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Sensitizing osteosarcoma stem cells to doxorubicin-induced apoptosis through retention of doxorubicin and modulation of apoptotic-related proteins

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ABSTRACT

Aims: Osteosarcoma is the most common pediatric bone malignancy with high propensity to metastasize and relapse. Emerging evidence suggest that osteosarcoma is sustained by a subset of self-renewing cancer stem-like cells (CSCs) relying on mechanisms to evade apoptosis and survive in response to drugs-induced DNA damage. We proposed to decipher the mechanisms underlying the resistance of CSCs to doxorubicin-induced apoptosis.

Main methods: CSCs were isolated using a sphere-forming assay and tested for sensitivity to doxorubicin-induced apoptosis, using MTT cell viability and BrdU proliferation assays, TUNEL staining and caspases 3/7 activity. Bcl-2 family proteins were analyzed by Western blot. Doxorubicin uptake was determined by confocal microscopy and bioluminescence imaging.

Key findings: We showed that osteosarcoma sphere stem-like cells expressed the multidrug-related efflux transporters P-glycoprotein and BCRP and are highly resistant to doxorubicin-induced apoptosis. Conversely after exposure to doxorubicin, these cells displayed an up-regulation of the anti-apoptotic proteins Bcl-2 and Bcl-xL with concomitant down-regulation of Bak and decreased caspase 3/7 activity. Inhibition of drug efflux transporters enhanced the cellular uptake of doxorubicin, being encompassed by an up-regulation the pro-apoptotic protein Bak and suppression of Bcl-2, favoring the commitment of CSCs towards apoptosis.

Significance: These results seemingly suggest that the high apoptotic threshold of CSCs to doxorubicin-induced cell dead stimuli is mainly dependent on the drug concentration reaching tumor cells that are governed by efflux transporter activity. Therefore, modulation of these transporters may be effective in potentiating the pro-apoptotic effects of doxorubicin, and emerges as an attractive strategy to sensitize osteosarcoma CSCs to chemotherapy.

1. Introduction

Osteosarcoma is an aggressive bone-forming tumor affecting mainly young people, comprising 20% of all bone sarcomas, and responsible for the second highest cause of cancer-related death in pediatric age [1,2]. Despite the aggressive therapies, overall survival rates of non-metastatic osteosarcoma stagnated at 60–70% and remain poor (<20%) for patients with metastasis [3]. Moreover, recurrences occur in 30–40% of non-metastatic patients [4]. This scenario remained unchanged over the last decades, underlying the need for novel therapeutic strategies.

The hypothesis that tumors are sustained by cells with stem-like properties, so-called cancer stem cells (CSCs) is changing the paradigm in cancer treatment, since they represent a minority of cells with distinct properties from those constituting the bulk of the tumor and are associated with metastasis, radio/chemo-resistance and poor clinical outcome [5,6].

Recent data, including our own, demonstrates that osteosarcoma contains a distinct self-renewing cell population exhibiting a stem-like phenotype and highly resistant to current therapies, further suggesting that they are implicated in tumor recurrence frequently observed in osteosarcoma patients [7–9]. The ability of these cells to enter in quiescence or slow proliferating rate together with the overexpression of ABC drug efflux transporters and enhanced DNA repair mechanisms are considered strategies of CSCs to escape chemotherapy [10]. In addition,

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along with the mechanisms controlling CSCs' self-renewal [11], the regulation of the apoptotic cell death represents another important aspect of CSCs' biology, being therefore a critical determinant in anticancer therapy efficacy [12]. It has been suggested that CSCs exploit several mechanisms to deregulate the apoptotic signaling pathway as a way to survive to chemotherapy induced-apoptosis [12,13].

The anthracycline doxorubicin (DOX) is one of the most active drugs employed in the treatment of osteosarcoma. This drug intercalates into the DNA of living cells and causes cell death via inhibition of topoisomerase II and generation of reactive oxygen species and free radicals by redox reactions. It has been reported that DOX induces apoptosis through the intrinsic pathway via disruption of the mitochondrial membrane potential with release of cytochrome c and subsequent activation of caspase cascade [14,15]. The Bcl-2 family proteins are key regulators of the mitochondrial apoptotic pathway, by controlling the outer mitochondrial membrane permeabilization by pore formation and release of cytochrome c [16,17]. An imbalance in the ratio of anti- and pro-apoptotic proteins dictate cells susceptibility to undergo apoptosis and a ratio tilted towards survival can render tumor cells more resistant to cell death stimuli [16]. The relevance of the anti-apoptotic Bcl-2 family as regulators of chemoresistance in CSCs was demonstrated in leukemia [18], colon [19] and glioma stem cells [20,21]. However the mechanisms underlying the expression of these proteins in CSCs as well as the defects in the apoptotic pathway remained largely unknown.

Herein, we observed that osteosarcoma contains a slowly proliferating pool of CSCs that failed to undergo apoptosis through activation of anti-apoptotic mediators Bcl-2 and Bcl-xL, downregulation of Bak and caspase-3/7 inactivation, as a result of impaired accumulation of DOX due to the efflux activity of drug transporters P-glycoprotein and breast cancer related protein. Moreover we found that pharmacological inhibition of these transporters enhanced the intracellular accumulation of DOX and importantly, up-regulation of the pro-apoptotic protein Bak and prevented the up-regulation of Bcl-2, favoring the commitment of CSCs towards apoptosis.

2. Material and methods

2.1. Cell culture and sphere-forming assay

The human MNNG/HOS osteosarcoma cell line was purchased from the American Type Culture Collection (Rockville, MD) and maintained in monolayer in RPMI-1640 medium (Gibco, Scotland, UK) supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS, Gibco® Invitrogen Life Technologies, USA) and 1% (v/v) antibiotic/antimicotic (100 units/ml penicillin, 100 mg/ml streptomycin and 0.25 μM amphotericin B, Sigma-Aldrich®) at 37 °C in a humidified incubator with 95% air and 5% CO_2.

For sphere-forming assay, single-cell suspensions were plated at a density of 60,000 cells/well in 6-well poly-HEMA-coated plates (Sigma, St. Louis, USA) in serum-free DMEM/F12 medium (Sigma) with 1% of methylcellulose (Sigma) containing 1% penicillin/streptomycin (Gibco) 20 nM progesterone (Sigma), 100 μM putrescine (Sigma), 1% insulin–transferrin–selenium A supplement (Gibco), 10 ng/ml basic fibroblast growth factor (bFGF, Peprotech EC, London, UK) and 10 ng/ml human recombinant epidermal growth factor (EGF, Sigma) as described elsewhere [7]. Fresh aliquots of growth factors were added twice a week. The formed sarcospheres were dissociated with StemPro®Accutase (Life Technologies) and re-seeded as single-cell in serum-free medium and under anchorage-independent conditions for generating secondary spheres. This procedure was repeated at least four times to evaluate the self-renewing capacity of sarcospheres growing under stressful growth conditions. The sphere-forming efficiency (%) for each generation was calculated by dividing the number of spheres formed by the total number of cells seeded. First generation spheres were used in subsequent experiments. Before use, spheres were collected and enzymatically dissociated with Accutase, counted and diluted at the indicated concentration for each experiment.

2.2. Cytotoxicity of doxorubicin

Cytotoxicity of DOX was evaluated by measuring drug effects on cell viability, proliferation and apoptosis.

2.2.1. Cell viability

Cell viability was analyzed using the 3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide (MTT, Sigma) assay. Cells (7.5×10^3 cells/well) were seeded on 96-well plates (Orange Scientific, Belgium), allowed to attach for 24 h and then treated with increasing concentrations of DOX (DOXO-cell®, 2 mg/ml) varying from 0.001 to 100 μM. Forty-eight hours after treatment, the culture medium was removed and 50 μl of MTT solution (0.5 mg/ml) (M2128, Sigma-Aldrich®) was added to each well, and the cells were incubated for 4 h at 37 °C in a CO_2 incubator. After that, 50 μl of acidified isopropanol (0.04 N HCl) was added to each well to dissolve the formed blue formazan crystals. The absorbance was measured in a microplate reader (Synergy™ HT, Biotek Instruments) at 570 nm using a 620 nm filter as reference. Cell viability was expressed as the percentage of surviving cells in relation to untreated cells. The drug concentration required to inhibit growth by 50% (IC_{50}) was estimated by fitting the dose–response curve to a sigmoidal function using the OriginPro8 (OriginLab Corporation).

2.2.2. Cell proliferation

Cellular proliferation was assessed using the colorimetric BrdU cell proliferation assay kit (Roche®, Germany) according to the manufacturer's instructions, which measures the incorporation of 5-bromo-2′-deoxyuridine (BrdU) into cellular DNA during cell proliferation. Cells were platted and treated as described above for the MTT assay. At the end of the 48 h treatment period, 10 μl of the BrdU solution (100 μM) was added to each well, and the cells were placed in a CO_2 incubator at 37 °C for 3 h to allow BrdU to incorporate into the DNA of proliferating cells. After this period, the labeling medium was removed and 200 μl of fixative solution (FixDenat) was added to each well and incubated for 30 min at room temperature (RT). Then, FixDenat was removed, and the cells were incubated with 100 μl of anti-BrdU-POD solution for 90 min at RT. After this period, the antibody solution was aspired and the wells were washed three times with 200 μl/well of washing solution. Lastly, 100 μl of substrate solution was added to each well and waited 20 min until reading the absorbance in a microplate reader (Synergy™ HT, Biotek Instruments) at 370 nm using a 492 nm filter as reference. Cell proliferation was expressed as the percentage of surviving cells in relation to untreated cells and the IC_{50} estimated using a sigmoidal dose–response curve fitting.

2.2.3. Apoptosis

Cellular apoptosis induced by DOX was quantified using the Terminal Deoxynucleotidyl Transferase-mediated Nick-End Labeling (TUNEL) assay (Roche Diagnostics GmbH, Mannheim, Germany) by fluorescence microscopy. Cells were incubated with 0.5, 1 and 5 μM of DOX for 48 h. A negative control without exposure to DOX was included. After treatment, the supernatant containing dead detached cells was collected, together with adherent cells (surviving cells) that were trypsinized to obtain the whole cell population. Tubes containing cells were centrifuged for removal of supernatant and the cells were fixed with 4% paraformaldehyde (Sigma-Aldrich®) and centrifuged in a Cytospin centrifuge (Cellspin I, TharmacGmbH, Waldsolms, Germany) at 1000 rpm for 5 min for cell deposition in superfrost microscope slides (Thermo Scientific, Menzel GmbH & Co. KG, Braunschweig, Germany). Fixed cells were rinsed with PBS and permeabilized with 0.25% Triton-X for 30 min at RT, followed incubation with a terminal deoxynucleotidyl transferase buffer for 1 h at 37 °C in a humidified chamber. After that,