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Exosomes from mesenchymal stromal cells enhance imatinib-induced apoptosis in human leukemia cells via activation of caspase signaling pathway

YING LIU[1,2], BAOQUAN SONG[3], YIMENG WEI[1], FANG CHEN[1], YING CHI[1], HUIFANG FAN[1], NA LIU[4], ZONGJIN LI[4,*], ZHONGCHAO HAN[1,*] & FENGXIA MA[1,*]

[1]*State Key Laboratory of Experimental Hematology, Institute of Hematology and Hospital of Blood Diseases, Chinese Academy of Medical Sciences & Peking Union Medical College, Tianjin, People's Republic of China,* [2]*Central Laboratory, The First Affiliated Hospital of Hebei North University, Hebei, Zhangjiakou, People's Republic of China,* [3]*Department of Hematology, The First Affiliated Hospital of Soochow University, Suzhou, Jiangsu, People's Republic of China, and* [4]*School of Medicine, Nankai University, Tianjin, People's Republic of China*

Abstract

Background aims. Imatinib (IM), a tyrosine kinase inhibitor targeting the BCR-ABL oncoprotein, remains a major therapeutic strategy for patients with chronic myelogenous leukemia (CML). However, IM resistance is still a challenge in the treatment of CML. Recently, it was reported that exosomes (Exo) were involved in drug resistance. Therefore, the present study investigated whether Exo secreted by human umbilical cord mesenchymal stromal cells (hUC-MSC-Exo) affected the sensitivity of K562 cells to IM. *Methods.* hUC-MSC-Exo were isolated and identified. K562 cells were then treated or not with IM (1 μmol/L) in combination with hUC-MSC-Exo (50 μg/mL). Cell viability and apoptosis were determined by cell counting kit 8 (CCK-8) and annexin V/propidium iodide (PI) double staining, respectively. Apoptotic proteins, caspase and their cleaved forms were detected by Western blot. *Results.* It was shown that hUC-MSC-Exo alone had no effect on cell viability and apoptosis of K562 cells. However, hUC-MSC-Exo promoted IM-induced cell viability inhibition and apoptosis. Moreover, hUC-MSC-Exo enhanced the increased Bax expression and the decreased Bcl-2 expression that were induced by IM. Compared with IM alone, caspase-9 and caspase-3 were further activated by combination of hUC-MSC-Exo with IM. Finally, the effects of hUC-MSC-Exo on K562 cells could be reversed by pretreatment of K562 cells with caspase inhibitor Z-VAD-FMK (30 μmol/L) *Discussion.* These results indicate that hUC-MSC-Exo enhanced the sensitivity of K562 cells to IM via activation of caspase signaling pathway. Therefore, combining IM with hUC-MSC-Exo could be a promising approach to improve the efficacy of CML treatment.

Key Words: *apoptosis, caspase, exosome, imatinib, mesenchymal stromal cells*

Introduction

Imatinib (IM) is the front-line drug for patients at early stage of chronic myelogenous leukemia (CML) [1,2]. However, IM resistance and side effects occur in 22% patients who generally went through an unfavorable long-term survival [3,4]. Thus, it is urgent to develop a novel strategy to overcome the resistance.

Mesenchymal stromal cells (MSCs) are multipotent adult stem cells that are considered a promising tools for regenerative medicine [5]. The effects of MSCs on solid tumors are controversial. Some studies have shown that tumor progression and metastasis were promoted by MSCs [6,7]. In contrast, other studies have shown that MSCs suppressed tumor growth [8,9]. For leukemia, research in our and other laboratories found that MSCs inhibited leukemia cell proliferation and induced their differentiation [10–12]. However, MSCs were shown to protect leukemia cells from IM-induced apoptosis [13,14]. The varied effects of MSCs on leukemia cells might be attributed to releasing different kinds of cytokines and microvesicles [6,10,12,14].

Exosomes (Exo) are microvesicles with diameters of 40–100 nm that are released by most cell types [15]. An increasing number of studies have shown that Exo plays key roles in intercellular communication because it contains a variety of RNAs, proteins and lipids [16]. Recent evidence indicates that Exo derived from MSCs (MSCs-Exo) exerts antitumor effects

*These authors contributed equally to this work.
Correspondence: **Fengxia Ma**, MD, 288 Nanjing Road, Heping, Tianjin 300020, People's Republic of China. E-mail: mafengxia@ihcams.ac.cn

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[17,18]. Furthermore, MSCs-Exo play important roles in drug resistance in gastric cancer and multiple myeloma [19,20]. However, whether MSCs-Exo have any effects on biological characteristics of leukemia cells, especially the sensitivity of leukemia cells to IM, has not been investigated.

It has been reported that functions of Exo are similar to those of their parental cells. The therapeutic application of Exo is even more promising than cells since Exo have no risk of aneuploidy, lower possibility of immune rejection and lung barrier following in vivo allogeneic administration [15,16]. Therefore, in the present study, we aim to investigate whether hUC-MSC-Exo have any effects on biological characteristics of K562 cells, especially the sensitivity of K562 cells to IM. Moreover, we will try to clarify the possible mechanisms.

Methods

Ethical statement

This study was approved by the ethical committee of the Institute of Hematology & Blood Diseases Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College. Human umbilical cords (n = 12) were obtained from healthy donors with written informed consent.

Reagents and antibodies

IM and polyethylene glycol (PEG) were purchased from Sigma. Z-VAD-FMK was purchased from Selleck. Exosome-human CD63 isolation/detection kit was from Invitrogen. The CCK-8 kit, annexin V/PI apoptosis detection kit, CD9 and CD63 antibodies were purchased from BD Biosciences. The following antibodies were from Cell Signaling Technology: anti-β-Actin anti-Bax, anti-Bcl-2, anti-caspase-9, anti-cleaved-caspase-9, anti-caspase-3, anti-cleaved-caspase-3, anti–poly ADP-ribose polymerase (PARP) and anti-cleaved-PARP.

Cell culture

hUC-MSCs were isolated and identified as previously described [6,10]. They were maintained in DMEM/F-12 (Gibco) supplemented with 10% FBS or Exo-free FBS (Gibco), 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mmol/L glutamine and 10 ng/mL epidermal growth factor (PeproTech). Cells were cultured in humidified atmosphere with 5% CO_2 at 37°C. hUC-MSCs at passage 3–6 were used for the following experiments.

Human leukemia cell line K562 was purchased from pathological cell bank in Institute of Hematology, Chinese Academy of Medical Sciences. Cells were cultured in the RPMI-1640 medium (Gibco), supplemented with 10% FBS, 2 mmol/L L-glutamine, 100 IU/mL penicillin, and 100 mg/mL streptomycin in humidified atmosphere with 5% CO_2 at 37°C.

Exosome isolation and identification

Exo was isolated as previous report and a patent (US9005888) [21,22]. PEG with molecular weight 8000 was dissolved in PBS at concentration of 500 mg/mL. hUC-MSCs culture media were harvested and centrifuged at 5000*g* for 40 min at 4°C to remove cells and debris. PEG solution was added to the supernatant at the volume proportion of 1:5. The mixture was incubated at 4°C overnight. After incubation, the supernatant was centrifuged at 5000*g* for 40 min at 4°C. After centrifugation, supernatant was aspirated, and the pellet at the bottom was resuspended with phosphate-buffered saline at room temperature for 20 min. Then Exo solution was stored at –80°C.

Exo was dropped onto the copper grid. After absorbing excessive liquid, Exo was negatively stained with 3% (w/V) sodium phosphotungstate solution (pH 6.8) for 5 min and washed gently with double distilled water. Exo were observed and photographed by transmission electron microscopy (TEM). Exo was selected randomly to measure the mean diameter.

For surface markers detection, $CD63^+$ Exo was first isolated from pre-enriched Exo solution using human CD63 isolation/detection kit. Exo bound beads were then stained with Phycoerythrin-labeled CD9 and CD63 antibodies. Finally, CD9 and CD63 expression was detected by flow cytometry (LSRII, BD Biosciences).

Cell viability assay

Cell viability was determined by CCK-8. Cells were seeded in 96-well plates (5000 cells/well) in 200 μL medium per well and incubated at 37°C for 60 h with or without IM/hUC-MSC-Exo. After incubation, CCK8 was added (20 μL/well) and incubated for 2 h at 37°C. The absorbance at 450 nm was determined with microplate reader.

Apoptosis assay

Annexin V/PI apoptosis detection kit (BD Biosciences) was used to evaluate apoptosis according to the manufacturer's instructions. K562 cells cultured with hUC-MSC-Exo (50 μg/mL), IM (1 μmol/L) or both for 60 h were harvested. Cells were incubated with 5 μL annexin V–fluorescein isothiocyanate (FITC) for 15 min. Subsequently, PI staining was performed. Cell apoptosis was detected by flow cytometry (LSRII, BD Biosciences).

Western blot analysis

The total protein was extracted using RIPA buffer. The protein concentration was quantified by the BCA Protein Assay kit (Thermo). Protein (30 μg) of each sample was loaded into a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel, separated by electrophoresis and transferred onto polyvinylidene fluoride membranes (Millipore). After blocking for 2 h with 5% nonfat milk, membranes were incubated with primary antibodies (1/1000 dilution) including β-actin, Bax, Bcl-2, caspase3/cleaved-caspase3, caspase9/cleaved-cCaspase9, PRAP/cleaved-PRAP overnight at 4°C. After three repeated washing steps, membranes were incubated with corresponding horseradish peroxidase–conjugated secondary antibodies (1/2000 dilution) for 2 h at room temperature. Blots were detected using enhanced chemiluminescence solution (Invitrogen) and visualized with the ImageQuant LAS-4010 (GE).

Z-VAD-FMK pretreatment

K562 cells were pretreated with caspase inhibitor Z-VAD-FMK (30 μmol/L) for 4 h. Then cells were treated with IM or IM combined with hUC-MSC-Exo for 60 h. Cell apoptosis was detected by flow cytometry using annexin V-FITC/PI double staining.

Statistical analysis

All data obtained from at least three independent experiments and were presented as mean ± SD. Differences between groups were analyzed by Student *t* test or one-way analysis of variance using GraphPad Prism 6. Differences were considered significant at $P < 0.05$.

Results

Identification and characterization of hUC-MSCs and their exosomes

hUC-MSCs showed fibroblast like morphology (Figure 1A). Adipogenic and osteogenic differentiation was identified by oil red O and von Kossa staining (Figure 1B,C). hUC-MSCs were positive for CD44, CD73, CD90, CD105, CD106 and HLA-ABC, but negative for CD11b, CD14, CD34, CD45 and HLA-DR(Figure 1D).

TEM showed that hUC-MSC-Exo exhibited a round-shaped morphology with a size ranging from 50 to 100 nm (Figure 1E). In addition, CD63 and CD9 were highly expressed by hUC-MSC-Exo (Figure 1F). Taking together, our results proved that hUC-MSC-Exo with high purity were successfully isolated. The yield of hUC-MSC-Exo was 10–40 ug/10^6 MSCs.

hUC-MSC-Exo enhanced cell viability inhibition and apoptosis of K562 cells induced by IM

In our preliminary experiment, K562 cells were treated with different doses of IM (0.5 and 1.5 μmol) and/or hUC-MSC-Exo (25, 50, 100 μg/mL) for 24, 36, 48, 60 and 72 h to define the optimal dose and time point. Finally, K562 cells were treated with IM (1 μmol) and/or hUC-MSC-Exo (50 μg/ml) for 60 h in the following experiments because these doses and time points were optimal and representative. Furthermore, phosphorylated BCR-ABL loss was confirmed in K562 cells treated with 1 μmol IM. It was shown that p-BCR-ABL decreased in a time-dependent manner (Figure 2A). As was shown in Figure 2B, hUC-MSC-Exo had no effect on the cell viability of K562 cells ($P = 0.1973$ versus K562 group), whereas IM significantly inhibited K562 cell viability ($P = 0.000086$ versus K562 group). Compared with cells treated with IM alone, K562 cells exhibited a slight cell viability inhibition when treated together with IM and hUC-MSC-Exo (P = 0.029 vs K562 + IM group).

To further verify the effect of hUC-MSC-Exo on IM-treated K562 cells, we analyzed cell apoptosis. hUC-MSC-Exo alone did not exhibit an effect on K562 cell apoptosis ($P = 0.65$ versus K562 group). Consistent with previous reports, apoptosis was significantly induced by IM ($P = 0.000087$ versus K562 group), and the average apoptosis rate was 15.42 ± 0.32%. In the presence of IM and hUC-MSC-Exo, apoptosis of K562 cells was further increased ($P = 0.000083$ versus K562 + IM group). The average apoptosis rate reached 31.12 ± 0.27% (Figure 2C,D). Meanwhile, we found that the expression of pro-apoptotic Bax was enhanced and the expression of anti-apoptotic Bcl-2 was suppressed when K562 cells were treated with IM. Accordingly, enhancement of Bax expression and suppression of Bcl-2 expression were further reinforced when cells were treated together with IM and hUC-MSC-Exo compared with those treated with IM alone (Figure 2F).

Effects of hUC-MSC-Exo on cleavage of caspase proteins

Caspase-9 (37 kDa), caspase-3 (34 kDa) and PARP (116 kDa) can be cleaved to produce the active fragments, cleaved caspase-9 (35 kDa), cleaved caspase-3 (15,17 kDa) and cleaved PARP (89 kDa). hUC-MSC-Exo itself did not activate cleavage of caspase proteins. However, when K562 cells were treated with hUC-MSC-Exo in combination with IM, the levels of cleaved form of caspase-9, caspase-3 and PARP were much higher than that of IM group (Figure 3A). These results

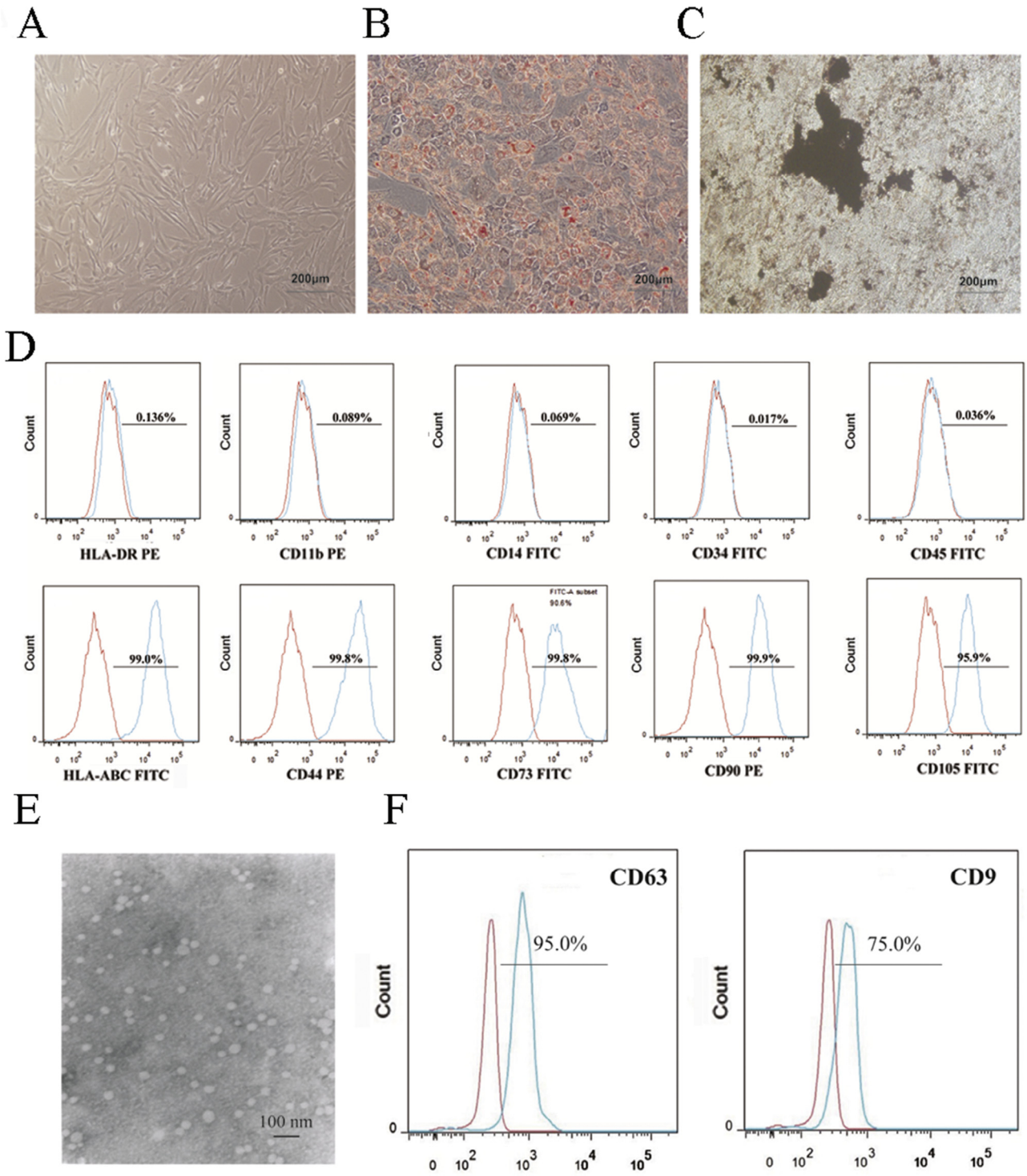

Figure 1. Identification and characterization of hUC-MSCs and their Exo. (A) The morphology of hUC-MSCs. (B) Adipogenic differentiation of hUC-MSCs. (C) Osteogenic differentiation of hUC-MSCs. (D) The immunophenotypic analysis of hUC-MSCs. (E) Representative transmission electron microscopy image of Exo derived from hUC-MSCs (scale bar = 100 nm). (F) Representative flow cytometric images of CD63 and CD9 expression, two Exo-specific markers.

indicate that hUC-MSC-Exo enhanced cleavage of caspase proteins induced by IM.

To further demonstrate whether the caspase signaling pathway was involved in increased apoptosis induced by IM and hUC-MSCs-Exo, K562 cells were treated with caspase inhibitor Z-VAD-FMK (30 μmol/L). Z-VAD-FMK alone showed no effect on K562 cell apoptosis. However, Z-VAD-FMK partially abolished apoptosis induced by IM ($P = 0.00032$ versus K562 + IM group), even

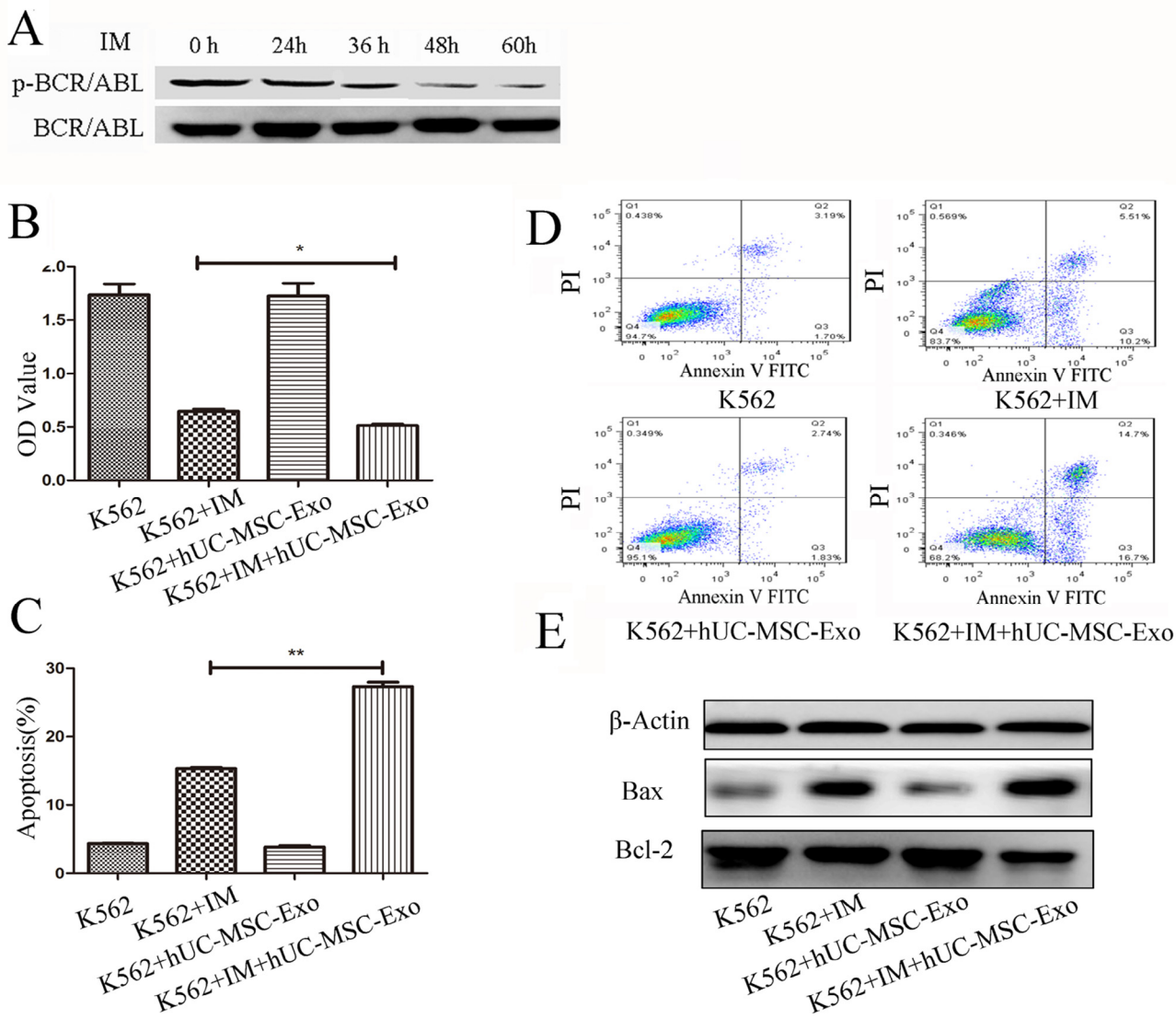

Figure 2. hUC-MSCs-Exo promoted K562 cell viability inhibition and apoptosis induced by IM. (A) Phosphorylated BCR-ABL loss was confirmed in K562 cells treated with 1 μmol/L IM. (B) Cell viability inhibition was induced by IM with or without hUC-MSC-Exo. *$P < 0.05$ versus K562 + IM group. (C) Apoptosis induced by IM and/or hUC-MSC-Exo was determined by flow cytometry. (D) Quantification of apoptosis rate. **$P < 0.01$ versus K562 + IM group. (E) The expression of apoptotic proteins Bax and Bcl-2 examined by Western blot.

by IM in combination with hUC-MSC-Exo ($P = 0.000071$ versus K562 + IM + hUC-MSC-Exo group) (Figure 3B). These results suggested that the caspase signaling pathway was involved in enhancement of IM-induced K562 cell apoptosis by hUC-MSC-Exo.

Discussion

MSCs are extensively used cells for cell therapy. MSCs are thought to exert their effects not only through differentiation potential but also through secreted products, including exosomes (Exo). Exo derived from MSCs have the capacity to mediate MSC interactions with multiple cell types. Therefore, MSC Exo can be used for cell-free therapy depending on their quality, reproducibility and potency [15,16]. UC-MSCs are ideal parental cells for Exo because they have several advantages such as abundant supply, painless collection, better expandability and secretory ability, immunoprivilege and immunosuppressive potential [23,24]. Previous work in our laboratory showed that UC-MSCs inhibited leukemia cell proliferation and induced their differentiation [10–12]. In the present study, it has been demonstrated that hUC-MSC-Exo alone had no effect on K562 cell viability and apoptosis. However, hUC-MSC-Exo enhanced the sensitivity of K562 cells to IM. The reason might be that cell response to Exo depended not only on cellular environment but also on the signaling pathway activated in recipient cells [25].

Compared with other reports, our apoptosis rates were lower in K562 cells after 60-h treatment with

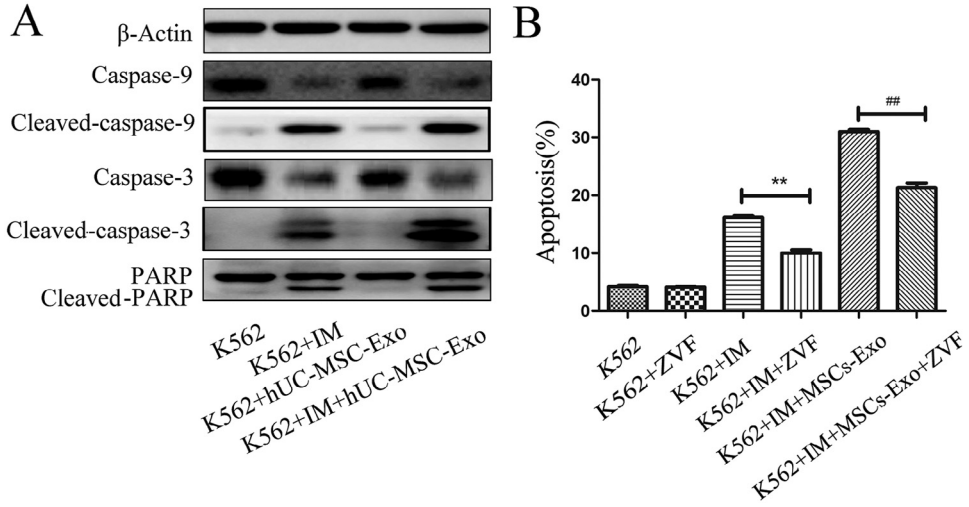

Figure 3. Caspase signaling pathway was involved in K562 cells apoptosis induced by IM and/or hUC-MSC-Exo. (A) Caspase-3, caspase-9, PARP and their cleaved forms were analyzed by Western blot. (B) Z-VAD-FMK reversed K562 cell apoptosis induced by IM and/or hUC-MSCs-Exo. $^{**}P < 0.01$ versus K562 + IM group, $^{\#\#}P < 0.01$ versus K562 + IM + hUC-MSC-Exo group.

1 μmol/L IM. The apoptosis rates depended on various factors, such as the sensitivity of K562 cells to IM, the quality of IM, the apoptosis assay kit and the apoptosis assay protocol. We thought the most important factor was the sensitivity of K562 cells to IM, which also depended on many factors, such as culture conditions and passages, among others. Although K562 cells are cell line, they are not absolutely homogeneous. When they are cultured in plastic flasks, some cells become adherent. It was found that adherent K562 cells were less sensitive to IM than suspended K562 cells [26]. Therefore, it was difficult to compare apoptosis rates between different studies.

As we knew, few reports have investigated the effects of MSC-Exo on leukemia cells. Hendijani *et al.* found that human Wharton's jelly–derived MSC secretome produced antiproliferative effect on K562 cells [27]. The contrasting results might be explained by the different components and dose of secretome. They further showed that combination of MSC secretome with doxorubicin displayed additive cytotoxic effect on K562 cells, which was similar to our results that hUC-MSC-Exo enhanced the sensitivity of K562 cells to IM. Previous study indicated that bone marrow MSC–derived Exo promoted multiple myeloma cell viability and proliferation by inducing drug resistance [20]. The variability of the results might be due to the different sources of Exo and different kinds of tumor [28]. Another study found that bone marrow MSC–derived Exo from leukemia patients significantly protected leukemia cells from chemotherapy [29]. The characteristics of MSCs from leukemia patients were much different from those of healthy person [30]; therefore, the contents of Exo were much different from each other.

It is well known that IM induces cell apoptosis by inhibiting BCR-ABL. However, the involved apoptotic proteins and signaling pathways still need to be elucidated. The Bcl-2 family proteins are key regulators of mitochondrial-mediated apoptosis and critical for the survival of leukemia and leukemia stem cells [31,32]. Reduced protein level of antiapoptotic Bcl-2 and increased proapoptotic protein level of Bax in K562 cells treated with IM alone or with hUC-MSC-Exo were identified in our experiment. Intrinsic (mitochondria) and extrinsic (death receptor) apoptotic pathways are two major pathways for inducing apoptosis [31]. Our results showed that the protein levels of caspase-9 and caspase-3 in K562 cells treated with IM with or without hUC-MSC-Exo were decreased. However, the cleaved form of caspase-9, caspase-3 and the caspase-3 substrate, PARP, increased. These results suggested that IM alone or with hUC-MSC-Exo could trigger the caspase-9/caspase-3 intrinsic signaling pathway. Furthermore, inhibition of the caspase signaling pathway partially reversed the apoptosis-inducing effect of IM and hUC-MSC-Exo, which suggests that another signaling pathway might be involved. Exo contained abundant microRNA (miRNA), and it was considered that miRNA might be more efficient in avoiding resistance or improving the efficiency of malignant tumors to chemotherapy [33]. Therefore, miRNA, which affected the sensitivity of K562 to IM, should be explored in prospective studies.

In conclusion, the present study demonstrated for the first time that hUC-MSCs-Exo enhanced the sensitivity of K562 cells to IM. These findings suggest that the application of hUC-MSCs-Exo in combination with

chemotherapeutical agents may be a potentially effective strategy.

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