In vitro characterization of human liver lysosomes isolated from fresh tissue

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Abstract

Evaluation of lysosomal catabolism is an integral part of the development of biologic drugs including antibody-drug conjugates (ADC). Biologics can enter the cell by fluid-phase or cell surface antigen-mediated endocytosis and can be degraded by multiple catabolic enzymes found in lysosomes. Purified rat liver tritosomes or human lysosomes are convenient test systems for an *in vitro* evaluation of lysosomal stability of antibodies, linkers and small molecule drugs comprising ADC. Hepatic lysosomes were isolated from fresh human liver donors according to the discontinuous density ultracentrifugation method based on Wang, et al (2012). Twelve individual fractions collected from the OptiPrep density gradient were characterized for acid phosphatase, cathepsin B, and cytochrome c oxidase activity in order to confirm separation of the lysosome- from the mitochondria-specific proteins. Acid phosphatase activity was distributed among multiple fractions while cathepsin B was higher in lighter lysosomal fractions in contrast to the heavier fractions containing more cytochrome c oxidase. Fractions were further characterized with Western blotting for distribution of lysosomal-associated membrane protein 1 (LAMP-1, CD107a) and microsomal cytochrome c oxidase subunit 4 (COX4). In agreement with the enzyme activity data, the blots demonstrated separation of the LAMP-1 signal, detected in the lighter lysosomal fractions from that of the COX4 signal found in the heavier lysosomal fractions of the OptiPrep density gradient. Expected enrichment of lysosome-specific cathepsins L and S in isolated lysosomes was demonstrated with sandwiched ELISA Human Protease Array (R&D Systems). This work provides a characterization of isolated human liver lysosomes that constitutes a test system for an *in vitro* assessment of catabolic stability of biologics drugs entering the cell by the endosomal–lysosomal pathway.

Introduction

Some biopharmaceutical drugs can enter the cell by fluid-phase or cell surface antigen-mediated endocytosis and can be degraded by multiple catabolic enzymes found in lysosomes. These drugs and products of their catabolism can be stored in lipofuscin-containing lysosomes or excreted via endocytic pathway into the extracellular spaces or bile. Proteases present in lysosomes affect fate of biologic drugs such as antibody drug conjugates and are of considerable interest in the drug development practice for their ability to degrade antibodies, small molecule drugs and their linkers (Sussman et al., 2014; Hong et al., 2015). Therefore, the purpose of the present study was to isolate human hepatic lysosomes and characterize them for the presence of specific lysosomal markers and proteolytic enzymes potentially involved in the catabolism of biopharmaceuticals.

Materials & Methods

Human tissue: Non-transplantable human hepatic tissue from multiple donors was used for lysosomal isolations.

Lysosome purification: Hepatic lysosomes were freshly isolated from human liver tissue (n = 4 donors) with a modified discontinuous density ultracentrifugation method based on experimental procedures described by Wang, et al. (2012). **Figure 1** shows the stepwise procedure of lysosome isolation from fresh human liver tissue and a representative OptiPrep gradient separation of the heavy membranes.

Immunoblotting: Proteins were separated by SDS-PAGE with a 10.5%-14% Criterion Tris-HCL gel (Bio-Rad) and transferred to PVDF membranes (Bio-Rad). Membranes were probed with anti-LAMP-1 and anti-COX4 antibodies purchased from Abcam. Proteins were probed with HRP-conjugated secondary antibodies (GE Healthcare) and visualized with chemiluminescence with a FluorChem System (Protein Simple).

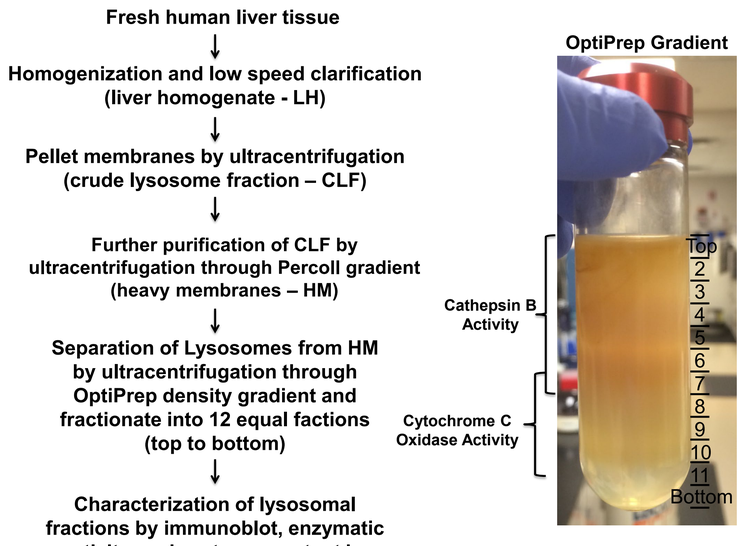
Enzymatic Activity: Cathepsin B activity was assayed as previously described with Z-RR-AMC (Z-Arg-Arg-7-amido-4-methylcoumarin hydrochloride, Sigma) as substrate (Giusti et al., 2008). Acid phosphatase was measured with a kit purchased from Sigma. Cytochrome c oxidase activity was assayed in accordance with a protocol provided by Sigma. All enzymatic assays were measured with a Synergy Neo plate reader (BioTek).

Protease array: Proteome Profiler Array, Human Protease Array Kit was purchased from R&D Systems. 200 µg of proteins were used per array following the manufacturer's protocol. Luminescence was visualized with a FluorChem System.

References

1. Giusti I, D'Ascenzo S, Millimaggi D, Taraboletti G, Carta G, Franceschini N, Pavan A, and Dolo V (2008) Cathepsin B mediates the pH-dependent proinvasive activity of tumor-shed microvesicles. *Neoplasia* **10:**481-488.
2. Hong EE, Erickson H, Lutz RJ, Whiteman KR, Jones G, Kovtun Y, Blanc V, and Lambert JM (2015) Design of Coltuximab Ravtansine, a CD19-Targeting Antibody-Drug Conjugate (ADC) for the Treatment of B-Cell Malignancies: Structure-Activity Relationships and Preclinical Evaluation. *Molecular pharmaceutics* **12:**1703-1716
3. Sussman D, Smith LM, Anderson ME, Duniho S, Hunter JH, Kostner H, Miyamoto JB, Nesterova A, Westendorf L, Van Epps HA, Whiting N, and Benjamin DR (2014) SGN-LIV1A: a novel antibody-drug conjugate targeting LIV-1 for the treatment of metastatic breast cancer. *Mol Cancer Ther* **13:**2991-3000.
4. Wang X, Zhang X, Dong XP, Samie M, Li X, Cheng X, Goschka A, Shen D, Zhou Y, Harlow J, Zhu MX, Clapham DE, Ren D, and Xu H (2012) TPC proteins are phosphoinositide- activated sodium-selective ion channels in endosomes and lysosomes. *Cell* **151:**372-383.

Figure 1: Hepatic lysosome isolation and image of a representative OptiPrep gradient.



Results

Table 1 illustrates that the location of highest cathepsin B activity fraction varies between positions 3 and 6. This variation may be due to intrinsic differences in density of specific organelles among human liver donors. A range of cathepsin B activity found in the highest activity fraction is also noted.

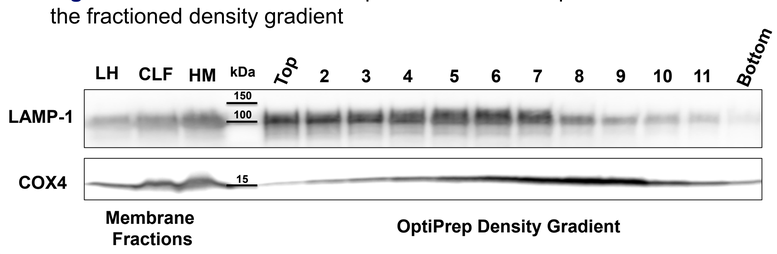
Table1: Acid phosphatase and cytochrome c oxidase activities in heavy membrane fractions containing peak cathepsin B activity

Donor	Fraction	Acid phosphatase, U/g*	Cytochrome c oxidase, U/g**	Cathepsin B, U/mg***
A	5	54.5 ± 1.43	158 ± 7.17	41.0 ± 0.486
B	3	77.1 ± 2.78	132 ± 3.88	23.7 ± 0.933
C	6	64.5 ± 2.12	468 ± 5.40	100 ± 1.22
D	3	126 ± 2.55	128 ± 7.92	143 ± 2.52

* One unit of acid phosphatase will hydrolyze 1µM of 4-nitrophenyl phosphate per minute at pH 4.8 at 37°C
** One unit of cytochrome c oxidase will oxidize 1µM of cytochrome c per minute at pH 4.8 at 25°C
*** One unit of cathepsin B will hydrolyze 1µM of AMC per minute at pH 5.5 at 38°C

Hepatic heavy membrane fractions collected from the OptiPrep density gradient separation were immunoblotted for lysosomal marker, LAMP-1, and mitochondria maker cytochrome c oxidase subunit IV, COX4, to identify fractions that contain enriched lysosomes. LAMP-1 and COX4 were detected in the initial liver homogenate (LH) and increased as the homogenate was sequentially processed to the crude lysosome fraction (CLF) and the heavy membranes (HM). The majority of LAMP-1 was detected in the lighter fractions (1 through 7, Fig 1.), while cytochrome c was concentrated in the heavier fractions (6 through 10), indicating a separation between lysosomes and mitochondria as HM resolved through the OptiPrep density gradient. The detection and position of the organelle markers throughout the OptiPrep gradient is representative of the lysosomes isolated from multiple livers. Lysosomes presented in this figure were isolated from non-transplantable liver from donor D.

Figure 2: LAMP-1 and COX4 expression levels and position within the fractioned density gradient



Hepatic lysosomal fractions (donor D) were assayed for lysosomal enzymes (acid phosphatase and cathepsin B) and mitochondrial marker (cytochrome c oxidase). Acid phosphatase and cathepsin B specific activities were highest in fractions 1 through 7 followed with a drop in activity in the subsequent fractions and remained low throughout the bottom half of the gradient. As expected, the inverse was found for cytochrome c oxidase activity. Low activity was detected in the top portion of the gradient, followed by an increase in fraction 7 and throughout the bottom portion of the gradient. The position of the enzymatic activities throughout the OptiPrep gradient of donor D is representative of the enzymatic activity isolated from lysosomes from multiple tissue donors. Values are the mean and standard deviation of triplicate analytical measurements.

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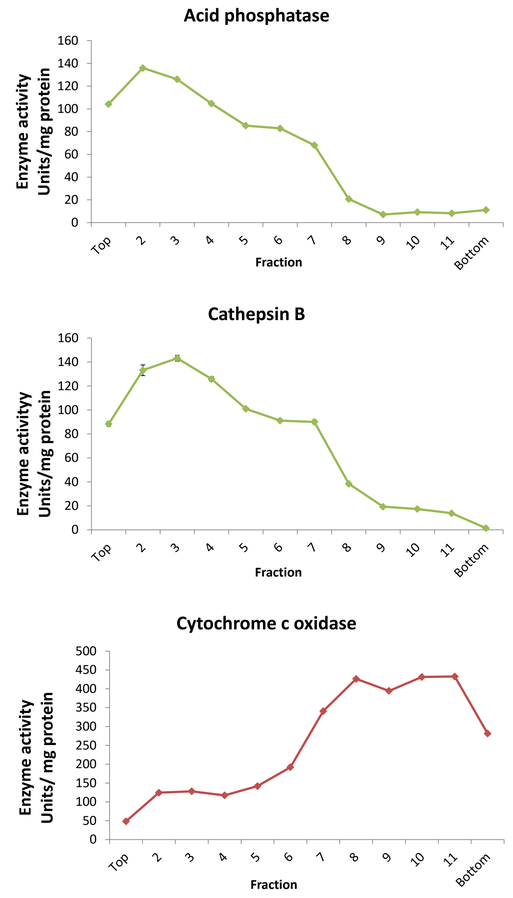
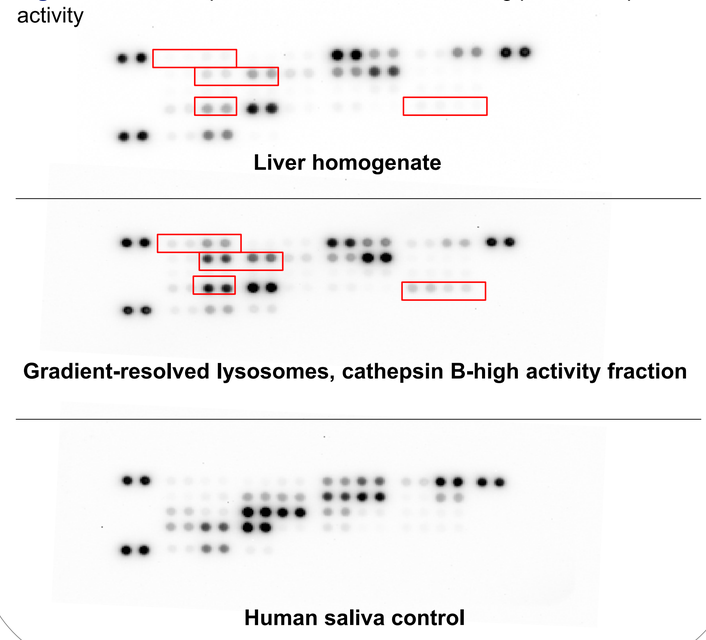
Figure 3. Enzymatic activity and position of acid phosphatase, cathepsin B and cytochrome c oxidase within the fractionated density gradient



Lysosomes were isolated from liver homogenate and assayed for proteolytic enzymes (donor D, top panel). Each pair of spots in **Figure 4** represents detection of one protease. Cathepsin B-high activity fraction (fraction 3, **Figure 1**) composed of disintegrin and metalloproteinase domain-containing proteins 8 and 9, cathepsins A, B, D, L, S and X/Z/P, dipeptidyl-peptidase 4, matrix metalloproteinases 8 and 9, neprilysin, presenilin and proteinase 3 (middle panel). Human saliva proteins served as a control (bottom panel). The gradient fractionation enriched cathepsins L and S, disintegrin and metalloproteinase domain-containing protein 8 and 9, matrix metalloproteinase 8, neprilysin, presenilin and proteinase 3 (boxed in red) but not the other proteases present in fraction 3.

Figure 4: Protease profile of the fraction containing peak cathepsin B activity



Conclusions

- Light fractions of OptiPrep-separated lysosomal membranes are enriched for the organelle-specific proteases and depleted of mitochondrial proteins.
- Liver lysosomes purified from fresh tissues constitute a convenient test system for evaluation of human-specific catabolism of biologic pharmaceuticals processed through endosomal-lysosomal pathway.