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ОЦЕНКА ВЛИЯНИЯ КУКУРБИТУРИЛОВ НА МОНОЦИТЫ И NK-КЛЕТКИ ЗДОРОВЫХ ДОНОРОВ

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Резюме. Нанотехнологии в иммунологии – перспективно развивающееся направление в фундаментальной и практической медицине. Кукурбитурилы являются макроциклическими кавитандами с определенным количеством гликолурильных фрагментов (n), определяющих размер полости данного соединения. На данный момент существует шесть синтезированных гомологов: 5, 6, 7, 8, 10 и 14. Отличаются друг от друга портальным размером и размером полостей. Характеризуются особыми физико-химическими и биологическими свойствами, такими как биосовместимость, стабильность, высокая способность к инкапсуляции химических соединений. Известно, что кукурбитурилы инкапсулируют молекулы путем формирования комплексов по типу «гость–хозяин», что позволяет высвобождать вещество из комплекса, повышать растворимость соединений и использовать кукурбитурилы как системы доставки лекарственных веществ. Иммуномодуляторная активность зависит от его специфических наноразмерных характеристик: функциональные группы, форма, размер, поверхность, растворимость в различных средах. Каждая наночастица, в зависимости от этих свойств, оказывает различные эффекты на клетки. Эффекты кукурбитурилов могут быть различными даже для одной субпопуляции клеток в зависимости от гомолога или дозировки. Взаимодействие клеток врожденного иммунитета с кукурбитурилами пока еще недостаточно изучены.

Целью этого исследования была оценка влияния кукурбит[n]урилов (n = 6, n = 7, n = 8) на клетки врожденного иммунитета – моноциты, NK-клетки, NKT-клетки.

Иммунологическое исследование включало выделение мононуклеарных клеток периферической крови условно здоровых доноров (n = 8) на градиенте плотности фиколл-урографина и проточную цитометрию с определением количества иммунокомпетентных клеток по классическим маркерам дифференциации этих клеток – $CD3^-CD16^+CD56^+$ для NK-клеток, $CD3^+CD16^+CD56^+$ для NKT-клеток и $CD3^-CD14^+$ для моноцитов. Активацию моноцитов определяли по экспрессию поверхностного маркера HLA-DR.

Клетки культивировали 72 часа с добавлением кукурбитурилов CB[6], CB[7] в концентрациях 0,1, 0,3, 0,5 мМ и CB[8] в концентрации 0,01 мМ, поскольку он имеет плохую растворимость.

Было отмечено достоверное снижение количества NK-клеток ($p < 0,01$ для исследуемых концентраций CB[7]), увеличение количества NKT-клеток ($p < 0,04$ и $p < 0,02$ для концентраций CB[6] и CB[7] соответственно). Также наблюдалась тенденция к повышению экспрессии HLA-DR на моноцитах ($p = 0,06$ для CB[6]).

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Учитывая такое разностороннее действие кукурбитурилов, в перспективе можно рассматривать возможность использования кукурбитурилов в качестве иммуномодуляторов, противоопухолевых агентов, при аутоиммунных заболеваниях.

Ключевые слова: кукурбитурилы, макроциклические соединения, иммунитет, врожденный иммунитет, моноциты, NK-клетки, NKT-клетки, проточная цитометрия

ASSESSING EFFECTS OF CUCURBITURILS ON MONOCYTES AND NK-CELLS IN HEALTHY VOLUNTEERS

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Abstract. Nanotechnology in immunology is a prospectively developing area in fundamental and practical medicine. Cucurbiturils are macrocyclic cavitands with a definite amount of glycoluril fragments (n) that determine the size of the cavity of these compounds. Nowadays, there are six synthesized homologues: 5, 6, 7, 8, 10 and 14. They differ from each other in the portal size and the size of the cavities. They are characterized by special physicochemical and biological properties, such as biocompatibility, stability, high ability to encapsulate chemical compounds. It is known that cucurbiturils encapsulate molecules by forming guest-host complexes, which allow the substance to be released from the complex and increase the solubility of the compounds. These advantages allow using cucurbiturils as drug delivery systems. Immunomodulatory activity of cucurbiturils depends on its specific nanoscale characteristics: functional groups, shape, size, surface, solubility in various media. Each nanoparticle depending on these properties has different effects on cells. The effects of cucurbiturils can be different even for one subpopulation of cells, depending on the homologue or dosage. The interaction of innate immune cells with cucurbiturils are not yet sufficiently characterized.

The aim of this study was to assess the effects of cucurbit[n]urils (n = 6, n = 7, n = 8) on innate immune cells – monocytes, NK-cells, NKT-cells.

The immunological recearch included the isolation of peripheral blood mononuclear cells from healthy donors (n = 8) on the density gradient of ficoll-urografin and flow cytometry with the determination of the amount of immunocompetent cells according to the classic markers of differentiation of these cells – $CD3^-CD16^+CD56^+$ for NK-cells, $CD3^+CD16^+CD56^+$ for NKT-cells and $CD3^-CD14^+$ for monocytes. Monocyte activation was determined by the expression of surface HLA-DR.

The cells were cultured for 72 hours with the addition of cucurbiturils CB[6], CB[7] at concentrations of 0.1 mM, 0.3 mM, 0.5 mM and CB[8] at concentration of 0.01 mM, due to its poor solubility.

There were a significant decrease in the quantity of NK-cells ($p < 0.01$ for the test concentrations of CB[7]), an increase in the quantity of NKT-cells ($p < 0.04$ and $p < 0.02$ respectively for the concentrations of CB[6] and CB[7]). There was a tendency to increase the expression of HLA-DR on monocytes ($p = 0.06$ for CB[6]).

Considering a variative effects of cucurbiturils, in the future it is possible to consider a possibility of using cucurbiturils as an immunomodulators, antitumor agents, in autoimmune diseases.

Keywords: cucurbiturils, macrocyclic compounds, immunity,innate immune system, monocytes, NK-cells, NKT-cells, flow cytometry

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Introduction

Cucurbiturils (CB[n]) consists of 5 to 14 glycoluril molecules, which are linked via methylene bridges. The quantity of glycoluril monomers determines the serial number (n) of cucurbiturils. CB[n] have a cavity surrounded by highly reactive oxygen atoms. Cucurbituril through oxygen atoms interact with positively charged compounds, which are placed into CB[n]. Thermal and chemical stability, non-covalent interactions with guest-molecules, the cavi-

ties of different sizes and the most importantly is a biocompatibility of these macrocyclic-hosts allow using of cucurbiturils as delivery systems. These properties distinguish cucurbiturils from other organic nanostructures [3]. For example, the solubility of poorly soluble drug molecules can be significantly increased by complexation with CB[n] [6]. Nanotoxicity is determined by the structure of the nanomolecule and its functional groups. The toxicity of the cucurbituril should be examined for its potential pharmacological application. Cucurbiturils mainly at the concentrations up to 1 mM are not toxic to host tissues and immune cells [2, 10, 14]. However, the immunomodulatory activity of cucurbiturils has not been well studied. For example, it is known that CB[n] has an immunostimulatory effect enhancing cell proliferative activity and increasing the expression of HLA-DR on lymphocytes [11]. However, few published data on the interaction of cucurbiturils with other cells of innate immunity are available.

Natural killer cells (NK-cells) are cytotoxic cells of innate immunity with the classic phenotype $CD3^-CD16^+CD56^+$. Functional NK-cells exert activity corresponding to that of adaptive cytotoxic T-lymphocytes (T-killers), however, NK-cells recognize infected cells without involving membrane major histocompatibility complex (MHC) molecules. NK-cells destroy infected cells and also actively take part in events of anti-tumor immunity. The main function of these cells is killing target cellsvia one of the two ways: through the secretion of perforin and granzymes or through membrane-bound death receptors. The molecular signatures of nanomaterials can be recognized by pattern recognition receptors (PRP) expressed on the surface of innate immune cells. The function of NK-cells can be altered by interaction with nanoparticles. For example, the interaction between NK-cells and nanocarbon tubes reduces viability of NK-cells and the production of perforins and granzymes, but elevates production of ROS and cell apoptosis, which led to the suppression of NK-cell functions [1].

Natural killer T-cells (NKTs) are a subpopulation of T-lymphocytes expressing NK-cells markers and T-cells differentiation antigens ($CD3^+CD16^+CD56^+$). NKTs are the most important regulators of the immune response that protect from emergence, growth and metastasis of tumors, intracellular infections of various nature, and development of autoimmune diseases. NKT-cells maintain the induction of anti-tumor immune response by producing IFN-g, which activates NK-cells, $CD8^+$T-lymphocytes and macrophages. Basically, nanoparticles are used to selectively activate NKT-cells. For example, PLGA-based nanoparticle systems designed to modulate the activity of NKT-cells to improve their antitumor function [5]. However, the effect of organic nanoparticles such as cucurbiturils on the viability of NK-cells and NKT-cells should be investigated to determine synergistic effects of cucurbiturils.

Monocytes are mononuclear cells of innate immunity displaying classic phenotype $CD14^+CD16^-$, expressing pattern recognition receptors and chemokine receptors allowing to migrate to the inflammation focus. Monocytes secrete pro-inflammatory cytokines in inflammation focus and participate in phagocytosis. The main functions of monocytes are to enable phagocytic and cytolytic activity. Monocytes express human leukocyte antigen-DR molecules (HLA-DR), which are responsible for presenting antigen to adaptive T-cells. The expression of HLA-DR indicates about cell activation and presence of foreign agents in the organism. Nanostructures can persist for some time in the systemic circulation and, apparently, affect monocytes function. Inorganic nanoparticles can be phagocytosed by monocytes and cause the production of pro-inflammatory cytokines, while the viability of monocytes is reduced [8]. However, modified inorganic nanoparticles by an organic component and organic nanoparticles can be used as darts for monocyte-targets to improve the diagnosis and treatment of diseases [7].

The purpose of the study is to assess an effect of cucurbiturils on monocytes, natural killer cells and nature killer T-cells.

Materials and methods

Heparinized peripheral blood was collected from 8 healthy donors (average 29±2.4 years) after obtaining their informed consent. Peripheral blood was placed on Ficoll-urografin density gradient medium according to the protocol. Cells were centrifuged at 2.7xrpm for 20 minutes, followed by washing out peripheral blood mononuclear cells (PBMCs) twice in the appropriate phosphate-buffered saline (PBS). The among of cells was counted in a hemocytometer added with 2% acetic acid.

After isolation, PBMCs at a quantity of 1×10^6 cells/ml were cultured with CB[6], CB[7] and CB[8] using the complete culture medium RPMI-1640, supplemented with 10% FCS, 50 mg/ml gentamicin and 25 mg/ml thienam in 24 well plate (TPP, Switzerland) for 72 h at 37 °C, 5% CO_2, 90% relative humidity. CB[6], CB[7] were added at the concentrations of 0.1 mM, 0.3 mM, 0.5 mM. CB[8] was added at a concentration of 0.01 mM due to its very low solubility in the media.

After 72h-incubation the cells were washed 2× in PBS. Fluorochrome-conjugated monoclonal antibodies were used to determined phenotypes of monocytes and NK-cells by $CD3^+$ (BioLegend, USA), $CD14^+$ (BioLegend, USA), $CD16^+CD56^+$ (BioLegend, USA) and HLA-DR (BioLegend, USA). The cells were labeled with 4 fluorochrome-conjugated antibodies by combining two monoclonal antibodies recognizing the human $CD16^+$ and $CD56^+$-cell surface antigens. NK-cells were identified by the classical phenotype $CD3^-CD16^+CD56^+$, NKT-cells – $CD3^+CD16^+CD56^+$ and monocytes by the $CD14^+$ marker.

Samples were analyzed by flow cytometry using cytometer FACS Canto II (BD, Franklin Lakes, NJ, USA) with Diva 6.0 software (BD). Expression of cell surface receptor HLA-DR was analysed gated on $CD3^-CD14^+$ monocytes.

Statistical analysis was performed using Statistica 6.0 software (StatSoft, USA). Differences between groups were determined using non-parametric Wilcoxon matched-pairs test. P value of < 0.05 level was considered statistically significant.

Results and discussion

First, we evaluated the effect of cucurbiturils on NK-cells obtained from peripheral blood mononuclear cells from 8 healthy donors in PBMCs cultures *in vitro*. The NK-cells frequency was decreased at all three tested concentrations of CB[7] compared to the control (0 mM) (Figure 1). Interestingly, the amount of NK-cells at a concentration of 0.3 mM is higher compared to concentrations of 0.1 and 0.5 mM of CB[7]. According to literature data, some nanoparticles also lower functional activity of NK-cells and even alter their phenotype [9]. On the contrary, other nanoparticles were able to increase NK-cell proliferative and cytotoxic activity [4, 12].

Next, we evaluated the effect of cucurbiturils on a subpopulation of T-lymphocytes associated with the innate immunity – NKT-cells. Our data showed that CB[6] at a concentration of 0.3 mM and CB[7] at a concentration of 0.5 mM increased the amount of NKT-cells compared to the control group (0 mM) (Figure 2). The frequency of NKT-cells was increased compared to NK-cells, probably due to the fact that NKT-cells express T-cell antigen receptors. In previous studies we found an increase in T-lymphocytes during PBMC treated with cucurbiturils [11]. According to available literature, nanoparticles are mainly used to stimulate the NKT-cells [13].

The next step was to assess the frequency of monocytes and their activation during cultivation with CB[n]. We found no significant differences in amount of monocytes (Figure 3A), however, there was a tendency ($p = 0.06$) to enhanced expression of HLA-DR on the surface of monocytes with the CB[6] at a concentration of 0.1 mM acting probably in a dose-dependent manner (Figure 3B). This may be due to the small size of cucurbituril and its ability to penetrate immunocompetent cells.

Conclusion

To summarize the data obtained, it is clear that nanoscale compounds interact with the innate immune system, which is important for potential deve-

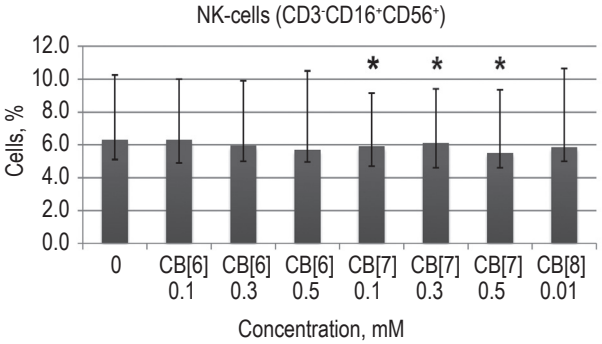

Figure 1. The frequency of NK-cells in the presence of CB[6], CD[7], CB[8]

Note. Human PBMCs were cultured with CB[6] and CB[7] at concentrations 0.1 mM, 0.3 mM, 0.5 mM and with CB[8] at a concentration of 0.01 mM for 72h. PBMCs cultured without CB[n] were used as a control (0 mM). The frequency of $CD3^-CD16^+CD56^+$ NK-cells were evaluated by flow cytometry. Data presented as median and interquartile range (n = 8). * – significant differences compared to control (Wilcoxon test), $p < 0.05$.

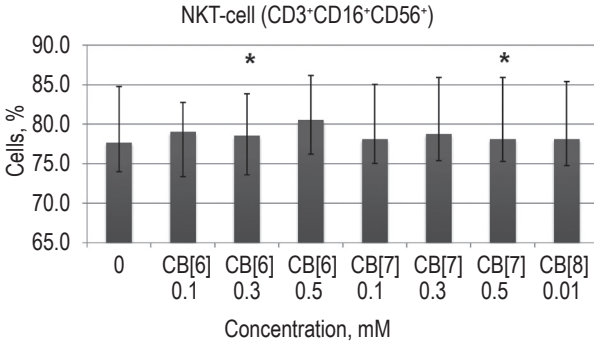

Figure 2. The frequency of NKT-cells in the presence of CB[6], CD[7], CB[8]

Note. Human PBMCs were cultured with CB[6] and CB[7] at concentrations of 0.1 mM, 0.3 mM, 0.5 mM and with CB[8] at a concentration of 0.01 mM for 72h. PBMCs cultured without CB[n] were used as a control (0 mM). The frequency of $CD3^+CD16^+CD56^+$ NKT-cells were evaluated by flow cytometry. Data presented as median and interquartile range (n = 8). * – significant differences compared to control (Wilcoxon test), $p < 0.05$.

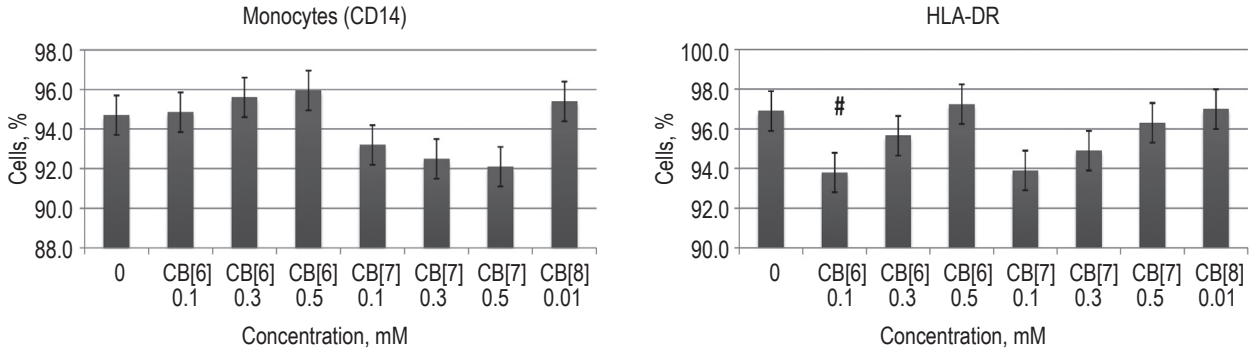

Figure 3. The frequency of monocytes and expression of HLA-DR molecules on monocytes in the presence of CB[6], CD[7], CB[8]

Note. Human PBMCs were cultured with CB[6] and CB[7] at a concentration of 0.5 mM and with CB[8] at a concentration of 0.01 mM for 72h. PBMCs cultured without CB[n] were used as a control (0 mM). The frequency of $CD3^{-}CD14^{+}$ monocytes (A) and HLA-DR expression (B) were evaluated by flow cytometry. Expression Data presented as median and interquartile range (n = 8). # – tendency compared to control (Wilcoxon test), p = 0.06

lopment and modification of immunocompatible nanomaterials. Nanoconstructions are mainly used for cancer immunotherapy, vaccines and treatment of autoimmune diseases. However, the mechanism and molecular pathways of how nanomaterials affect the innate immune system are not clear. Therefore, more research is needed to understand the interaction of cucurbiturils with the innate immune system to develop new prospective strategies for the prevention, diagnosis or treatment of human diseases.

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