Hacettepe University Journal of the Faculty of Pharmacy
Volume 33 / Number 1 / January 2013 / pp. 59-74

Macroautophagy-Lysosomal System (Mals) in Gaucher Patients Carrying L444P and N370S Mutations

Received : 16.07.2013
Revised : 06.01.2014
Accepted : 07.01.2014

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Introduction

Recent studies were focused on the possible involvement of macroautophagy-lysosomal system (MALS) in pathophysiology of Gaucher disease (GD)[1-4]. MALS is a vacuolar, self-digesting system responsible for removal of long lived cytosolic proteins (general/ structural), damaged organelles (mitochondria and peroxisomes) and misfolded aggregated proteins[5-7]. MALS starts with engulfment of cytoplasmic materials by a phagophore, followed by sequestration through formation of a double-membrane vesicle (autophagosome), maturation by fusion of the autophagosome with late endosomes and then with the lysosomes to form the autolysosome and ultimate degradation of their cargo by lysosomal hydrolases[8]. Due to the vital role of the lysosome in autophagy, MALS is an obvious candidate in lysosomal storage disease (LSD) pathogenesis[9]. MALS can be measured by changes in LC3 localization. The ubiquitin-like protein Atg8/ LC3 is the only protein marker that is reliably associated with complete autophagosomes and also localized to phagophores[1,10]. Soluble LC3-I is conjugated to phosphatidylethanolamine (PE) to become LC3-II which associates with phagophore- and autophagosome- membranes. On fusion with lysosomes, LC3-II is degraded by lysosomal proteases. In particular the levels of LC3-II correlate with autophagosome formation, due to its association with the autophagosome membrane. Conversion of LC3-I to LC3-II provides an indicator of autophagic activity. To analyze the levels

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of autophagy induction and flux in GD we monitored LC3B-I and LC3B-II in Gaucher fibroblasts carrying the most prevelant mutations L444P and N370S. There is a general tendency that the amount of LC3-II increases when autophagy is induced. However, once the increase of LC3-II is observed, the maturation of autophagosomes to autolysosomes must be further examined to determine whether or not autophagy is induced and whether or not it goes to completion. Glucosylceramide and glucosylsphingosine accumulation in lysosomes may disrupt the normal autophagic flux and/or the completion of the autophagic pathway in GD[8]. DQ-BSA is used to measure the rate of substrate clearance from lysosomes, thus the turnover rate in lysosomes. DQ-BSA is a bovine serum albumin derivative conjugated to a self-quenched fluorophore and internalized from outside the cell through phagocytosis. Intracellular proteolysis of this compound results in dequenching and release of brightly fluorescent fragments, allowing us to detect the turnover of substrate with in an active proteolytic compartment, lysosome. It is also an indication of the potential flux (turn-over) rate of other substrates delivered to lysosomes by autophagy including LC3-II. Cellomics KSR is a high throughput instrument which is capable of resolving spatially and temporally separated fluorescent labels to quantitatively measure multiple features simultaneously. We used the "Cellomics Total Fluorescent Detector BioApplication" to quantify the total and average fluorescent intensities of DQ-BSA in live cells for the determination of the intracellular proteolytic activity and the completion of the autophagic pathway.

In this study, we aimed to determine MALS, increased levels of autophagy and/ or decreased flux rates, whether or not autophagy is induced and whether or not it goes to completion by the monitoring of LC3B-I, LC3B-II and proteolysis of DQ-BSA in clinically different types of Gaucher patients fibroblasts carrying L444P and N370S mutations.

Material and Methods

Phenotypes of Gaucher Patients

Three patients (L1, L2 and L3) were homozygous for the L444P mutation with different phenotypes, L1 displays a chronic nonneuropatic form (Type I) (died at 7 years old), L2 displays an acute neuronopathic form

(Type II) (died at 1 year old) and L3 displays an acute neuronopathic form (Type II) (died at 2 years old). L2 patient has 2 additional base substitutions (1483G>C and 1497G>C) in one allele. Two patients (N1 and N2) were homozygous for the N370S mutation together with mild nonneuropatic form (Type I).

Cell Lines and Tissue Culture Conditions

Fibroblast cell lines homozygous for the L444P GD Type I and Type II (1448T>C) and homozygous for the N370S GD Type I (1226A>G) mutations and wild type controls were obtained from Coriell. Fibroblast cells were grown in complete alpha-minimal essential medium from Wisent Inc. (Canada) in the presence of 5% antibiotics (penicillin and streptomycin, Invitrogen), supplemented with 10% fetal bovine serum (FBS, Wisent Inc,. Canada) and incubated at 37 °C in a humidified atmosphere with 5% CO2.

GCase activity assay

Activities from cell lysates were determined using the fluorogenic substrates 4-methylumbelliferyl-beta-D-glucopyranoside (MubGlc). Reactions were initiated by the addition of 25 µl of MUbGlc (in CP 20 mM, plus TDC 0.4% (w/v), pH 5.5)(5 mM final concentration) to 25µl cell lysates (diluted in CP 20 mM, plus TDC 0.4% (w/v), pH 5.5), incubated in 37 °C for 2 hours and terminated by the addition of 200 µl of 2-amino-2-methyl-propanol (MAP; 0.1 M, pH 10.5). The fluorescence was measured with an M2 Microplate spectrofluorometer (Molecular Devices Inc.) with excitation and emission wavelengths set at 365 and 450nm, respectively.

Western blot analysis

Cells were scrapped with ice cold PBS and total cell lysates with membrane fractions were obtained by using ice cold lysis buffer (50mM TrisHCl, pH 7.4, 150 mM NaCl, 1% Triton X-100) containing 1% Proteinase inhibitor coctail (Sigma).

Western blots were performed with 30 ug of protein on NuPAGE 4-12% Bis-Tris and/or 16% Tris-glycin Gels (Invitrogen). Proteins were transferred to Polyvinylidene fluoride (PVDF) membranes which were blocked in 5% non-fat dry milk. Membranes were probed with 1:1000 dilution of rabbit GCase, 1:1000 dilution of rabbit LC3B, peroxireductase and 1:10000 dilution of GAPDH primary antibodies. Secondary antibodies

(horseradish peroxidase-conjugated) were diluted 1:5000–1:10000 and visualized using chemiluminescent substrate with Amersham ECL system.

Cellomics, turn over of DQ-BSA and labeling with Hoechst dye

The day before applying fluorescent DQ-BSA, fibroblast cells were splitted into 24 well plates to reach a confluency lower than 60%. Two hours before, the α-MEM was changed (with FCS, without antibiotic) and DQ-BSA was applied in a final concentration of 10 ug/ml and left overnight. The media was changed (with FCS, without antibiotic). two hours before the application of the nuclear dye (*Hoechst*). The nuclear dye (*Hoechst*) was aplied from the the stock (10 mg/ml) in a 1/10000 dilution in α-MEM without antibiotic, left for 10 minutes. After 2 times with PBS washing, total and average intensities were measured with Cellomics. All samples were studied in duplicate or triplicate.

Results

GCase deficiency in fibroblasts of Gaucher patients

We first, confirmed the deficiency of GCase in fibroblasts of Gaucher patients. As expected, significantly very low levels of GCase specific activities were observed in patient fibroblasts compared to wild types, 2.72-3.46% and 11.46-15% in L444P and N370S mutations, respectively (Table I). Lysates from the fibroblasts also showed the deficiency of Gcases in Western Blot analyses which were correlated with specific activities (Fig. 1).

Increased levels of LC3B-II in fibroblasts of Gaucher patients

In western blot analysis, LC3 is detected as two bands; cytosolic LC3B-I and LC3B-II which is bound to PE in the autophagosome membrane. This makes the molecular weight of LC3B-II greater than LC3B-I. However due to its hydrophobicity, LC3B-II migrates faster and therefore displays a lower apparent molecular weight (14 kDa). We used commercial rabbit antibody LC3B (D11) XP®(cell signalling), to detect and measure endogenous levels of LC3B-I and LC3B-II (relative to GAPDH) in patient and wild type fibroblasts. These are well separated on 16% Tris-Glycin gel. Elevated levels of LC3B-II in patient fibroblasts were observed, more significant in L2, L3 and N1 patients (Fig.2). LC3B-II protein levels were normalized to GAPDH signals in the same sample and presented as ratio relative to W (Wild fibroblasts). In L2, L3 and N1 patients 2.8,

TABLE I
GCase specific activities in the fibroblasts of Gaucher patients

	Mutation type	GCase Specific activity (nmol/mg protein/hour)	%
L1	L444P	28± 1	3.5
L2	L444P	22± 1	2.7
L3	L444P	30± 2	3.6
N1	N370S	94± 3	11
N2	N370S	123± 3	15
Wild 55	-	850± 28	Average of 2 wilds 100
Wild 57	-	791± 25	

Specific activity is given in nmol/mg protein/hour ± standart deviation

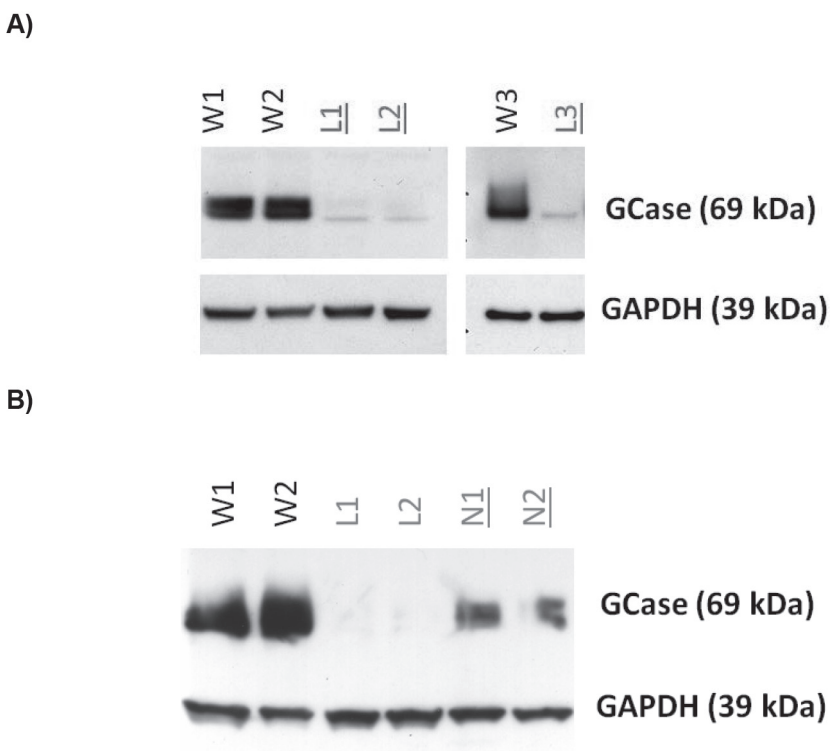


Figure 1
Significantly very low GCase protein bands were observed in L1, L2, L3 (L444P mutation) (A) and N1, N2 (N370S mutation) (B) Gaucher patients fibroblasts compared to wild types (W). The results were correlated with specific GCase activities. GAPDH was used as loading control.

1.4 and 2.2 fold increases in LC3B-II were observed, respectively. When LC3B-II protein levels were normalized to LC3B-I signals in the same sample and presented as ratio relative to W (Wild fibroblasts), L2, L3 and N1 patients showed 2.2, 1.7 and 2.7 fold increases in LC3B-II, respectively. In other patient, increases in LC3B-II were also observed relative to LC3B-I and GAPDH. The increased levels of LC3B-II relative to LC3B-I and GAPDH demonstrate an increase in the number of autophagosomes in patient fibroblasts. Next we investigated whether those increases were due to an induction of autophagy or a decrease in autophagic flux due to impaired lysosomal function and/or autophagosome-lysosome fusion.

Lysosomal turnover of DQ-BSA in fibroblasts of Gaucher patients

We investigated the rate of lysosomal turnover in Gaucher patient fibroblasts by monitoring the degradation of DQ-BSA by lysosomal proteases. Intracellular proteolysis of this compound results in dequenching and release of brightly punctuated fluorescent fragments, thus clearance of this substrate is a direct measurement of lysosomal function (Fig.3).

We feeded the cells with fluorescent DQ-BSA and left overnight. We measured DQ-BSA fluorescent and turnover of DQ-BSA in live patient and wild type fibroblast cells with 'Cellomics Total Fluorescent Detector BioApplication'. To identify the individual cells we labeled cells with nuclear dye (Hoechst) (Fig.4).

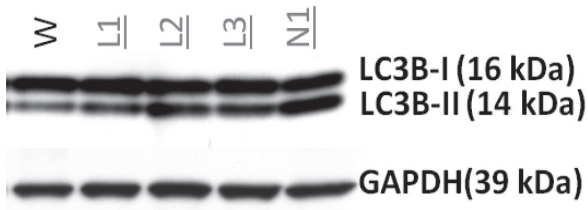
After quantifying the total and average intensities of DQ-BSA in live cells, turnover of the DQ-BSA were found lower in the patients (significantly lower in L2 and N1 patients) compared to wild type (Fig.5).

These results suggest that the increase in LC3B-II in patient cells is also a result of decreased turn-over of this macromolecule and that the glucosylceramide and glucosylsphingosine accumulation in lysosomes is likely responsible for this, since the decrease roughly correlates with the clinical severity of the Gaucher patients.

Increased levels of peroxiredoxin in fibroblasts of Gaucher patients

Autophagy also plays a crucial role in the degradation and turnover of cellular organelles like mitochondria. Indeed, it has been suggested that autophagy selectively degrades dysfunctional mitochondria (5). Accumulation of dysfunctional mitochondria subject cells to increasing oxidative stress when autophagy is dysfunctional (11). Increases of peroxiredoxin

A)



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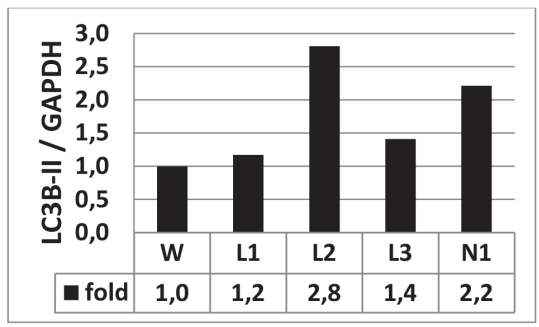

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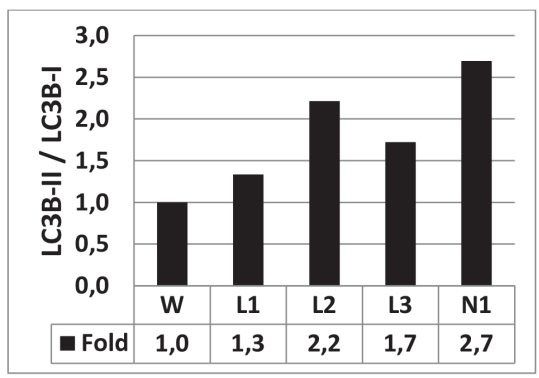


Figure 2

A) Western Blot analysis of lysates from L1, L2, L3 (L444P mutation) and N1 (N370S mutation) patient fibroblasts. (B) LC3B-II protein levels were normalized to GAPDH signals in the same sample and presented as ratio relative to W (Wild fibroblasts). In L2 and N1 patients 2.8 and 2.2 fold increases in LC3B-II were observed, respectively. (C) LC3B-II protein levels were normalized to LC3B-I signals in the same sample and presented as ratio relative to W (Wild fibroblasts). In L2 and N1 patients 2.2 and 2.7 fold increases in LC3B-II were observed, respectively. In other patients, increases in LC3B-II were also observed relative to LC3B-I and GAPDH. Densitometry was performed using Image J analysis software (NIH).

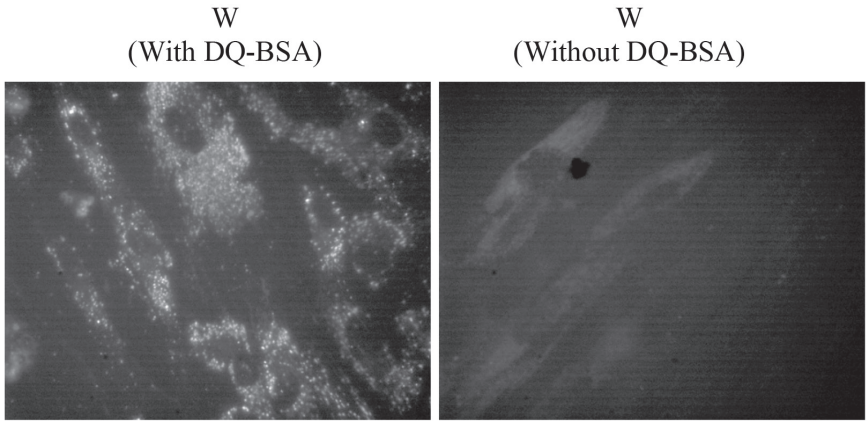


Figure 3
Intracellular proteolysis of DQ-BSA resulted release of brightly punctuated fluorescent fragments. The clearance of this substrate in wild type fibroblasts (W) was visualized under fluorescent microscope.

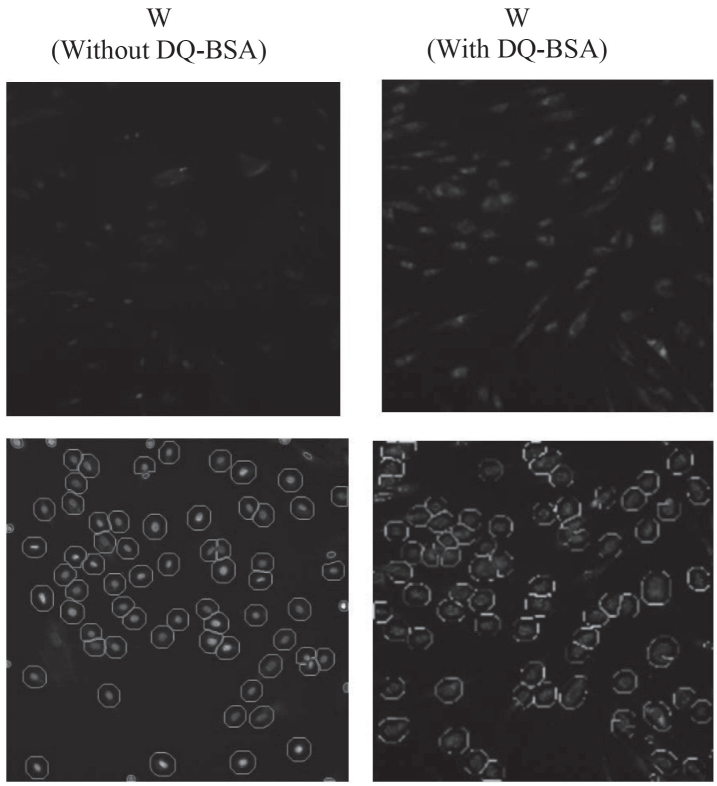
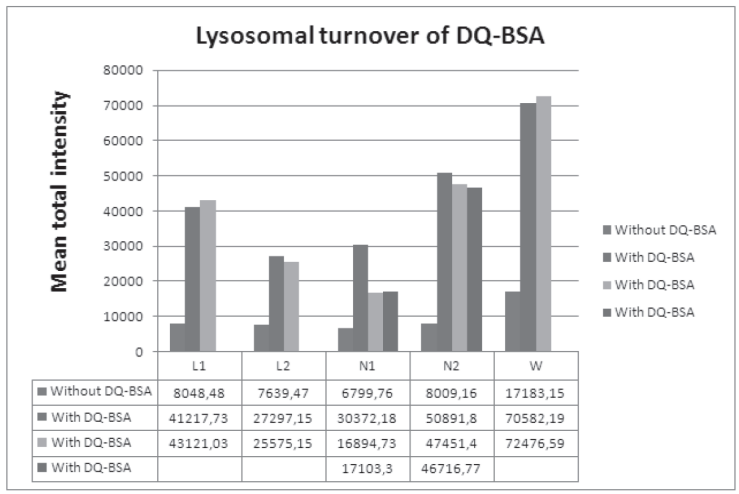


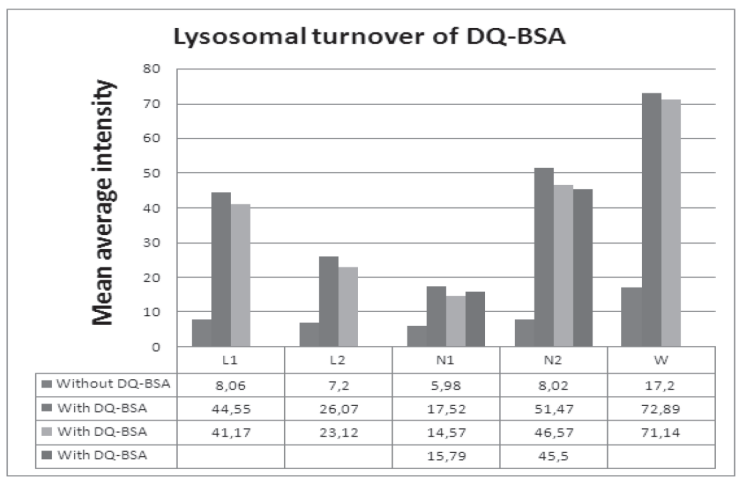
Figure 4
Fluorescent from the proteolysis of DQ-BSA was visualized in pink under Cellomics microscope. Individual cells were identified by labeling cells with nuclear dye Hoescht which was seen in blue.

A)



	L1	L2	N1	N2	W
Without DQ-BSA	8048,48	7639,47	6799,76	8009,16	17183,15
With DQ-BSA	41217,73	27297,15	30372,18	50891,8	70582,19
With DQ-BSA	43121,03	25575,15	16894,73	47451,4	72476,59
With DQ-BSA			17103,3	46716,77	

B)



	L1	L2	N1	N2	W
Without DQ-BSA	8,06	7,2	5,98	8,02	17,2
With DQ-BSA	44,55	26,07	17,52	51,47	72,89
With DQ-BSA	41,17	23,12	14,57	46,57	71,14
With DQ-BSA			15,79	45,5	

Figure 5
Mean total and mean average intensities of DQ-BSA in live cells, turnover of the DQ-BSA were found lower in the patients (significantly lower in L2 and N1 patients) compared to wild type.

levels, antioxidant enzyme, were detected by western blotting in L1, L2, N1 and N2 patient fibroblasts, more in L2 and N1 patients (2.8 and 3.3 fold, respectively) in our study (Fig.6).

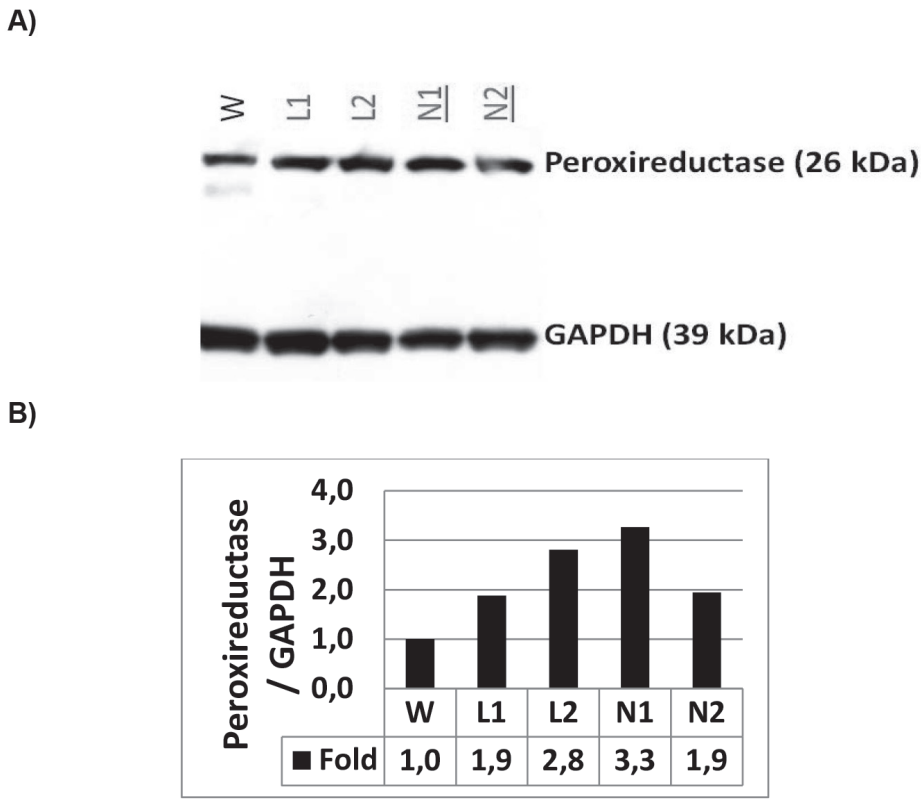


Figure 6

A) Western Blot analysis of lysates from L1, L2 (L444P mutation) and N1, N2 (N370S mutation) patient fibroblasts show increased levels of peroxireductase levels, more in L2 and N1 patients. GAPDH was used as a loading control. B) Peroxireductase protein levels were normalized to GAPDH signals in the same sample and presented as ratio relative to W (Wild fibroblasts). In L2 and N1 patients 2.8 and 3.3 fold increases in peroxireductase levels were observed, respectively. In other patients, increases in peroxireductase levels were also observed relative to GAPDH. Densitometry was performed using Image J analysis software (NIH).

Discussion

In this study, Gaucher disease was hypothesized that is associated with a lysosomal dysfunction which impairs the MALS ultimately leading to cell death. Our results revealed an increase in the number of autophagosomes which is due to a decrease in autophagic flux, probably resulting from glucosylceramide and glucosylsphingosine accumulations

in lysosomes. Furthermore the decrease in autophagic flux seems to be correlated with genotypes and phenotypes in our patients. Our data in L444P mutations indicated that, type II acute neuronopathic form that is associated with severe neurodegeneration has a significant block of autophagy.

Previous reports provide autophagosome accumulation in other types of LSDs, Danon disease, neural ceroid lipofuscinoses, Pompe, Mucolipidosis IV (12-16). Noting that these previous studies did not resolve the issue, if the autophagosome accumulation was due to increased formation of autophagosomes (which would lead to increased degradation of autophagic substrates) or decreased autophagic flux (which results in decreased degradation of such substrates), we aimed to determine MALS, whether or not autophagy is induced and whether or not it goes to completion by monitoring of LC3B-II and intralysosomal proteolysis of DQ-BSA considering the clinical phenotypes of Gaucher patients carrying L444P and N370S mutations. Therefore, we used LC3B-II, the only known protein that specifically associates with autophagosomes and not with other vesicular structures, complemented by appropriate degradation of DQ-BSA to analyze the autophagic flux in MALS. For this purpose, the release of fluorescent from DQ-BSA dequenching was measured by using high-throughput instrument Cellomics. The ratio between LC3B-I and LC3B-II was also considered as it correlates with changes in autophagy and provides a more accurate measure of autophagic flux than ratios based on the total level of LC3B-II (1). We revealed that the accumulation of autophagosomes in Gaucher fibroblasts is due to defective clearance caused by impaired lysosome function and correlated with the phenotypes in L444P mutations.

Contradictory and rare results of Gaucher studies exist in the literature. Tatti et al. demonstrated an activation of autophagy in Gaucher patient fibroblasts due to saposin C deficiency and observed a correlation among genotype, phenotype and increased autophagy (17). They observed normal autophagy in nonneuropathic phenotype. In contrast, enhanced autophagic response was found in the fibroblasts from chronic neuronopathic phenotype and from another phenotype that had not presented neurological manifestations yet. In our study, L2 and L3 patients with, type II acute neuronopathic form of the L444P mutation also revealed more significant increases in LC3B-II relative to LC3B-I

and GAPDH, thus increased autophagosomes and more significant decreases in the lysosomal turnover of DQ-BSA in L2 patient compared to L1, and more impaired lysosomal function. Even though defects in autophagy are less detrimental in fibroblasts than in neurons, which cannot dilute stored material by cell divisions, we could postulate that our findings could have a more significant impact if the same results could be obtained in the neurons of neuronopathic Gaucher patients. Pathological dysregulation of MALS could contribute to neurodegeneration and neuronal cell death, as recently reported in a mouse model with combined selective saposin C deficiency and mutant glucosylceramidase (19). Tatti et al. also speculated that the increased autophagy observed in the fibroblasts from chronic neuronopathic phenotype and from another phenotype that had not presented neurological manifestations yet, may worsen the patient's health conditions in the future. In N370S mutations in our study, N1 patient also revealed a decrease in lysosomal turnover of DQ-BSA compared to N2 patient, consistent with the results of LC3B-II. We could speculate that the decreased autophagic flux could worsen the patient's health conditions in the future.

The induction of autophagy is achieved through either the dephosphorylation of mTOR or the activation of the Beclin-1 pathway. Beclin-1, participates in autophagosome formation. Pacheco et al. showed wild-type basal levels of Beclin-1 and starvation induced LC3-II expression in Gaucher disease fibroblasts, which traffic sphingolipids normally (19). Our results, consistent with Pacheco et al. in the way that an autophagy induction doesn't exist in Gaucher patients and rather lysosomal accumulation of undegraded substrates result a block of the autophagic pathway.

Cells that display increased levels of autophagy and/ or decreased flux rates can lead to an accumulation of dysfunctional mitochondria (5). Accumulation of dysfunctional mitochondria subject cells to increasing oxidative stress when autophagy is dysfunctional (11,20). Peroxiredoxins are antioxidant enzymes that reduce hydrogen peroxide and alkyl hydroperoxides to water and alcohol, respectively. Consistently, increases of peroxireductase levels were detected by western blotting in L1, L2, N1 and N2 patient fibroblasts, much more in L2 and N1 patients, correlating with the severity of autophagy dysfunction in these patients.

Our results revealed a block of autophagic pathway occuring as a consequence of decreased ability of lysosomes to fuse with autophagosomes

in L444P and N370S mutations, more significant in type II acute neuronopathic form exacerbating the clinical phenotype. Glucosylceramide and glucosylsphingosine accumulations in lysosomes decreased autophagic flux and is a crucial component in the pathogenesis of Gaucher disease. However, further studies will be necessary to understand the underlying mechanism in MALS, as this pathway is complex and requires different steps and may be a promising therapeutic approach for several LSDs.

Conclusion

We can conclude that L444P and N370S mutations results in an increase in the number of autophagosomes which is due to a decrease in autophagic flux exacerbating the clinical phenotype.

Summary

Gaucher disease is caused by defects in the activity of the lysosomal enzyme, glucocerebrosidase (EC 3.2.1.45). Since lysosomes ultimately are responsible for turning over macromolecules transported to them from both outside (endocytosis and phagocytosis) and inside the cell (macroautophagy-lysosomal system), we explored the possibility that the glucosylceramide and glucosylsphingosine accumulation resulting from Gaucher disease may disrupt the normal autophagic fusion process and/or autophagic flux exacerbating the clinical phenotype. To discriminate between an induction of autophagy and defective maturation/fusion of autophagosomes to autolysosomes, versus autophagic flux (turnover of cargo in the autolysosome), LC3B-II levels and proteolysis of internalized fluorescent DQ-BSA in lysosomes (with the use of high throughput instrument Cellomics KSR) were monitored in Gaucher fibroblasts carrying the most prevelant mutations L444P and N370S. Increased levels of LC3B-II relative to LC3B-I and GAPDH demonstrated an increase in the number of autophagosomes and/or a decrease rate of lysosomal turn-over (flux) in fibroblasts of patients which were correlated with the phenotypes. Lower turnover of the DQ-BSA in patient fibroblasts indicated that the flux rate was decreased in Gaucher disease. The decrease

of lysosomal flux was more significant in the Type II, acute neuropha-thic form of L444P mutation. We can conclude that L444P and N370S mutations decreased the the rate of turn-over of autophagy and other substrates, including LC3B-II, in the lysosome and that the decreases roughly correlated with the clinical severity.

Key words: Macroautophagy-lysosomal system, Gaucher, LC3B-II, DQ-BSA

Özet

L444P ve N370S mutasyonları taşıyan gaucher hastalarında makrootofaji-lizozomal sistem (mals)

Gaucher hastalığı, lizozomal bir enzim olan glukoserebrosidazın (EC 3.2.1.45) aktivitesinin bozukluğu sonucu ortaya çıkar. Lizozomlara hücre dışından (endositoz ve fagositoz) ve hücre içinden (makrootofaji-lizozomal sistem) makromoleküller taşınmaktadır. Bu çalışmada, Gaucher hastalığında biriken glukozilseramid ve glukozilsfingozinin normal otofajik füzyon süreci ve/veya otofajik akışı bozabilme ve klinik fenotipi şiddetlendirme olasılığı araştırıldı. Otofaji indüksiyonu ve otofagozomların otolizozomlara bozuk maturasyonu/füzyonu durumunun, otofajik akış (otolizozomlara kargonun taşınması) bozukluğundan ayırt edilmesi için, en sık gözlenen L444P ve N370S mutasyonlarını taşıyan Gaucher fibroblastlarında LC3B-II düzeyleri ve lizozomlarda floresan DQ-BSA'nın proteolizi (Cellomics KSR kullanılarak) izlendi. LC3B-I ve GAPDH'a göre artmış düzeydeki LC3B-II, hastaların fibroblastlarında otofagozomların sayısında artışı ve/veya azalmış lizozoma akış hızını gösterdi. Bu durum hasta fenotipleri ile uyumlu bulundu. Hasta fibroblastlarında DQ-BSA'nın düşük proteolizi, Gaucher hastalığında otofajik akış hızının azaldığını göstermektedir. Azalmış lizozoma akış hızı, L444P mutasyonu taşıyan Tip II, akut nöropatik formda daha belirgindi. Sonuç olarak, L444P ve N370S mutasyonlarında otofajik ve LC3B-II'yi de içeren diğer substratların lizozomlara akış hızı azalmıştır ve bu azalma klinik şiddet ile kabaca uyumludur.

Anahtar kelimeler: Makrootofaji-lizozomal sistem, Gaucher, LC3B-II, DQ-BSA

Acknowledgements

This work was supported by the Scientific and Technical Research Council of Turkey

(TUBITAK).

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