[Frontiers in Bioscience, Landmark, 22, 157-167, January 1, 2017]

Lysosomal alpha-mannosidase and alpha-mannosidosis

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1. ABSTRACT

Lysosomal alpha-mannosidase is ubiquitous in human tissues where is expressed in two major forms, A and B that are the product of a single gene located on chromosome 19. Mutations in the gene encoding for alpha-mannosidase cause alpha- mannosidosis, an autosomal recessive disease resulting in the accumulation of unprocessed mannose-containing oligosaccharide material. This rare disease has an estimated incidence of 1/500.000 live births and is clinically divided into three subgroups. Today the most promising therapy for this disease is the enzyme replacement therapy. To develop this strategy a mouse model for alpha-mannosidosis has been generated and a recombinant human alpha-mannosidase has been produced from Chinese Hamster Ovary cells. Interestingly, it has been shown that the recombinant enzyme, used in high dose, can cross the blood brain barrier. The recombinant enzyme has been tested in the first randomized study investigating the efficacy of the enzyme replacement therapy in patients with alpha-mannosidosis. This review contains the scientific progresses on lysosomal alpha-mannosidase from the cloning to the beginning of the therapy.

2. INTRODUCTION

Mammalian alpha-mannosidases are expressed in multiple forms in human tissues and body fluids. These enzymes play a prominent role in the glycoproteins metabolism. Alpha-mannosidases are involved in biosynthesis and processing of N-linked and free oligosaccharides, as well as in their catabolism.

Alpha-mannosidases that act in the Endoplasmic Reticulum (ER) and in Golgi compartments are referred as *processing* alpha-mannosidases, whereas the others that are involved in the breakdown of glycans are known as *catabolic* enzymes. The classification of the various alpha-mannosidases occurs as the outcome of the progress of biochemical characterization methods. Based on the results of subcellular fractionation and biochemical properties, alpha-mannosidases were classified into three groups according to their pH optimum and subcellular location: acid/lysosomal, intermediate/ Golgi and neutral/cytosolic (1). Alpha-mannosidases can be also classified into two broad classes because of their inhibition by mannose analogues. Alpha-mannosidases that are inhibited by pyranose analogues, such as 1-deoxymannojirimycin (DMM) or kifunensine (KIF), belong to Class I alpha-mannosidases (e.g. Golgi mannosidase I and Endoplasmic reticulum alpha-1,2mannosidase I) whereas alpha-mannosidases that are inhibited by furanose analogues, such as swainsonine (SW) and l,4-dideoxy-1,4-imino-D-mannitol (DIM), belong to Class II alpha-mannosidases (e.g. lysosomal and neutral alpha-mannosidase) (2-4). The two classes differ in biochemical characteristics of the enzymes, and also in the kinetic parameters for the hydrolysis of their specific substrates. Class I alpha-mannosidases hydrolyze alpha-1,2 mannoside bonds inverting the configuration of the anomeric carbon, while the Class II alpha-mannosidases retain the configuration by a mechanism that involves two catalytic side chains, the nucleophile and the general acid/base (3, 5-8). In contrast to Class I alpha-mannosidases, Class II alpha-mannosidases are able to

hydrolyze alpha-1,2, alpha-1,3 and alpha-1,6-mannoside linkages and the aryl substrate (2-3, 5). Further alpha-mannosidase classification is based on sequence homologies and separates alpha-mannosidases in two non related families: glycoside hydrolase 47 (CAZy GH47) and glycoside hydrolase 38 (CAZy GH38) (CAZy: Carbohydrate Active Enzymes database, http://www.cazy.org) (5, 9-10). Class I alpha-mannosidases belong to family GH47. GH47 mannosidases are exoglycosidases involved in processing of N-glycans and in the quality control, during the early secretory pathway. Based on substrate specificities three GH47 subfamilies have been identified: ER-alpha-mannosidase I (ERMI) that hydrolyzes a single residue from Asn-linked glycans in the ER, Golgi mannosidase I (Golgi MI) involved in glycan maturation and ER degradation-enhancing mannosidase-like (EDEM) proteins implicated in the degradation of misfolded glycoproteins (11-13). GH38 family is composted of enzymes from archea, bacteria, and eukaryotes. Class II alpha-mannosidases belong to this family. The GH38 alpha-mannosidases are exoglycosidases, which hydrolyze the terminal mannose residues from the non-reducing end of the oligosaccharides. In mammalian, five GH38 alpha-mannosidases have been identified: membrane bound Golgi II (GIIAM) and the homologous Golgi IIx (GIIxAM) alpha mannosidase, neutral alpha-mannosidase (MAN2C1), core specific lysosomal alpha-1,6 mannosidase (MAN2B2) and lysosomal alpha-mannosidase (MAN2B1) (14-19).

Lysosomal alpha-mannosidase takes part in the sequential degradation of complex, hybrid and high-mannose N-linked oligosaccharides. A deficiency of this enzyme causes alpha-mannosidosis, a lysosomal storage disorder (LSD) described in humans, cattle, cats and guinea pigs (20-23).

3. LYSOSOMAL ALPHA-MANNOSIDASE

Lysosomal alpha-mannosidase (LAMAN, EC 3.2.1.2.4.) has an acidic pH optimum of 4.5, is activated by Zn^{2+} and is ubiquitous in eukaryotic cells. In mammalian, lysosomal alpha-mannosidase is expressed in two immunologically identical isoenzymes, A and B, which can be separated by ion-exchange chromatography on DEAE-cellulose (24-25). The difference in the pI of the two forms may be the result of differences in post-translational processing and degradation of the common precursor (24). The A and B isoenzymes have similar thermal stabilities, molecular masses and broad specificities towards natural and synthetic substrates, but differently from alpha-mannosidase A, alpha-mannosidase B is taken up into fibroblast in colture by receptor-mediate endocytosis (26-27). The proportion of the two forms vary from tissue to tissue (28-29). The A and B forms are the product of a single gene (MAN2B1) as demonstrated in the lysosomal storage disease alpha-mannosidosis in which both A and B forms are lacking (30-31).

Lysosomal alpha-mannosidase is synthesized as a single chain-precursor which is processed into several smaller polypeptides. Biochemical studies have revealed discrepancy in the number and composition of the enzyme subunits among different human tissues. However these differences may be the consequence of the different purification methods. In human fibroblasts, lysosomal alpha-mannosidase is expressed as a 110 kDa precursor which is processed in glycopeptides with molecular weights ranging from 40 to 67 kDa (32). In human placenta, the enzyme is synthesized as a single-chain precursor which is processed into three glycopeptides of 70, 42 and 15 kDa. The 70 kDa peptide is further partially hydrolyzed into three additional, heavily glycosilated peptides (a:39 kDa, b:8-10 kDa, c:20-22 kDa) joined by disulfide bridges (33-35). In human liver, the form A is composted of equimolar proportion of 62 and 26 kDa subunits which form a 280 kDa native glycoprotein, while the isoenzyme B has a native molecular weight of 260 kDa and is composted of subunits of 58, 62 and 26 kDa (24).

The MAN2B1 gene is localized close to the centromere of human chromosome 19 (19p13,2-q129), spans 21.5 kb of genomic DNA and contains 24 exons (36-38). The MAN2B1 gene encodes for 1011 amino acids sequence containing a putative signal peptide of 48 amino acids followed by a polypeptide sequence of 962 amino acids and 11 potential N-glycosilation sites. Human cDNA of alpha-mannosidase is 80% identical to the bovine. The three-dimensional structure of bovine alpha-mannosidase has been resolved (PDB ID:1O7D), providing a close structural model for the human enzyme (39). Lysosomal alpha-mannosidase consists of four domains: a N-terminal alpha/beta –domain, and three all-beta-domains. The N-terminal alpha/beta domain contains the active site and is formed by the a- and b-peptides. It is followed by a three-helix bundle joining the b and c-peptides, and three mainly β-sheet domains formed by peptides c, d and e (39). The mature enzyme is a dimer (33, 40).

The cloning and characterization of the gene have been of fundamental importance in identifying mutations causing alpha-mannosidosis. Two full lenght cDNAs have been isolated and sequenced (30, 33). Multiple transcription start sites have been identified at positions -191, -196, and -309 and at positions -28, and -20 (37-38). The 5' flanking region contains several GC-rich regions and putative binding sites for the transcription factors Sp1, AP-2 and ETF, but neither TATA nor CAAT boxes have been identified. Promoter analysis showed that only 150 bp of the 5' flanking region from the first ATG was required for promoter activity. Northern blotting analysis showed a main transcript of about 3 kb in all tissues and a minor of 3.6 kb only in some adult tissues (30). A shorter 2.3 kb mRNA was also identified by Sun *et al* by RT-PCR (41). The level of MAN2B1 gene expression is highest in lung followed by kidney, pancreas

and liver (30). A much lower expression was observed in other tissues. In the brain MAN2B1 is expressed at very low levels. The highest levels of expression appear to be in corpus callosum and the spinal cord whereas lower levels were observed in cerebellum, cerebral cortex, frontal and temporal lobes, and occipital pole (33).

4. MOUSE LYSOSOMAL ALPHA-MANNOSIDASE

The mouse alpha-mannosidase is two amino acids longer than the human. Comparison of the deduced amino acid sequences of the mouse and human alpha-mannosidase showed that they have 75% identity and 83% similarity. As in human, also in mouse tissues two isoforms of alpha-mannosidase can be identified on DEAE-cellulose: a basic form (A) and a more acidic component (B) similar to those seen in human under identical conditions. Both forms have been found in testis, brain, spleen and kidney, whereas in epididymis and liver only the B form was present (26).

The gene encoding the lysosomal alpha-mannosidase in mouse is located on the chromosome 8, is composted by 24 exons and spans approximately 16 kb (40,42-44). cDNA have been isolated, sequenced and used to map the alpha-mannosidase gene (26-27, 43). The cDNA contains an open reading frame of 3036 bp. A single polyadenilation signal (ATTAAA) 14 bp upstream from the poly(A)+ tail has been identified. This occurs in only 10% of mRNAs and the same polyadenilation signal is conserved in the human alpha-mannosidase cDNA sequence (43). In the murine Man2b1gene, three different transcription start sites have been identified at position -131, -149 and -174 (44). In both human and mouse gene two possible ATG codons have been identified, but only the sequence that precedes the first ATG corresponds closely to the consensus sequence described by Kozak (45). Thus, the first ATG probably corresponds to the real start codon. The 5'-flanking sequence of the mouse gene contains GC-rich regions, TATA and CAAT motifs and potential Sp1, AP2 and PEA3 binding sites (44).

5. ALPHA-MANNOSIDOSIS AND MUTATIONS IN THE MAN2B1 GENE

Alpha-mannosidosis (MIM 248500) is an autosomal recessive inherited disorder that occurs in 1/500.0.00 live births and according to the European Union regulation, is designed as an "orphan" disease (46). It is caused by the lack of the lysosomal alpha-mannosidase activity, which results in the accumulation of unprocessed mannose-containing oligosaccharide in tissues.

Alpha-mannosidosis was described for the first time by the Swedish physician Öckerman (20). He found large amounts of oligosaccharide material, with the dominance of mannose, stored in tissues from a 4 years old boy with a Hurler-like phenotype. Based on it, *Mannosidosis* was suggested as the name of the disorder (47).

Alpha-mannosidosis has been described in humans, cattle, domestic cats and guinea pigs (21-23, 48-49). It is characterized by immune deficiency, recurrent infections, facial and skeletal deformation, hearing impairment, and mental retardation. Other common findings are elevation of serum and urine oligosaccharides levels, enlargement of lysosomes in most type of cells, lens opacities, muscular hypotonia, macroglossia and pancitopenia. The human disease has been described as three distinct phenotypes according to the age of onset and the symptoms. The mild form affects patients older than 10 years and is characterized by very slow progression and by the absence of skeletal abnormalities; the moderate form, clinically recognized before 10 years of age, is characterized by skeletal abnormalities and slow progression with development of ataxia, and the severe infantile form that leads to an early death. The classification is often not straightforward, since the patients have a variety of symptoms (46).

Alpha-mannosidosis diagnosis is made by measuring lysosomal alpha-mannosidase activity in leukocytes or other nucleated considered cells and can be confirmed by genetic testing (46). The assay of acid alpha-mannosidase activity is the most efficient and reliable method for the alpha-mannosidosis diagnosis. This is performed a pH 4.5 with the substrate 4-methylumbelliferyl-alpha-D-mannopyranoside.In peripheral blood leukocytes of affected people the acid alpha-mannosidase enzyme activity is 5%-15% of normal activity whereas in carriers it is usually 40-60% of normal. Thus, due to the overlap between carriers and non-carriers, the detection of carriers is difficult to perform. The presence of mannose-rich oligosaccharides in the urine can be also investigated by thin-layer chromatography or high performance liquid chromatography. Nevertheless this finding cannot be considered as diagnostic, but only as suggestive of alpha-mannosidosis (50-51). To confirm the diagnosis, the identification of the disease causing mutations is also carried-out by DNA sequencing.

The currently known 155 alpha-mannosidosis causing mutations are scatterated over the whole length of the MAN2B1 gene and are highly heterogeneous (e.g. frame shifting small insertions, deletions, missense, nonsense and splicing site mutations, duplications and large deletions) (52). A total of 125 different disease causing mutations have been reported in the literature (33, 53-62). Over 40 missense mutations have been described which represent the 27,7% of the all known MAN2B1 gene mutations (http://amamutdb.no). A recent study has identified 96 disease-associated sequence variants in 130 unrelated alpha-mannosidosis

patients from 30 countries. Most of these mutations were unique to each family, but three mutations c.2248C>T (p.R750W), c.1830+1G>C and c.2426T>C (p.L809P), were relatively frequent, and accounted for approximately 27%, 5% and 3% respectively of the disease alleles (62).

Depending on the causative mutation, mutant lysosomal alpha-mannosidase can be folded incorrectly and arrested in the ER or can be folded correctly and transported to the lysosomes in the inactive form (63-64). Some studies analyzed the consequences of the missense mutations on the structure and folding of the enzyme using the resolved 3D structure of the bovine alpha-mannosidase. The human p.R750W mutation affects the interactions between the d and e-domain interface, whereas the mutations resulting in p.H200L or p.H200N remove hydrogen bonding and destabilize the active site (39, 57, 60, 63, 65). It has been found that the variant enzymes p.R22H and p.H72L are transported to the lysosome, but they do not show alpha-mannosidase activity. In particular it has been shown that the mutation p.H72L affects zinc binding and leads to the loss of enzymatic activity (33, 39, 63, 65).

Despite the available genetic data, the phenotypic variability in alpha-mannosidosis patients is high even between siblings with identical genotype. Thus the phenotype-genotype correlations are difficult to identify in alpha-mannosidosis (66-69). Recently a correlation between genotype/phenotype and subcellular localization was published on patients affected by alpha-mannosidosis type II (70). Forty-five known mutations and 11 novel mutations were identified in 66 patients from 57 families. In accordance with previous reports the p.R750W mutation resulted the most frequent (54, 62). Furthermore it has been found that genotypes that allowed to the mutant MAN2B1 proteins to enter into the lysosomes, correlated positively with several clinical and biochemical parameters. On the basis of the mutation analysis, and the predicted effect of mutation, the patients were divided into three subgroups showing three different genotypes/subcellular localizations. In the subgroup 1 nonsense, frameshift mutations and truncations are present, whereas in subgroup 2 all mutations that modify the folding of the enzyme (missense, in–frame mutations, duplications and deletions) are present. These mutations do not allow the movement of the alpha-mannosidase to the lysosomes. Only the mutations in subgroups 3 (missense, in-frame, duplication/deletion mutations), allow the localization of the mutant MAN2B1 protein to the lysosomes. It seems that they lead to develop a milder phenotype in alpha-mannosidosis patients.

More recently an alpha-mannosidosis mutation database (http://amamutdb.no) has been constructed as a publicly accessible online resource for recording and analyzing MAN2B1 variants (52). The database provides a complete overview of all nucleotide variants identified in alpha-mannosidosis and corresponding demographic and clinical data. It could facilitate and increase the understanding of the clinical and molecular aspects of alpha-mannosidosis. It provides a wide range of molecular information for pathophysiological studies and patient descriptions for clinical use, allowing the improvement of the interactions between the clinical and molecular sciences involved in the disease. Availability of clinical data will be beneficial for molecular scientists, who aim to solve genotype-phenotype correlations, consequences of accumulation of the unhydrolyzed oligosaccharides, and attempt to develop specific treatments for alpha-mannosidosis. For physicians, the molecular data will, in the future, provide tools for personalized treatment options, depending on the molecular pathophysiology of the patient.

6. ANIMAL MODELS

Alpha-mannosidosis naturally occurs in cattle, cats and guinea pigs. In cattle alpha-mannosidosis was first described in New Zealand and Australia where about the 10% of Angus cattle are heterogeneous carriers of abnormal MAN2B1 gene (21, 49). The majority of the affected calves are stillborn or fail to survive the immediate postnatal period. The animals that survive show severe and progressive neurological disease characterized by tremors of the head, ataxia and aggression (71-72).

Feline alpha-mannosidosis shows similar clinical, pathological, and biological manifestations including action tremors, intention tremors of the head and neck, loss of balance, spinal ataxia, corneal and lenticular opacitiese. All signs are progressive (22, 73-75). Feline affected by alpha-mannosidosis are used to study the effects of different therapy strategies in large animal models (76-78).

Alpha-mannosidosis has been found also occurring in guinea pigs where it resembles the clinical, and molecular features of the human disease. The affected animals show stunted growth, apathy and behavioral abnormalities. Based on these traits they result useful models to investigate efficacy of enzyme replacement therapy as a treatment for alpha-mannosidosis (23, 79-80).

In 1999 an alpha-mannosidosis mouse model has been generated by targeted disruption of MAN2B1 gene (81). This model expresses neither specific alpha-mannosidase mRNA nor shows alpha-mannosidase activity, and develops a biochemical and morphological phenotype closely resembling that of naturally occurring in human and in the other affected animal models. Alpha-mannosidase-deficient mice did not exhibit differences in growth and weight development when compared to wild type mice. They were fertile and did not show an elevated mortality. In alpha-mannosidosis mouse model an elevated urinary secretion of mannose-containing oligosaccharides similar to that observed in human patients has been found. In liver, kidney, spleen, brain

and testis of alpha-mannosidase-deficient mice, an increased amount of neutral sugars was observed. This was only moderate in liver, whereas a pronounced content of neutral sugars was observed in the other tissues. A prominent lysosomal storage in different cells of liver, kidney, spleen, pancreas, testis, eye, thyroid gland, smooth muscle, bone and the central and the peripheral nervous systems was also observed. This model has been used to study the pathogenesis of the disease and evaluate new therapeutic approaches (82-83).

7. TOWARDS A THERAPY FOR ALPHA-MANNOSIDOSIS

Several approaches have been evaluated for the treatment of alpha-mannosidosis and most in general for the therapy of the lysosomal storage disorders (LSD). These methods include enzyme replacement therapy (ERT), bone marrow transplantation (BMT) and gene therapy (76, 80, 82, 84). Today the most promising therapy results to be the ERT which is based on the administration of the recombinant active enzyme in the blood stream of patients. The enzyme is internalized by the cells and reaches the lysosomes replacing the missing endogenous enzyme. Although ERT can give significant positive results, the involvement of the central nervous system is a problem for this therapy. The blood brain barrier prevents the crossing of lysosomal enzymes from the blood to the interstitial space surrounding neuronal and glial cells of the central nervous system (85). Nevertheless it has been found that ERT can have some beneficial effects on brain function in LSD (86).

The production of the recombinant lysosomal alpha-mannosidase (LAMAN) and the generation of the alpha-mannosidosis mouse model have made possible to study the efficacy of ERT in this LSD (82). In one study LAMAN has been purified from three different species, bovine, human and mouse and used for ERT in alpha-mannosidosis mice. A remarkable correction of storage (reduction of 80-90% of the stored material) was observed in spleen and kidney after the injection of 250mU of recombinant human LAMAN/g body weight. A subsequent injection after 3.5 days was sufficient to clean liver, kidney and heart from neutral oligosaccharides. The decrease of neutral oligosaccharides concentration in brain was also observed. It cannot be attributed to an uptake of LAMAN into neural cells because it has been demonstrated that the lysosomal enzymes administrated intravenously in mice after the first 2 weeks of life do not cross the blood-brain barrier (87). Thus the alpha-mannosidase activity has been attributed to extra-neural cells, such as endothelial cells and choroid plexus epithelium. Another hypothesis is that the clearance of oligosaccharides via blood circulation or via an improved flow of the cerebrospinal fluid can contribute to the decrease of neutral oligosaccharide storage in brain (82). More recently Blanz and coworkers have shown the dose-dependent effect on visceral and nervous system pathology using low phosphorilated rhLAMAN. They found that the storage in peripheral tissues was efficiently reduced with low-dose injections (25U/Kg), whereas in neurons of the central nervous system significant reduction of the stored material was appeared after high-dose treatment (250U/Kg). In the hippocampal neurons the injected enzyme was detected after the administration of very high dose of the recombinant enzyme (1000U/Kg) indicating that in this conditions the enzyme is able to cross the blood-brain barrier and to be taken up by the cells. Uptake of rhLAMAN in the hippocampal neurons seems to be independent of mannose-6-phosphate receptors. It has been hypothesized that other receptors than mannose-6-phosphate or even micropinocytosis may be responsible for the uptake of rhLAMAN *in vitro* and *in vivo* (83).

For the ERT of alpha-mannosidosis, a recombinant human alpha-mannosidase has been also developed from Chinese Hamster Ovary cells from Zymenex. The rhLAMAN has been successfully used *in vitro* and *in vivo* preclinical pharmacological studies. This recombinant enzyme has been used in the first randomized study investigating the efficacy of ERT in patients with alpha-mannosidosis (88). The obtained results show improvements both concerning peripheral somatic manifestation and in parameters of integrity and cerebral function. RhLAMAN was well tolerated; the frequency of the infusion related reactions (IRR) and the development of IgG antibodies were low compared to other ERT (89-91). Furthermore a significant reduction of mannose-contain oligosaccharides, GFAP (Glial Fibrillary Acidic Protein) and p-Tau was observed in cerebrospinal fluid of the treated alpha-mannosidosis patients. These results may indicate that the recombinant human alpha-mannosidase passes the blood-brain barrier as observed by Blanz and coworkers in the alpha-mannosidosis –knock-out mice model (83). It has been also observed that rhLAMAN have a long T1/2 (91-92). This may be the result of a low mannose-6-phosphate content of the rhLAMAN, which may reduce the uptake based on the presence of mannose-6-phosphate into the extra-cerebral tissues and increase the concentration of rhLAMAN at the blood-brain barrier facilitating absorptive endocytosis (83, 93). Thus it is possible to speculate that there is not a unique uptake mechanism or a defined degree of post-translational modification of the therapeutic lysosomal enzyme needed for an efficient correction of storage in the brain.

8. ACKNOWLEDGEMENTS

We are grateful to Fondazione Cassa di Risparmio di Città di Castello, Città di Castello, Perugia, Italy for the financial support. Maria Rachele Ceccarini is currently a PhD student in “Public health and veterinary sciences” XXIX cycle.

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Key Words: alpha-mannosidase; alpha-mannosidosis; MAN2B1; alpha-mannosidosis causing mutations; animals models; ERT.

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