Mesenchymal stem cells desensitize castration-resistant prostate cancer to docetaxel chemotherapy via inducing TGF-β1-mediated cell autophagy

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Research

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Abstract

**Background:** Mesenchymal stem cells (MSCs) have been proved to accelerate prostate cancer (PCa) castration resistance progression. The purpose of this study is to investigate the contribution of MSCs to the development of docetaxel resistance in castration-resistant prostate cancer (CRPC) cells and its potential mechanisms.

**Methods:** The effect of MSCs on CRPC cells resistance to docetaxel was determined using in-vivo and in-vitro approaches. CCK8 and PI/Annexin V-FITC assay were used to examined the cell viability and apoptosis. The concentration of transforming growth factor-β1 was measured by enzyme-linked immunosorbent assay and small interfering RNA was used for functional analyses.

**Results:** MSCs significantly reduced the sensitivity of CRPC cells to docetaxel-induced proliferation inhibition and apoptosis promotion in vivo and in vitro. CRPC cells cocultured with MSCs under docetaxel administration have an increased autophagy activation, while autophagy inhibitor could effectively reversed MSCs-induced resistance to docetaxel. Additionally, MSCs-induced CRPC cell autophagy increase under docetaxel administration depends on MSCs secreting TGF-β1 and inhibition of TGF-β1 secretion in MSCs could consequently increase the sensitivity of CRPC cells to docetaxel.

**Conclusions:** These results suggest that docetaxel administrated CRPC cells may elicit MSCs secreting TGF-β1 increase, which desensitizes CRPC to docetaxel chemotherapy accelerating chemoresistance occurrence via inducing cell autophagy.

Introduction

Prostate cancer (PCa) is the most common cancer and the second leading cause of cancer-related mortality among men in the United States[1]. In China, PCa shows an increasing incidence and mortality in recent years[2]. Early staged and localized PCa can be well controlled by prostatectomy or radiotherapy. For locally advanced and metastatic PCa, androgen deprivation therapy (ADT) is currently considered as the most effective treatment, giving a 70% initial effective rate[3]. However, almost all of them with initially castration-sensitive prostate cancer (CSPC) would eventually develop into castration-resistant prostate cancer (CRPC) after a period of ADT. Currently, docetaxel-mediated antimitotic chemotherapy is typically used as the first-line standard treatment in metastatic CRPC patients[4]. However, it only gives a moderate survival advantage as patients eventually acquire resistance resulting in therapeutic failure. Clarifying the mechanism of docetaxel chemoresistance in CRPC can provide a basis for new treatment approaches.

Resistance to docetaxel attributes to numerous different mechanisms and many of them are related to abnormal molecular regulation, which involved in cell survival and death[5]. Recently, increasing evidence suggests that tumor microenvironment (TME) may play a key role in occurrence of docetaxel resistance[6]. TME is comprised of tumor epithelial cells and diverse non-malignant stromal cell types. Mesenchymal stem cells (MSCs), as a heterogeneous subset of stromal stem cells, are an important
component of TME. MSCs originate from the mesodermal germ layer and main exist in bone marrow[7]. MSCs can migrate into PCa tumor sites and perform critical roles ranging from supporting tumor cell proliferation to inducing tumor cell metastasis, accelerating tumor development[8-10]. Besides that, numerous data from clinical studies have shown a strong association between MSCs density and poor prognosis in various types of human cancer[11]. MSCs have been also reported to be used as a predictor of reduced cancer-free survival interval in PCa[12]. Our previous studies have demonstrated that MSCs could promote androgen-dependent PCa cells form a tumor xenograft in castrated mice, suggesting that MSCs could promote PCa cells growth from androgen-dependent into androgen-independent manner and contribute to PCa castration resistance progression[13]. However, the role of MSCs in the development of chemoresistance to docetaxel in CRPC cells during tumor growth remains unclear.

In the present study, we used human CRPC cell lines PC3 and DU145 to investigate the effect of MSCs on the occurrence of chemoresistance to docetaxel and explore the potential mechanisms. The results indicate that MSCs could desensitize CRPC cells to docetaxel chemotherapy and contribute to chemoresistance via inducing TGF-β1-mediated cell autophagy.

Materials And Methods

Cell lines and culture

Human prostatic carcinoma cell lines, including PC3, DU145 cells, and bone marrow-derived MSCs were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Shanghai Institute of Cell Biology, Chinese Academy of Sciences. PC3, DU145 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS). MSCs were cultured in MSCs basal medium (all from Invitrogen, Carlsbad, CA, USA). MSCs were transfected with the adenoviral vector GFP-mock (Invitrogen). After transfection about 48 hours, MSCs-GFP were collected for further experiments. All cells were cultured at 37°C in a 5% CO₂ humidified atmosphere.

In vivo xenograft experiment

Nude mice, 6–8 weeks old, were obtained from the Shanghai Experimental Animal Center of the Chinese Academy of Sciences, Shanghai, China, and housed in pathogen-free conditions. All aspects of the animal care and experimental procedures were in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Chinese Academy of Sciences’ Committee on Animals. PCa cells were prepared as single-cell type suspensions (1×10⁶ cells in 200μL PBS) and subcutaneously administrated in the armpit area of nude mice. When tumors grew to approximate 200 mm³ size, nude mice were randomly divided into three groups: with solvent or docetaxel treatment (15 mg/kg/week via intraperitoneal injection, 5 mice per group). With that, mice were injected with the green fluorescent protein (GFP)-labeled MSCs (MSCs-GFP) through tail vein every 3 days. Mice were examined every day and tumor growth was evaluated by measuring the length and width of tumor mass. All tumor-bearing
mice survived until they were sacrificed at the end of the experiment, then tumors were removed and dissected quickly for frozen section preparation, while others were stored at -80°C.

**Cell apoptosis assay**

Cells (2×10^5 cells/well) were cultured in 6-well plates to 70–80% confluence. The cells were then treated with docetaxel for 48 hours. PI/Annexin V-FITC assay was used to measure apoptotic cells by flow cytometry according to the manufacturer’s instruction (Keygen Biotech. Co., Ltd, Nanjing, China, Cat.KGA108). The cells were collected by trypsinization and were washed with ice cold phosphate buffered saline (PBS). Cells were then incubated in 300 µL of 1× binding buffer containing 5 µL Annexin V and 5 µL PI for 30 min at room temperature in the dark. Apoptosis of cells was measured on a BD FACScan flow cytometer (BD Biosciences). At least 30,000 gated events were acquired from each sample. Results are expressed as the percentage of apoptotic cells (PI and Annexin V positive) in the gated cell population.

**Proliferation assays**

Cells were plated at a density of 2×10^5 cells per well in 6-well plates cocultured with MSCs and treated with docetaxel (20 µM) for 48 h. CCK8 test was performed to evaluate the extent of cell proliferation (OD values) according to the manufacturer's instructions.

**Real-time quantitative PCR (RT-PCR)**

To quantify mRNA expression of PCNA, caspase-3 and TGF-β1, total RNA was isolated using Trizol reagent (Invitrogen) and cDNA synthesis was performed using the Prime Script RT reagent Kit (Takara, Kyoto, Japan) according to the manufacturer’s specifications. Quantitative PCR was performed using SYBR Green PCR Kit (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer’s instructions. β-actin was used as an internal control for RNA integrity and loading normalization.

**Western blot analysis**

Cells were lysed in RIPA lysis buffer (Beyotime) with 1 mM PMSF. Equal amount of protein was separated by SDS-PAGE and transferred to NC membrane. The membranes were washed, blocked and incubated with specific primary anti-human antibodies against p62/SQSTM1 (all from Cell Signaling Technology, Inc., Danvers, MA, USA), LC3 (Novus Biologicals, Littleton, CO), TGF-β1 and β-actin (Abcam, Cambridge,
MA, USA), followed by incubation with horseradish peroxidase-conjugated secondary antibodies (Hangzhou HuaAn Biotech). Signals were visualized by chemiluminescent detection (Beyotime).

**Enzyme linked immunosorbent assay (ELISA)**

ELISA assays were performed using commercial ELISA kits (R&D Systems, Minneapolis, MN) according to manufacturer instructions. Assays were performed in duplicates, and readings were compared with standard curves obtained with standard protein provided with the kit. Means and standard deviations of concentrations in triplicate samples were compared by t-test.

**RNA interference**

Cells (1×10^6) growing to 50%-60% confluence in 10 cm petri dishes were transfected with TGF-β1 siRNA sequences (sense: 5'-CACUGCAAGUGGACAUCAATT-3'; antisense: 5'-UUGAUGUCACAUUGCAGUGTT-3') or their corresponding mock sequences (sense: 5'-UUCUCGGAACGU-3'; antisense: 5'-ACGUGACACGUUCGGAGAATT-3') using a Lipofectamine 2000 kit (Invitrogen, Cat.11668-019) with the procedure provided by the manufacturer. Cells were observed under a fluorescence microscope and harvested 48 h after transfection.

**Transient transfection**

Fugene HD transfection reagent (Calbiochem, La Jolla, CA) was used to transfect cells with GFP-LC3 expressing plasmids according to the manufacturer’s instructions. After initial treatment, autophagy was detected by counting the number of GFP-LC3-positive dots per cell under fluorescence microscope (Olympus IX71).

**Electron microscopic analysis**

Cells were fixed in 2.5% glutaraldehyde in PBS (pH = 7.4) for 2 hours at room temperature, then postfixed in 1% osmium tetroxide in water for 1 hour, dehydrated in an ascending series of ethanol, and at last embedded in araldite (Basel, Switzerland). After solidified, 50 nm sections were cut on a LKB-I ultramicrotome and picked up on copper grids, post-stained with uranyl acetate and lead citrate, and observed in a Philips CM-120 TEM.
**Statistical analysis**

All of the experiments were repeated at least three times. Final data were expressed as mean ± standard deviation (SD). Statistical analysis of the data was done by using GraphPad Prism 5. Student's t-test was used to compare between mean values of two groups. Value of at least \( p<0.05 \) was considered statistically significant.

**Results**

**MSCs accelerate CRPC cells resistance to docetaxel**

Firstly, we infected MSCs with an adenovirus vector to obtain GFP-labeled MSCs (Figure 1A). Then studies were performed in PC3 xenograft mouse model. As shown in Figure 1B and C, docetaxel could effectively inhibit prostate tumor growth. However, when MSCs-GFP were injected through nude mouse tail vein, the docetaxel-induced inhibition of PC3 cell growth was attenuated and the tumor would grow faster than before. The volume and weight of tumor were consequently both increase (Figure 1B and C). To investigate whether MSCs could migrate into PCa sites, we also performed frozen sections to detected GFP signals in tumors. High numbers of GFP signals in frozen sections were detected in tumors removed from mice injected with MSCs-GFP (Figure 1D). The results showed that MSCs desensitize CRPC cells to docetaxel accelerating chemoresistance in vivo.

**MSCs alleviate docetaxel-induced apoptosis in CRPC cells**

To evaluate the tumor cells proliferation and apoptosis induced by docetaxel, the mRNA expression of PCNA (a cell proliferation indicator) and Caspase-3 (a cell apoptosis indicator) were measured by real-time PCR. As shown in figure 2A and B, docetaxel treatment group induced a lower expression level of PCNA and a higher expression level of Caspase-3 than those of PC3 group. However, when MSCs-GFP were injected, the docetaxel-induced PCNA low expression and Caspase-3 high expression were significantly attenuated. We also analyzed tumor tissues sections with Ki67 and TUNEL, markers for proliferative and apoptotic response respectively. Docetaxel treatment group with MSCs-GFP injection showed a marked increase in number of Ki67-positive cells and an obvious decrease in number of TUNEL-positive cells compared to those group without MSCs-GFP injection (Figure 2C).

In addition, we also performed *in vitro* experiment for cell proliferation and apoptosis using two human CRPC cell lines (PC3 and DU145) to verify the development of MSCs-induced docetaxel chemoresistance in these cells. As shown in Figure 3A and C, CRPC cell proliferation was significantly inhibited by docetaxel, while MSCs could effectively improve CRPC cell proliferation in the presence of docetaxel. Correspondingly, MSCs caused a significant reduction of docetaxel-induced apoptosis in CRPC cells (Figure 3B and D). Together, these results indicate that MSCs could alleviate docetaxel-induced CRPC cell proliferation inhibition and apoptosis increase.
**MSCs promote CRPC cells docetaxel chemoresistance via inducing cell autophagy**

Several studies have reported that autophagy contributes to tumor cell resistance to chemotherapeutic agents[14]. We then explored if autophagy was involved in MSCs-induced docetaxel chemoresistance in CRPC cells. Firstly, analysis of autophagy in PC3 and DU145 cells was performed using an expression vector encoding GFP-LC3 which is concentrated in autophagic vacuoles, resulting in punctate fluorescence within cells undergoing autophagy. As shown in Figure 4A and B, MSCs effectively induced autophagy in PC3 and DU145 cells, which exhibited a significantly high number of punctate GFP. As we know, the conjugated form of LC3 called LC3-II is targeted to the autophagosomal membrane and its accumulation is a symbol of autophagy activation. Therefore, LC3-II used as a marker to monitor autophagic flux was analyzed by western blotting. As shown in Figure 4C, the amount of LC3-II in PC3 cells with MSCs coculture was significantly increased compared to cells administrated with docetaxel only. We also examined the expression of P62, as another specific autophagy indicator, and obtained the consistent consequence. Electron microscopic analysis was also employed to observe the autophagosome formation, suggesting the presence of characteristic double-membrane organelles in cells with MSCs coculture when docetaxel administration performed.

Furthermore, addition of autophagy inhibitors CQ or 3-MA to the cell culture was performed. The results showed that autophagy inhibitor could effectively restore the sensitivity of CRPC cell cocultured with MSCs to docetaxel, resulting in inhibition of proliferation and increase in apoptosis of CRPC cells following treatment with docetaxel (Figure 5). Our results suggest that MSCs promote CRPC cells docetaxel chemoresistance via inducing cell autophagy.

**MSCs induce CRPC cells autophagy and docetaxel chemoresistance depending on TGF-β1 secretion**

Previous studies have reported that TGF-β1 plays an important role in induction of autophagy[15]. So we tested TGF-β1 level in the conditioned medium (CM) obtained from CRPC cells treated with MSCs-CM or cocultured with MSCs in response to docetaxel administration. As is shown in Figure 6A, TGF-β1 was dramatically increased in CM obtained from docetaxel administrated CRPC cells cocultured with MSCs. Additionally, TGF-β1 secretion was increased in time-dependent manner in MSCs when cocultured with docetaxel administrated PC3 cells (Figure 6B). We then investigated the effect of docetaxel on secretion of TGF-β1 in MSCs and PC3 cells, and found that docetaxel administrated PC3 cells caused a significant up-regulation of TGF-β1 expression at both mRNA and protein levels in MSCs (Figure 6C and D). We also validated significantly increased TGF-β1 expression by immunohistochemistry in MSCs-GFP injection group (Figure 6E). These results indicate that MSCs cocultured with CRPC cells secret TGF-β1 increase when docetaxel administration performed.
In order to define the role of TGF-β1 in MSC-mediated autophagy induction and docetaxel chemoresistance in CRPC cells, TGF-β1 was silenced in MSCs by siRNA. We found that the increased autophagy activation of docetaxel administrated PC3 cells upon MSCs coculture was obviously suppressed when MSCs suffered TGF-β1 knockdown (Figure 7A). Effects of si-TGF-β1 on blocking MSCs-induced PC3 cells proliferation increase and apoptosis inhibition were also demonstrated (Figure 7B-D). In addition, results in xenotransplant tumor model showed that TGF-β1 knockdown significantly inhibited the effect of MSCs on increasing the volume of prostate tumor in docetaxel administration (Figure 7E and F). Combined with above, TGF-β1 is essential for MSCs-induced CRPC cells autophagy and docetaxel chemoresistance.

**Discussion**

Docetaxel chemoresistance remains an important obstacle to the cure of metastatic CRPC today. Stroma cells in tumor microenvironment were recognized as important contributors in facilitating the development of chemotherapy resistance[16]. MSCs, also called multipotent mesenchymal stromal cells, are a heterogeneous subset of stromal stem cells contributing to tissue homeostasis and regeneration. Previous studies have reported that MSCs can promote cisplatin chemotherapy resistance by secreting protective cytokines to help cancer cells overcome the anticancer effect of chemotherapeutic agents[17]. In current study, we evaluated the role of MSCs in the development of CRPC cells chemoresistance to docetaxel. We found that MSCs desensitize CRPC cells to docetaxel and accelerate chemoresistance to docetaxel both in vivo and in vitro experiments (Figure 1 and Figure 3). Further study showed that MSCs could alleviate docetaxel-induced CRPC cell proliferation inhibition and apoptosis increase (Figure 2).

As we know, autophagy is a critical cell survival mechanism for both prokaryotic and eukaryotic cells in response to stress. Previous studies have reported that autophagy is a lysosomal degradation process that diminishes long-lived cellular proteins and removes damaged or excess organelles selectively[18-20]. Energy, amino acids, and other precursor molecules for maintaining cellular homeostasis and facilitating cell survival are produced through this process. Autophagy associates with various physiological and pathological processes, including differentiation, tumorigenesis, chemoresistance and adaptation to changed environmental conditions[21, 22]. Recently, autophagy has been reported to perform critical roles for cancer progression, such as promoting tumor cell proliferation, angiogenesis, and metastasis. Autophagy has been reported to decrease the sensitivity of hepatoma cells to chemotherapeutic agents by affecting their apoptotic potential[23]. Evidence has also suggested that autophagy plays critical roles in docetaxel chemoresistance[5]. In present study, we performed studies in PC3 and DU145 cells confirmed that MSCs could significantly induce autophagy in CRPC cells (Figure 4). In addition, we have shown that inhibition of autophagy could restore CRPC cell sensitivity to docetaxel (Figure 5), suggesting MSCs-induced autophagy serves as a protective mechanism for CRPC cells to resist the cell toxicity of docetaxel.

It is reported that MSCs communicate with cancer cells mostly by secreting soluble factors. Besides that, MSCs could secrete multiple cytokines when exposure to various local microenvironment. TGF-β1, as a
key cytokine secreted by MSCs, is related to various tumor cell invasion and migration by regulating some cytokines expression. TGF-β1 also plays an important role in tumor cell autophagy activation[15]. In present study, we found that TGF-β1 was dramatically increased in CM obtained from docetaxel administrated CRPC cells cocultured with MSCs. Further study found that TGF-β1 secretion was increased in time-dependent manner in MSCs when cocultured with docetaxel administrated PC3 cells (Figure 6). Inhibition of TGF-β1 expression of MSCs diminished the ability of MSCs in inducing autophagy and chemoresistance in CRPC, indicating that TGF-β1 is essential for MSCs induced CRPC cells autophagy and docetaxel chemoresistance. Meanwhile, we found that MSCs secreting TGF-β1 would increase when cocultured with docetaxel administrated PC3 cells (Figure 6B and C). However, detail mechanisms about the difference of TGF-β1 secretion need a further investigation. We also found that MSCs and their conditioned medium were significantly different in improving CRPC cell proliferation and reducing cell apoptosis after docetaxel treatment. As we know, MSCs as an important mediator in TME play important roles in tumor progression via secreting various cytokines as well as direct contact with adjacent cancer cells. In our previous study, we also found that MSCs in mixed co-culture system showed a more enhancement of PC3 cell stemness than that in transwell-culture system, which implies that MSCs can accelerate PCa growth through their secretory effects, as well as in a cell-cell contact manner. Therefore, the difference between MSCs and their conditioned medium in improving CRPC cell proliferation and reducing cell apoptosis after docetaxel treatment would be reasonable.

In conclusion, major finding of present study is that MSCs could desensitize CRPC to docetaxel chemotherapy and accelerate chemoresistance occurrence via secreting TGF-β1 and inducing cell autophagy. The results suggest that docetaxel treatment in clinical PCa therapy may elicit the expression of TGF-β1 in MSCs, which will result in docetaxel chemoresistance occurrence. We expect that our findings will offer insights into further explore on the mechanism of prostate cancer development and provide a theoretical basis for finding new therapies for prostate cancer.

**Abbreviations**

PCa: prostate cancer; MSCs: mesenchymal stem cells; ADT: androgen deprivation therapy; CRPC: castration-resistant prostate cancer; TME: tumor microenvironment; FBS: fetal bovine serum; GFP: green fluorescent protein; RT-PCR: Real-time quantitative PCR; siRNA: Small interfering RNA; ELISA: Enzyme linked immunosorbent assay; GFP, green fluorescent protein; LC3, microtubule-associated protein 1 light chain 3; DTX, docetaxel.

**Declarations**

**Ethics approval and consent to participate**

All mouse experiments and procedures were approved by the Chinese Academy of Sciences’ Committee on Animals and all experiments were performed in accordance with the approved guidelines and regulations.
Consent for publication

All authors reviewed and approved the final manuscript. All authors supported publication of this manuscript.

Availability of data and materials

Please contact the corresponding author for data requests.

Competing interests

The authors declare no conflict of interest.

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Authors' contributions

Yang Yu, Lin Ye and Xu-dong Yao conceived the study and drafted the paper, Yang Yu, Wen-tao Zhang and Fu-han Yang performed the experiments, Yang Yu and Ya-dong Guo gathered and analyzed the data.

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