All-trans retinoic acid inhibits HOXA7 expression in leukemia cell NB4

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Abstract: Leukemia is a malignant proliferative disease of blood system, which is caused by hyperplasia of white blood cells and infiltration into other tissues and organs with blood flow, leading to a series of clinical manifestations. In this study, we detected the expression of HOXA7 gene in human acute promyelocytic leukemia cell line NB4. The expression level of HOXA7 decreased in the presence of ATRA, which was able to inhibit the proliferation of NB4 cells. Furthermore, ATRA altered the morphology of NB4 cells. The study suggested that HOXA7 might be a new gene candidate that influences the maturation of acute myeloid leukemia, and provided the molecular basis for the treatment for acute promyelocytic leukemia.

Key words: NB4 cell line, HOXA7 gene, All-trans retinoic acid, acute promyelocytic leukemia.

Introduction

Leukemia is a malignant proliferative disease of blood system, which is caused by hyperplasia of white blood cells and infiltration into other tissues and organs with blood flow, leading to a series of clinical manifestations. Leukemia can occur in any age, and with the highest incidence in children, especially in preschool and school-age children (1, 2). The incidence rate was about 3-4/100000 in children less than 10 years old. In which, more than 90% leukemia were acute leukemia and few were chronic leukemia (3). According to the main infected cells, acute leukemia is divided into acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL).

Homeobox (HOX) gene was first discovered in Drosohila (4), which was also found in human body, and located on four chromosomes: No.7, 17, 12 and 2, thereby designated as A, B, C, D clusters (5, 6). Each cluster was consisted of multiple gene fragments with different size; in turn they were named numerically. More than thirty HOX genes were found, and most of them contained homeobox fragments of 180 nucleotides and transcribed sequences with 60 amino acids; which was called homeodomain with high conservatism.

HOX gene plays a regulatory role in growth and development, and its abnormal expression may lead to many diseases (7). Meanwhile, HOX gene plays an important role in the regulation of proliferation and differentiation of hematopoietic cells, which could participate in the development of the hematopoietic system and promote HSPC differentiate into erythroid, myeloid, lymphoid, etc (8, 9). HOX gene was not only expressed in normal hematopoietic cells, but also expressed in leukemia cells, and resulting in occurrence of leukemia by combining other transcription factors or other mutant gene (10, 11). In recent years, it has been found that all-trans retinoic acid (ATRA) could induce HOX gene expression in vivo and in vitro (12, 13). Further research found that the main inducers were retinoic acid receptor (RAR) (14). Retinoic acid (RA) and its receptor was combined with retinoic acid response element (RARE), leading to the activation or inhibition of gene transcription. RARE was present in the promoter region of HOX A1, A7, A9 genes (15). Treatment of acute promyelocytic leukemia (M3 type) using RA was the success cases of molecular targeted cancer therapy (16). Treatment of leukemia with ATRA had been confirmed to be associated with regulation of ATRA and HOX genes (17). However, few studies reported whether the HOXA7 gene was associated with leukemia and whether ATRA could regulate the human acute promyelocytic leukemia NB4 cell proliferation and the expression of HOXA7 gene in NB4 cells. In this study, we detected the effect of ATRA on NB4 cells, and the dynamic expression alteration of HOXA7 gene in the NB4 cells with the treatment of ATRA.

Materials and Methods

NB4 cell culture

The NB4 cell line was purchased from DSMZ. NB4 cells were cultured in RPMI1640 medium with 10% fetal bovine serum, added with 100 μg/ml penicillin-streptomycin, and placed in 5% CO2 incubator at 37°C.

Cell proliferation assay

Cell Counting Kit-8 (CCK-8) was used to test the cell proliferation. The kit was purchased from Beyotime Biotechnology, Shanghai, China. The protocol was strictly followed by the Kit manual. Briefly, 10μl CCK-8 solution was added into each well. The cells were incubated for 1 hour, and analyzed by a microplate reader (Bio-Rad). The cells were treated with ATRA to determine the optimal concentration. The OD values at 450nm were measured.

ATRA treatment

NB4 cells were divided into four groups. 1) Control

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group: without the ATRA treatment; 2) Group 4 h: 1 μmol/L ATRA were added into cells for 4 h; 3) Group 48 h: 1 μmol/L ATRA were added into cells for 48 h; 4) Group 96 h: 1 μmol/L ATRA were added into cell for 96 h.

**Wright Giemsa staining**

The Wright-Giemsa Stain (Sigma-Aldrich) was placed upon the cells to cover the entire surface for 3 minutes. Then, the cells were rinsed with PBS until the edges show faintly pinkish red. The slides were allowed to dry in the air, and mounted with mounting medium. The cell differentiation and apoptosis were identified by morphological changes.

**Immunocytochemistry**

The cells were fixed in 4% paraformaldehyde in PBS pH 7.4 for 10 min at room temperature. PBS containing 0.1% Triton X-100 was used to permeabilize the cells for 10 min, and incubated in H2O2 for 10 min. Then, the cells were incubated with 1% bovine serum albumin (BSA) for 30 min to block unspecific binding of the antibodies, with diluted primary rabbit anti-HOXA7 antibody (1:1000, Abcam) overnight at 4°C. The slides were incubated in the secondary antibody for 1 hour at room temperature, then in the staining solution for 5 min to develop the color. Finally, the slides were mounted in resinene.

**Real Time PCR**

The protocol for Real Time PCR was reported previously (18). Briefly, the total RNA was extracted by Trizol (Life Technologies). Synthesis of cDNA was performed by using one-step RT-PCR kit from Takara. A mock control was carried out to exclude DNA contamination. Primers used are as follows: HOXA7, 5’-CCCTGCAGCGGTCTT-3’ and 5’-CCTTCGTTA-TGCTCTTTC-3’. GAPDH, 5’-ATGCTGCGC-GTGAATGTCCGC-3’ and 5’-GGAGTGATTCCCTT-CACGATA-3’. SYBR Green (Toyobo) RT-PCR amplification and real time fluorescence detection were performed using the PRISM 7300 sequence detection system (Applied Biosystems). Relative gene expression was calculated by the ΔΔCt method. The calibrator was the unstimulated control sample. At the end of the experiments, the products were analyzed by gel electrophoresis to confirm the presence and assess the purity of the amplicons of interest. Each sample was analyzed in triplicate.

**Western Blotting**

The protocol for Western Blotting was reported previously (19). Briefly, 2 μg cell lysates were loaded on each lane of 10% polyacrylamide gel, and then blotted onto a polyvinylidenedifluoride (PVDF) membrane. After blocking with a PBST containing 5% nonfat dry milk, the blots were incubated with antibodies against HOXA7 (1:500, Abcam). Peroxidase-linked anti rabbit IgG (Invitrogen) were used as secondary antibodies. These proteins were visualized by using an ECL western blotting detection kit (Amersham Biosciences).

**Statistical analysis**

All data were expressed as mean±standard deviation (x±s) and analyzed with ANOVA by SPSS17.0 software. The normalized density for immunocytochemistry and western blotting results were analyzed by Image J. The normalized density for cell staining = total intensity/cell number. The normalized density for western blotting band = total intensity/area of selected region. Statistical differences were analyzed with LSD pairwise comparisons. A P<0.05 was considered as statistically significant.

**Results**

**Treatment of ATRA altered the morphology of the NB4 cells**

First, we used the CCK-8 to determine the optimal ATRA concentration. As shown in the Figure 1, 10 and 5μmol/L ATRA showed high cytotoxicity to NB4, while 1,0.5,0.1μmol/L did not have apparent cytotoxicity. Since the lower concentration may not have the biological effect on NB4 cells, we used the 1μmol/L ATRA in the following experiments.

After the treatment of 1μmol/L ATRA, we observed the apparent morphological alteration in the NB cells. Compared to the normal NB cells with round and large nuclei (Fig.2A), the cellular nuclei started to shrink and size of cytoplasm was increasing at 4 hours after ATRA treatment (Fig.2B). 24 hours after ATRA treatment, apparent changes were observed in the nuclei and cytoplasm (Fig.2C). The nuclei continued to shrink, while the cytoplasm expanded further. The edge between nuclei and cytoplasm became obvious, and nuclei-cytoplasm ratio decreased.

**Figure 1.** The effect of ATRA at different concentrations on the viability of NB4 cells.

**Figure 2.** Treatment of ATRA altered the morphology of the NB4 cells by Giemsa staining. (A) NB4 cells without the treatment of ATRA. (B-D) The NB4 cells were treated with ATRA for 4h, 24h, and 96h. Scale bar, 10 μm.
Statistical analysis for the change in HOXA7 mRNA detected the expression of stem cells, and the expressions would be increased. Liu found that HOXB8 were expressed in human umbilical cord blood, indicating the expression of HOX gene in blood system. In order to overcome and cure leukemia, numerous studies have been made in the pathogenesis and treatment of leukemia.

Discussion

Acute leukemia is a common cancer in children. At present, the basic treatment was chemotherapy, while the peripheral blood stem cell transplant, cord blood stem cell transplant, gene therapy are emerging. Although these methods have been used in clinic, the prognosis of leukemia is still not ideal with high recurrence rate. In order to overcome and cure leukemia, numerous studies have been made in the pathogenesis and treatment of leukemia.

In our previous experiments, we also studied the expression of HOX gene in blood system. Liu found that HOXB8 were expressed in human umbilical cord blood stem cells, and the expressions would be increased.

Figure 3. Treatment of ATRA decreased the expression of HOXA7 detected by immunocytochemistry. (A) NB4 cells without the treatment of ATRA. (B-D) The NB4 cells were treated with ATRA for 4h, 24h, and 96h. (E) Negative control without the incubation of primary antibody. (F) Statistical analysis for the immunocytochemistry data. *p<0.05, **p<0.01. Scale bar, 10 µm.

Figure 4. Statistical analysis for the change in HOXA7 mRNA level in the presence of ATRA. *p<0.05, **p<0.01.

Figure 5. Treatment of ATRA decreased the HOXA7 protein level in the NB4 cells. (A) Western blotting analysis indicated HOXA7 expression level was decreased in the presence of ATRA. (B) Statistical analysis for the western blotting data. *p<0.05, **p<0.01.
analyze the gene expression, and found that the expression of HOXB3, HOXB4, HOXA7-11 were increased, suggesting that disorders of HOX genes might be related to the incidence of AML (11).

Using FQ-RT-PCR, Western blot, immunohistochemistry and other techniques, human acute promyelocytic leukemia NB4 cells as leukemia model, ATRA as inducer, we found that 1 μmol/L ATRA could promote NB4 cell differentiation and maturation, HOXA7 gene and protein were stably expressed in NB4 cells, and ATRA could decrease the expression of HOXA7 gene and protein (P<0.05), which was consistent with previous studies. The expression of HOXA7 was increased in AML and down-regulated treating with ATRA, suggesting that the molecular mechanisms of treatment of leukemia with ATRA might be related to regulation of HOXA7 expression.

In summary, the molecular mechanisms of treatment of leukemia with ATRA might be related to regulation of HOXA7 gene and protein expression. Our study showed that treatment of promyelocytic leukemia with ATRA might be via down-regulation of HOXA7 gene. Studies also suggested that ATRA was an effective drug in treatment of acute promyelocytic leukemia. ATRA could reduce the proliferation of cancer cells and promote their differentiation to block the proliferation of malignant diseases. Thereby we speculate that ATRA can be used to treat leukemia through intervention of HOXA7 gene expression.

References