ПУПОВИННАЯ КРОВЬ КАК ПЕРСПЕКТИВНЫЙ ИСТОЧНИК NK-КЛЕТОК ДЛЯ ИММУНОТЕРАПИИ

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Резюме. В настоящее время большое количество исследований по генной модификации NK-клеток пуповинной крови (UCB-NK) проводится как на клиническом, так и доклиническом уровне. Иммунотерапия на основе UCB-NK-клеток обладает большим терапевтическим потенциалом для использования в противоопухолевой терапии. Однако, несмотря на известные преимущества перед PB-NK-клетками, такие как высокая концентрация в пуповинной крови, низкий процент передачи вируса от донора, а также возможность сохранения фенотипа после криоконсервации, UCB-NK-клетки преимущественно характеризуют в научной литературе как незрелые и низкофункциональные NK-клетки. В данной работе были изучены фенотипические характеристики UCB-NK-клеток и возможность стимуляционной компенсации сниженной функциональной активности UCB-NK-клеток. Проведенные исследования выявили, что фенотипически UCB-NK-клетки можно охарактеризовать как малодифференцированные и слабоактивированные клетки, экспрессирующие высокий уровень ингибирующего рецептора NKG2A, низкий уровень активирующего рецептора NKG2C и молекулы активации HLA-DR, что соответствовало литературным данным. Для стимуляции свежевыделенных UCB-NK-клеток было выбрано два вида стимулов: 1) 100 ед IL-2; 2) комбинация 100 ед IL-2 и фидерных клеток K-562, экспрессирующих мембраносвязанный IL-21 (K562-mbIL21). Было показано, что при стимуляции UCB-NK-клеток в течение 7 дней комбинацией IL-2 и K562-mbIL21 уровень дегра-

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нуляции (LAMP-1) и пролиферативная активность этих клеток была выше, чем у параллельно культивируемых в тех же условиях *Ex vivo* PB-NK-клеток, при этом стимул в виде IL-2 и K562-mbIL21 оказался более перспективным способом получения большого количества пролиферативно активных UCB-NK-клеток, по сравнению со стимуляцией только IL-2. Поскольку генетическая модификация NK-клеток является перспективным направлением улучшения противоопухолевых свойств NK-клеток, для дальнейшего изучения полученных UCB-NK-клеток была проведена процедура ретровирусной трансдукции. UCB-NK-клетки, стимулированные комбинацией IL-2 и K562-mbIL21, трансдуцировались на 8-й день культивирования. В данной работе применялась направленная оверэкспрессия адапторной молекулы DAP12, участвующей в сигналинге активирующих NK-клеточных рецепторов. PB-NK-клетки и UCB-NK-клетки трансдуцировали параллельно, в одинаковых экспериментальных условиях при равном объеме вирусных частиц. В результате было выявлено, что эффективность трансдукции вирусными частицами, несущими ген адапторной молекулы DAP12, в более чем 4 раза выше для UCB-NK-клеток по сравнению с PB-NK-клетками. Таким образом UCB-NK-клетки представляются перспективным инструментом для дальнейших исследований в области иммунотерапии рака.

Ключевые слова: UCB-NK-клетки, фенотип, стимуляция NK-клеток, функциональная активность, трансдукция, DAP12

UMBILICAL CORD BLOOD AS A PROMISING SOURCE OF NK CELLS FOR IMMUNOTHERAPY

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Abstract. Currently, a large number of studies on genetic modification of cord blood NK cells (UCB-NK) are carried out at both clinical and preclinical levels. Immunotherapy based on UCB-NK cells has great potential for antitumor therapy. However, despite having known several advantages over peripheral blood NK cells (PB-NK), including a high concentration in cord blood and low virulence rate, UCB-NK cells are predominantly characterized in the scientific literature as immature and low-functioning NK cells. In this work, we studied the phenotypic characteristics of UCB-NK cells and the possibility of stimulatory compensation of the decreased functional activity of UCB-NK cells. Our studies revealed UCB-NK cells can be characterized as poorly differentiated and weakly activated cells with high level of inhibitory receptor NKG2A and low level of activating receptor NKG2C and HLA-DR, accordingly with the literature data. Two types of stimuli were chosen to stimulate freshly isolated UCB-NK cells: 1) 100 units of IL-2; 2) combinations of 100 units IL-2 and K-562 feeder cells expressing membrane-bound IL-21 (K562-mbIL21). It was shown the degranulation (LAMP-1) and proliferative activity was higher than for parallel cultured *ex vivo* PB-NK cells under the same conditions for UCB-NK cells stimulated for 7 days with IL-2 + K562-mbIL21. Moreover, stimulation in the way of IL-2 + K562-mbIL21 seemed to be a more perspective way to obtain a large number of proliferatively active UCB-NK cells compared to stimulation with IL-2 only. Since genetic modification of NK cells is a promising way to improve the antitumor properties of NK cells, retroviral transduction procedure was performed to study of the stimulated UCB-NK cells. UCB-NK cells stimulated with IL-2 + K562-mbIL21 were transduced on day 8 of cultivation. In this study, we used targeted overexpression of the adaptor molecule DAP12, which is involved in

the signaling of activating NK cell receptors. PB-NK cells and UCB-NK cells were transduced under the equal experimental conditions in same volume of viral particles. As a result, the transduction efficiency was found to be more than 4-fold higher for UCB-NK cells compared to PB-NK cells. Thus, UCB-NK cells appear to be a promising tool for further research in cancer immunotherapy.

Keywords: UCB-NK cells, phenotype, NK cell stimulation, functional activity, transduction, DAP12

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Introduction

NK cells are lymphocytes of the innate immune system with great cytotoxic potential against tumor cells as well as cells infected by viruses [14]. Unlike T cells, NK cells do not require prior sensitization by antigen or presentation of antigen by HLA-I molecules to recognize "targets" [7], making them an attractive tool for tumor immunotherapy. Therapeutically useful NK cells can be derived from various sources, but currently the greatest number of clinical studies are focused on NK cells from peripheral blood (PB-NK) [9] and NK cells from cord blood (UCB-NK) NK cells [13]. Thus, UCB-NK cells have several advantages: 1) low risk of virus transmission from donor to recipient; 2) high concentration and, as a consequence, high availability of NK-cells and rapid receipt of the "ready" product; 3) possibility to preserve phenotypic characteristics and functional activity after cryopreservation [12]. Nevertheless, the use of cord blood as a source of NK cells for immunotherapy has its limitations: 1) difficult blood sampling; 2) UCB contains low numbers (between 10-100-fold fewer) nucleated cells blood [2] 3) UCB have specific phenotypic characteristics compared to PB-NK. It has been shown that UCB-NK cells have an immature phenotype [8], which is comparable to data reporting about decreased expression of the activating receptor NKG2C and increased expression of the inhibitory receptor NKG2A compared to PB-NK cells [15], although some groups of researchers have found that UCB-NK cells are sufficiently mature and functional in their phenotypic characteristics [11]. Currently, a large number of studies on UCB-NK gene modification are being conducted at both clinical and preclinical levels. One potentially promising area is genetic modification to improve the anti-tumor properties of NK cells. A variety of strategies aimed to enhance the cytotoxicity, survival and migration activity of NK cells have been developed for clinical application. One the approach is targeted overexpression of the adaptor molecule DAP12, which is involved in the signaling of activating NK cell receptors such as NKG2C, NKp44 and activating receptors of the KIR family [3].

Materials and methods

Peripheral blood samples were collected from healthy volunteers agreed to participate in the study. The collection of umbilical cord blood samples of volunteer healthy adults was carried out based on the Federal Research and Clinical Center for Specialized Types of Medical Care and Medical Technologies (FMBA).

To isolate the umbilical cord mononuclear cells were collected in EDTA-containing test tubes and centrifuged in a Ficoll gradient with a density of 1.077 g/cm^3. NK cells were isolated from by negative magnetic separation from mononuclear cells using the NK cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. Freshly isolated NK cells were grown in NK MACS Medium (Miltenyi Biotech, Bergisch Gladbach, Germany) supplemented with 10% FCS (HyClone, USA), 2 mM L-glutamine, 2 mM sodium pyruvate (PanEco, Moscow, Russia), 2 mM antibiotic-antimycotic (Sigma-Aldrich, St. Louis, MO, USA), 1% of NK supplement (Miltenyi Biotech, Bergisch Gladbach, Germany).

To determine the most effective method of activation, peripheral blood or cord blood NK cells were cultured with stimuli in two variants: 1) 100 U/mL IL-2 (Hoffmann La-Roche, Basel, Switzerland) 2) in combination of 100 U/mL IL-2 (Hoffmann La-Roche, Basel, Switzerland) and irradiated K562-mbIL21 feeder cells with 1:2 ratio. The cells were cultured at 37 °C with 5% of CO_2. Cultivated NK cells were counted on eighth and tenth days by cell counter TC20 (Bio-Rad Laboratories, Hercules, CA, USA).

The proliferative activity of NK cells was evaluated using the internalizable fluorescent label CFSE passively penetrating the cells. The cells were incubated in a solution supplemented with 5 μmol/mL CFSE for 15 minutes in heat. The cells were washed three times with FBS serum medium to inactivate CFSE and cultured in complete medium with the addition of stimuli. The level of proliferation was measured at 7 and 10 days after staining using flow cytometry. Actively proliferating cells were detected by decreased CFSE levels.

NK cell degranulation was determined by the level of expression on the cell surface of the lysosomal marker LAMP-1 in the presence of brefeldin A and K562 target cells. For this purpose, NK cells were mixed with K562 in a 1:1 ratio and incubated for 2.5

hours in complete medium with CD107a antibodies and brefeldin. Cell surface staining and flow cytometry measurements were then performed.

UCB-NK were analyzed using a MACSQuant 10 flow cytometer (Miltenyi Biotec, Bergisch Gladbach, Germany) equipped with lasers $\lambda = 405$ nm, $\lambda = 488$ nm, $\lambda = 635$ nm; threshold was set to cut-off events with low CD45 staining. The following mouse anti-human fluorescent-labeled antibodies were used for surface cell staining: NKG2C-FITC (clone REA205, Miltenyi Biotec, Bergisch Gladbach, Germany), HLA-DR-PE (clone L243, Sony Biotechnology, San Jose, CA, USA), CD56-APC-Vio770 (clone REA196, Miltenyi Biotec, Bergisch Gladbach, Germany), KIR2DL2/L3-APC (clone DX27, Sony Biotechnology, San Jose, CA, USA), CD57-APC (clone HNK-1, Sony Biotechnology, San Jose, CA, USA).

The data was analyzed using FlowJo, GraphPad Prism X 10.0.7r2. Statistical analysis of differences in the data was conducted by Mann–Whitney one-tailed U test. $P < 0.05$ were considered significant.

Transduction of PB-NK and UCB-NK was performed in 24-well plates pre-treated with retronectin solution (Takara, USA) and concentrated viral particles containing the gene for the adaptor molecule DAP12 (TYROBP). To determine the infectious titer of the obtained retroviral particles, we preliminarily transduced the Raji cell line. Next, the number of transducing units (TU) was calculated using the formula (N cells * % transduction)/Vvir.particles) to predict the parameters for efficient PB-NK and UCB-NK transduction. Cytometric analysis of GFP luminescence was used to evaluate the efficiency of transduction, the measure was performed on day 3 after staging the experiment.

Results and discussion

To study the phenotypic features of NK cells, the portion of the UCB mononuclear cell fraction obtained during isolation was taken for analysis. Expression of major NK cell surface markers, including the activation marker HLA-DR, inhibitory receptors KIR2DL2/3, NKG2A, activating receptor NKG2C, and differentiation marker CD57 were measured by flow cytometry. Phenotypic data characterize UCB-NK as poorly differentiated and weakly activated cells (Figure 1, see 3rd page of cover). However, it is worth noting that in comparison with the *ex vivo* PB-NK phenotype data we obtained earlier, a significant difference in the analyzed phenotypes of peripheral and cord blood NK cells was observed specifically in the expression of NKG2 family receptors; apparently, UCB-NK express higher levels of NKG2A and lower levels of NKG2C (Figure 1A, see 3rd page of cover), which corresponds to literature data [8, 15]. In addition, it has been shown decreased HLA-DR expression observed in UCB-NK cells (Figure 1, see 3rd page of cover) compared with PB-NK cells [5] suggests a priority stage of activation of peripheral blood NK cells over cord blood NK cells. However, also comparing with our earlier data, it has not been revealed significant differences in the percentage of $CD56^{bright}$, $CD57^{+}$ and KIR^{+} subpopulations (Figure 1B, see 3rd page of cover) between PB-NK and UCB-NK cells were observed in the analysis [6].

The immaturity of UCB-NK cells makes it difficult to obtain clinically relevant antitumor therapy agents because such NK cells have reduced cytotoxicity compared to PB-NK cells [10]. However, it has been shown that various cytokine activation methods used, such as IL-2 or IL-15, or the combination of IL-15 with IL-2 or IL-18 can increase the functional activity of UCB-NK cells to the level observed for PB-NK cells [1]. Low number per unit of donor blood and immaturity of UCB-NK cells are the main obstacle to obtain a sufficient number of NK cells to create effective genetically modified antitumor therapy tools, so optimization of effective activation and genetic engineering processes is the basis for obtaining clinically meaningful results

To measure the level of proliferative activity, isolated PB-NK and UCB-NK cells were cultured under two types of stimulation: 1) with 100 units of IL-2; 2) in combination of IL-2 and K562-mbIL21 feeder cells. Cell counts were performed on days 8 and 14. The data obtained indicate that a steadily increasing induction of UCB-NK proliferation occurred in response to all stimuli added, and differences in proliferative activity were also revealed depending on the stimulation for PB-NK and UCB-NK stimulation (Figure 2, see 3rd page of cover). It was shown that only UCB-NK actively divided under conditions of stimulation with IL-2, whereas PB-NK had weak proliferative activity during the first 8 days of cultivation (Figure 2A, see 3rd page of cover), then the dynamics of the cell division process slowed and reached a plateau by day 21, in contrast to UCB-NK, which continued to proliferate actively (data not shown). In the stimulation way with K562-mbIL21 feeder cells, UCB-NK was found to outperform its own proliferation dynamics compared with the IL-2 only, whereas for PB-NK a high level of proliferation was observed only during the first 8 days after followed by a sharp decline in proliferative activity (Figure 2A, see 3rd page of cover). According to CFSE levels in cells on day 7 after IL-2 + K562-mbIL21 stimulation, the highest proportion of UCB-NK was involved in division (26.7%) compared with IL-2 and stimulation (10.6%) (Figure 2B, C, see 3rd page of cover). At day 10 after stimulation, there was a significant increase in proliferating UCB-NK stimulated by IL-2 + K562-mbIL21 (95.3%), whereas for IL-2, the proportion of divided cells was 62.2% (Figure 2B, C, see 3rd page of cover). In addition to the

analysis of NK proliferative activity, the cytotoxicity of UCB-NKs activated with combination of IL-2 and K562mbIL-21 was evaluated by measuring of the level of degranulation. Degranulation was determined by the level of expression of lysosomal marker LAMP-1 (CD107a) on the surface of UCB-NK. The baseline degranulation of NK cells without targets was taken as a negative control. It was shown that UCB-NK stimulated for 7 days with a combination of IL-2 and K562-mbIL21 had increased and even exceeded level of degranulation compared of PB-NK (Figure 2D, E, see 3rd page of cover).

Thus, UCB-NK cells proliferate in response to stimulation by both IL-2 and a combination of IL-2 and K562-mbIL21. Moreover, UCB-NK cells showed a more pronounced positive proliferative potential in response to cytokine stimulation, which is supported by literature data [1, 4]. Cultivation of *ex vivo* UCB-NK cells with IL-2 and K562-mbIL21 stimulation is a promising way to obtain a large number of proliferatively active UCB-NK cells with stable cytotoxicity.

After several transfection procedures of Phoenix Ampho cell line, sufficient volume of viral particles was accumulated to perform transduction of PB-NK and UCB-NK stimulated by a combination of IL-2 and K-562mbIL-21 for 7 days. Transduction of the Raji cell line (Figure 3A, see 3rd page of cover) was preliminarily performed and the infecting concentration of viral particles was determined using the formula TU = (Ncells*%transduction)/Vvir. particles) at a rate of 2TU for PB-NK and UCB-NK. All cells were transduced in parallel and with strictly the same volume of viral particles to compare the infection efficiency of PB-NK and UCB-NK. As a result, it was found that the transduction efficiency of viral particles carrying the DAP12 gene was more than 4-fold higher for UCB-NK compared to PB-NK (Figure 3B, C, see 3rd page of cover). Experimental data were confirmed in three independent repeats using a single accumulated stock of viral particles containing the DAP12 gene.

Conclusion

The obtained data indicate that depending on the selection of cultivation conditions, stimulation can contribute to an increase in the efficiency of gene modification for UCB-NK cells compared to PB-NK cells. And despite the limitations [12] in use, UCB-NK cells remain a promising tool for application in antitumor therapy. In addition, the choice of a target gene for transduction did not fall by chance, since the adaptor molecule DAP12 is involved in signaling from a number of activating NK-cell receptors, such as NKG2C, NKp44, activating receptors of the KIR family [3]. In the future, we plan to evaluate the effect of DAP12 overexpression on the ratio of NKG2A and NKG2C receptors on the surface of UCB-NK cells, as well as on the way for "switching" the phenotype of NK cells from $NKG2A^+$ to $NKG2C^+$, thereby increasing the phenotypic and functional characteristics of genetically modified UCB-NK cells with further possibility of their use in clinical practice.

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