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Autophagic cell death induced by 5-FU in *Bax* or *PUMA* deficient human colon cancer cell

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ABSTRACT

Autophagy is a membrane process that results in the transporting of cellular contents to lysosomes for degradation. Autophagic cell death is another way of programed cell death called type II PCD, which has complicated connection with apoptosis, both of these two types of cell death play an important role in tumor development. In this study, we investigated chemotherapeutic agent induced cell death pathway in wild type (WT), $Bax^{-/-}$ and $PUMA^{-/-}$ HCT116 cells. *Bax* or *PUMA* deficient cells had similar chemosensitivity to WT cells but were defective in undergoing apoptosis. The results of electron microscopy and GFP–LC3 localization assay showed that autophagy was induced in *Bax* or *PUMA* deficient cells but not in WT cells. mTOR activity was decreased in *Bax* or *PUMA* deficient cells which further indicated the up-regulation of autophagy. Inhibition of autophagy by 3-Methyladenine (3-MA) decreased the cell death in *Bax* or *PUMA* deficient cells. Taken together, these results suggest that autophagic cell death can be used as an alternative cell death pathway in apoptosis defective cells and may bring a new target for cancer therapy.

1. Introduction

Programmed cell death (PCD) is important for cancer cells to support malignant growth. Thus, understanding the mechanisms of programmed cell death and designing specific therapeutic approaches to induce cell death in cancer cells are significant for disease treatment [1,2]. There are two morphologically distinctive forms of programmed cell death, apoptosis and autophagic cell death [3].

Autophagic cell death (type II PCD) is an important physiological cell death process beside apoptosis. This type of cell death is characterized by massive degradation of cellular contents, including essential organelles such as mitochondria. These intracellular contents are sequestered in a membrane vesicle autophagosome, and then fuse to lysosomes to form autolysosomes for degradation. Autophagy is believed to have an important role in tumor development. When baseline levels were compared, the amount of autophagic degradation in cancer cells was less than that of their normal counterparts [4–6]. Many researches show that apoptosis and autophagy may be interconnected in some settings [7], and even can be simultaneously induced by the same stimulus resulting in different cellular outcomes in some cases [8]. Depending on the trigger and cellular context, autophagy may be indispensable for apoptosis by preceding and further turning on apoptosis. In other settings, autophagy can resist or suspend apoptosis, and there are also examples where the two processes

Abbreviations: WT, wild type; PCD, programmed cell death; 5-FU, 5-fluorouracil; LC3, microtubule associated protein 1 light chain 3; DAPI, 4′,6′-diamidino-2-phenylindole dihydrochloride; 3-MA, 3-methyladenine.

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may be mutually exclusive acting as backup mechanisms of each other [9–11]. We expect more discoveries on the relationship of the two cell death pathways, including the effect of apoptosis deficiency on autophagic cell death pathway.

The molecular pathways which regulate autophagy are complicated. PI3K-Akt-mTOR signaling pathway is a negative regulation mechanism of autophagy induction, inhibition of mTOR has been consistently associated with indicators of autophagy in cancer cells [12]. 3-MA, an inhibitor of phosphatidylinositol 3-kinase, has been shown to inhibit autophagy [13].

Traditional cancer therapy is mainly targeting on enhancing cell apoptosis, however, it is well established that many cancer cells are chemo-resistant and defective in apoptosis induction. Evidence indicates that the modulation of autophagy is an important component of tumorigenesis [14,15], and recent research report that treatment with radiation and chemotherapeutic agents can induce autophagy in cancer cells [16,17]. So if we can induce autophagic cell death in apoptosis defective cancer cells, it may bring a new therapeutic strategy for cancer.

Bax and PUMA are critical apoptosis regulators, deficiency in Bax and PUMA or expression of Bcl-2 blocks apoptosis, and this failure to execute apoptotic cell death impacts development and promotes tumorigenesis. When apoptotic cell death is deficient, other ways of cell death are critical for cancer therapy [18–20]. In this study, we treated WT, $Bax^{-/-}$ and $PUMA^{-/-}$ HCT116 cells with 5-FU, reaching the conclusion that there was another death pathway—autophagic cell death, in *Bax* or *PUMA* deficient cells. This type of cell death is up-regulated with the deactivation of mTOR signaling pathway and can be inhibited by 3-MA.

2. Materials and methods

2.1. Cell culture and drug treatments for cell line

The colon cancer adenocarcinoma cell line HCT116, WT, $Bax^{-/-}$ and $PUMA^{-/-}$ cells (gifts from Dr. Chuanshu, Huang) were maintained in DMEM plus M5A, and supplemented with 10% fetal bovine serum. The cells were incubated at 37 °C with 5% CO_2. 5-FU was purchased from QILU PHARMA (jinan,Shandong,China). 3-Methyladenine (3-MA) was obtained from Sigma–Aldrich (Shanghai, China) and it was dissolved in heated sterile double distilled water to make a 400 mM stock solution and then added to the medium after heating for a final concentration of 5 mM, and 2 h later 5-FU was added for treatment. GFP–LC3 plasmid was a kind gift from Dr. shengkan Jin.

2.2. Growth inhibition assay

The measurement of viable cell mass was performed with a Cell Counting Kit (Dojin Laboratories, Kumamoto, Japan) to count living cells by WST-8. Five thousand cells were seeded in a 96-well plate. Twenty-four hours later cells were treated with no drug as control, 5, 10, 20, 40 μg/ml 5-FU. As soon as drug treatment was completed, 10 μl of a solution from Cell Counting Kit-8 was added for each well, and then the plate was incubated for 2 h at 37 °C in a humidified CO_2 incubator. The absorbance was measured on a microplate reader (Synergy HT, Bio-Tek, USA) at 450 nm. The percent of surviving cells at each concentration relative to the untreated group was plotted.

Cell death was assessed by trypan blue staining. Cells (2×10^4 cells/well) were seeded in 24-well flat-bottomed plates. 24 h later, 5-FU was added at the concentrations indicated for 24 h. Then cells were detached from the substrate by trypsinization, resuspended in trypan blue solution (0.4% in PBS) and the number of dead cells was counted in a hemocytometer under a light microscope. 200 cells at least were counted for each sample, and the experiment was processed for three repeats.

2.3. Apoptosis analysis

The morphological observation of apoptosis was measured by DAPI staining. Cells were seeded in 24-well flat-bottomed plate, and the cells were treated with or without 20 μg/ml 5-FU for 24 h, after which the cells were fixed with 4% paraformaldehyde for 30 min at room temperature. After cells were washed with PBS for 5 min, 2 μl DAPI (5 μg/ml) was added to the fixed cells for 5 min, after which they were visualized by fluorescence microscopy (Olympus IX71). Cells in which the nucleus contained clearly condensed chromatin or cells exhibiting fragmented nuclei were taken as apoptotic.

The apoptotic cells were determined by Annexin V-PI dual staining. Cells (2×10^5 per well) were cultured in six-well plates to 70–80% confluence, and cells were treated with the indicated concentrations of chemotherapeutic agent for 24 h, then cells were collected and the annexin V-PI dual staining assay was performed according to the manufacturer's instructions (Nanjing Keygen Biotech, China). Collected cells were briefly washed with ice-cold phosphate-buffered saline (PBS) twice and resuspended in 300 μl 1 × binding buffer containing 5 μl Annexin V and 5 μl propidium iodide (PI) for 30 min at room temperature in the dark. After incubation, the cells were analyzed using a FACS Aria flow cytometer (Becton Dickinson; San Jose, CA).

2.4. Transmission electron microscopy

Cells were fixed with 2.5% glutaraldehyde in phosphate buffer and stored at 4 °C until embedding. Cells were post-fixed with 1% osmium tetroxide followed by an increasing gradient dehydration step using ethanol and acetone. Cells were then embedded in araldite, after which ultrathin section were obtained (50–60 nm), placed on uncoated copper grids, and stained with 3% lead citrate–uranyl acetate. Images were examined with a CM-120 electron microscope (Philips).

2.5. GFP–LC3 transient transfection

Green fluorescent protein (GFP)-tagged microtubule associated protein light chain 3 (LC3)-expressing cells have recently been utilized to detect autophagy [21]. HCT116