Abstract. Chondrosarcoma is a malignant cartilage-forming cancer composed of cells derived from transformed cells that produce cartilage. Conventional chemotherapy and radiotherapy have very limited efficacy in patients with advanced chondrosarcoma. In the present study, we reported a novel therapeutic approach in the treatment of chondrosarcoma cells. We detected that lactate dehydrogenase-A (LDHA) is highly active in chondrosarcoma cells and chondrosarcoma patient samples compared with normal chondrocyte cell lines and primary human chondrocyte. Moreover, chondrosarcoma cells exhibited elevated levels of LDHA expression under doxorubicin treatment. To further explore the mechanisms, we generated doxorubicin-resistant cells from SW1353 chondrosarcoma cell line. Notably, the activity and expression of LDHA are upregulated in doxorubicin-resistant cells. Moreover, our data showed a strong correlation between glucose metabolism and doxorubicin resistance in chondrosarcoma cells; doxorubicin-resistant cells displayed highly activated glucose metabolism and depended more on glucose supply. Finally, we reported a synergistic effect produced by incorporating doxorubicin with glycolysis inhibitors-oxamate in the combined treatment of chondrosarcoma cells in vitro and in vivo. In summary, the present study may aid in the development of new approaches using the combination of chemotherapeutic agents for the treatment of chondrosarcoma patients.

Introduction

Chondrosarcoma is a malignant cartilage-forming tumor which accounts for ~20% of bone malignancies (1). The conventional chondrosarcoma is associated with a significant rate of morbidity and the 10-year survival rate is low (29-83%) (1,2). In addition to surgical resection which is the primary treatment option for patients with chondrosarcoma, conventional chemotherapy and radiotherapy are still under investigation for treatment options (2-4). It is known that most chemotherapy drugs for the treatment of chondrosarcoma are associated with strong toxicities for normal cells, yet, tumor cells remain drug-resistant (3). Currently, chondrosarcomas are relatively chemotherapy- and radiotherapy-resistant. A recent study reported that the growth of chondrosarcoma cells can be inhibited by mTOR inhibitor in an in vivo syngeneic rat model (5), suggesting a putative chemotherapeutic approach for clinical applications.

Doxorubicin (Dox) is an antitumor drug that is used frequently in chemotherapy for a variety of solid tumors (6). Similar to other chemotherapeutic agents, the efficacy of doxorubicin treatment is limited by drug resistance (7). Despite considerable clinical responses initially, the majority of patients develop resistance to doxorubicin. Although the underlying mechanism of doxorubicin resistance is not fully understood, researchers have determined several factors that influence cellular doxorubicin toxicity. It has been reported that doxorubicin can induce ROS generation in various tumor cells (8,9) and inhibition of P-glycoprotein by 20(S)-Rh2 attenuates adriamycin resistance (10).

Cancer cells, unlike their normal counterparts, use aerobic glycolysis with reduced mitochondrial oxidative phosphorylation for glucose metabolism (11). Therefore, the metabolic switch of cancer cells suggests that targeting metabolic pathway could be a selective approach not only to treat cancer patients, but also to override the chemoresistance. It has been reported that the combination of WZB117 which is a Glut1 inhibitor and cisplatin or paclitaxel displayed synergistic anticancer effects (12). Another GLUT1 inhibitor phloretin significantly enhanced daunorubicin's anticancer effects under hypoxia (13). Moreover, 3-BrPA which is a glycolysis inhibitor partially reversed the resistant phenotype and re-sensitized cells to oxaliplatin and 5-fluorouracil (14). Lactate dehydrogenase-A (LDHA) is one of the main isoforms of LDH expressed in breast tissue, controlling the conversion of pyruvate to lactate of the cellular glycolytic process.
Studies have shown that the LDHA expression in cancer cells is associated with radiotherapy sensitivity (15). In addition, it has been reported LDHA contributed to paclitaxel resistance in breast cancer, inhibition of LDHA by oxamate overrides the chemoresistance (16).

In the present study, we reported a novel therapeutic approach in the treatment of chondrosarcoma cells. LDHA is highly active in chondrosarcoma cell lines compared with normal chondrocytes. Doxorubicin treatment induced the LDHA expression which contributes to the doxorubicin resistance. Meanwhile, the activity and expression of LDHA were upregulated in doxorubicin-resistant cells. Moreover, our data showed a strong correlation between glucose metabolism and doxorubicin resistance in chondrosarcoma cells. Doxorubicin-resistant cells displayed highly activated glucose metabolism and depended more on glucose supply. Finally, we reported a synergistic effect produced by incorporating doxorubicin with glycolysis inhibitors-oxamate in the combined treatment of chondrosarcoma cells in vitro and in vivo.

Materials and methods

Cell lines and cell culture. Hs 819.T and SW1353 human chondrosarcoma cells were purchased from ATCC. CHON-001 and CHON-002 human normal chondrocyte cell lines were purchased from ATCC. Primary human chondrocyte was purchased from Promocell.com. All cells were cultured in RPMI-1640 supplemented with 10% FBS and 1X penicillin-streptomycin-glutamine (10378-016; Invitrogen) at 37°C in a humidified incubator with 95% air and 5% CO₂. All primary human chondrosarcoma patient specimens were obtained from patients undergoing surgery for tumor from 2009 to 2012 at the Cancer Research Center, The 101st Hospital of PLA, Wuxi, Jiangsu, China and stored in liquid nitrogen until analysis. All patients provided written informed consent. The study was approved by the Ethics Committee of the Cancer Research Center, The 101st Hospital of PLA, Wuxi.

Antibodies and reagents. Antibodies used in this study were purchased from: LDHA (Cell Signaling Technology #2012); β-actin (Cell Signaling Technology #4967); doxorubicin and oxamate were purchased from Sigma-Aldrich (St. Louis, MO, USA).

siRNA transfections. Transfection was performed using the Oligofectamine Transfection reagent (Invitrogen) according to the manufacturer's protocol. siRNA oligonucleotides for LDHA were purchased from Sigma, with a scrambled siRNA (Sigma) used as a control. Forty-eight hours after transfection, whole-cell lysates were prepared for further analysis.

Western blot analysis. Whole cells were lysed in 1X SDS sample buffer and resolved by electrophoresis using SDS-PAGE and transferred to nitrocellulose membranes. The membranes were probed with primary antibodies overnight, and then incubated with appropriate horseradish peroxidase conjugated secondary antibodies for 3 h followed by detection with a SuperSignal enhanced chemiluminescence kit (Pierce, Rockford, IL, USA). For sequential blotting, the membranes were stripped with Stripping Buffer (Pierce) and re-probed with proper antibodies.

Cell viability. Cell viabilities were determined using trypan blue dye exclusion assays. A total of 5x10⁴-1x10⁵ cells/well were seeded in 12-well plates. Twenty-four hours later, the medium was replaced with different concentrations of doxorubicin or oxamate. After treatments with multiple concentrations of drugs, cells were trypsinized and resuspended in PBS. Viable cell numbers were determined by trypan blue staining.

Generation of doxorubicin-resistant cell line. SW1353 cells were treated with gradually increasing concentrations of doxorubicin in regular cell culture conditions for the selection of resistant cells. After successive treatments for up to 3 months, resistant cell clones were pooled and used for all subsequent experiments in the present study. The resistant cells were treated by doxorubicin each month for repeating selection.

Real-time PCR. RNA was extracted from cancer cells using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The cDNA synthesis was performed using a SuperScript First-Standard Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's protocol. Quantitative PCR analyses were performed using Assay-on-Demand primers and the TaqMan Universal PCR Master Mix reagent (Applied Biosystems, Foster City, CA, USA). The samples were analyzed using an ABI Prism 7700 Sequence Detection System (Applied Biosystems). The primers for q-PCR were: LDHA: forward, 5'-TGGAGTGGAATGAATGTTGC-3' and reverse, 5'-ATAGCCCAGGATGTGAGCC-3'. The expression levels of β-actin were used to normalize the relative expression levels. Experiments were triplicated.

Glucose uptake assay. Cells were seeded in 12-well plates at 1x10⁴-3x10⁴ cells/well. Culture media was collected at 48 h and stored at -20°C until assayed. Glucose uptake was measured using an Amplex Red Glucose/Glucose Oxidase assay kit (Molecular Probes). Absorbance was measured at 563 nm using a SpectraMax M5 plate reader (Molecular Devices) and the results were normalized to the amount of total protein compared with the control cells.

Lactate production assay. Lactate production in the medium was detected by using a Lactate assay kit (BioVision). Results were normalized to the amount of total protein compared with the control cells.

LDHA activity assay. The total LDHA activity in cell lysates was examined according to the manufacturer's instructions of the LDH cytotoxicity assay kit (BioVision). Briefly, 2x10⁵ cells were seeded in a 24-well plate one day before assaying and all samples were analyzed in triplicate. Then, cells were collected, washed and extracted for protein to measure LDHA activity. Results were normalized to the amount of total protein compared with the control cells.

Colony formation assay. For chondrosarcoma cell colony formation assay, 500 cells were seeded on a 10-cm dish
with drugs in regular cell culture medium. The medium was refreshed every two days with drugs. Cells were grown for three weeks and the surviving colonies were stained with gentian violet after methanol fixation, and visible colonies (>50 cells) were counted. Colonies from randomly-selected image areas of three replicate wells were enumerated.

**Animal experiments.** Athymic BALB/c nude mice (5-8 weeks old) were housed in the Biological Resource Centre of the Department of Orthopaedics, The 101st Hospital of the People's Liberation Army. Mice were implanted subcutaneously in both sides of the flank with 3x10^6 chondrosarcoma cells without or with doxorubicin resistance, respectively. When the tumor reached a size of >150 mm^3, the mice were randomly divided into groups (8 mice per group) with the indicated treatments. Mice were weighed weekly and tumor diameters were measured with calipers twice per week for >4 weeks. Tumor progress was monitored by tumor size measurements every other day. All the experiments involving mouse models complied with both Chinese laws and the guidelines of the Ethics Committee of Jiangsu Institutes for Biological Sciences.

**Statistical analysis.** The unpaired Student's t-test was used for the data analysis. All data are shown as mean ± standard error (SE). P<0.05 was considered to indicate a statistically significant difference.

**Results**

**LDHA is highly activated in chondrosarcoma cells.** Cancer cells, unlike their normal counterparts, use aerobic glycolysis with reduced mitochondrial oxidative phosphorylation for glucose metabolism (11). Since LDHA is an important key enzyme of glycolysis, we first measured the LDHA expression in two malignant chondrosarcoma cell lines compared with benign chondrocyte. When the tumor reached a size of >150 mm^3, the mice were randomly divided into groups (8 mice per group) with the indicated treatments. Mice were weighed weekly and tumor diameters were measured with calipers twice per week for >4 weeks. Tumor progress was monitored by tumor size measurements every other day. All the experiments involving mouse models complied with both Chinese laws and the guidelines of the Ethics Committee of Jiangsu Institutes for Biological Sciences.
through Oncomine.com revealed the expression of LDHA was upregulated in previously published chondrosarcoma microarray database (Fig. 1D). We further compared the activities of LDHA in chondrosarcoma cells and normal chondrocytes. As we expected, the activities of LDHA were higher in malignant chondrosarcoma cells than in normal chondrocytes (Fig. 1E). Taken together, our results suggested the increased activity of LDHA might be an important biomarker for the clinical treatment of chondrosarcoma patients.

Doxorubicin treatment at low-toxic dosage induces LDHA expression and activity. It has been reported that doxorubicin acts as a chemotherapeutic agent against chondrosarcoma cells (17). However, resistance to doxorubicin represents a major obstacle to successful treatment. To explore the biological significance of elevated LDHA in chondrosarcoma cells, we treated chondrosarcoma cells with doxorubicin at a low concentration which does not induce apoptosis sharply for 72 h followed by the measurement of LDHA expression. Fig. 2A shows the protein expressions of LDHA were significantly upregulated by doxorubicin treatments at low-toxic concentrations in two chondrosarcoma cell lines. The mRNA levels as well as the kinase activities of LDHA in response to multiple doxorubicin treatments were also upregulated (Fig. 2B and C) suggesting LDHA and glucose metabolism are involved in chemotherapy in chondrosarcoma cells.

Doxorubicin-resistant chondrosarcoma cells are more dependent on glucose. To further explore the biological significance of elevated LDHA in chondrosarcoma cells, we generated doxorubicin-resistant cell line using SW1353 parental cells by gradually increasing concentrations of doxorubicin in cell culture medium for a selection of resistant cells. After successive treatments for a duration of three months, several doxorubicin-resistant cell clones were developed and pooled for the following experiments. To verify the resistance, parental cells and resistant pool cells were treated with doxorubicin at multiple concentrations for 72 h. As expected, cell viability assays showed that SW1353 doxorubicin-resistant cells could tolerate much higher concentrations of doxorubicin compared with sensitive cells which exhibited significant inhibition of viability at 100 and 200 µM (Fig. 3A). As our above results showed glucose metabolism was highly correlated with doxorubicin treatments (Fig. 3), we investigated the susceptibility of doxorubicin-resistant cells under glucose starvation. SW1353 doxorubicin-resistant cells exhibited more sensitivity to glucose depletion compared with SW1353 parental cells (Fig. 3B). Under low glucose conditions, cell viability of
doxorubicin-resistant cells was decreased more than parental cells (~25%), suggesting the glucose metabolism in resistant cells was higher than in sensitive cells and might be targets for clinical therapeutic agents.

**Increased expression and activity of LDHA in doxorubicin-resistant cells.** To examine the role of LDHA in mediating doxorubicin resistance in human chondrosarcoma cells, the expression of LDHA was examined in SW1353 parental and doxorubicin-resistant cells (DoxR). We found that LDHA levels were markedly increased in SW1353 DoxR cells, compared to their parental cells (Fig. 4A, left panel). The mRNA and activity of LDHA were also increased ~2-fold in resistant cells, compared to parental cells (Fig. 4A, middle and right panel). These results indicated that doxorubicin resistance was correlated with the increased LDHA expression and activity. Notably, SW1353 DoxR cells showed upregulated glucose metabolism. The glucose uptake and lactate product were significantly increased in resistant cells (Fig. 4B) indicating the metabolic changes contributed to doxorubicin resistance in chondrosarcoma cells.

**Inhibition of LDHA re-sensitizes doxorubicin-resistant cells.** The increased LDHA expression and LDHA activity detected in doxorubicin-resistant cells suggested that LDHA might play a critical role in doxorubicin resistance. We hypothesized that the downregulation of LDH-A by siRNA might re-sensitize resistant cells to doxorubicin. Therefore, to further verify the effects of LDHA downregulation in the doxorubicin-induced chemotherapy, we knocked down LDHA by specific siRNA in SW1353 parental cells and DoxR cells (Fig. 5A). Consistently, knockdown of LDHA significantly enhanced the sensitivity
of chondrosarcoma cells to doxorubicin treatments in both parental and resistant cells (Fig. 5B). Knockdown of LDHA decreased the cell viability in parental cells by 30% at 30 µM and the suppression of cell viability in doxorubicin-resistant cells was 40% at 200 µM. Since LDHA is a critical enzyme in the glycolytic pathway, our results demonstrated LDHA plays...
The combination of glycolysis inhibitor with doxorubicin shows synergistic inhibitory effects on chondrosarcoma cells in vitro and in vivo. Oxamate is a pyruvate analog that directly inhibits the converting process of pyruvate to lactate by LDHA to inhibit cell glycolysis (16). Since downregulation of LDHA by siRNA significantly inhibited the viability of the doxorubicin-resistant chondrosarcoma cells, we further investigated the effects of combining doxorubicin with glycolysis inhibitor oxamate on the treatment of doxorubicin-resistant chondrosarcoma cells. In both SW1353 parental cells and doxorubicin-resistant cells, doxorubicin combined with oxamate was considerably more effective in inhibiting cell viability compared with either agent administered alone (Fig. 6A). To further strengthen our results from the cell viability assay, we performed colony formation assay for the detection of clonogenicity of chondrosarcoma cells under treatments of doxorubicin alone, oxamate alone and the combination of doxorubicin and oxamate. Consistent results from Fig. 6B show significantly increased colony formation inhibition in fourteen days under the treatments of the combination of oxamate and doxorubicin compared with control and treatment with doxorubicin or oxamate alone.

To verify our in vitro results that inhibition of LDHA by siRNA and inhibitor resulted in the re-sensitization of doxorubicin-resistant cells to doxorubicin, we studied the effects of the treatments by the combination of doxorubicin with oxamate on the xenograft tumor growth in nude mice. We injected SW1353 parental cells or DoxR cells into nude mice and treated mice with doxorubicin alone, oxamate alone and doxorubicin with oxamate. After five weeks, mice with the combination treatment had significantly attenuated tumor sizes compared with the treatment alone (Fig. 7), indicating the combination of doxorubicin and oxamate result in strong tumor growth inhibition. Collectively, these results obtained from both in vitro and in vivo experiments established that the combination of doxorubicin with LDHA inhibitor has a greater capacity to inhibit doxorubicin-resistant chondrosarcoma cells compared to either agent alone and might lead to a therapeutic aspect for human chondrosarcoma patients.

Discussion

Approximately 20% of skeletal system cancers are chondrosarcomas derived from transformed cells that produce cartilage (1). However, conventional chemotherapy has very limited efficacy in patients with advanced chondrosarcoma and it is not considered active treatment in clinical trial in addition to surgery which is the primary treatment for this chondrosarcoma (2,3). To date, the highest benefit from chemotherapy observed is in mesenchymal and dedifferentiated chondrosarcoma. Therefore, developments of effective and low-toxic therapeutic approaches are required to improve chondrosarcoma clinical management. Evasion of programmed cell death or apoptosis has been recognized as one of the main alterations that dictate malignant growth and is a hallmark of most types of cancer. In the present study, we first reported glucose metabolism is highly active in chondrosarcoma cells compared with normal chondrocytes which may lead us to induce cancer cell apoptosis by inhibiting the key enzymes in the glucose metabolism pathway.

Doxorubicin is frequently used in chemotherapy for a variety of solid tumors, but the efficacy of doxorubicin treatment is limited by drug resistance. Multiple mechanisms responsible for the drug-resistant phenotype in cancer cells have been recognized. The most common is characterized by the enhanced expression of the P-glycoprotein, ABCB1 which is a transmembrane pump responsible for drug efflux from cells (18). Recently, another study described that loss of HuR, which is an RNA binding protein involved in the post-transcriptional regulation of a wide spectrum of mRNAs, is responsible for the doxorubicin resistance in breast cancer (19). Restoration of HuR expression in breast cancer cells re-sensitized resistant cells to doxorubicin (19). Therefore, the purpose of the present study was to find new, targeted treatment strategies to overcome chemoresistance for clinical chondrosarcoma patients.

Cancer cells depend mostly on glycolysis, the anaerobic breakdown of glucose into the energy-storing molecule ATP, even in the presence of available oxygen while normal cells rely primarily on the process of mitochondrial oxidative phosphorylation (11). Therefore, we sought to use these unique bioenergetic properties to improve the therapeutic efficacy to inhibit cancer cells. The combination of Taxol and oxamate was previously reported to enhance the therapeutic effects on
human breast cancer cells (16). In this study, we investigated the role of LDHA in the acquired doxorubicin resistance in human chondrosarcoma cells. We identified that compared to parental cells, doxorubicin-resistant cells possess an increased expression and activity of LDHA. Downregulation of LDHA resulted in an increased sensitivity of doxorubicin-resistant cells. In addition, compared to parental cells, doxorubicin-resistant cells showed a higher sensitivity to the LDHA inhibitor oxamate. Notably, we found inhibition of LDHA significantly inhibited chondrosarcoma cell viability when combined with doxorubicin in vitro and in vivo, demonstrating the important roles of LDHA in overcoming chemoresistance in chondrosarcoma cells. In our next study, we will focus on the mechanisms by which highly active glucose metabolism makes chondrosarcoma cells evade apoptosis in response to doxorubicin treatment. Proteomic approaches have been applied to identify the putative LDHA interaction proteins under the treatment of doxorubicin, and, more importantly, we will explore more novel glycolysis inhibitors which contribute to the therapeutic effects on the treatment of chondrosarcoma patients. In general, the results of the present study demonstrated that LDHA plays an important role in doxorubicin resistance and that it may potentially serve as a therapeutic target for overcoming chemoresistance in chondrosarcoma patients.

Acknowledgements

The authors thank the staff and faculty working in the Department of Orthopaedics, The 101st Hospital of the People’s Liberation Army. We thank Dr Xiangyong Li from the Department of Hematology and Oncology, The 101st Hospital of the People’s Liberation Army for the editorial assistance.

References