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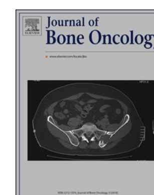

Research Paper

Hypoxia promotes drug resistance in osteosarcoma cells via activating AMP-activated protein kinase (AMPK) signaling

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Purpose: Drug resistance has been recognized to be a major obstacle to the chemotherapy for osteosarcoma. And the potential importance of hypoxia as a target to reverse drug resistance in osteosarcoma has been indicated, though the mechanism underlining such role is not clarified. The present study aims to investigate the role of hypoxia in the drug resistance in osteosarcoma cells via activating AMP-activated protein kinase (AMPK) signaling.
Experimental design: We investigated the promotion of the resistance to doxorubicin of osteosarcoma MG-63 and U2-os cells in vitro, and then determined the role of hypoxia-inducible factor-1 (HIF-1)α and HIF-1β, the activation and regulatory role of AMPK in the osteosarcoma U2-os cells which were treated with doxorubicin under hypoxia.
Results: It was demonstrated that hypoxia significantly reduced the sensitivity of MG-63 and U2-os cells to doxorubicin, indicating an inhibited viability reduction and a reduced apoptosis promotion. And such reduced sensitivity was not associated with HIF-1α, though it was promoted by hypoxia in U2-os cells. Interestingly, the AMPK signaling was significantly promoted by hypoxia in the doxorubicin-treated U2-os cells, with a marked upregulation of phosphorylated AMPK (Thr 172) and phosphorylated acetyl-CoA carboxylase (ACC) (Ser 79), which were sensitive to the AMPK activator, AICAR and the AMPK inhibitor, Compound C. Moreover, the promoted AMPK activity by AICAR or the downregulated AMPK activity by Compound C significantly reduced or promoted the sensitivity of U2-os cells to doxorubicin.
Conclusion: The present study confirmed the AMPK signaling activation in the doxorubicin-treated osteosarcoma cells, in response to hypoxia, and the chemical upregulation or downregulation of AMPK signaling reduced or increased the chemo-sensitivity of osteosarcoma U2-os cells in vitro. Our study implies that AMPK inhibition might be a effective strategy to sensitize osteocarcoma cells to chemotherapy.
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1. Introduction

Osteosarcoma is the most frequent primary bone malignancy, mainly attacks adolescents [1]. The combined chemotherapy with intensive dose has greatly improved the overall survival for osteosarcoma patients to over 70% [2]. However, the prognosis remains poor for those with metastasis, or for those who relapse, and survival rates only reach 20–30% [3,4]. Doxorubicin, methotrexate and Cisplatin are commonly used as anticancer drugs in osteosarcoma [5] for the last 20 years, and there has been no improvement in the survival of those osteosarcoma patients, who acquisite the drug-resistant phenotype. Thus, it is urgent to recognize the drug-resistance mechanism of osteosarcoma and to provide novel therapeutic options for this disease.

Hypoxia-induced drug resistance has been confirmed for a variety of anti-tumor agents in various types of tumors [8,6,7], and even in osteosarcoma [9]. Such hypoxia markers as hypoxia-inducible factor-1 (HIF-1), vascular endothelial growth factor (VEGF) and carbonic anhydrase IX (CA IX) are detectable in osteosarcomas [10], and correlate with poor progress of osteosarcoma patients, suggesting the important role of hypoxia in the survival of osteosarcoma cells [11,12], and implying the potential importance of hypoxia as a target to antagonize drug resistance in osteosarcoma. However, it is indicated that the drug resistance in osteosarcoma is independent on the upregulated HIF-1α, suggesting other hypoxia-related signaling may be more relevant in the drug resistance to osteosarcoma.

Multiple other signaling pathways are deregulated in hypoxia and may exert regulatory roles in the hypoxia-induced drug resistance. Wild-type p53 is confirmed to be inactivated in some

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tumor cells by hypoxia [14,13]. c-jun, activator protein-1 (AP-1), Phosphoinositol-3-kinase (PI3K) pathway and nuclear factor kappa-B (NF-κB) have also been indicated to involve in the hypoxia-induced drug resistance, mainly by inhibiting the drug-induced apoptosis [16,15,17]. And the target inhibiting of these signaling pathways sensitizes cells to cytotoxic agents under the condition of hypoxia, implying these markers as possible targets to counteract the hypoxia-induced drug resistance.

AMP-activated protein kinase (AMPK) is the most important sensor of cellular energy [19,18], and is also activated by hypoxia as a compensatory response to the reduced mitochondrial respiration [20]. The heterotrimeric AMPK composes of two regulatory subunits and one catalytic subunit, which is activated the increased AMP/ATP ratio [21]. Then the activity was promoted of upstream kinases and phosphatases that control AMPK phosphorylation and dephosphorylation at the Thr-172 [22,23], particularly by the tumor suppressor LKB1, which phosphorylates the catalytic subunit of AMPK in an AMP-dependent manner [24,25]. AMPK is also activated by the Ca^2 /calmodulin-dependent protein kinase kinase (CaMKK) [27,26], independently of cellular AMP levels. In addition, the activation of AMPK can also be induced in response to oxidant stress [28,29] with or without hypoxia. However, the role of the AMPK activation in hypoxia in osteosarcoma is still unclarified.

In the current study, we investigated the resistance of osteosarcoma cells to the widely utilized in clinic cytotoxic drug, doxorubicin under hypoxia, and then examined the association of such resistance with the activation of AMPK signaling. On the other side, we investigated the influence by the chemical inhibition of AMPK signaling on the hypoxia-induced resistance to doxorubicin. The present study indicated the key regulatory role of AMPK in the hypoxia-induced resistance to doxorubicin in osteosarcoma cells, suggesting a possible target of AMPK against the drug-resistance of osteosarcoma cells.

2. Materials and methods

2.1. Cell culture and treatment

Human osteosarcoma cell line MG-63 and U2-os were purchased from American Type Culture Collection (ATCC) (Rockville, MD, USA). MG-63 cells were cultured in Eagle's minimal essential medium (EMEM), supplemented with 2 mM Glutamine, 1% Non Essential Amino Acids (NEAA) and 10% Fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA), and were routinely incubated at 37 °C with 5% CO_2. U2-os cells were grown in RPMI-1640 medium (Ameresco, Framingham, MA, USA) supplemented with 10% FBS (Invitrogen, Carlsbad, CA, USA). For hypoxia culture, MG-63 or U2-os cells were incubated in a hypoxia incubator infused with a gas mixture of 5% CO_2, 2% oxygen and nitrogen, whereas cells were incubated in an ordinary incubator with 5% CO_2. For the doxorubicin treatment, MG-63 or U2-os cells with more than 85% confluence were updated with medium containing 2% FBS and the docorubicin with various concentrations. To induce or inhibit the AMPK activity, AICAR and Compound C (Sigma-Aldrich, St. Louis, MO, USA) were added to the medium with a concentration of 1 mM and 20 μM respectively.

2.2. MTT assay and apoptosis assay

The viability of MG-63 or U2-os cells which were seeded in 96-well plates with more than 85% confluence post various treatments were measured with MTT assay according to the standard protocol. The absorbance was measured at 570 nm with a reference wavelength of 750 nm using a spectrophotometer.

Apoptosis of U2-os cells was examined with an Annexin V-FITC apoptosis detection kit (Sigma-Aldrich, USA). Briefly, 6×10^5 U2-os cells were stained with Annexin V-FITC and propidium iodide and then were detected by a FACScan flow cytometer (Bio-Rad, Hercules, CA, USA). The results were presented as a percentage of apoptotic cells from total cells.

2.3. Western blot analysis

U2-os cells, post treatment, were harvested and were homogenized in an ice-cold Cell lysis buffer (Bio-Rad, Hercules, CA, USA). Cellular lysates was centrifugated with $12{,}000 \times g$ for 30 min at 4 °C, and the supernatant was collected. Protein samples were separated with 8–12% SDS-PAGE gel and were transferred to a nitrocellulose membrane (Millipore, Bedford, MA, USA). The membrane was blocked for non-specific binding targets with 5% Skimmed milk powder overnight at 4 °C, and were incubated with the rabbit polyclone antibody (against caspase 3, Poly (ADP-ribose) polymerase (PARP), HIF-1α, HIF-1β, AMPKα with or without phoshorylated Thr 172, ACC with or without phoshorylated Ser 79, or β-actin) overnight at 4 °C, and then were incubated with HRP-linked secondary anti-rabbit antibody (New England Biolab, Ipswich, UK) for 1 h at room temperature. The specific binding band was scanned and quantified according to the band density by Image J software.

2.4. HIF-1α knockdown via RNA interference

The HIF-1α specific siRNA (siRNA- HIF-1α) (25 or 50 nM) or the scrambler oligonucleotides as control (siRNA-Con) (25 or 50 nM) were purchased from Thermo Fisher (Waltham, MA, USA), and were transfected into U2-os cells with Opti-MEM containing Lipofectamine RNAiMax (Invitrogen, Carlsbad, CA, USA). 6 h post transfection, cells were updated with fresh RPMI-1640 medium, which was supplemented with 2% FBS, and were subject to other treatment or were assayed for the knockdown efficiency post another inoculation of 24 h.

2.5. DCFH-DA assay ELISA for AMPK activity

Reactive oxygen species (ROS) was assessed using 2,7-dichlorofluorescin diacetate (DCFH-DA). U2-os cells were added with BSS containing DCFH-DA (5 μM), and fluorescence signaling was collected at 535 nm using excitation at 484 nm. Cellular fluorescence density from individual cell well was collected and was averaged to provide an overall assessment for each group.

The AMPK activity was examined with AMPK Kinase Assay Kit AMPK Kinase Assay Kit (CY-1182; CycLex, Nagano, Japan) according to the manufacturer's manual. U2-os cells post various treatments were directly lysed with ice-cold lysis buffer; then the cellular lysates were serially diluted in Kinase buffer and supplemented with phosohorylation substrate, and the amount of phosphorylated substrate specifically bonded to an anti-phospho-mouse IgG, which then assayed with anti mouse Ig-G conjugated with horseradish peroxidase and its substrate, by absorbance 450 nm.

2.6. Statistical evaluations

Quantitative results are presented as mean ± SE. For the analysis between two groups, the Student's t test was performed. A p value less than 0.05 was considered significant.

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