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ЭКСПРЕССИЯ ПОВЕРХНОСТНЫХ МОЛЕКУЛ И ФУНКЦИОНАЛЬНЫЕ ХАРАКТЕРИСТИКИ ЭНДОТЕЛИАЛЬНЫХ КЛЕТОК: ВЛИЯНИЕ БЕЛКОВЫХ ФРАКЦИЙ ЛИЗАТА МИКРОВЕЗИКУЛ ЕСТЕСТВЕННЫХ КИЛЛЕРОВ В СИСТЕМЕ *IN VITRO*

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Резюме. Микровезикулы – мембранные образования размером от 100 до 1000 нм, продуцируемые различными клетками в состоянии покоя и активации, – могут передавать компоненты своего содержимого клеткам-мишеням, регулировать физиологические процессы, участвовать в развитии патологий. Среди множества клеток-источников микровезикул особый интерес представляют естественные киллеры – субпопуляция лимфоцитов, осуществляющих контактный цитолиз вирус-инфицированных и опухолевых клеток, а также участвующих в регуляции ангиогенеза. Продуцируя различные стимуляторы и ингибиторы этого процесса, естественные киллеры способны изменять функциональную активность эндотелиальных клеток путем контактного взаимодействия с ними собственных микровезикул. Учитывая недостаточность имеющихся в литературе сведений о способности экстраклеточных везикул влиять на функциональное состояние эндотелия в зависимости от баланса передаваемых ими про- и антиангиогенных факторов, целью данного исследования явилось изучение влияния белковых фракций лизата микровезикул, продуцируемых клетками естественных киллеров линии NK-92, на фенотип и функциональные характеристики эндотелиальных клеток линии EA.hy926 в модельном эксперименте *in vitro*. В результате микропрепаративного разделения лизата микровезикул клеток линии NK-92 было получено двенадцать белковых фракций (индукторы). Установлено, что пролиферация и миграция клеток линии EA.hy926 после их культивирования в присутствии десяти из двенадцати полученных фракций, в зависимости от концентрации содержащихся в них активных

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компонентов, изменялись разнонаправленно и дозозависимо или оставались неизменными. Показан преимущественно стимулирующий эффект воздействия индукторов на пролиферацию клеток-мишеней, что свидетельствует о наличии в этих фракциях белков, регулирующих функции эндотелия. При этом остаточная площадь, не занятая мигрировавшими эндотелиальными клетками после их культивирования в присутствии индукторов, не всегда коррелировала с интенсивностью миграции и не была обратно пропорциональна количеству мигрировавших клеток. Дополнительно было установлено, что ни одна из полученных белковых фракций не оказывала влияния на экспрессию рецепторов CD54 (ICAM-1), CD34, CD31 (PECAM-1), CD119 (IFNγR1) клетками линии EA.hy926. Полученные данные об изменении функциональных характеристик клеток линии EA.hy926 под влиянием белковых фракций лизата микровезикул, продуцируемых клетками линии NK-92, подтверждают вовлеченность этих субклеточных образований в обеспечение коммуникации естественных киллеров с клетками эндотелия и указывают на различное участие эффекторных белков, переносимых микровезикулами, в механизмах ангиогенеза.

Ключевые слова: естественные киллеры, эндотелий, иммунный ответ, ангиогенез, фенотип, пролиферация, миграция

PHENOTYPIC AND FUNCTIONAL CHARACTERISTICS OF ENDOTHELIAL CELLS: THE *IN VITRO* EFFECTS OF PROTEIN FRACTIONS FROM THE LYSATE OF NATURAL KILLER-DERIVED MICROVESICLES

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Abstract. Microvesicles are membrane-derived formations ranging in size from 100 to 1000 nm, being produced by a variety of resting and activated cells. They can transfer their cargo to target cells, regulate physiological processes, and participate in the development of clinical disorders. Among the microvesicles of different origin, natural killers are of special interest. They represent a subpopulation of lymphocytes that eliminate aberrant cells, including virally infected and malignant cells, and participate in regulation of angiogenesis. By producing various stimuli and inhibitors of the latter process, natural killers are able to change functional activity of endothelial cells by means of microvesicle-mediated contacts. There are only scarce literature data on ability of the extracellular vesicles to influence endothelial functions, depending on the intrinsic balance of pro- and anti-angiogenic factors. Therefore, the aim of our study was to evaluate the effect of protein fractions derived from microvesicle lysate of the NK-92 natural killer cell line upon phenotype and functional characteristics of EA.hy926 endothelial cell line under *in vitro* experimental conditions. Using chromatographic micro-preparatory separation, twelve protein fractions (inducers) were obtained from the lysate. It was found that proliferation and migration of EA.hy926 cells after their cultivation with 10 of 12 protein fractions, were changed in different directions. These effects were dose-dependent, or remained unchanged, at distinct concentrations of active components in the fractions. The inducing factors from these fractions exerted predominantly stimulating effects on proliferation of the target cells, thus suggesting presence of proteins which are able of regulating endothelial functions. However, the size of residual area free of migrating endothelial cells treated by the inducers did not always correlate with the migration intensity and did not inversely correlate with the number of migrating cells. Moreover, it was found that the obtained protein fractions had no effect upon expression of CD54 (ICAM-1), CD34, CD31 (PECAM-1) and CD119 (IFNγR1) receptors by EA.hy926 cells. The data obtained confirm an involvement of microvesicles in communications between natural killer cells and endothelial cells, and presume different participation modes of microvesicle-derived effector proteins in the angiogenesis machinery.

Keywords: natural killer cells, endothelium, immune response, angiogenesis, phenotype, proliferation, migration

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Introduction

Natural killer (NK) cells are part of the innate arm of the immune system. They eliminate aberrant cells, including virally infected and tumorigenic cells. The main function of NK cells is implemented via the following mechanisms: exocytosis of lytic granules, ligand-mediated interaction with death receptors on target cells, secretion of cytokines, and antibody-dependent cellular cytotoxicity. NK cells are involved in physiological and pathological processes through the production of cytokines. It has been shown previously that NK cells are involved in all processes occurring in the area of uteroplacental contact during pregnancy, including implantation of a blastocyst into the endometrium, regulation of trophoblast invasion, remodeling of uterine and decidual arteries, as well as formation of the placental vascular bed [9, 14]. A significant part of research was aimed at studying the properties of decidual NK cells with the $CD56^{bright}CD16^{-}$ phenotype, which produce a large number of pro-angiogenic factors, such as VEGF, PlGF, and CXCL8 [6, 18]. These cytokines can influence endothelial cells (ECs) and their microenvironment, controlling angiogenesis. It is believed that NK cells prepare uterine spiral arteries for remodeling, inducing Fas-dependent apoptosis of smooth muscle cells and ECs [3].

In vitro model experiments have yielded conflicting data on the effect of NK cells on angiogenesis. There is evidence in favor of stimulating endothelial cell migration and formation of vessels [18, 22], and in favor of inhibiting the processes of angiogenesis [12, 15]. It was found that IL-15 enhances the production of VEGF and PlGF by NK cells [19, 25] and, on the contrary, NK cells activated by IL-12 suppress vascular growth due to the production of IFNγ, IP-10, perforin, and granzyme [38]. Thus, depending on the experimental model used or characteristics of NK cells obtained from different sources, researchers have come to opposite conclusions regarding the effect of NK cells on the endothelium.

In addition to contact interactions or cytokine production, NK cells can also implement their cytotoxic and regulatory functions through the microvesicles (MVs) they produce. MVs are membrane formations ranging in size from 100 to 1000 nm and are produced by a variety of resting and activated cells. These formations can transfer their cargo to target cells, regulate inflammation, coagulation, antigen presentation, and apoptosis, as well as participate in the pathogenesis of diseases and inflammatory processes [7, 17]. MVs of leukocyte origin, as a minor part of MVs in the blood flow under physiological conditions [11], remain the least studied population. With pathologies, their level in the blood plasma increases sharply, therefore, leukocyte MVs are considered as markers of various diseases [31, 35]. The role of MVs (including those produced by NK cells) in angiogenesis, inflammation, and the immune response has been insufficiently studied.

Previously, we have shown altered expressions of the CD54 (ICAM-1), CD34, CD31 (PECAM-1) and CD119 (IFNγR1) receptors by the EA.Hy926 endothelial cell line, as well as the appearance of the pan-leukocyte marker molecule CD45 on the target cell membrane after co-cultivation of ECs with NK-92 cell derived MVs [29]. In particular, incubation of the target cells in the presence of the MVs reduced the relative number of ECs expressing the CD34, CD31 and CD119 receptors. Besides, the decreased number of ECs with the $CD34^{+}$ phenotype after incubation with the MVs correlated with the increased intensity of the receptor expression by the target cells as compared to intact cells. Despite the fact that no differences were found in the number of ECs with the $CD54^{+}$ phenotype, the intensity of this receptor expression by the cells was higher after their incubation with the MVs compared to intact cells [29]. We have also shown increased dose-dependent proliferation of the target cells after cultivation in the presence of the same MVs as compared to intact cells [29]. Additionally, decreased endothelial cell migration under the same conditions was found, and that was caused by a decrease in the number of migrated ECs compared to the cultivation of the target cells in the absence of the MVs [29].

The literature data on the ability of MVs to influence the functions of the endothelium, depending on the balance of pro- and anti-angiogenic factors they carry, are still scarce. Therefore, considering the ability of NK-92 cell derived MVs to change the functionality of ECs and their response to external signals, **the aim of this study** was to evaluate the effect of protein fractions of the NK-92 cell derived MV lysate on the phenotype and functional characteristics of EA.hy926 cells in an *in vitro* experiment.

Materials and methods

Cells and cell culture

The cells of the NK-92 cell line and the EA.hy926 cell line (American Tissue Culture Collection, USA)

were cultured using standard cell culture procedures under the damp atmosphere at 37 °C and 5% CO_2 as per instructions provided by the manufacturer. NK-92 cells reproduce the main phenotypic and functional characteristics of activated NK cells [16], while EA.hy926 cells reproduce the main morphological, phenotypic and functional characteristics inherent in macrovascular ECs [37]. Cell vitality was evaluated by trypan blue staining and was not less than 96%.

Inducers

Protein fractions of the NK-92 cell derived MV lysate obtained using micropreparative size exclusion liquid chromatography of medium pressure were used as inducers of EA.Hy926 cells (see below). Phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich Chem. Co., USA) at a concentration of 10 ng/ml was used as a positive control for stimulation of EA.Hy926 cells.

Isolation of biomaterial

There being no single standard protocol available for MV isolation and characterization, a variety of methodological approaches are currently used to obtain MV fractions with proper purity and enrichment [26]. Therefore, the MVs shed from NK-92 cells were isolated by the modified step-wise centrifugation method [36] in Hanks's solution without Ca^{2+} and Mg^{2+} (Sigma-Aldrich Chem. Co., USA), for which the supernatants were sequentially centrifuged at 200 g (4 °C, 10 min.) and 9,900 g (4 °C, 10 min.). After the second centrifugation, the pellet was washed twice with cold phosphate buffer solution (PBS; Sigma-Aldrich Chem. Co., USA) and was recentrifuged at 19,800 g (4 °C, 20 min.). The supernatant was discarded, with the pellet washed several times with cold PBS, each time precipitating the MVs by centrifugation at 19,800 g (4 °C, 20 min.). The purified pellet was resuspended in MilliQ deionized water, with the protease inhibitor mixture (cOmplete, EDTA-free; Roche Diagnostics GmbH, Germany) added at the concentration specified by the manufacturer, and was stored at -80 °C until being analyzed. This protocol allows for isolating MVs with a diameter of 100-200 nm with sufficient purity and minimal biomaterial loss, as MVs are sequentially separated from coarse particles of cellular debris and large apoptotic bodies, as well as from exosomes [24].

On the day of the experiment, the frozen MVs were thawed and subjected to repeated "freeze-thaw" cycles five times, followed by being intensively homogenized in a glass homogenizer for 5 min. The debris was removed by centrifugation at 16,000 g (4 °C, 10 min.), the supernatant being collected for further investigation.

Preparative chromatography

To obtain protein fractions of the NK-92 cell derived MV lysate, a medium pressure liquid NGC chromatograph with ChromLab™ Software (Bio-Rad Laboratories, USA) was used. Chromatographic separation was carried out under non-denaturing conditions on an ENrich™ SEC 650 High-Resolution Size Exclusion Column, 10 × 300 mm, 24 ml, 10 μm (Bio-Rad Laboratories, USA). Fresh PBS supplemented with 0.5 M sodium chloride (Sigma-Aldrich Chem. Co., USA) (pH 7.4) was used as a mobile phase. The analysis was performed in an isocratic mode at room temperature with a mobile phase flow rate of 0.5 ml/min and a detector wavelength of 280 nm. The analysis run time was 60 min. Protein fractions of the lysate of the MVs derived from 6.5×10^8 NK-92 cells were isolated and sterilized through a Millex-GV4 membrane filter with a pore diameter of 0.22 μm (Merck Millipore, USA), followed by being frozen at -80 °C and stored for no more than two weeks until being analyzed.

Electrophoresis

Samples of the obtained MV lysate fractions were separated by microarray electrophoresis under non-denaturing conditions using commercial High Sensitivity Protein Chips (Agilent Technologies, USA) in an Agilent 2100 bioanalyzer (Agilent Technologies, USA) as per instructions provided by the manufacturer. The intensities of the bands were assessed using the Agilent 2100 Expert software (Agilent Technologies, USA). All samples were run in triplicate.

Assessment of cytotoxicity

To determine the minimum toxic doses of the mobile phase for chromatography and the obtained MV lysate fractions, EA.hy926 cells were pipetted and transferred into the wells of a 96-well cell clear flat bottom culture plate (BD, USA) at a concentration of 3.5×10^3 cells per well in 100 μl of complete culture medium supplemented with 10% inactivated fetal calf serum (FCS). To avoid the edge effect in this and all further experiments, the outer wells along the entire perimeter were filled with the medium and were not used subsequently. The cells were then cultured for 24 h using standard cell culture procedures under the damp atmosphere at 37 °C and 5% CO_2. Following that, the culture medium in the wells was replaced with the mobile phase for chromatography or the obtained protein fractions in several dilutions. For this, by sequential titration on complete culture medium supplemented with 10% FCS, series of dilutions of the mobile phase and the protein fractions were prepared in the following ratios: 50%, 25%, 20%, 10%, 5%, 2.5%, 1.25%, 0.625%, 0.313%, 0.156%, and 0.078%. To obtain statistically reliable results, each dilution was prepared in six replicas. The culture medium supplemented with 10% FCS was used as a control. Then, the cells were cultured for 24 h using

standard cell culture procedures under the damp atmosphere at 37 °C and 5% CO_2. On the following day, the culture medium was removed and the cells were stained with 0.2% crystal violet solution (Sigma-Aldrich Chem. Co., USA) containing 5% methanol, for which 0.1 ml of the dye solution was added to each well and incubated for 10 min. After washing the wells four times with distilled water, the plate was dried up to completely remove moisture, and the dye was extracted by adding 0.1 ml of 50% acetic acid solution to each well. The optical density was measured using an ELx808 microplate photometer (BioTek Instruments Inc., USA) at a wavelength of 540 nm (cutoff wavelength of 630 nm). The decrease in cell viability was judged by optical density changes compared to the control.

Evaluation of the minimum toxic dose of the mobile phase for chromatography in relation EA.hy926 cells showed that the mobile phase was toxic when undiluted or diluted with the culture medium in ratios of 50% and 25% (the difference from the viability of intact cells: $p < 0.001$). When diluted with the culture medium in ratios starting from 12.5%, the mobile phase had no toxic effect on EA.hy926 cells (the difference from the viability of intact cells: $p > 0.05$).

The toxicity of the obtained MV lysate fractions towards EA.hy926 cells was also preliminary assessed: undiluted and diluted fractions were not toxic towards the cells.

Evaluation of cell proliferation

To assess the effect of protein fractions of the NK-92 cell derived MV lysate on proliferation of EA.hy926 cells, we used a method based on staining the protein components of the cell cytoplasm with the vital dye crystal violet. This method is comparable in sensitivity to other methods for assessing proliferation [2]. For this, the cells were pipetted and transferred into the wells of a 96-well cell clear flat bottom culture plate at a concentration of 5.0×10^3 cells per well in 0.1 ml of complete culture medium supplemented with 10% FCS for 24 h to adhere to the plate surface. On the following day, the culture medium was removed and the wells were washed with an excessive amount of pre-warmed HBSS solution (Sigma-Aldrich Chem. Co., USA), followed by the solution removal. Then, 0.1 ml of inducer solutions (diluted protein fractions of the NK-92 cell derived MV lysate) prepared in the culture medium without FCS were added to the wells, the final concentrations of each fraction being 10%, 5%, 2.5%, 1.25%, 0.625%, 0.313%, 0.156%, and 0.078%. Following that, FCS was added to each well in an amount equal to 2.5% of the well contents, with the outer wells filled with pre-warmed HBSS solution. After incubation for 72 h, the culture medium was removed and the cells were stained with 0.2% crystal violet solution, as described above. After the plate was dried up, the dye was extracted by adding 0.1 ml of 50% acetic acid solution to each well.

The intensity of cell proliferation was assessed by the change in the optical density of the stained solutions, which was measured using an ELx808 microplate photometer at a wavelength of 540 nm (cutoff wavelength of 630 nm). The obtained optical densities were matched with the number of cells using the titration curve, and the results were expressed in the number of cells. The change in cell proliferation was judged by the change in both the optical density of the sample and the number of cells compared to incubation in the culture medium supplemented with 2.5% FCS (control). The culture media supplemented with 0% and 10% FCS were used as additional controls. All samples were run in triplicate. Each protein fraction was analyzed in four repetitions for each dilution.

Assessment of cell migration

To assess the effect of protein fractions of the NK-92 cell derived MV lysate on migration of EA.hy926 cells, the cells were pipetted and transferred into the wells of a 96-well cell clear flat bottom culture plate at a concentration of 3.5×10^4 cells per well in 0.1 ml of complete culture medium supplemented with 10% FCS for 24 h. On the following day, a vertical line was drawn from the upper edge to the lower one of each well using a 300 μl thin plastic tip, and the wells were washed three times with pre-warmed HBSS solution. Then, 0.1 ml of inducer solutions (diluted protein fractions of the NK-92 cell derived MV lysate) prepared in the culture medium supplemented with 10% FCS were added to the wells, the final concentrations of each fraction being 10%, 5%, 2.5%, 1.25%, 0.625%, 0.313%, 0.156%, 0.078%, 0.039%, and 0.020%.

Control wells containing cells without the protein fractions added were photographed using an Axio Observer Z1 microscope and an AxioCam MRc 5 camera (Carl Zeiss Industrielle Messtechnik GmbH, Germany), capturing the initial line width. The prepared plates were then incubated for 24 h. On the following day, the culture medium was removed, and the cells were stained with 0.2% crystal violet solution, as described above. After washing the wells five times with distilled water, the plates were dried up for 24 h at 37 °C to completely remove moisture and were photographed to obtain three fields of view from each well. In each plate, the initial line width was measured and the average value was calculated.

The data obtained were analyzed using the patented MarkMigration software (Russia) [28], which automatically accounts both the number of cells that migrated into the zone of the disturbed monolayer and

the residual area within the boundaries of the zone. Cell migration was assessed by the change in these two parameters in comparison with the control (complete culture medium supplemented with 10% FCS). All samples were run in triplicate. Each protein fraction was analyzed in four repetitions for each dilution.

Flow cytometry

To evaluate the effect of protein fractions of the NK-92 cell derived MV lysate on the phenotype of EA.hy926 cells, the cells were transferred into the wells of a 24-well cell clear flat bottom culture plate (BD, USA) at a concentration of 1.8×10^5/ml of complete culture medium supplemented with 10% FCS and were cultured for 24 h before reaching confluence. The culture medium was then removed so that when the protein fractions were added, the total volume in the well was 0.45 ml. After that, 0.05 ml of the undiluted protein fractions were added to some of the wells. To control the effect of the mobile phase for chromatography on the phenotype of EA.hy926 cells, the mobile phase at a concentration of 12.5%, which was not toxic to the cells, was added to the other wells. Part of EA.hy926 cells was incubated in the wells in complete culture medium containing no inducers. As a positive control, the cells were cultured in complete culture medium in the presence of PMA (10 ng/ml). On the following day, the cell monolayer was disintegrated with pre-warmed Versene solution and was washed three times in Cell Wash solution (BD, USA). The cells were then treated with antibodies to the CD54 (ICAM-1), CD34, CD31 (PECAM-1) adhesion receptors and the CD119 IFNγ receptor in accordance with the manufacturer's recommendations (BD, USA). Isotypic antibodies were used to control nonspecific binding of antibodies as per the manufacturer's recommendations (BD, USA). The relative number and the intensity of the CD54, CD34, CD31 and CD119 receptor expression by EA.hy926 cells were assessed using a FACS Canto II flow cytometer (BD, USA). All samples were run four times. Each protein fraction was analyzed in duplicate for each dilution.

Statistical analysis

Statistical processing of the obtained data was carried out using the nonparametric Mann–Whitney and Kruskal–Wallis tests in the STATISTICA 10 and GraphPad Prism 8 programs. The data are presented as median (first quartile - third quartile). Figures, tables, and diagrams illustrate the changes in the studied parameters compared to the control, error bars showing the minimum and maximum data values. A p value of less than 0.05 was considered statistically significant.

Results

Micropreparative separation of the NK-92 cell derived MV lysate and electrophoresis of the obtained protein fractions

A lysate protein profile of the MVs produced by NK-92 cells that consisted of twelve fractions was obtained using chromatographic separation (Figure 1).

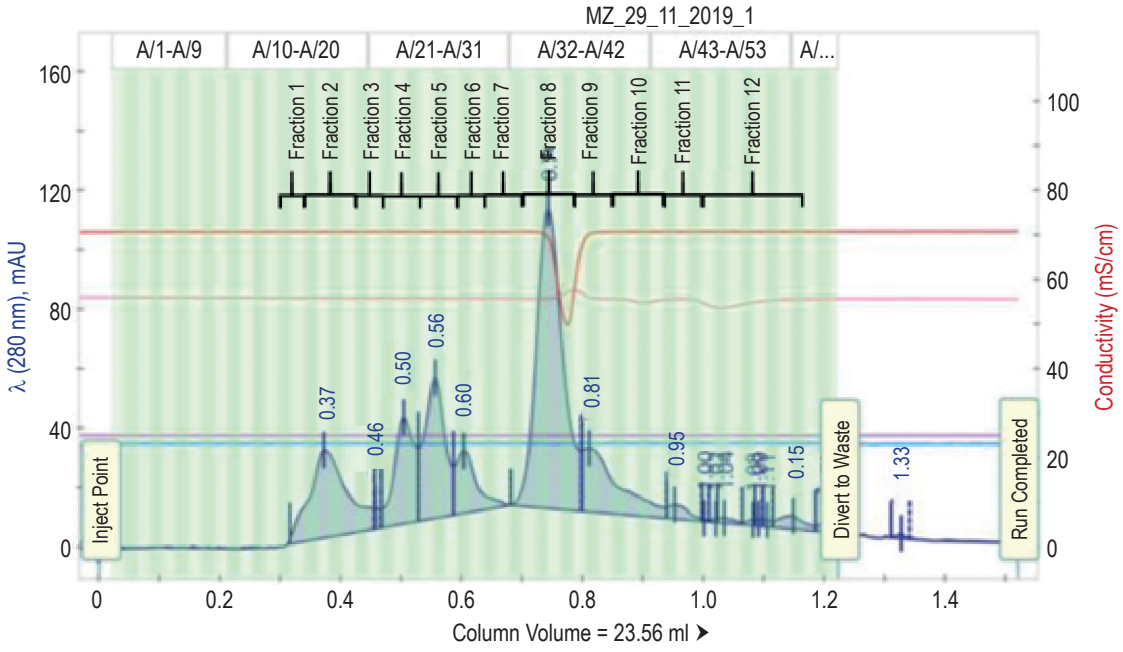

Figure 1. Chromatographic profile of the NK-92 cell derived MV lysate

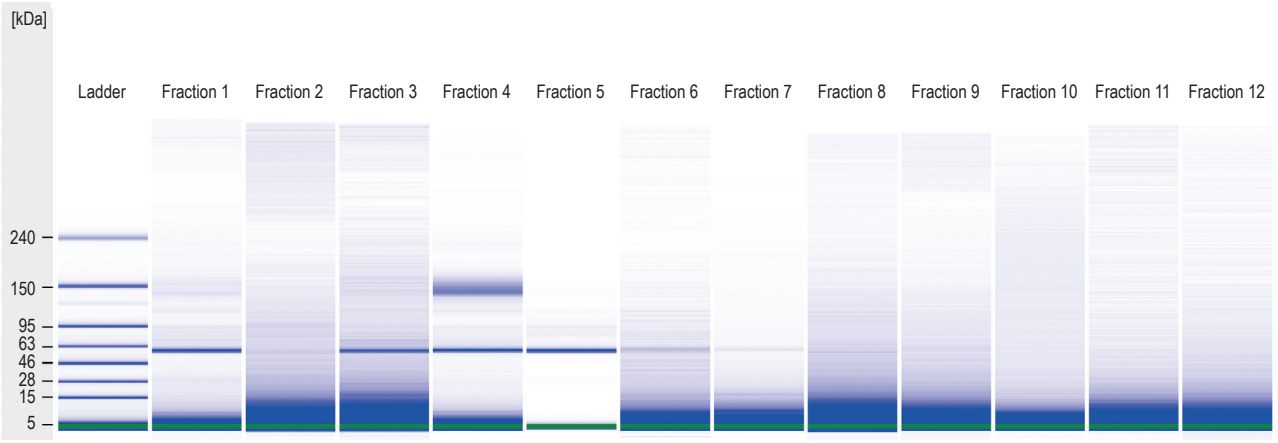

Figure 2. Electropherograms of the protein fractions of the NK-92 cell derived MV lysate

The following electrophoretic separation of proteins in the obtained fractions showed the molecular weight (MW) distribution of major (constituting more than 5% of the total profile) bands, as presented below. Fractions 1 and 3 contained a group of proteins with an average MW 59-60 kDa (100%); fraction 4 comprised groups of proteins with MW 60 kDa (55.9%) and 143 kDa (44.1%); fraction 5 contained a more diverse composition, in which groups of proteins with MW 62 kDa (88.5%), 84 kDa (6.4%) and 95 kDa (5.1%) were distinguished; fraction 6 included two major bands with MW 10 kDa (85.1%) and 14 kDa (10.6%), as well as fraction 7 with MW 10 kDa (91.5%) and 17 kDa (7.5%). The rest of the protein fractions contained minor components with an unknown average MW (fraction 2) or a MW lesser than 15 kDa (fractions 8-12) (Figure 2).

Evaluation of the effect of protein fractions of the NK-92 cell derived MV lysate on proliferation of EA.hy926 cells

In a preliminary experiment undertaken to verify the correctness of the data obtained, it was shown that proliferation of non-activated EA.hy926 cells cultured in complete growth medium supplemented with 2.5% inactivated FCS (control: 19721 (18662-21686) cells per well) differed from that of the cells cultured in the absence of FCS (first additional control: 4301 (3863-5159) cells per well; $p < 0.001$) and in the presence of 10% FCS (second additional control: 34837 (32356-36018) cells per well; $p < 0.001$), which indicated the optimal conditions for the subsequent tests.

It was found that ten out of twelve protein fractions of the NK-92 cell derived MV lysate changed the EA.hy926 cell proliferation pattern in comparison with the spontaneous cultivation (Figure 3). When compared to the control (non-activated ECs), the number of proliferated ECs increased 1.04-1.62 times after cultivation in the presence of the protein fractions at concentrations: 10% (fractions 1, 6, 9, 10, and 12); 5% (fractions 1, 3, 4, 6, 9, and 12); 2.5% (fractions 2-4, 6, and 9); 1.25% (fractions 2-4, 6, and 9); 0.625% (fractions 2-4, and 9); 0.313% (fractions 1-4); 0.156% (fractions 1, 3, and 4) and 0.078% (fractions 1, 3, and 4). Besides, it was found that this parameter decreased 1.03-1.16 times after culturing ECs in the presence of the fractions at concentrations: 10% and 5% (fraction 8); 0.313% and 0.078% (fraction 11).

In addition, it was found that after culturing EA.hy926 cells in the presence of fractions 1, 2, 6, 8-10, and 12, proliferation of the cells changed in a dose-dependent manner until the observed effect disappeared (Figure 3). This parameter was 1.11-1.62 times higher after cultivation of ECs in the presence of proliferation-stimulating fractions 1, 2, 6, 9, 10, and 12 at initial concentrations of 10%, 5% and 2.5%, than after cultivation in the presence of the same fractions in stronger dilutions. A similar tendency was shown in relation to proliferation-inhibiting fraction 8, the successive dilution of which gradually neutralized the observed effect.

Fractions 5 and 7 had no effect on proliferation of the target cells (Figure 3).

To sum up, the data obtained in this part of the experiment indicate a multidirectional (mainly stimulating) effect of protein fractions of the NK-92 cell derived MV lysate on proliferation of the endothelium.

Evaluation of the effect of protein fractions of the NK-92 cell derived MV lysate on migration of EA.hy926 cells

In a preliminary experiment undertaken to check the correctness of the data obtained, it was shown that the median (first quartile - third quartile) of the

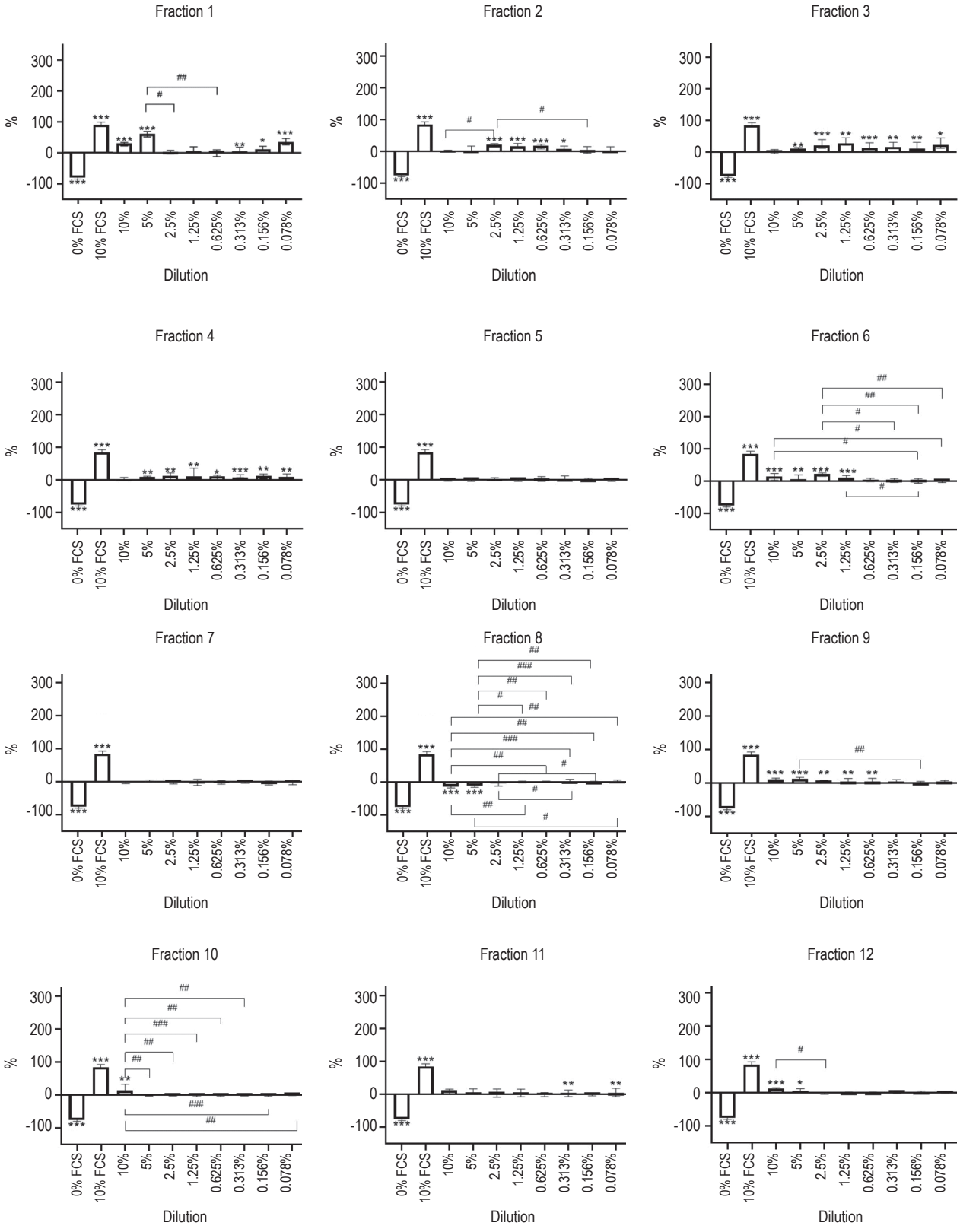

Figure 3. Proliferation of EA.hy926 cells under different cell cultivation conditions in the presence of the protein fractions of the NK-92 cell derived MV lysate (% of proliferation of the non-activated cells)

Note. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (compared to the non-activated cells); # $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$ (compared to the cells after culturing in the presence of the same fraction at two different concentrations).

area of the initial line after scraping the monolayer was 1.07 (1.02-1.15) mm^2, with no cells found in the area of the disturbed monolayer. The culture medium supplemented with 10% FCS was used as a control: the median (first quartile - third quartile) of the number of migrated cells was 373.6 (298.8-450.1), that of the residual area being 0.46 (0.37-0.56) mm^2, which differed from the residual area of the monolayer immediately after drawing the line ($p < 0.001$).

As in the case of the proliferation study, migration of EA.hy926 cells after their being cultured in the presence of the inducers changed in different directions or remained unchanged (Figure 4). When compared to the control (non-activated ECs), the number of migrated ECs increased 1.13-1.27 times after cultivation in the presence of the protein fractions at concentrations: 10% (fraction 3); 5% (fraction 11); 2.5% (fraction 5); 1.25% (fraction 11); 0.625% (fractions 1 and 11); 0.313% (fractions 10 and 11); 0.156% (fraction 1); 0.039% (fractions 1, 3, and 9) and 0.020% (fraction 5). It was also found that this parameter decreased 1.08-1.57 times after cultivation of ECs in the presence of the fractions at concentrations: 10% (fractions 2, 6, 10, and 12); 5% (fractions 2, 6, and 12); 2.5% (fraction 6); 1.25% (fractions 6 and 12); 0.625% (fraction 6); 0.313% (fractions 4 and 6); 0.156% (fractions 6 and 12); 0.078% (fractions 2, 6, and 12) and 0.020% (fraction 6).

It should also be noted that when fractions 3-5, 9, and 10 were used as inducers, only single changes in migration of EA.hy926 cells were found depending on the concentration of the inducer, while when fractions 1, 2, 6, 11, and 12 were used, more significant and frequent variations of this parameter were observed. Moreover, of all the fractions, fraction 6 showed the most prominent effect: all dilutions, with the exception of 0.039%, decreased the number of migrated cells.

Dose-dependent effects of the inducers on migration of EA.hy926 cells were observed only, when fraction 10 was used (Figure 4). Upon reaching the minimum endothelial cell migration after cultivation in the presence of the fraction at a concentration of 10%, its further dilution caused a sequential increase in the number of migrated cells by 1.35-1.45 times.

Fractions 7 and 8 had no effect on migration of the target cells (Figure 4).

It was also found that the residual area not occupied by migrated EA.hy926 cells after culturing them in the presence of the inducers did not always correlate with the migration rate and was not inversely proportional to the number of the migrated cells (Figure 5). When compared to the control (non-activated ECs), the area not occupied by migrated cells decreased 1.15-1.46 times after their cultivation in the presence of the fractions at concentrations: 10% (fraction 3); 5% (fraction 11); 2.5% (fraction 5); 1.25% (fraction 11); 0.625% and 0.313% (fractions 3, 10, and 11); 0.156% (fraction 1); 0.078% (fraction 9); 0.039% (fractions 1, 7, and 9) and 0.020% (fraction 5). An increase in this parameter by 1.04-1.41 times was observed after cultivation of ECs in the presence of the fractions at concentrations: 10% (fractions 6-9 and 12); 5%, 1.25%, 0.625%, and 0.313% (fraction 6), and 0.078% (fractions 2 and 6), with this effect observed in more than half of the studied concentrations when using fraction 6.

Fraction 4 did not affect the residual area (Figure 5).

Serial dilution of the inducers during cultivation of EA.hy926 cells also revealed some dose-dependent effects on the residual area not occupied by the migrated cells (Figure 5). For example, the area decreased by 1.49 times, when fraction 12 was diluted in the concentration range from 10% to 2.5%. This effect gradually leveled off with a further sequential decrease in the concentration of the inducer down to 0.020%, reaching a value of 81.7% compared to that, when using the fraction at a concentration of 10%.

To sum up, the data obtained in this part of the experiment indicate a multidirectional effect of the protein fractions of the NK-92 cell derived MV lysate on migration of the endothelium.

Evaluation of the effect of protein fractions of the NK-92 cell derived MV lysate on the phenotype of EA.hy926 cells

In a preliminary experiment undertaken to select the optimal conditions for subsequent tests, it was shown that the mobile phase for chromatography had no effect on the autofluorescence of EA.hy926 cells. Cultivation of the target cells in the presence of the mobile phase did not alter their expression of the CD54, CD34, CD31 and CD119 receptors.

After culturing EA.hy926 cells in the presence of PMA, the expression intensity of the CD54, CD34 and CD119 receptors was higher, and that of the CD31 receptor was lower, when compared to the non-activated cells (Table 1). The same changes were observed, when determining the relative number of cells expressing these receptors (Table 2).

It was found that the protein fractions of the NK-92 cell derived MV lysate did not affect the expression of the CD54, CD34 and CD119 receptors by the target cells (Tables 1, 2).

Discussion

In this study, using size exclusion chromatography, protein fractions of the NK-92 cell derived MV lysate

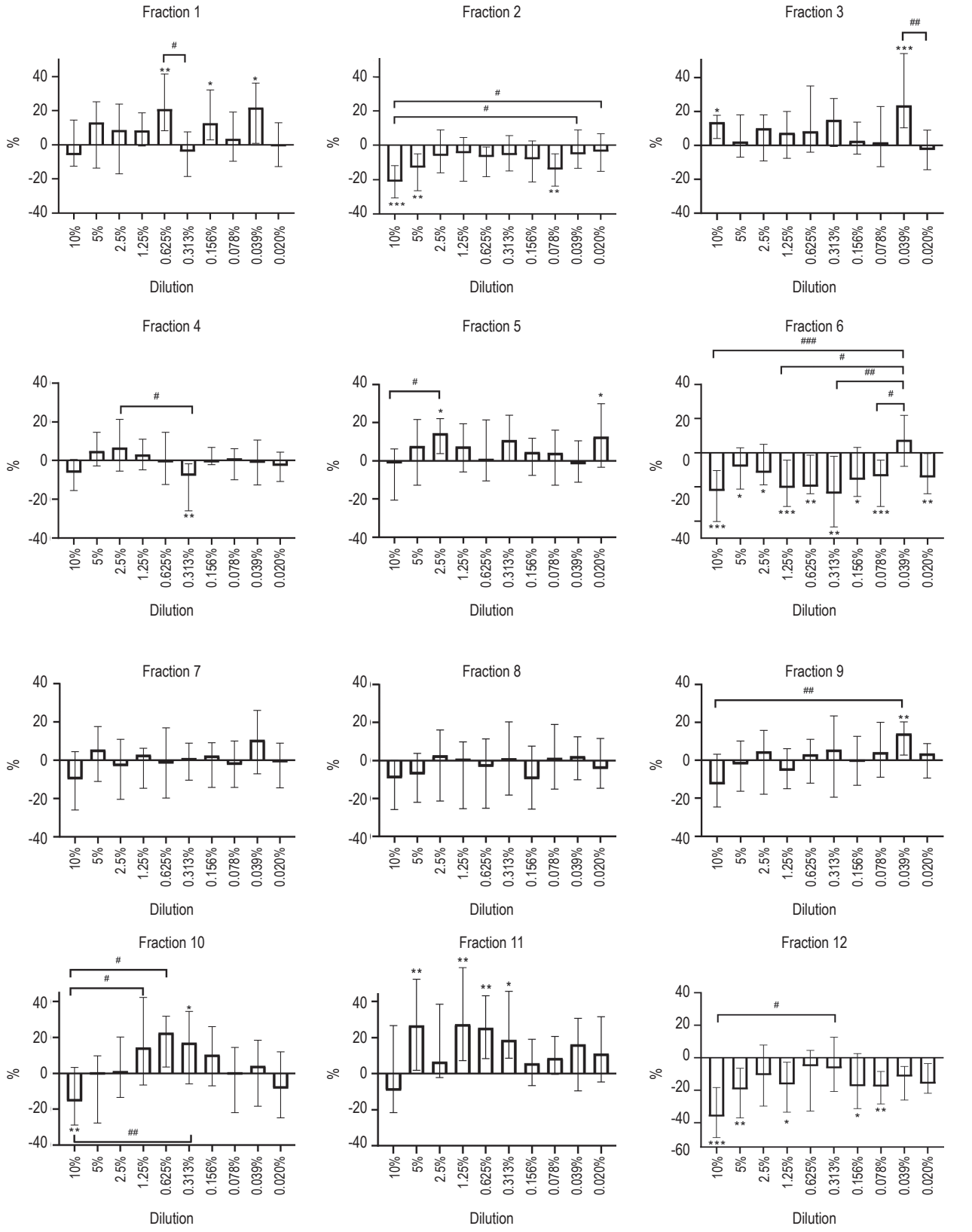

Figure 4. Number of EA.hy926 cells that migrated to the area of the disturbed monolayer under different cell cultivation conditions in the presence of the protein fractions of the NK-92 cell derived MV lysate (% of the non-activated cells; the abscissa shows the content of the fraction in the culture medium in serial dilutions)

Note. As for Figure 3.

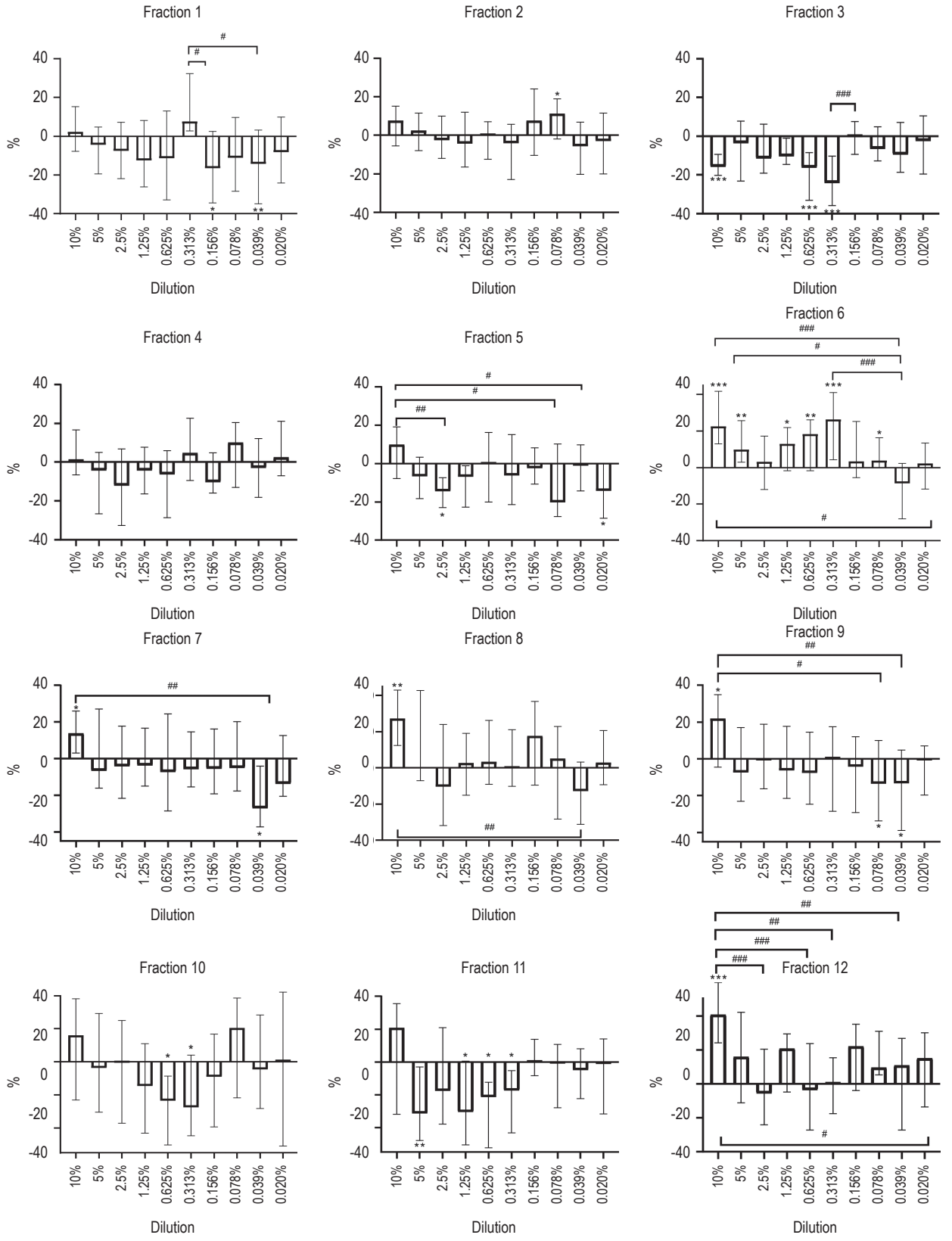

Figure 5. Residual area after EA.hy926 cells migrated to the area of the disturbed monolayer under different cell cultivation conditions in the presence of the protein fractions of the NK-92 cell derived MV lysate (% of the residual area not occupied by the non-activated cells; the abscissa shows the content of the fraction in the culture medium in serial dilutions)

Note. As for Figure 3.

TABLE 1. MEAN FLUORESCENCE INTENSITY OF CD54, CD34, CD31 AND CD119 RECEPTOR EXPRESSION BY EA.hy926 CELLS UNDER DIFFERENT CELL CULTIVATION CONDITIONS IN THE PRESENCE OF PROTEIN FRACTIONS OF THE NK-92 CELL DERIVED MICROVESICLE LYSATE

Cell cultivation conditions	Receptor expression intensity (MFI)			
	CD54	CD34	CD31	CD119
ECs (control)	2568 (1862-3200)	0 (0-0)	877 (790-1002)	489 (330-568)
ECs + mobile phase for chromatography	2468 (2292-2503)	0 (0-0)	505 (480-922)	25 (3-723)
ECs + PMA	5604 (5196-6224)***	188 (55-459)**	148 (108-171)***	756 (606-1081)*
ECs + fraction 1	2615 (2288-3409)	2 (0-13)	659 (581-1549)	563 (302-604)
ECs + fraction 2	2478 (2467-2924)	14 (0-65)	630 (559-1761)	417 (352-648)
ECs + fraction 3	2548 (2196-2645)	0 (0-137)	636 (542-1248)	471 (356-661)
ECs + fraction 4	2584 (2410-2761)	45 (0-141)	768 (623-1708)	605 (324-660)
ECs + fraction 5	2948 (2731-3041)	0 (0-27)	731 (647-1883)	453 (289-630)
ECs + fraction 6	2646 (2256-2846)	76 (0-111)	704 (606-1967)	540 (221-681)
ECs + fraction 7	2596 (2545-2821)	98 (0-253)	731 (604-1496)	640 (469-694)
ECs + fraction 8	2443 (2310-2698)	0 (0-71)	622 (494-1874)	512 (338-634)
ECs + fraction 9	2720 (2682-3079)	44 (0-82)	723 (560-1844)	611 (411-634)
ECs + fraction 10	2675 (2380-2804)	46 (0-89)	622 (547-2217)	598 (416-651)
ECs + fraction 11	2770 (2482-2998)	19 (0-142)	668 (553-2013)	592 (264-709)
ECs + fraction 12	2748 (2363-3089)	0 (0-39)	663 (554-2038)	506 (209-703)

Note. ECs, non-activated EA.hy926 cells; PMA, phorbol 12-myristate 13-acetate (10 ng/ml). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (compared to the non-activated cells expressing the same receptor).

TABLE 2. RELATIVE NUMBER OF EA.hy926 CELLS EXPRESSING CD54, CD34, CD31 AND CD119 RECEPTORS UNDER DIFFERENT CELL CULTIVATION CONDITIONS IN THE PRESENCE OF PROTEIN FRACTIONS OF THE NK-92 CELL DERIVED MICROVESICLE LYSATE

Cell cultivation conditions	Relative number of cells expressing receptors (%)			
	CD54	CD34	CD31	CD119
ECs (control)	59.6 (53.2-62.9)	0.0 (0.0-1.1)	44.0 (40.4-50.1)	30.8 (19.6-35.9)
ECs + mobile phase for chromatography	59.5 (59.4-59.7)	0.0 (0.0-0.9)	35.0 (2.8-48.3)	16.2 (4.9-19.9)
ECs + PMA	80.7 (79.5-82.8)***	17.6 (2.9-34.5)**	4.1 (2.9-5.5)***	49.9 (38.0-70.5)*
ECs + fraction 1	62.4 (58.4-66.2)	1.0 (0.0-1.4)	31.2 (28.0-65.6)	32.2 (17.5-37.1)
ECs + fraction 2	62.5 (59.7-64.5)	3.2 (0.0-4.2)	30.6 (27.6-69.7)	25.8 (21.5-42.9)
ECs + fraction 3	60.3 (56.3-62.2)	0.0 (0.0-11.7)	31.0 (25.3-55.8)	29.5 (23.0-40.4)
ECs + fraction 4	61.1 (59.1-63.3)	1.1 (0.0-5.9)	34.1 (30.7-69.0)	36.8 (18.6-39.1)
ECs + fraction 5	64.7 (62.7-68.8)	0.0 (0.0-4.2)	34.6 (31.5-73.0)	27.9 (17.0-43.2)
ECs + fraction 6	61.2 (57.1-66.5)	4.1 (0.0-9.7)	32.8 (28.7-72.2)	28.9 (11.6-48.4)
ECs + fraction 7	61.1 (59.6-65.3)	4.8 (0.0-10.3)	33.2 (27.9-59.7)	35.3 (24.4-40.5)
ECs + fraction 8	59.1 (57.1-63.8)	0.0 (0.0-2.2)	29.8 (25.1-69.8)	33.6 (16.8-40.8)
ECs + fraction 9	61.3 (58.9-67.1)	4.3 (0.0-5.6)	30.9 (25.3-68.7)	36.8 (22.4-41.4)
ECs + fraction 10	59.2 (58.0-68.0)	3.5 (0.0-4.3)	29.2 (26.1-76.5)	34.0 (23.7-40.2)
ECs + fraction 11	61.4 (59.8-67.2)	0.9 (0.0-2.7)	31.9 (27.0-74.7)	32.9 (14.8-40.3)
ECs + fraction 12	62.3 (57.0-69.0)	0.0 (0.0-3.2)	31.5 (24.9-76.2)	27.1 (10.2-40.5)

Note. As for Table 1.

were obtained, which were further characterized by weight using capillary gel electrophoresis on microchips. The obtained fractions are represented by groups of proteins with different molecular weights, the average values of which are in the range of 5-250 kDa. The quality of the distribution of protein groups by fractions indicates a satisfactory chromatographic separation of the lysate, which is proven by broad absorption and fluorescence bands spanning several neighboring fractions. The obtained result also reflects a wide variety of proteins in the composition of the NK-92 cell derived MV lysate (including cytokines, cell adhesion regulators, intracellular signaling proteins), which we previously identified as candidate proteins using MALDI-TOF mass spectrometric analysis [23].

The aim of this study was to evaluate the effect of the obtained protein fractions of the NK-92 cell derived MV lysate on the phenotype and the functional characteristics of the EA.Hy926 cell line. Previously, we have found that the cultivation of ECs in the presence of NK-92 cell derived MVs increased endothelial cell proliferation in a dose-dependent manner, when compared with cultivation in a medium without MVs [29]. Besides, we have found decreased endothelial cell migration under the same conditions, which was caused by a decrease in the number of migrated ECs compared to the cultivation of the target cells in the absence of MVs [29]. Therefore, the next stage of our study was the assessment of the effect of the protein fractions of the NK-92 cell derived MV lysate on proliferation and migration of EA.Hy926 cells.

In this study, it was found that ten out of the twelve protein fractions of the MV lysate cause a multidirectional change in endothelial cell proliferation as compared to cultivation in a medium that did not contain the inducers. This fact indicates the presence in these fractions of proteins that regulate endothelial proliferation. In this case, the predominant increase in proliferation of the target cells was observed in the presence of fractions 1-4, 6, and 9-12. Moreover, the most significant effect in a wide range of concentrations was exerted by fraction 1 (high and low concentrations; dose-dependent effect), as well as fractions 3 and 4 (all concentrations except 10%), while the other fractions only exerted their influence in several dilutions. The inhibitory effect on endothelial cell proliferation was exerted by fraction 8 (high concentrations; dose-dependent effect) and fraction 11 (low concentrations). The rest of the fractions (5 and 7) did not change proliferation of the target cells.

The data obtained by us are consistent with both the literature data on the multidirectional effect of NK cells on angiogenesis in *in vitro* systems [12] and our previously obtained data regarding MVs produced by NK-92 cells [29].

When assessing the effect of the protein fractions of the MV lysate on endothelial cell migration, multidirectional effects were also found. For example, fractions 1, 3, 5, 9, and 11 increased the number of cells that migrated into the zone of the disturbed monolayer, which may imply the production by NK cells and the subsequent transfer by means of MVs of a variety of cytokines that can affect the formation of blood vessels [4]. Despite this, in other cases, i.e. during cultivation of ECs in the presence of fractions 2, 4, 6, and 12, decreased migration of the target cells was observed, which can be accounted for by increased dying of ECs in the presence of specific proteins from the MV cargo, including antiangiogenic factors. At the same time, the predominant suppression of migration of the target cells was observed in the presence of fraction 6 (all concentrations except 0.039%) and fraction 12 (high and low concentrations). Cultivation of ECs in the presence of fraction 10 revealed opposite effects depending on dilution: for example, at a high concentration (10%), the fraction dose-dependently decreased the number of cells in the zone of the disturbed monolayer, and in a low concentration (0.313%), on the contrary, it increased this parameter. The observed multidirectional effect of this fraction indicates different limiting concentrations of factors that stimulate or inhibit migration of the target cells, at the cumulative achievement of which the influence of some inducer proteins begins to dominate, while the effective action of the others is gradually leveled out. The rest of the fractions (7 and 8) did not affect endothelial cell migration.

It was also found that cultivation of ECs in the presence of fractions 6-9 increased the residual area in the zone of the disturbed monolayer, with the number of the migrated target cells elevated or not changed significantly. In the case of fraction 4, the residual area did not change with a simultaneous decrease in the number of the cells, and in the case of fraction 10, this parameter lowered with a simultaneous decrease in the number of the target cells. The data obtained, which not in all studied cases reflect the negative correlation between migration and the filling area, may indicate changes in the morphology of ECs under the influence of both pro-angiogenic and anti-angiogenic factors contained in the inducers. At the same time, low endothelial cell migration and proliferation decreased by some factors propagated by NK cells by means of their MVs can, *in vivo*, contribute to the disintegration of the vascular endothelial lining. This, in turn, leads to a disruption of blood vessel permeability and

can contribute to other pathologies associated with endothelial dysfunction [32].

The multidirectional dose-dependent effects of different concentrations of the NK-92 cell derived MV lysate fractions in relation to the regulation of endothelial proliferation and migration indicate the presence of cytokines in MVs that control these processes. Such cytokines in the MV cargo may be cytokines with opposite effects: VEGF [20], a stimulator of angiogenesis, as well as IL-10 [10] and TGF-β [21], inhibitors of angiogenesis. Previously, we have obtained data that NK cells upon contact cultivation with ECs lengthen capillary-like structures formed by ECs [30]. NK-92 cells are a source of VEGF and carry the VEGFR1 receptor on their surface [27]. VEGF is one of the main and most important factors that regulate all stages of angiogenesis, stimulating endothelial cell migration, proliferation and viability [1]. Moreover, NK-92 cells are a source of the matrix metalloproteinases MMP-2 and MMP-9 [13], which play an important role in angiogenesis [5]. Matrix metalloproteinases provide degradation and remodeling of the extracellular matrix [34] and stimulate endothelial cell migration [8]. There is evidence in the literature that, due to their proteolytic activity, matrix metalloproteinases play an important role in the regulation of signaling pathways that control cell proliferation, invasion, and viability [5].

Previously, using the MALDI-TOF mass spectrometry method, we have found 986 proteins in MVs produced by NK-92 cells, including semaphorins, TGF-β, RANTES, IP-10, CXCL11, etc. (Table 3) [23], which also indicates the active influence of NK cells on angiogenesis and the functions of ECs. Table 3 includes the cytokine receptors found in MVs, which could competitively bind to cytokines, reducing their autocrine effect on ECs.

Comparison of data on the effect of the protein fractions of the NK-92 cell derived MV lysate on proliferation and migration of EA.Hy926 cells also revealed a heterogeneous relationship. It is logical to assume that when cells proliferate, the migration stops. However, such an effect was observed only in a part of the obtained fractions: when fractions 2, 4, 10, and 12 were added, the number of proliferating cells increased but the number of migrated cells decreased. At the same time, when fractions 1, 3, 6, and 9-11 were added, the number of proliferating and migrated cells increased simultaneously. Fraction 5 did not change proliferation, but it increased migration, while fraction 8, on the contrary, decreased proliferation and did not change endothelial cell migration. Fraction 7 did not affect the numbers of migrated and proliferated cells.

Proliferation and the migration rates have different effects on the vasculature, as they can be weakened in different ways by selective agents in proliferative diseases such as cancer; or these parameters can be selectively increased in ischemic diseases, wound healing or regenerative medicine. Using computer modeling, it was shown that proliferation is of great importance in the development of the tumor

TABLE 3. FUNCTIONS OF SEVERAL PROTEINS OF MICROVESICLES PRODUCED BY NK-92 CELLS AND THEIR PRESUMPTIVE DISTRIBUTION IN THE OBTAINED PROTEIN FRACTIONS

Protein	MW, kDa	Function / Biological process	Protein fractions
C-C chemokine receptor type 3	7.8	CCL5, CCL7, CCL11, CCL13, CCL15, CCL24, CCL26, and CCL28 receptor	8, 9, 10, 11,12
Prostaglandin E2 receptor EP4 subtype (EP4)	53.1	PGE2 receptor, inhibition of cell cytotoxicity	3, 4, 5
C-C motif chemokine 5 (CCL5, RANTES)	10.0	chemokine, inflammatory mediator	8, 9, 10, 11,12
C-X-C motif chemokine 10 (CXCL10, IP-10)	10.9	chemokine, inflammatory mediator control of cell migration	8, 9, 10, 11,12
C-X-C motif chemokine 11 (CXCL11)	10.4	chemokine, inflammatory mediator, control of cell migration	8, 9, 10, 11,12
Interferon β	22.3	cell activation	5
Semaphorin-4D (SEMA4D, CD100), isoform 1	96.1	activated cell surface receptor, mediating of cell cytotoxicity by binding to CD72 on the target cell	4, 5
Transforming growth factor β-1	44.3	control of cell activity, regulation of differentiation	3, 4, 5

vasculature [33]. When proliferation is low, the vessels reach the tumor inner space, but the capillaries still do not cover most of the area. Thus, cell migration allows the vasculature to spread in the tumor space, but with fewer cells. When proliferation is moderate, the tumor vasculature spreads throughout the area. In this case, there is a balance between proliferation and migration, in which proliferation is too low to allow the proliferation of end cells, but it is high enough for vessels to grow. This leads to an expansion of the tumor space due to cell migration and better coverage due to the branching of new cells. When proliferation is high, the resulting vasculature is minimal but very tortuous [33]. The heterogeneous effect of the protein fractions of the NK-92 cell derived MV lysate on proliferation and migration of EA.Hy926 cells found by us confirms the multifunctional role of NK cells in controlling the development of the vascular network in health and disease.

In this study, we also analyzed the effect of the protein fractions of the NK-92 cell derived MV lysate on the expression of the CD54 (ICAM-1), CD34, CD31 (PECAM-1) and CD119 (IFNγR1) receptors by EA.Hy926 cells. The choice of the receptors for assessing their expression by the target cells was due to the fact that previously, it was found by us that the expression of these receptors changed after cultivation of ECs in the presence of MVs produced by NK-92 cells [29]. Namely, incubation of ECs in the presence of MVs decreased the number of the cells expressing the CD34, CD31 and CD119 receptors. A decrease in the number of the target cells with the $CD34^+$ phenotype after incubation with MVs produced by NK-92 cells was accompanied by an increased expression of this receptor by ECs as compared to intact cells. Despite no differences in the number of ECs with the $CD54^+$ phenotype, the intensity of CD54 expression on ECs was higher after their incubation with MVs compared to intact cells [29].

We found that proteins of the NK-92 cell derived MV lysate fractions did not alter the expression of the CD54, CD34, CD31 and CD119 receptors. No changes in the expression indicate an insufficient concentration of proteins capable of influencing the relevant gene expression and the probable presence of active phosphorylated forms of signal proteins in the lysate (one cannot exclude a possible contribution to the observed effects of miRNAs and membrane bound proteins). Besides, it may indicate no contamination of the protein fractions with lipopolysaccharides after their being filtered for the purpose of sterilization (this necessary procedure also lowers the concentration of active components of the fractions).

Taking into account all the information presented, one can conclude that the our data obtained on altered proliferation and migration of EA.hy926 cells under the influence of the protein fractions of the NK-92 cell derived MV lysate and on insignificant contribution of the several inducers to the total lysate effect confirm the involvement of a variety of pro- and anti-angiogenic factors of leukocyte origin and associated signaling pathways in blood vessel formation [38].

Thus, MVs produced by NK-92 cells, in addition to cytotoxic proteins, carry other protein components (regulators of the immune response, inflammation, and angiogenesis) that can provide communication of NK cells with ECs, causing their activation and changing their proliferative and migratory activity in different directions. Comparison of the obtained data with the results of previous studies allows for proposing specific proteins that may be involved in these processes: calcium/calmodulin-dependent protein kinase type II, serine/threonine kinase Akt, sphingosine-1-phosphate lyase 1, α-catulin, CXC chemokines, profilin-3, relaxin-3, etc. However, to elucidate the immunological and biochemical mechanisms underlying proliferation and migration of ECs, further research is needed to identify effector proteins in the cargo of MVs produced by NK cells and to search for direct evidence of their relation to the effects observed during an interaction of the MVs with ECs.

Conclusion

NK cells can perform their functions with the help of MVs, a new poorly studied object of cell communication. Further study of the role and the protein cargo of MVs produced by NK cells will expand the existing knowledge of the mechanisms of participation of these cells not only in the implementation of cytotoxic effects, but also in the regulation of various physiological processes, in particular angiogenesis.

References

1. Abhinand C.S., Raju R., Soumya S.J., Arya P.S., Sudhakaran P.R. VEGF-A/VEGFR2 signaling network in endothelial cells relevant to angiogenesis. *J. Cell Commun. Signal., 2016, Vol. 10, no. 4, pp. 347-354.*

2. Amchislavskii E.I., Sokolov D.I., Sel'kov S.A., Freidlin I.S. Proliferative activity of human endothelial cell line EA.hy926 and its modulation. *Tsitologiia, 2005, Vol. 47, no. 5, pp. 393-403.* (In Russ.)

3. Ashton S.V., Whitley G.S., Dash P.R., Wareing M., Crocker I.P., Baker P.N., Cartwright J.E. Uterine spiral artery remodeling involves endothelial apoptosis induced by extravillous trophoblasts through Fas/FasL interactions. *Arterioscler. Thromb. Vasc. Biol., 2005, Vol. 25, no. 1, pp. 102-108*.

4. Bassani B., Baci D., Gallazzi M., Poggi A., Bruno A., Mortara L. Natural killer cells as key players of tumor progression and angiogenesis: old and novel tools to divert their pro-tumor activities into potent anti-tumor effects. *Cancers, 2019, Vol. 11, no. 4, 461*. doi: 10.3390/cancers11040461.

5. Bauvois B. New facets of matrix metalloproteinases MMP-2 and MMP-9 as cell surface transducers: outside-in signaling and relationship to tumor progression. *Biochim. Biophys. Acta, 2012, Vol. 1825, no. 1, pp. 29-36.*

6. Blois S.M., Klapp B.F., Barrientos G. Decidualization and angiogenesis in early pregnancy: unravelling the functions of DC and NK cells. *J. Reprod. Immunol., 2011, Vol. 88, no. 2, pp. 86-92.*

7. Burger D., Schock S., Thompson C.S., Montezano A.C., Hakim A.M., Touyz R.M. Microparticles: biomarkers and beyond. *Clin. Sci. (Lond.), 2013, Vol. 124, no. 7, pp. 423-441.*

8. Carmeliet P., Jain R.K. Molecular mechanisms and clinical applications of angiogenesis. *Nature, 2011, Vol. 473, no. 7347, pp. 298-307.*

9. Chazara O., Xiong S., Moffett A. Maternal KIR and fetal HLA-C: a fine balance. *J. Leukoc. Biol., 2011, Vol. 90, no. 4, pp. 703-716*.

10. Clark S.E., Burrack K.S., Jameson S.C., Hamilton S.E., Lenz L.L. NK cell IL-10 production requires IL-15 and IL-10 driven STAT3 activation. *Front. Immunol., 2019, Vol. 10, 2087*. doi: 10.3389/fimmu.2019.02087.

11. Dasgupta S.K., Abdel-Monem H., Niravath P., Le A., Bellera R.V., Langlois K., Nagata S., Rumbaut R.E., Thiagarajan P. Lactadherin and clearance of platelet-derived microvesicles. *Blood, 2009, Vol. 113, no. 6, pp. 1332-1339.*

12. Dondero A., Casu B., Bellora F., Vacca A., de Luisi A., Frassanito M.A., Cantoni C., Gaggero S., Olive D., Moretta A., Bottino C., Castriconi R. NK cells and multiple myeloma-associated endothelial cells: molecular interactions and influence of IL-27. *Oncotarget, 2017, Vol. 8, no. 21, pp. 35088-35102.*

13. Edsparr K., Johansson B.R., Goldfarb R.H., Basse P.H., Nannmark U., Speetjens F.M., Kuppen P.J., Lennernas B., Albertsson P. Human NK cell lines migrate differentially *in vitro* related to matrix interaction and MMP expression. *Immunol. Cell Biol., 2009, Vol. 87, no. 6, pp. 489-495.*

14. el Costa H., Tabiasco J., Berrebi A., Parant O., Aguerre-Girr M., Piccinni M.P., Le Bouteiller P. Effector functions of human decidual NK cells in healthy early pregnancy are dependent on the specific engagement of natural cytotoxicity receptors. *J. Reprod. Immunol., 2009, Vol. 82, no. 2, pp. 142-147.*

15. Fraser R., Whitley G.S., Thilaganathan B., Cartwright J.E. Decidual natural killer cells regulate vessel stability: implications for impaired spiral artery remodelling. *J. Reprod. Immunol., 2015, Vol. 110, pp. 54-60.*

16. Gong J.H., Maki G., Klingemann H.G. Characterization of a human cell line (NK-92) with phenotypical and functional characteristics of activated natural killer cells. *Leukemia, 1994, Vol. 8, no. 4, pp. 652-658.*

17. Halim A.T., Ariffin N.A., Azlan M. Review: the multiple roles of monocytic microparticles. *Inflammation, 2016, Vol. 39, no. 4, pp. 1277-1284.*

18. Hanna J., Goldman-Wohl D., Hamani Y., Avraham I., Greenfield C., Natanson-Yaron S., Prus D., Cohen-Daniel L., Arnon T.I., Manaster I., Gazit R., Yutkin V., Benharroch D., Porgador A., Keshet E., Yagel S., Mandelboim O. Decidual NK cells regulate key developmental processes at the human fetal-maternal interface. *Nat. Med., 2006, Vol. 12, no. 9, pp. 1065-1074.*

19. Hanna J., Wald O., Goldman-Wohl D., Prus D., Markel G., Gazit R., Katz G., Haimov-Kochman R., Fujii N., Yagel S., Peled A., Mandelboim O. CXCL12 expression by invasive trophoblasts induces the specific migration of $CD16^-$ human natural killer cells. *Blood, 2003, Vol. 102, no. 5, pp. 1569-1577.*

20. Hawke L.G., Whitford M.K.M., Ormiston M.L. The production of pro-angiogenic VEGF-A isoforms by hypoxic human NK cells is independent of their TGF-beta-mediated conversion to an ILC1-like phenotype. *Front. Immunol., 2020, Vol. 11, 1903*. doi: 10.3389/fimmu.2020.01903.

21. Jiang Y., Yang M., Sun X., Chen X., Ma M., Yin X., Qian S., Zhang Z., Fu Y., Liu J., Han X., Xu J., Shang H. IL-10(+) NK and TGF-beta(+) NK cells play negative regulatory roles in HIV infection. *BMC Infect. Dis., 2018, Vol. 18, 80*. doi: 10.1186/s12879-018-2991-2.

22. Kalkunte S.S., Mselle T.F., Norris W.E., Wira C.R., Sentman C.L., Sharma S. Vascular endothelial growth factor C facilitates immune tolerance and endovascular activity of human uterine NK cells at the maternal-fetal interface. *J. Immunol., 2009, Vol. 182, no. 7, pp. 4085-4092.*

23. Korenevsky A.V., Shcherbitskaia A.D., Berezkina M.E., Markova K.L., Alexandrova E.P., Balabas O.A., Selkov S.A., Sokolov D.I. MALDI-TOF mass spectrometric protein profiling of microvesicles produced by the NK-92 natural killer cell line. *Medical Immunology (Russia), 2020, Vol. 22, no. 4, pp. 633-646*. doi: 10.15789/1563-0625-mms-1976.

24. Kowal J., Arras G., Colombo M., Jouve M., Morath J.P., Primdal-Bengtson B., Dingli F., Loew D., Tkach M., Thery C. Proteomic comparison defines novel markers to characterize heterogeneous populations of extracellular vesicle subtypes. *Proc. Natl Acad. Sci. USA, 2016, Vol. 113, no. 8, pp. E968-E977.*

25. Leonard S., Murrant C., Tayade C., van den Heuvel M., Watering R., Croy B.A. Mechanisms regulating immune cell contributions to spiral artery modification – facts and hypotheses – a review. *Placenta, 2006, Vol. 27, Suppl., pp. 40-46.*

26. Li P., Kaslan M., Lee S.H., Yao J., Gao Z. Progress in exosome isolation techniques. *Theranostics, 2017, Vol. 7, no. 3, pp. 789-804.*

27. Male V., Trundley A., Gardner L., Northfield J., Chang C., Apps R., Moffett A. Natural killer cells in human pregnancy. In: Campbell K. (eds) Natural Killer Cell Protocols. *Methods Mol. Biol., 2010, Vol. 612, pp. 447-463.*

28. Markov A.S., Markova K.L., Sokolov D.I., Selkov S.A., *MARKMIGRATION*. Russia, 2019.

29. Markova K.L., Mikhailova V.A., Korenevsky A.V., Milyutina Y.P., Rodygina V.V., Aleksandrova E.P., Markov A.S., Balabas O.A., Selkov S.A., Sokolov D.I. Microvesicles produced by natural killer cells of the NK-92 cell line affect the phenotype and functions of endothelial cells of the EA.Hy926 cell line. *Medical Immunology (Russia), 2020, Vol. 22, no. 2, pp. 249-268.* doi: 10.15789/1563-0625-MPB-1877.

30. Markova K.L., Stepanova O.I., Sheveleva A.R., Kostin N.A., Mikhailova V.A., Selkov S.A., Sokolov D.I. Natural killer cell effects upon angiogenesis under conditions of contact-dependent and distant co-culturing with endothelial and trophoblast cells. *Medical Immunology (Russia), 2019, Vol. 21, no. 3, pp. 427-440.* doi: 10.15789/1563-0625-2019-3-427-440.

31. Mikhailova V.A., Ovchinnikova O.M., Zainulina M.S., Sokolov D.I., Sel'kov S.A. Detection of microparticles of leukocytic origin in the peripheral blood in normal pregnancy and preeclampsia. *Bull. Exp. Biol. Med., 2014, Vol. 157, no. 6, pp. 751-756.*

32. Morris G., Puri B.K., Olive L., Carvalho A., Berk M., Walder K., Gustad L.T., Maes M. Endothelial dysfunction in neuroprogressive disorders-causes and suggested treatments. *BMC Med., 2020, Vol. 18, 305.* doi: 10.1186/s12916-020-01749-w.

33. Norton K.A., Popel A.S. Effects of endothelial cell proliferation and migration rates in a computational model of sprouting angiogenesis. *Sci. Rep., 2016, Vol. 6, 36992.* doi: 10.1038/srep36992.

34. Papetti M., Herman I.M. Mechanisms of normal and tumor-derived angiogenesis. *Am. J. Physiol. Cell Physiol., 2002, Vol. 282, no. 5, pp. 947-C970.*

35. Sedgwick A.E., D'Souza-Schorey C. The biology of extracellular microvesicles. *Traffic, 2018, Vol. 19, pp. 319-327.*

36. Sokolov D.I., Ovchinnikova O.M., Korenkov D.A., Viknyanschuk A.N., Benken K.A., Onokhin K.V., Selkov S.A. Influence of peripheral blood microparticles of pregnant women with preeclampsia on the phenotype of monocytes. *Transl. Res., 2016, Vol. 170, pp. 112-123.*

37. Thornhill M.H., Li J., Haskard D.O. Leucocyte endothelial cell adhesion: a study comparing human umbilical vein endothelial cells and the endothelial cell line EA-hy-926. *Scand. J. Immunol., 1993, Vol. 38, no. 3, pp. 279-286.*

38. Yao L., Sgadari C., Furuke K., Bloom E.T., Teruya-Feldstein J., Tosato G. Contribution of natural killer cells to inhibition of angiogenesis by interleukin-12. *Blood, 1999, Vol. 93, no. 5, pp. 1612-1621.*

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