Cytotoxicity of Human Cord Blood Natural Killer Cells is Enhanced by Recombinant Interleukin-15

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ABSTRACT

Hematopoietic cord blood (CB) stem cell transplantation has more advantages to other cell sources because of lower Graft Versus Host Disease (GVHD). Interleukin-15 (IL-15) is an immunoregulatory cytokine, known to enhance cytolytic function of cord Natural Killer (NK) cells. The aim of this study was to investigate the effect of IL-15 on NK cytotoxicity simultaneously in different cell death stages.

We compared the ability of IL-15 to enhance the NK cytotoxicity of CB in comparison to adult blood Mononuclear Cells (MNCs) against K562 target cells by co-staining with AnnexinV-FITC and Propidium Iodide after 3.5 h incubation at 37°C and 5% CO2 by using flow cytometric method. We also evaluated phenotypic changes after treatment by IL-15 in both cell sources.

Our results indicated that CB samples had lower level of apoptosis, while necrosis was negligible; also by escalating Effector: Target (E: T), we got higher level of apoptosis and necrosis in peripheral blood (PB). NK activity of cord and adult MNCs was enhanced by incubation with IL-15 (10 ng/ml) for 72 h with significantly higher results of PB in comparison to CB (p<0.0001). Moreover, IL-15 increased the percentage of CD3-CD56+ and CD25+ cells after 72 h incubation. Results showed incubation with human recombinant (hr) IL-15 for 3 days increased NK activity.

Taken together, these results indicated that NK cytotoxicity of CB MNCs could be augmented by human recombinant (hr) IL-15, but this activity did not reach to same level of PB counterparts.

We established that CD25 expression on CB MNCs could be increased with IL-15, in 72-hour cultures, but to a lesser degree compared to that on corresponding adult PB MNCs.

Key words: Apoptosis; Cord blood; Cytotoxicity; IL-15; Natural Killer; Necrosis
INTRODUCTION

Natural killer (NK) cells are a distinct lineage of lymphoid cells, defined as being membrane CD3−, CD16+ and/or CD56+, providing the first-line defense by lysing tumor and virus-infected cells in a non-major histocompatibility restricted fashion and without the need of prior sensitization. Decreased graft versus-host disease (GVHD), easier accessibility, and sustained engraftment encourage the use of umbilical cord blood (CB) as an alternative source to bone marrow for immune reconstitution for children with immunological and hematological disorders.

IL-15 has been found to play an important role in enhancing various lymphocyte functions, including NK and T-cell cytotoxicity. Cytokines, such as IL-2 and IL-15, increase the cytotoxicity of neonatal NK cells against K562 targets. Compared with adult NK cells, neonatal NK defects decreased NK and antibody-dependent cell-mediated cytotoxicity (ADCC). A number of researchers have confirmed up-regulation of neonatal Natural Killer Cytotoxicity (NKC) and ADCC to adult levels with IL-2-like cytokines, such as IL-12 and IL-15. We performed this study to show whether IL-15 enhances NK cytotoxicity of neonatal cells and also increases the percentage and absolute number of NK cells in culture for 3 days. We also investigated the effect of immunoregulatory cytokine: interleukin-15 (IL-15), on NK cytotoxicity and CD25 expression of mononuclear cells (MNCs) obtained from umbilical cord blood (CB) and adult peripheral blood (PB).

PATIENTS AND METHODS

The method of this study was adapted from the study by Choi et al with some modification.

Cord Blood Sample

MNCs were obtained from healthy adult volunteers and from the umbilical cord of term newborn infants after normal vaginal delivery. Cord blood was collected into sterile heparinized tubes and processed within 12 h of birth. Cord blood was used to obtain neonatal lymphocyte populations.

Isolation of Mononuclear Cells from Heparinized Blood

MNCs were separated from heparinized blood using Ficoll-Hypaque density gradient centrifugation and removing the turbid layer from the interphase, nucleated red blood cells (NRBC) of the cord blood were removed by NRBC lysis buffer. The isolated cells were resuspended at a density of 10⁶ cells/ml in RPMI 1640 medium (Sigma-Aldrich, St. Louis, Mo) supplemented with 10% heat-inactivated fetal calf serum (FCS) (GIBCO, Grand Island, N Y). MNCs were then phenotyped by flow cytometric methods (as described) or used as effector cells in cytotoxicity assays.

Cell Culture

MNCs were then placed in a 15-ml culture tube (Falcon 2095, Becton Dickinson, Oxnard, Calif.) or a 25-cm² tissue culture flask (Nunc, Inc., Newton, N.C.) with no cytokines or with recombinant IL-15 (R&D Systems, Minneapolis, Minn.). The cells were incubated at 37°C in 5% CO₂ without cytokine or with 10 ng/ml cytokine concentration for 72 h. Interleukin dosage for optimal in vitro NK activity were used in 10 ng/ml concentration. The cytokine was added at the same dosage every other day to the cultures to maintain optimal concentrations in the longer experiments. After incubation, the MNCs were washed and resuspended in RPMI to a density of 1 × 10⁶ cells/ml for cytotoxicity assays.

Evaluation of Surface Markers using Flow Cytometry

For Flow cytometric analysis, healthy adult and umbilical cord MNC were washed in cold phosphate-buffered saline containing 2% FCS and 0.1% sodium azide and then stained with fluorescein isothiocyanate- or phycoerythrin-conjugated mouse anti-human monoclonal antibodies. Five microliters of the appropriate fluorescent reagent was incubated with 5 × 10⁴ cells for 20 to 30 min at 4°C in the dark. The antibodies used were anti-CD3 FITC/CD56 PE and anti-CD25 FITC (all purchased from DAKO, Glostrup, Denmark). The cells were then washed twice with 2 ml of phosphate-buffered saline pH 7.2-7.4 at 4°C. The fluorescence staining was analyzed on a FACStar (Becton Dickinson) flow cyrometer after acquiring 10000 cells. Electronic gates were set to enable analysis of the fluorescence of the lymphocytes or monocytes in each preparation. The percentage of cells staining with each monoclonal antibody was determined by comparing each histogram with one from control cells stained with fluorescein isothiocyanate- or phycoerythrin-labeled anti-gamma-1
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monoclonal antibodies. Data were evaluated by Lysis II software (Becton Dickinson).

Cytotoxic Assay of Natural Killer Cells

For NK cytotoxicity assays, the erythroleukemic tumor cell line; K562 (cells obtained from the cell Bank of the Pasteure Institute of Iran) were used as targets, \(10^4\) cells in 10 \(\mu\)l were added to each tube (final concentration, \(1 \times 10^6/\text{ml}\)) in RPMI 1640 medium containing 10% FCS. To achieve a 20:1 effector:target cell ratio, \(2.0 \times 10^5\) isolated MNCs were added to each tube as effector cells and to preparing a 10:1 (E:T ratio) ; \(1.0 \times 10^5\) MNCs were added to targets. The final volume was adjusted to 0.2 ml for all tubes. After 3.5h incubation, the cells were stained with AnnexinV-FITC (IQ. Product, Groningen, Netherlands) and Propidium Iodide (Sigma-Aldrich, St. Louis, MO) made up at 100mg/ml in Phosphate Buffer Saline (PBS).

The percentage of cytotoxicity was assessed by analyzing Propidium Iodide (PI) incorporation by K562 cells for necrosis and AnnexinV-FITC (AXV-FITC) for apoptosis using flow cytometry.

Statistical Analysis

The Student’s t-test was used to compare the means with standard deviation (SD) for the different variables; n refers to the number of experiments performed. Data were considered significant if the p-value was <0.05.

RESULTS

Phenotype Analysis of MNCs in Cord and Peripheral Blood prior to IL-15 Treatment

To study the phenotype of cord blood NK cells and those in adult peripheral blood, the cell samples were stained and analyzed using flow cytometry (Figure 1).

As shown in Table 1, the difference observed in expression of CD56 surface marker in CB and adult PB was not significant. In contrast, average percentage of CD25 cells in CB was significantly decreased compared to adult PB (p<0.05).

Table 1. Phenotypic analysis of some lymphocyte subpopulations in adult peripheral and cord blood prior to IL-15 treatment (sample number 15)

<table>
<thead>
<tr>
<th>Lymphocyte subpopulation</th>
<th>Cord Blood (average percent ±SD)</th>
<th>Adult Peripheral Blood (average percent ±SD)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3-CD56+</td>
<td>3.46±2.33</td>
<td>5.21±3.04</td>
<td>p=0.14</td>
</tr>
<tr>
<td>CD 25+</td>
<td>1.38±0.72</td>
<td>2.75±1.84</td>
<td>p=0.015 *</td>
</tr>
</tbody>
</table>

(*: P Values lower than 0.05 were considered significant)

Figure 1. Frequency of CD3-CD56+ cells after staining with monoclonal antibodies CD3-FITC/CD56-PE is shown. A; lymphocytes have been gated based on (Forward Scatter) FSC and (Side Scatter) SSC. B; Respective isotype control in lymphocyte gate.C; Q1region: CD3-CD56+ cells in lymphocyte gate (Q1 = 7.8%) D; FL1 Histogram of isotype control in lymphocyte gate, E; total number of CD3+ cells in lymphocyte gate. F; FL2 Histogram of isotype control in lymphocyte gate, G; Total number of CD56+ cells in lymphocyte gate.

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Phenotype Analysis of MNCs in Cord and Peripheral Blood after IL-15 Treatment

Figures 2 and 3 show average percent of different lymphocyte phenotypes after the effect of IL-15 in 10 ng/L concentration for 72 hours. As shown in Figures 2 and 3, there was a significant difference in expression of CD56 and CD25 surface antigens on CB and adult PB lymphocytes after the effect of IL-15 compared to control (P<0.05).
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Figure 4. Evaluation of cytotoxic activity of mononuclear cord blood NK cells using AXV-FITC and PI. Top left; box of target cells K562 (R2) has been separated from the box of effector mononuclear cells (R1) based on FSC and SSC and selection of appropriate voltage. Top right; Dot plot of target cells after incubation with effector cells in 10:1 ratio (effector:target) that the Q1 box shows the AXV-FITC-/PI+ necrotic cells, Q2 AXV-FITC+/PI+ cells in late stages of apoptosis and necrosis, Q3 live AXV-FITC-/PI- cells, Q4 AXV-FITC+/ PI- target cells in early stage of apoptosis. Lower left histogram; Total AXV-FITC + cells in the target gate. Lower right histogram; total number of PI+ cells in the target gate.

Figure 5. Cytotoxic activity of mononuclear NK cells in adult peripheral blood NK cells compared with similar cells in cord blood. Mononuclear cells as effector cells were incubated in 10:1 and 20:1 ratios with K562 cells as target cells for 3.5 hours at 37°C. Mean cytotoxicity percent of K562 cell apoptosis at early stage of apoptosis and late apoptosis /necrosis stage are shown (samples number=15, p<0.05),(* P Values of lower than 0.05 were considered significant)
NK Cells early Apoptosis Prior to IL-15 Treatment

After incubation of effector and target cells and staining of cell suspension using AXV-FITC and PI, the samples were analyzed using Flow cytometry. Figure 4 shows the cytotoxicity of mononuclear NK cells using AXV-FITC and PI.

Average percent of cytotoxicity induced in early apoptosis pathway by NK cells in adult PB in 10:1 and 20:1 ratios were 20.23±3.39 and 26.04±4.34, respectively. The same percentages in late stages of apoptosis and necrosis were 14.53±4.37 and 20.27±2.42, respectively, while comparison of average percents in similar ratios in CB indicated that the cytotoxicity pathway induced by NK cells in CB was mainly apoptosis; and there was negligible necrosis (Figure 5). Increased ratio of effector:target cell increased target cell destruction only through apoptosis to some extent (p= 0.06) which was not significant, and no considerable difference was observed in late apoptosis and necrosis stages (p>0.05).

In addition, comparison of induced cytotoxicity from early apoptosis stage by PB NK cells compared to those in CB in both ratios of effector:target cells showed significant difference (p<0.05).

NK Cells early Apoptosis after IL-15 Treatment

The effect of hr IL-15 on cytotoxic activity of adult PB and CB NK cells as effector against K562 target cells is shown in figure 6. Comparison of the results in both cell sources indicated that hr IL-15 considerably increased the cytotoxicity of mononuclear NK cells in both cell sources compared to control sample (MNCs in complete culture medium without cytokine). This increase is important in mononuclear CB cells, but these cells are less active than adult PB cells. Therefore, there was a significant difference in cytotoxic activity of NK cells in early apoptosis stage against K562 cells after the effect of IL-15 (p<0.05).

NK Cells late Apoptosis/ Necrosis Prior to IL-15 Treatment

As shown in Figure 5, increased ratio of effector:target cells in adult PB increased the induced cytotoxicity compared to CB (p<0.05), while increased ratio of effector:target cells showed no significant change in cytotoxicity induction by CB MNCs in this pathway. Comparison of mean percent of cytotoxicity induced by CB MNCs in both pathways indicated no significant difference in any ratio.

Figure 6. Effect of hr IL-15 on cytotoxic activity of NK cells in adult peripheral blood and cord blood compared with control. Mononuclear cells were cultured with IL-15 (10 ng/ ml) or without this cytokine for 72 hours in 37 °C and 5% CO2. Mononuclear cells as effector cells were incubated with K562 cells as target in a ratio of 20:1 for 3.5 hours. Mean cytotoxicity percent of K562 cell at early apoptosis stage and late apoptosis /necrosis stage are shown (sample number= 15, p<0.05).

(*: P Values of lower than 0.05 were considered significant)
NK Cells Late Apoptosis/ Necrosis after IL-15 Treatment

Comparison of the results in both cell sources indicated that hr-IL-15 considerably increased the NK cytotoxic activity in late apoptosis and necrosis stages against K562 cells compared to control (Figure 6). Comparison of induced destruction by adult MNCs shows considerable increase relative to similar cells in CB (p<0.05).

Comparison of average percent of NK cytotoxicity from both apoptosis and necrosis pathways (figure 6) in both groups showed that adult PB MNCs after treatment with hr-IL-15 induced mainly apoptosis in K562 cells and induce necrosis to a lesser extent.

DISCUSSION

IL-15 is crucial for development and survival of NK cells and is also an important cytokine for survival and activation of memory T cells. It has biological functions and shared receptor components with Interleukin-2. IL-15 increases cytotoxic activity of NK and CD8+ T cells and also is able to dramatically increase the percentage and numbers of NK cells in cord blood and adult MNC cultures.

In this study, the expression of CD56 and CD25 were evaluated at first, no significant difference in CD56 of CB in comparison with adult PB was observed. This is in agreement with most other research groups. Although the results of our research are in agreement with most other research groups, there are some controversies in this regard indicated deceased expression of CD56 antigen on CB lymphocytes relative to adult blood. This difference might be due to different methods used by groups of researchers, as in partly lysing buffer and in some other magnetic beads was used for isolating NK cells. In this study, CD25 in CB showed significant decrease relative to CD25 expression of adult PB, similar to results of most researches. Decreased expression of CD25 in CB cells may be due to lack of prior stimulations. The role of CB cells in lower incidence of GVHD can be due to decreased CD25 expression in these cells.

Following the effect of IL-15 on CB and PB MNCs, the results showed that there was a significant difference in expression of CD25 and CD56 surface antigens on these cells compared to control (MNCs in complete culture medium without cytokine).

Gaddy and Broxmeyer also showed that IL-15 caused maturation of intermediate precursor CD56-CD16+ CB cells to phenotypically mature NK cells with high lytic property. Precursor NK cells of CB can develop under the effect of IL-2, IL-12, IL-7 and IL-15.

After the effect of IL-15, CD25 expression on CB and PB cells increased compared to control due to increasing effect of this cytokine on T cell activation.

The mechanism of the low cytotoxicity of cord NK cells is not well understood. Many studies indicated that CB NK activity is most likely suppressed by specific cells and others have also showed increased suppressor cells in CB.

In our study, increased effector:target ratio in adult PB samples increased two populations of dead target cells: AXV-FITC+/PI- cells involved in early apoptosis stages and AXV-FITC+/PI+ cells in late apoptosis/necrosis stages, while there was no significant increase in CB samples by increasing effector:target ratio.

Induction of cytotoxic activity in K562 cells by adult PB effector cells proceeds in both apoptosis and necrosis pathways, whereas induction of this activity by effector cells in CB was mainly induced through early apoptosis pathway with negligible necrosis in these cells.

In our results, increased effector:target ratio in cord blood samples caused no significant increase in cytotoxic activity of NK cells from early apoptosis pathway (p<0.05).

In addition, as the results of this study have indicated, despite lack of significant difference in CD56 marker expression in CB and PB cells, lower level of cytotoxic activity was observed in CB, confirming no relationship between low cytotoxic activity of cord blood by number and percentage of NK cells. This is confirmed considering the results of this research, since no significant increase in cytotoxicity of these cells was observed by increased effector:target ratio in cord blood samples.

In evaluation of the effect of hr IL-15 on cytotoxic activity of CB and PB NK cells, the results of this study showed that this cytokine has considerably increased cytotoxicity in both cell sources compared to control. This increase, while high for CB MNCs, was lower than PB cells and was statistically significant.
Various studies showed that using cytokines; IL-2, IL-12 and IL-15 alone or in combination increased cytolytic activity of CB and PB NK cells against K562 target cells are induced\textsuperscript{26, 27}. The utilization of multi-parameter flow cytometry to evaluate K562 cytotoxicity as we used in our study –such as other flow cytometric based studies\textsuperscript{28-30} – may prove to be even more sensitive as the methods based on the changes in plasma membrane permeability like Chromium release which only detects necrotic cells. Flow cytometry co-staining makes possible to evaluate cytotoxicity simultaneously in different cell death stages; apoptosis and necrosis.

Cytotoxic activity of CB and PB NK cells increased by treatment with cytokines IL-2, IL-12 and IL-15 for 24 hours, but this level of NK activity in cord blood never attains that of adult blood in the same culture conditions. NK activity level in 3 days is higher than activity in 24 hours, thus increased incubation time of cells with hrIL-15 causes increase cytotoxic activity of NK cells.\textsuperscript{13}

On the other hand, induction of killing activity in CB NK cells after treatment of the cells with hrIL-15 was done in both apoptosis and necrosis pathways, while fresh CB MNCs mainly eliminated cell targets through apoptotic pathway, as stated in results section.

Therefore, it can be stated that apoptosis, the physiological form of cell death in vivo, is a primary cytotoxic mechanism of cellular cytotoxicity. This does not seem to be true for all cells and is related to cell source of effector, because Gardiner, while reporting and confirming the existence of two NK cytotoxicity pathways for CB MNCs after induction by rIL-2, demonstrates that IL-2 is not capable of inducing apoptotic mechanism in activated NK cells in bone marrow.\textsuperscript{31}

In conclusion, cytotoxic activity of NK cells in CB is lower than NK cells in PB. Recombinant IL-15, as a stimulating factor on NK cells, increases cytotoxic activity of these cells but this increase is more prominent in PB NK cells. The reason of lower increase in weaker cytotoxic activity of CB NK cells in comparison to PB NK cells could be attributed to; lower density in CD56 expression, specific inhibitory cells present in cord blood or the existence of intrinsic inhibitory factors or immaturity of these cells (low level of perforin).

Considering the insignificant difference of CD56 expression between CB and PB and the washing step of mononuclear cells during this experiment supports, the hypothesis regarding intrinsic inhibitory factor is stronger. Therefore, there is the possibility that effector NK cells in CB are different from PB NK cells, in as yet undefined property.

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