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Autophagy sustains the survival of human pancreatic cancer PANC-1 cells under extreme nutrient deprivation conditions

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ABSTRACT

Pancreatic ductal adenocarcinomas are an extremely aggressive and devastating type of cancer with high mortality. Given the dense stroma and poor vascularization, accessibility to nutrients is limited in the tumor microenvironment. Here, we aimed to elucidate the role of autophagy in promoting the survival of human pancreatic cancer PANC-1 cells exposed to nutrient-deprived media (NDM) lacking glucose, amino acids, and serum. NDM inhibited Akt activity and phosphorylation of p70 S6K, and induced AMPK activation and mitochondrial depolarization. NDM also time-dependently increased LC3-II accumulation, number of GFP-LC3 puncta, and colocalization between GFP-LC3 and lysosomes. These results suggested that autophagy was progressively activated through Akt- and AMPK-mTOR pathway in nutrient-deficient PANC-1 cells. Autophagy inhibitors (chloroquine and wortmannin) or silencing of Atg5 augmented PANC-1 cell death in NDM. In cells exposed to NDM, chloroquine and wortmannin induced apoptosis and Z-VAD-fmk inhibited cytotoxicity of these inhibitors. These data demonstrate that autophagy is anti-apoptotic and sustains the survival of PANC-1 cells following extreme nutrient deprivation. Autophagy modulation may be a viable therapeutic option for cancer cells located in the core of solid tumors with a nutrient-deficient microenvironment.

1. Introduction

Despite its relatively low epidemiological ranking, pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive and devastating types of cancer, with a poor survival rate [1]. Because surgical resection, radiation, and chemotherapy are minimally effective, early-stage detection is crucial for therapy. Aggressive behavior and resistance to therapies by PDAC are responsible for altered cellular metabolism [2,3]. Oncogenic *KRAS* mutation, found in most PDAC, accounts for metabolic alterations in PDAC, including increased glycolysis, altered glutamine metabolism, and autophagy activation [2,4]. Moreover, PDAC cells efficiently recycle various metabolic substrates through the activation of different salvage pathways, such as autophagy and macropinocytosis [2].

Autophagy is a cellular process of clearance and recycling of cytoplasmic components, including protein aggregates and obsolete organelles, through lysosomal degradation [5]. In response to starvation and hypoxia, autophagy serves as a cellular pro-survival mechanism through the generation of alternative energy resources that fuel cellular metabolism, but excessive autophagy could contribute to cell death through autophagic (type II) cell death [6]. Autophagy plays opposing roles in tumors depending on their progression stage. Autophagy suppresses tumorigenesis through the clearance of toxic cytoplasmic cargos in the early stage of tumor development, whereas many established tumor cells utilize autophagy for survival in tissue regions that lack nutrients and oxygen [5,7].

Nutrient starvation is the primary inducer of autophagy [8,9]. For autophagy activation, researchers have generally used nutrient-deficient conditions that lack any or all nutrients such as glucose, amino acids, growth factors, or serum. Depending on the cell type,

Abbreviations: AMPK, 5′ adenosine monophosphate-activated protein kinase; CQ, chloroquine; LC3, microtubule-associated protein 1A/1B-light chain 3; mTOR, mammalian target of rapamycin; PDAC, pancreatic ductal adenocarcinoma; PI, propidium iodide; PI3K, phosphatidylinositol 3-kinase; ROS, reactive oxygen species; WM, wortmannin; Z-VAD, Z-VAD-fmk.

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cancer cells display diverse vulnerabilities to specific deprivation conditions. PDAC cell lines, including PANC-1, have been reported to exhibit remarkable tolerance against nutrient starvation conditions such as glucose, amino acid, or serum deficiency [10]. Despite the possible intervention of autophagy, the precise role of autophagy as a survival mechanism of PANC-1 cells in extreme nutrient deprivation is still elusive. Because deprivation of all vital nutrients is frequently found in solid tumors with poor vasculature, it is necessary to explore the mechanism or role of autophagy induced by a complete blockade of diverse nutrient availability.

Here, we report that extreme nutrient starvation leads to progressive autophagy activation in human pancreatic cancer PANC-1 cells. Inhibition of autophagy by inhibitors and RNA silencing both sensitized cells to starvation-induced cell death through activation of caspase-dependent apoptosis. Our study suggests that autophagy sustains PANC-1 cell survival in extreme nutrient starvation, and that autophagy inhibition is a promising therapeutic option for PDAC.

2. Materials and methods

2.1. Reagents and chemicals

Information about culture medium, supplements, drugs, antibodies, plasmid, siRNA and other reagents was described in supplementary material.

2.2. Cell culture and treatment

Human cancer cell lines (A549; lung carcinoma, HCT116; colorectal carcinoma, Mia PaCa-2 and PANC-1; pancreatic carcinoma) were obtained from Korean Cell Line Bank (Seoul, Korea). RPMI 1640 was used for A549 and HCT116 cell culture, and DMEM was used for Mia PaCa-2 and PANC-1. All growth media were supplemented with 10% FBS and antibiotics (50 units/mL penicillin G and 50 μg/mL streptomycin). Nutrient deprived media (NDM) was prepared following the method of Izuishi et al. with slight modification [10]. NDM was composed of 265 mg/L $CaCl_2 \cdot 2H_2O$, 0.1 mg/L $Fe(NO_3) \cdot 9H_2O$, 400 mg/L KCl, 200 mg/L $MgSO_4 \cdot 7H_2O$, 6400 mg/L NaCl, 3500 mg/L $NaHCO_3$, 125 mg/L NaH_2PO_4, 15 mg/L phenol red, and 25 mmol/L HEPES buffer (pH 7.4), supplemented with MEM vitamin solution (Life Technologies. Rockville, MD, USA). Cells (45,000 cells/cm^2) were plated on microplates or culture dishes using appropriate growth media for each cell line and incubated for 24 h. Cells were treated with drugs by replacing the media with drug-containing media (growth media or NDM).

2.3. Cell death assay

Cell death was assessed following the method of Kim et al. with slight modification [11]. Cells were seeded in a black 96-well plate. After cells were stained with PI (30 μM, 20 min, 37 °C), fluorescence of PI (Fl_{dead}), which represents the signal of dead or dying cells was measured with a microplate reader (535 nm/617 nm; Synergy H1; BioTek, Winooski, VT, USA). After permeabilization of plasma membranes in the presence of digitonin (200 μM, 20 min, 37 °C), fluorescence of PI (Fl_{total}), which represents the signal of total cells, was measured. Cell death was calculated by the following equation: % Cell death = $Fl_{dead}/Fl_{total} \times 100$. Microscopic imaging of live and dead cells was assessed using Hoechst and PI staining. Cells in a 24-well plate were stained with Hoechst (10 μg/mL) and PI (1 μg/mL) for 20 min at 37 °C. Stained cells were visualized by fluorescence microscopy (Eclipse Ti–U, Nikon, Tokyo, Japan).

2.4. Cell viability assay (MTT assay)

Cell viability of PANC-1 cells in NDM condition was assessed by MTT assay [12]. At the end of incubation, cells were treated with 0.5 mg/mL of MTT reagent for 3 h at 37 °C (adding an equal volume of 1.0 mg/mL MTT dissolved in 2-fold concentrated DMEM containing 20% FBS). Cell viability was expressed as the percentage viability of the treated cells relative to that of the control cells.

2.5. Transfection and RNAi

PANC-1 cells were transfected with GFP-LC3 plasmid or siRNA (Atg5 and scrambled control) using Lipofectamine 2000 and Opti-MEM media, following the manufacturer's protocols. After 1 day of recovery following GFP-LC3 transfection and 2 days of recovery following siRNA transfection, the transfected cells were used for further experiments.

2.6. Imaging

GFP-LC3 transfected PANC-1 cells were treated with NDM for 1, 2, 4, 8, and 24 h. Cells were stained with LysoTracker Red (50 nM) and visualized by confocal microscopy (C2 Plus, Nikon, Tokyo, Japan). GFP-LC3 positive cell counts and colocalization between GFP-LC3 and lysosomes (Pearson's correlation) were determined using NIS-elements software (Nikon). At least ten cells were used for quantitation in each group.

2.7. Western blotting

Cell lysate was prepared using RIPA buffer containing protease inhibitors, phosphatase inhibitors, and 1 mM dithiothreitol. Protein concentration was determined using the Bradford protein assay kit (Thermo Scientific, Rockford, IL, USA). Samples containing equal amounts of protein were boiled in SDS sample buffer, resolved on SDS-PAGE gels, and transferred onto PDVF membranes (Millipore, Billerica, MA, USA). Blots were incubated with primary antibodies and a secondary antibody conjugated with horseradish peroxidase. Blot images were obtained using a chemiluminescent substrate reagent and an imaging instrument (ImageQuant LAS 4000 mini, GE Healthcare, Pittsburgh, PA, USA). Quantitation was performed by densitometric analysis using Image J software (National Institutes of Health, Baltimore, MD, USA). Densitometry values for each protein were normalized to those for the loading control (GAPDH or β-tubulin).

2.8. Statistical analyses

Bars or symbols in the graph represent means ± standard errors of the mean generated from at least 3 independent experiments. Significant differences were determined by one-way analyses of variance (ANOVA) or *t*-tests at the indicated *P* value.

3. Results

3.1. PANC-1 cells exhibited remarkable tolerance to extreme nutrient deprivation

We first examined the tolerance of PANC-1 cells to extreme nutrient starvation. Various human cancer cell lines (A549, HCT116, Mia PaCa-2, and PANC-1) were cultured in growth media or nutrient-deprived media (NDM) for 3 days. Cell death was assessed by PI-digitonin assay and Hoechst-PI staining. Cell death was at a minimal level when cells were cultured in growth media for 2 days, but A549, HCT116, and Mia PaCa-2 cells displayed increased cell

death at 3 days (Fig. 1A). This might have been due to metabolic stress generated from the exhaustion of nutrients when cells reached a high density. However, A549, HCT116, and Mia PaCa-2 cells died within 2 days after NDM treatment. PANC-1 cells exhibited the greatest survival among the cell lines, with survival at up to 3 days. Around 20% of PANC-1 cells died after exposure to NDM for 1 day and cell death reached around 60% after 3 days. Similar results were also observed in the nuclear dye stained PANC-1 and Mia PaCa-2 cells exposed to NDM for 24 h (Fig. 1B). PANC-1 cells exhibited normal morphology and only a small population of cells was stained with PI, whereas most of the Mia PaCa-2 cells exhibited morphology indicating cell death and were stained with PI. These data suggested that PANC-1 cells exhibited strong survival in NDM. Mia PaCa-2 cells were the most vulnerable of the tested cell lines to NDM exposure.

3.2. NDM induced progressive activation of autophagy in PANC-1 cells

Nutrient starvation is the primary stimulus that activates autophagy through the mTOR pathway [8]. We first tested the activities of Akt and AMPK following exposure of PANC-1 cells to NDM. As expected, NDM exposure induced Akt activation and AMPK inactivation (Supplementary Fig. 1). Because these are upstream effectors that inhibit mTOR activity, we next examined whether NDM induces time-dependent activation of autophagy in PANC-1 cells. NDM treatment significantly reduced the protein expression of p70 S6K and suppressed the phosphorylation of p70 S6K (Thr-389), a surrogate marker for mTOR activity (Fig. 2A–C). NDM slightly reduced beclin-1 levels and significantly increased conversion of LC3-I to LC3-II in a time dependent manner (Fig. 2D and E). GFP-LC3-transfected PANC-1 cells were used for determining the formation of autophagosomes and autolysosomes. The number of GFP-LC3 puncta and colocalization between GFP-LC3 and lysosome were significantly increased by NDM exposure in a time dependent manner (Fig. 2F–H). The percentage area and mean intensity of GFP-LC3 and LysoTracker staining within cells were also significantly increased (data not shown). AMPK activation can indicate a decrease in ATP levels and crisis in the cellular bioenergetic status. Therefore, we tested whether NDM induces mitochondrial dysfunction. As expected, NDM reduced the mitochondrial membrane potential, as assessed by JC-1 staining (Supplementary Fig. 2). Taken together, our findings suggest that NDM induced progressive activation of autophagy through the mTOR inhibition and also induced mitochondrial depolarization.

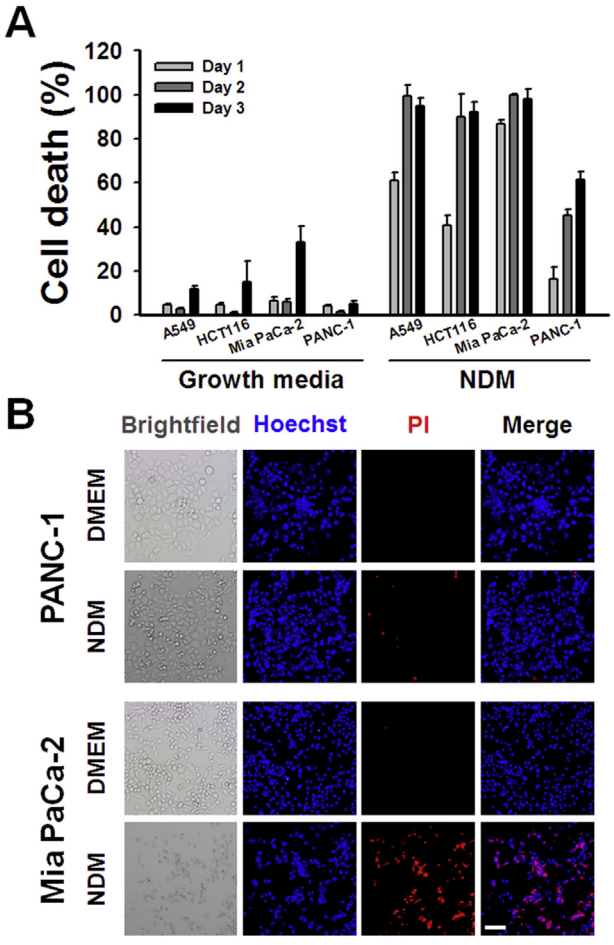

Fig. 1. PANC-1 cells exhibit remarkable survival under extreme nutrient deprivation. A549, HCT116, Mia PaCa-2, and PANC-1 cells were cultured in either growth media or nutrient deprived media (NDM) for 3 days. On each day of incubation, cell death was assessed by PI-digitonin assay (A). PANC-1 and Mia PaCa-2 cells were cultured in either growth media or NDM for 24 h, and then stained with Hoechst and PI. Stained cells were visualized by fluorescence microscopy (B). Merged images were generated by overlaying Hoechst and PI images. The scale bar represents 100 μm.

3.3. Autophagy inhibition augmented PANC-1 cell death in NDM condition

Because autophagy is induced in diverse stress conditions, increased autophagy might be casual or an epiphenomenon [13,14]. To identify the role of autophagy activation on PANC-1 survival in NDM, we examined whether autophagy inhibition affects PANC-1 cell death in NDM. The efficacy of drugs in inhibiting autophagy was confirmed by western blotting (Supplementary Fig. 3). Treatment of PANC-1 cells with wortmannin (WM, a PI3K inhibitor, an inhibitor of autophagosome formation) led to decreased levels of LC3-I and LC3-II [15]. Chloroquine treatment (CQ, a lysosomotropic agent, an inhibitor of autophagosome degradation) led to increased levels of LC3-II [16]. CQ induced a dose-dependent loss of PANC-1 cell viability in both DMEM and NDM (Fig. 3A). WM had no effect on cell viability in DMEM, whereas it specifically induced toxicity in NDM (Fig. 3B). Of note, NDM significantly sensitized the toxicity of CQ (GI_{50} = 49.8 μM) and WM (GI_{50} = 3.2 μM) on PANC-1 cells. To exclude non-specific actions of inhibitors, we also tested autophagy inhibition by RNA silencing using Atg5 siRNA. Expression of Atg5 was reduced to around 50% at 2 days after transfection (Fig. 3C). Cell death in NDM was significantly increased in Atg5 silenced PANC-1 cells compared with those treated with a scrambled siRNA control (Fig. 4C). Thus, autophagy inhibition augmented PANC-1 cell death in NDM, implying that autophagy was protective and sustained the survival of PANC-1 cells exposed to NDM.

3.4. Chloroquine and wortmannin activated caspase-dependent apoptosis in NDM-treated PANC-1 cells

In most cancers, autophagy is cytoprotective and anti-apoptotic. Autophagy and apoptosis closely interact with each other through functional crosstalk under certain stress conditions [17,18]. We examined whether apoptosis contributes to the enhancement of cell death by autophagy inhibition in cells subjected to extreme nutrient starvation. Because a 20% loss of cell viability was found in non-drug-treated PANC-1 cells exposed to NDM, we investigated the basal cell death modality in cells cultured under the NDM condition using various modulators of cell death. Calpeptin (calpain inhibitor) and Z-VAD (caspase inhibitor) had no effects on cell viability, whereas rapamycin (allosteric inhibitor of mTOR) and PP242 (catalytic inhibitor of mTORC1 and mTORC2) increased the

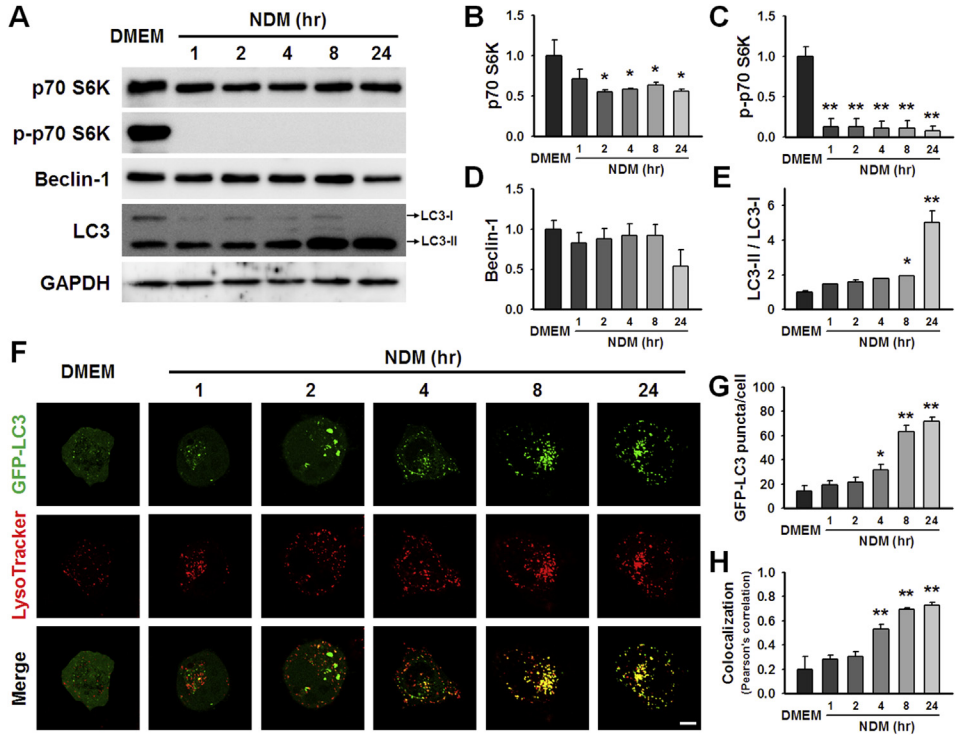

Fig. 2. Extreme nutrient deprivation induces progressive activation of autophagy in PANC-1 cells. PANC-1 cells were treated with NDM and harvested at the indicated incubation times. Expression levels of p70 S6K, phosphorylated p70 S6K (p-p70 S6K), Beclin-1, LC3, and GAPDH were determined by western blotting (A) and quantitated by densitometric analysis (B–E). GFP-LC3 transfected PANC-1 cells were treated with DMEM or NDM and stained with LysoTracker Red. Cells were visualized by confocal microscopy (F). The scale bar represents 10 μm. Punctate dots of GFP-LC3 in each cell were counted (G), and colocalization (Pearson's correlation) between GFP-LC3 and lysosomes was assessed using imaging software (H). * represents a significant difference compared with DMEM control (one-way ANOVA; *, $P < 0.05$; **, $P < 0.01$).

viability of PANC-1 cells in the NDM condition (Fig. 4A). These findings suggest that calpain- and caspase-independent cell death occurred in PANC-1 cells exposed to NDM, and that autophagy might be protective against cell death. To verify the functionality of the apoptotic machinery of PANC-1 cells exposed to NDM, we tested the induction of apoptosis in NDM using staurosporine (positive control for apoptosis). Staurosporine induced robust cell death in PANC-1 cells exposed to NDM, and Z-VAD inhibited cell

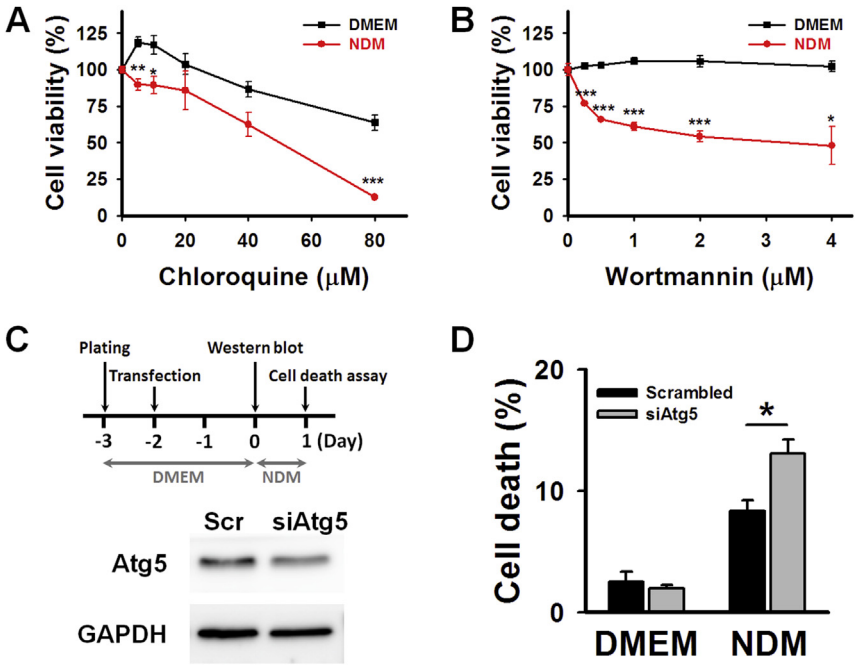

Fig. 3. Inhibition of autophagy augments NDM-induced cell death in PANC-1 cells. PANC-1 cells were treated with chloroquine or wortmannin under either DMEM or NDM conditions for 24 h. Cell viability was assessed by MTT assay (A and B). A scheme of the siRNA transfection experiments and western blotting to assess Atg5 silencing is shown (C). Atg5 silenced PANC-1 cells were treated with NDM for 24 h. Cell death was assessed by PI-digitonin assay (D). * represents a significant difference compared with the corresponding concentration of drugs or scrambled siRNA control (*t*-test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

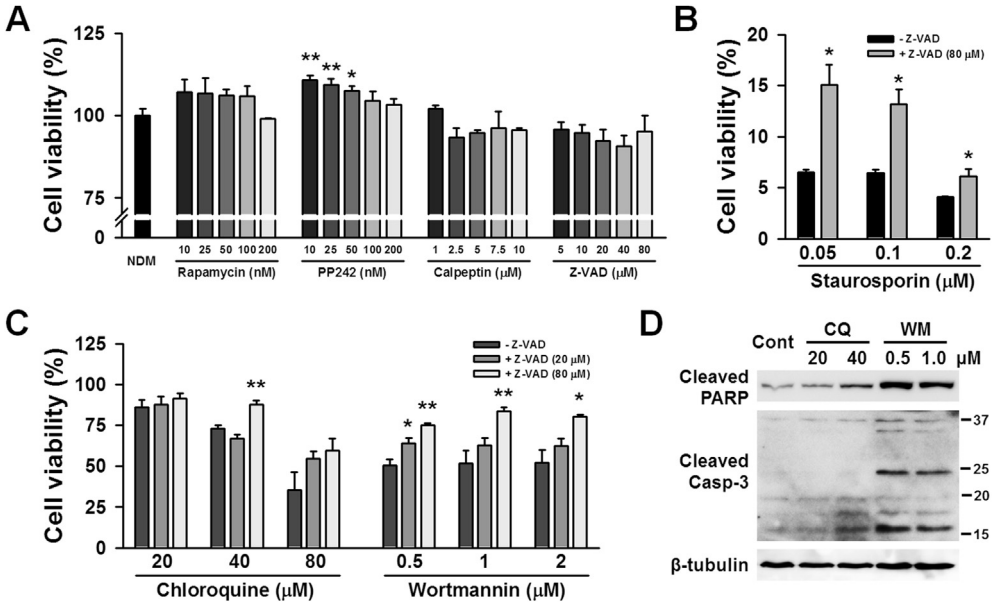

Fig. 4. Chloroquine and wortmannin induce caspase-3 dependent apoptotic cell death in PANC-1 cells under the NDM condition. All experiments were carried out under the NDM condition and PANC-1 cells were treated with drugs for 24 h. Cell viability was assessed by MTT assay. The cell viability of PANC-1 cells treated with rapamycin, PP242, calpeptin, and Z-VAD-fmk (Z-VAD) (A), staurosporine with/without Z-VAD (80 μM) (B), and chloroquine or wortmannin with/without Z-VAD (20 or 80 μM) (C) was determined using MTT assay. PANC-1 cells were treated with chloroquine or wortmannin in NDM for 24 h. Expression levels of cleaved PARP, cleaved caspase-3 (casp-3), and β-tubulin were assessed by western blotting (D).

death, suggesting that the apoptotic machinery of PANC-1 cells was still intact under nutrient-deprived conditions (Fig. 4B). We next tested whether the induction of increased cell death by CQ and WM is a consequence of apoptosis activation. Z-VAD dose-dependently inhibited CQ- and WM-induced cell death (Fig. 4C), and this inhibition by Z-VAD was statistically significant for WM. Apoptosis activation was also confirmed by western blotting. After treatment with CQ and WM for 24 h, we measured levels of cleaved caspase-3 and cleaved PARP (executioner of apoptosis and substrate of caspase-3, respectively). CQ and WM activated apoptosis, as assessed by robust increases in the levels of cleaved caspase-3 and PARP (Fig. 4D). Taken together, our findings indicate that autophagy was anti-apoptotic and promoted the survival of PANC-1 cells exposed to NDM, and that increased cytotoxicity of CQ and WM in NDM was responsible for the activation of apoptosis.

4. Discussion

In the core of aggressive solid tumors, chronic nutrient starvation and hypoxia are frequent features of the cellular microenvironment. Thus, tumor cells develop altered metabolic pathways through genetic mutations or the activation of innate recycling machinery [2,14]. Dense stroma and hypovascularization, typical characteristics of PDAC, result in metabolically stressed cells. Many PDAC cells exhibited metabolic alteration by a *KRAS* mutation that leads to increased glycolysis, glutamine metabolism, or autophagy activation [19,20]. Autophagy also plays critical roles in PDAC, not only in tumor growth, progression, and invasion, but also in resistance to chemotherapy [20–22]. These studies were performed under physiological or starvation conditions, with cells cultured in balanced salt solution containing glucose. Because hypovascularization induces global nutrient deficiency rather than the deprivation of any specific nutrients, complete starvation of essential nutrients should be considered as an experimental approach to understand the adaptive survival strategies of cancer cells in the tumor microenvironment.

We demonstrated that autophagy rescues PANC-1 cells from extreme nutrient starvation, which mimics the poor microenvironment. The NDM used in our study was first designed by Esumi group [10]. They found that high expression levels of Akt and AMPK were responsible for the remarkable tolerance of PANC-1 cells to NDM [10,23]. In our study, NDM inhibited Akt and increased AMPK activity, and thereby induced robust activation of autophagy in PANC-1 cells through mTOR inhibition. Autophagy inhibitors (CQ and WM) and silencing of Atg5 increased the vulnerability of PANC-1 cells in NDM to cell death. In contrast to the cytotoxicity of CQ, that of WM in NDM almost reached a plateau at 0.5 μM. The short half-life of WM might be involved in the mild dose–response [10]. Of note, enhanced cell death by CQ and WM in NDM was responsible for caspase-3 dependent apoptosis. However, the mediators that regulate the crosstalk between autophagy and apoptosis in cells exposed to NDM are still elusive.

Autophagy-mediated mitochondrial quality control is most likely linked to the tolerance of PANC-1 cells to being exposed to NDM. Dysfunctional mitochondria are not only the major cellular source of ROS but also initiate intrinsic apoptosis through apoptogenic protein release and the subsequent activation of executioner caspases [24]. Mitophagy, the selective autophagic clearance of obsolete mitochondria, is the mechanism maintaining mitochondrial quality and dynamics, together with mitochondrial fission and fusion [25,26]. Therefore, autophagy could reduce oxidative stress and maintain the functional pool of mitochondria in metabolically stressed cells [5,27]. NDM treatment of PANC-1 cells induced robust activation of AMPK and mitochondrial depolarization, suggesting that cellular ATP levels and mitochondrial oxidative phosphorylation were affected in the absence of nutrients. We also found that colocalization between mitochondria and lysosomes was significantly increased even after an hour of NDM exposure and the increase was maintained up to 24 h (Supplementary Fig. 4). These results all supported that NDM-induced autophagy may largely account for the clearance of damaged mitochondria. It was previously reported that CQ treatment of PDAC cells increased

mitochondrial ROS and decreased oxidative phosphorylation [20]. WM has been reported to regulate the mitochondrial permeability transition pore and Bax activation under stress conditions, and thereby triggers mitochondrial apoptosis [28,29]. Indeed, mitochondrial inhibitors or drugs exerted preferential cytotoxicity on metabolically stressed tumor cells [12,30]. Thus, modulatory effects on mitochondrial function and autophagy might underlie the sensitization of cell death in response to CQ and WM treatment in cells exposed to NDM. However, it should be robustly investigated in our further studies whether these inhibitors directly affect mitochondrial function in cells exposed to NDM. Hypoxic stress and nutrient deficiency should also be considered together to understand cellular adaptations to the tumor microenvironment as a whole.

In summary, we demonstrated that autophagy is anti-apoptotic and sustains the survival of PANC-1 cells in extreme nutrient starvation. Our study helps to expand our understanding of the role of autophagy as a survival strategy of tumor cells in nutrient-poor environments and may provide insights to aid the discovery of new therapeutic targets or the development of innovative therapies for such a deadly disease.

Conflict of interest

We declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.05.022.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.05.022.

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