Медицинская иммунология
2018, Т. 20, № 2, стр. 251-254
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Краткие сообщения
Short communications

Medical Immunology (Russia)/
Meditsinskaya Immunologiya
2018, Vol. 20, № 2, pp. 251-254
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ОПРЕДЕЛЕНИЕ МИКРОВЕЗИКУЛ, ОБРАЗУЕМЫХ NK-КЛЕТКАМИ, МЕТОДОМ ПРОТОЧНОЙ ЦИТОФЛУОРИМЕТРИИ

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Резюме. В результате активации и/или апоптоза клетки могут образовывать микровезикулы (МВ) размером от 100 нм до 1000 нм. В настоящее время все больше внимания уделяется детекции и оценке динамики содержания МВ лейкоцитарного происхождения. В этой связи большой интерес представляет определение МВ, образуемых NK-клетками, основной функцией которых является индукция апоптоза вирус-инфицированных и опухолевых клеток. В настоящее время нет прямых свидетельств о способности NK-клеток продуцировать МВ. Целью настоящей работы явилась оценка содержания МВ NK-клеток при помощи метода высокоточной проточной цитофлуориметрии. Установлено, что с помощью метода высокоточной проточной цитофлуориметрии возможно выявление МВ размером от 200 нм до 1000 нм, образуемых NK-клетками. Показано, что инкубация NK-клеток в присутствии TNFα не влияла на относительное количество МВ, однако приводила к повышению интенсивности экспрессии CD95 на МВ. Таким образом, метод высокоточной проточной цитофлуориметрии может быть использован для определения МВ и их фенотипа.

Ключевые слова: NK-клетки, проточная цитофлуориметрия, микровезикулы, микрочастицы, клеточная культура, NK-92, поверхностные рецепторы

EVALUATION OF MICROVESICLES FORMED BY NATURAL KILLER (NK) CELLS USING FLOW CYTOMETRY

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Abstract. As a result of activation and/or apoptosis, the cells can form microvesicles (MV) from 100 nm up to 1000 nm in size. Nowadays, the attention is being increasingly focused on dynamic detection and evaluation of leukocyte-derived microvesicles by their contents. In this regard, determination of microvesicles formed by NK cells is of utmost interest. The main function of these population is to induce apoptosis of virus-infected

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Образец цитирования:
В.А. Михайлова, К.Л. Белякова, Л.П. Вязьмина, А.Р. Шевелева, С.А. Сельков, Д.И. Соколов «Определение микровезикул, образуемых NK-клетками, методом проточной цитофлуориметрии» // Медицинская иммунология, 2018. Т. 20, № 2. С. 251-254. doi: 10.15789/1563-0625-2018-2-251-254

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For citation:
V.A. Mikhailova, K.L. Belyakova, L.P. Vyazmina, A.R. Sheveleva, S.A. Selkov, D.I. Sokolov "Evaluation of microvesicles formed by natural killer (NK) cells using flow cytometry", Medical Immunology (Russia)/Meditsinskaya Immunologiya, 2018, Vol. 20, no. 2, pp. 251-254. doi: 10.15789/1563-0625-2018-2-251-254

DOI: 10.15789/1563-0625-2018-2-251-254

and tumor cells. At the present time, there is no direct evidence of the NK cells ability to produce microvesicles. This investigation was performed in order to estimate contents of NK cell-derived microvesicles using high-precision flow cytometric approach. It has been shown that the high-precision flow cytometry allows to detect microvesicles formed by NK cells, ranging from 200 to 1000 nm in size. It was demonstrated that incubation of NK cells in the presence of TNFα did not affect the relative value of microvesicles, however, being associated with increased intensity of CD95 expression on microvesicles. Hence, the high-precision flow cytometry can be used to detect microvesicles and to determine their phenotype.

Keywords: NK cells, flow cytometry, microvesicles, microparticles, cell culture, NK-92, cell surface receptors

Работа поддержана грантом РФФИ № 17-04-00679 (культивирование клеток) и грантом РНФ № 17-15-01230 (оценка фенотипических характеристик линии NK-92 и их микрочастиц).

The present work was supported by the Russian Foundation for Basic Research grant No. 17-04-00679 (cell cultures) and the Russian Science Foundation grant No. 17-15-01230 (evaluation of the NK-92 cell line phenotype and their microvesicles).

Introduction

Cell communication is an integral process of cells functioning and is carried out by contact interactions between cells and by the transmission of activation or inhibitory signals due to secreted cytokines. It is assumed that microvesicles can participate in cell communication, since along with the membrane molecules expressed by a source cell, they contain cytoplasmic molecules: lipids, MHC molecules of I and II class, chemokines, cytokines, growth factors, transcription factors, microRNA [3, 4, 6, 8], and template RNA [8]. The possibility of a signal transmission from cell to cell with the help of microvesicles and thus formation of a distant cell interaction is under discussion. Microvesicles can be formed as a result of cells activation and/or apoptosis. They are present in the peripheral blood in healthy donors; the number and composition of microvesicle membrane receptors may vary under pathological conditions [10]. Nowadays, the attention is being increasingly focused on the detection and evaluation of the dynamics of the content of leukocyte-derived microvesicles. One of the leukocyte populations is the population of NK cells, the main function of which is the induction of apoptosis of virus infected and tumor cells. At present, there is no direct evidence of the ability of NK cells to produce microvesicles.

The detection of microvesicles in biological fluids is possible through several methods: transmission electron microscopy [12], detection of changes in flow resistance through micropores (TRPS – Tunable Resistive Pulse Sensing) [12, 13], atomic force microscopy [11], flow cytometry [12]. In comparison with other methods of microvesicle detection, flow cytometry is the most applicable one, since it allows to obtain data on microvesicle concentration in biological fluids, for example, in peripheral blood, as well as to determine the receptors on their surface [12, 13]. Attempts to detect leukocyte-derived microvesicles have been made using the FacsCanto II device (BD, USA) [7, 9]. However, the sensitivity of the device allows to detect particles of only 300 nm and up in size, which leads to the undercount of microvesicle content in samples. The use of filtered solutions and calibration particles of a certain size (200 nm, 500 nm, 1000 nm) improves the accuracy of microvesicle detection [7, 9].

The Cytoflex flow cytometer (Beckman Coulter, USA) equipped with three lasers: 488 nm, 638 nm, and 405 nm, allows to detect side scatter from a 405 nm laser using a 405/10 filter and makes it possible to determine particles of 200 nm and up in size. The use of additional protocols for cleaning the flowing liquid and sample washing buffers can increase the sensitivity of the device.

The aim of this work was to estimate microvesicle content in NK cells using high-precision flow cytometry, namely the Cytoflex device.

Materials and Methods

NK cells of the NK-92 cell line (ATCC, USA) were selected as source cells for microvesicles. NK-92 cell line is a suspension culture reproducing the basic phenotypic and functional characteristics of activated NK cells. NK-92 cell line was cultured according to the manufacturer's directions (ATCC, USA) in the presence of recombinant IL-2 (500 U/ml) (Roncoleukin, Biotech LTC, Russia). TNFα (10 U/ml) (Sigma, USA) was used as an inducer.

To evaluate the phenotype of NK cells of the NK-92 cell line and the microvesicles they formed, the cells were placed into a 24-well plate in 1 ml of complete cell culture medium at a concentration of 400,000 cells/ml, cultured at 37 °C under the damp atmosphere with 5% CO_2 for 24 hours in the presence of an inductor. After 24 hours, the plates were centrifuged under 200 g and 22 °C for 10 minutes to settle the cells. To separate the microvesicles, we used the differential centrifugation method by M.P. Gelderman, J. Simak [5] in Hanks' solution without Ca^{2+} and Mg^{2+}, for which the obtained supernatants had been consecutively centrifuged under 500 g 10 °C for 10 min, 15500 g 10 °C for 90 min, 20000 g 10 °C for 20 min. Thus, we obtained the NK-92 cell line and its microvesicles and processed them with monoclonal antibodies to CD95 according to the manufacturer's instructions (BD, USA). The expression of receptors by NK cells (Figure 1, see 2nd page of cover) and microvesicles (Figure 2, see 3rd page of cover) was assessed using the high-precision flow cytometry method, namely the Cytoflex flow cytometer (Beckman Coulter, USA), which allows to detect particles from 0.2 μm in size.

TABLE 1. EXPRESSION PARAMETERS OF CD95 (Fas) SURFACE RECEPTOR OF NK CELLS AND THEIR MICROVESICLES

NK cells and microvesicle parameters				
Object	**NK cells expressing CD95 (Fas)**		**Microvesicle expressing CD95 (Fas)**	
Incubation conditions	Constitutive	In the presence of TNFα (10 U/ml)	Constitutive	In the presence of TNFα (10 U/ml)
Relative value, %	98.9±0.1	98.9±0.01	2.2±0.3	2.5±0.2
Expression intensity (MFI)	10064±277	14113±258**	82.0±8. 0	147±50*

Note. Differences in CD95 expression levels by NK cells or microvesicles are significant, as compared to the constitutive expression levels: *, $p < 0.05$; **, $p < 0.001$.

To adjust the device we used calibration particles of 0.1 μm, 0.2 μm, 0.5 μm, and 1.0 μm (Invitrogen, USA) in size in accordance with the device manufacturer's indications (Figure 2, see 3rd page of cover). To work with microvesicles, we filtered all solutions through filters with 0.2 μm pore size [71]. The statistical analysis was performed in Statistica 10 software using Student's t-test.

Results and Discussion

CD95 (Fas) is a proapoptotic receptor inducing cell death when binding to CD95L ligand (FasL). The expression of CD95 by cells indicates their readiness for apoptosis and reflects the mechanisms controlling the functional activity of the cell population, and therefore the presence of this receptor on all cells of a body is suggested. The analysis of CD95 expression by NK cells, both with incubation without inducers and in the presence of TNFα, the proinflammatory cytokine, revealed that 99% NK cells of the NK-92 cell line expressed CD95 on their surface. The incubation of cells in the presence of TNFα did not result in a change in the number of $CD95^{+}$NK cells. At the same time, the intensity of CD95 expression by NK cells was increased (Table 1). The increase in the intensity of CD95 proapoptotic receptor expression by NK cells as a result of TNFα action indicates the strengthening of control of NK cells population, which prevents their excessive activation.

It has been established that the high-precision flow cytometry method, namely the Cytoflex flow cytometer (Beckman Coulter, USA), allows to detect microvesicles from 200 nm to 1000 nm in size formed by NK cells. It was demonstrated that the relative value of microvesicles formed by NK cells of the NK-92 cell line expressing CD95 was significantly lower compared to the source cells. The high-precision flow cytometry method, namely the Cytoflex flow cytometer, showed that the activation of NK cells by TNFα did not affect the relative value of microvesicles. However, it increased the intensity of CD95 expression on microvesicles (Table 1).

Literature tells us about CD95L and CD95 presence in the membrane of microvesicles of various origin [1, 2]. The data obtained on CD95 presence on microparticles of NK cells are consistent with the literature data. It is assumed that the formation of $CD95^{+}$ on microvesicles by tumor cells represents one of the mechanisms of avoiding the recognition of tumor cells by cells of the immune system [2]. The expression of CD95 microvesicles of NK cells of the NK-92 cell line in the intact state and in the presence of TNFα, revealed by us, can also be a representation of this mechanism.

Thus, Cytoflex flow cytometer (Beckman Coulter, USA) implementing the method of high-precision flow cytometry can be used to detect microvesicles of a cell origin from 200 nm to 1000 nm in size, as well as to assess their phenotypic characteristics.

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Поступила 25.09.2017
Отправлена на доработку 10.10.2017
Принята к печати 12.10.2017

Received 25.09.2017
Revision received 10.10.2017
Accepted 12.10.2017