Autophagy is increased in prostate cancer cells over-expressing acid ceramidase and enhances resistance to C₆-ceramide

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Abstract

Acid ceramidase (AC) over-expression has been observed in prostate cancer cell lines and primary tumors, and contributes to resistance to chemotherapy and radiation. The consequence of AC over-expression is the ability to convert ceramide, which is often produced as a pro-apoptotic response to stress, to sphingosine, which can then be converted to the pro-survival molecule sphingosine-1-phosphate. In addition to their ability to metabolize ceramide produced in response to stress, we show here that prostate cancer cell lines over-expressing acid ceramidase also have increased lysosomal density and increased levels of autophagy. Furthermore, pre-treatment with 3-methyladenine restores sensitivity of these cells to treatment with C₆-ceramide. We also observed increased expression of the lysosomal stabilizing protein KIF5B and increased sensitivity to the lysosomotropic agent LCL385. Thus, we conclude that acid ceramidase over-expression increases autophagy in prostate cancer cells, and that increased autophagy enhances resistance to ceramide.

Keywords
autophagy; acid ceramidase; sphingolipids; ceramide; prostate cancer; apoptosis

Introduction

Prostate cancer is the second leading cause of cancer-related death in men in the United States. Statistical estimations for 2010 project 217,730 new cases and 32,050 deaths.¹ Nearly 1 in 6 men in the United States will be diagnosed with prostate cancer in their lifetime.² While localized prostate cancer is often treated effectively, advanced disease, whether local or metastatic, is resistant to treatment and often fatal. These statistics highlight

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Conflict of Interest
The authors report no conflicts of interest relating to the current study.
the need for elucidation of the mechanisms of resistance of prostate cancer to current treatment protocols and development of new treatment modalities.

Acid ceramidase (AC) is a ceramide-metabolizing enzyme primarily localized to lysosomes. Ceramide is the basic building block of the complex sphingolipids, which are major structural and signaling components of cells. Ceramide is produced in response to many cellular stresses and functions as a bioactive signaling lipid in processes including apoptosis, inflammation, and cell cycle arrest. Alterations in ceramide metabolism have been shown to contribute to apoptosis resistance in many types of cancer [reviewed in 3,4]. Increased transcription of AC has been observed in multiple prostate cancer cell lines compared to a benign prostatic hyperplasia cell line and in over 60% of primary tumors analyzed compared to matched normal tissue controls.5 In addition, our group has shown that AC over-expression contributes to resistance of prostate cancer cells to both chemotherapy6 and radiation.7 These results suggest that the increased clearance of ceramide by over-expression of AC allows cancer cells to escape ceramide-induced apoptosis, and highlight a novel target for cancer treatment. This hypothesis is supported by a study from Morales, et al., who showed that daunorubicin treatment increased acid ceramidase activity in hepatoma cell lines, protecting them from daunorubicin-induced cell death.8

Autophagy is a mechanism of recycling cellular proteins and organelles as a function of cellular homeostasis, development, or in response to stress.9 The process involves the formation of an autophagosome to sequester cytoplasmic material, fusion with a lysosome to form an autolysosome, and subsequent degradation of sequestered components by lysosomal hydrolases (reviewed in 10). Evidence that many types of cancer utilize autophagy as a survival mechanism has greatly increased research interest in this field in the last decade (reviewed in 11,12). Autophagy has been shown to be critical in survival of colorectal cancer cells under low-nutrient conditions,13 and increased autophagy in pancreatic cancer cells promotes tumor cell survival and is correlated to poor outcome.14,15 Recently, autophagy has been implicated in the development of resistance of breast cancer cells to the growth inhibitory effects of the anti-HER2 monoclonal antibody Trastuzumab.16

Our group has shown previously that AC over-expression contributes to resistance of prostate cancer cells to chemotherapy and radiation, and that inhibition of AC increases susceptibility to treatment;6,7,17-19 however, the role of autophagy in prostate cancer cells over-expressing AC has not been elucidated. In this study we investigated the effects of AC over-expression on autophagy in prostate cancer cells. We show that AC over-expression results in increased autophagy and lysosomal density, which confers a survival advantage in these cells. We also observed increased expression of the lysosomal stabilizing protein KIF5B in prostate cancer cells over-expressing AC which consequently increased their susceptibility to a lysosomal destabilizing agent. Our results suggest that prostate cancer cells over-expressing AC maintain a higher level of autophagy than parental cell lines, possibly creating an “insult-ready” phenotype, whereby cells have a higher resistance to initial insult and can rapidly metabolize any ceramide produced.
Materials and Methods

Cell lines, culture, and reagents

DU145 prostate cancer cell line (ATCC; Manassas, VA) and PPC1 prostate cancer cell line\textsuperscript{20} (a kind gift of Dr. Yi Lu at the University of Tennessee) were cultured at 37°C in 5% CO\textsubscript{2} in RPMI 1640 (Thermo Scientific HyClone; Logan, UT) containing 10% bovine growth serum (Thermo Scientific HyClone) and antibiotic-antimycotic solution (Mediatech; Manassas, VA). Generation of the DU145-AC-EGFP and DU145-EGFP cell lines has been described previously.\textsuperscript{6} PPC1 cells were transfected with a pEF6/V5-His-TOPO plasmid (Invitrogen; Carlsbad, CA) containing either lacZ-V5 or AC-V5 (kindly provided by Youssef Zaidan, Medical University of South Carolina) and stable clones were obtained by long-term culture in media containing blasticidin (Invivogen; San Diego, CA). LC3 cDNA was obtained from Dr. Noboru Mizushima (Tokyo Medical and Dental University, Japan) and inserted into pEGFP-C1 (Clontech; Mountain View, CA). Cell lines expressing EGFP-LC3 were obtained by transfection with the resulting plasmid and stable clones were obtained by long-term culture in media containing G418 (Mediatech;). Cell transfection was performed using TransIT-Prostate Transfection kit (Mirus Bio; Madison, WI). 3-methyladenine (3-MA) was obtained from Sigma (St. Louis, MO). C\textsubscript{6}-ceramide and LCL385 were synthesized by the Medical University of South Carolina Lipidomics Core Facility Synthetic Subcore (Charleston, SC).

Cytotoxicity Assays

Cell viability was assessed using the CellTiter 96 AQ\textsubscript{ueous} One Solution Cell Proliferation Assay (Promega; Madison, WI). Cells were plated at approximately $5 \times 10^3$ cells per well in 96-well plates and incubated overnight. The following day media was replaced with desired treatment and following incubation the assay was carried out according to the manufacturer’s instructions.

Confocal and Fluorescent microscopy and Flow Cytometry

Confocal microscopy was performed using a Leica Laser Scanning Confocal Microscope maintained by the Medical University of South Carolina Hollings Cancer Center Cell and Molecular Imaging Core (1P30 CA138313–01). EGFP was detected using 488\textsubscript{λ} excitation laser. For Lysotracker Red and Acridine Orange staining, cells were plated at $5 \times 10^5$ cells per 60mm culture plate and incubated overnight. The following day, media was replaced containing Lysotracker Red (Invitrogen) diluted 1:2,000, or Acridine Orange (Sigma) at a final concentration of 0.1μg/ml. Cells were incubated for 15 or 30 minutes (Acridine Orange and Lysotracker Red, respectively) at 37°C, then trypsinized and washed twice with PBS. Staining was visualized using an Olympus Inverted Fluorescent Microscope with exposure times of 200ms (bright field) and 800ms (red fluorescence). Lysotracker and acridine orange staining was quantified using a Becton Dickinson FACSCalibur Analytical Flow Cytometer by the Medical University of South Carolina Hollings Cancer Center Flow Cytometry & Cell Sorting Core Facility.
siRNA Transfection

Cells were seeded at $2 \times 10^5$ cells per well in a 6-well plate and incubated overnight. The following day, the cells were transfected with siRNA using Oligofectamine (Invitrogen) according to manufacturer’s protocol. The scramble siRNA sequence has been described previously. The AC siRNA sequences (designed by Donald Rao, Gradalis; Dallas, TX) were designed based on the published sequence of AC mRNA (NM_004315). AC siRNA sequence #1 corresponds to bases 2052 to 2076 in the 3' UTR (5'-AAGAAGTAATCAGTATGCAAAGCAA-3') and sequence #2 corresponds to bases 800 to 824 in the coding region (5'-AACAGTGAATTTGGATTTCCAAAGA-3'). Both siRNA sequences were purchased from Qiagen (Valencia, CA).

Protein preparation and western blot

Cells were seeded in 60mm plates as described above and treated accordingly. Cells were lifted by gently scraping the plates, washed once with ice cold PBS and then lysed by incubation on ice for 30 minutes in PBS containing 1% Triton X-100, 10% glycerol, and Complete Mini Protease Inhibitor Cocktail Tablet (Roche; Indianapolis, IN). Insoluble material was removed by centrifugation at 14,000 rpm for 15 minutes at 4°C. The supernatants were supplemented with SDS at a final concentration of 2% and stored at −80°C. Protein concentration was determined using the BCA Protein Assay kit (Pierce; Rockford, IL) according to the manufacturer’s instructions. Fifty μg of protein per sample (unless otherwise indicated) were separated on NuPAGE 4–12% Bis-Tris gels (Invitrogen) and transferred to nitrocellulose membranes (BioRad; Hercules, CA). Membranes were blocked with 5% milk in Tris-buffered saline (TBS) containing 0.1% Tween-20 and incubated overnight at 4°C with primary antibodies: actin (Sigma); AC (BD Biosciences; San Jose, CA); LC3 (Abcam; Cambridge, MA); KIF5B (Abcam). Following primary antibody incubation, membranes were washed and incubated 1 hour at room temperature with secondary antibodies: goat anti-rabbit-HRP and goat anti-mouse-HRP (Santa Cruz Biotechnology; Santa Cruz, CA). Proteins were visualized using Immobilon Western HRP Substrate (Millipore; Billerica, MA).

Results

Prostate cancer cells over-expressing AC are resistant to C6-ceramide treatment and nutrient depletion

We have shown previously that prostate cancer cell lines that over-express acid ceramidase are resistant to chemotherapy and radiation, both treatments that can induce ceramide. To confirm these observations, DU145 prostate cancer cells over-expressing AC (DU145-AC-EGFP) and control cells (DU145-EGFP) and PPC1 prostate cancer cells over-expressing AC (PPC1-AC-V5) and control cells (PPC1-lacZ-V5) were cultured in 96-well plates and treated with C6-ceramide. Cell viability was measured by MTS assay 72 hours after treatment. Consistent with our previous observations, over-expression of AC conferred resistance to C6-ceramide treatment compared to control cells on both cell types (Figures 1A and 1B). DU145 prostate cancer cells over-expressing AC also exhibited improved survival following 72 hours in serum-free media, a condition known to induce autophagy (Figure 1C).
**Autophagy is increased in PPC1 cells over-expressing AC**

As autophagy has been shown to be involved in the progression of many types of cancer, we wanted to determine if autophagy was involved in the increased resistance to stress observed in AC over-expressing cells. We observed that PPC1-AC-V5 cells had increased expression of the autophagy protein LC3 by western blot (Figure 2A). Following transfection with a construct coding for an EGFP-LC3 fusion protein, we observed that in cells over-expressing AC there was an increase in the number of cells with punctate distribution of EGFP-LC3, an indicator of autophagy, versus diffuse cytoplasmic staining in control cells (Figure 2B). In order to confirm that increased LC3 expression in the AC cells was due to AC over-expression, we knocked down AC expression using siRNA. Reduction in AC expression resulted in decreased expression of LC3 (Figure 2C), confirming a link between AC expression and autophagy in these cells.

**AC over-expression results in increased lysosomal density**

As AC is primarily expressed in lysosomes, and autophagy involves fusion of autophagosomes with lysosomes, we wanted to determine if AC over-expression altered lysosomal density in prostate cancer cells. DU145 and PPC1 prostate cancer cells over-expressing AC were stained with Lysotracker Red and analyzed by flow cytometry and fluorescent microscopy. In both cell types, AC over-expression resulted in a significant increase in lysosomal staining compared to control cells when analyzed by flow cytometry (Figure 3A). This increase was also visualized using fluorescent microscopy (Figure 3B). Next, we stained PPC1-AC-V5 and PPC1-lacZ-V5 cells with acridine orange and analyzed fluorescent intensity by flow cytometry. Acridine orange stains nucleic acids and acidic organelles, resulting in green and red emission spectra, respectively, when analyzed using fluorescence. Thus, an increase in the ratio of red staining to green staining indicates an increase in acidic vesicular organelles in the cells. PPC1-AC-V5 cells showed an increase in the red:green staining ratio compared to PPC1-lacZ-V5 cells (Figure 3C), indicating an increase in acidic vesicular organelles in these cells.

**Inhibition of autophagy improves treatment response**

We next wanted to analyze if increased autophagy in AC over-expressing cells contributes to resistance to ceramide treatment. DU145 and PPC1 cell lines were treated with 25μM C6-ceramide for 72 hours, with or without 3-methyladenine (3-MA), an inhibitor of autophagy. As observed previously, DU145-AC-EGFP and PPC1-AC-V5 cells had reduced sensitivity to C6-ceramide treatment compared to DU145-EGFP and PPC1-lacZ-V5 cells. However, pre-treatment with 3-MA increased sensitivity of both AC over-expressing cell lines to ceramide to levels comparable to control cells (Figure 4).

**AC over-expression results in the increased expression of the lysosomal stabilizing protein KIF5B**

Kinesin 5B (KIF5B) is a microtubule motor protein that associates with lysosomes and plays a role in autophagy. Cardoso, et al., showed that KIF5B has increased association with lysosomes in the highly invasive MCF-7 breast cancer cells that over-express an active form of ErbB2, and that KIF5B depletion resulted in perinuclear accumulation of
autophagosomes and increased lysosomal membrane permeabilization.\textsuperscript{21} The invasive MCF-7 cells also exhibited increased expression of lysosomal proteases and altered lysosomal trafficking. Therefore, we next wanted to determine if KIF5B expression was altered in prostate cancer cells over-expressing AC. Control cells and cells over-expressing AC were analyzed by western blot for KIF5B expression. We observed an increase in KIF5B expression in cells over-expressing AC (Figure 5A), and knockdown of AC expression with siRNA reduced KIF5B expression (Figure 5B).

**Prostate cancer cells over-expressing AC have increased sensitivity to lysosome destabilization**

It has been shown previously that alterations in lysosome density and structure in transformed cells can actually sensitize such cells to lysosomal cell death pathways.\textsuperscript{22} Since we observed significant alterations in lysosome density as well as increased expression of the lysosome-associated protein KIF5B, we wanted to determine if our cells were more sensitive to lysosome destabilization. For this study we used LCL385, a lysosomotropic agent designed and developed by the Lipidomics Core Facility at the Medical University of South Carolina and described previously.\textsuperscript{7,23} DU145-AC-EGFP and DU145-EGFP cells or PPC1-AC-V5 and PPC1-lacZ-V5 cells were cultured in 96-well plates in media with or without LCL385 (15 μM). Cell viability was measured by MTS assay 48 hours after treatment. Compared to cells cultured in media alone, DU145-AC-EGFP and PPC1-AC-V5 cells were significantly more sensitive to LCL385 than DU145-EGFP and PPC1-lacZ-V5 cells (Figure 6).

**Discussion**

The involvement of autophagy in cancer cell biology is widely recognized and extensively published. Autophagy has been shown to serve as a protective mechanism against cell death following radiation, a treatment known to increase cellular ceramide levels. Irradiated MCF-7 cells exhibited an increased number of autophagic vacuoles, and inclusion of an inhibitor of autophagy decreased the surviving fraction by 30–40%.\textsuperscript{24} In addition, inhibition of autophagy in radiation-resistant breast, pharyngeal, cervical, lung, and rectal carcinoma cells sensitizes these cells to radiation therapy.\textsuperscript{25}

Acid ceramidase over-expression has been observed in multiple cancer cell lines and primary samples. Our group has shown previously that AC contributes to resistance to chemotherapy and radiation, and that AC inhibition improves response to treatment.\textsuperscript{6,7,17,26} We have shown here that, in addition to having an altered ceramide profile,\textsuperscript{6} prostate cancer cells that over-express acid ceramidase have enhanced stress resistance through increased autophagy and lysosomal density (Figures 2–4). Increased lysosomal density likely means an increase in the amount of lysosomal cathepsins present within a cell; however, the consequence of high levels of cathepsins in the cells is an increased likelihood of cell death should the cathepsins be released.\textsuperscript{22} Therefore, it follows that such cells would increase the stability of the lysosomes in order to prevent unwanted release of cathepsins into the cytosol. In addition to our unpublished observations of increased activity of lysosomal cathepsins in cells over-expressing AC, the results presented here indicate that cells over-expressing AC...
have increased expression of the lysosome-associated protein KIF5B (Figure 5) and are more sensitive to lysosome destabilization (Figure 6). Our observations are consistent with a report by Fehrenbacher, et al., which showed that alterations to lysosomes induced by transformation of murine embryonic fibroblasts sensitize cells to lysosomal cell death pathways induced by various chemotherapies.\textsuperscript{22} Based on the results presented here and on a previous report that KIF5B knockdown in MCF-7 cells limited autophagosome formation in response to rapamycin,\textsuperscript{21} we believe that AC over-expression in prostate cancer cells increases the basal level of autophagy in these cells through a pathway that includes KIF5B, a motor protein associated with lysosome transport.\textsuperscript{27} The increase in autophagy complements the already enhanced resistance to stress in these cells.

As ceramide has been shown to starve cells,\textsuperscript{28} it makes sense that mechanisms to evade this fate (over-expression of a ceramide-metabolizing enzyme and an increased activity of a cellular mechanism to buffer metabolic stress) would go hand in hand. Guenther, et al., showed that cellular metabolic state affects the response to ceramide: forcing cells to adapt to growth in lower nutrient levels, conditions known to induce autophagy, actually increased their resistance to ceramide treatment.\textsuperscript{28} Therefore, increased basal autophagy may confer a cellular advantage in response to stress. Vazquez-Martin, et al., reported autophagy-associated development of resistance in a breast cancer cell line chronically exposed to an anti-HER2 monoclonal antibody. SKBR3 cells cultured in the antibody ultimately increased the basal level of autophagy, and continually relied on autophagy in order to survive in the presence of the antibody.\textsuperscript{16}

Our data demonstrate that AC over-expression in PPC1 and DU145 prostate cancer cells results in increased lysosomal density and autophagy. In addition, we have shown that AC over-expression increases expression of the motor protein KIF5B, which may contribute to lysosomal stability in these cells. We believe that in addition to the increased capacity to metabolize ceramide produced in response to insult, the increased basal level of autophagy results in cells that have a heightened resistance to cellular stress. Furthermore, increased stability of lysosomes due to increased expression of KIF5B may protect cells from lysosomal membrane permeabilization and release of lysosomal contents into the cytoplasm, which would result in apoptosis. However, if this is true, this increased protection creates a therapeutic target: we have shown the AC over-expressing cells to be more sensitive to lysosome destabilizing agents than control cells. In summary, acid ceramidase over-expression, previously shown to confer resistance to chemotherapy and radiation, also increases autophagy in prostate cancer cells, enhancing the ability of these cells to respond to stress, but possibly increasing sensitivity to therapeutic agents that specifically target lysosomes.

**Acknowledgments**

The authors would like to thank the Lipidomics Core Facility Synthetic Subcore (MUSC) for ceramide and LCL385, Yousef Zeidan (Department of Biochemistry and Molecular Biology, MUSC) for the AC-V5 and lacZ-V5 plasmid constructs, Dr. Noboru Mizushima (Tokyo Medical and Dental University, Japan) for the LC3 cDNA, Dr. Christina Voelkel-Johnson (MUSC) for use of the fluorescent microscope, Rick Peppler (MUSC Hollings Cancer Center Flow Cytometry & Cell Sorting Core Facility) for assistance with flow cytometry, Margaret Kelly (MUSC Hollings Cancer Center Cell and Molecular Core Facility) for assistance with confocal microscopy, Donald

Prostate Cancer Prostatic Dis. Author manuscript; available in PMC 2014 October 20.
Rao (Gradalis; Dallas, TX) for assistance with siRNA design, and Janie Nelson (Department of Microbiology and Immunology, MUSC) for secretarial assistance.

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Figure 1. AC over-expression reduces sensitivity to stress

DU145 prostate cancer cells stably expressing AC-EGFP or EGFP (A) or PPC1 prostate cancer cells stably expressing AC-V5 or lacZ-V5 (B) were incubated for 72 hours with
25µM C₆-ceramide and analyzed by MTS assay. (C) DU145 prostate cancer cells stably expressing AC-EGFP or EGFP were incubated in serum-free media for 72 hours and analyzed by MTS assay. Results are representative of two or three separate experiments. **p<0.01.
Figure 2. Over-expression of AC increases expression and punctate staining of the autophagy protein LC3

(A) Whole cell lysates from PPC1 prostate cancer cells stably expressing AC-V5 or lacZ-V5 fusion proteins were separated by SDS-PAGE and analyzed by western blot. Results are representative of at least two separate experiments. (B) PPC1-AC-V5 cells (upper panel) and PPC1-lacZ-V5 cells (lower panel) were transfected with a vector containing EGFP-LC3 and analyzed by confocal microscopy. Results are representative of multiple fields of two separate experiments. (C) PPC1-AC-V5 cells were transfected with scramble siRNA or AC siRNA, separated by SDS-PAGE and analyzed by western blot. Results are representative of at least two separate experiments.
Figure 3. Lysosomal density increases with AC over-expression

Cells were stained with Lysotracker Red and analyzed by flow cytometry (A) and fluorescent microscopy (B). Results are representative of three (A) or two (B) separate experiments. (C) PPC1-AC-V5 and PPC1-lacZ-V5 cells were stained with Acridine Orange and analyzed by flow cytometry. Results represent the mean red staining (acidic vesicles) divided by the mean green staining (nucleic acids). Values are averages of three separate experiments. **p<0.01; *p<0.05.
A

![Graph showing cell viability](image)
Figure 4. Inhibition of autophagy restores sensitivity to ceramide

DU145-AC-EGFP and DU145-EGFP cells (A) or PPC1-AC-V5 and PPC1-lacZ-V5 cells (B) were treated with C₆-ceramide, with or without pre-treatment with 3-MA, and analyzed by MTS assay after 48 hours. Results are presented as percent viability compared to controls (no C₆-ceramide). ***p<0.001; *p<0.05.
Figure 5. Expression of KIF5B is increased with AC over-expression and reduced following AC inhibition

(A) Whole cell lysates from PPC1-AC-V5 and PPC1-lacZ-V5 cells were separated by SDS-PAGE and analyzed by western blot. (B) PPC1-AC-V5 cells were transfected with scramble or AC siRNA (50 nM). Whole cell lysates were collected 48 hours after transfection, separated by SDS-PAGE, and analyzed by western blot. Results are representative of at least two separate experiments.
Figure 6. AC over-expression increases sensitivity to lysosomal destabilization

DU145-AC-EGFP and DU145-EGFP cells (A) or PPC1-AC-V5 and PPC1-lacZ-V5 cells (B) were cultured in LCL385 (15μM) for 48 hours. Cell viability was analyzed by MTS assay. Results are representative of three separate experiments. ***p<0.001.