Original Article

Lysosomal enzymes and mannose 6-phosphate receptors in the lacrimal drainage system: Evidence and its potential implications

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Purpose: To investigate the presence and patterns of lysosomal enzymes and mannose 6-phosophate receptor (MPRs) in human lacrimal drainage system. **Methods:** The study was performed on healthy lacrimal sacs and nasolacrimal ducts obtained from exenteration samples immediately after surgery and frozen at −80°C for subsequent analysis. Soluble proteins' extract was used for enzyme assays, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (PAGE), native PAGE, activity staining, and western blot analysis. Membrane proteins were separately assessed for detection of mannose 6-phosphate receptors, MPR 46. Sepharose gels, 4-methylumbelliferyl substrates, and antibodies against common lysosomal enzymes and MPRs were used. Enzyme assays were carried out in triplicate to ascertain the results. **Results:** Differential lysosomal enzyme activities were documented, and among them acid phosphatase and β-hexosaminidase were found to be high. Western blot analysis using enzyme antibodies and subsequent activity staining confirmed strong signals for moderately expressed enzymes such as fucosidase, glucuronidase, and mannosidase. Membrane extracts demonstrated the presence of MPR 46, which indicates the possible roles of cation-dependent MPRs in lysosomal targeting in human lacrimal drainage system. **Conclusion:** This study provides a proof of principle for the presence of differential lysosomal activity and mannose 6-phosphate ligand transport receptors in human lacrimal drainage system and hypothesizes the potential implications of their dysfunctions.

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Lysosomes are intracellular organelles composed of acidic compartments with more than 50 membrane proteins and 60 hydrolases.[1] They together play a major role in degradation of extracellular materials through endocytosis and intracellular wastes by autophagy.[1-3] The hydrolases; glycosidases, proteases, and lipases are involved in the catabolic degradation of polysaccharides, complex proteins, and lipids, and the products are exported out of lysosomes for excretion or reutilization in biological pathways.[1-3] Lysosomes are hence energy and nutrient sensors and involved in intracellular ion conductances. Disturbances of these functions commonly lead to lysosomal storage disorders. However, lysosomes are also a focus of increasing attention because of their role in regulation of inflammatory glucocorticoid pathways and other inflammatory signaling mechanisms.[4-7] Lysosomal enzymes are known to widely express in ocular tissues and lacrimal gland, with uvea and retina showing high concentrations of glycosidases, acid phosphatases, and cathepsins.[8-13] These enzymes have been potentially implicated in pathogenesis of ocular storage disorders, retinal degenerations, uveitis, and glaucoma.[8] To the best of the authors' knowledge, no exploration had been carried out for lysosomal enzymes and their receptors in lacrimal drainage system. This study explores the differential expressions of lysosomal enzymes and their mannose 6-phosphate transport receptors in normal lacrimal sacs and nasolacrimal ducts.

Methods

Institutional review board approval was obtained and the study complied with the tenets of Declaration of Helsinki. The study was performed on healthy lacrimal sacs and nasolacrimal ducts obtained from exenteration samples (n = 3, 2 females, 1 male; age range: 54–67 years) immediately after surgery and frozen at −80°C for subsequent analysis. None of the exenteration patients had a history of lacrimal or nasal disorders, trauma, or nasal surgery. Irrigation of the lacrimal drainage system before exenteration was patent. The substrates used for lysosomal enzyme activities and the sugars phenyl Sepharose CL-4B, 5-bromo 4-choloro 3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) reagents, and Con A-Sepharose gels were from Sigma Chemicals (St. Louis, MO, USA). 4-Methylumbelliferyl substrates, namely, 4-methylumbellifery

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l-β-glucuronide, 4-methylumbelliferyl α-D-mannopyranoside, and 4-methylumbelliferyl α-L-fucopyranoside (Carbosynth, Berkshire, UK) were used for activity staining. The details of each antibody used for western blot are listed in Table 1.

Lysosomal enzyme assays

Enzyme assays with soluble extracts of human lacrimal sac at pH 5.0 and pH 7.0 were carried out with techniques described previously.[14] The substrates used for the assays were *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide for β-hexosaminidase; *p*-nitrophenyl α-L-fucopyranoside for α-fucosidase; *p*-nitrophenyl α-D-mannopyranoside for α-mannosidase; *p*-nitrophenyl α-D-galactopyranoside for α-galactosidase; *p*-nitrocatechol sulfate dipotassium salt for arylsulfatase A; *p*-nitrophenyl β-D-glucuronide for β-glucuronidase; and *p*-nitrophenyl phosphate for acid phosphatase. The absorbance of the released *p*-nitrophenol was measured at 405 nm. One unit of enzyme activity was defined as the absorbance equivalent of 1 μmol *p*-nitrophenol released per minute, per milliliter of enzyme solution under experimental conditions. Each enzyme assay was carried out in triplicate.

Activity staining

Activity staining was performed in 10% native polyacrylamide gel electrophoresis (PAGE) as described previously[14] using 4-methylumberlliferyl substrates, and the active protein bands were visualized by illuminating the gel under ultraviolet light.

Table 1: Details of the antibodies used

Antigen	Host	Clonality	Source
Hexosaminidase	Rabbits	Polyclonal	In-house[15]
Fucosidase	Rabbits	Polyclonal	In-house[16]
Mannosidase II	Goat	Polyclonal	Santa Cruz
Arylsulfatase A	Goat	Polyclonal	Sigma
Acid phosphatase 2	Goat	Polyclonal	Abcam
β-Glucuronidase	Rabbit	Polyclonal	Abcam
MSC1 (for MPR46)	Rabbit	Polyclonal	In-house[17]

MSC1: Mammalian synthetic cytoplasmic tail 1, MPR: Mannose 6-phosphate receptor

Western blot analysis

Aliquots of the soluble extract and membrane extracts were subjected to western blot analysis for each of the lysosomal enzymes and receptors [mannose 6-phosphate containing ligand transport receptor (MPR) 46] separately, with their respective antibodies [Table 1] The antibodies to enzymes – hexosaminidase and fucosidase – and MPR receptors were raised in rabbits and affinity-purified in the laboratory as per senior author's (NSK) prior publications.[14-18] After sodium dodecyl sulfate– PAGE, the proteins were transferred to a polyvinylidene difluoride membrane. Each membrane was incubated separately with each antibody (1:1,000 dilution). The membranes were subsequently washed and incubated separately with alkaline-phosphatase-conjugated anti-rabbit IgG for fucosidase, hexosaminidase, arylsulfatase, acid phosphatase, glucuronidase, MPR 46, and anti-goat IgG (1:1,000 dilutions in PBST) Phosphate buffer saline with tween 20 for mannosidase as secondary antibody. The membrane was finally developed using BCIP/NBT reagents (Sigma Chemicals).

Results

Lysosomal enzyme assays

The soluble extracts of the human lacrimal sac obtained by sodium acetate (pH 5.0) and Tris–HCl (pH 7.4) buffer extraction exhibited several lysosomal enzyme activities [Fig. 1], and among them acid phosphatase and β-hexosaminidase activities were found to be high at both the pH concentrations [Fig. 1]. When pH 8.0 eluates were assayed, acid phosphatase activity was found to be high followed by hexosaminidase activity similar to earlier assays. However, when pH 9.0 eluates were assayed, higher activity of glucosidase followed by hexosaminidase and mannosidase was found. And when pH 10.0 eluates were assayed, activity of glucosidase alone was observed to be very high [Fig. 2]. These results clearly demonstrate the strong binding of the enzymes indicating the highly hydrophobic nature of the lacrimal drainage lysosomal

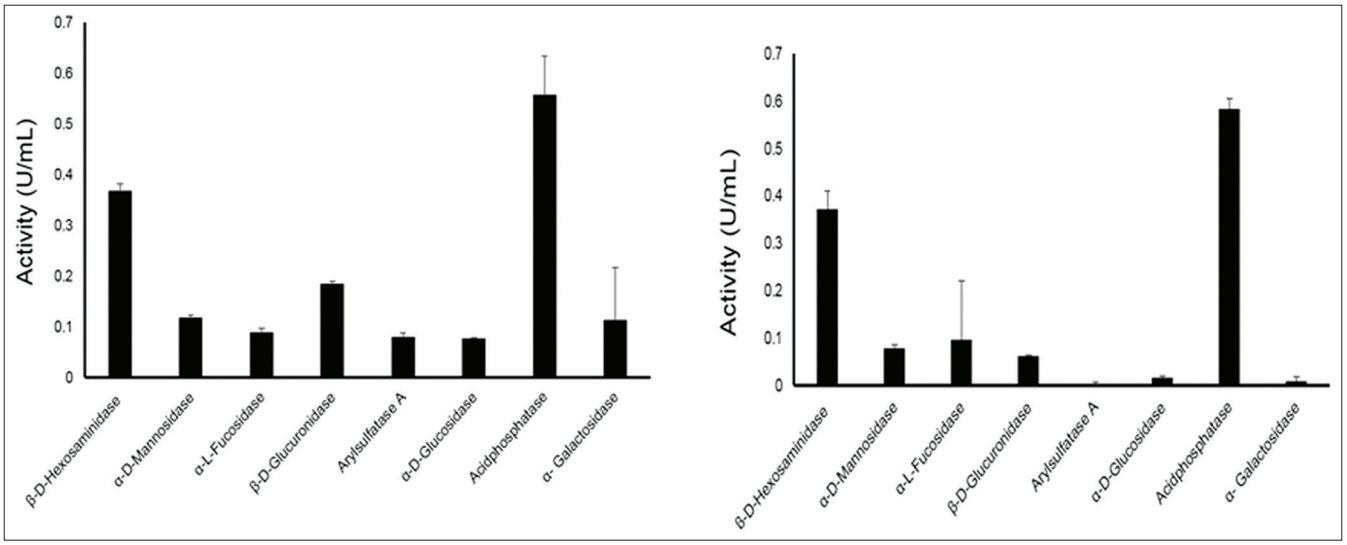

Figure 1: Lysosomal enzyme activities of human lacrimal sac soluble extract. Lysosomal enzyme activities of 25 mM Tris–HCl buffer pH 7.4 extract (left panel) and lysosomal enzyme activities of 50 mM sodium acetate buffer pH 5.0 extract (right panel)

enzymes, and among the lysosomal enzymes assayed, glucosidase was found to be most hydrophobic.

Activity staining

When the native gel containing the soluble extract of lacrimal sac was subjected to activity staining for moderately expressed lysosomal enzymes, with respective 4-methylumbelliferyl-conjugated substrates separately, as described above, strong fluorescence was observed with 4-methylumbelliferyl-β-glucuronide, 4-methylumbelliferyl α-D-mannopyranoside, and 4-methylumbelliferyl α-L-fucopyranoside, confirming the presence of α-fucosidase [Fig. 3a], β-glucuronidase [Fig. 3b], and α-mannosidase [Fig. 3c] in the human lacrimal drainage system.

Western blot analysis

Western blot analysis confirmed the presence of lysosomal enzymes in the human lacrimal drainage system and showed strong signals for α-fucosidase [Fig. 4a], glucuronidase [Fig. 4b], arylsulfatase A [Fig. 4c], mannosidase [Fig. 4d], β-hexosaminidase [Fig. 4e], and acid phosphatase [Fig. 4f]. Membrane extract analysis with western blot using specific receptor antibodies [mammalian synthetic cytoplasmic tail 1 antibody, MSCI for MPR 46, Table 1] showed presence of MPR 46 receptors [Fig. 4g], which indicates the possible roles of cation-dependent lysosomal targeting in human lacrimal drainage system.

Discussion

This study has provided a proof of principle of the differential presence of several lysosomal enzymes and mannose 6-phosphate ligand transport receptors in the lacrimal drainage system. In the light of current evidence of lysosomal involvement in several inflammatory pathways, lysosomal dysfunctions may add another potential dimension to multifactorial etiopathogenesis of primary acquired nasolacrimal duct obstruction (PANDO), which needs to be further investigated in detail.

Lysosomes are degradation centers of the cell and are aided in this function by its numerous membrane proteins and hydrolase enzymes. They degrade extracellular molecules and phagocytosed pathogens and also aid in intracellular proteins turnover.[1,2] In addition, lysosomes are also involved in immune functions, pigmentation, cell signaling pathways, cell adhesions, and membrane repair mechanisms.[3,4] Mannose 6-phosophate receptors (MPR) are transmembrane glycoproteins that play a crucial role in the transport of newly synthesized lysosomal enzymes.[18-20] Targeting of lysosomal enzymes depend on the presence of mannose 6-phosphate moiety within them and their recognition by two specific MPR proteins: the cation-independent MPR 300 and the cation-dependent MPR 46. Their functions are to some extent distinct but mostly overlapping.[18-20]

Lysosomes are known to negatively regulate the anti-inflammatory actions of glucocorticoids.[4,5] Cytoplasmic glucocorticoid receptors (GRs) mediate anti-inflammatory effects through inhibiting synthesis of cytokines, prostaglandins, and prostacyclins. Lysosomal autophagy degrades the cytoplasmic GR and enhances inflammation by negating their actions. The lysosomes can also positively or negatively regulate inflammatory pathways by secreting or degrading numerous cytokines such as interleukins (IL-1 β, IL-6, IL-18, and IL-8), β-interferons, tumor necrosis factor-alpha, and transforming growth factor-β.[4,6,7] A lysosomal membrane protein, trans-membrane protein 9B, is also involved in activation of nuclear factor "kappa-light-chain-enhancer" activated B cells and mitogen-activated protein kinase pathways, the central signaling pathways of inflammation, and hence reflects on the potent regulatory role of lysosomes in inflammation.[21]

These findings may have potential implications for further studies to explore the etiopathogenesis of PANDO. The hypothetical role of lysosomal dysfunctions in PANDO should be assessed in the light of their major influences in core inflammatory cascades. Since lysosomes can negatively or positively regulate inflammation, a critical pro- and anti-inflammatory balance is maintained. Disturbance of this equilibrium can lead to chronic inflammation and lysosomes have been implicated in pathogenesis of autoimmune, metabolic, cardiovascular, and neurodegenerative disorders.[4] The widespread presence of lysosomal hydrolases and their transport receptors within the healthy lacrimal drainage system points toward optimal functions with pro- and anti-inflammatory equilibrium. Hypothetically, it is possible that numerous exogenous or endogenous triggering agents within the lacrimal drainage system can create lysosomal instability and lysosomes may respond to them. Prolong presence of triggering agents or repeated assaults may lead to severe instability of lysosomal functions and these dysfunctions may have a potential role in mediating, coordinating, and enhancing the inflammatory pathways leading to chronic inflammation of the lacrimal sac and nasolacrimal duct, followed by the response of fibrosis and subsequent acquired obstructions (PANDO).

Selective autophagic clearance of bacteria is an important host defense mechanism where lysosomes play an important role, and dysfunctions in this regard have been implicated in pathogenesis of sepsis and inflammation.[22,23] Severe inflammation and tissue destruction in experimental toxoplasma infestation, in part, have been attributed to inability of the lysosome to fuse with phagosome (*Toxoplasma gondii*), resulting in abundant lysosomal enzymes intended against the organism, instead, destroying the tissues in the vicinity.[24] Similarly, lysosomal dysregulation of selective autophagy of mitochondria and cilia has been implicated in chronic obstructive pulmonary disease and lung fibrosis.[22,23] The lacrimal sac and nasolacrimal ducts also have numerous cilia on their luminal surfaces and the adluminal mucosa acts as an important first-line defense against sustained bacterial and exogenous triggers that attack the ocular surface and are subsequently washed into the lacrimal drainage through tears. Hypothetically, lysosomal autophagic dysfunctions in the lacrimal drainage system have the potential to trigger chronic inflammation, tissue destruction, and subsequent fibrosis and finally resulting in acquired obstructions (PANDO).

Lysosomal hydrolases are widely distributed in ocular tissues with higher concentrations in the retina and uvea.[8-13] Every tissue has its own differential expression levels of various enzymes, for example, ciliary body shows higher expression of fucosidase, mannosidase, and cathepsin B; retinal pigment epithelium shows higher concentration of cathepsins; tear analysis shows higher concentration of acid phosphatase,

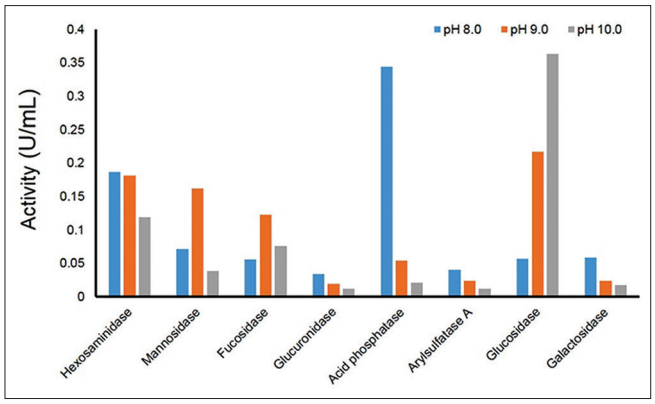

Figure 2: Chromatography on phenyl Sepharose matrix. Elution was carried out at pH 8.0, pH 9.0, and pH 10.0. Note the differential activities of various lysosomal enzymes at various pH elution

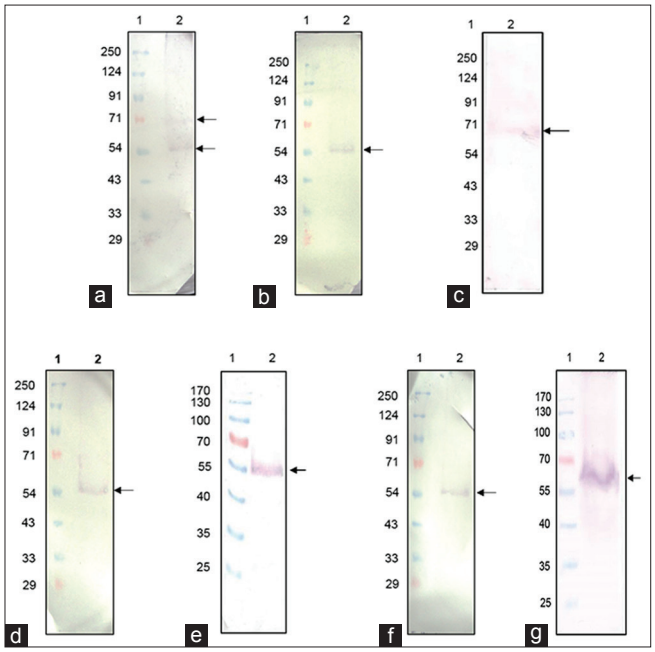

Figure 4: Western blot analysis of human lacrimal sac soluble extract. Strong signals were noted for fucosidase (a), glucuronidase (b), arylsulfatase (c), mannosidase (d), hexosaminidase, (e) and acid phosphatase (f). Membrane extracts showed strong signal for mannose 6-phosophate receptor 46 (g). Lane 1: molecular weight marker, Lane 2: crude soluble extract. Arrows indicate the signals of respective enzyme in the blot

fucosidase, and glucosaminidase.[8,10] Heterogeneity in regional and differential distribution of these enzymes could potentially be involved in various tissue-specific disease involvement and their variable clinical presentations.[8] In concurrence with this, it has also been found that the receptor proteins involved in the targeting of lysosomal enzymes are also differentially expressed in different tissues in humans and other vertebrates.[25] High activity of acid phosphatase and β-glucuronidases was found in Behcet's disease,[26] and similarly release of lysosomal enzymes from macrophages has been implicated in chronic inflammation in sarcoidosis.[27] Glucocorticoids have been demonstrated to suppress acid phosphatase activity.[28] The assessment of various lysosomal

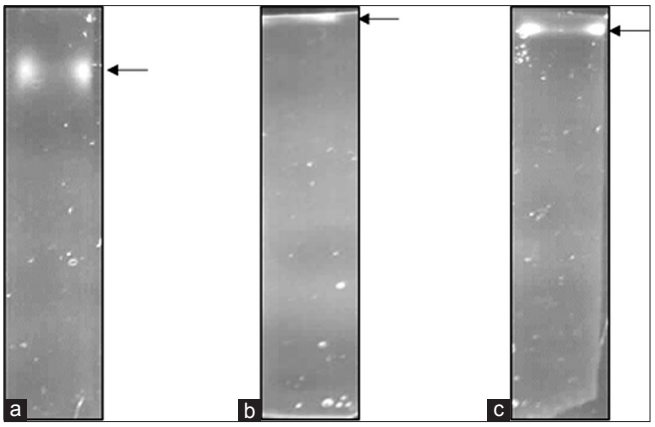

Figure 3: Activity staining of human lacrimal sac soluble extract. Enzymes were detected using respective 4-methylumbelliferyl substrates. Fucosidase (a), glucuronidase (b), and mannosidase (c). Arrows indicates the position of the enzymes

enzymes in diseased lacrimal sacs and nasolacrimal duct would be useful in nailing the overexpressed hydrolases with possible therapeutic solutions.

The limitations of this study include lack of assays of other lysosomal enzymes, lack of comparisons with diseased models, and the current speculative nature of the hypotheses that needs further validation. However, the strengths of the study include wide assessment of common lysosomal enzymes within a focused area of lacrimal drainage system and providing the proof of principle of presence of their MPRs.

Conclusion

The hypothesis suggested in this study needs further investigations, including the identification of MPR 300 proteins. It is also important to understand that such lysosomal pathways of inflammation are one among the many that could be involved in etiopathogenesis of PANDO. The widespread presence of lysosomal enzymes, particularly glucosidases, and their MPRs within the lacrimal drainage system opens up exciting newer avenues for further exploration to demystify etiopathogenesis of PANDO.

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Conflicts of interest

There are no conflicts of interest.

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