisossomos na Doença de Gaucher e MPS I seria a presença de alterações nos canais TPC, devido ao acúmulo de material dentro da organela. Como a identificação destes canais como receptores de NAADP é recente (Pitt et al., 2010), ainda não existem relatos de estudos destes canais nas DDL.
Em relação à menor quantidade de H⁺ encontrada nos lisossomos dos esplenócitos *Idua* -/- (Artigo 1, Figura 3, p.47), é possível que o aumento de pH comprometa a atividade de outras enzimas lisossômicas, levando ao acúmulo intracelular de diversas moléculas. O aumento de pH lisossômico foi também observado em fibroblastos de pacientes com Mucolipidose tipo IV, mas não em pacientes com Niemann-Pick tipo A e C, MPS II e Doença de Farber (Bach et al., 1999). Esses resultados sugerem que os processos envolvidos na fisiopatologia das DDL devem ser diferentes entre as doenças, além de tecido-específicos.

A diferença na distribuição de H⁺ entre lisossomos e citosol observada nos camundongos com MPS I (Artigo 1, Figura 2, p.46) também reforça a evidência de PML, pois fisiologicamente o pH ácido dos lisossomos (pH≤5,0) é mantido por H⁺ATPases, que transportam os prótons do citosol para o interior do lisossomo (Mellman et al., 1986; Luzio et al., 2007). Ainda não se sabe se o processo de permeabilização ocorre por meio de uma ruptura não específica da membrana ou se alguns poros transientes são formados, permitindo uma translocação específica de moléculas (Boya e Kroemer, 2008). Em alguns casos nos quais algumas catépsinas foram detectadas no citosol, o gradiente de pH entre lisossomos e citosol ainda estava mantido (Bidere et al., 2003), mas neste presente estudo foi observada uma diferença na distribuição de H⁺ entre os compartimentos celulares, além da presença de cisteíno proteases no citosol (Artigo 1, Figura 4, p.48). Assim, é provável que esses dois mecanismos coexistam.

Já foi demonstrado que lisossomos grandes são mais suscetíveis à indução de PML do que os de menor tamanho (Ono et al., 2003), corroborando o
envolvimento deste processo na fisiopatologia das DDL. Porém, além do artigo apresentado nesta tese (Pereira et al., 2010), há apenas um relato na literatura de associação do processo de PML a outras DDL (Niemman-Pick tipo A e B). Nesse estudo com fibroblastos de pacientes com Niemman-Pick tipo A e B, os autores demonstraram que os lisossomos são menos estáveis e mais susceptíveis à ruptura, e a administração de uma chaperona Hsp70 recombinante foi capaz de reverter o fenótipo da doença nas células, por estabilizar a membrana lisossômica (Kirkegaard et al., 2010).

A translocação de proteases para o citosol durante o processo de PML leva à clivagem de diversos substratos, o que pode ativar vias de sinalização para apoptose (Stoka et al., 2001; Stoka et al., 2005; Boya e Kroemer, 2008). De fato, nos esplenócitos Ldua -/- foi observada uma maior taxa de apoptose (Artigo 1, Figura 5, p.49), provavelmente em conseqüência da PML.

A integridade da membrana lisossômica, pH baixo e liberação de Ca²⁺ dos lisossomos são necessários para o processo de fusão de vesículas (Pryor et al., 2000). Portanto, alterações no processo de autofagia provavelmente estão também envolvidos na fisiopatologia da MPS I. Para a avaliação dos marcadores de autofagia foram estudados dois outros órgãos além do baço – cérebro e fígado.

Em relação à Beclina-1, foi encontrada uma quantidade aumentada da proteína no cérebro dos camundongos Ldua -/- (Artigo 2, Figura 1, p.62), sugerindo uma ativação do processo autofágico. Quantidades aumentadas de Beclina-1 também foram encontradas no cérebro de camundongos com GM1-Gangliosidose (Takamura et al., 2008) e em fibroblastos de pacientes com
Niemman-Pick C, Doença de Sandhoff, Mucolipidose IV e MPS VI (Pacheco et al., 2007; Vergarajauregui e Puertollano, 2008; Tessitore et al., 2009), mas não em fibroblastos de pacientes com Doença de Gaucher (Pacheco et al., 2007), sugerindo que o mecanismo deve ser comum a apenas algumas DDL.

Além do aumento de Beclina-1, os fibroblastos de pacientes com Mucolipidose IV e MPS VI apresentaram acúmulo de p62/SQSTM1, e os autores especulam que devido à falha na degradação dos componentes celulares (demonstrada pelo aumento de p62/SQSTM1), ocorre uma ativação da autafagia para tentar remover o material acumulado (Vergarajauregui e Puertollano, 2008; Tessitore et al., 2009). Porém, nos cérebros de camundongos com MPS I não foi observado acúmulo de p62/SQSTM1, sugerindo que a ativação da autafagia pelo aumento de Beclina-1 deve ocorrer antes do acúmulo neste tecido.

Não foram observadas diferenças na quantidade de p62/SQSTM1 em nenhum dos tecidos estudados, porém com a identificação de uma isoforma menor da proteína foi possível demonstrar que há uma diferença na proporção das isoformas entre os tecidos (Artigo 2, Figura 2, p.63). Curiosamente, a isoforma menor não é citada na maioria dos artigos, apesar de ter sido observada pelos autores que descreveram técnicas de detecção da p62/SQSTM1 (Waguri e Komatsu, 2009), que especulam que essa isoforma pode representar a proteína parcialmente degradada. Neste sentido, os resultados observados neste presente estudo sugerem que há uma diferença no fluxo de degradação entre os tecidos. Além disso, foi observada uma diferença na razão das isoformas no baço dos camundongos com MPS I (Artigo 2, Figura 2, p.63), sugerindo que a degradação da p62/SQSTM1 deva estar aumentada neste tecido.
É importante ressaltar que para a avaliação dos parâmetros descritos no “Artigo 1” foram utilizados camundongos com 6 meses de idade, que apresentam uma patologia completamente instalada e manifestações bastante exacerbadas. A avaliação dos marcadores de autofagia (Artigo 2) foi realizada utilizando camundongos com 3 meses de idade, que já apresentam sintomas de MPS I, porém menos exacerbados. Assim, é possível que aos 3 meses algumas das cascatas patogênicas ainda não tenham sido ativadas. O acúmulo de p62/SQSTM1 representa o bloqueio do processo autofágico, portanto, é possível que esse bloqueio seja conseqüência de alterações celulares anteriores (como permeabilização da membrana lisossômica e alteração na homeostase iônica), e que ainda não seja evidente nas células de camundongos mais novos.

De acordo com essa hipótese, um estudo com células musculares de pacientes com Doença de Pompe demonstrou que pacientes com a forma infantil da doença apresentam lisossomos grandes e sem bordas delimitadas, o que sugere a permeabilização e ruptura da membrana lisossômica, mas as células não apresentam acúmulo de vacúolos autofágicos, que é observado na forma adulta da doença. Porém, após 6 meses de acompanhamento e tratamento, os pacientes com a forma infantil apresentaram um número aumentado de vacúolos autofágicos, sugerindo que a terapia de reposição enzimática não tem efeito sobre o bloqueio da autofagia e que esse processo ocorre mais tardiamente na Doença de Pompe (Raben et al., 2010).

A alteração da β-tubulina observada no fígado dos camundongos com MPS I foi um resultado inesperado inicialmente, pois esta proteína estava sendo utilizada como referência para normalizar a quantificação por Western blot.
Portanto, para a quantificação de proteínas no fígado foi utilizada outra proteína, a gliceraldeído 3-fosfato desidrogenase (GAPDH), como referência. Nos demais tecidos, a expressão da β-tubulina foi uniforme entre os grupos, sendo então utilizada como normalizadora.

Fisiologicamente, a β-tubulina forma heterodímeros com a α-tubulina para compor os microtúbulos, que estão envolvidos em diversos processos celulares como divisão, diferenciação, manutenção da estrutura, segregação cromossômica, organização citoplasmática e transporte intracelular (Desai e Mitchison, 1997). Já foi demonstrado que drogas desagregadoras de microtúbulos (como a colchicina) inibem o transporte entre endossomos e lisossomos em vários tipos celulares e reduzem a entrega de hidrolases às organelas, resultando no acúmulo de vacúolos autofágicos (Aplin et al., 1992; Monastyrska et al., 2009).

Os autofagossomos são geralmente formados na periferia da célula, enquanto os lisossomos e endossomos localizam-se na região perinuclear. Assim, para a fusão eficiente das organelas, os autofagossomos são transportados para a proximidade dos endossomos e lisossomos pela interação com os microtúbulos. Alterações nesse mecanismo de transporte causam atraso da autofagia, mas não bloqueiam o processo (Jahreiss et al., 2008).

Portanto, os baixos níveis de β-tubulina observados no fígado dos camundongos Idua -/- (Artigo 2, Figura 3, p.64), sugerem uma possível desorganização do citoesqueleto e alteração no processo de transporte vesicular, que por sua vez pode levar a alterações no processo de autofagia.

Alterações na α-tubulina foram observadas em células musculares de pacientes com Doença de Pompe, que demonstraram uma desorganização da
estrutura dos microtúbulos. Como o acúmulo de vacúolos autofágicos também é observado na Doença de Pompe, os autores sugerem que a desorganização da estrutura microtubular ocorre devido à fusão ineficiente das organelas (Fukuda et al., 2006). Porém, nos hepatócitos Idua -/- não foi observado o acúmulo de p62/SQSTM1 apesar da alteração na β-tubulina, sugerindo que a alteração na estrutura dos microtúbulos precede o bloqueio da autofagia nestas células.

Analisados de maneira conjunta, os resultados observados neste presente estudo demonstram que diversas alterações bioquímicas e celulares secundárias à deficiência enzimática estão envolvidas na fisiopatologia da MPS I, e que essas alterações são tecido-específicas. Além disso, para uma melhor compreensão dos mecanismos patogênicos é necessário identificar: (1) a existência de alterações comuns entre os tecidos; (2) a existência de alterações comuns a todas as DDL, ou pelo menos em subgrupos específicos; e (3) a sequência de ativação das diferentes cascatas patogênicas.
4. CONCLUSÕES
4. Conclusões

- Esplenócitos provenientes de camundongos com MPS I apresentaram uma maior quantidade de Ca\(^{2+}\) no retículo endoplasmático e nos lisossomos, menor quantidade de H\(^+\) nos lisossomos, permeabilização da membrana lisossômica e maior taxa de apoptose.

- Células de cérebro de camundongos com MPS I apresentaram uma maior quantidade de Beclina-1, indicando ativação da autofagia.

- Hepatócitos de camundongos com MPS I apresentaram uma menor quantidade de β-tubulina, indicando uma desorganização da estrutura de microtúbulos.

- Os tecidos avaliados (cérebro, fígado e baço) em camundongos com MPS I não apresentaram acúmulo de p62/SQSTM1, porém a taxa de degradação da proteína foi diferente entre eles.
5. ANEXOS
5. Anexos

5.1. Aprovação do Comitê de Ética em Pesquisa da UNIFESP

Ilmo(a), Sr(a).
Respeito e deferência.

PESQUISADOR(a): VANESSA GONÇALVES PEREIRA
Co-Investigador: Vanessa Gonçalves Pereira, Vanja D'Almeida (orientadora), Elaine Mendias Lauro, Lara Cheliz Rodrigues, Marcos Leoni Gazzarrini
Disciplina/Departamento: Pediatria Geral e Comunitária/Pediatria da Universidade Federal de São Paulo/Hospital São Paulo
Participação da CEP: Recursos Próprios.

PARECER DO COMITÊ DE ÉTICA INSTITUCIONAL

Ref: Projeto de pesquisa intitulado: “Avaliação de marcadores de estresse oxidativo em modelo animal de mucopolissacaridose tipo I”.

CARACTERÍSTICA PRINCIPAL DO ESTUDO: Experimental, categoria B - estudo agudo.
RISCOS ADICIONAIS PARA O PACIENTE: Não se aplica.
OBJETIVOS: Estudar marcadores bioquímicos de estresse oxidativo em diversos tecidos de um modelo animal de MPS I para avaliar o possível envolvimento das espécies reativas de oxigênio e a fisiopatologia da doença.
RESUMO: Estudo com 60 camundongos C57BL/6. Eutanásia: decapitação. Serão utilizados camundongos que apresentam modelo de MPS I. A colônia será expandida no biotério do Departamento de Psicobiologia da UNIFESP. Os animais serão genotipados por PCR. Será realizada análise bioquímica sérica. Será retirado o tecido cerebral e fígado para análise dos níveis de glutatona total e atividade das enzimas antioxidantes. O baço será retirado para extração de linfócitos e análise de mobilização de Ca²⁺ intracelular.
FUNDAMENTOS E RACIONAL: Vários estudos demonstram o envolvimento das espécies reativas de oxigênio na fisiopatologia da doença mucopolissacaridose tipo I. Este estudo visa estudar marcadores bioquímicos de estresse oxidativo em diversos tecidos de um modelo animal de MPS I.
MATERIAL E MÉTODO: Estão descritos os procedimentos, estando o projeto inserido na linha de pesquisa da orientadora.

CRONOGRAMA: 48 meses.
OBJETIVO ACADÊMICO: Doutorado.

O Comitê de Ética em Pesquisa da Universidade Federal de São Paulo/Hospital São Paulo ANALISOU e APROVOU o projeto de pesquisa referenciado.
1. Comunicar toda e qualquer alteração do projeto.
2. Comunicar imediatamente ao Comitê qualquer evento adverso ocorrido durante o desenvolvimento do estudo.
3. Os dados individuais de todas as etapas da pesquisa devem ser mantidos em local seguro por 5 anos para possível auditoria dos órgãos competentes.

Atenciosamente,

Rua Botucatu, 572 - 1º andar – conj. 14 - CEP 04023-062 - São Paulo / Brasil
Tel.: (011) 5671-1062 - 5509-7162
5.2. Artigos publicados no período de 2007 – 2011


Mutational and oxidative stress analysis in patients with mucopolysaccharidosis type I undergoing enzyme replacement therapy

Vanessa Gonçalves Pereira a,*, Ana Maria Martins a, Cecilia Micheletti a, Vânia D’Almeida a,b

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Available online 19 September 2007

Abstract

Background: Mucopolysaccharidosis type I (MPS I) patients present a wide range of clinical manifestations, which could be due to the high molecular heterogeneity of the IDUA gene and to pathological events besides the enzyme deficiency. The aim of this study was to identify the most common MPS I causing mutations and to evaluate some oxidative stress markers in Brazilian patients.

Methods: 3 common mutations in the IDUA gene were searched in 11 MPS I patients by PCR-RFLP. Activities of antioxidant enzymes catalase and superoxide dismutase, and levels of total glutathione and thiobarbituric acid reactive substances were evaluated by spectrophotometric and colorimetric methods, during different periods of enzyme replacement therapy.

Results: The most common mutations were P533R and W402X, with allelic frequencies of 33.33% and 27.8% respectively. MPS I patients presented high levels of lipid peroxidation and enzyme replacement therapy led to an increase of catalase and a decrease of superoxide dismutase activities.

Conclusions: P533R and W402X accounted for more than 60% of the alleles, but no genotype-phenotype correlation could be established. The alterations in antioxidant enzyme activities suggest that oxidative stress may be an important event among MPS I patients, which could contribute to the physiopathology of the disease.

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Keywords: Mucopolysaccharidosis type I; α-L-Arabinidase; IDUA gene; Enzyme replacement therapy; Oxidative stress

1. Introduction

Mucopolysaccharidosis type I (MPS I) is a rare autosomal recessive lysosomal storage disorder, caused by a deficiency of α-L-arabinidase (IDUA, EC. 3.2.1.76), which is responsible for the degradation of 2 glycosaminoglycans, dermatan sulfate and heparan sulfate. Partially degraded glycosaminoglycans accumulate in the lysosomes, leading to cell, tissue and organ dysfunction [1].

Patients with MPS I present a wide range of clinical manifestations, and although 3 phenotypes have been described (Hurler — severe, Hurler–Scheie — intermediate, Scheie — attenuated), it is well recognized that Hurler and Scheie syndromes represent both ends of a continuous spectrum of clinical heterogeneity. Hurler syndrome is characterized by onset in infancy or early childhood and death before 10 y, with severe somatic and neurological manifestations, whereas Scheie syndrome is characterized by survival until adulthood, with milder somatic manifestations and normal intelligence [1,2].

To date, >100 MPS I causing mutations have been described in the IDUA gene (Human Mutation Database — http://www.hgmd.org), and this high degree of molecular heterogeneity is thought to contribute to phenotypic diversity of the disease. 2 mutations, W402X and Q70X, have been shown to be the most frequent among European patients, accounting for 70% of the alleles in some countries [3–5]. In a study among Brazilian patients, Mate et al. [6] demonstrated that the 2 most common mutations were W402X and P533R, W402X and Q70X have been associated with a more severe clinical presentation, whereas P533R has been correlated to an attenuated phenotype [7–9].

Letter to the Editor

Chitotriosidase deficiency in Brazil: Evaluation of enzyme activity and genotypes

To the Editors:

Chitotriosidase (CT) is expressed by activated macrophages and often referred as a biochemical marker on diagnosis and prognosis for Gaucher Disease (GD), the most common lysosomal storage disorder [1]. It is well recognized that CT activity is increased in GD patients, and that activity usually decreases after enzyme replacement therapy. However, the reason and/or the role for these alterations is still not clear. Nevertheless, CT activity has been the best and most frequently used parameter to evaluate the efficacy of GD treatment and macrophage cell burden [2]. CT is encoded by CHIT1, which is a 20 kb gene with 12 exons on chromosome 1 (1q31-32). A 24 bp duplication in exon 10 causes the deletion of amino acids 334-337, abolishing enzyme activity. Homozygosis for this mutation occurs in about 6% of Caucasian populations and it has not yet been associated with any pathological condition [3,4].

A study in the European population suggests a relation among improved environmental conditions, decreased incidence of parasitic diseases and the presence of the 24 bp duplication. A higher prevalence of parasitic diseases in association with social economical problems might have contributed to the maintenance of the wild type allele in Sub-Saharan regions [5]. Another study comprising populations with African, European, and Asian ancestry observed the following 24 bp duplication allele frequencies: 74, 17%, and 56%, respectively. This same study suggested that a selective advantage for CT deficiency is confirmed by the existence of other polymorphisms that are associated with reduced enzyme activity [6]. In this work, we evaluated CT deficiency in Brazilian healthy volunteers (HV) concerning enzyme activity and genotype for the 24 bp mutation. Dried blood spots (DBS) on filter paper were obtained from 122 unrelated HV (52 males, among 18-55 years old, being 13% non-whites). Race was assigned based on morphological phenotype and family origin as used by the 1953 W180 Latin America Study of Congenital Malformations. All volunteers have signed an informed written consent (Research Ethical Committee of UNIFESP #1007/07; Research Grant FAPESP #2008/02994-4).

CT activity determination was performed in a fluorimetric assay with 30 min of incubation at 37 °C [7]. Enzyme activities below the first quarter (5.69 nmol/mL/h) were observed in 31 HV and these samples were re-assayed in a reaction with 4 h of incubation. Enzyme deficiency was characterized by CT activities below 1.0 nmol/mL/h in the 4-hour assay and it was found in 14 individuals, establishing 11.47% of CT deficient activity. The mean CT activity obtained with the exclusion of the 14 CT deficient HV was 12.63 nmol/mL/h.

All the 122 HV were genotyped in order to evaluate the frequency of 24 bp duplication. DNA was extracted from DBS and polymerase chain reaction was performed as described previously [8]. Of the 14 individuals with CT activity under 1 nmol/mL/h, 10 were identified as homozygotes for the 24 bp duplication and the other 4 were heterozygous. Another case of CT deficient genotype was found among the individuals that presented CT activities higher than the first quarter in the 30-minute assay. This subject presented a CT activity of 5.78 nmol/mL/h in the 30-minute assay and for this reason, he was not previously selected for the 4-hour assay. As to that, we decided to perform the 4-hour assay in this sample and we found a CT activity value of 0.11 nmol/mL/h, compatible with CT deficiency. As CT activity determination was firstly validated for GD patients diagnosis and prognosis, a 30-minute assay has been considered enough to correctly discriminate GD patients (very high CT activities) from healthy subjects (normal CT activities) [7]. For the purpose of CT deficiency identification, the 30-minute assay requires a complement of a 4-hour assay to confirm or exclude the enzyme deficiency (Chamois, personal communication). However, it is difficult to establish a cut-off in the 30-minute assay that would correctly select individuals for the 4-hour assay. As to that, we arbitrarily chose first quarter to segregate individuals with CT deficiency suspicion from those without. The chosen strategy failed to identify one individual and for this reason, performing the 4-hour assay directly would better investigate CT deficiency. Another important tool in cases of CT deficiency suspicion is CHIT1 genotyping. DBS is an eligible sample for CT deficiency investigation because it allows the determination of enzyme activity and it is also a source of genetic material.

CT activity assay determined a percentage of 11.47% of enzyme deficiency in our sample, while homozygosity for the 24 bp duplication was found in 9.01%.

<table>
<thead>
<tr>
<th>CHIT1 genotype</th>
<th>CT activity (nmol/mL/h)</th>
<th>WT/Mut (n=39)</th>
<th>Mut/Mut (n=52)</th>
<th>Mut/Mat (n=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Range</strong></td>
<td>1.28-48.87</td>
<td>0.00-26.44</td>
<td>0.00-6.78</td>
<td></td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td>15.72</td>
<td>10.51</td>
<td>1.25</td>
<td></td>
</tr>
<tr>
<td><strong>Median</strong></td>
<td>12.94</td>
<td>8.97</td>
<td>0.49</td>
<td></td>
</tr>
</tbody>
</table>

(WT = wild type; Mut = mutated.)
Reference values for lysosomal enzymes activities using dried blood spots samples - a Brazilian experience

Karen B Müller1, Mayra D.B. Rodrigues2, Vanessa G. Pereira1, Ana M. Martins1, Vânia D'Almeida1,*

Abstract

**Background:** Lysosomal storage diseases (LSD) are inherited disorders caused by deficiency of lysosomal enzymes in which early diagnosis is essential to provide timely treatment. This study reports interval values for the activity of lysosomal enzymes that are deficient in Mucopolysaccharidosis type I, Fabry, Gaucher and Pompe disease, using dried blood spots on filter paper (DBS) samples in a Brazilian population.

**Results:** Reference activity values were obtained from healthy volunteers samples for alpha-galactosidase A (457 ± 1.37 umol/L/h), beta-glucosidase (3.06 ± 0.99 umol/L/h), alpha-glucosidase (ratio: 13.19 ± 4.26 % inhibition: 70.66 ± 7.60), alpha-iduronidase (3.45 ± 1.21 umol/L/h) and beta-galactosidase (14.09 ± 4.36 umol/L/h).

**Conclusion:** Reference values of five lysosomal enzymes were determined for a Brazilian population sample. However, as our results differ from other laboratories, it highlights the importance of establishing specific reference values for each center.

Introduction

Lysosomal storage diseases (LSD) comprise a group of more than 40 inherited disorders caused by deficiency of specific lysosomal hydrolases, which results in the accumulation of different macromolecules in the lysosome, leading to cell dysfunction and progressive clinical manifestations [1,2].

Most LSD are inherited in an autosomal recessive manner, with the exception of Hunter, Fabry and Danon diseases that are X-linked. As a group, LSD have an incidence of about 1 per 7700 live births [3].

For some LSD as Fabry, Gaucher and Pompe diseases (FD, GD and PD respectively) and Mucopolysaccharidosis type I (MPS I), enzyme replacement therapy has been widely used as a treatment option, improving the quality of life and prognosis of these patients. Since those diseases are all progressive, early diagnosis is an essential tool for a successful treatment [2].

Biochemical diagnosis of LSD is performed by determination of enzymatic activities in different biological fluids (as plasma, leukocytes, fibroblasts and most recently dried blood spots on filter paper (DBS)), using fluorimetry, immunocapture and mass spectrometry assays [4-8]. One of the advantages for the use of DBS in the diagnosis of LSD is that these samples may be transported safely through long distances, including mailing in regular envelopes, because enzyme activities remain stable for months at room temperature [8]. In addition, the preparation of a DBS sample requires only a small amount of blood [9]. These advantages are especially important in large countries like Brazil, in which there are several reference laboratories to perform these assays.

Several studies have demonstrated the feasibility of using DBS to diagnose LSD [9-11], but it is important to establish specific reference values to each center, since enzyme activity may vary due to specific characteristics of each population and mainly because different assay conditions. Therefore, the aim of this study was to establish a reference interval value for the activities of the following lysosomal enzymes in a Brazilian population sample alpha-galactosidase A (egal A), beta-glucosidase (bglu), acid alpha-glucosidase (aglu), alpha-iduronidase (aidua), which are used for the diagnosis of FD, GD, PD
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