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Spectral properties and mechanisms that underlie autofluorescent accumulations in Batten disease

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Abstract

Neuronal Ceroid Lipofuscinoses (NCLs) have an incidence of 1 in 12,500 live births. These devastating neurodegenerative lysosomal storage diseases are characterized by the lysosomal accumulation of autofluorescent storage material (AFSM) similar to that seen in aging cells. Using patient derived lymphoblasts from three genetically distinct NCLs we report that AFSM for each NCL has distinct spectral properties. Moreover, by using pharmacological inhibitors to disrupt various biochemical pathways in normal control lymphoblasts we have determined that disruptions in microtubule assembly and non-muscle myosin II function results in accumulation of lysosomal AFSM. Interestingly, inhibition of autophagy did not result in AFSM. We conclude that cellular disturbances outside the lysosome in addition to compromised function of this organelle can result in accumulation of lysosomal AFSM in NCLs and possibly as a result of cellular aging.

Keywords

autofluorescent storage material; Neuronal Ceroid Lipofuscinosis; microtubule assembly; non-muscle myosin II

Introduction

Neuronal Ceroid Lipofuscinoses or Batten disease (NCLs) are the most prevalent family of pediatric neurodegenerative diseases. This family of disorders is inherited in an autosomal recessive manner with an occurrence of 1 in 12,500 live births worldwide [1]. There are nine

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reported variants of NCL of which eight have been confirmed to be genetically distinct [2]. Symptoms include progressive blindness, seizures, mental and cognitive decline, and which ultimately results in premature death [3].

The three most studied NCLs are Infantile NCL (INCL), Late Infantile NCL (LINCL) and Juvenile NCL (JNCL). INCL has an age of onset between 0–2 years old and results from mutations in *CLN1*, which encodes for the soluble lysosomal enzyme palmitoyl protein thioesterase-1 (PPT1). LINCL has an age of onset of 2–4 years old and results from mutations in *CLN2* that encodes the soluble lysosomal enzyme tripeptidyl peptidase (TPP1). JNCL, the most common variant of NCL, has an age of onset of 5–10 years old and results from mutations in the *CLN3*. *CLN3* encodes for the lysosomal/endosomal membrane protein whose function is currently unknown. CLN3 has been suggested to function in a wide array of pathways (reviewed in [4]).

NCLs are characterized by the presence of autofluorescent storage material (AFSM) in the lysosomes. AFSM has been characterized to be composed of 60% protein [5]. In INCL the predominant stored protein is sphingolipid activating proteins A & D (reviewed in [6]). For LINCL and JNCL, the major protein component is subunit c of the mitochondria ATPase [7; 8]. Other components of AFSM reported are: neutral lipids, phospholipids, dolichol pyrophosphate linked oligosaccharides, lipid linked oligosaccharides, dolichol esters, and metals such as Fe (reviewed in [6]).

We have studied the spectral characteristics of AFSM in immortalized lymphoblast cell lines from INCL, LINCL, and JNCL patients by fluorimetry as a means to distinguish AFSM in NCLs. In addition, by treating normal control lymphoblasts with biochemical inhibitors of a number of pathways, we have been able to identify biological pathways that when disrupted can result in characteristic accumulation of AFSM. Surprisingly, blocking microtubule assembly or inhibiting non-muscle myosin II resulted in a fluorescence signal over and above the AFSM accumulated in NCL lymphoblasts. This is the first study to suggest that AFSM accumulation can result from a cell biological insult that is not primarily associated to lysosomal function.

Materials and methods

Cell Culture

Lymphoblast cell lines were established from blood samples collected from patients with JNCL and LINCL as previously described [9]. Lymphoblasts for age, sex, split ratio matched controls and INCL, Niemann Pick C (NPC) were obtained from Coriell Cell Repository. These cell lines were established using a similar protocol as described in [9]. Lymphoblast cell lines used in this study are shown in Table 1. Lymphoblasts were maintained in T75 cm² flask with RPMI media (Sigma, St.Louis, MO) containing 20% Fetal Clone I (Hyclone, Logan, UT) and antibiotic-antimycotic (Sigma) at 37°C and 5% CO₂. After 3–5 days cells were split continuously until they reached log phase of growth. Cells for all experiments were seeded at 500,000 cells/ml in normal growth media. NCL lymphoblasts and corresponding control lymphoblasts were exposed to 30 µM cytosine-β-D-arabinofuranoside (Ara-C) (Sigma), a mitotic inhibitor, for 24 hr, then reseeded at 500,000

cells/ml and examined after 3 days of growth for the presence of autofluorescent storage material (AFSM). For the pharmacological inhibitor studies (shown in Table 3 and Figure 3), control lymphoblasts were treated with each drug once daily for 3 days and examined on the fourth day, with the exception of brefeldin A and trichostatin A, which were only once, on day one, due to their toxicity to control lymphoblasts. Drug treatments were compared to appropriate vehicle controls, and cell viability was determined in cell lines using the trypan blue exclusion assay.

Fluorescent Microscopy

Cells were counted on the day of imaging and resuspended at a concentration of 7.5 million cells/ml in Hank's Buffer Salt Solution, (HBSS) (Invitrogen, Carlsbad, CA). Cells were imaged on a fluorescence microscope (BX60, Olympus, Melville, NY) equipped with a CCD camera (Spot RT, Diagnostic Instruments, Sterling Heights, MI) using a U-MWU filter cube (Ex 330–385BP Em 420LP, Olympus) under identical exposure conditions. Thirty images were taken at random locations on two slides for each sample per day, with over 100 images analyzed in total, with each field containing an average of 15 to 50 cells. Threshold Analysis was performed on the fluorescent photomicrographs in Image J (NIH) to determine the mean fluorescence per photomicrograph. The amount of AFSM was expressed as mean fluorescence per cell.

For localization of AFSM to the lysosome, cells were resuspended at a concentration of 5 million cells/ml and loaded with 200 nM LysoTracker Red (Invitrogen) for 1 hr, followed by two washes in HBSS. Lymphoblasts were imaged on a Olympus BX61 microscope equipped with a CCD camera (CoolSNAP HQ, Photometrics, Tucson, AZ) using appropriate filters for AFSM and LysoTracker Red visualization. Multiple images in the z-plane were obtained for each cell and subsequently deconvolved using AutoQuant X2 software (Media Cybernetics, Bethesda, MD). Images of AFSM were pseudo-colored green for clarity and images were overlaid using ImageJ.

Fluorimetric detection of AFSM

Lymphoblasts were resuspended at 7.5 million cells/ml and placed in triplicate into individual wells of a black 96-well plate (Corning, Corning, NY). Fluorometric Scans for both emission and excitation wavelengths, at 1 nm intervals, were performed on a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA) to determine excitation and emission maxima. Spectral profiles from individual cell lines were then performed using these specific excitation and emission wavelengths, and are expressed as relative fluorescence units (RFU) normalized to background fluorescence of HBSS.

Immunocytochemistry

Control lymphoblasts were seeded on the poly-L-lysine coated coverslips at a density of 500,000 cells/ml and grown for two days at 37°C with 5% CO₂. On the third day, cells were treated with nocodazole and blebbistatin for 2 hours, and then fixed with 2% paraformaldehyde (PFA) solution for 30 min. The cells were incubated in 3%PFA/3% Sucrose/0.1% Triton-X solution 1 hr, followed by three washes with TBS with 0.1% Triton-X (TBS-T). Each well was blocked overnight at 4°C with TBS-T containing 5% fat-free

milk (Blotto-TBS-T). Primary antibodies for non-muscle myosin IIB (1/250, CMII23, DSHB, Iowa City, IA) and β -tubulin (1/500, T9028, Sigma) were diluted in Blotto-TBS-T and incubated overnight at 4°C. Cells were washed with TBS-T and incubated in Goat-anti-mouse Alexa-fluor 568 secondary antibody (A11004, Invitrogen) for 1 hour at 25°C. Cell nuclei were stained by incubating in 4',6-diamidino-2-phenylindole (DAPI) for 5 mins, then coverslipped with anti-fade mounting media [10]. Each coverslip was imaged as described for lysosomal localization.

Statistical analysis

Results comparing two samples were analyzed with a student *t*-test using Prism 4 software (Graphpad Software, La Jolla, CA). *p*-values values <0.05 were considered significant using a confidence interval of 95%.

Results and Discussion

Autofluorescent storage material (AFSM) in NCL lymphoblast cells

Breaking cells open to isolate AFSM results in a loss of its spectral properties presumably due to dissociation of the AFSM or loss of aggregate properties necessary for the fluorescence (unpublished data). While JNCL lymphoblasts accumulate AFSM without the use of a mitotic inhibitor, it took approximately 15 days in culture (data not shown). As lymphoblasts are suspension cells that cannot be grown to a high enough concentration to cause complete mitotic arrest, to optimize detection of AFSM a mitotic inhibitor, β -cytosine-arabinofuranoside (Ara-C), was utilized.

Both JNCL and control lymphoblasts were treated with Ara-C for 24 hours then reseeded at 500,000 c/mL. After three days in culture, JNCL and control lymphoblasts were examined by fluorescent microscopy using the DAPI filter (Figure 1A). While fluorescence was seen under other fluorescence filters, the DAPI filter with excitation 330–385 nm and emission 420 nm exhibited the most robust fluorescence (data not shown). Photomicrographs were quantified using threshold analysis to determine mean fluorescence per cell (Figure 1B). JNCL cells have accumulation of AFSM that is significantly higher than matched control cell lines ($p < 0.001$). Control cells have a mean fluorescence per cell of approximately 0.007 this is attributed to the aging pigment, lipofuscin, having a similar excitation and emission spectra. A comparison of lymphoblasts from a patient with Niemann Pick type C disease, a lysosomal storage disorder not known to accumulate AFSM and an age and sex matched control had no significant differences (Figure 1B). A cell death assay using trypan blue exclusion indicated no differences between control and disease lymphoblasts (data not shown).

Threshold analysis and the mean fluorescence per cell was also determined the presence of AFSM in INCL and LINCL lymphoblasts (Figure 1A, B). INCL and LINCL had significantly higher mean fluorescence per cell than control lymphoblasts ($p < 0.01$). AFSM has been reported to be lysosomal in localization in NCLs [8]. The AFSM we were studying in JNCL lymphoblast cells was confirmed to be partially localized to the lysosome by wide-field deconvolution imaging (Supplementary Figure 1). INCL and LINCL lymphoblasts showed similar colocalization (data not shown).

Spectral characteristics of autofluorescent storage material (AFSM) in NCL lymphoblasts

Previous studies have shown that INCL has different accumulations than LINCL and JNCL [11]. Most microscopes have a set of fluorescence filter with broad ranges of excitation light and a single emission readout point. We used the U-MWU DM400 filter (DAPI) with an excitation range of 330–385 nm and the 420 nm emission readout point. This filter set up was unable to determine a difference in spectral properties in the AFSM between JNCL, LINCL and INCL. Fluorimetry with its ability to scan in 1 nm increment and adjust wavelength for both excitation and emission was therefore used to resolve the characteristic spectral properties of INCL, LINCL and JNCL cells.

The previously reported range of excitation and emissions for AFSM in NCL is 350–380 nm and 400–600 nm, respectively (Reviewed in [6]). Numerous combinations of different excitation and emission wavelength were used to conduct spectral scans to determine the most robust excitation and emissions to obtain spectral emission profiles of AFSM for JNCL, LINCL, and INCL. It was determined that the following scans would be examined for each: emission scan (ex360) and emission scan (ex370). The excitation 360 nm scans of NCL lymphoblast cells and their corresponding control lymphoblast cells indicated that each NCL variant had a similar spectral profile with the emission maximum approximately 458–459 nm (Figure 2A, B, C; Table 2). It is noted that the excitation of 360 nm was the midpoint of the excitation from the microscopy previously shown. However, the excitation 370 nm emission scans showed a different profile. Upon examination of the emission maximums it is noted that LINCL has an emission maximum at 446.36 ± 2.36 ; which significantly shifted in the red wavelength direction compared to JNCL and INCL ($p < 0.01$) (Figure 3A, B, C; Table 2). It should be noted that while the shift was shown in emission maximums, that only INCL has a fluorescence signal seen above control levels (Figure 3B). Thus, excitation at 370 nm is the optimal excitation to examine INCL. LINCL and JNCL lymphoblasts had distinguishable spectral differences at excitation 370 nm. Overall, INCL does exhibit a more intense AFSM signal as seen by fluorimetry.

Differences in storage material in NCLs has been illustrated previously by ultrastructural examination of AFSM by electron microscopy [12]. INCL have granular osmiophilic deposits, while LINCL has curvilinear profiles [12]. JNCL has been reported to have several different ultrastructures of AFSM such as fingerprint profiles and rectilinear-complex profiles [13]. Thus, in addition to the ultrastructural differences, we now report that spectral differences in AFSM in NCLs. However, it should be noted that as it is known that accumulations are different in INCL and JNCL, but the spectral measures we report cannot differentiate between these two diseases.

Possible mechanisms that underlie AFSM accumulation

We tested inhibitors known to affect lysosomal function for their ability to induce AFSM, Table 3. Sub-lethal concentrations of lysosomal inhibitors were determined by a dosage curve using the highest concentration of each inhibitor that did not induce mitotic arrest after a week of treatments (data not shown). NCL lymphoblasts exhibited accumulation of AFSM after three days in culture (data not shown) and this method gave a time scale for drug treatment that did not cause cell death or morphological changes in the lymphoblasts.

Pepstatin A, a general acid protease inhibitor [14], and bafilomycin, which inhibits V-ATPase, [15] did not induce AFSM (Figure 4). However, chloroquine which raises lysosomal pH did induce AFSM significantly over vehicle/no drug control ($p<0.001$) [16].

Mutations in CLN6 and CLN8 result in variant forms of LINCL that also bear the characteristic accumulation of lysosomal AFSM (reviewed [6; 17]. Interestingly, CLN6 and CLN8, although not studied in this paper, localize to the ER and ERGIC, respectively, suggesting that AFSM could result from a disruption in a protein or pathway independent of lysosomal function. Therefore we decided to test inhibitors of a variety of different cellular pathways for the ability to induce accumulation of AFSM. In accordance with our established methods for lysosomal inhibitors, inhibitor dosage curves were carried out on lymphoblasts to determine the highest concentration of each inhibitor that did not induce mitotic arrest. A daily dosage scheme for three days was again unless otherwise indicated.

MG132, an inhibitor of the proteasome [18], Brefeldin A, which blocks endoplasmic reticulum trafficking to Golgi apparatus, Trichostatin A, which inhibits histone deacetylases [19] and 3-methyladenine, which blocks the sequestration step in autophagy [20] did not induce AFSM (Figure 4).

Disruption of the cellular highways or cytoskeletal network was blocked with nocodazole, which inhibits microtubule assembly, and the actin depolymerizing agent, latrunculin B [21; 22]. Blebbistatin an inhibitor of non-muscle myosin II was also tested [23]. Interestingly, nocodazole and blebbistatin did induce an AFSM signal higher than that observed in JNCL lymphoblasts ($p<0.001$) (Figure 3A). However, latrunculin did not induce AFSM (Figure 4).

In summary, nocodazole, chloroquine, and blebbistatin all induced AFSM and were scanned for emission profiles at excitation 370 nm (Supplementary Figure 2; Table 3). The emission maximums of nocodazole, blebbistatin and chloroquine treatments are statistically different from LINCL but indistinguishable from those observed in INCL and JNCL. To determine that the fluorescence signal seen in drug treated control lymphoblasts was due to NCL-like AFSM, LysoTracker was used to confirm lysosomal localization of the induced AFSM. It was shown that both nocodazole and blebbistatin induced AFSM partially colocalized to the lysosome (Supplementary Figure 3A). AFSM induced by chloroquine could not be localized by this method because the associated increase lysosomal pH disrupts lysosomal uptake of LysoTracker into the lysosome. Immunocytochemistry (ICC) with a β -tubulin antibody confirmed that lymphoblasts treated with nocodazole or blebbistatin, did manifest disrupted microtubule networks (Supplementary Figure 3B and C).

While there is no clear association in disrupting the cytoskeleton with lysosomal dysfunction, our study implies that AFSM accumulation can result from a cell biological disruption that emanates from outside of the lysosome. This raises the intriguing possibility that while INCL, LINCL and JNCL, lack lysosomal proteins that presumably results in altered lysosomal function, that AFSM may accumulate indirectly from lacking these proteins. Abnormal lysosomal function could result in suboptimal cytoskeletal function that ultimately could disturb many cellular pathways and feed back to the lysosome, resulting in the accumulation of lysosomal AFSM. Thus, in any NCL, whether the defect ultimately

resides initially in the lysosome or not, the AFSM may be a secondary consequence resulting from other cellular disturbances.

In summary, this paper highlights the spectral differences between AFSM stored in INCL, LINCL and JNCL patient lymphoblasts. Moreover, the different functions of these proteins associated to the NCLs likely result in somewhat different accumulations in the lysosome. It is likely that there are many routes that lead to AFSM and that NCL-proteins, while sharing the common theme of resulting in AFSM when mutated, could be at very different points in a host of processes that when disrupted can lead to AFSM. Similarly, AFSM as a result of aging could result from disruptions in any combination of these or other cellular processes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig 1.

NCL lymphoblasts accumulate autofluorescent storage material (AFSM). Panel A illustrates representative photomicrographs for AFSM in control JNCL, JNCL, control INCL/LINCL, INCL and LINCL lymphoblasts. Each fluorescent photomicrograph underwent threshold analysis to determine the mean fluorescence over the photomicrograph followed by cell counts using the brightfield photomicrographs. The quantification of photomicrographs is expressed in mean fluorescence per cell. B) Threshold analysis for control JNCL, JNCL, control INCL/LINCL, INCL, LINCL, Niemann Pick C (NPC), and control NPC lymphoblast cells. JNCL, INCL, and LINCL lymphoblasts all exhibited a significantly higher mean fluorescence per cell when compared to its corresponding control cell line ($p < 0.0001$; $p < 0.01$; $p < 0.001$ respectively).

Fig 2.

Spectral characterization of NCL lymphoblasts. Using a SpectraMax M5 microplate reader cells were scanned for emission profiles from 420 nm to 600 nm at excitation 360 nm. A) Representative scans of JNCL and control lymphoblasts showing the characteristic wide peak for AFSM at excitation 360nm. B) Representative scans of INCL, LINCL and control lymphoblasts showing a similar wide peak for AFSM at excitation 360nm. C) Emission maximums for all NCL variants were shown to have a similar emission maximum at approximately 458–459 nm when excited at 360 nm.

Fig 3.

Spectral characterization of NCL lymphoblasts. Using a SpectraMax M5 microplate reader cells were scanned for emission profiles from 420 nm to 600 nm at excitation 370 nm. Representative scans of JNCL and control lymphoblasts illustrating that this is not the optimal excitation for JNCL lymphoblasts however it does show a different spectral profile from control lymphoblasts at excitation 370 nm. B) Representative scans of INCL, LINCL and control lymphoblasts show a different spectral profile for each NCL variant at excitation 370 nm. INCL appears to be best examined at excitation 370 nm. C) Emission maximums for all NCL variants have different emission maximum at excitation 370 nm.

Fig 4.

Induction of AFSM. Control lymphoblasts were treated with a single pharmacological inhibitor daily for three days at the concentration shown in Table 3. Fluorescent and brightfield photomicrographs were taken for each drug treatment and no drug control. A) Threshold analysis of drug treatments with mean fluorescence per cell normalized to the no drug control. JNCL line is a reference from Figure 1. Nocodazole (disrupts microtubule assembly), blebbistatin (inhibits myosin II), and chloroquine (increases lysosomal pH) induce AFSM in control lines significantly over the no drug/vehicle control ($p < 0.0001$).

Table 1

Lymphoblasts cell lines used in this study

Disease	Disease Mutations	Age	Sex
JNCL	1.02kB/1.02kB	15	M
JNCL	1.02kB/1.02kB	10	M
JNCL	1.02kB/1.02kB	9	M
JNCL	1.02kB/1.02kB	14	M
JNCL	1.02 kB/ivs11+5 G to A	14	F
JNCL	1.02 kB/R334H	25	M
INCL	R151T/R151T	3	M
LINCL	R208 X/splicing	4	M
Niemann-Pick C	P237S/I1061T	9	F
Healthy Control	None	9	M
Healthy Control	None	4	M
Healthy Control	None	10	F
Healthy Control	None	15	M
Healthy Control	None	14	F
Healthy Control	None	25	M

Table 2

AFSM emission maximums

Disease	Excitation 360 (nm)	Excitation 370 (nm)
JNCL	459 ± 1.15	438.62 ± 1.383 [#]
INCL	458.42 ± 1.26	439.95 ± 2.03 [*]
LINCL	458.89 ± 0.19	446.36 ± 2.36 ^{# *%@&}
Nocodazole	NA	437.45 ± 2.51 [%]
Blebbistatin	NA	440.91 ± 3.5 [@]
Chloroquine	NA	438.11 ± 1.41 ^{&}

(One way ANOVA, Bonferroni *post hoc* test:^{*}
p<0.01[#]
p<0.01[%]
p<0.001[@]
p<0.01[&]
p<0.001)

Table 3

Concentration of biochemical inhibitors used.

Inhibitor	Pathway altered	Concentration
3-Methyadenine (3-MA)	Autophagy	67 uM
Bafilomycin (Bafilo)	V-ATPase	10 nM
Blebbistatin (Blebb)	Non-muscle Myosin II	10 uM
Brefeldin A (BFA)	Trafficking from Golgi apparatus to ER	36 nM
Chloroquine (Chl)	Lysosomal homeostasis	20 nM
Latrunculin B (Lat B)	Actin dynamics	0.1 nM
MG132	Proteosome	5 uM
Nocodazole (Noco)	Microtubule dynamics	16 nM
Pepstatin A (Pepst)	Lysosomal enzymes	730 nM
Trichostatin A (TSA)	Histone deacetylase Class I & II	33 nM