Expression and effect of serum interleukin-24 level on bone marrow mononuclear cells in children with acute leukemia

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ABSTRACT. To investigate the expression of interleukin-24 (IL-24) in the children with acute leukemia (AL) and its effect on the apoptosis of bone marrow mononuclear cells (BMMNCs) *in vitro*. Four groups were assessed: acute lymphocytic leukemia (ALL), acute myeloid leukemia (AML), non-leukemia, and healthy groups, 20 children in each group. ELISA was used to measure IL-24 serum level. The bone marrow was taken from patients and controls. BMMNCs were isolated and the DNA was analyzed by glucose electrophoresis. Flow cytometry was used to determine BMMNC apoptosis. The serum level of IL-24 in the ALL and AML groups were significantly higher than in the other two groups. There was no statistical difference between ALL and AML groups, either between non-leukemia and healthy groups. BMMNCs were exposed to IL-24 for 48 h, and the apoptotic rate of the group treated with 50 ng/ml IL-24 was obviously higher than that of control group (0 ng/mL). The serum IL-24 level of AL children decreased comparing to non-leukemia

and healthy children, indicating that IL-24 can induce BMMNCs apoptosis of AL children *in vitro*.

Key words: Acute leukemia; Children; Interleukin-24; Mononuclear cell; Apoptosis

INTRODUCTION

Acute leukemia (AL) is a serious disease in children, with immune factors playing an important role in its occurrence and development (Chada et al., 2004). Chemotherapy is the most important treatment, but it has considerable side effects. Therefore, it is utmost important to find an effective alternative method for the treatment of AL. Interleukin-24 (IL-24) is a novel potential cytokine for cancer therapy, since it can selectively induce the apoptosis of tumor cells, while having no effect on normal cells (Chada et al., 2004; Dong et al., 2008). It is prospective to treat acute leukemia with IL-24. In this study, the serum levels of IL-24 in AL, non-leukemia, and healthy control groups of children were determined. We discuss its effect on inducing bone marrow mononuclear cells (BMMNC) apoptosis in children with AL by flow cytometry *in vitro*, in order to provide a theoretical basis for exploring the pathogenesis and treatment of children with AL.

MATERIAL AND METHODS

Patients

All samples were of newly diagnosed children from our hospital, diagnosed according to the international guidelines (Zhang, 2007), or healthy children, all of which have not received any treatment of cytokines, hormones, or any other chemical drugs. There were 40 cases of children with acute leukemia, 25 male and 15 female, with an average age of 6.8 years (within 4-14). These cases included 20 children with acute lymphoblastic leukemia (ALL; 12 male and 8 female) and 20 with acute myeloid leukemia (AML; 13 male and 7 female). There were 20 cases of non-leukemia patients, 13 male and 7 female, with an average age of 5.6 years (within 2-13), among which 10 had iron deficiency anemia, 4 had megaloblastic anemia, 3 had glucose-6-phosphate dehydrogenase (G6PD) deficiency, and the last 3 had thalassemia. Finally, there were 20 cases of normal children (control group), 10 male and 10 female, with ages between 4 and 10 years old. All patients provided a written informed consent.

Extraction of samples

Peripheral blood (5 mL) was extracted from each subject and it was centrifuged at 2000 *g* for 5 min, after which the supernatant serum was transferred to another tube and stored at -20°C. Bone marrow (3-5 mL) was extracted from each subject and mixed in the heparin anticoagulation tube. Then, it was diluted in Iscove's Modified Dulbecco's Medium (IMDM) with a 1:1 ratio to separate mononuclear cells with lymphocytes from the rest of the bone marrow, taking care to keep all the process aseptic. Specimens collected were stored at 4°C.

Measurement of IL-24

The separated serum thawed at room temperature for 20 min and 10 µL of serum was used to measure the IL-24 according to ELISA Kit instructions, which was purchased from Shanghai Yan Hui Biotechnology Co. Ltd.

Leukemia BMMNCs cultured *in vitro*

Bone marrow mononuclear cells were subjected to a density gradient centrifugation protocol using lymphocyte separation medium (d = 1.077 g/mL) (P8610-200, Solarbio Technology Co., Ltd., Beijing, China). Then, it BMMNC phase was washed twice with PBS and the cell concentration was adjusted to 2 x 10^6 cells/mL using RPMI1640 culture medium, which contains 10% inactivated fetal bovine serum (FBS) and 1% L-glutamine. A volume of 1.0 mL of cell suspension was added to each well of a 24-well culture plate and the plate was incubated at 37°C, 5% CO_2, and 100% humidity for 72 h. The cells were inoculated with 0, 5, 10, 20, 50, and 100 ng/mL IL-24, in triplicate and collected after being cultured for 0, 12, 24, 36, 48, 60, and 72 h. The percentage of viable cells was calculated using trypan blue staining 0.05%. When the cells entered the logarithmic growth phase, they were collected for the MTT assay.

BMMNC inhibitory rate using the MTT assay

Cells cultured for 48 h were collected and added to 96-well plates. The cell number per well was adjusted to 2 x 10^5 cells/mL by adjusting the serum concentration to 10%. A volume of 200 µL was used per well, with 20 µL MTT added to each well, and the plate was cultured at 37°C for 4 h. The supernatant was discarded and 200 µL DMSO were added to each well, after which the plate was oscillated in a micro-oscillator for 10 min or until the purple crystals completely dissolved. A microplate reader was used to determine the concentration from the absorbance values at a wavelength of 570/630 nm. Using the cells inoculated with no IL-24 (0 mg/mL) as the control group, the BMMNC inhibition rate (IR) by IL-24 was calculated as [1 - (treatment group value) / (control group value)] x 100%. Then, a dose-effect relationship curve was used to find the concentration at which the highest inhibition rate of IL-24 occurs. The MTT assay was also used to determine the BMMNC growth inhibition rate curves, in order to understand the cell proliferation under those conditions.

BMMNC apoptosis detection

Agarose gel electrophoresis was used to detect DNA. After 48 h, cultured cells inoculated with 50 ng/mL IL-24 (treatment cells) and with no IL-24 (control cells) were collected and washed twice with PBS, after which the cell lysate was added. The DNA samples were electrophoresed on a 2% agarose gel with TBE electrophoresis buffer at constant voltage of 100 V for 1 h.

Flow cytometry

Treatment cells (50 ng/mL IL-24) and control cells (no IL-24) were collected after being cultured for 48 h. They were fixed for 24 h with ethanol 75% at 4°C, then washed twice with PBS and added 50 µg/mL propidium iodide (PI), and left dyeing for 30 min at 4°C. Flow cytometry was

used to analyze the cell cycle and calculate the percentage of apoptotic cells. To exclude dead cells, a few cells were stained with PI and Hoechst 33342 before samples were fixed. PI-negative rate was over 95%.

Statistical analysis

SPSS statistical software (version 11.5) was used for statistical analysis, namely pairwise comparisons using a paired Student *t*-test.

RESULTS

Serum level of IL-24

The IL-24 serum levels in the different groups are summarized in Table 1. IL-24 serum concentrations of the AML group were significantly lower than the control group ($P < 0.05$). IL-24 serum concentrations of ALL and AML groups were significantly lower than the non-leukemia group ($P < 0.05$). The differences between ALL and AML groups, and between control and non-leukemia groups were not statistically significant ($P > 0.05$).

Table 1. Serum levels of IL-24 in different groups.

Group	Number (N)	Serum level of IL-24, pg/mL (mean ± SD)
ALL	20	28.25 ± 2.6*
AML	20	26.32 ± 3.2▲
Non-AL	20	105.82 ± 4.2#
Normal control	20	113.15 ± 3.7Δ

Compared ▲ with # and Δ, q = 3.92, 4.15, $P < 0.05$. Compared * with # and Δ, q = 4.26, 3.82, $P < 0.05$.

BMMNC inhibition rate reacted to IL-24 in AL patients

Increasing concentrations of IL-24 up to 50 ng/mL lead to increasing IR, while the inhibition effect is reduced for greater concentrations (Figure 1). The differences between the group inoculated with 50 ng/mL IL-24 and the other groups were statistically significant ($P < 0.05$), which is consistent with the cell count results. When one considers BMMNCs cultured with 50 ng/mL IL-24, the IR increases with time within the first 48 h of growth, while after that time the inhibition effect is weakened (Figure 2). The differences between the time point at 48 h and the other time points were statistically significant ($P < 0.05$), consistent with the cell count results.

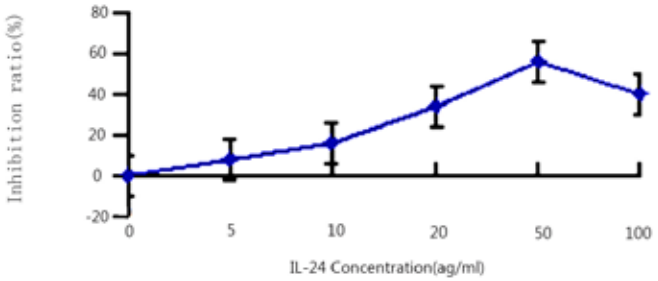

Figure 1. Inhibition rate of AL BMMNCs cultured *in vitro* at various IL-24 concentrations.

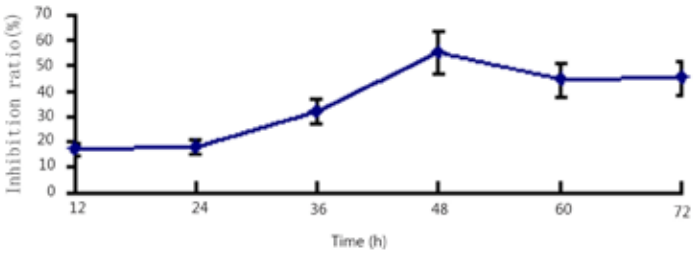

Figure 2. Inhibition rate of AL BMMNCs cultured *in vitro* with 50 ng/mL IL-24 at different growth times.

In vitro effect of IL-24 on BMMNC apoptosis in AL children

The main biochemical features of apoptosis is that the chromatin-concentrated junction between the nucleosome units of chromatin is broken, forming a long DNA fragment of 50-300 kbp or 180-200 bp fragments of an integral multiple of oligonucleotides. Trapezoid electrophoresis patterns (DNA ladder) are visible on the electrophoresis gel (Figure 3).

Figure 3. Electrophoresis pattern on agarose gel of DNA extracted from AL BMMNCs. *Lane 1* = untreated group; *lane 2* = group treated with 50 ng/mL IL-24.

Flow cytometry

Flow cytometry with PI staining showed that the apoptotic peak appeared before the G0/G1 phase, after the staining. The percentage of apoptotic cells in the control group (no IL-24) was significantly lower than in the treatment group (50 ng/mL IL-24), with statistical significance (Table 2, Figure 4).

Table 2. Morphological change and apoptosis rate of BMMNCs cultured with IL-24 for 48 h.

Group	N	Morphological change (%)	Apoptosis rate (%)
Control group (no IL-24)	40	9.5 ± 1.5$^{\Delta}$	9.8 ± 1.38$^{\Delta\Delta}$
Treatment group (50 ng/mL IL-24)	40	22.5 ± 6.21	23.35 ± 7.25

Compared Δ with 50 ng/mL IL-24, $P < 0.01$. Compared ΔΔ with 50 ng/mL IL-24, $P < 0.05$.

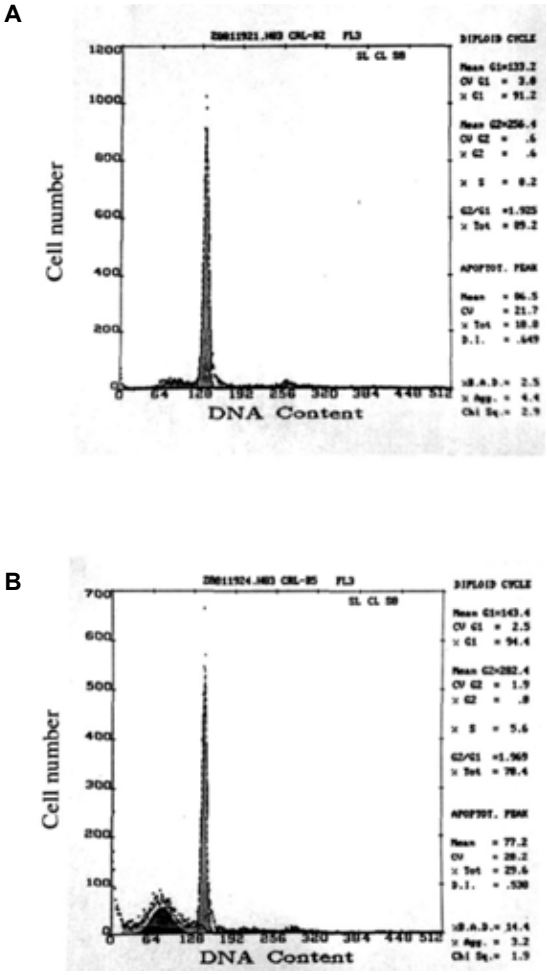

Figure 4. Apoptosis inducing effect of IL-24 on BMMNCs from children with AL. **A.** Untreated BMMNCs from AL patients (control). **B.** BMMNCs treated with 50 ng/mL IL-24 for 48 h, in which the peak of the sub-diploid population is seen.

DISCUSSION

In this study, we found the serum levels of IL-24 in ALL and AML patients are lower than in non-leukemia and normal control groups. However, the serum levels of IL-24 between ALL and AML have no statistically significant difference. This indicates that the IL-24 expression levels in children with AL are lower than in normal children.

IL-24 (also known as mda-7) is a recently found cytokine that is very promising as a potential tumor suppressor, and IL-24, IL-19, and IL-20 have the same receptors (Conti et al., 2003). In the immune system, IL-10 shows an immunosuppressive effect, while IL-24 shows immunostimulatory effects (Chada et al., 2004). Since IL-24 has an immunostimulatory effect, its level decline may cause an immune dysfunction in children with AL. Are the serum levels of IL-24

in AL children associated with the development and prognosis of the disease? By adjusting or stimulating IL-24 levels *in vitro*, can it show a clear anti-tumor effect? All of these questions remain unanswered. Therefore, continuous observing of IL-24 levels in AL children may help monitor the progress of the disease, and give hints about the prognosis and prediction of recurrence.

Application of exogenous IL-24 can inhibit various types of tumor cell growth, such as malignant glial cells, human breast cancer cells, and lung cancer cells, as well as induce apoptosis while leaving normal cells unharmed (Yacoub et al., 2003; Zheng et al., 2007; Sarkar et al., 2007; Sauane et al., 2008). Not only IL-24 induces solid tumor cell apoptosis, but it can also inhibit K562 (erythroleukemia cell line) and Namalwa (lymphoma cell line) growth and colony formation. There is another study suggesting that IL-24 has anti-tumor activity in chronic myelogenous leukemia and lymphatic tumors (Dong et al., 2008). Recent studies show that purified IL-24 can inhibit various types of tumor cell proliferations and induce apoptosis without mediation by adenovirus (Yang et al., 2007; Xiao et al., 2009). Malignant cells in acute leukemia (primarily white blood cells) are characterized by uncontrolled growth, which may be related to the dropping of IL-24 levels, failing to effectively inhibit the growth of malignant cells and induce apoptosis. Perhaps applying exogenous IL-24 may be effective to induce apoptosis in leukemia cells while keeping the normal cells unscathed.

In this study, we found that exogenous IL-24 can inhibit the proliferation of BMMNCs from AL patients. IL-24 inhibits AL BMMNC proliferation *in vitro* while its concentration is in the 0-50 ng/mL range, with a dose-dependent IR. AL BMMNC proliferation status is the highest when cultured for 48 h.

Apoptosis, also known as programmed cell death, is induced by factors *in vivo*, a physiological process of cell suicide strictly controlled by genes. DNA fragmentation can occur during apoptosis. There are two types of double-stranded DNA break when apoptosis occurs: the formation of high molecular DNA fragments of 50 or 300 kb, maybe caused by chromatin DNA loop fracture; and the breaking of DNA double-strands occurring in the nucleosome connecting section, forming a 180-200 bp fragment or multiple fragments. Therefore, “ladder”-shaped strips in an agarose gel electrophoresis are an indicator of apoptosis. This study shows that there is a clear ladder after treatment with IL-24, confirming the pro-apoptotic effect of IL-24. In this study, IL-24 is shown to induce the apoptosis of AL BMMNCs *in vitro*. Compared with the control group, 50 ng/mL IL-24 induced significant apoptosis of AL BMMNCs *in vitro*, suggesting that IL-24 may be able to improve and reverse AL. Similarly, Yang et al. (2007) also reported that 50 ng/mL IL-24 can inhibit the proliferation of A549 cells.

Through regulation of apoptosis-related genes, cytokines achieve their effect on apoptosis. IL-24, as a secreted protein, has both anti-tumor and immune stimulation roles. Nonetheless, this subject requires further investigation.

Conflicts of interest

The authors declare no conflict of interest.

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