CXCR4 Blockade Enhances The Sensitivity Of Osteosarcoma To Doxorubicin By Inducing Autophagic Cell Death Via PI3K-Akt-Mtor Pathway Inhibition

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Abstract

Background: Doxorubicin is one of the most frequent used chemotherapy drugs in the treatment of osteosarcoma (OS), but the emergence of chemoresistance often leads to its treatment failure. CXCR4 has been proved to regulate osteosarcoma progression and metastasis, however whether CXCR4 is also implicated in OS chemoresistance and its molecular mechanisms have not yet been fully elucidated. In this study, we investigated CXCR4-mediated autophagy for OS chemotherapy.

Methods: Cell viability, western blot and apoptosis were performed to evaluate doxorubicin resistance after CXCR4 regulation (downregulation or upregulation) and treatment with autophagy modulators (bafilomycin A1 or rapamycin) in two OS cell lines (LM8 and Dunn). Autophagy-related proteins (Beclin1 and LC3B) detected by western blot, autophagosomes and autolysosomes observed by transmission electron microscopy, and autophagic flux monitored by confocal microscopy were utilized to comprehensively assess autophagy level changes. A C3H mouse osteosarcoma orthotopic model was established to determine anti-tumor effect of CXCR4 antagonist AMD3100 and doxorubicin in vivo.

Results: CXCR4 inhibition enhanced doxorubicin-induced apoptosis by reducing p-glycoprotein, while CXCR4 overexpression promoted OS doxorubicin resistance. Moreover, CXCR4 inhibition combined with or without doxorubicin increased Beclin1 and LC3B expression, numbers of autophagosome and autolysosome, and induced autophagic flux activation by suppressing PI3K-Akt-mTOR pathway. In addition, pretreatment with autophagy inhibitor bafilomycin A1 attenuated CXCR4 abrogation-induced cell death. Finally, CXCR4 antagonist AMD3100 synergistically reinforced anti-tumor effect of doxorubicin in orthotopic OS mouse model.

Conclusions: This study revealed CXCR4 inhibition sensitizes OS to doxorubicin by inducing autophagic cell death. Targeting CXCR4/autophagy axis may be a promising therapeutic strategy to overcome OS chemotherapy resistance.

Background

Osteosarcoma (OS) is the most frequent primary malignant bone tumor which predominantly affects children and adolescents, and accounts for approximately 15% all bone malignancies(1, 2). The current standard clinical treatment for OS includes preoperative chemotherapy followed by surgical removal of primary tumor, combined with postoperative chemotherapy. Since chemotherapy was introduced in 1970s, the 5-year survival rate of OS dramatically improved from less than 20–70% (3).Doxorubicin, cisplatin and methotrexate are the most commonly used first-line chemotherapy drugs in the treatment of OS(4, 5). Despite great advance in chemotherapy for OS, survival rate has reached a plateau and remained unsatisfactory in the past three decades largely because of chemotherapy resistance(6, 7). More than 40% OS patients are not sensitive to chemotherapy drugs with the 5-year survival rate is only 16–20%(8). The emergence of chemoresistance often leads to treatment failure and poor prognosis. It

has become a major obstacle to improve OS therapeutic effect. Therefore, it is urgently required to elucidate the underlying molecular mechanisms implicated in OS chemoresistance.

Chemokine receptor 4(CXCR4) is a G protein-coupled receptor to which chemokine 12(CXCL12) bind with high affinity(9). Accumulating evidence has indicated that CXCR4 plays a crucial role in OS progression and metastasis (10–12). In addition, CXCR4 has been shown to be associated with poor survival of OS patients and considered as an important clinical prognosis indicator(13). Recently, much more attentions have been paid to CXCR4-mediated chemotherapy resistance in various kinds of tumor(14–17). However, the correlation between CXCR4 and OS chemoresistance remains unknown.

Autophagy is a catabolic process via which cells eliminate and recycle its own damaged proteins and organelles to provide energy. It has been proved to play the dual role in the regulation of OS resistance. On the one hand, moderate autophagy can lead to drug resistance due to its cytoprotective effect. On the other hand, excessive autophagy reverses drug resistance by inducing cell death(18). In our previous work, we found that CXCR4 promoted OS growth and metastasis by activating AKT pathway(10). Due to PI3K-AKT-mTOR is one of key regulators in autophagy, we hypothesize that CXCR4 is involved in OS doxorubicin resistance by regulating autophagy. In the present study, we investigate whether CXCR4 blockade could enhance the sensitivity of OS to doxorubicin by inducing autophagic cell death and CXCR4 may be potential therapeutic target to reverse OS doxorubicin resistance.

Methods

Cell lines and culture

The murine LM8 and Dunn OS cell lines were kindly donated by Dr Eugenie Kleinerman (M.D. Anderson Cancer Center, Houston, TX, USA). The cell lines were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM-h; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS), 100U/ml penicillin and 100µg/ml streptomycin. The cultures were incubated at 37°C in a humidified atmosphere containing 5% CO₂.

Reagents and antibodies

Doxorubicin, Rapamycin and Bafilomycin A1 were purchased from Selleckchem (Houston, TX, USA). Antibodies against CXCR4, Beclin1 and LC3B were purchased from Abcam (Cambridge, MA, USA). Antibodies against MDR1, caspase-3, PI3K, p-PI3K, Akt, p-Akt, mTOR, p-mTOR, GAPDH were purchased from Cell Signaling Technology (Danvers, MA, USA).

Cell viability assay

Cells (2×10⁴/ml) were seeded in 96-well plates overnight and then treated with various concentrations of doxorubicin(0,0.2,0.4,0.8,1,10µg/ml). After the 48h incubation, 10µl Cell Counting Kit-8 (CCK-8) solution (Dojindo, Tokyo, Japan) was added to each well for 1h at 37°C. The optical density (OD) was then

measured using a model EL×800 microplate reader (Bio-Tech Instruments Inc.) at 450nm. The cell viability was calculated using the equation: Cell viability(%)=(OD_{450nm} of treatment/OD_{450nm} of control) ×100%. IC₅₀ was calculated by GraphPad Prism8.0.

Plasmid and siRNA transfection

Transfection was used by Lipofectamine 2000 Transfection Reagent according to manufacturer's protocol. LM8 and Dunn cells were transfected with mouse CXCR4 siRNA and plasmids encoding CXCR4 respectively. Both LM8 and Dunn cells were transfected with mRFP-GFP-LC3 adenovirus (Asia-Vector Biotechnology, Shanghai, China). Transfection efficiency was assessed by western blotting.

Flow cytometry

Cells (5×10⁴/ml) were cultured in six-well plates for 24h and treated with 0.2μg/ml doxorubicin or siCXCR4/CXCR4 overexpression or 0.2μg/ml doxorubicin combined with siCXCR4/CXCR4 overexpression. After the 48h incubation, cell apoptosis was assayed using the Annexin V-FITC apoptosis detection kit (BD Biosciences, San Jose, CA, USA).

Western blotting

Protein was extracted from cells using RIPA lysis buffer containing phosphatase inhibitors and quantified with BCA protein assay kit (Beyotime Institute of Biotechnology, Shanghai, China). Equivalent amounts of protein (40μg) were separated by 10-12% SDS-PAGE at 80V for 1.5h and transferred to polyvinylidene difluoride (PVDF) membranes. After blocking with TBST containing 5% skim milk for 1h at room temperature, the membranes were incubated overnight at 4°C with 1:1000 dilution of primary antibodies (CXCR4, Beclin1, LC3B, MDR1, cleaved-caspase3, caspase3, phosphor-PI3K, PI3K, phosphor-Akt, Akt, phosphor-mTOR, mTOR). The membranes were rinsed with TBST three times and subsequently incubated with 1:1000 dilution of secondary antibodies for 1h at room temperature, then visualized using an ECL kit. Quantitative analysis of protein was used by Image J software.

Confocal microscopy analysis

Cells were cultured in six-well plates and transfected with mouse CXCR4 siRNA or CXCR4-encoding plasmids using Lipofectamine 2000. Then cells were simultaneously treated with 0.2μg/ml doxorubicin and mRFP-GFP-LC3 adenovirus for 48h. The cells were fixed with 4% formaldehyde for 30min and incubated with DAPI for 5min. Images were obtained using a confocal laser scanning microscope (Olympus, Japan).

Transmission electron microscopy

Cells were fixed with 2.5% glutaraldehyde at 4°C overnight and then fixed in 1% buffered osmium tetroxide for 1.5h. The cells were dehydrated, embedded and stained with uranyl acetate. Representative

areas were chosen for ultrathin sectioning detected by transmission electron microscopy (HITACHI, Japan).

Mouse tibia orthotopic tumor model

Thirty-two 4-week-old female C3H mice (Shanghai Slack Laboratory Animal Co. LTD, Shanghai, China) were purchased and housed under standard conditions with a 12-h light-dark cycle and fed with sufficient water and food. All the animal procedures were performed in accordance with a protocol approved by the Animal Care and Use Committee of Shanghai tenth people's hospital. LM8 cells (5×10^5) in 10 μ l of PBS was injected into the tibia medullary cavity to establish an orthotopic OS model. Two weeks after injection of tumor cells, the mice were randomly allocated to four groups: control (n=8), 5mg/kg AMD3100 (n=8), 1mg/kg doxorubicin (n=8) and 5mg/kg AMD3100 plus 1mg/kg doxorubicin (n=8). Each mouse in the treatment groups received 100 μ l AMD3100 or doxorubicin by tail vein injection every two days. The control mice were injected with 100 μ l PBS in the same manner. Meanwhile, the tumor volume and body weight of each mouse was measured at every injection using the formula: tumor volume = $(\text{length} \times \text{width}^2)/2$. After 8 continuous injections, the mice were euthanized. Tumors were dissected, weighed and stored in liquid nitrogen or fixed in formalin for immunohistochemistry analysis.

Immunohistochemical staining

Tumor samples were fixed overnight, embedded in paraffin and sliced to 4- μ m thick sections, and then deparaffinized in xylene and rehydrated with graded alcohol and incubated in 3% H_2O_2 to block endogenous peroxidase activity. The slides were boiled for 30min in 10mM sodium citrate for antigen retrieval, blocked in 5% BSA for 30min, incubated with antibodies against CXCR4, Beclin1, LC3B and p-gp at 4°C overnight. The slides were washed three times with PBS and incubated with secondary antibodies for 30 min at room temperature. Immunoreactivity was visualized using DAB Kit. Areal density of each image was quantified by Image-Pro Plus 6.0 software for statistical analysis.

Statistical analysis

All data are presented as means $\pm$ standard deviations from at least three independent experiments. Statistical analysis between two groups was performed using the unpaired, two-tailed Student's t-test with GraphPad Prism8.0. $P < 0.05$ was considered statistically significant.

Results

CXCR4 inhibition sensitizes OS cells to doxorubicin by regulating apoptosis and p-glycoprotein

To determine whether CXCR4 affects OS doxorubicin resistance, we first down-regulated CXCR4 expression in CXCR4-positive LM8 cells and up-regulated CXCR4 expression in CXCR4-negative Dunn cells by siRNA and lentiviral transfection respectively. The differential expression of CXCR4 in these two

cells has been described previously(10). As shown in Fig. 2A, CXCR4 was significantly decreased by siCXCR4 in LM8 cells, and increased by lentiviral transfection in Dunn cells. Then LM8 and Dunn cells were treated with various concentrations of doxorubicin for 48 h, and cell viability was measured by CCK-8 assay. The results showed that half maximal inhibitory concentration (IC_{50}) values of CXCR4-knockdown LM8 cells (0.3879 $\mu\text{g/ml}$) was obviously reduced compared with LM8 cells (0.5891 $\mu\text{g/ml}$). Conversely, IC_{50} values of CXCR4-overexpression Dunn cells (0.6661 $\mu\text{g/ml}$) was much higher than Dunn cells (0.4581 $\mu\text{g/ml}$) (Fig. 1A).

We then detected the expression of apoptosis-related protein caspase-3 and multidrug resistance-related protein p-glycoprotein (p-gp). As shown in western blotting, both CXCR4 knockdown and doxorubicin increased cleaved caspase-3 and reduced p-gp in LM8 cells. Furthermore, highest expression of cleaved caspase-3 and lowest expression of p-gp were found in doxorubicin combined with CXCR4 knockdown group in LM8 cells. Inversely, CXCR4 upregulation reduced doxorubicin-induced cleaved caspase-3 activation and increased p-gp in Dunn cells (Fig. 1B).

To further explore CXCR4-mediated OS doxorubicin resistance, we investigate the effect of CXCR4 regulation on apoptosis induced by doxorubicin in LM8 and Dunn cells by flow cytometry analysis. LM8 and Dunn cells were cultured for 48 h in the presence of 0.2 $\mu\text{g/ml}$ doxorubicin, CXCR4 regulation (silencing or overexpression), 0.2 $\mu\text{g/ml}$ doxorubicin combined with CXCR4 regulation (silencing or overexpression). The percentage of apoptotic LM8 cells in doxorubicin group was $8.12 \pm 0.12\%$, and $10.2 \pm 0.35\%$ in doxorubicin combined with siCXCR4 group which indicated that CXCR4 knockdown increased doxorubicin-induced apoptosis in LM8 cells. On the contrary, the percentage of apoptotic Dunn cells in doxorubicin group was $32.52 \pm 1.14\%$, and $28.19 \pm 0.20\%$ in doxorubicin combined with CXCR4-overexpression group which indicated that CXCR4 upregulation partially reversed doxorubicin-induced apoptosis in Dunn cells (Fig. 1C). These findings suggested that CXCR4 inhibition enhanced the sensitivity of LM8 to doxorubicin by inducing apoptosis and reducing p-gp. On the contrary, CXCR4 upregulation reduced the sensitivity of Dunn to doxorubicin by inhibiting apoptosis and inducing p-gp.

CXCR4 inhibition induces autophagy, whereas CXCR4 upregulation inhibits autophagy in OS cells

To investigate the role of autophagy in CXCR4-mediated OS doxorubicin resistance, western blotting was performed to detect autophagy-related proteins Beclin1 and LC3B after CXCR4 regulation in LM8 and Dunn cells. In LM8 cells, it was showed that both CXCR4 inhibition and doxorubicin increased Beclin1 and LC3B-II expression, and highest expression of Beclin1 and LC3B-II in doxorubicin combined with CXCR4 inhibition group. Inversely, CXCR4 upregulation reduced Beclin1 and LC3B-II expression in Dunn cells compared with control group, and partially reversed Beclin1 and LC3B-II expression induced by doxorubicin (Fig. 2A).

Transmission electron microscopy (TEM), the golden standard for autophagy detection, was used to observe the ultrastructure of autophagosome and autolysosome in OS cells. In LM8 cells, compared with control group, numbers of autophagosomes and autolysosomes were obviously increased in both CXCR4

inhibition group and doxorubicin-treated group and were further increased in doxorubicin combined with CXCR4 inhibition group. In Dunn cells, numbers of autophagosomes and autolysosomes were dramatically decreased in doxorubicin combined with CXCR4 upregulation group compared with doxorubicin group (Fig. 2B).

Given that autophagy is dynamic process, mRFP-GFP-LC3 was utilized to observe autophagic flux via confocal microscopy. To be specific, autophagosomes are labeled as yellow puncta, and autolysosomes are labeled as red puncta. In LM8 cells, percentage of red fluorescence in CXCR4 inhibition group ($7.40 \pm 1.11\%$) was higher than control group ($4.07 \pm 0.72\%$). And percentage of red fluorescence in doxorubicin combined with CXCR4 inhibition group ($27.77 \pm 5.75\%$) was further augmented compared with doxorubicin group ($15.40 \pm 1.92\%$). In Dunn cells, percentage of red fluorescence was reduced in CXCR4 upregulation group ($1.17 \pm 0.51\%$) compared with control group ($5.10 \pm 1.60\%$). And percentage of red fluorescence was dramatically decreased in doxorubicin combined with CXCR4 upregulation group ($1.67 \pm 0.64\%$) compared with doxorubicin group ($9.73 \pm 4.81\%$) (Fig. 2C). These results indicated that CXCR4 inhibition induced autophagic flux activation in LM8 cells and oppositely CXCR4 upregulation suppressed autophagic flux activation in Dunn cells.

Cxcr4-mediated Autophagic Cell Death Reverses Os Doxorubicin Resistance

Due to the dual role of autophagy in the regulation of OS chemoresistance. On the one hand, cytoprotective autophagy leads to drug resistance. On the other hand, autophagic cell death reverses drug resistance(18). To further determine CXCR4 inhibition augments doxorubicin sensitivity by either inhibiting cytoprotective autophagy or inducing autophagic cell death, we used autophagy inhibitor bafilomycin A1 and autophagy activator rapamycin to observe the effect of autophagy on chemoresistance before CXCR4 regulation. It was showed by western blotting that pretreated with bafilomycin A1 reduced Beclin1, LC3B-II and cleaved caspase-3 and increased p-gp in LM8 cells. Conversely, pretreated with rapamycin increased Beclin1, LC3B-II and cleaved caspase-3 and reduced p-gp in Dunn cells (Fig. 3A). Additionally, CCK-8 assay and flow cytometry indicated that pretreatment with bafilomycin A1 promoted cell proliferation in vitro and reversed apoptosis induced by CXCR4 inhibition with or without doxorubicin in LM8 cells. On the contrary, rapamycin inhibited cell proliferation in vitro and enhanced doxorubicin-induced apoptosis in Dunn cells (Fig. 3B and 3C). These results demonstrated that CXCR4 inhibition enhance OS doxorubicin sensitivity by inducing autophagic cell death.

OS doxorubicin resistance regulated by CXCR4/autophagy axis is dependent on PI3K-Akt-mTOR pathway

To further determine whether PI3K-Akt-mTOR pathway, one of the most important regulators of autophagy, by which CXCR4/autophagy axis modulate OS doxorubicin resistance, we performed western blotting to detect the phosphorylation levels of PI3K, Akt and mTOR in LM8 and Dunn cells after doxorubicin treatment and CXCR4 regulation. In LM8 cells, CXCR4 inhibition reduced the phosphorylation

of PI3K, Akt and mTOR. In Dunn cells, CXCR4 upregulation induced the phosphorylation of PI3K, Akt and mTOR (Fig. 4). These findings indicated that CXCR4 inhibition-induced autophagic cell death to reverse OS doxorubicin resistance by suppressing PI3K-Akt-mTOR pathway.

AMD3100 enhances anti-tumor effect of doxorubicin in orthotopic OS mouse model

To investigate whether CXCR4 inhibition could reinforce the cytotoxicity of doxorubicin in vivo, we established a C3H mouse orthotopic model through intratibial injection of LM8 cells. Mice were randomized to four groups and then treated by tail vein injection with PBS, 5 mg/kg AMD3100, 1 mg/kg doxorubicin, AMD3100 plus doxorubicin. After 8 continuous injections, compared with PBS group, both AMD3100 and doxorubicin resulted in significantly tumor growth inhibition, and interestingly AMD3100 showed a markedly synergistic anti-tumor effect with doxorubicin (Fig. 5A). In the dynamic observation of mice body weight, different variation tendency was found in the four groups: the body weight in PBS group rose linearly, the weight gain was delayed in both AMD3100 and doxorubicin group, and no obvious weight change in AMD3100 combined with doxorubicin group (Fig. 5B). Our results showed that compared with PBS group, after 8 continuous administrations of AMD3100, doxorubicin, AMD3100 plus doxorubicin resulted in notably decreased tumor volume to 50.3%, 66.3% and 89.9% respectively and induced 45.9%, 67.9% and 82.4% weight loss respectively. (Fig. 5C). Immunohistochemical staining demonstrated that prominently increased Beclin1 and LC3B expression and decreased p-gp expression in AMD3100 plus doxorubicin-treated tumor tissues (Fig. 5D). These encouraging findings indicated that AMD3100 exerted a significant synergistic effect with doxorubicin in tumor growth inhibition.

Discussion

Tumor recurrence, distant metastasis and chemoresistance are three important factors contributing to treatment failure and poor prognosis in OS(7, 19). It has already been proved that CXCR4 plays a crucial role in OS survival and metastasis, and targeting CXCR4 is an effective strategy for OS(10–12). However, whether CXCR4 is involved in regulation of OS chemoresistance and its concrete mechanism have not yet been elucidated. In the present study, we first reported that CXCR4 inhibition could augment the sensitivity of LM8 cells to doxorubicin by inducing autophagic cell death, while CXCR4 upregulation oppositely increased chemoresistance of Dunn cells to doxorubicin by inhibiting autophagic cell death. And further revealed that negative correlation between CXCR4 and autophagy was dependent on PI3K-Akt-mTOR pathway. These findings indicate that CXCR4 abrogation could overcome the chemoresistance of OS cells via autophagic cell death.

To observe the effect of CXCR4 on OS doxorubicin resistance, we first calculated half inhibitory concentration of doxorubicin in OS LM8 and Dunn cells after CXCR4 regulation. As shown in our previous work, CXCR4 is highly expressed in LM8 cells and lowly expressed in Dunn cells(10). Therefore, CXCR4 inhibition and upregulation were performed in LM8 and Dunn cells respectively. In LM8 cells, IC₅₀ values of doxorubicin decreased from 0.5891 µg/ml to 0.3879 µg/ml when CXCR4 expression was inhibited. In Dunn cells, IC₅₀ values of doxorubicin increased from 0.4581 µg/ml to 0.6661 µg/ml when CXCR4

expression was upregulated. Apoptosis and caspase family protein activation induced by chemotherapy drugs is another method to evaluate its chemosensitivity. Based on the observation that CXCR1 knockdown increased cisplatin-induced apoptosis and caspase-3 activation in Saos2 and Saos2-lung cells, Han et al (20) concluded that CXCR1 knockdown enhanced the sensitivity of OS to cisplatin. Consistent with their findings, we found that CXCR4 inhibition facilitated doxorubicin-induced apoptosis and caspase-3 activation in LM8 cells and CXCR4 upregulation partially reversed doxorubicin-induced apoptosis and caspase-3 activation in Dunn cells. P-gp, also known as multidrug resistance protein 1 (MDR1), which is encoded by ATP-binding cassette subfamily B member 1 (ABCB1), contributes to chemoresistance in various kinds of cancer(21). Wang et al (22) demonstrated that Raddeanin A restored doxorubicin chemosensitivity in OS drug-resistant U2OSR and KHOSR cells by downregulating MDR1. To further identify whether MDR1/p-gp is involved in CXCR4-mediated doxorubicin resistance, western blotting was utilized to detect the changes of p-gp expression after CXCR4 regulation. Positive correlation between CXCR4 and p-gp was found in two OS cells. To be specific, both CXCR4 inhibition alone and CXCR4 inhibition combined with doxorubicin reduced p-gp expression compared with its counterparts in LM8 cells. On the contrary, both CXCR4 upregulation alone and CXCR4 upregulation combined with doxorubicin increased p-gp expression compared with its counterparts in Dunn cells. It can be concluded that combination treatment of CXCR4 inhibition and doxorubicin exerts synergistic cytotoxic effect on LM8 cells, while CXCR4 upregulation partially abrogates doxorubicin-induced cell death in Dunn cells.

Autophagy is a catabolic process through which cells eliminate and recycle its own damaged proteins and organelles to provide energy. It can be activated under stressful conditions such as hypoxia, starvation, cytotoxicity induced by chemotherapeutic drugs to maintain cell survival(23). For a long time, autophagy has been only regarded as a cytoprotective process contributing to OS chemoresistance and amounts of studies has been focusing on the role of autophagy inhibition in OS chemosensitization. Huang et al (24) discovered that chemotherapeutic drugs doxorubicin, cisplatin and methotrexate induced high mobility group box1 (HMGB1) expression in MG-63, SaOS-2 and U-2OS cells. And downregulation of HMGB1 sensitized OS cells to chemotherapeutic drugs by suppressing autophagy-related protein Beclin1. Kim et al (25) demonstrated that glial cell line-derived neurotrophic factor receptor $\alpha 1$ (GFRA1) promoted OS cisplatin resistance by inducing autophagy. However, with the in-depth research, it has been confirmed that dual role of autophagy in OS chemoresistance: cytoprotective autophagy contributing to chemoresistance or autophagic cell death reversing chemoresistance(18). Recently, much more attentions have been paid to the diametrically opposite autophagy: autophagic cell death, defined as cell death mediated by autophagy, rather than by apoptosis or necrosis(26). Intricate crosstalk between autophagy and apoptosis is widely discussed in cell death mechanism. Generally, interaction between autophagy and apoptosis is mostly in negative regulating manner that autophagy blocks apoptosis induction and apoptosis-related caspase activation suppresses autophagic process. Interestingly, induction of autophagic cell death inversely facilitates apoptosis activation(26). Majority of studies reveal that autophagy inhibition could induce apoptosis. Wang et al(27) reported that an anti-tumor drug combretastatin A-4 (CA-4) could induce cytoprotective autophagy and combined with autophagy inhibitor chloroquine exerted synergistic cytotoxic effect on OS cells as chloroquine further enhanced CA-4-

induced apoptosis by elevating apoptosis-related protein PARP and caspase-3. It was found by another study that a microtubule-disrupting agent CYT997 induced both apoptosis and autophagy in OS. Furthermore, pretreatment with autophagy inhibitor 3-methyladenine(3-MA) augmented anti-tumor effect of CYT997 by increasing cell apoptosis and apoptosis-related protein PARP(28). Different from above researches, honokiol extracted from *Magnolia trees* was found to exhibit anti-tumor effect on HOS and U-2OS cells by inducing both apoptosis and autophagy. And honokiol-induced cell death was more obviously reversed by autophagy inhibitor 3-MA compared with apoptosis inhibitor Z-VAD-FMK which indicated that honokiol-induced cell death was largely dependent on autophagic cell death(29). Besides, autophagic cell death induced by tanshinone IIA and diallyl disulfide exerted inhibitory effect on 143B and MG-63 cells respectively(30, 31). Consistent with these findings, we observed that CXCR4 inhibition with or without doxorubicin induced autophagy as shown by higher expression of Beclin1 and LC3B, larger numbers of autophagosome and autolysosome, and autophagic flux activation. On the contrary, CXCR4 upregulation blocked autophagy as shown by lower expression of Beclin1 and LC3B, fewer number of autophagosome and autolysosome, and autophagic flux inactivation. To determine whether CXCR4-mediated autophagy is pro-survival or pro-death, we used autophagy inhibitor bafilomycin A1 and autophagy activator rapamycin to detect the effect of CXCR4-mediated autophagy on cell death induced by doxorubicin. It was revealed by our results that bafilomycin A1 reversed apoptosis induced by CXCR4 inhibition with or without doxorubicin in LM8 cells, and rapamycin enhanced doxorubicin-induced apoptosis in Dunn cells indicating that enhanced doxorubicin cytotoxicity by CXCR4 inhibition was, at least in part, dependent on autophagic cell death.

The mechanisms by which G protein coupled receptors regulate autophagy include second messengers such as cAMP and Ca^{2+} and downstream signal molecules such as ERK1/2 and mTORC1 modulation(32). It is intrigued that the role of CXCR4 in regulation of autophagy is paradoxical. On the one hand, positive CXCR4-autophagy loop is involved in drug resistance and metastasis. CXCR4 induced by reactive oxygen species stimulated autophagy formation, which further contributed to drug resistance in mantle cell lymphoma(14). This result was consistent with another study that CXCR4 activation decreased the sensitivity of acute myeloid leukemia cells to cytarabine by inducing autophagy(16). In addition, autophagy inhibitor polymeric chloroquine reduced CXCR4-mediated metastasis in U2OS cells by promoting the surface CXCR4 internalization which blocked its binding with extracellular CXCL12(33). On the other hand, negative regulation of CXCR4 in autophagy has been confirmed as well. Coly et al (34) found that CXCR4 activation led to a decrease in the number of autophagosomes in HEK-293 and U87 glioblastoma cells. They indicated that CXCR4 exerted its anti-autophagy effect by activating calpains which prevented pre-autophagosomal vesicles formation. Similar to their results, we also observed anti-autophagy effect of CXCR4 on two OS cells. CXCR4 inhibition accelerated autophagy activation in LM8 cells by inhibiting PI3K-Akt-mTOR pathway which was a well-known negative regulator of autophagy. Inversely, CXCR4 upregulation suppressed autophagy in Dunn cells by activating PI3K-Akt-mTOR pathway.

As tumor recurrence, distant metastasis and drug resistance are three main reasons contributing to OS treatment failure(7). In our previous study, AMD3100, a most widely used CXCR4 specific antagonist, has been proved to inhibit OS growth and metastasis in vivo(10).However, whether CXCR4 is also involved in OS chemoresistance remains unknown. In the present work, it was surprisingly found that AMD3100 synergistically increased doxorubicin-induced tumor suppression in OS orthotopic mouse model.

Conclusion

Our studies show that CXCR4 blockade enhances the sensitivity of OS to doxorubicin by inducing autophagic cell death via PI3K-Akt-mTOR pathway inhibition (Fig. 6). Taken together, our findings elucidate that a novel molecular mechanism of CXCR4 in OS doxorubicin resistance regulation, and targeting CXCR4/autophagy axis may be a promising therapeutic strategy to overcome OS chemotherapy resistance.

Abbreviations

OS, osteosarcoma; CXCR4, chemokine receptor 4; CXCL12, chemokine 12; IC₅₀, half maximal inhibitory concentration; p-gp, p-glycoprotein; TEM, transmission electron microscopy; MDR1, multidrug resistance protein 1; ABCB1, ATP-binding cassette subfamily B member 1; HMGB1, high mobility group box1; GFRA1, glial cell line-derived neurotrophic factor receptor α1; CA-4, combretastatin A-4; 3-MA, 3-methyladenine;

Declarations

Ethics approval and consent to participate

All the animal procedures were performed in accordance with a protocol approved by the Animal Care and Use Committee of Shanghai tenth people's hospital.

Consent for publication

Not applicable

Availability of data and materials

All data that support the findings of this study are available from corresponding authors upon reasonable request.

Competing interests

The authors declare that they have no financial conflicts of interest.

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Authors' contributions

YXL designed the study and wrote the manuscript. HYY, JYL and ZFZ performed the in vitro experiments. TYX and DY performed the animal experiments. QMG and LF collected and analyzed the data. KYL and GDL revised the manuscript critically. All of the authors read and approved the final manuscript.

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Figures

Figure 1

CXCR4 is implicated in the regulation of OS doxorubicin resistance. A) CXCR4 was down-regulated in CXCR4-positive LM8 cells by siRNA and up-regulated in CXCR4-negative Dunn cells by lentiviral transfection. LM8 and Dunn cells were treated with various concentrations of doxorubicin (0.2,0.4,0.8,1,10 μ g/ml) for 48h, and cell viability was measured by CCK-8 assay. Dose-response curves were draw by GraphPad Prism 8.0 software, and half inhibitory concentrations of doxorubicin were obtained for each group. B) LM8 and Dunn cells were cultured with 0.2 μ g/ml doxorubicin (with or without CXCR4 knockdown/overexpression) for 48h, expression levels of apoptosis-related protein cleaved-caspase3, caspase3 and drug resistant-related protein p-gp were determined using western blot and the protein bands were quantified and subjected to statistical analysis. C) LM8 and Dunn cells were cultured with 0.2 μ g/ml doxorubicin (with or without CXCR4 knockdown/overexpression) for 48h, apoptosis ratios for each group (percent of Annexin V+ cells) were determined by flow cytometry. (*:p<0.05;***:p<0.001;****:p<0.0001)

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CXCR4 inhibition induces autophagy in LM8 cells and CXCR4 upregulation suppresses autophagy in Dunn cells. A) Expression levels of CXCR4, the autophagy-related proteins Beclin1 and LC3B were determined using western blot and the protein bands were quantified and subjected to statistical analysis. B) Autophagosomes and autolysosomes were detected by transmission electron microscopy in LM8 and Dunn cells after treatments. Red arrows indicated the autophagosomes, blue arrows indicated the autolysosomes. The numbers of autophagosomes and autolysosomes were calculated and subjected to statistical analysis. C) LM8 and Dunn cells were transfected with mRFP-GFP-LC3 adenovirus before treatments. Colocalizations of RFP and GFP puncta was examined by confocal microscopy. Yellow puncta represented autophagosomes, red represented autolysosomes. Calculation of percentage of red fluorescence was analyzed using ImageJ software. (*:p<0.05;**p=0.001;***p<0.001;****p<0.0001)

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CXCR4 inhibition reverses OS doxorubicin resistance by inducing autophagic cell death A) LM8 (CXCR4 knockdown) and Dunn (CXCR4 overexpression) cells were pretreated with bafilomycin A1 (200nM) and rapamycin (200nM) for 6h respectively, then treated with or without 0.2μg/ml doxorubicin for 48h. Expression levels of Beclin1, LC3B, cleaved-caspase3, caspase3 and p-gp were determined using western blot and the protein bands were quantified and subjected to statistical analysis. B) Cell proliferations for

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Beclin1

LC3B-I

LC3B-II

C-Caspase3

Caspase3

p-gp

GAPDH

52 kDa

16 kDa

14 kDa

17 kDa

35 kDa

140 kDa

36 kDa

Relative expression of protein

Beclin1

LC3B-II

C-Caspase3/Caspase3

p-gp

CXCR4-OE

CXCR4-OE+RAP

CXCR4-OE+Dox

CXCR4-OE+Dox+RAP

LM8

Cell proliferation in vitro(OD value)

0h

24h

48h

72h

siCXCR4

siCXCR4+BAF

siCXCR4+Dox

siCXCR4+Dox+BAF

Cell proliferation in vitro(OD value)

0h

24h

48h

72h

CXCR4-OE

CXCR4-OE+RAP

CXCR4-OE+Dox

CXCR4-OE+Dox+RAP

LM8

siCXCR4

siCXCR4+BAF

siCXCR4+Dox

siCXCR4+Dox+BAF

PI1815-Annexin V-A

Annexin V-B525-FITC-A

Q2-UL(19.10%)

Q2-UR(6.39%)

Q2-LI(72.09%)

Q2-LR(2.42%)

CXCR4-OE

CXCR4-OE+RAP

CXCR4-OE+Dox

CXCR4-OE+Dox+RAP

PI1815-Annexin V-A

Annexin V-B525-FITC-A

Q2-UL(1.73%)

Q2-UR(3.74%)

Q2-LI(91.59%)

Q2-LR(2.73%)

% of Annexin V(+) cells

**

siCXCR4

siCXCR4+BAF

siCXCR4+Dox

siCXCR4+Dox+BAF

% of Annexin V(+) cells

*

*

CXCR4-OE

CXCR4-OE+RAP

CXCR4-OE+Dox

CXCR4-OE+Dox+RAP

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Figure 5

AMD3100 enhances anti-tumor effect of doxorubicin in orthotopic OS mouse model A) Macroscopic appearance of OS tumors in the tibia of C3H mice after 8 continuous injections of PBS, 5mg/kg AMD3100, 1mg/kg doxorubicin, AMD3100 plus doxorubicin. B) Body weight of mouse in each group was measured at every injection, and growth curve was obtained. C) Tumor volume and weight of tumors in the four treatment groups were measured. D) Expression of CXCR4, Beclin1, LC3B and p-gp in tumor tissues were detected by immunohistochemical staining and areal density was quantified and subjected to statistical analysis. (*:p<0.05;**p=0.001;****p<0.0001)

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Figure 6

Schematic diagram of mechanism and function of CXCR4 in OS doxorubicin resistance CXCR4 blockade by siRNA or AMD3100 enhances the sensitivity of OS to doxorubicin by inducing autophagic cell death via PI3K-Akt-mTOR pathway inhibition.

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