Biochemical and Biophysical Research Communications xxx (2018) 1–7

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

CXCL8 is associated with the recurrence of patients with acute myeloid leukemia and cell proliferation in leukemia cell lines

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ARTICLE INFO

Article history:
Received 23 March 2018
Accepted 24 March 2018
Available online xxx

Keywords:
Acute myeloid leukemia
CXCL8
Cytokines
Gene expression profiling
Proliferation

ABSTRACT

Acute myeloid leukemia (AML) blasts release a wide range of chemokines in which CXCL8 has recently been recognized to be important for tumor progression. To find out the function of CXCL8 in AML, we compared blood serum of AML patients and healthy donors and found that the average level of CXCL8 was higher in AML patients. Among patients, higher expression of CXCL8 was also a positive recurrence indicator which illustrated the critical role of CXCL8 in AML. Knocking down of CXCL8 in leukemic cell lines led to significant reduction of proliferation via inducing G0/G1 cell cycle arrest and apoptosis, which was accompanied by the inactivation of ERK1/2. Furthermore, inhibition of ERK1/2 by specific chemical inhibitors reconstructed the CXCL8 knocking down phenomenon. Overall, we demonstrated that expression level of CXCL8 had a positive relationship with recurrence probability in AML. And CXCL8 was strongly implicated in AML cells growth by activating the ERK1/2 signal pathway.

1. Introduction

AML is a heterogeneous disease characterized by rapid proliferation and accumulation of immature myeloid cells in the bone marrow, which leads to hematopoietic dysfunction and an extremely poor prognosis [1]. Approximately 50% to 75% of patients with AML realize complete remission with chemotherapy and around 20% to 30% of them enjoy long-term disease-free survival [2]. Nevertheless, a considerable fraction of patients shows recurrence and chemoresistance [3]. To identify genes which might be associated with therapeutic results will further help us to optimize the treatment of AML.

CXCL8 is a member of the CXC chemokine family. Its main physiological function is to promote inflammation and to regulate the activation and migration of granulocytes from peripheral blood to other tissues by binding to its receptor CXCR1 and CXCR2 [4]. In recent years, it has been found that the concentration of CXCL8 in the serum and tumor tissue had a close relationship with the occurrence and development of many kinds of tumors [5,6]. CXCL8 also plays an important role in modulating angiogenesis, migration and cell survival in both neutrophils and cancer cells by inducing downstream intracellular signal pathway activation, such as PI3K/Akt and MAPK signal pathway [7–9].

In peripheral blood of patients with primary myelofibrosis, increased CXCL8 represents higher circulating blasts, more constitutional symptoms, and transfusion requirement. And these are evaluable diagnostic parameters highly predictive of leukemic transformation [10,11]. Primary AML cells release several chemokines cultured in vitro. One most abundant chemokine is CXCL8, which could be investigated as a possible prognostic parameter [12]. In subsequent research, the CXCL8-CXCR2 pathway has been proposed as an attractive therapeutic target against MDS (Myelodysplastic Syndromes) and AML stem cells [13]. Owing to its prognostic value for hematological malignancies and involvement in leukemic progression, the mechanism of how this CXCL8-CXCR2 axis works needs to be further studied. And its value as a drug target has not yet reached expectation. In this research, we further investigated the function of CXCL8 in the growth of AML cell lines to provide a theoretical basis for the therapeutic target of AML.

2. Materials and methods

2.1. Patient samples & patient database and cell culture

16 healthy donors' and 57 AML patients' serum (Table 1, Supplementary Table 1) were collected in Hospital of Blood Diseases (Tianjin China). Inclusion criteria were based on the European

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https://doi.org/10.1016/j.bbrc.2018.03.181
0006-291X/

Table 1
Characteristics of the patients.

	Normal	AML
Number of samples	16	57
Age (years)		
Median	36.3	39.6
Range	5–61	4–73
Sex		
Female	5	26
Male	11	31

Abbreviations: AML, Acute Myeloid Leukemia.

Leukemia Net (ELN) criteria. All the patient samples follow the principles of the Declaration of Helsinki.

Gene expression data from 19 AML samples were obtained from GEO (GSE9476) [14] and were divided into "CXCL8 high" and "CXCL8 low" groups based on their expressions of CXCL8. Then gene expression chip information of two groups was applied to Ingenuity® Pathway Analysis (IPA®) (QIAGEN, Hilden, Germany) to identify the most significant pathways involved by these genes and to further discover potential novel regulatory networks and causal relationships associated with CXCL8. In Canonical pathways, P-value≤0.01 was considered to be statistically significant.

HL60 cells, NB4 cells, and THP1 cells were purchased from the State Key Laboratory of Experimental Hematology (Tianjin China) and were cultured in RPMI 1640 (Gibco-BRL Life Technologies, Inc. Burlington, ON, Canada) with 10% fetal bovine serum (HyClone, Logan, UT), 100U/ml penicillin and 100 μg/ml streptomycin at 37 °C and 5% CO2.

2.2. Lentiviral shRNA vectors and transfection

The two different shRNA lentiviral vectors and empty vector were transfected respectively into 293T producer cells with Lipofectamin™ 3000 (Invitrogen, CA, USA) according to the protocol. CXCL8-shRNAs and the control vector were then transfected into HL60 cells, NB4 cells and THP1 cells individually. The transfected cells were selected with puromycin. Knock-down efficiency of CXCL8 was measured by real-time PCR and ELISA.

2.3. Quantitative real-time PCR and western blot

Total RNA was extracted with Trizol Reagent (Invitrogen, Grand Island, NY) and reverse transcripted to cDNA by using the Superscript II RT (Invitrogen, Grand Island, NY). Quantitative real-time PCR was performed with SYBR Green PCR kit (Takara, Japan) on the ABI PRISM 7500 Sequence Detection System.

Total protein was extracted with RIPA lysis buffer and 1 mM PMSF (Sigma, USA) and resolved by SDS-PAGE, then transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). Indicated antibodies and enhanced chemiluminescence ion reagent (GE Healthcare, UK) was used to detect the protein bands with a Lynx video densitometer (Biological Vision). Antibodies against GAPDH, anti-phosphospecific ERK1/2 and anti-nonphosphorylated ERK1/2 were from Santa Cruz Biotechnology (Santa Cruz, CA); Cell cycle and Apoptosis Antibody Sampler Kit from Cell Signaling Technology (Cell Signaling Technology, USA). PD98059 (Beyotime, China) was used to inhibit phosphorylation of ERK1/2.

2.4. Enzyme-linked immunosorbent assay (ELISA)

The expression of CXCL8 by patients and cell lines was assessed by ELISA kits (RayBiotech, USA). The supernatant was collected and immediately processed for ELISA as instructed by the manufacturer.

2.5. Cell proliferation assay

Cells were seeded in 96-well plates at a density of 5×10^3 cells/well. At 0, 24, 48, 72 h after plating, 20 μl MTT labeling reagents (Sigma, America) were added to each well and 100 μl solubilization buffer (10% SDS in 0.01 m HCl) were added following incubating at 37 °C for 4 h. After incubating at 37 °C overnight, the absorbance at 575 nm was detected by BioRad model 550 Microplate Reader (BioRad, USA).

2.6. Methylcellulose colony formation assay

Cells were seeded in 24-well plates at a density of 1×10^3 cells/well in semisolid methylcellulose medium (Stem Cell Technologies, Canada) with 10% FBS. After 7 days incubation, colonies were counted with an inverted microscope.

2.7. Cell cycle analysis

Cells were harvested at the number of 1×10^6 and washed with PBS, then resuspended in 1 ml PBS. After fixed in cold 75% ethanol at 4 °C overnight, cell cycle distribution was evaluated with PI/RNase staining solution (Tian-jin Sungene Biotech Co., Ltd., China) according to the manual by flow cytometry (Accuri C6, BD Biosciences).

2.8. Apoptosis assay

Cells were harvested at the number of 1×10^6 and washed with PBS, then resuspended in 1 ml PBS. The apoptosis of the cells was measured with Annexin V -APC/PI Apoptosis Analysis Kit (Tianjin Sungene Biotech, China) as recommended by the manufacturer and analyzed by flow cytometry.

2.9. Statistical analysis

Every experiment was repeated at least three times. All data were presented as mean value ± Standard error of the mean (SEM). All statistical analyses were analyzed with Student's t-test using GraphPad Prism software (San Diego, CA, USA). $P < 0.05$ was considered as statistically significant (*).

3. Results

3.1. Increased expression of CXCL8 in AML patients and cell lines

We compared CXCL8 expression in the serum of patients with AML and of healthy donors by ELISA. The results exposed that the expression of CXCL8 in AML patients was much higher ($P < 0.05$) (Fig. 1A). And the expression of CXCL8 in patients is heterogeneous. The patients who have suffered relapses expressed higher levels of CXCL8 than patients with primary AML ($P < 0.05$) (Fig. 1B). Moreover, we found that the higher level of CXCL8 in patient's sample had a consequent indication of relapse after complete remission. According to the average expression of CXCL8 in patients' sample, the high expression group had shorter complete remission time before recurrence ($P < 0.05$) (Fig. 1C). To find out the differentially expressed genetic pathways in peripheral blood mononuclear cells with high expression of CXCL8, we divided the transcripts of 19 AML samples into CXCL8 high and low groups using the median CXCL8 expression as the cutoff in the cohort (Fig. 1D). Ingenuity® Pathway Analysis (IPA) revealed significant dysregulation of pathways including PI3K/AKT signaling pathway, molecular

Please cite this article in press as: Y. Li, et al., CXCL8 is associated with the recurrence of patients with acute myeloid leukemia and cell proliferation in leukemia cell lines, Biochemical and Biophysical Research Communications (2018), https://doi.org/10.1016/j.bbrc.2018.03.181