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Sensitizing effect of 3-methyladenine on radiation-induced cytotoxicity in radio-resistant HepG2 cells in vitro and in tumor xenografts

Hsien-Chun Tseng [a,b], Wen-Shan Liu [b], Yeu-Sheng Tyan [c], Huei-Ching Chiang [d], Wu-Hsien Kuo [e,f,*,1], Fen-Pi Chou [a,*,1]

[a] Institute of Biochemistry and Biotechnology, College of Medicine, Chung Shan Medical University, No. 110, Sec. 1, Jianguo N. Road, Taichung City 40201, Taiwan
[b] Department of Radiation Oncology, Chung Shan Medical University Hospital, No. 110, Sec. 1, Jianguo N. Road, Taichung City 40201, Taiwan
[c] Department of Medical Image, Chung Shan Medical University Hospital, No. 110, Sec. 1, Jianguo N. Road, Taichung City 40201, Taiwan
[d] Department of Pathology, Chung Shan Medical University Hospital, No. 110, Sec. 1, Jianguo N. Road, Taichung City 40201, Taiwan
[e] Department of Medicine, Armed-Forces Taichung General Hospital, No. 348, Sec. 2, Chungshan Rd., Taiping Dist., Taichung City 41152, Taiwan
[f] General Education Center, Central Taiwan University of Science and Technology, No. 666, Buzih Road, Beitun District, Taichung City 40601, Taiwan

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ABSTRACT

Many recent efforts have focused on targeting cell death pathways for discovering new cancer therapies. The relative resistance of liver cancer cells to ionizing radiation (IR) and chemotherapeutic agents due to autophagic response limits the available treatment options for this type of cancer. In this study, 3-methyladenine (3-MA), an autophagy inhibitor, was investigated for its potential to enhance radio-sensitivity under radio-resistant conditions both in vitro and in vivo. Hep3B and HepG2 cells were used to examine the radio-resistance of liver cancer cells. The results show that Hep3B cells respond to irradiation with increased apoptotic cell death and that HepG2 is radio-resistant due to the IR-induced autophagy, as verified by DNA fragmentation, electron microscopy, acidic vesicular organelle formation, and Western blot analysis. Application of IR with 3-MA to inhibit autophagy simultaneously suppressed the expression of LC3 and enhanced cell death. The tumor xenograft model in nude mice verified the synergistic cytotoxic effect of 3-MA and IR, which resulted in significant repression of tumor growth. The results demonstrate that IR-induced autophagy provides a self-protective mechanism against radiotherapy in HepG2 cells. In addition, 3-MA enhances the cytotoxicity of IR in cell models and suppresses tumor growth in animal models. Based on the results, application of 3-MA, or other autophagy inhibitors, could be used as an adjuvant for radiotherapy when radio-resistance develops as a result of autophagy response.

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common form of cancer in the world and the second leading cause of cancer death in Taiwan. Surgical resection remains the only curative action. However, the majority of patients are not eligible for optimal resection at diagnosis. Advances in non-surgical treatment modalities, including transcatheter arterial chemoembolization, percutaneous ethanol injection therapy, microwave coagulation therapy, radiofrequency ablation, and 3-dimensional conformal radiotherapy, may improve the prognosis of HCC patients [1–5]. Although advanced irradiation techniques, such as intensity modulated RT and image-guided RT with the aid of respiratory gating, deliver a more conformal and higher dose to the tumor without damaging normal tissue [6]; the use of radiotherapy to treat HCC is limited by inherent tumor radio-resistance and low radiation tolerance of the surrounding normal liver. Therefore, strategies that increase tumor radiosensitivity and reduce normal tissue complications are urgently needed.

Ionizing radiation (IR) can result in lethal cell damage, which is characterized by the induction of DNA double-strand breaks if left unrepaired [7]. Radiation-induced apoptosis, namely type I programmed cell death, has been widely investigated over the past decade. However, apoptosis is not the predominant form of cell death, accounting for only 20% of cases [8]. Another type of programmed cell death, autophagy, has been reported to be

Abbreviations: 3-MA, 3-methyladenine; AO, acridine orange; AVOs, acidic vesicular organelles; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HCC, hepatocellular carcinoma; IR, ionizing radiation; mTOR, mammalian target of rapamycin; PFTα, pifithrin-α; PI, propidium iodide; PI3K, phosphoinositide 3-kinase.

* Corresponding authors. Present address: Department of Medicine, Armed-Forces Hualien General Hospital, No. 163, Jiali Rd., Xincheng Township, Hualien County 97144, Taiwan. Tel.: +886 4 38266805; fax: +886 4 38261370 (W.-H. Kuo), Tel.: +886 4 24730022x1676; fax: +886 4 23248195 (F.-P. Chou).

E-mail addresses: rad.tseng@msa.hinet.net (H.-C. Tseng), p53_tw@yahoo.com.tw (W.-S. Liu), ystyan@ms15.hinet.net (Y.-S. Tyan), kcm@csmu.edu.tw (H.-C. Chiang), wuhsienk@gmail.com (W.-H. Kuo), fpchou@csmu.edu.tw (F.-P. Chou).

[1] These authors contributed equally to this work.

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initiated by irradiation [9]. Autophagy, an evolutionarily conserved response to nutrient deprivation in all eukaryotic cells, is a multistep process that involves sequestration of bulk cytoplasm and organelles in double-membrane vesicles called autophagosomes [10]. Autophagosomes fuse with lysosomes to form autolysosomes in which acid-dependent enzymatic degradation ensues. The digested constituents are recycled to generate ATP for cell survival in case of starvation. Autophagy can also be triggered by other perturbations including inhibition of mTOR (mammalian target of rapamycin) [11], activation of mitogen-activated protein kinase signaling [12], accumulation of intracellular calcium [13] and reactive oxygen species [14], and endoplasmic reticulum stress [15]. When the magnitude of stress is excessive or persists for an extended period of time, autophagy can lead to cell death. Radiation-induced cell death is enhanced by the upregulation of autophagy through the inhibition of apoptosis and the promotion of autophagy [16]. In addition to promoting cells into an irreversible mode of cell death, paradoxically, autophagy can be activated to protect cancer cells from death. In studies on cell lines defective in apoptosis, autophagy is triggered in response to hypoxia, deprivation of growth factors, or nutrient depletion to confer survival advantage for tumor cells [17,18]. Most of the current anti-cancer therapies, such as chemotherapy [19,20], radiotherapy [9,21], targeted therapy with kinase inhibitors [22,23], and hormone therapy [24], activate the autophagy process. Therefore, autophagy is a reversible damage response that can be modulated to enhance cell death. Many clinical trials have rigorously attempted to elucidate the specific role of autophagy in cancer treatments [25].

Suppression of cell death pathways is closely related to tumor initiation, progression and resistance to anticancer treatment. To increase the therapeutic window between normal tissue complications and tumor cytotoxicity, it is important to investigate how different cell types respond to radiation. The radiobiological relevance of autophagy in the treatment of cancer remains obscure. Autophagy presents a paradox of opposing effects of cell death and cell survival in radiation treatment [26]. A thorough understanding of this multifaceted relationship is crucial for developing anti-cancer strategies that modulate cell death pathways.

The liver is indispensable for normal metabolic functions. In addition, there is much that needs to be determined regarding the relationship of autophagy and cell death in the development and treatment of hepatobiliary neoplasia [27]. Thus, our interest is to identify the impact of radiation-induced autophagy on treatment outcome in liver cancer cells. In this study, the effect of 3-methyladenine (3-MA), an inhibitor of autophagy, on radiosensitivity was investigated in HepG2 cells, which are resistant to radiation due to autophagy. From both an in vitro and in vivo point of view, our study is the first to use a radio-resistant hepatoma model to demonstrate the importance of autophagy and provide a useful strategy to improve the efficacy of radiotherapy.

2. Materials and methods

2.1. Cell lines, cell cultures and chemicals

HepG2 and Hep3B human liver cancer cell lines were obtained from ATCC (Manassas, VA, USA). The p53 status of HepG2 and Hep3B cells is well documented. While HepG2 cells express p53-wt, Hep3B cells are characterized as p53 null [28]. Cells were cultured in DMEM (Life Technologies, Grand Island, NY) and maintained at 37 °C in a humidified atmosphere of 5% CO_2. For treatment, the medium was removed and replaced with fresh medium containing DMSO (final concentration <0.1%) or different concentrations of 3-MA, bafilomycin A1 or pifithrin-α (all purchased from Sigma, St. Louis, MO). 3-Methyladenine (3-MA) is a class III phosphoinositide-3-kinase inhibitor and is widely used as an autophagy inhibitor. Bafilomycin A1, a specific inhibitor of the vacuolar type H^+-ATPase, was used to block fusion of autophagosomes and lysosomes. Pifithrin-α is a potent inhibitor of p53.

2.2. Ionizing radiation modalities

Cells were cultured in phenol red-free medium and irradiated with a 6MV-X ray linear accelerator (Varian 21-EX). The radiation was delivered in a single dose of 5–30 Gy over an appropriate field size at a dose rate of 400 cGy/min. A 3-cm polystyrene block was placed under the petri dishes during each irradiation to allow for homogeneous backscattering radiation. Control cells were removed from the incubator and placed for the same period of time under the IR source without radiation exposure. In the combined treatment modality studies, indicated concentrations of 3-MA were added before applying IR.

2.3. Determination of cell viability

Cell viability was determined by the MTT assay. Cells were treated with the indicated concentrations of 3-MA alone or in combination with IR at 37 °C for 24 or 48 h. Thereafter, the medium was changed, and the cells were incubated with 100 μl of MTT (5 mg/ml) (Sigma, St. Louis, MO)/well for 4 h and then solubilized in isopropanol. The absorbance at 563 nm was measured.

2.4. Cell cycle analysis

Flow cytometry was performed by propidium iodide staining (Roche Molecular Biochemical, Indianapolis, IN) according to the manufacturer's instructions. The cells were stained with propidium iodide and analyzed for cell-cycle distribution by flow cytometry with a FACStar caliber (Becton Dickinson) cell sorter. The percentage of each phase was evaluated using the software CellQuest.

2.5. Quantification of acidic vesicular organelles (AVOs)

Cell staining was performed according to published procedures [29]. Acridine orange (AO) (Polysciences, Warrington, PA) was added to cells at a final concentration of 1 mg/ml for 15 min. Images were obtained with a fluorescent microscope (Nikon TE-300) equipped with 490-nm band-pass blue excitation filters, a 500-nm dichroic mirror, and a 515-nm-long pass-barrier filter.

2.6. Detection of nucleosomal fragmentation of genomic DNA and DAPI stain

DNA extraction and electrophoresis on agarose gels were performed as described previously [30]. After the indicated treatments, cells were fixed with 3 ml of 4% paraformaldehyde. To stain the cells, 500 ml of a 0.5 mg/ml chilled solution of DAPI (Invitrogen) stain was added for 5 min. The cells were then rinsed with phosphate-buffered saline and counted under a fluorescent microscope.

2.7. Electron microscopy

Cells were harvested by trypsinization and fixed with 2% glutaraldehyde, 4% paraformaldehyde, and 1% tannic acid in 0.1 mol/L cacodylate buffer, pH 7.4, for 25 h. The cells were stained with osmium-thiocarbohydrazide-osmium. After staining, the cells were dehydrated in a series of graded EtOH concentrations (70–100%) and were then immersed serially in 1:1 hexamethyldisilazane and absolute ethanol. One-micrometer thin sections were cut,