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Research Paper

Hypoxia-induced autophagy as an additional mechanism in human osteosarcoma radioresistance

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ABSTRACT

Osteosarcoma (OS) responds poorly to radiotherapy, but the mechanism is unclear. We found OS tumor tissues expressed high level of protein HIF-1α, a common biological marker indicative of hypoxia. It is known that hypoxic cells are generally radioresistant because of reduced production of irradiation-induced DNA-damaging reactive oxygen species (ROS) in the anaerobic condition. Here we report another mechanism how hypoxia induces radioresistance. In MG-63 human osteosarcoma cells, hypoxic pretreatment increased the cellular survival in irradiation. These hypoxia-exposed cells displayed compartmental recruitment of GFP-tagged LC3 and expression of protein LC3-II, and restored the radiosensitivity upon autophagy inhibition. The following immunohistochemistry of OS tumor tissue sections revealed upregulated LC3 expression in a correlation with HIF-1α protein level, implying the possibly causative link between hypoxia and autophagy. Further studies in MG-63 cells demonstrated hypoxic pretreatment reduced cellular and mitochondrial ROS production during irradiation, while inhibition of autophagy re-elicited them. Taken together, our study suggests hypoxia can confer cells resistance to irradiation through activated autophagy to accelerate the clearance of cellular ROS products. This might exist in human osteosarcoma as an additional mechanism for radioresistance.

1. Introduction

Osteosarcoma (OS) is the most common type of primary bone cancer that mainly affects younger populations [1,2]. Current therapies combining surgery with chemotherapy (doxorubicin and cisplatin with or without methotrexate) yield 60–70% of the 5-year survival rate. However, the effective cure for patients with metastatic or relapsed osteosarcoma is still challenging [3]. Therefore, improvement of the existing therapies and exploitation of other approaches are highly anticipated.

Radiotherapy is an alternative combinatory therapy for OS. The incorporation of radiotherapy significantly improved the efficiency of chemotherapy by certain anticancer drugs (e.g., ifosfamide, cisplatin, HDMTX, etc.) [4], which even led to a long-term remission in some patients [5]. Locally complete cure could also be observed in unresectable or partially resected cases by radiotherapy when applied at high intensity [6]. Nevertheless, OS is generally considered radioresistant with poorly understood mechanism [7].

In this study, we found HIF-1α was overexpressed in human OS tissues. HIF proteins are often indicators of hypoxia which is common in solid tumors like OS where blood supply in the microenvironment is usually limited [8–11]. In cancer stem cells, HIF proteins promote tumor aggressiveness and confer resistance to certain therapies including irradiation [12–15].

The mechanism that tumor with hypoxia has reduced sensitive to radiotherapy is well studied [16]. It is known that irradiation generates free radicals on DNA. At the normal condition, these radicals can be fixed by oxygen (O_2) to generate DNA-damaging ROS products which will initiate cellular death. However, this death-inducing effect is compromised when the oxygen availability is low in hypoxic cells and ROS production is therefore limited [17].

Here, we found an additional mechanism that involves

Abbreviations: HIF-1α, hypoxia-inducible factor 1-alpha; ROS, reactive oxygen species; OS, osteosarcoma; OC, osteochondroma; LC3, microtubule-associated protein-1 light chain 3; CQ, chloroquine; 3-MA, 3-methyladenine

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autophagy in the mechanism of OS radioresistance, which is independent of oxygen at the time during irradiation. Autophagy is a process in which subcellular organelles or complex of proteins are sequestrated by intracellular membranes and then fused with lysosomes for degradation. This process is an important to eliminate damaged cellular components and maintain cellular survival [18]. Autophagy has been evidenced to be involved in cancer [19–21], and recent studies suggest its contribution to radioresistance in various tumors. Lomonaco et al. have found the induction of autophagy contributes to the radioresistance of glioma stem cells [22]. The Rodemann group also reported that autophagy also caused resistance to ionizing radiation in breast cancer cell lines [23]. The similar phenomenon was additionally evidenced in pancreatic cancer cells [24]. Another study also thoroughly support the role of autophagy in mediating radioresistance [25].

In this study, we propose that autophagy induced by hypoxia is another important mechanism that accounts for the radioresistance of OS.

2. Materials and methods

2.1. Patient samples

Histopathologically confirmed paraffin-embedded tissue sections from 89 osteosarcoma (51 males and 38 females) and 28 age-matched osteochondroma patients (16 males and 12 females) were recruited from the Fourth Hospital of Hebei Medical University. Clinical stages were evaluated according to the 2002 American Joint Committee on Cancer (AJCC). This study complied with the Declaration of Helsinki and was approved by the Human Ethics and Research Ethics Committees of the hospital. Written informed consents were obtained from all patients.

2.2. Tissue section and cell immunostaining

Paraffin-embedded tissue sections (4 μm) were incubated sequentially with primary antibodies and HRP-conjugated secondary antibodies. The signal was developed by EnVisionTM Peroxidase/DAB detection kit (Dako, UK). For immunocytochemical staining, MG-63 cells were washed with PBS and then received common processes like fixation (4% paraformaldehyde), permeabilization, blocking, and antibody incubation. Antibodies used in this study included anti-HIF-1α (Abcam, USA), anti-LC3 (Novus Biologicals, USA) and anti-γH2AX (Cell Signaling, USA). DAPI, Hoechst 33,258 and dichlorofluorescin diacetate (DCF-DA) were purchased from Sigma-Aldrich of USA. MitoSOX Red was from Thermo Fisher Scientific of USA.

2.3. Cell culture and irradiation procedure

Human MG-63 osteosarcoma cells were cultured in the DMEM medium (10% FBS, 50 U/ml penicillin, 50 μg/ml gentamicin, 2.5 μg/ml amphotericin B, 1% glutamine and 2% HEPES) at 37 °C in atmosphere with 5% CO_2. ELEKTA Synergy Linear Accelerator (Cravoley, UK) was used to treat the cells at 6 Gy (350 cGy/min) unless otherwise indicated. Culture medium was replaced with fresh medium without serum or antibiotics at 6 h before irradiation. Cellular viability was measured by the trypan blue exclusion method.

2.4. Western blot

Cell lysate with equal amount of protein was resolved by SDS-PAGE, and then transferred to NC membrane. After being blocked by 5% nonfat milk, the membrane was incubated with primary and secondary antibodies sequentially. Signals were developed by Pico Chemiluminescent Substrate (Thermo Fisher Scientific, USA) on films.

2.5. Statistical analysis

ANOVA, Tukey's test, and regression analysis were performed by software SPSS 21.0.

3. Results

3.1. HIF-1α expression is increased in osteosarcoma and is associated with the survival rate

It is established that hypoxia is common in most solid tumors due to limited blood supply in the microenvironment. This low oxygen condition and cellular adaptive responses often cause tumor aggressiveness and resistance to treatments including irradiation. Osteosarcoma (OS) is commonly known to be radioresistant. To determine whether radioresistance of this solid tumor could possibly involve hypoxia, we recruited osteosarcoma tissues from 89 cases to stain the typical hypoxia marker, HIF-1α, by immunohistochemistry, using 28 control samples from osteochondroma (OC), the most common benign bone tumor.

When compared to OC controls, most OS tissue samples expressed higher level of HIF-1α. Much more cells demonstrated positive staining and had stronger intensity in OS sections (Fig. 1A). Because the staining intensity was largely correlated with the number of positively stained cells, we simply counted the number of cells with observable staining and calculated the percentage of HIF-1α positive cells to grade the expression level ranges. 5%, 15% and 45% were used as the cutoff values for expression ranges of "−", "+", "++" and "+++"accordingly. We found 82 out of 89 (92.1%) OS sections expressing HIF-1α in positive ranges ["+": 16 (18.0%); "++": 25 (28.1%); and "+++": 41 (46.1%)] (Fig. 1B). In contrast, most OC samples have no or relatively low HIF1α expression ["−": 23 (82.1%); "+": 5(17.9%)].

HIF-1α expression in cancer often results from hypoxia and predicts poor prognosis because it is involved in tumor aggressiveness and intractability such as chemoresistance, radioresistance, angiogenesis, vasculogenesis, invasiveness and metastasis [9,26]. We therefore looked into the case medical history records and found the overall survival rate of these patients was correlated with HIF-1α expression: cases in the "+++" range had significantly lower survival rate than those in the "− or +", "++" ranges (Fig. 1C, Kaplan-Meier curve, the log rank test, $p=0.019$). "The positive correlation of HIF-1α expression with the post-operative treatment (mainly chemotherapy) indicates HIF-1α expressed in the tumor tissue exerts a biological effect. Because HIF-1α can contribute to resistance of both chemotherapy and irradiation [9,17,27,28], therefore although none of these patients received irradiation after surgery, the poorer chemotherapeutic efficiency on patients with higher HIF-1α expression might implicate a insensitive response of these cases to irradiation as well".

3.2. Hypoxia pretreatment protects osteosarcoma cells from irradiation

It is commonly known that hypoxic cells generally are less sensitive to irradiation because of insufficient oxygen to generate toxic ROS. We found another mechanism how hypoxia leads to radioresistance in a cellular model. This mechanism requires hypoxia not during the irradiation, but prior to the irradiation.

The human osteosarcoma cell line MG-63 was used to demonstrate in this study. We first determined the optimal