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ORIGINAL ARTICLE

Cytotoxic Effect of Cord Blood Derived Natural Killer Cells on Breast Cancer Cells

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OBJECTIVE

Human natural killer (NK) cells are cluster of differentiation 3 (CD3)-CD56 + lymphocytes. NK cells can be obtained from various sources such as peripheral blood and cord blood. Among those, cord blood (CB) is an important cellular resource. The aim of this study is to determine the cytotoxic effect of freshly isolated CB derived NK cells toward breast cancer cells *in vitro*.

METHODS

CB mononuclear cells were isolated by Ficoll-Paque and NK cells were selected by with magnetic activated cell sorting (MACS) technique. CB-NK cell's surface markers were quantified by flow cytometry analysis. Water-soluble tetrazolium salt cytotoxicity assay was used to measure cell viability; Annexin V/7-amino actinomycin D assay was used to measure apoptosis and necrosis. Enzyme-linked immunosorbent assay was used to determine perforin and Granzyme B activity of CB-NK cells.

RESULTS

Here, we show that CD56+ cells within the NK cell population were measured as 99.59% and CD314+ cells surface marker expression was measured as 99.48% after MACS selection. CB-NK cells exerted significant cytotoxicity toward Michigan cancer foundation 7 (MCF-7), MDA-231, and K562 tumor cells. Importantly, we show that CB-NK cells were able to kill MCF-7 and MDA-231 cells through apoptosis and necrosis, respectively. The amount of Granzyme B and perforin produced by CB-NK cells was measured as 50 ng/ml and 80 ng/ml, respectively.

CONCLUSION

Our findings confirm that freshly isolated CB-NK cells can be expanded *in vitro* with supplementation of various cytokines. We provide evidence that in these conditions, CB-NK cells exert efficiently cytotoxic effect and induced apoptotic and necrotic cell death toward breast cancer cells.

Keywords: Breast cancer; cancer immunotherapy; fresh cord blood natural killer cells.
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Introduction

Natural killer (NK) cells are produced in the bone marrow and settled in various lymphoid and non-lymphoid tissues including lymph nodes, spleen, peripheral blood, lung, and liver.[1] Human NK cells are cluster of differentiation 3 (CD3)-CD56 + lymphocytes.[2] Having many cytotoxic receptors that are effective in recognizing malignant cells. These receptors are called NKp46, NKp44, NKp30, NKG2A/B/C/CD94, NKG2D/CD314, KIR-2DS, KIR3DS, KIR-2DL, and KIR-3DL.[3] NK cells are activated by cytokines such as interleukin 2 (IL 2), IL 12, IL 15, and IL 18 and can induce apoptosis in tumor cells by several mechanisms. The

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most common tumor cell death mechanism is the induction of apoptosis in cancer cells through cytotoxic proteins secreted by NK cells such as perforin and granzyme B and cell death receptor and ligand interaction (Fas/FasL, etc.).[4] NK cells can be obtained from various sources including peripheral blood cord blood (CB) and bone marrow hematopoietic stem cells and can be differentiated from embryonic and induced pluripotent stem cells (IPS) which are important in cellular therapy today.[5]

CB is an important cellular resource of allogenic NK cells and can be preserved for many years with the cryopreservation technique.[6] CB has important advantages such as being suitable for allogenic use and being easily obtained.[7] CB derived NK cells displays the same quality as peripheral blood derived NK cells in terms of the amount of interferon gamma (INFγ) and tumor necrosis factor alpha production as well as expression of cytotoxic receptors.[8,9] In addition, CB and peripheral blood derived NK cells can be induced by same IL2, IL7, IL15, IL18, and IL21 for *in vitro* expansion.[10]

Breast cancer is one of the leading cancer deaths among women;[11] thus, alternative treatment approaches are in development in addition to chemotherapy and radiotherapy.[12] Recently, immunotherapy approach in breast cancer treatment has attracted considerable attention.

Here, in this study, we isolated NK cells from umbilical CB and expanded them *in vitro* by cytokine supplementation and eventually assessed the cytotoxic ability of CB-derived NK cells on breast cancer cells *in vitro*.

Materials and Methods

Ethics Statement

CB samples were obtained from cesarean deliveries after informed consent. The study complied with all provisions of the Declaration of Helsinki and were approved by non-interventional clinical research ethics committee of Aydin Adnan Menderes University Medicine Faculty, Aydın, Turkey (Ethics Committee Apr. No. 2018/1354).

Cell Culture

Michigan Cancer Foundation-7 (MCF-7) (ATCC® HTB-22™), MDA-231 (ATCC® HTB-26™) breast cancer cells, and the leukemia cell line K562 (ATCC® CCL-243™) were obtained from American Type Culture Collection cultured in Roswell Park Memorial Institute (RPMI) Medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA) and 1% penicillin/streptomycin (Gibco, USA).

Mononuclear Cell (MNC) Isolation and Culture

Six freshly collected CB (50 ml/unit) were layered over Ficoll Histopaque (Sigma, USA) and centrifuged at 400 g for 30 min. MNCs were collected at the interface and washed in phosphate-buffered saline (PBS) (Gibco, USA). CB MNCs were cultured in RPMI-1640 medium supplemented 500 IU/mL IL2 (ProSpec, Israel) for 1 week.[13]

Isolation and Expansion of NK Cells

CD56 positive NK cells from CB MNCs were isolated by immunomagnetic bead selection using the magnetic activated cell sorting (MACS) system according to the manufacturer's instructions. CD56+ CB-NK cells were cultured in RPMI-1640 medium supplemented 500 IU/mL IL2, 50 ng/ml stem cell factor (SCF) (ProSpec, Israel), 50 ng/ml fms-like tyrosine kinase 3 (FLT3) (ProSpec, Israel), and 40 ng/ml IL15 (ProSpec, Israel) cytokines for 1 week.[14]

Flow Cytometry Analysis

3×104 CB-NK cells were washed with PBS and incubated with phycoerythrin (PE) conjugated anti-CD56 antibody (1:10 dilution; BioLegend, USA), PE conjugated anti-CD314 antibody (1:10 dilution; BioLegend, USA), and isotype control antibody PE conjugated IgG1 antibody (1:10 dilution; BioLegend, USA) for 20 min at 4°C. Then, the cells were washed with PBS and surface markers expressions were analyzed by flow cytometry (Beckman Coulter, Life Sciences, USA).

Cytotoxicity Assay Water-soluble Tetrazolium Salt (WST1)

CB-NK, MCF-7, MDA-231, and K562 control cells or CB-NK cells mixed with tumor cells (MCF-7, MDA-231, and K562) as 1:1 and 1:2 (effector:target/E:T) ratios were plated at 10^4 cells/per well and in 96 well plates in a final volume of 100 µl/well culture medium in a humidified atmosphere (37°C and 5% CO_2). Cells were incubated for 4 h and following 10 µl Cell Proliferation Reagent WST-1 (Sigma, USA) was added to each well and incubated for 30 min at 37°C and 5% CO_2. The wavelength to measure absorbance was 450 nm on the microplate reader. The percentage of cytotoxicity was calculated from the following equation using corrected absorbance: % Cytotoxicity=(×100 (Control-Sample))/Control.

Apoptosis Analysis (Annexin V/7-Amino Actinomycin D [7AAD] Assay)

CB-NK cells were mixed with tumor cells (MCF-7, MDA-231, and K562) at 1:1 and 1:2 ratios. MCF-7, MDA-231, and K562 tumor cells were plated at 105

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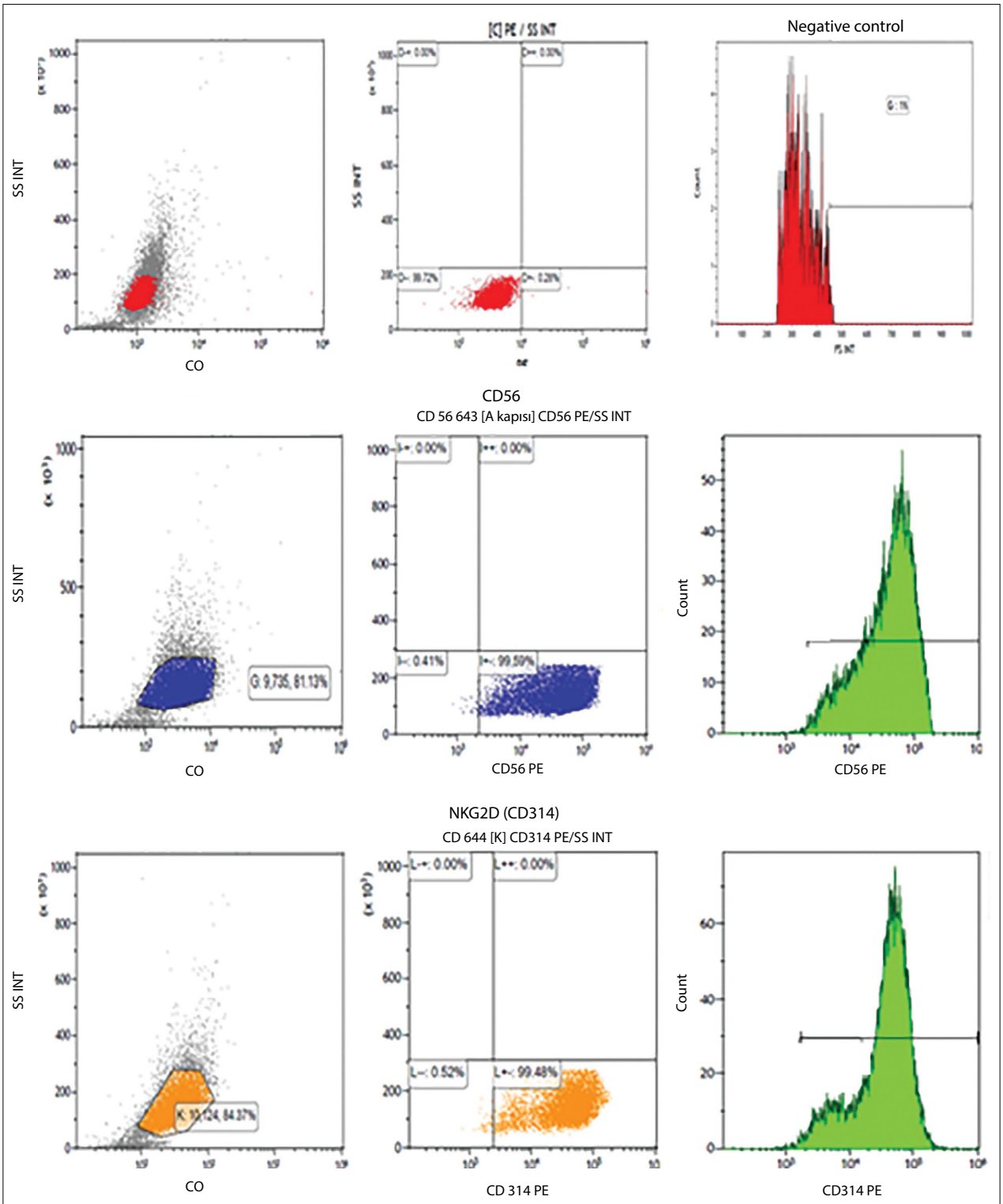

Fig. 1. The surface markers expression on CB-MNC and CB-NK cells. Flow cytometry was used to assess surface expression of CD56 and NKG2D after magnetic activated cell sorting (MACS) separation. Negative Control, CB-NK cell CD56 exp. after MACS, CB-NK cell CD314 exp. after MACS.

CO: Control (Ig G), PE: Phycoerythrin.

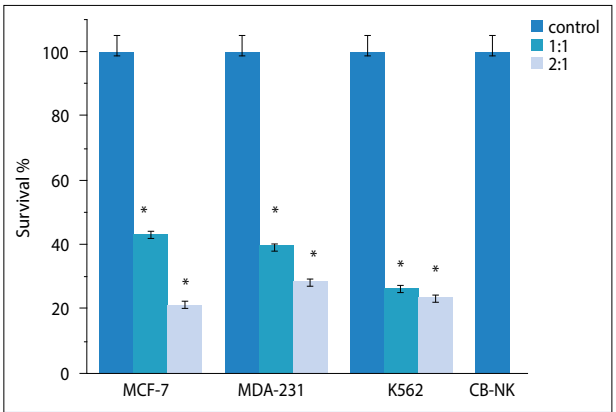

Fig. 2. Cytotoxic activity of CB-NK cells. Michigan cancer foundation 7, MDA-231, and K562 cells were incubated with CB-NK cells for 4 h at 1:1 and 1:2 Effector: Target ratios. WST-1assay used for measurement of cell viability. (*p<0.0001, Target vs. Targer+CB-NK).
MCF: Michigan cancer foundation, MDA: MD Anderson, CB-NK: Cord blood derived natural killer cells.

cells/per well and then CB-NK cells were added and mixed in 46 well plates and cells were incubated for 4 h at 37°C in a humidified atmosphere in a CO_2 incubator. Determination of apoptosis profile was achieved using Muse® Annexin V and Dead Cell Assay according to the manufacturer's instructions. Quantitative analysis of apoptotic and necrotic cells was evaluated by Muse Cell Analyzer (Millipore, Austin, TX, USA).

Measurement of Cytokine Production by Enzyme-linked Immunoabsorbent Assay (ELISA)

CB-NK cells were plated at 10^5 cells/per well in 46 well plates and cells were incubated for 24 h at 37°C in humidified atmosphere in a CO_2 incubator. Supernatants were collected from CB-NK cells after incubation and Granzyme B and perforin levels were measured using commercial ELISA kits (Booster, China), according to the manufacturer's instructions. Granzyme B and perforin levels in the supernatant were determined by measuring absorbance at 450 nm on a microplate reader.

Statistical Analysis

Statistical analysis was performed using Origin 8, Graph software. Paired sample student's t-test was used where indicated (*p<0.001; *p<0.0001). Data are expressed as means of three independent experiments±SD (standard deviation).

Results

CB-NK Cells Characterization

To examine the efficacy of CB-NK cells on breast cancer cells *in vitro*; initially, we isolated that NK cells characterization of CB-NK cells was performed with flow cytometry after immunomagnetic bead MACS selection. CD56+ cells were measured as 99.59% within NK cell population after MACS selection (Fig. 1). In addition, CD314+ cells surface marker expression was measured as 99.48% after MACS selection (Fig. 1).

CB-NK Cells Exert Cytotoxicity Towards Breast Cancer Cell Lines

We tested the cytotoxicity of *in vitro* expanded CB-NK cells toward MCF-7, MDA-231 cells using WST1 cytotoxicity assay. K562 cells were used as positive control which is known from literature.[15] As shown in Figure 2, our results demonstrate that CB-NK cells were able to lyse MCF-7, MDA-231 cell. CB-NK cells show variable cytotoxicity toward MCF-7, MDA-231, and K562 tumor cells. CB-NK cells lyse MCF-7 cells by 57% and MDA-231 cells by 60%. It was also 74% effective against K562 leukemia cells (Fig. 2).

CB-NK Cells Induced Apoptosis and Necrosis in Breast Cancer Cell Lines

To determine whether CB-NK cells induced cytotoxicity is mediated by cell death, we used Annexin V/7AAD assay. As shown in Figure 3, our results demonstrate that CB-NK cells were able to kill MCF-7 cell, by apoptotic cell death and apoptosis were detected as 50%. Interestingly, in MDA-231 cells, we detected mainly necrotic cell death. CB-NK induced necrotic cell death rate was 35% in MDA 231 breast cancer cells. In addition, when we measure the cell death in K562 cell line, we detected 63% of apoptosis. Thus, these results confirmed that CB-NK cells were able to kill breast cancer cell lines effectively (Fig. 3).

CB-NK Cells Produced Cytotoxic Proteins

Since we measured considerable amount of cell death against breast cancer cell lines, we measured whether CB-NK cell produced cytotoxic proteins Granzyme B and perforin. We measured the amount of Granzyme B and perforin by ELISA. Our results show that CB-NK cells produce different levels of Granzyme B and perforin. As shown in Figure 4, CB-NK cells produced 50 ng/ml Granzyme B and 80 ng/ml perforin cytotoxic proteins (Fig. 4).

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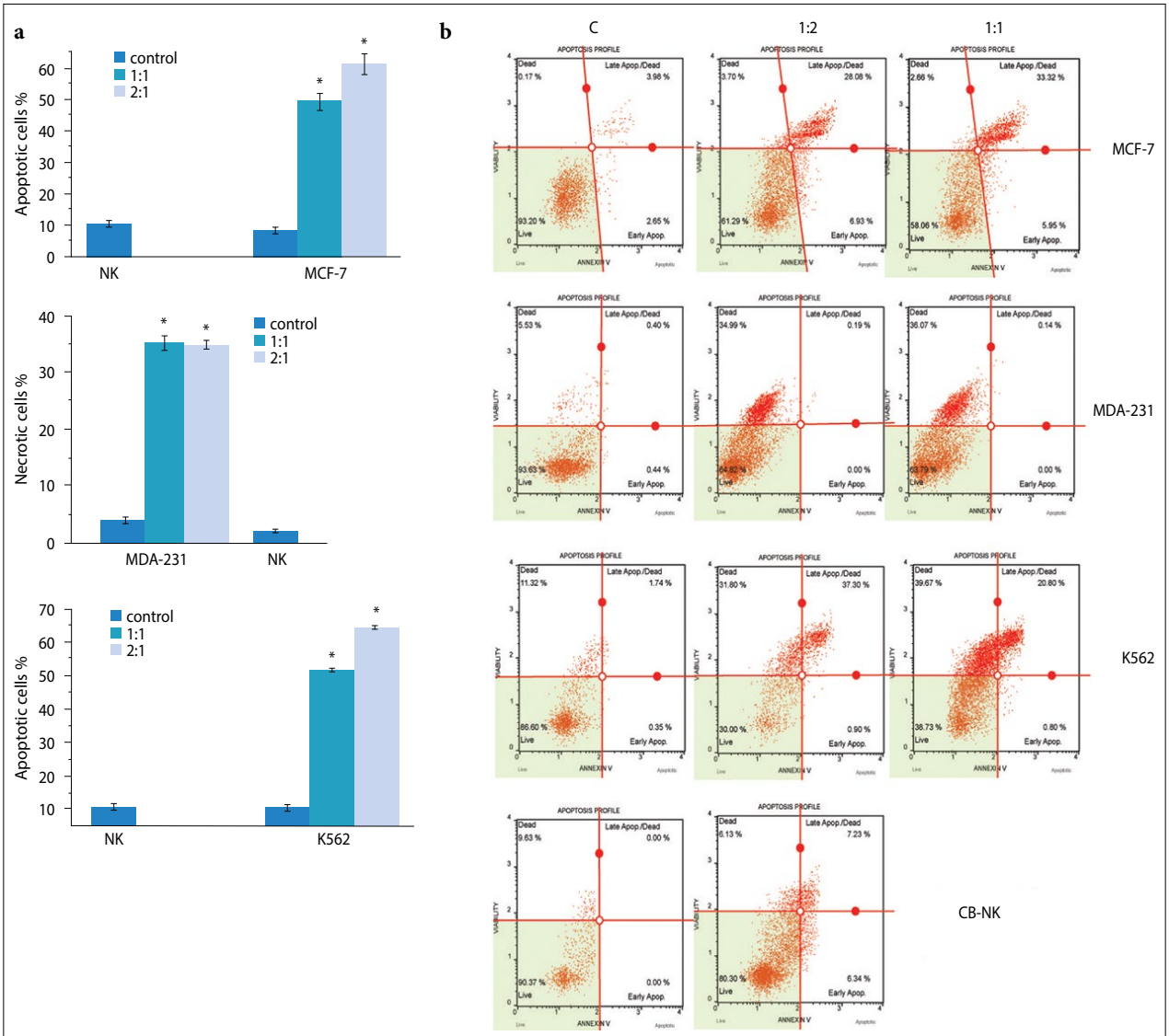

Fig. 3. (a) Quantification of apoptotic cells. (b) Flow cytometry plots. CB-NK cells induce apoptosis Michigan Cancer Foundation 7 (MCF-7) and K562 cells while induce necrosis MDA-231 cells. CB-NK cells and tumor target cells were incubated for 4 h at 1:1 and 2:1 E:T ratios and used Muse® Annexin V and Dead Cell Assay. Apoptotic cell death MCF-7, Necrotic cell death MDA-231, Apoptotic cell death K562, and Control CB-NK cell (*p<0.0001).
MCF: Michigan cancer foundation, MDA: MD Anderson, CB-NK: Cord blood derived natural killer cell, NK: Natural killer.

Discussion

NK cells are important mediators of immunotherapy approach and can be obtained from several sources including peripheral blood, CB and bone marrow hematopoietic stem cells, and embryonic or IPS.[5] Among those CB contains considerable amount of NK cells and has been considered as a promising source of NK cells for cellular immunotherapy. CB offers unique advantages, which are directly applicable to NK cell-directed allore-activity. The ease of collection of CB and cryopreservation possibilities makes them attractive source for NK cell immunotherapy. However, it has not been easy to expand a large number of high purity NK cells from CB; thus, various methods have been developed.[7-9]

Breast cancer is the most common type of cancer in women.[15] At present, cellular immunotherapy approach has gained great importance as significant therapeutic option in many other cancers including breast cancer. Although significant advances have

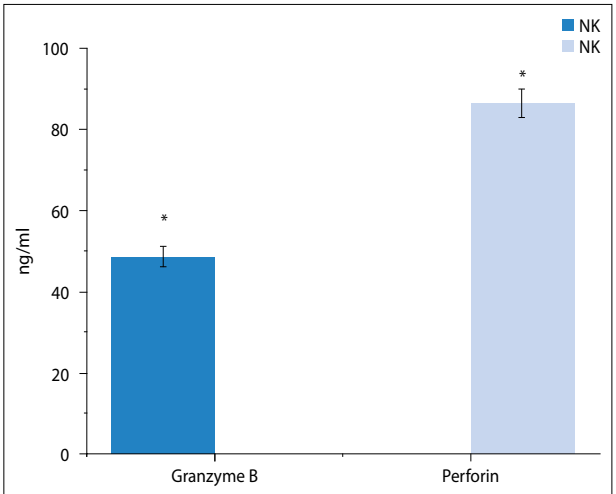

Fig. 4. CB-NK cell produced cytotoxic granules Granzyme B and perforin. Supernatants were collected from CB-NK cells after incubation, Granzyme B and perforin levels using the enzyme-linked immunosorbent assay kits. Granzyme B and perforin enzyme-linked immunoabsorbent assay (*p<0.001).
NK: Natural killer.

been made in immunotherapy, more studies are still needed in breast cancer. We are interested in using human CB derived NK cells as an alternative source of immunotherapy against human breast cancers. Here, we show that freshly isolated and *in vitro* expanded CB-NK cells can efficiently kill MCF-7 and MDA-231 breast cancer cells *in vitro*.

NK cells found in peripheral blood at rate of 10% whereas in CB it is 30%.[8] NK cells are characterized by their CD3-CD56+ surface marker expressions.[1] In our study, we isolated MNCs from CB according to CD56 positivity by magnetic separation and show that we can obtain a pure NK cell population by this technique (Fig. 1). Inhibitory and activator receptor expressions are of great importance in the regulation of NK cell functions. Peripheral blood and CB NK cells show a similar rate of NKG2D activator receptor (also known as CD314) expression.[16] Our result shows that NKG2D activator receptor is the high level of expression pattern (Fig. 1).

Many culture protocols have been developed for the growth of CB NK cells *in vitro*. Among these protocols; using different cytokine combinations SCF, FLT3 ligand, IL2, IL7, IL12, IL15, and IL18 and special feeder layers (CD3dep PBMC, antigen-presenting cell, mbIL-21 K562, etc.) are more common.[17-20] In our study, we used cytokine cocktail including SCF, FLT3 ligand, IL2, and IL15 for CB-NK cells culture.

In a previous study, Nham et al. have shown that CB NK cells have different cytotoxic effects on triple negative breast cancers and hormone positive breast cancer cells [14]. The presence of factors such as human leukocyte antigen-I (HLAI) and HLAG antigens and immunosuppressive factor transforming growth factor-beta expression or inhibitory receptor NKG2A expression, has been shown as limiting factors of the cytotoxic effect of NK cells.[14] In line with the previous studies, we also show that CB-NK cells have different levels of cytotoxic effects on breast cancer cells (Fig. 2). However, whether or not the same factors might be responsible for the differences will be future subject of our studies. One of the important finding of our study is that CB-NK cells induce apoptosis in MCF-7 cell (Fig. 3) but necrosis in MDA-231 cells (Fig. 3). Recently, Backes et al. show that NK cells are capable of inducing apoptosis, necrosis, and other mix forms of cell death in tumor cells [21,22]. Furthermore, it was found that mainly two major cytotoxic pathways perforin/granzyme release or FasL/FasR interaction mediates the cell death. On the other hand, it was shown that these pathways may suppress each other's effect depending on the amount of calcium entering to the cell.[21] In our case, the reason why CB-NK cells induced different types of cell death in different cell lines may be dependent on cell type or context. At present, we do not know the exact mechanism; further studies are needed to clarify these issues.

Our results demonstrating CB-NK cells produced perforin and Granzyme B cytotoxic proteins suggest that CB-NK mediated cytotoxic activity may be related to granzyme and perforin activity (Fig. 4). Previously, it was shown that NK cells exert cytotoxic activity by Granzyme B and perforin pathway.[1]

Limitations of the Study

In this study, the cytotoxic activity of CB derived NK cells on breast cancer cells was determined. However, by which mechanisms CB-NK cells induce apoptosis (INFγ or death cell receptor/ligand interactions) has not been evaluated in tumor cells.

Conclusion

CB is an important resource for cancer immunotherapy approaches because of its NK cell content. In our study, we investigated the effect of fresh CB-NK cells with common methods. Our findings confirm that freshly isolated CB-NK cells can be *in vitro* expanded

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solely with various cytokine combinations including SCF, Flt3 IL2, and IL15. In addition, we provide evidence that CB-NK cells exert efficiently cytotoxic effect and induced apoptotic and necrotic cell death toward breast cancer cells. Our study suggest that fresh CB can be used as an alternative resource for NK cell isolation which can be further expanded and used for cancer immunotherapy approaches. Future studies are needed to determine exact mechanism of cytotoxic activity of CB-NK cells.

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Conflict of Interest: The authors declare that they have no conflict of interest.

Ethics Committee Approval: The study was approved by the Non-interventional Clinical Research Ethics Committee of Aydın Adnan Menderes University Faculty of Medicine (No: 24, Date:12/04/2018).

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