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Article

Hyperthermia Induces Apoptosis through Endoplasmic Reticulum and Reactive Oxygen Species in Human Osteosarcoma Cells

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Abstract: Osteosarcoma (OS) is a relatively rare form of cancer, but OS is the most commonly diagnosed bone cancer in children and adolescents. Chemotherapy has side effects and induces drug resistance in OS. Since an effective adjuvant therapy was insufficient for treating OS, researching novel and adequate remedies is critical. Hyperthermia can induce cell death in various cancer cells, and thus, in this study, we investigated the anticancer method of hyperthermia in human OS (U-2 OS) cells. Treatment at 43 °C for 60 min induced apoptosis in human OS cell lines, but not in primary bone cells. Furthermore, hyperthermia was associated with increases of intracellular reactive oxygen species (ROS) and caspase-3 activation in U-2 OS cells. Mitochondrial dysfunction was followed by the release of cytochrome c from the mitochondria, and was accompanied by decreased anti-apoptotic Bcl-2 and Bcl-xL,

and increased pro-apoptotic proteins Bak and Bax. Hyperthermia triggered endoplasmic reticulum (ER) stress, which was characterized by changes in cytosolic calcium levels, as well as increased calpain expression and activity. In addition, cells treated with calcium chelator (BAPTA-AM) blocked hyperthermia-induced cell apoptosis in U-2 OS cells. In conclusion, hyperthermia induced cell apoptosis substantially via the ROS, ER stress, mitochondria, and caspase pathways. Thus, hyperthermia may be a novel anticancer method for treating OS.

Keywords: osteosarcoma; hyperthermia; endoplasmic reticulum (ER) stress; reactive oxygen species (ROS)

1. Introduction

Osteosarcoma (OS) is the most common type of bone cancer characterized by locally aggressive growth and early metastatic potential [1]. Although OS is a relatively rare form of cancer, it is the most commonly diagnosed bone cancer in children and adolescents [2]. In recent decades, the combination of surgical resection and chemotherapy has improved the five-year survival rate [3]. Nevertheless, these treatments can cause side effects and induced drug resistance in OS [4]. An effective adjuvant therapy was insufficient for treating OS. Therefore, researching novel and adequate remedies is critical.

Accumulated evidence has suggested that hyperthermia can induce apoptosis in normal and tumor cells [5,6]. Normal tissues subjected to hyperthermia are accompanied by some of the normal physiological responses, such as microvascular expansion, a rapid breathing, a rapid heartbeat, increased cardiac output, increased blood flow, and increased kidney manufacturing of urine to dissipate heat [7]. Nevertheless, the damage caused by hyperthermia is not obvious in normal tissues. In cancer tissues, the blood vessels have an irregular architecture [8]. The microvascular permeability and blood circulation differ from those of normal tissues as the temperature increases, and damage becomes markedly obvious [9]. Moreover, a high temperature can denature protein, DNA, and RNA damage and interrupt vital cellular processes, ultimately causing cell death [10]. Previous studies have reported that hyperthermia is a suitable candidate for cancer therapy. Shellman (2008) stated that hyperthermia may be a promising treatment for basal cell carcinoma and melanoma by eliciting the intrinsic and extrinsic apoptosis pathways [11]. In 2013, Pawlik *et al.* provided evidence suggesting that one mode of heat-induced cell death in H1299 cells was mitotic catastrophe, which probably caused apoptosis [12].

Hyperthermia can induce endoplasmic reticulum (ER)-triggered apoptosis in numerous cancers, such as breast cancer, melanoma, skin cancer, OS, colon cancer and lung cancer [11–13]. The ER is responsible for protein modifications, protein folding, protein synthesis, and lipid synthesis. When cells are exposed to various stimuli (oxidation, heat, drug, damage, or infection), the ER homeostasis is disrupted, and unfolded or misfolded proteins accumulate in the ER. Cells then activate several signaling pathways, including the unfolded protein response (UPR) or ER-associated protein degradation [14]. These responses protect cells, but intense ER stress eventually causes cell apoptosis [15].

The ER chaperone proteins glucose-related protein 78 (GRP78)/Bip and GRP94, are the marker and key regulators of ER stress [16]. The GRP78 protein has anti-apoptotic properties, and can attenuate the UPR [17]. In addition, hyperthermia induces reactive oxygen species (ROS) and the functional disorders of the mitochondria in various cancer cell lines [18–20]. The ROS and mitochondria dysfunction play vital roles in the apoptotic process.

In this study, we demonstrate that hyperthermia markedly increased the cytotoxicity on OS cell lines. For the first time, we observed that hyperthermia activated ROS, mitochondria dysfunction, and ER stress, thereby activating caspase-dependent apoptotic pathways.

2. Results

2.1. Hyperthermia Induced Apoptosis in Human Osteosarcoma Cells

To investigate the potential for hyperthermia to induce cell death in human OS cells, we first examined the effect of hyperthermia on cell survival in human OS cells (U-2 OS, MG63 and HOS) using the sulforhodamine B (SRB) assay. The cells with hyperthermia-induced cell death were treated in a temperature-dependent manner (Figure 1A–C). The inhibition of cell proliferation was observed when the cells were exposed with hyperthermia for 60 or 90 min at 43–48 °C. Hyperthermia did not affect the viability of normal bone cells (hFOB 1.19, Figure 1D). We then confirmed that hyperthermia induced cell death through an apoptotic mechanism by performing 4,6-diamidino-2-phenylindole (DAPI) staining, a cell cycle, Annexin V/PI assay, and the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay. The U-2 OS cells were treated with hyperthermia conditions and, after 24 h, the nuclei of the cells were stained with DAPI (a typical marker of apoptosis). DAPI staining revealed that hyperthermia induced substantial chromatin condensation (Figure 1E). Because continual incubation with hyperthermia caused a substantial reduction in viable cells, we examined the effect of hyperthermia on the induction of cell death in cells by using the cell cycle progression in flow cytometric analysis of propidium iodide (PI) staining. The results shown in Figure 1F–H indicated that hyperthermia induced an increase in the percentage of cells in the sub-G1 phase. In addition, compared with sham-treated U-2 OS cells, hyperthermia-treated cells increased TUNEL fluorescence intensity in a temperature-dependent manner (Figure 2A). We then analyzed whether hyperthermia induced cell death through an apoptotic mechanism. Compared with sham-treated cells, a high proportion of Annexin V labeling was detected in cells treated with hyperthermia (Figure 2B–D). These data indicate that hyperthermia induced cell death through an apoptotic mechanism.

2.2. Hyperthermia Induced Mitochondrial Dysfunction and ROS Accumulation

To determine whether hyperthermia induced apoptosis by triggering the mitochondrial dysfunction, we measured the mitochondrial membrane potential by applying a mitochondria-sensitive dye, JC-1 using flow cytometry. As shown in Figure 3A, the treatment of U-2 OS cells with hyperthermia conditions and after 24 h diminished the mitochondrial membrane potential in a temperature-dependent manner. To determine whether hyperthermia induced apoptosis by triggering the mitochondrial apoptotic pathway, we measured changes in the expression of cytochrome c and Bcl-2 family proteins.