

Original Article

Human umbilical cord mesenchymal stem cells restore imatinib and doxorubicin sensitivity in drug-resistant chronic myeloid leukemia cells

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Received September 6, 2017; Accepted January 4, 2018; Epub March 15, 2018; Published March 30, 2018

Abstract: Mesenchymal stem cells (MSCs) have therapeutic effects in different types of cancers. The physical contact of chronic myeloid leukemia (CML) cells with MSCs potentially induces apoptosis by activating Bax and downregulating Bcl-2. Herein, we elucidated whether human umbilical cord MSCs (hUC-MSCs) can modulate chemosensitivity in a CML cell line (K562) and a K562-derived imatinib-resistant cell line. Imatinib-resistant K562 cells were generated from sequential imatinib treatment of K562 cells. hUC-MSCs were isolated from the Wharton's jelly of an umbilical cord. After co-culture with hUC-MSCs, imatinib-sensitive and imatinib-resistant K562 cells were exposed to either imatinib or doxorubicin. Inhibition of tumor cell growth was based on cell viability, which was determined using annexin V/propidium iodide flow cytometry and MTS assays. Protein expression of Bax, Caspase3, CD44, FoxO3a, and MDR1 was measured using Western blotting. We found the presence of hUC-MSCs resulted in the loss of CD44 and inactivation of the FoxO3a-MDR1 (multidrug resistant gene ABCB1) signaling pathway that is dominant in imatinib-resistant K562 cell monoculture. Importantly, co-culture of hUC-MSCs with CML cells restored imatinib and doxorubicin sensitivity of the latter *in vitro*, as evidenced by a decrease in viability and an increase in apoptotic subpopulations. Our findings indicated that hUC-MSCs restore sensitivity to imatinib and doxorubicin in drug-resistant K562 cells by downregulating FoxO3a-MDR1 expression, and may thus serve as a therapeutic tool to overcome drug resistance in CML.

Keywords: Mesenchymal stem cells, imatinib, drug-resistant, chronic myeloid leukemia

Introduction

Chronic myeloid leukemia (CML) is a hematologic neoplasm associated with a chromosomal translocation that gives rise to the Philadelphia (Ph) chromosome and the BCR-ABL fusion gene [1]. The BCR/ABL gene results in deregulated, constitutively activated tyrosine kinase activity. Tyrosine kinase inhibitor (TKI) therapy has succeeded in altering the course of the disease and extending life spans to potentially near-normal for many patients with CML. TKI therapy is highly effective for treating patients in the chronic phase of CML; however, in patients with different phases of CML progression, 74% of those who relapse after a good response have a detectable cytogenetic mechanism of resistance [2]. Thus, the challenge of overcoming resistance to TKI therapy persists in the management of CML.

The promotion of cellular survival through suppression of apoptotic pathways is a fundamental characteristic of tumor cells that enables resistance to anticancer therapies [3]. As the substrates of survival kinases, the FoxO family of transcription factors, particularly FoxO3a, have emerged as key players in the cell cycle arrest and apoptosis of hematopoietic cells [4].

Umbilical cord tissue-derived mesenchymal stem cells (UC-MSCs), also called Wharton jelly stromal cells, can differentiate into osteogenic, adipogenic, and chondrogenic lineages when placed in the appropriate environments [5]. Accumulating evidence demonstrates UC-MSCs play complex roles in tumor development and progression, by impeding tumor cell migration, promoting apoptosis, and reducing drug resistance [6]. Therefore, comprehensive knowledge

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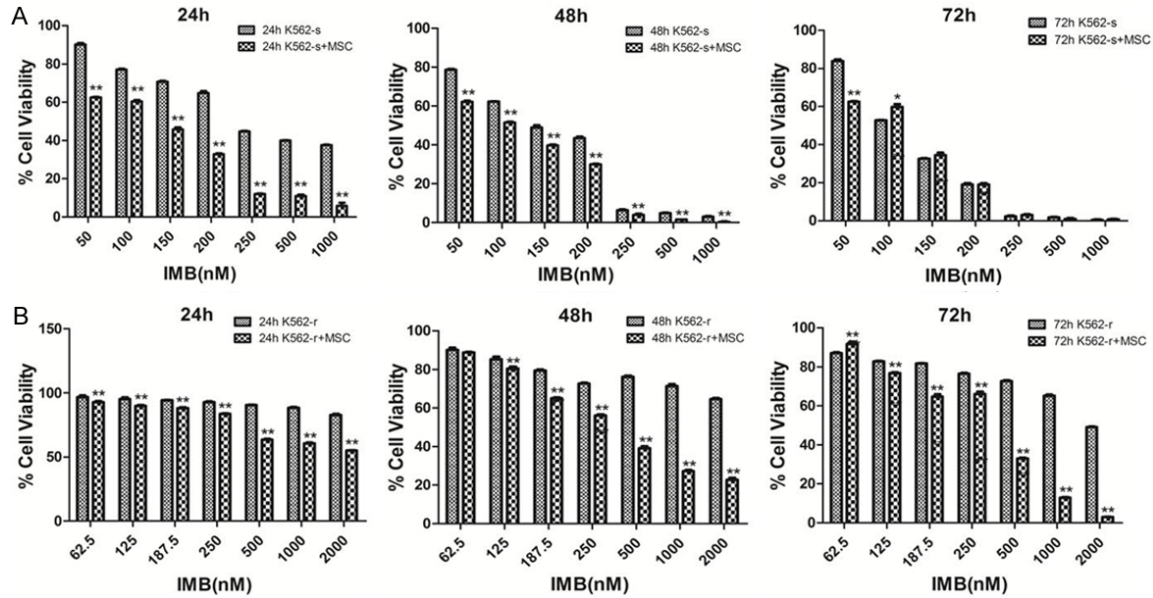


Figure 1. Imatinib sensitivity of (A) imatinib-sensitive K562 (K562-s) and (B) imatinib-resistant K562 (K562-r) cells after co-culture with umbilical cord mesenchymal stem cells (UC-MSCs). Cell viability of resistant and parental K562 cell lines following co-culture with UC-MSCs in the presence of indicated concentrations of imatinib, as assessed by MTS uptake at 24 h, 48 h, and 72 h. * $P < 0.05$ and ** $P < 0.01$, K562-s + MSC vs. K562-s cells or K562-r + MSC cells vs. K562-r cells at each time point.

of the mechanism(s) of interaction between tumor cells and UC-MSCs is critical.

In this study, we aimed to investigate whether human UC-MSCs (hUC-MSCs) can modulate chemosensitivity in a CML cell line (K562), as well as determine the underlying mechanism(s).

Materials and methods

Cell culture

The K562 cell line was purchased from the Shanghai Cell Repository Bank and maintained in RPMI 1640 medium (Gibco, Paisley, UK) supplemented with standard antibiotics, and 10% fetal bovine serum (FBS), herein referred to as K562-s (sensitive). The imatinib-resistant K562 cell line (K562-r) was generated by gradually exposing K562-s cells to increasing concentrations of imatinib at a rate of 0.1 $\mu\text{mol/L}$ increment every 10 days of culture, as described previously [7]. After approximately 3 months, sublines of cells growing in 1 $\mu\text{mol/L}$ imatinib were maintained continuously in culture at this dose of the inhibitor. Parental, sensitive cell line K562-s was maintained in parallel cultures without imatinib to be used in controls.

Human UC-MSCs were isolated from full-term delivery patients. In brief, umbilical cords were cut into 3-5 mm^3 pieces and plated in 150 mm tissue-culture dishes for growth at 37°C in a humidified CO_2 atmosphere in MEM medium with 10% FBS. After 2 weeks of incubation, adherent cells were detached with trypsin and then expanded.

For direct co-culture, 1×10^6 UC-MSC cells were plated into 100-mm Petri dishes, followed by seeding with 5×10^6 CML cells on d2. CML cells were allowed to grow for 96 h in the presence of UC-MSCs. For patterned monoculture, 5×10^6 CML cells were seeded into 100-mm Petri dishes and allowed to grow for 96 h.

Cell viability assay

Cell viability was determined using MTS tetrazolium (Promega), which measures numbers of viable cells. Between 5×10^3 and 2×10^4 cells were washed twice in PBS and plated in triplicate into wells of microtiter plates in 100 μL RPMI 1640 complete medium plus various doses of imatinib and doxorubicin. Controls using the same concentrations of drugs without cells were set up in parallel. A total of 20 μL MTS was added to the wells at daily intervals. Two hours after MTS was added, the plates

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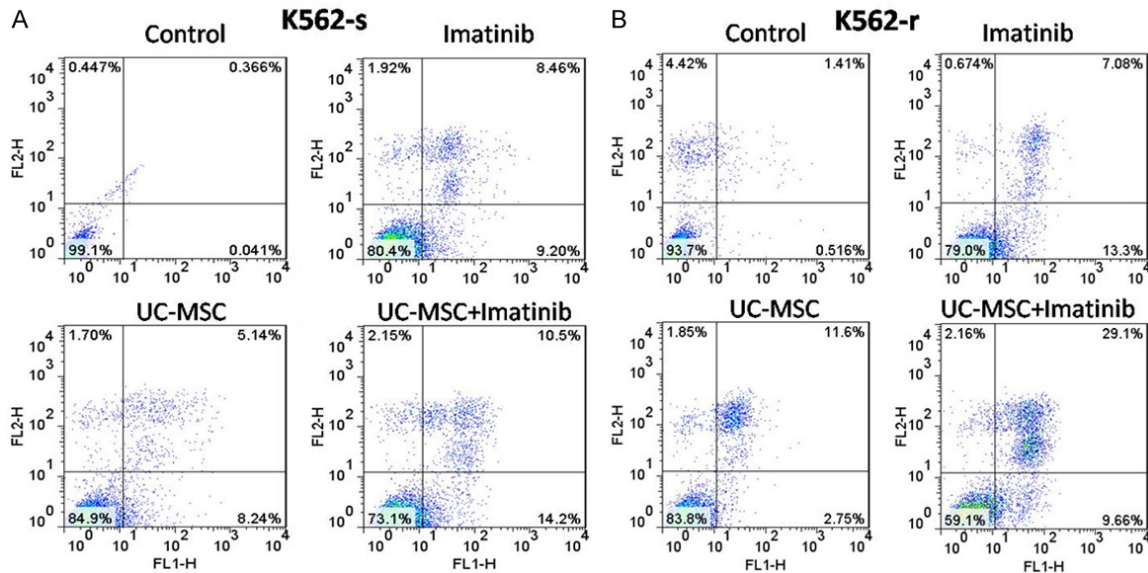


Figure 2. Flow cytometric analysis using annexin V/propidium iodide (PI) to assay the restoration of imatinib sensitivity following co-culture with UC-MSCs. A. Dot plot of imatinib-sensitive K562 (K562-s) cells following co-culture with UC-MSCs in the presence or absence of 200 nM imatinib. B. Dot plot of imatinib-resistant K562 (K562-r) cells following co-culture with UC-MSCs in the presence or absence of 1000 nM imatinib.

were read in a microplate autoreader (Bio-Rad, USA) at 490 nm. Results are expressed as the mean optical density of the three-well set for each agent dose. All experiments were repeated at least three times.

Apoptosis assay

To determine the extent of apoptosis, annexin V (R&D Systems) staining was used according to the manufacturer's instructions. Briefly, imatinib-treated cells were washed in PBS containing 2% bovine serum albumin, before being stained in fluorescein isothiocyanate-labeled annexin V and propidium iodide (PI; R&D Systems). Cells were analyzed by flow cytometry using a FACScan (BD Biosciences), and data were processed using CellQuest software (BD Biosciences).

Western blot analysis

Western blotting was performed on whole cell extracts prepared by lysing cells in mammalian cell lysis buffer (MCL1; Sigma) supplied with protease inhibitors (Roche Applied Science) on ice for 15 min. Insoluble material was removed by centrifugation, and protein concentration was determined by WST-8 protein assay (Sigma). A total of 50 μ g of protein was size-fractionated using SDS-PAGE, and electrotransferred onto PVDF membranes (Minipore). Membranes were incubated with specific antibodies

recognizing FoxO3a (Cell Signaling Technologies), MDR1 (Abcam), CD44 (Abcam), Bcl-2 (Abcam), Bax (Abcam), Caspase3 (Abcam) and GAPDH (Abcam). Primary antibodies were detected using horseradish peroxidase-linked anti-rabbit conjugates as appropriate (Santa Cruz), and visualized using the ECL detection system (GE).

Results

UC-MSCs restore imatinib sensitivity in K562CML cells

Sensitive and resistant CML cell lines (K562-s and K562-r) were co-cultured with UC-MSCs, and then treated with a range of concentrations of imatinib and assayed for their drug response in short- and long-term cell viability assays. Cell proliferation (MTS) assays showed that K562-s cells became more sensitive to imatinib with UC-MSC co-culture (**Figure 1**). In addition, in the imatinib-resistant K562-r cell line, we observed a more dramatic effect of co-culture with UC-MSCs on drug response, particularly at 1000 and 2000 nM imatinib treatment (**Figure 1**).

UC-MSCs promote imatinib-induced apoptosis

Treatment of K562-s and K562-r cells with UC-MSCs with or without the IC₅₀ of imatinib

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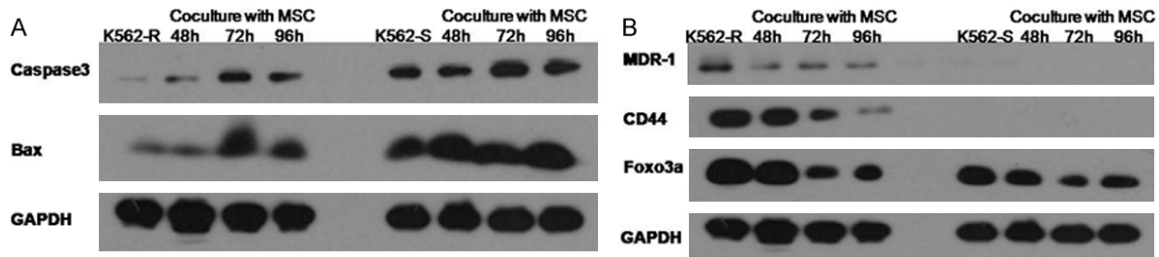


Figure 3. Western blot analysis of the differences in FOXO3-associated signaling between imatinib-sensitive and -resistant K562 cells following co-culture with UC-MSCs. A. Caspase3 and Bax expression in imatinib-sensitive and -resistant K562 cells following treatment with UC-MSCs. B. MDR-1, CD44, and FOXO3a expression in imatinib-sensitive and resistant K562 cells following treatment with UC-MSCs. GAPDH was used as negative control.

increased the apoptotic cell fraction (annexin-V⁺/PI⁻ and annexin-V⁺/PI⁺ cells) compared to untreated controls (**Figure 2**). Compared with single-agent treatment, combined UC-MSC and imatinib treatment resulted in higher induction of apoptosis both in sensitive and resistant cells. In imatinib-resistant K562-r cells, a dramatic increase of the apoptotic cell fraction to 37.55% occurred following combination treatment, indicating synergistic action compared to single imatinib administration (17.71%) or UC-MSC treatment (12.28%). Comparable combination effects could be observed in K562-s cells (24.2% vs. 17.71%), showing synergistic activity in this cell line as well (**Figure 2**).

UC-MSCs modulate imatinib sensitivity by regulating apoptosis and Foxo3a signaling

Modulation of Bax can cause permeabilization of the mitochondrial outer membrane and hence the release of soluble molecules responsible for the activation of caspase cascade. To assess the role of Bax/Caspase3 in UC-MSC-mediated chemosensitivity, we analyzed expression in imatinib-sensitive and -resistant cell lines following co-culture with UC-MSCs. K562-r cells showed decreased expression of Bax and Caspase3 compared to K562-s cells; however, Bax and Caspase3 expression was significantly elevated when the resistant cells were treated with UC-MSCs (**Figure 3A**).

FoxO3a is an essential gene for initiation of CML and has been linked to downstream effector molecules of drug resistance, such as MDR1 (multi-drug resistant gene ABCB1). We found elevated expression of FoxO3a in imatinib-resistant K562 cells compared to sensitive cells, whereas exposure to UC-MSC treatment resulted in a time-dependent decrease in

FoxO3a protein levels (**Figure 3B**). Similar tendencies were observed for the expression of MDR1 and CD44 in imatinib-resistant K562. Meanwhile, MDR1 and CD44 were not detected in the imatinib-sensitive K562 cells.

UC-MSCs restore doxorubicin sensitivity

To measure the sensitivity of imatinib-resistant cells to traditional cytotoxic drugs, we used doxorubicin (0-15 μ M) to treat imatinib-resistant and -sensitive K562 cells. After 72 h of treatment, the IC₅₀ value of doxorubicin in the resistant cells was 11.18 μ M, which was significantly higher than that in the sensitive cell line (0.2426 μ M). This indicates imatinib-resistant cells show cross-resistance to doxorubicin. After treatment with UC-MSCs, the IC₅₀ of doxorubicin decreased to 1.571 μ M and 0.1396 μ M in the imatinib-resistant and -sensitive K562 cells, respectively (**Figure 4**). The increased sensitization to both imatinib and doxorubicin suggests UC-MSC co-culture could be used as a common sensitization approach.

Discussion

Treatment with imatinib has dramatically improved the 8-year overall survival rate of CML, to 80-90% [8]. However, imatinib resistance has emerged as a major problem in the treatment of CML. Therefore, novel approaches are urgently needed to overcome imatinib resistance in CML. As promising antitumor candidates, hUC-MSCs have received attention because of their capacity to migrate towards malignant lesions [9]. However, how hUC-MSCs influence drug resistance in CML is poorly understood.

In the present study, a resistant CML cell line (K562-r) exhibited a decrease in imatinib sen-

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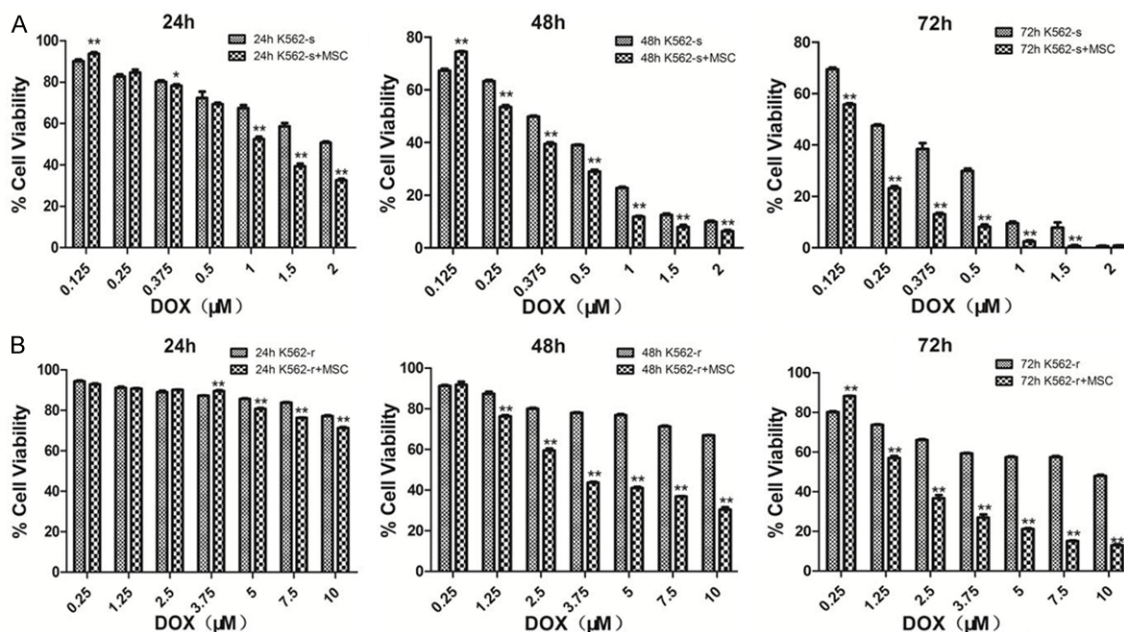


Figure 4. Doxorubicin (DOX) sensitivity of (A) imatinib-sensitive (B) and imatinib-resistant K562 cells after co-culture with UC-MSCs. Cell viability of imatinib-sensitive and-resistant K562 cell lines following co-culture with UC-MSCs in the presence of indicated concentrations of doxorubicin, as assessed by MTS uptake at 24 h, 48 h and 72 h. *P < 0.05 and **P < 0.01 between K562-s + MSC and K562-s cells and between K562-r + MSC cells and K562-r cells at each time point.

sitivity and showed cross-resistance to doxorubicin. This chemoresistance was linked to the overexpression of FoxO3a, CD44, and MDR1 in K562-r cells. These findings strongly suggested that resistance of K562 cells is independent of BCR-ABL, but instead due to activation of the FoxO3a-MDR1 pathway. Indeed, Bax and Caspase 3 expression were reduced in imatinib-resistant versus -sensitive K562 cells. The preferential expression of CD44 in resistant cells is currently not understood [10].

As an approach to modulate chemosensitivity, we tested whether hUC-MSC co-culture decreased the constitutive activation of the FoxO3a-MDR1 pathway, thereby restoring sensitivity of imatinib-resistant K562 cells. Co-culture with hUC-MSCs reduced expression of FoxO3a, MDR1, and CD44 in imatinib-resistant K562 cells, indicating that hUC-MSCs target a BCR-ABL independent pathway to restore sensitivity. In addition, increased expression of Bax and Caspase3 was observed in both imatinib-sensitive and imatinib-resistant K562 cells, implying hUC-MSCs may promote apoptosis. These effects provide insight into the contribution of FoxO3 signaling to imatinib resistance.

In summary, we have shown that hUC-MSCs are capable of inhibiting FoxO3a-MDR1 signaling, thereby improving the sensitivity of CML cells to imatinib and doxorubicin. Our findings provide preclinical evidence that targeting FoxO3a using hUC-MSCs might be a promising therapeutic strategy for treating CML. Understanding the impact of UC-MSCs on leukemia cells may pave the way for new molecular therapies that overcome the problem of drug resistance in CML.

Acknowledgements

This study was supported by Natural Science Foundation of Gansu Province for Distinguished Young Scholars (No. 1308RJYA065) and Medical Health Profession Plan Project of Gansu Province (No. GSWSKY-2015-64).

Disclosure of conflict of interest

None.

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