Cytokine expression patterns and mesenchymal stem cell karyotypes from the bone marrow microenvironment of patients with myelodysplastic syndromes

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Abstract

The purpose of this study was to explore cytokine expression patterns and cytogenetic abnormalities of mesenchymal stem cells (MSCs) from the bone marrow microenvironment of Chinese patients with myelodysplastic syndromes (MDS). Bone marrow samples were obtained from 30 cases of MDS (MDS group) and 30 healthy donors (control group). The expression pattern of cytokines was detected by customized protein array. The karyotypes of MSCs were analyzed using fluorescence *in situ* hybridization. Compared with the control group, leukemia inhibitory factor, stem cell factor (SCF), stromal cell-derived factor (SDF-1), bone morphogenetic protein 4, hematopoietic stem cell (HSC) stimulating factor, and transforming growth factor-β in the MDS group were significantly downregulated (P<0.05), while interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and programmed death ligand (B7-H1) were significantly upregulated (P<0.05). For chromosome abnormality analysis, the detection rate of abnormal karyotypes (+8, –8, –20, 20q–, –Y, –7, 5q–) was 30% in the MDS group and 0% in the control group. In conclusion, the up- and downregulated expression of these cytokines might play a key role in the pathogenesis of MDS. Among them, SCF and SDF-1 may play roles in the apoptosis of HSCs in MDS; and IFN-γ, TNF-α, and B7-H1 may be associated with apoptosis of bone marrow cells in MDS. In addition, the abnormal karyotypes might be actively involved in the pathogenesis of MDS. Further studies are required to determine the role of abnormal karyotypes in the occurrence and development of MDS.

Key words: Bone marrow microenvironment; Cytokine; Mesenchymal stem cell; Chromosome; Pathogenesis

Introduction

Myelodysplastic syndromes (MDS) are a group of diseases derived from malignant amplification of hematopoietic stem cells (HSCs), with typical features of abnormal myeloid cell differentiation and maturation, ineffective hematopoiesis, and a high risk of progression to acute myeloid leukemia (1). The incidence and prevalence of MDS are higher in males than in females and increase sharply with age (2,3). With the aging population and improving awareness of the disease, the documented disease burden is predicted to escalate in the near future. Moreover, the age of onset is younger in Asia than in Western countries, (4,5). Therefore, there is an urgent need to develop new therapeutic options and clinical management of MDS, which must be based on diagnostic efficiency and knowledge of the pathology of MDS.

Currently, the diagnosis of MDS is complex (6), because signs and symptoms for MDS are nonspecific and are generally related to blood cytopenias (7,8). MDS must be distinguished from anemia, thrombocytopenia, and leukopenia (9,10). Hence, it is important to find specific pathological hallmarks for improving diagnostic efficiency.

Based on previous studies, MDS are characterized by complicated pathological hallmarks such as abnormal HSCs (11), changes in the hematopoietic microenvironment (12), and immune system defects (13). As the niche for HSCs, the bone marrow hematopoietic microenvironment is a complicated network system containing the bone marrow microcirculation and mesenchymal stromal cellular components and cytokines (14). These cytokines are important for the growth and survival of HSCs (15,16)

and antagonistically or synergistically induce excessive apoptosis of HSCs during the occurrence and development of MDS (17). However, the specific roles of these cytokines are still unclear. In addition, an abnormal karyotype is the key genetic feature of MDS pathogenesis. Although cytogenetic abnormalities of MSCs in MDS have been observed in some previous studies (18-22), it is still necessary to further investigate the karyotypic features of Chinese MDS patients, because there are population differences in genetic makeup, especially between Asian and Western peoples (5,22). We, therefore, studied 30 Chinese patients with MDS and observed the expression pattern of cytokines and abnormal karyotypes of MSCs in their bone marrow microenvironment.

Patients and Methods

All patients gave their written informed consent prior to inclusion in this study, which was approved by the Clinical Research Center, Chinese Academy of Sciences, Shanghai Xuhui District Central Hospital Ethics Committee, and performed in accordance with their ethical standards.

Patients

A total of 30 Chinese patients with presumptively diagnosed MDS (15 males and 15 females, age ranging from 40 to 68 years with a median of 50 years) were evaluated in this study. According to World Health Organization criteria (23), the patients were classified as follows: 8 cases of refractory anemia, 7 cases of refractory anemia with ringed sideroblasts, 7 cases of refractory anemia with excess blasts, and 8 cases of refractory anemia with excess blasts in transformation. None of the patients were diagnosed as having had other diseases, except myelodysplastic syndromes, previously. Moreover, none of them had received either chemotherapy or immunosuppressant treatments.

In addition, 30 healthy donors (15 males and 15 females, age ranging from 36 to 66 years with a median of 49 years) were included as normal controls.

Bone marrow samples

Bone marrow samples were derived from 30 MDS patients (MDS group) and 30 healthy donors (control group). Bone marrow aspiration and biopsy were performed under conscious sedation with intravenous propofol and local infiltration with lidocaine. Bone marrow aspirates were obtained from the posterior superior iliac crest and collected into the alpha medium containing 10 U/mL preservative-free heparin. Then the mononuclear cells (MNCs) were isolated by Ficoll density gradient centrifugation (GE Healthcare, USA), the supernatant was used for protein array hybridization, and the isolated MNCs were collected and cultured.

Protein array preparation and hybridization

The supernatant of bone marrow was used for protein array hybridization with a customized cytokine antibody array from RayBio Human Cytokine Antibody Array (RayBiotech, Inc., USA) according to standard operational protocols. The protein array map is shown in Table 1. Pretest results showed that there was no cross-reaction among the cytokine antibodies. Chemiluminescent signals were detected using a Fuji LAS1000 imaging system (Fujifilm, Japan) and then analyzed using the Advanced Image Data Analyzer software (AIDA, 3.28; Raytest Izotopenmessgeraete, Germany). Expression levels of cytokines are reported as signal intensity values.

Cell cultures

Isolated MNCs from the MDS group and the control group were cultured in MesenCult MSC basal medium (Human, Stem Cell Technologies, Canada) at 37°C in a humidified incubator with 5% CO_2 in air. The cells were subcultured to passage three (P3), and the P3 cells were then obtained by trypsin digestion and centrifugation at 1082 g for 5 min.

Flow cytometry analysis

Flow cytometric analysis was performed to confirm the types of cells using specific surface antigens of MSCs (CD29 and CD90) and HSCs (CD34 and CD45). The cells in culture were trypsinized and stained with fluorescein isothiocyanate (FITC) or phycoerythrin (PE) conjugated antibodies, including FITC-CD34, PE-CD90, FITC-CD45, and PE-CD29 monoclonal antibodies (Immunotech, USA). Ten thousand labeled cells were acquired and analyzed using a Becton Dickinson flow cytometer (USA). Data (collected in list mode) were analyzed using the EXPO32 ADC software (Beckman Coulter, Germany).

Fluorescence *in situ* hybridization (FISH) detection

The P3 bone marrow MSCs were collected for interphase FISH analysis using a FISH kit (Beijing GP Medical Technologies, China). The following probes were used for hybridization: LSI EGR1 (5q31)/D5S721, D5S23 (5p15.2), LSI D7S486 (7q31)/CEP 7 SG (CSP7), LSI D20S108 (20q12)/CSP8, and CSPX/CSPY (Beijing GP Medical Technologies). Procedures were performed according to standard Abbott/Vysis protocols. Briefly, the cells were collected by centrifugation at 128,778 g for 10 min. The cell pellets were gently mixed with a small volume of paraformaldehyde, and then 5 µL of the cell mixtures was placed on the slide and dried naturally. The slides were then fixed in an oven at 56°C for 20 min, and afterwards soaked consecutively in 100% methanol for 5 min, 100% ethanol for 30 min, 70% ethanol for 2 min, 85% ethanol for 2 min, and 100% ethanol for 2 min. After they were soaked, the slides were dried naturally and heated to 56°C again. Then, 10 µL of the probe mixtures was added on the surface of the slides and immediately

Table 1. Map of the protein array.

	A	B	C	D	E	F	G	H	I
1	Pos1	Pos2	Pos3	Neg	LIF	SCF	EGF	CCL2	SDF-1
2	Pos1	Pos2	Pos3	Neg	LIF	SCF	EGF	CCL2	SDF-1
3	BMP-4	IL-2	IFN-γ	Neg	TNF-α	CXCR4	FLT-3	MIG	TGF-β
4	BMP-4	IL-2	IFN-γ	Neg	TNF-α	CXCR4	FLT-3	MIG	TGF-β
5	XIAP	Notch1	IGF-I	IL-8	B7-H1	Neg	ANG	Neg	IL-1
6	XIAP	Notch1	IGF-I	IL-8	B7-H1	Neg	ANG	Neg	IL-1

LIF: leukemia inhibitory factor; SCF: stem cell factor; SDF-1: stromal cell-derived factor; BMP-4: bone morphogenetic protein 4; FLT-3: HSC-stimulating factor; TGF-β: transforming growth factor-β; IFN-γ: interferon-γ; TNF-α: tumor necrosis factor-α; B7-H1: programmed death ligand; Pos: photoreceptor outer segment proteins; Neg: endo-1,6-beta-D-glucanase; EGF: epidermal growth factor; CCL2: monocyte chemoattractant protein 1; CXCR4: CXC chemokine receptor-4; IL-2: interleukin-2; MIG: membrane immunoglobulin; XIAP: X-linked inhibitor of apoptosis; Notch1: neurogenic locus notch homolog protein 1.

covered with a cover glass. Hybridization was performed on the hybridization apparatus for 16 h. After they were washed, the slides were dried naturally in darkness. The slides were then counterstained with 15 μL of the fluorescent dye 4′,6-diamidino-2-phenylindole 2HCl in darkness for 20 min and observed under a fluorescence microscope (Olympus BX 50, Japan). In total, 200 HSC nuclei and 500 MSC nuclei were scored.

Statistical analysis

All data were analyzed with the SPSS 11.0 software. Statistical differences were determined using Student's t-test for independent samples. A P value <0.05 was considered significant.

Results

Expression patterns of cytokines in the bone marrow microenvironment

Compared to the control group, there were significantly lower levels of leukemia inhibitory factor (LIF), stem cell factor (SCF), stromal cell-derived factor (SDF-1), bone morphogenetic protein 4 (BMP-4), HSC-stimulating factor (FLT-3), and transforming growth factor-β (TGF-β; $P<0.05$) and higher levels of interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and programmed death ligand (B7-H1; $P<0.05$) in the MDS group (Figure 1).

Immune phenotypic analysis

The expression levels of cell surface markers, including bone marrow MSC surface markers (CD29 and CD90), HSC surface marker CD34, and leukocyte surface marker CD45, were detected using a flow cytometer. The results indicated that the HSC surface marker CD34 and leukocyte surface marker CD45 were not expressed in the MDS group nor in the control group. Meanwhile, the MSC markers (CD29 and CD90) were expressed in both the MDS and the control groups. Our results suggested that the cells isolated from bone marrow in MDS and control groups were confirmed as MSCs (Figure 2).

Chromosome abnormality analysis

As expected, no chromosomal abnormalities were detected in MSCs derived from the 30 healthy donors in the control group. However, 9 of the 30 MDS patients (abnormal karyotype rate of 30%) showed abnormal karyotypes (+8, −20, −8, 20q−, −7, −Y, 5q−) in MSCs (Figure 3, Table 2).

Discussion

Cytokines in the bone marrow microenvironment have been reported to play an important role in supporting the growth and survival of HSCs (24). In this study, we found that the expression levels of cytokines including LIF, SCF, SDF-1, BMP-4, FLT-3, and TGF-β were significantly downregulated in the bone marrow microenvironment of MDS patients. Among these cytokines, SCF is the key in
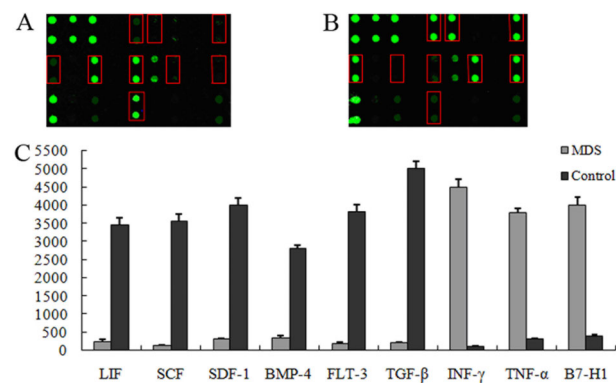

Figure 1. Expression analysis of cytokines through protein array hybridization (Student t-test). *A*, Signal map of myelodysplastic syndromes (MDS) group. *B*, Signal map of normal control group. *C*, Quantization of the expression level of cytokines by histogram plotting. LIF: leukemia inhibitory factor; SCF: stem cell factor; SDF-1: stromal cell-derived factor; BMP-4: bone morphogenetic protein 4; FLT-3: HSC-stimulating factor; TGF-β: transforming growth factor-β; IFN-γ: interferon-γ; TNF-α: tumor necrosis factor-α; B7-H1: programmed death ligand.
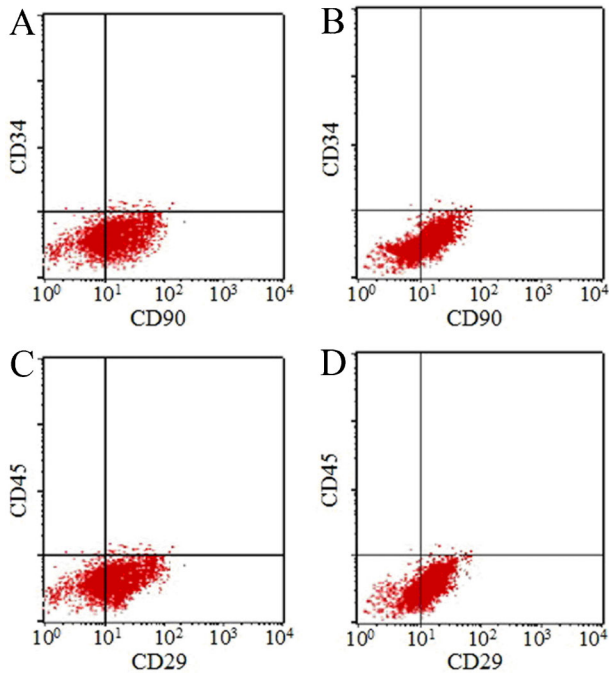
Figure 2. Immune-phenotypic analysis through flow cytometry detection. *A*, *C*, myelodysplastic syndromes (MDS) group; *B*, *D*, normal control group.

survival, proliferation, and differentiation of early HSCs (25,26). Meanwhile, SCF was associated with the apoptosis of expanded HSCs. Also, as the specific receptor of CXCR4, SDF-1 is secreted by the stromal cells in bone marrow (27). Normally, SDF-1 and CXCR4 are involved in the regulation of migration, survival, and development of HSCs (28). In MDS, it has been reported that the SDF-1/CXCR4 axis and its correlation were associated with the angiogenesis and apoptosis of HSCs (29). Therefore, we inferred that SCF and SDF-1 may play roles in the apoptosis of HSCs in MDS, and that there may be some correlation between them. In addition, upregulated expression of IFN-γ, TNF-α, and B7-H1 was found in MDS patients in this study, which indicated that they may play some role in the occurrence and development of MDS. Kondo et al. (30) reported that the expression of B7-H1 was enhanced after adding IFN-γ and TNF-α to the MDS cell culture medium. Cachaço et al. (31) discovered that TNF-α could induce apoptosis of bone marrow cells *in vitro*. Thus, we speculated that there may be some correction among the three cytokines for the apoptosis of bone marrow cells in MDS. In summary, the up- and downregulated expressions of these cytokines might play a key role in the pathogenesis of MDS, especially the apoptosis of cells.

The bone marrow microenvironment is formed mainly of MSCs, which can support hematopoiesis and differentiate along multiple mesenchymal lineages (32). In the chromosome abnormality analysis of this study, we found that 30% of the 30 MDS patients displayed abnormal karyotypes for MSCs, which may be a genetic marker of MSCs in MDS. However, the rate of cytogenetic aberrations in MSCs was 44% in the study by Blau et al. (20) and 64% in the study by Song et al. (18). Meanwhile, there were some differences
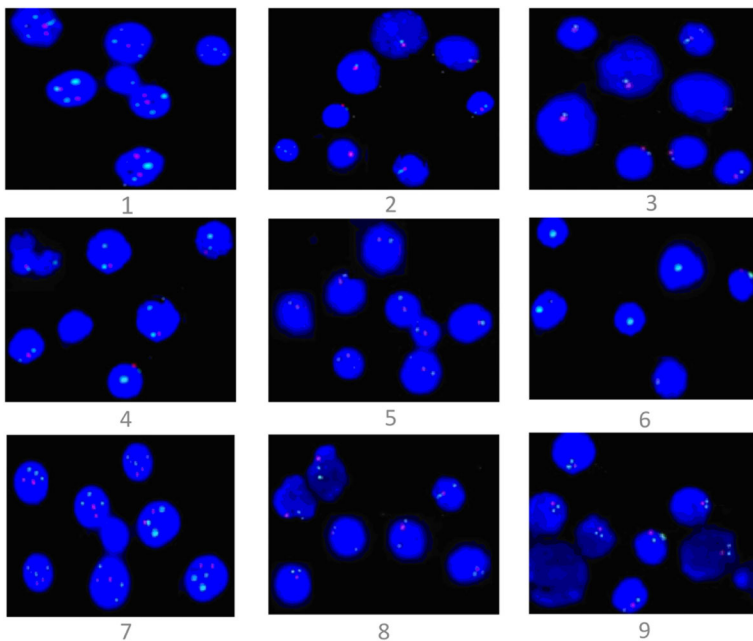

Figure 3. Karyotypic analysis by FISH detection. Various probes were designed for abnormal karyotype detection. *1* and *7*, karyotype of trisomy (+8); *2*, deletion of chromosome 20 (–20); *3*, deletion of chromosome 8 (–8); *4* and *8*, deletion of long arm in chromosome 20 (20q–); *5*, *in situ* fluorescent signal pattern for the deletion of chromosome 7 (–7); *6*, deletion of Y chromosome (–Y); *9*, deletion of long arm in chromosome 5 (5q–).

Table 2. Abnormal karyotype of mesenchymal stem cells (MSCs) from the myelodysplastic syndromes group.

No.	Gender	Age	MDS type	Karyotype
1	Male	48	RA	+8
2	Male	53	RARS	−20
3	Female	65	RAEB	−8
4	Male	60	RAEB	20q−
5	Female	63	RAEB-T	−7
6	Male	45	RA	−Y
7	Male	55	RAEB	+8
8	Female	40	RAEB-T	20q−
9	Male	42	RARS	5q−

MDS: myelodysplastic syndromes; RA: refractory anemia; RARS: refractory anemia with ringed sideroblasts; RAEB refractory anemia with excess blasts; RAEB-T: refractory anemia with excess blasts in transformation.

between the chromosome abnormalities (+8, −8, −20, 20q−, −Y, −7, 5q−) in this study and those of previous studies (18-20,33). Among them, the deletion of chromosome 8 (−8) was not found in the studies by Blau et al. (20,33) and Pinheiro et al. (34), but it was identified in the studies by Flores-Figueroa et al. (12,19) and our study. These inconsistent results may be attributed to the different detection methods (Song et al. (18), Flores-Figueroa et al. (12,19), and Pinheiro et al. (34): trypsine-giemsa banding) or to the different populations (Flores-Figueroa et al. (12,19): Mexico; Blau et al. (20,33): Germany; Pinheiro et al. (34): Brazil). Thus, it is necessary to explore different karyotype abnormalities in different populations, and more studies are needed to verify the syndrome of deletion of chromosome 8 (−8) in MDS. For the diagnosis of MDS, different karyotype abnormalities among ethnicities should be considered. In addition, deletion of the long arm of chromosome 5 (5q−) was found both in our study and in studies by Blau et al. (20,33). It has been reported that the MDS patients with syndrome 5q− showed a specific genetic profile, which could play an important role in the pathogenesis and disease characteristics (35). Therefore, these chromosome abnormalities may be associated with the pathogenesis of MDS. Further studies are required to determine the roles of abnormal karyotypes in the occurrence and development of MDS.

In addition, in this study we only aimed to explore cytogenetic abnormalities of MSCs in MDS, so total bone marrow karyotyping was not performed. Further studies are required to investigate whether the karyotype abnormalities of HSCs and MSCs exist in MDS. Furthermore, since FISH analysis occurred in MSC cells during the third passage, contamination with clonal HSCs could be not possible in this study.

In conclusion, nine potentially key cytokines (downregulated cytokines: LIF, SCF, SDF-1, BMP-4, FLT-3, and TGF-β; upregulated cytokines: IFN-γ, TNF-α, and B7-H1) in MDS pathogenesis were found. Among them, SCF and SDF-1 may play roles in the apoptosis of HSCs in MDS; and IFN-γ, TNF-α and B7-H1 may be associated with the apoptosis of bone marrow cells in MDS. Abnormal karyotypes (+8, −8, −20, 20q−, −Y, −7, 5q−) in MSCs were detected in 30% of Chinese MDS patients. These results indicated that these abnormal karyotypes might be actively involved in the pathogenesis of MDS. Furthermore, the up- and downregulation of cytokines as well as abnormal karyotypes could serve as indicators for MDS diagnosis and prognosis.

Acknowledgments

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