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

Sang Eun Kim [a, 1], Hye-Jin Park [a, 1], Hye Kyoung Jeong [a, 1], Mi-Jung Kim [a], Minyeong Kim [a], Ok-Nam Bae [b], Seung-Hoon Baek [a, *]

[a] College of Pharmacy, Ajou University, Suwon, Gyeonggido 443-749, Republic of Korea
[b] College of Pharmacy, Hanyang University, Ansan, Gyeonggido 426-791, Republic of Korea

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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.

* Corresponding author. Fax: +82 31 219 3435.
E-mail address: shbaek@ajou.ac.kr (S.-H. Baek).

[1] These authors contributed equally to this work.

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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 colocalization 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

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