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Original Article

Role of CXCR4/STAT3 pathway in mesenchymal stromal cell-mediated drug resistance of acute leukemia cells

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Abstract: Our aim is to explore the role of CXCR4/STAT3 in mesenchymalstromal cell (MSC)-mediated drug resistance of acute myeloid leukemia (AML) from the version of tumor mieroenvironment. AML cell lines U937 and KG1a and primary AML cells were co-cultured with MSC. The AML celllines cultured alone was used as controls. Apoptosis induced by mitoxantrone was measured by flow cytometry and Annexin V/PI double and 4'-6-diamidino-2-phenylindole (DAPI) staining. CXCR4 and STAT3 protein were detected by Western blot under both culturing conditions. Apoptosis of AML cells (U937and KG1a) significantly decreased during co-culturing with MSC (U937: 10.08%±1.53% vs 45.33%±1.03%, *P*=0.02; KG1a: 21.6%±1.82% vs 40.33%±3.29%, *P*=0.020) which suggested that drug resistance was induced after co-culture with MSC. The co-culturing of AML cells with MSC significantly induced the expression of phosphorylated STAT3 and CXCR4 protein. The inhibitor of STAT3 Cucurbitacin I could induce apoptosis of AML cells. After addition of Cucurbitacin I into the co-culture system, the apoptotic rates of primary AML cells significantly increased. Similarly, the apoptotic rates were also increased when AMD3100, the inhibitor of CXCR4, were added to overcome the stromal cell-mediated drug resistance. And AMD3100 induces an up-regulation of phosphorylated STAT3. Therefore targeting on STAT3 or CXCR4 protein could be a novel approach for the treatment of AML.

Keywords: Acute myeloid leukemia, CXCR4, STAT3, mesenchymalstromal cell, drug resistance

Introduction

Acute myeloid leukemia (AML) is a clonal malignant hyperplasia of myeloid blast cells derived from the hematopoietic system. It is also the most common type of adult acute leukemia. Although chemotherapy can induce complete remission in many AML patients, most patients will relapse or switch to refractory leukemia after remission [1, 2]. Bone marrow microenvironment provides "niche" for AML cells, protecting them from killing by chemotherapy drugs [3, 4]. Ligation of the membrane-spanning G-protein associated receptor CXCR4 is very important in the cross-talk between leukemia cells and the tumor microenvironment [5]. And Signal transducer and activator of transcription 3 (STAT3), a member of the STAT protein family, also plays a pivotal role in chemotaxis, proliferation, and cell survival of leukemia cells [6]. But very little is known about the relationship between CXCR4, STAT3 and bone marrow microenvironment [7]. In this study, we examined the expression changes of phosphorylated STAT3 and CXCR4 in AML cells after co-cultured with mesenchymal stem cells (MSC), and discussed their effects on the resistance of AML cells to chemotherapy mediated by MSC.

Materials and methods

Cell lines and patient samples

AML cell lines U937 and KG1a were provided by Cell Bank of Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI-1640 medium supplemented with 20% fetal bovine serum (FBS). BM samples were obtained from adult AML patients without any treatment at diagnosis. Patients with antecedent hematological disease or therapy-related AML were excluded. All subjects gave informed consent,

Table 1. Patient sample information

Patient	Diagnose[#]	Disease status	Sex	Age	Cell source	CD33 cells (%)[$]
1	M2a	Newly Diagnosed	Male	28	BM	98
2	M2a	Newly Diagnosed	Male	42	BM	97
3	M4	Newly Diagnosed	Male	40	BM	90
4	M5b	Relapsed	Female	25	PB	96
5	M5a	Relapsed	Female	33	PB	98
6	M5a	Newly Diagnosed	Female	30	BM	95
7	M5b	Relapsed	Female	29	BM	98
8	M4	Newly Diagnosed	Female	14	BM	99
9	M2b	Newly Diagnosed	Female	18	PB	95
10	M2b	Newly Diagnosed	Male	45	PB	91
11	M2a	Relapsed	Female	50	PB	88
12	M2b	Relapsed	Male	48	PB	90
13	M2b	Relapsed	Male	45	BM	89

[#]Criteria for the FAB classification of acute myeloid leukemia; [$]CD33 leukemia cells were quantified by flow cytometry; PB: Peripheral blood; BM: Bone marrow.

and the study protocol was approved by the Ethics Committee of Tianjin Medical University Cancer Institute and Hospital. Analysis of baseline morphology, cytogenetics, molecular markers and cell surface antigens were performed as part of the routine clinical evaluation of the patients.

Culture of bone marrow stromal cells

Bone marrow mononuclear cells were separated by Ficoll from BM samples of normal donors and then cultured with DMEM/10% FBS. After 72 h, non-adherent cells were removed and adhereTnt cells were cultured for 3-5 weeks.

Co-culture system and drug treatment

First, MSC were cultured in 6-well plates. When the confluence reached 90%, AML cells were added and then cocultured for 24 h. After 6 h for coculture, 0.5 mg/ml mitoxantrone (Sigma-Aldrich, USA) was added. Then AML cells were separated with MACS upon CD33 staining (**Table 1**).

Real-time PCR

RNA was used to synthesize complementary DNAs. Reverse transcription was achieved using QuantiTect Reverse Transcription Kit (Qiagen). Real-time PCR was performed using an ABI-Prism 7500 Sequence Detector (Applied Biosystems). The primers for CXCR4 were upper: AAA CTG AGA AGC ATG ACG GAC AA, lower: GCC AAC ATA GAC CAC CTT TTC AG. β-actin was used as housekeeper. The primers for β-actin were: 5'-ATGGAGGGGAATACAGCC-C-3' (forward) and 5'-TTC-TTTGCAGCTCCTTCGTT-3' (reverse).

Western blot analysis

Proteins were extracted from AML cells. Then protein samples (at 30 g per lane) were analyzed by SDS-PAGE. Immunoblotting was performed using antibodies against CXCR4, p-STAT3 and GAPDH (Abcam, USA).

Flow cytometry analysis

Cells were stained with PE-CXCR4 and then analyzed the expression rate of CXCR4 using flow cytometry. AML cells were stained with Annexin-V-PE and 7AAD (BD Pharmingen) for 20 min at room temperature. The rate of apoptosis was also assessed by flow cytometry.

Statistical analysis

Data were summarized as means ± standard deviations (SD). The significance of differences was assessed using the Student's t-test. *P* values less than 0.05 were considered significant. Statistical analyses were performed using Prism version 4.0 software (GraphPad).

Results

Apoptosis of AML cells decreased after coculture with MSC

48 hours later after coculture with MSC, the apoptosis rates of U937 and KG1a were 10.08%±1.53% and 21.6%±1.82% respectively, while that of U937 and KG1a cultured alone were 45.33%±1.03% and 40.33%± 3.29% (**Figure 1**), suggessting that MSC could protect AML cells from apoptosis induced by mitoxantrone.

p-STAT3 and CXCR4 expression in AML cells was upregulated after coculture with MSC

We used western blot to compare the expression of p-STAT3 of AML cells culture alone and

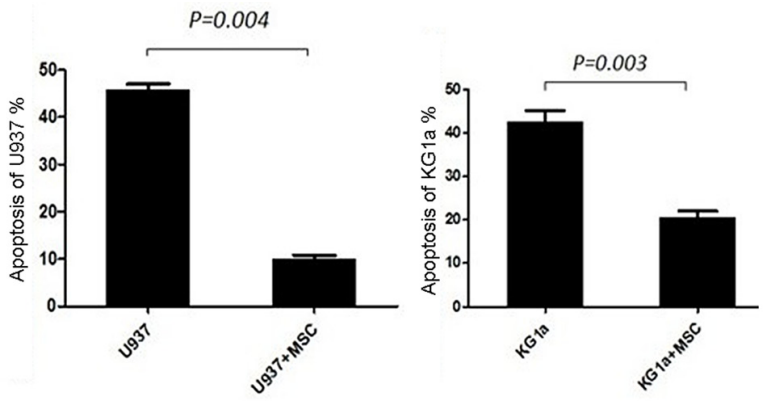

Figure 1. Apoptosis of AML cells decreased after coculture with MSC. Apoptosis of AML cells (U937 and KG1a) significantly decreased during co-culturing with MSC (U937: 10.08%±1.53% vs 45.33%±1.03%, *P*=0.02; KG1a: 21.6%±1.82% vs 40.33%±3.29%, *P*=0.020).

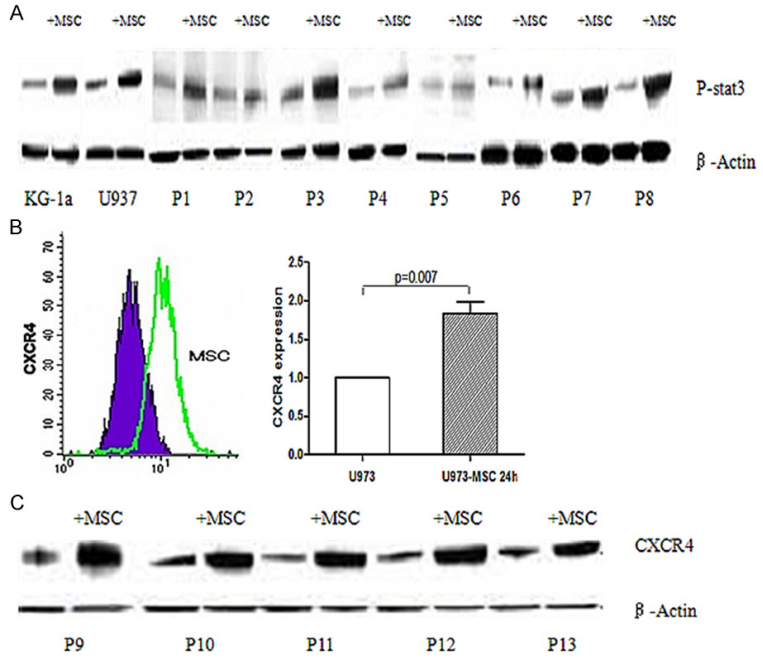

Figure 2. p-STAT3 and CXCR4 expression in AML cells was upregulated after coculture with MSC. 24 hours after coculture with MSC, the expression of p-STAT3 was upregulated compared to AML cells cultured alone (A). Flow cytometry analysis (B) and western blot (C) results showed that the expression of CXCR4 was also upregulated after coculture with MSC.

coculture with MSC. Results showed that after coculture with MSC, the expression of p-STAT3 was upregulated compared to AML cells cultured alone (**Figure 2A**). Besides, flow cytometry analysis (**Figure 2B**) and western blot (**Figure 2C**) results showed that the expression of CXCR4 was also upregulated after coculture with MSC.

Apoptosis increased after addition of Cucurbitacin I

After addition of Cucurbitacin I (100 nmol/L), the inhibition of p-STAT3, the apoptosis of AML cells increased. In the coculture system, the apoptosis also increased after treatment with Cucurbitacin I (**Figure 3**), suggessting that Cucurbitacin I could induce the apoptosis of AML cells.

Treatment of AMD3100 overcomes the drug resistance induced by MSC

After treatment of AMD3100, the apoptosis of AML cells increased significantly regardless of cultured with or without MSC (**Figure 4A**). Moreover, the expression of p-STAT3 was downregulated (**Figure 4B**).

Discussion

Several studies have confirmed that the interaction between leukemia cells and bone marrow microenvironment is vital for AML disease progression and treatment of drug-resistance [8-10]. Leukemia stem cells (LSC) co-cultured with MSCs can promote cellular self-renewal and proliferation, retard differentiation, and protect leukemia cells from chemotherapy. Drug resistance is the main cause of minimal residual disease (MRD) after primary treatment [11, 12]. MRD finally leads to disease progression which is difficult tocure [13, 14]. Therefore, systematic studies on the pathogenesis of AML, especially the molecular mechanism of the interaction between AML cells and bone marrow microenvironment, can clarify the drug resistance mechanism induced by bone marrow microenvironment, and provide novel ideas and strategies for cure.

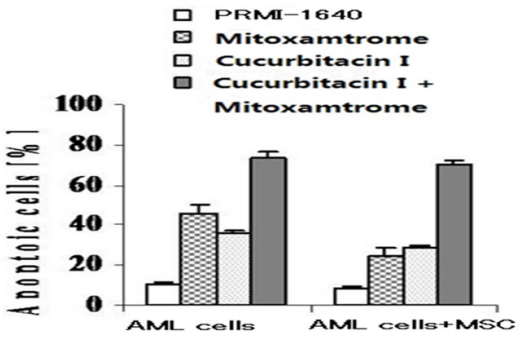

Figure 3. Cucurbitacin I inhibits MSC-induced leukemia cell drug resistance. Cells alone or co-cultured with MSCs at ratio of 5:1. Bath of the rates of apoptosis increased after treatment with Cucurbitacin I (100 nmol/L) in the two culture systems.

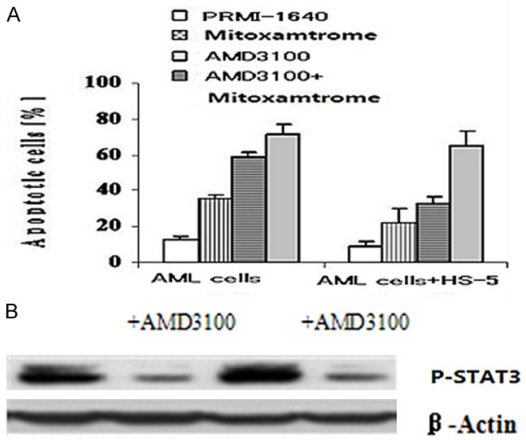

Figure 4. Treatment of AMD3100 overcomes the drug resistance induced by MSC and induces the downregulation of p-STAT3 protein. After treatment of AMD3100, the apoptosis of AML cells increased significantly regardless of cultured with or without MSC (A). The expression of p-STAT3 was downregulated (B).

Signal transducer and activator of transcription 3 (STAT3) is the key signaling molecule for many cytokines and growth factor receptors, and is persistently inactivated in human tumor cells [15]. And it is closely related to anti-apoptosis and tumor angiogenesis [16]. CXCR4, the specific receptor for stromal cell-derived factor-1 (SDF-1), is highly expressed in human AML cells and animal models, and is closely related to the occurrence, progression and prognosis of AML [17-19]. SDF-1 combined with CXCR4 can activate non-receptor tyrosine kinases (JAK2 and JAK3) via self-conformational change, further activating the JAK-STAT3 signaling pathway. JAK-STAT3 and the regulated anti-apoptosis proteins (BCL-2 and BCL-xl) are critical for the survival of leukemia cells [20].

In this study, we found that after co-incubation with MSC, the apoptosis of leukemia cell lines U937 and KG1a, and primary AML cells induced by mitoxantrone (a chemotherapy drug) was significantly reduced as compared tocells under single incubation alone. After adhering to bone marrow MSCs, AML is protected by the bone marrow microenvironment, which produces drug resistance to chemotherapy. Additionally, the expression levels of phosphorylated STAT3 (p-STAT3) and CXCR4 in AML cells were greatly increased after co-incubation. When specific inhibitor cucurbitacin I was used to block STAT3 or AMD3100 was used to block CXCR4, they induced apoptosis of AML cells under incubation alone, and more importantly partly removed the “niche” of bone marrow stroma on AML cells, which partly restored the toxicity of mitoxantrone on AML and increased the apoptosis rate of AML cells. Moreover, p-STAT3 was downregulated after CXCR4 was blocked with AMD3100, suggesting that reduced CXCR4 expression could further regulate the expression of p-STAT3. Activation of SDF-1/CXCR4 axis could downregulate signaling pathways such as JAK2/STAT3 and further regulate the proliferation, differentiation and apoptosis of AML cells. Adhering of AML cells to stromal cells might be a multi-step and multifactor process [21, 22]. These results indicated that CXCR4 and p-STAT3 proteins played a role in this process, but the detailed mechanism requires further study.

The bone marrow hematopoietic microenvironment is an internal environment for supporting and regulating the settlement, proliferation, differentiation, development and maturity of hematopoietic cells, and is closely related to the occurrence, progression and prognosis of leukemia [23]. After AML cells adhere to stromal cells, expression of CXCR4 is upregulated via a series of complicated mechanisms. Further, it activates STAT3 and reduces the apoptosis of AML induced by chemotherapy drugs. CXCR4 protein plays a very important role in the pathogenesis of many tumors, including AML [24]. Activation of SDF-1 and CXCR4can activate downstream cell signaling pathways, such as JAK-STAT3 [25]. However, the mechanisms by which CXCR4 regulates STAT3 downstream and the effects on anti-apoptosis, growth-promotion and drug resis-

tance induced by stroma after STAT3 activation need further study. Therefore, understanding the effect of CXCR4/STAT3 on the drug resistance mediated by AML stroma will provide novel ideas for AML pathogenesis research and target therapy.

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Disclosure of conflict of interest

None.

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