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Diagnostic Application of Lysosomal Exoglycosidases

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Abstract

Lysosomal exoglycosidases gradually degrade oligosaccharide chains of glycoconjugates (glycoproteins, glycolipids, glycosaminoglycans) in cell lysosomes. Defect in the activity of suitable lysosomal exoglycosidase stops degradation of oligosaccharide chains on sugar molecules not released by deficient exoglycosidase, and nondegraded oligosaccharide chains are stored in enlarged lysosomes. Enlarged lysosomes damage remaining cell structures and disturb the function of involved tissues, causing storage diseases. An increase in the activity of exoglycosidases in tissues and body fluids is observed in the reconstruction of damaged tissues. Exoglycosidase activity is an inexpensive and sensitive marker in diagnostics and monitoring of many diseases.

Keywords: lysosomal exoglycosidases, fucosidase (FUC), β -D-galactosidase (GAL), β -D-glucuronidase (GLU), N-acetyl- β -hexosaminidase (HEX), α - and β -mannosidases (MAN)

1. Introduction: lysosomes

Inside lysosomes, more than 50 hydrolytic enzymes (glycosidases, proteases, lipases, nucleases, phosphatases, sulfatases, etc.) that are able to degrade all types of cell macromolecules are located. Lysosomal enzymes are active at acidic (pH ~5.0) water environment. High intralysosomal $[H^+]$ (about 100x higher than in cytoplasm) is maintained by vacuolar H^+ , V-type ATPase, located in the lysosomal membrane, which uses the energy of ATP hydrolysis to pump protons into lysosomes [1–3]. Lysosomal unique highly acidic environment creates some sort of protection for cytoplasmic components against noncontrolled autodigestion, additionally reinforced by integral proteins of the lysosomal membrane that are highly glycosylated to protect both

lysosomal membrane and cytosolic elements against autodigestion [2, 4, 5]. Furthermore, some of the membrane glycoproteins function as specific receptors for molecules destined to degradation in lysosomes [3].

Designated for autodigestion, extracellular high-molecular substances reach lysosomes by endocytosis, pinocytosis, and phagocytosis [6]. Intracellular high-molecular substances are digested by autophagy [7]; autophagy eliminates waste or damaged parts of the living cells. There are many types of autophagy: macroautophagy, microautophagy, chaperone-dependent autophagy, and specific autophagy. Autophagy may also be classified according to the digested material, e.g., mitophagy (digestion of mitochondria) or nucleophagy (digestion of nuclear debris) [8–10]. The best described is macroautophagy, where the cellular region destined for digestion is surrounded by the phospholipid membrane creating autophagosome. Then autophagosome merges with lysosome, where acid hydrolases degrade autosome contents into simple organic compounds, ready for utilization by the cell [11]. Additionally, autophagy provides the cells with energy [12]. Autophagy may be induced by hypoxia-caused stress, hunger, radiation, inflammation, and so on [8]. In the case of pathological autophagy, cells exposed to intracellular toxins suffer from defective metabolism and die. Some of the researchers suspect that deficient autophagy may initiate many diseases such as diabetes or Alzheimer's disease [8], or even cancerogenesis. On the other hand, excessive autophagy may facilitate the survival of neoplastic cells during harmful conditions (e.g. chemotherapy). Therefore, autophagy in neoplasia may have dual biological sense [13].

2. Lysosomal enzymes

In autophagy, lysosomal acid proteases and glycosidases play a main role. Proteases cleave peptide bonds in the middle (endopeptidases) or outside (exopeptidases) of polypeptide chains. Main group of lysosomal proteolytic enzymes constitute cathepsins [14, 15], having aspartate (cathepsin D and E), cysteine (cathepsins B, C, H, K, and L), or serine (cathepsins A and G) in the active site [16, 17]. Proteases (PROT) (**Figure 1**) facilitate the action of three groups of glycosidases that gradually degrade tissue glycoconjugates (glycoproteins, glycolipids, and glycosaminoglycans): **aminohydrolases** that hydrolyze the N-glycosidic linkage between amino group of polypeptide chain asparagine and N-acetylglucosamine of oligosaccharide chain of glycoprotein, **endoglycosidases** that hydrolyze O-glycosidic bonds inside of oligosaccharide chains, and **exoglycosidases** that hydrolyze O-glycosidic bonds releasing sugars from nonreducing and reducing terminals of oligosaccharide chains [18].

Aminohydrolases as well as endo- and exoglycosidases create a sequence of reactions where the product of the previous enzyme is the substrate for the subsequent enzyme (**Figure 1**), and oligosaccharide is digested from reducing and nonreducing ends. When neuraminidase (NEU) releases N-acetylneuraminic acid (NANA) from the nonreducing ends of oligosaccharide chains, PROT degrade protein cores of glycoproteins, releasing reducing ends of oligosaccharides with attached asparagines. Oligosaccharides deprived of NANA are substrates for appropriate exoglycosidases depending on oligosaccharide composition.

Figure 1. A sequential degradation of glycoproteins in lysosomes based on Ref. [19]. **Asn**—asparagine, **Fuc**—fucose, **Gal**—galactose, **GlcNAc**—N-acetylglucosamine, **Man**—mannose, **NANA**—N-acetylneuraminic acid, **Asp-GlcNAc**—aspartylglucosaminidase, **E-GlcNAc**—endo N-acetylglucosaminidase, **FUC**— α -fucosidase, **GAL**— β -galactosidase, **HEX**—N-acetyl- β -hexosaminidase, **MAN**— α - and β -mannosidases, **NEU**—neuraminidase (sialidase), **PROT**—proteases.

Oligosaccharides with β -D- galactose on non-reducing ends are substrates for β - galactosidase and oligosaccharides with α -L-fucose near reducing ends are substrates for α -L-fucosidase (**Figure 1**). Then, oligosaccharide chains are degraded by aspartylglucosaminidase (Asp-GlcNAc) that hydrolyses N-glycosidic bond between N-acetylglucosamine of the reducing end of oligosaccharide and asparagine remained from polypeptide as well as endo-N-acetylglucosaminidase (E-GlcNAc) releasing N-acetylglucosamine from the reducing end

of oligosaccharide chains. N-acetylhexosaminidase (NAG, N-acetyl- β -hexosaminidase (HEX)) releases N-acetylglucosamine and N-acetylgalactosamine from a nonreducing end of the remaining part of oligosaccharide chains. Oligosaccharides containing mannose are substrates of α - and β -mannosidases (**Figure 1**). Lack or deficiency of a particular exoglycosidase blocks catabolism of oligosaccharide chains on a nondetached sugar residue [19]. Disorders in the activity of lysosomal enzymes are closely related to autophagy and reflect intensity of development and course of many diseases, for example, infections, inflammations, cancers, heart diseases, Crohn's disease, myopathy, liver diseases, and neurodegenerations. Autophagy is induced in cells by numerous factors: bacterial or viral infections, oxidative stress, and lack of nutrients. Some of the literature data also indicate the protective effect of autophagy [8, 20–23]. Increase in the activity of exoglycosidases in tissues [24–27] and body fluids [28–32] is observed in autophagy combined with the reconstruction of damaged tissues. In addition, determination of exoglycosidase activity is inexpensive and sensitive [33]. In joint diseases (osteoarthritis, rheumatoid arthritis, and Lyme arthritis), progressive destruction of joint cartilages occurs. Destruction of cartilage is a multifactorial process caused by concerted action of lysosomal hydrolases (**Figure 2**). Proteases digest polypeptide chains of glycoconjugates exposing glycopeptides. Endoglycosidases (hyaluronidases, chondroitinases, keratanases, etc.) break down glycosidic bonds inside glycoconjugates and release oligosaccharide chains from the protein core. Lysosomal exoglycosidases, HEX, GAL, β -D-glucuronidase (GLU), and so on, release monosaccharides from the nonreducing terminals of oligosaccharide chains of glycoproteins, glycolipids, and glycosaminoglycans of synovial tissue, articular cartilage, and synovial fluid (**Figure 2**) [34].

2.1. Lysosomal exoglycosidases

Lysosomal exoglycosidases include GAL, GLU, FUC, HEX, as well as MAN. Among lysosomal exoglycosidases, the most active is **HEX** [35] that releases N-acetylglucosamine and

Figure 2. Cartilage destruction by proteases and glycosidases based on [34]. GAG—glycosaminoglycans, GAL— β -galactosidase, GLU— β -glucuronidase, and HEX— β -hexosaminidase.

N-acetylgalactosamine from nonreducing ends of oligosaccharide chains of glycoconjugates (glycoproteins, glycolipids, and glycosaminoglycans). HEX is a glycoprotein composed of two (α and β) subunits. HEX subunits create three isoenzymes: HEX A ($\alpha\beta$), HEX B ($\beta\beta$), and HEX S ($\alpha\alpha$) [36]. Predominant are HEX A and HEX B present in different proportions in the particular tissue [19]. Higher concentration of HEX A in comparison to HEX B may be connected with selective degradation of glycosaminoglycans, because HEX A releases N-acetylhexosamines from acid oligosaccharide chains. Some glycolipids (e.g., gangliosides GM2 and GA2) also are degraded exclusively by HEX A. HEX A is thermolabile and undergoes inactivation at pH ~5 after 3 h of incubation at 50°C. Such conditions does not change the activity of HEX B significantly and thus thermal inactivation is used for the differential determination of HEX A and HEX B in biological materials [37].

Human FUC is a glycoprotein occurring in different molecular forms. During separation on Sephadex G-200 column, FUC is eluted in two peaks: α -L-fucosidase I and α -L-fucosidase II. Both isoforms of α -L-fucosidase differ in molecular mass, pH optimum, and susceptibility on heat denaturation (both isoforms are thermolabile, but α -L-fucosidase I undergoes thermal inactivation in basic environment) [38].

Human GAL possesses three isoenzymes: A, B, and C (GAL C activity is small). Isoenzymes A and B absorb at 95% on concanavaline A (ConA) column, and at 60% on wheat germ agglutinin (WGA) column. Absorbance on ConA indicates the presence of mannose, and absorbance on WGA indicates the presence of N-acetylgalactosamine and N-acetylglucosamine in oligosaccharide chains of GAL [39].

Human MAN has three isoenzymes: A, B, and B2 that differ in sialic acid content and spatial arrangement of atoms in macromolecules [40]. Human GLU is a tetrameric glycoprotein with MW 310–380 kDa. In humans, there are two forms of β -D-glucuronidase: endogenic and exogenic (bacterial GLU derived from intestinal bacteria: *Escherichia coli*, *Peptostreptococcus*, *Bacteroides*, *Clostridia*) [41, 42]. Increase in the activity of bacterial β -D-glucuronidase in gastrointestinal tract increases detoxication by hydrolysis of glucuronides combined with drugs and other toxic substances, secreted with bile [43, 44]. It was reported that the activity of bacterial β -D-glucuronidase in gastrointestinal tract of persons on high meat diet is significantly higher than that in persons on vegetarian diet [45].

2.1.1. Decrease in the activities of lysosomal exoglycosidases

Both deficiency and excessive HEX activity may have clinical significance. Inherited deficiency in **HEX A** (Tay-Sachs disease) causes storage of nondigested ganglioside GM2 in lysosomes of the neural cells. Enlarged lysosomes oppress remaining organelles of neurons that undergo apoptosis [46]. Up to 5–6 months of life, infants with Tay-Sachs disease develop normally but eventually show impairment of vision, hearing, and movement as well as growing mental retardation. Tay-Sachs children usually live up to 4–5 years of age [35]. Tay-Sachs disease may (but much less often) occur in teenagers and adults, generating milder symptoms [47]. Crucial for the diagnosis of Tay-Sachs disease is the significant decrease in HEX A activity in blood serum, leukocytes, and skin fibroblasts. Tay-Sachs disease may be diagnosed during pregnancy by determination of HEX A activity in amniotic fluid [35].

Deficit in **HEX A and HEX B** activity induces Sandhoff's disease that has symptoms similar to Tay-Sachs disease, with additional storage of oligosaccharides in internal organs, mainly liver and spleen. Children with Sandhoff's disease also live up to 4–5 years of age [35]. Sandhoff's disease may appear in older children and adolescents as well as adults, but in older people it occurs extremely rarely. Symptoms of Sandhoff's disease in older children, adolescents, and adults are unspecific and include muscular weakness, lack of motoric coordination (ataxia), speech disorders, and mental retardation. For confirmation of Sandhoff's disease, determination of HEX in blood serum, leukocytes, tears, and skin fibroblasts of potential patients is recommended [35].

Absence or deficiency of **α -L-fucosidase** leads to mucopolysaccharidosis, called fucosidosis. Fucosidosis is characterized by lysosomal storage of glycoproteins, glycolipids, and oligosaccharides containing fucose residues. Fucosidosis may be confirmed by the decrease in α -L-fucosidase activity, in fibroblasts or leukocytes of suspected persons [35].

Decreased activity of α -L-fucosidase in breast tissue may be a predisposing factor for the appearance of breast cancer, because high levels of cell surface-associated α -L-fucose are related to neoplastic progression [48].

Deficiency of **β -D-galactosidase** results in generalized gangliosidosis with lysosomal storage of keratan sulfate, oligosaccharide chains of glycoproteins, and gangliosides GM1. Generalized gangliosidosis is manifested by mental retardation, liver enlargement, and bone deformation. Absence of GAL leads to Krabbe disease with storage of galactosylceramide [35]. Deficiency of **α -L-mannosidase** (mannosidosis) is characterized by primary immune deficiency, skeletal abnormalities, facial dysmorphism, and mental retardation [49]. An inherited lack of **β -D-glucuronidase** activity results in metabolic disease called Sly syndrome or mucopolysaccharidosis VII. Sly syndrome may cause generalized edema of fetus before delivery. Fetuses that survive generalized edema before delivery frequently suffer from mental retardation, littleness, thick facial features, as well as liver and spleen enlargement [35].

2.1.2. Increase in lysosomal exoglycosidase activities

Intensive inflammatory processes, for example tonsillitis, usually are accompanied by increase in lysosomal glycoconjugate catabolism [50]. Hashimoto et al. [51] reported that pancreatic inflammation increases autophagy in the pancreatic inflamed cells. During autophagy, there is observed increase in the activity of lysosomal enzymes characteristic to the involved tissue. The most active of lysosomal exoglycosidases is **HEX**. Therefore, HEX activity in tissues and body fluids is particularly sensitive and is a specific diagnostic parameter for diseases proceeding with increased glycoconjugate degradation. HEX in serum and its isoenzyme B (HEX B) in urine became sensitive and specific markers of alcoholic abuse [52, 53]. A significant increase in serum HEX A activity in smokers may be a marker of risk for arteriosclerosis [54], and an increased HEX A in saliva may be a marker of periodontitis in persons addicted to ethanol [28]. In infectious diseases (e.g., Lyme arthritis), a significant increase in the activity of lysosomal exoglycosidases (including HEX) in serum [55] and synovial fluid was observed [30]. In chronic exposition of rats to cadmium, damage to the proximal renal canaliculi and significant increase in HEX and HEX A activities in urine of exposed rats were observed [29].

Determination of HEX in neoplastic tissues presents ambiguous results that depend on circumstances [56]. Generally, in cancerous tissue, increase in the activity of hydrolytic enzymes including HEX should be observed. In tissues of benign neoplasm of human salivary gland a significant increase in HEX and its isoenzymes was observed, in comparison to healthy salivary gland [57]. A significant increase in the activity of lysosomal enzymes (including HEX, HEX A, and HEX B) was reported in malignant brain tissue in comparison to brain tissues without neoplastic changes [58]. But also significant decrease in HEX, HEX A, and HEX B activities in renal cancer tissue in comparison to healthy renal tissue was reported, followed by a significant increase in HEX and its isoenzymes in urine of neoplastic patients in comparison to healthy persons [27]. Therefore, determination of urinary HEX and its isoenzymes may be particularly useful in diagnostics of neoplasms derived from renal epithelial cells of proximal contorted canalicules. Activity of urinary HEX and other exoglycosidases may be helpful in the diagnostics of pancreatic [31] and colon [32] cancers. Detection of HEX and its isoenzyme activity in stools may be used in elaboration of screening markers for detection of the colon cancer [59]. Determination of HEX activity in serum and saliva may be used for the diagnostics and control of salivary gland tumors [25]. The activity of lysosomal α -L-fucosidase (FUC) [60] reflects the intensity of degradation the α -L-fucose containing glycoproteins and glycolipids [25]. The activity of β -galactosidase (GAL) reflects intensity of degradation glycoproteins, glycolipids and glycosaminoglycans containing galactose [39] and activity of β -glucuronidase (GLU) reflects intensity of glycosaminoglycans catabolism [61, 62].

Determination of the activities of FUC, GAL, and GLU may be applied for the diagnostics and monitoring of diseases proceeding with an increase in catabolism of oligosaccharide chains containing sugars released by appropriate exoglycosidases [33]. Increase in the activity of **α -L-fucosidase** in patients with liver cirrhosis seems to be a promising marker for detecting small focuses of liver cancer, particularly when currently used markers (α -fetoprotein and des- γ -carboxy-prothrombin) seem to be less useful than it was primarily expected [63–66]. In the case of **β -D-galactosidase**, there are suggestions that increase in serum GAL activity that may be applied for the diagnostics of glandular colon cancer [59] and larynx cancer [67]. Serum **β -D-glucuronidase** may be a useful marker for recurrence of liver inflammation [68] and increase in proteoglycans degradation in diabetes [69]. Increased activity of serum and tissues β -D-glucuronidase was found in joint inflammation, dermatoses, liver diseases, AIDS, and breast, stomach, rectum, and pancreatic cancers [70].

Activities of the lysosomal exoglycosidases in body fluids are good markers of neoplasms, inflammations, and infections. Determination of exoglycosidase activities in tissues may be helpful in establishing pathogenesis and treatment of some diseases, for example, nasal polyps. Nasal polyps are grape-shaped smooth structures, arising from the inflammatory nasal mucous membrane. Nasal polyps bulge to interior of the nose, restricting nasal patency [71]. There are different pathogenesis theories of nasal polyps, however, none was satisfactorily confirmed, and the lack of understanding nasal polyp pathogenesis impedes therapy. It is known that untreated nasal polyps may cause intra- and extracranial complications. Currently used pharmacological and surgical treatments of nasal polyps do not provide satisfactory results [72]. In nasal polyp tissue, a significant decrease in the concentration of activities of particular exoglycosidases was found in comparison to control, with simultaneous increase in specific

activity of HEX A [73, 74]. A decrease in concentration of lysosomal exoglycosidases in nasal polyp tissue, without significant changes in their specific activities, denies the theory of full symptomatic inflammation in nasal polyp pathogenesis and may indicate neoplastic theory.

The activities of lysosomal exoglycosidases may be helpful in the selection of a proper method for treatment of hypertrophied and inflammatory palatal tonsils. Healthy palatal tonsils are important elements of immunological barrier against infections of the respiratory tracts [75, 76]. In the case of hypertrophy of lymphoidal tissue or chronic inflammation of palatal tonsils, otorhinolaryngologists very often face situations where palatal tonsils fail to serve as an immunological barrier and cause complications such as: impeded breathing and swallowing as well as speech disturbance. Palatal tonsils hypertrophy and inflammation are indication for tonsillotomy (trimming) or tonsillectomy (removal of palatal tonsils) [77, 78]. However, some otorhinolaryngologists claim that indications for tonsillo- and tonsillectomy should be limited, especially in younger children (6–7 years old), because the role of palatine tonsils and the possibility of surgical complications are not fully known [77–79]. Popko et al. [80] reported that the activity of lysosomal exoglycosidases in palate tonsils is independent of patients' age and she concluded that probably chronic inflammatory processes of the connective tissue of palate tonsils have the same intensity in childhood and in mature persons, and therefore she recommend tonsillectomy even in childhood.

3. Preparation of tissues and body fluids for determination of lysosomal exoglycosidases

Tissues for exoglycosidase determination were collected during surgery, rinsed in tap water, and then in 0.9% saline. After drying with sterile swab, the tissue was weighed, suspended in 0.154-M KCl with 0.2% Triton X-100 (9 mL of fluid for 1 g of tissue) and homogenized. The homogenate was centrifuged at 40°C for 20 min at 12,000 xg. The supernatants were used to determine exoglycosidase activity [25, 72].

Cell culture: Isolated tissue was cut into small pieces (about 0.5 mm²) and incubated in a mixture prepared in the following proportions: 1.5 mg of collagenase (*Clostridium histolyticum* type I-A) and 1 mg of hyaluronidase in 1 mL of DMEM (Dulbecco's modified Eagle's medium), for 1–2 hours at a temperature of 37°C. After incubation the cells were centrifuged and washed with medium (DMEM), the rinsed cells were then cultivated in plastic bottles (25-cm² culture surface) on DMEM with: 10% of calf serum in 25-mM Hepes buffer, 10,000-U/mL penicillin, and 1-mg/mL streptomycin, at 37°C and an atmosphere saturated with 5% CO₂ for –3–5 days, when cells grow up to ~80% of confluence [80].

Articular fluid was collected with sterile syringe needle during local anesthesia, usually at USG control. Collected articular fluid was centrifuged, and exoglycosidases were determined in the supernatant [62].

Urine collection from midstream (after night and after hygiene of intimate places) for determination of exoglycosidases was performed at the same way as for general examination of urine. Exoglycosidase activity determination was performed in supernatants from centrifuged urine [32].

Figure 3. (HEX) determination in the serum of hemolyzed (upper part) and nonhemolyzed blood (lower part). Reprinted from [82], with permission from Elsevier: order number: 4042581223257. TCA — trichloroacetic acid.

Saliva secreted to the bottom of the oral cavity was spitted off to the plastic containers placed in crushed ice and then centrifuged; salivary exoglycosidases were determined in the supernatant [74].

Plasma: Blood was collected typically to anticoagulant from cubital vein and then centrifuged for 5 min at 4000 rev/min. Plasma was collected in plastic tubes [81].

Serum: Blood was taken typically from cubital vein, without an anticoagulant. After clotting in room temperature (30–60 min), the clot was centrifuged off at 4000 rev/min for 5 min. Serum was collected in plastic tubes [82].

Tissues immediately after resection, rinsing, and drying were frozen and stored at -80°C for a very long time. In homogenates and supernatant fluids, exoglycosidases should be determined without delay. Synovial fluids, urine, saliva, plasma, and serum may be stored at -80°C .

Determination of lysosomal exoglycosidases is usually performed by modifications of the Chatterjee et al. [83] method based on incubation of suitable tissue homogenates or body fluids with 4-nitro-derivatives of adequate sugar as substrates, in buffered incubation mixture of appropriate pH (4.7 for HEX, 4.5 for GLU, and 4.3 for GAL, FUC, and MAN), at optimal temperature (36°C), and optimal incubation time (60 min). Enzymatic reaction is terminated by alkalization of the incubation mixture (usually by borate buffer at pH 9.8). Chatterjee et al. [83] method was adopted for the determination of exoglycosidases in synovial fluid [62] and saliva [74]. Recently we have published the method for determination HEX [82] in serum from hemolyzed blood (**Figure 3**) [84].

4. Conclusion

The above literature review indicates the activities of lysosomal exoglycosidases in tissues and body fluids as the markers for detection and monitoring of many human diseases.

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