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Interferon-gamma induces autophagy-associated apoptosis through induction of cPLA2-dependent mitochondrial ROS generation in colorectal cancer cells

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ABSTRACT

Colorectal cancer (CRC) is the second most commonly diagnosed cancer in females and the third in males. In this work, we aim to investigate the possible anti-cancer effects of interferon-gamma (IFN-γ) in CRC cells. We observed that IFN-γ induced mitochondria-derived reactive oxygen species (ROS) production in a time-dependent manner in SW480 and HCT116 cell lines. The IFN-γ-induced mitochondrial ROS generation was dependent on the activation of cytosolic phospholipase A2 (cPLA2). In addition, a mitochondria-targeted antioxidant SS31 and/or cPLA2 inhibitor AACOCF3 abolished the IFN-γ-induced ROS production and subsequent autophagy and apoptosis. Moreover, suppression of autophagy by CQ was able to reduce IFN-γ-induced cell apoptosis. Beclin-1 gene silencing resulted in caspase-3 inactivation, decreased Bax/Bcl-2 ratio and less population of apoptotic cells. Collectively, our results suggested that IFN-γ induces autophagy-associated apoptosis in CRC cells via inducing cPLA2-dependent mitochondrial ROS production.

1. Introduction

Colorectal cancer (CRC) is a severe health problem all over the world. More than 1.2 million patients are diagnosed with CRC, and almost 0.6 million died per year, making CRC the second most common cancer in females and the third in males [1]. An important family of potential anti-cancer agents are the interferons (IFNs), which play important roles in the regulation of cell cycle and apoptosis as well as the induction of cell differentiation [1,2]. However, the potential usefulness of type I IFN is less evident for CRC in animal studies. It is reported that type I IFN signaling promotes tumorigenesis and mice deficient in type I IFN signaling do not show altered intestinal polyp formation [3]. Unlike the type I IFNs, type II IFNs contribute to cancer surveillance and suppression [4,5]. Slattery and colleagues reported that variations in IFN-γ (type II) and IFN-γR are closely associated with the risk of CRC and survival [6]. In addition, Wang and others suggested that IFN-γ administration inhibited CRC cell proliferation and IFN-γ/IFN-γ R1 act as a rate-limiting factor in the development of CRC [7]. Reactive oxygen species (ROS) generated during inflammation was believed to play critical roles in various diseases including different types of cancer [8]. Yang and others reported that pro-inflammatory cytokines including TNF-α, IL-1β and IFN-γ increased mitochondrial- and NADPH oxidase-generated ROS productions [9]. In addition, IFN-γ induces marked augmentation of ROS and apoptosis in lymphoblast cell lines [10] and hepatocytes [11]. However, the ability of IFN-γ to stimulate ROS production in CRC cell lines has not yet been reported.

The balance between the reactive oxygen species (ROS) and antioxidants production is necessary for physiological state. However, this balance has been broken under pathological conditions, and excessive level of ROS accumulation may lead to different kinds of diseases including CRC [12]. Considerable evidences suggested that CRC risk factors like smoking and alcohol consumption were involved in ROS production [13,14]. Furthermore, studies also revealed that more ROS may increase colon cancer risk [15]. On the other side, anti-cancer agents that induce ROS accumulation have long been recognized as an important class of drugs that induce cancer cell apoptosis and death [16,17]. Since mitochondria are the major source of ROS production, mitochondria play important roles in the ROS-mediated damages to cancer cells. In addition, mitochondria also play important roles in calcium homeostasis and regulation of apoptosis through their effectors such as cytochrome

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c, which induces apoptosis by activating caspases [18]. Moreover, it has been reported that mitochondria dysfunction and its subsequent ROS accumulation induces autophagic cell death in transformed and cancer cells [19]. Cytosolic phospholipase A2 (cPLA2) is one of the PLA2 families that catalyze the hydrolysis of the sn-2-ester bond of phospholipids. An important feature of cPLA2 is its link to receptors that stimulate signaling pathways associated with activation of protein kinases and production of ROS [20]. In addition, cPLA2 has been reported to be required for the mitochondrial ROS generation in infection-related diaphragm dysfunction [21]. Wu and colleagues reported that IFN-γ induced synthesis and activation of cPLA2 in human bronchial epithelial cell line [22], and others found that IFN-γ induced a rapid but transient activation of PLA2 in BALB/c 3T3 fibroblasts [23] and human neuroblastoma cell line [24]. However, it is unclear whether IFN-γ induces cPLA2 activation and subsequent mitochondria-derived ROS production in CRC cells. In the present study, we aim to investigate whether IFN-γ induces cPLA2-dependent mitochondria-derived ROS production and increase cell autophagy and apoptosis in CRC cells.

2. Materials and methods

2.1. Cell culture, plasmid and transfection

The colon cancer cell line SW480 and HCT116 were purchased from Chinese Academy of Typical Culture Collection Cell Bank and were cultured in RPMI-1640 (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS), 100 IU/mL penicillin and 100 μg/mL streptomycin, and grown at 37 °C in a humidified 5% CO_2 atmosphere. Cells were treated with IFN-γ at various concentrations (0.1, 1, 10, 100, 500 and 1000 units/mL) for 24, 48 and 72 h, respectively. In order to investigate the possible roles of cPLA2 and mitochondrial oxidative stress in the IFN-γ-induced inhibitory effects in CRC cells, AACOCF3 (20 μM), a cPLA2 inhibitor, and a mitochondria-targeted antioxidant, SS31 (30 μg/mL) was added into the cell culture medium. Measurements of ROS were performed at 1 h after IFN-γ (1000 units/mL) administration. Cells were transfected with siRNA oligonucleotides/plasmids using Lipofectamine 2000. Cells were seeded in 60 mm culture dish/6 well plates for 24 h in culture medium. After 40–50% confluence, cells were transfected with respective siRNA and plasmid, subsequently IFN-γ treatment for 24 h. After incubation, cells were harvested for further analysis.

2.2. Reagents and antibodies

The primary antibodies used in the present study including anti-p-cPLA2, cPLA2, caspases-3, Bax, and Bcl-2 were purchased from Abcam (Shanghai, China). Anti-Beclin-1 and LC3I/II were purchased from Sigma-Aldrich (St. Louis, MO, USA). cPLA2 activity assay kit was purchased from Abcam (Shanghai, China). AACOCF3 was purchased from APEXBIO (B6748, Shanghai, China). SS31 was purchased from Chinapeptides (Shanghai, China). MitoSOX™ Red Mitochondrial Superoxide Indicator was purchased from Yaesen (#40778ES50, Shanghai, China). siRNA targeting human Beclin-1 (#6222) and non-targeted sequence were obtained from Cell Signaling Technology (Danvers, MA, USA).

2.3. Cell viability assay

SW480 and HCT116 cells were seeded in 96-well plates and cultured overnight. Then, cells were treated with different agents at indicated concentrations. Afterwards, cell viability was evaluated using MTT assay. Data were collected from triplicate determinations.

2.4. Cell apoptosis analysis

Cell apoptosis was evaluated using a fluorescein isothiocyanate Annexin V Apoptosis Detection Kit (BD Bioscien, NJ, USA). Cells were resuspended in binding buffer (400 μl), incubated with Annexin V-fluorescein isothiocyanate (10 μl) and PI (5 μl) for 15 min at 4 °C and then 5 min in dark, and then followed by flow cytometry detection within 1 h.

2.5. cPLA2 activity assay

Cells were collected by centrifugation (1000 × g, 10 min, 4 °C) and cell pellet were homogenized in 2 mL of cold buffer (50 mM Hepes, pH 7.4, containing 1 mM EDTA). Then, homogenates were centrifuged at 10,000 × g for 15 min at 4 °C, and remove the supernatant for assay and store on ice. cPLA2 activity was measured using a commercial assay kit according to the manufacturer's instructions.

2.6. Detection of intracellular ROS generation

Intracellular ROS production was detected using a DCFH-DA based reactive oxygen species assay kit as previously described [25]. In detail, cells were seeded into plates at a density of 1.0×10^5 cells/mL and incubated overnight. After incubation with DCFH-DA (10 μM) at 37 °C for 30 min, then cells were treated with IFN-γ at various concentrations for 1 h. Finally, ROS levels were determined by flow cytometry.

2.7. Mitochondrial ROS detection using MitoSOX

Mitochondrial reactive oxygen species (ROS) generation was assessed using a MitoSOX™ Red Mitochondrial Superoxide Indicator. In detail, apply 2.0 mL of 5 μM MitoSOX™ reagent working solution to cover CRC cells adhering to coverslips. Incubate cells for 10 min at 37 °C in dark. Then, wash cells gently 3 times with warm buffer. Finally, stain cells with counterstains as desired and mount in warm buffer for imaging.

2.8. Immunofluorescence analysis of LC3

Cells were seeded in glass coverslips, grown to required confluence and treated. Cells were fixed with 4% PFA in PBS, permeabilized in 0.5% Triton X-100 and blocked with 2% BSA in PBS. Cells were incubated with anti-LC3 antibody overnight at 4,washed thrice with PBS, incubated with secondary antibody for 1 h and imaging in a confocal microscope.

2.9. Western blot analysis

Cells were collected, washed with ice-cold phosphate buffer saline (PBS) and then solubilized in radioimmunoprecipitation assay lysis buffer containing protease inhibitors. Protein concentrations were measured using a bicinchoninic acid (BCA) protein assay (Pierce, Rockforsd, WI, USA) and standardized among the samples. Proteins were then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to membranes (Millipore, Billerica, MA, USA). The membranes were incubated with primary antibodies including anti-p-cPLA2, anti-cPLA2, anti-caspase-3, anti-Bax, anti-Bcl-2, anti-Beclin-1, anti-LC3 at 4 °C overnight and followed by incubating with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature (RT). GAPDH was used as a loading control.