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Molecular Mechanisms of Pathogenesis in a Glycosphingolipid and a Glycoprotein Storage Disease

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Abstract

The lysosomal system comprises a specialized network of organelles crucial for the sorting, digestion, recycling and secretion of cellular components. With their content of hydrolytic enzymes lysosomes regulate the degradation of a multitude of substrates that reach these organelles via the biosynthetic or the endocytic route. Gene defects that affect one or more of these hydrolases lead to lysosomal storage diseases (LSD). This underscores the apparent lack of redundancy of these enzymes and the importance of the lysosomal system in cell and tissue homeostasis. Some of the lysosomal enzymes may form multiprotein complexes, which usually work synergistically on substrates and in this configuration, may respond more efficiently to changes in substrate load and composition. A well-characterized lysosomal multienzyme complex is the one comprised of the glycosidases, β -galactosidase (β -GAL) and neuramidase-1 (NEU1), and of the serine carboxypeptidase protective protein/cathepsin A (PPCA). Three neurodegenerative LSDs are caused by either single or combined deficiency of these lysosomal enzymes. Sialidosis (NEU1 deficiency) and galactosialidosis (combined NEU1 and β -gal deficiency, secondary to a primary defect of PPCA) belong to the glycoprotein storage diseases, whereas GM1-gangliosidosis (β -GAL deficiency) is a glycosphingolipid storage disease. Identification of novel molecular pathways that are deregulated because of loss of enzyme activity and/or accumulation of specific metabolites in various cell types has shed light on mechanisms of disease pathogenesis and may pave the way for future development of new therapies for these LSDs.

A Multifunctional Lysosomal Enzyme Complex

The lysosomal multienzyme complex specified by PPCA, NEU1 and β -GAL is tailored to efficiently control the hydrolysis of complex glycoconjugates, starting at their non-reducing end with the cleavage of sialic acids and β -galactosyl moieties. At least another glycosidase, N-acetylgalactosamine-6-sulfate sulfatase (GALNS), is loosely associated with this complex, which may include additional hydrolases needed for the subsequent removal of sugar nucleotides, albeit this has not been experimentally proven [1–4]. Besides their canonical degradative capacity towards specific classes of glycoconjugates, it is becoming apparent that each of the enzymes may exert additional functions either within or outside the complex.

PPCA and Galactosialidosis

Mammalian PPCA is a member of the serine protease family of enzymes and in this capacity exerts carboxypeptidase, deamidase and esterase activities on selected bioactive peptides, like substance P, oxytocin, and endothelin 1 [5,6]. The enzyme precursor is a zymogen that gets activated in the endosomal/lysosomal compartment by partial proteolysis. The 3D structure of the PPCA zymogen, the first determined for a lysosomal enzyme precursor, unraveled the structural mechanism of its catalytic activation [7]. Distinct from its catalytic activity, PPCA has a protective function towards at least two other lysosomal glycosidases,

β -GAL and NEU1, with which it is found in complex. Early studies using subcellular fractionation, co-purification and co-immunoprecipitation methods in cultured cells and mammalian tissues have demonstrated that interaction of the PPCA zymogen with β -GAL and NEU1 occurs soon after synthesis of the proteins [2]. This early interaction assures the regulated trafficking of the two glycosidases to the lysosome as well as their intralysosomal activation and stability. More recently, the use of analytical ultracentrifugation and surface plasmon resonance techniques has enabled a better assessment of the influence of PPCA/NEU1 assembly on the overall biochemical and structural properties of NEU1 [8]. This study suggests a new and complex mechanism in which PPCA competes with NEU1 for the same binding sites, preventing premature oligomerization and catalytic activation of the glycosidase, but allowing the formation of a heterodimeric complex suited for lysosomal targeting [8]. The surface plasmon resonance data of the two enzymes led to the proposition of a structural model of the PPCA/NEU1 heterodimeric complex (Fig. 1). These studies also define PPCA as a molecular chaperone for NEU1's lysosomal localization and activation [8]. The determination of the 3D structure of the two enzymes in complex should ultimately explain their interdependent mode of action on target substrates.

In humans, only mutations that interfere with the protective function of PPCA have been identified [9,10]. They result in complete loss of NEU1 activity and reduced β -GAL activity, hence providing the genetic evidence of PPCA interaction with the two glycosidases. The combined β -GAL/NEU1 deficiency is the biochemical hallmark of the neurodegenerative LSD galactosialidosis (GS). It is still unclear, however, whether an isolated loss of cathepsin A activity could have pathological consequences, although some authors have demonstrated that an enzyme hydrolyzing the C-terminus of endothelin-1 was deficient in tissues from a GS patient, and implicated PPCA as the major endothelin-1-degrading enzyme present in human tissues [10]. GS patients are clinically heterogeneous and differ widely in severity and age of onset of the symptoms. The severe, early onset forms develop a systemic condition associated with foetal hydrops, skeletal dysplasia, visceromegaly, renal and cardiac failure, variable neurological involvement and early death [9]. They have phenotypic manifestations similar to those found in patients with the severe form of sialidosis, a related disorder of glycoprotein metabolism that is caused by an isolated deficiency of NEU1 (see below). The loss of NEU1 activity in both diseases accounts for the storage of essentially the same type of sialylated oligosaccharides and glycopeptides in patients' tissues and body fluids, which likely contribute to disease pathogenesis. Correlation of genotype with disease severity has emerged from structure/function studies of mutant PPCA variants [7].

The mouse model of GS was the first generated in my laboratory and one of the first genetically engineered animal models of an LSD [4]. *Ppca*-null mice have pathologic manifestations reminiscent of patients with GS; symptoms include progressive nephropathy, hepatosplenomegaly, and shortened lifespan (~12 months) [4]. In addition, *Ppca*^{-/-} mice develop severe and progressive ataxia that is associated with gradual loss of cerebellar Purkinje cells [4,11]. This phenotype could either be due to the combined deficiency of NEU1 and PPCA or the isolated deficiency of cathepsin A, although no specific neuronal targets for this enzyme that would explain the loss of Purkinje cells in the GS model have been identified to date. However, the potential physiological esterase/deamidase activity of PPCA in platelets [12], endothelial cells [6], heart [13] and kidney towards several bioactive peptides that regulate blood circulation and blood pressure, could justify the occurrence of cardiomyopathy and arterial hypertension that are often diagnosed in patients with GS [9]. A cardiovascular role of *Ppca* has been recently suggested by a study in a knockin mouse model of PPCA deficiency that carries an amino acid substitution abrogating the catalytic activity but not the protective function of the protein [14]

NEU1 and sialidosis

Neuraminidase 1 (NEU1) belongs to the family of sialidases or neuraminidases, which are widely distributed in nature. These enzymes have similar substrate specificity and catalyze the cleavage of ketosidically-linked sialic acid residues on glycoproteins, glycolipids (gangliosides), and oligo- or poly-saccharides [15]. The existence of four mammalian sialidases (NEU1–4) that differ in their subcellular localization, substrate specificity, and pH optimum underscores the importance of these enzymes in basic cellular processes, including cell proliferation, differentiation and immunity. These processes are strongly influenced by the extent of sialic acid residues on receptors and/or ligands. NEU1 is the most abundant and widely expressed mammalian sialidase, being present in nearly all vertebrate tissues and cell types. Curiously, NEU1 has a higher sequence identity to several well-characterized bacterial sialidases than to the other three mammalian sialidases, which are more similar to each other than to NEU1 [16,17].

Lysosomal NEU1, the second component of the multi-enzyme complex, is the only mammalian sialidase that is involved in two LSDs: sialidosis, caused by structural mutations at the *NEU1* locus, and GS. Sialidosis patients with the early onset form of the disease have a mucopolysaccharidosis-like phenotype with visceromegaly, dysostosis multiplex and mental retardation [18]. Late onset, milder cases are characterized by ocular cherry-red spot and generalized myoclonus, but some of them may suffer from seizures and ataxia [18].

Neu1^{-/-} mice manifest a multi systemic disease similar to severe sialidosis [19]. One of the phenotypes that these mice share with sialidosis patients is a time-dependent enlargement of the spleen, with features consistent with splenic extramedullary hematopoiesis (EMH). While investigating the molecular bases of this phenotype, we discovered a new function of NEU1 as a negative regulator of lysosomal exocytosis [20]. We found that in hematopoietic cells NEU1 controls the extent of sialylation and turnover of the lysosomal associated membrane protein LAMP-1. Loss of NEU1 function results in hypersialylation of LAMP-1 at the lysosomal membrane, which changes the fate of these organelles making them poised to preferentially dock at the PM and engage in lysosomal exocytosis (Fig. 2). The downstream effect is excessive exocytosis of lysosomal content, particularly serine proteases, in the bone niche, which culminates in the premature degradation of VCAM-1, and loss of bone marrow retention [20]. These findings identify sialidosis as the only known genetic disease in which lysosomal exocytosis is exacerbated [20]. Given the multi-systemic nature of the disorder and the fact that lysosomal exocytosis is a basic physiological process in many cell types we predicted that abnormal exocytosis may underlie other phenotypic abnormalities in sialidosis.

In fact, enhanced lysosomal exocytosis in *Neu1*-null mice could contribute to the profound hearing loss and muscle atrophy characteristic of the disease in sialidosis children [21,22]. Progressive morphological abnormalities and vacuolization of the marginal cells of the stria vascularis in the cochlea of these mice is associated with oversialylation of LAMPs, most importantly LAMP-1. The increased expression and apical localization of LAMP-1 in these cells suggests exacerbation of lysosomal exocytosis into the endolymph, resulting in reduced endolymphatic potential and failure to transduce sound through sensory hair cells [21].

In skeletal muscle tissue, loss of Neu1 activity and consequent exacerbation of lysosomal exocytosis of fibroblasts may underlie the observed abnormal remodeling of the extracellular matrix (ECM) and excessive proliferation of connective tissue [22]. Muscle fibers juxtaposed to the expanded perimysium and epimysium become infiltrated by fibroblasts and components of the ECM, leading to their progressive fragmentation and

ultimately destruction [22]. Together, these findings may offer the molecular basis for the muscle weakness and atrophy associated with sialidosis in children.

β -Galactosidase and GM1-gangliosidosis

While both genetic and biochemical studies support the strict requirement of NEU1 for PPCA, it is less clear to which extend β -GAL, the third component of the multienzyme complex, rely on PPCA for its function. Although its interaction with PPCA aids the trafficking of the enzyme to the lysosomes and influences its stability, the partial deficiency of β -GAL in GS patients and the apparent normal β -GAL activity in the GS mouse model suggest a more loose dependence of this enzyme on PPCA. In fact, β -GAL maintains catalytic activity outside the complex. The enzyme is largely responsible for the intralysosomal degradation of GM1-ganglioside (GM1), a major glycosphingolipid of neuronal membranes, and keratan sulfate [23,24]. Deficiency of β -GAL in human's leads to GM1-gangliosidosis, a severe, generalized neurodegenerative disorder affecting primarily the CNS, and to Morquio B syndrome, a milder condition characterized by skeletal dysplasia and corneal clouding. The major clinical signs of GM1-gangliosidosis include progressive neurologic deterioration, macular cherry red spot, facial dysmorphism, hepatosplenomegaly and generalized skeletal dysplasia [23].

The mouse model of this disease closely recapitulates the CNS phenotype seen in patients and develop tremor, ataxia, abnormal gait and paralysis of the hind limbs [25]. Studies on this mouse model brought to the discovery of previously unknown molecular pathways that are induced by GM1 accumulation and result in neurodegeneration and neuronal apoptosis. We found that defective lysosomal degradation of GM1 leads to an increased amount of this ganglioside at the ER membranes, where it induces depletion of ER Ca^{2+} stores and activation of the unfolded protein response [26,27]. We subsequently demonstrated that GM1 accumulates specifically in glycosphingolipid-enriched fractions (GEMs) of the mitochondria-associated ER membranes (MAMs), which are the sites of apposition between ER and mitochondria. This finding suggests that accumulation of GM1 favors the formation of contact sites between the ER and the mitochondrial membranes (Fig. 3). At these microdomains GM1 interacts with the IP3R Ca^{2+} channel and, in turn, influences Ca^{2+} flux between these organelles. The ensuing mitochondrial Ca^{2+} overload ultimately activates the mitochondrial leg of apoptosis. These exciting results enabled us to propose a novel mechanism of Ca^{2+} -mediated apoptotic signaling, where a lipid rather than a protein acts as molecular effector of both ER stress-induced and mitochondrial-mediated apoptosis [28]. Overall these studies may help us to uncover new functions of GM1 and β -GAL in normal cell physiology, and to design more efficacious therapies for this devastating disease.

Therapy

Therapy for LSDs is based on the mechanism of cross-correction: i.e. on the capacity of soluble lysosomal enzyme precursors to be secreted and recaptured by cells, be routed to the lysosomes and function normally (reviewed in Beck) [29]. Based on their molecular and biochemical bases, sialidosis, galactosialidosis and GM1-gangliosidosis bring in different challenges from the standpoint of therapy. However, the mouse models of these diseases have made possible to test different therapeutic approaches, including transplantation of normal or overexpressing bone marrow (BMT), enzyme replacement therapy (ERT), substrate reduction therapy (SRT), as well as ex vivo and in vivo gene therapy [29]. The results of these studies have reiterated that the same therapeutic approach might not be successful in all LSDs and that any therapeutic attempt in different LSDs should be accompanied by a thorough understanding of the molecular bases of pathogenesis. For instance, in our model systems we found that BMT improved the phenotype of *Ppca*^{-/-}

mice, but failed in *Neu1*^{-/-} mice because of lack of engraftment of the donor BM [4,30]. ERT studies with recombinant enzyme in the GS model resolved lysosomal storage in their systemic organs [31], while the same approach used in the sialidosis model was only partially successful because the recombinant enzyme elicited an immune response [32]. SRT has proven efficacious for ameliorating disease symptoms in the GM1-gangliosidosis mouse model [33].

BM-mediated ex vivo gene therapy was also tested in both *Ppca*^{-/-} and *β-Gal*^{-/-} mice with very encouraging results, including partial correction of the CNS phenotype [11,34]. An interesting observation was that in the *β-Gal*^{-/-} mice the microgliosis that ensues at the site of neuronal apoptosis [35] initiates an inflammatory response accompanied by activation of specific cytokines and chemokines. We found that this pathologic feature creates a local microenvironment for the recruitment into the CNS of genetically-modified BM monocytes overexpressing β-Gal. The resulting cross correction of neighboring neurons led to reduction of GM1 storage, and hence reversal of activation of the unfolded protein response and partial rescue of the CNS phenotype.

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Figure 1. Structural model of PPCA-NEU1 heterodimer. PPCA-NEU1 heterodimeric complex: NEU1 peptides 33, 34 and 42 [34] interact with the PPCA excision peptide, and NEU1 peptide 48 [34] interacts with the PPCA peptide 65 (red). Adapted from Bonten et al, 2009 [8].

Figure 2. Immunofluorescence detection of Lamp-1 and confocal microscopy of Neu1-deficient macrophages. Non-permeabilized cultured macrophages were incubated with a Lamp-1 primary antibody and a FITC-conjugated secondary antibody. Nuclei were labeled with DAPI. Confocal microscopy shows the exclusive presence of Lamp-1 at the cell surface (arrows) in KO macrophages. Magnification is 400x. Adapted from Yogalingam et al, 2008 [20]

Figure 3. GM1 accumulation in β -Gal-null neurons promotes the formation of MAMs. Representative electron micrograph of mitochondria isolated from β -Gal^{-/-} brains shows multiple contact sites between ER (asterisks) and a mitochondrion. Direct magnification is 20,000x. Adapted from Sano et al., 2009 [28].