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МИКРОВЕЗИКУЛЫ ЕСТЕСТВЕННЫХ КИЛЛЕРОВ ЛИНИИ NK-92 ВЛИЯЮТ НА ФЕНОТИП И ФУНКЦИИ ЭНДОТЕЛИАЛЬНЫХ КЛЕТОК ЛИНИИ EA.Hy926

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Резюме. Микровезикулы (МВ) – субклеточные структуры размером от 100 до 1000 нм, продуцируемые клетками в состоянии покоя и активации. МВ могут передавать молекулы клеткам-мишеням, регулировать физиологические процессы, участвовать в патологиях. Микровезикулы лейкоцитарного происхождения, в частности МВ NK-клеток, остаются наименее изученной популяцией МВ. NK-клетки способны изменять функциональную активность эндотелиальных клеток (ЭК), участвуют в регуляции ангиогенеза. Недостаточно изучена способность МВ NK-клеток влиять на функциональное состояние ЭК. Целью настоящего исследования явилось изучение влияния МВ, образуемых естественными киллерами линии NK-92, на фенотип, активность каспаз, пролиферацию и миграцию ЭК линии EA.Hy926. ЭК культивировали в присутствии МВ клеток линии NK-92. При помощи проточной цитофлуориметрии оценивали изменение фенотипа ЭК, передачу внутриклеточного белка из МВ в ЭК, относительную гибель ЭК. При помощи Western blot analysis оценивали экспрессию гранзима B в NK-клетках и их МВ, появление гранзима B в ЭК, экспрессию каспаз, Erk, AKT в ЭК. Также оценивали пролиферацию и миграцию ЭК в присутствии МВ клеток линии NK-92. Установлено значимое различие протеомных профилей клеток линии NK-92 и образуемых ими МВ. Контакт ЭК с МВ клеток линии NK-92 сопровождается развитием следующих событий: 1) экспрессией в ЭК гранзима B; 2) активацией каспазы-9, каспазы-3 и частичной гибелью ЭК; 3) появлением на ЭК панлейкоцитарного маркера CD45; 4) снижением экспрессии CD105 и повышением экспрессии CD34 и CD54; 5) ингибированием миграции ЭК. Передача эндотелиальным клеткам Erk, но не AKT, в составе МВ клеток линии NK-92 в концентрации в 10 раз ниже концентрации, вызывающей гибель ЭК, способствует повышению пролиферации ЭК.

Ключевые слова: NK-клетки, эндотелий, микровезикулы, гранзим B, каспазы, пролиферация, миграция

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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

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Abstract. Microvesicles (MVs) are small (100-1000 nm) subcellular structures produced by both motionless and activated cells that can transfer molecules to target cells, and regulate physiological and pathological processes. MVs of leukocyte origin, in particular those produced by natural killer cells (NK cells), remain the least studied population of MVs. NK cells can change the functional activity of endothelial cells (ECs) and are involved in regulating angiogenesis. The ability of NK cell-derived MVs to influence the functionality of ECs is understudied currently. We aimed to study the effect of MVs produced by NK cells of the NK-92 cell line on the phenotype, caspase activity, proliferation and migration of ECs of the EA.Hy926 cell line. We cultured ECs in the presence of MVs derived from the NK-92 cell line, and then used flow cytometry to assess changes in EC phenotype, intracellular protein transfer from MVs to ECs, and the relative death of ECs. We used western blot analysis to evaluate the expression of granzyme B in NK cells and in the MVs that they produced, as well as the expression of granzyme B, caspases, extracellular-regulated kinase (*ERK)* and protein kinase B (AKT) in ECs. We also assessed the proliferation and migration of ECs in the presence of MVs derived from cells of the NK-92 cell line. The results revealed significant differences in the proteomic profiles of cells of the NK-92 cell line and their MV product. Contact between ECs and MVs derived from cells of the NK-92 cell line is accompanied by the following events: a) expression of granzyme B in ECs, b) activation of caspase-9 and caspase-3, with partial EC death, c) appearance of the panleukocyte marker CD45 on ECs, d) decrease in CD105 expression, and increase in CD34 and CD54 expression, and e) inhibition of EC migration. Transfer of ERK (but not AKT) from MVs derived from cells of the NK-92 cell line to ECs, at a concentration 10 times lower than that which causes EC death, leads to an increase in EC proliferation.

Keywords: natural killer cells, endothelium, microvesicles, granzyme B, caspases, proliferation, migration

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Introduction

Natural killer cells (NK cells) are CD3 negative lymphocytes that constitute 10-15% of all circulating lymphocytes in human blood [12, 49]. The major function of NK cells is to protect against pathogens in infected and transformed cells. This function is implemented by exocytosis of lytic granules, ligand-mediated interaction with death receptors on target cells, and peptide and cytokine secretion. The first two mechanisms of target cell cytolysis act directly to cause cell apoptosis [51]. Exocytosis of lytic granules on contact with a target cell is the most common mechanism for cytolysis induced by NK cells. NK cell granules contain amines, proteoglycans, catecholamines, enzymes and hormones. The main components are perforin, granzymes and granulysin [51, 58]. The second most important cytolytic process is the start of target cell apoptosis by the receptor mechanism. This is mediated through the interaction between molecules of the Tumor necrosis factor-α (TNFα) family (FasL (CD95L) and TRAIL) that are expressed on the NK cell surface and death receptors on target cells, being Fas (CD95) and TRAIL-R1/TRAIL-R2, respectively [51]. The third mechanism for cytolysis is associated with production of TNFα, interferon gamma (IFNγ) dependent differentiation of Th1 lymphocytes, and stimulation of the cellular immune response accompanied by potent antibacterial, antiviral and proliferation inhibiting effects [55]. NK cells also carry the Fc receptor (CD16) on their surface, which enables them to perform antibody-dependent cellular cytotoxicity [55].

In addition to innate immunity reactions, NK cells participate in various physiological and pathological processes due to production of a wide range of cytokines. In previous work, we showed that NK cells are involved in all processes of the uteroplacental bed in pregnancy, including blastocyst implantation into the endometrium, regulation of trophoblast invasion, remodeling of uterine arteries and decidua vessels, and formation of placenta vasculature [10, 19, 48]. Human NK cells in the uteroplacental bed are sources of various cytokines and other proteins including IL-15, IFNγ, VEGF-A, VEGF-C, IL-8, TGF-β, PlGF, Ang1, Ang2 [33], uPA, uPAR, MMP [56], MIP1α, GM-CSF, and CSF1 [57]. These cytokines can affect ECs and their microenvironment by controlling angiogenesis. NK cells help prepare uterine spiral arteries for remodeling [39], which causes Fas-dependent apoptosis of smooth muscle cells and ECs [4, 63, 69, 77]. Conflicting findings on the role of NK cells in angiogenesis have been seen in various model experiments conducted *in vitro*. There is evidence that they stimulate vessel EC migration and formation [25, 29], and that they inhibit angiogenesis processes [16, 21]. It has been established that IL-15 increases production of VEGF and PlGF by NK cells [26, 40]. In contrast, NK cells activated by IL-12 inhibit vascular growth through production of IFNγ, IP-10, perforin and granzyme [81]. Thus, depending on the model or characteristics of NK cells derived from different sources, researchers have obtained conflicting data on the effect of NK cells on ECs.

The regulatory and cytotoxic functions of NK cells can be performed not only by cytokines or contact interactions but also by the MVs that they produce. MVs are subcellular structures that range in size from 100 to 1000 nm and are found in almost all human biological fluids [13, 65, 71]. MVs can transfer molecules to target cells, and regulate inflammation, coagulation, antigen presentation, and apoptosis, as well as participate in the pathogenesis of diseases and inflammatory processes [2, 8, 15, 24, 54]. MVs of leukocyte origin are the least studied population of MVs. This may be because they constitute only a minor fraction of the MVs in the bloodstream under normal physiological conditions [13]. In pathological processes, the level of leukocyte-derived MVs in blood increases dramatically, so that they are considered to be markers for development of various diseases [7, 65]. The phenotype, composition and functions of MVs produced by NK cells are inadequately defined. It has been established that NK cell exosomes possess cytotoxic properties [47]. NK cell-derived MVs probably have similar properties. We have shown previously that MVs isolated from peripheral blood expressed the NK cell markers, CD45, CD16 and CD56. We showed that the level of MVs produced by NK cells was lower in the peripheral blood of women with preeclampsia compared with healthy pregnant controls [54]. A study of MVs derived from cell line cultures used flow cytometry to show that NK cells of the NK-92 cell line formed MVs ranging in size from 200 to 1000 nm. Some of these MVs expressed CD95. Expression intensity increased with preliminary culturing of NK cells with TNFα [53]. The role of MVs, including those produced by NK cells, in angiogenesis, inflammation and the immune response remains understudied.

In this research, we studied the effect of MVs produced by NK cells of the NK-92 cell line on the phenotype, caspase activity, proliferation and migration of ECs of the EA.Hy926 cell line.

Materials and methods

Cell cultures

ECs of the Ea.Hy926 cell line (American Type Culture Collection (ATCC), USA) were used as they reproduce the main morphological, phenotypic and functional characteristics of the endothelium [18, 62, 74]. ECs were cultured in DMEM/F12 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) (Sigma-Aldrich Chem. Co., USA) that was free of MVs, and 100 μg/mL streptomycin, 100 U/mL penicillin, 2 mM L-glutamine and HAT (Sigma-Aldrich Chem. Co., USA). The cell monolayer intended for subcultivation was disintegrated using Versene solution (BioloT, Russia). Cells of the NK-92 cell line (ATCC, USA) that reproduce the phenotypic and functional characteristics of activated NK cells were used as the source of MVs [23, 34]. Cells were cultured in complete minimum Eagle's medium (α-MEM) (Biolot, Russia) that was free of MVs and contained 12.5% heat-inactivated FCS, 12.5% inactivated donor horse serum, 0.2 mM myoinositol, 0.02 mM folic acid, 2 mM L-glutamine, 100 μg/ml streptomycin, 100 U/mL penicillin, 10 mM HEPES buffer solution, 0.1 mM 2-mercaptoethanol (Sigma-

Aldrich Chem. Co., USA), and 500 U/ml recombinant IL-2 (Roncoleukinum, Biotech LLC, Russia). Cell viability was assessed using Trypan blue solution and was at least 94%. Cells were cultured in an incubator in a humid environment at 37 °C under 5% CO_2. All experiments involving cell culturing were performed under the same incubation conditions.

Isolation of NK cell MVs

Cells of the NK-92 cell line were cultured in 75 cm^2 flasks (BD, USA) in complete α-MEM (changed once on the day prior to MV separation). The volume was adjusted to 40 mL with a cell concentration of 4×10^5 per ml. Unstimulated cells of the NK-92 cell line served as controls. Cell viability was assessed the day after culture initiation. As there is no single standard for isolation and characterization of MVs, various methodological approaches are used that allow MV fractions to be obtained that differ in purity and enrichment level [41, 80]. We used the modified differential centrifugation method, using Hanks' solution without Ca^{2+} and Mg^{2+} (Sigma-Aldrich Chem. Co., USA), to isolate MVs from cells of the NK-92 cell line [67, 71]. The obtained supernatants were centrifuged consecutively at 200 g (room temperature, 10 minutes) and at 9900 g (4 °C, 10 minutes). After the second centrifugation, the sediment was discarded and the supernatant was centrifuged several times at 19800 g (4 °C, 20 minutes), sedimenting and concentrating the MVs each time. This procedure allows MVs of 100-1000 nm diameter to be isolated with sufficient purity and minimal loss of biomaterial; the MVs are successively separated from coarse particles of cellular debris and apoptotic bodies, as well as from exosomes [17, 36].

Laser correlation analysis

To control the size of the isolated MVs, granulometric analysis was carried out using the dynamic light scattering method and the Zetasizer NanoZS laser correlation spectrometer (Malvern Instruments, UK). The particles ranged from 0.3 nm to 10 μm. MV diameter was calculated using Zetasizer 7.11 software (Malvern Instruments, UK). The size of the MVs produced by NK cells of the NK-92 cell line ranged from 210-490 nm, and the peak of the MV quantity distribution was 315 nm. These data complied with our previous work [35] and that of other research groups that ascertained the size of MVs produced by various cells [20, 71, 75]. The reproducibility of the MV granulometric analysis results obtained in our laboratory at different times has led us to recommend laser correlation analysis as a standard for assessing the isolation purity of these extracellular objects.

Evaluation of the protein profiles of cells of the NK-92 cell line and their MV products

The sediment containing MVs obtained above was resuspended in deionised MilliQ water with addition of a protease inhibitor mixture (cOmplete, EDTA-free; Roche Diagnostics GmbH, Germany) at the concentration specified by the manufacturer, and then stored at -80 °C until assay. The cell and MV membranes were disrupted with five freeze-thaw cycles and mechanical disruption in a glass homogenizer, and the obtained lysates were then centrifuged at 16 000 g (4 °C, 10 minutes). The sediment was discarded, and the proteins in the obtained supernatants were sedimented with a triple volume of icy acetone (OSTsch; Himmed, Russia), incubated at -20 °C for 30 minutes and centrifuged at 16 000 g (4 °C, 10 minutes). The obtained supernatants were discarded, and the sediment dried at room temperature. Next, the dry residue was dissolved in a minimal amount of 0.1 M sodium bicarbonate (Sigma-Aldrich Chem. Co., USA). The total protein content was determined and concentrations of the obtained protein solutions were aligned by adding the required volume of 0.1 M sodium bicarbonate, focusing on the lowest value of the measured protein. Purified proteins in the obtained solutions were separated by molecular weight using electrophoresis on microchips under non-denaturing conditions and commercial High Sensitivity Protein Chip kits (Agilent Technologies, USA) with an Agilent 2100 bioanalyzer (Agilent Technologies, USA) as per the manufacturer's instructions. The intensity of the bands obtained by electrophoresis was assessed using Agilent 2100 Expert software (Agilent Technologies, USA). All experiments were repeated six times independently.

Analysis of total protein content

The protein content of the cell and MV lysates was determined by the Bradford method [6] using a NanoDrop One spectrophotometer (Thermo Scientific, USA). After culturing for 24 h (as described above), the total protein content in NK cells of the NK-92 cell line and in their MVs was 60.2 ± 6.1 μg/10^6 cells and 2.5 ± 0.3 μg/10^6 source cells, respectively. The obtained data allowed us to calculate the protein load of the microchips and to align it between the cells and their MVs.

Isoelectric focusing of proteins in the liquid phase was used to separate proteins from the cells and from the MVs derived from these cells. The analysis was performed on OFFGEL High Resolution IPG strips (24 cm) with an immobilised pH gradient of 3-10 in a 3100 OFFGEL Fractionator under denaturing conditions. This was conducted as per the manufacturer's protocol (Agilent Technologies, USA) in the active rehydration and subsequent separation mode at a voltage of 200-3400 V (20 °C, 24 hours). Loading of test strips with lysates of the cells and their MVs was performed so that the protein content on all of the strips was equal and sufficient for obtaining valid results (4.5-5.0 mg). With isoelectric protein separation, 24 fractions were obtained each from cell lysate and from MV lysate. The pH step between fractions was ~0.3.

MALDI mass spectrometry analysis of tryptic peptide mixtures

After isoelectric focusing, protein fractions were mixed with icy acetone (3:1, v/v) and incubated at -20 °C for 30 minutes. Sedimented proteins were centrifuged at 15 000 g (4 °C, 10 minutes). The supernatant was discarded, and the sediment was washed with a cooled acetone:water mixture (4:1, v/v), centrifuged again and dried at room temperature. Modified bovine trypsin solution 10 μL (20 ng/mL, Promega, USA) was added to the samples and incubated on ice for 1 hour and then at 37 °C for 18 hours. Tryptic peptide mixtures in the resultant solutions were air dried at 4 °C, then dissolved in 50 μL of an acetonitrile solution (50%) containing trifluoroacetic acid (0.1%, Sigma-Aldrich Chem. Co., USA) and stirred until complete dissolution. The solutions were next applied to metal plates for MALDI analysis at a ratio of 2 × 0.5 μL of matrix solution and 5 × 0.5 μL of protein sample solution. A 2,5-dihydroxybenzoic acid (10 mg/mL) in sodium chloride solution (10 mM, Sigma-Aldrich Chem. Co., USA) was used as the matrix. The resultant mixtures were air dried. Mass spectra of the tryptic peptides were obtained using the Axima Resonance MALDI mass spectrometer (Shimadzu/Kratos Analytical Ltd., UK). Measurements were carried out under positive ion shooting. Spectra were obtained in the 200-3000 m/z (mass-to-charge ratio) mass range by choosing the laser power optimal for resolution. Protein identification was undertaken relative to the SwissProt databases using taxonomic constraints for the Homosapien species and Mascot software (www.matrixscience.com), and the peptide fingerprinting method. A parallel search was performed using a database of inverted and random (decoy) amino acid sequences. After peptide identification, the correspondence between an identified protein and its actual position on a strip was checked.

Evaluation of the effect of MVs derived from cells of the NK-92 cell line on the migration of ECs of the EA.Hy92 and Ea.Hy926 cell lines

The day before the experiment, ECs were added to wells of a 96-well flat bottom plate (3.5×10^4 cells per well in 0.1 mL of medium, 10% FCS) and cultured for 24 hours. The monolayer was then disrupted by partial cell scraping. For this purpose, we used a 200 μL pipette tip to draw a vertical straight line in the middle of each well from edge to edge and then washed the line with warm Hanks' solution. The width of the obtained line of the disrupted monolayer was photographed (Supplementary Figure 1, see 2nd page of cover).

Next, the medium was replaced with dilutions of MVs derived from cells of the NK-92 cell line that were prepared using the EC medium i.e. 2.5% FCS. The cells were then cultured for 24 hours. ECs were incubated with 100 μL of crystal violet solution (0.2%, Sigma-Aldrich Chem. Co., USA) containing 5% methanol for 10 minutes. After that, the plate was washed with distilled water and dried. Three fields of view were photographed in each well. Analysis of the obtained data was carried out using MarkMigration (Russia) software [50], which automatically considers the residual area of the disrupted monolayer line after migration. In each photograph, two parallel lines of the disrupted monolayer (mm^2) were run and the number of cells that migrated to the zone of the disrupted monolayer line was specified. Change in cell migratory activity was assessed by evaluating the change in the number of cells that migrated during the experiment, compared with controls; it was also assessed by evaluating the change in area of the disrupted monolayer line after cell migration in a well, compared with controls. Experiments determining EC migratory activity in the presence of MVs were performed three times. Each MV concentration was analysed four times. Culture medium containing 2.5% FCS was used as the control, while that containing 10% FCS was used as the positive control. The area of the initial line after monolayer scraping was a median (interquartile range) of 0.53 (0.48, 0.53) mm^2. No cells in the zone of the disrupted monolayer were revealed. We noted an increase in the number (470.5 (438.3, 522.3), $p < 0.001$) of migrated ECs and a decrease in the area (0.21 (0.18, 0.24), $p < 0.001$) mm^2 of the disrupted monolayer line after cell migration in the presence of 2.5% FCS. An increase in the FCS concentration of the culture medium to 10% (positive control) caused an increase in the number of migrated ECs (521.3 (470.8, 592.3), $p < 0.01$) and a decrease in the residual area of the disrupted monolayer line after cell migration (0.16 (0.14, 0.22), $p < 0.05$) mm^2. Thus, within the framework of the model used, cells of the EA.Hy926 cell line responded to a higher FCS concentration with increased migratory activity, which is consistent with results described previously [22, 66]. This allows evaluation of changes in cell migratory activity in the presence of MVs derived from cells of the NK-92 cell line.

Evaluation of the fluorescent tag transfer from MVs derived from cells of the NK-92 cell line to ECs of the Ea.Hy926 cell line

The day before the experiment, ECs were added to a 96-well plate (3.5×10^4 cells per well in 100 μL of medium) and cultured for 24 hours. To stain intracellular protein, cells of the NK-92 cell line were treated with a 5(6)-carboxyfluorescein diacetate succinimidyl ether (CFSE) solution at concentrations of 5 and 50 μM (three repetitions for each concentration) according to the manufacturer's instructions (Sigma-Aldrich Chem. Co., USA) for use as positive controls. Some of the cells of the NK-92 cell line were left unstimulated. Unstimulated stained cells of the NK-92 cell line were then cultured in 75 cm^2 flasks (BD, USA) in 40 mL of complete α-MEM for 24 hours. The cell concentration was 4×10^5 per mL. MVs were then isolated (as described above), added to ECs (20 μg of total protein in 100 μL of medium) and incubated for 24 hours. The ECs were washed three times with Versene solution and then removed from the plate surface. The ECs were then resuspended twice in Hanks' solution without Ca^{2+} and Mg^{2+}, and centrifuged at 200 g for 10 minutes to discard the supernatant. Fluorescent CFSE inclusions into ECs were evaluated using the FACS Canto II flow cytometer (Becton Dickinson, USA) (Supplementary Figure 5, p. 263). The experiments were repeated three times.

Evaluation of the effect of MVs derived from cells of the NK-92 cell line on the phenotype of ECs of the Ea.Hy926 cell line

The day before the experiment, ECs were added to wells of a 96-well flat bottom plate (3.5×10^4 cells per well in 100 μL of medium) and cultured for 24 hours. Next, the medium was removed from the plate along with the EC monolayer. MVs derived from cells of the NK-92 cell line were then added at a concentration of 20 μg of total protein in 100 μL of medium (in three repetitions). Unstimulated ECs were used as controls. ECs incubated with phorbol-12-myristate 13-acetate (10 ng/mL, Sigma-Aldrich Chem. Co., USA) were used as positive controls. One day later, ECs were washed three times with warm Hanks' solution and removed from the plate with Versene solution. Hanks' solution was again used to wash the Versene solution from the cells. To control survivability, the ECs were stained with 7-AAD dye (Biolegend, USA), and the cell death rate was assessed using the FACS Canto II flow cytometer by 7-AAD inclusion, as described above [59, 79]. The pool of nonviable ECs after culturing with MVs from cells of the NK-92 cell line was a median (interquartile range) of 29.9% (26.3, 54.5). Viability experiments were repeated four times. After incubation with MVs, ECs were treated with monoclonal antibodies to CD31, CD119, CD54, CD34 (Becton Dickinson, USA), VEGFR1, VEGFR2 and CD105 (R&D Systems, USA), as well as with isotypic antibodies according to the manufacturer's instructions. The fluorescence was analysed using the FACS Canto II flow cytometer (Supplementary Figure 2, 3, see 3rd page of cover). Analysis of the receptor expression by ECs was repeated four times.

Evaluation of the effect of MVs derived from cells of the NK-92 cell line on the proliferative activity of ECs of the EA.Hy92 cell line

The day before the experiment, ECs were added to wells of a 96-well flat bottom plate (2.5×10^3 cells per well in 0.1 mL of medium, 10% FCS) and cultured for 24 hours. After that, the medium was replaced with dilutions of MVs derived from unstimulated cells of the NK-92 cell line that were prepared using the EC medium of 2.5% FCS. The cells were then cultured for 24 hours. Medium containing 2.5% FCS was used as the control, while medium containing 10% FCS was the positive control. ECs were next stained with 0.2% crystal violet solution containing 5% methanol, which was added (100 μL) to each well and incubated for 10 minutes. After staining, wells were washed with distilled water four times. The plate was dried and the dye was extracted with 50% acetic acid solution. Optical density was calculated using a Labsystems Microplate Reader at a wavelength of 540 nm (cutoff 620 nm), and converted to the cell number using a titration curve. Optical density results are presented as the cell number. Change in proliferation level was assessed by comparing the change in sample optical density and cell number with that of the ECs incubated in culture medium with added 2.5% FCS that was free of MVs. When culturing ECs in the presence of 10% FCS (positive control), stimulation of EC proliferative activity [11169.1 (10612.69, 11362.76) cells per well] was observed, compared with EC cultured with 2.5% FCS (38577.2 (16583.2, 39818.4) cells per well, $p < 0.001$). Experiments were carried out three times. MV concentrations were analysed four times.

Evaluation of the effect of MVs derived from cells of the NK-92 cell line on caspase expression in ECs of the Ea.Hy926 cell line using the western blot method

The day before the experiment, ECs were added to wells of a 6-well plate (17.5×10^4 cells in 5 mL of medium) and cultured for 24 hours. The next day, MVs in the medium (200 μg/mL of total protein) were added to a portion of cells and cultured for 24 hours. The cells were then washed three times with Versene solution and removed from the plate surface before resuspending twice in Hanks' solution without Ca^{2+} and Mg^{2+}, and centrifuging at 200 g (10 minutes) to discard the supernatant. Caspase content and activity were assessed in the obtained cells via western blot analysis.

Western blot analysis

Cells of the NK-92 cell line, the sediment containing MVs derived from cells of the NK-92 cell line (obtained as described above), and unstimulated ECs or ECs treated with MVs derived from cells of the NK-92 cell line were washed three times with a cooled phosphate buffer (0.01 M PBS, pH 7.4) and lysed in RIPA buffer (50 mM, Tris-HCl, pH 8.1, Triton X-100 (1%), sodium dodecyl sulfate (0.1%), sodium deoxycholate (0.5%), EDTA (1 mM), sodium chloride (150 mM)) containing a protease inhibitor mixture (Roche, Switzerland) with intermittent shaking for 30 minutes. Cellular debris was removed by centrifugation at 16 000 g (4 °C, 10 minutes). Proteins from the obtained supernatant were separated by their molecular weight through electrophoresis in 10% polyacrylamide gel under denaturing Laemmli conditions using commercial Mini-Protean TGX™ Stain-Free Precast Gels (Bio-Rad Laboratories, USA) in a Mini-Protean Tetra System, which is a chamber for vertical electrophoresis (Bio-Rad Laboratories, USA). Proteins were separated in a TGS alkaline buffer solution containing 25 mM Tris, 192 mM glycine and 0.1% sodium dodecyl sulfate (Bio-Rad Laboratories, USA), at a voltage of 200 V. Gel separated proteins were transferred onto polyvinylidene fluoride membranes (Bio-Rad Laboratories, USA), which were then blocked with 2% albumin (Sigma-Aldrich Chem. Co., USA) in a TBST buffer solution containing 50 mM Tris-HCl (Bio-Rad Laboratories, USA), 150 mM sodium chloride (analytical grade reagent; Vekton, Russia) and 0.1% Tween 20 (Bio-Rad Laboratories, USA). The proteins were then incubated with primary monoclonal antibodies to granzyme B (Purified anti-Granzyme B, mouse Ab, 1:1000; Biolegend, USA), caspase-8 (Caspase-8 (1C12), mouse Ab, 1:1000; Cell Signaling, USA), caspase-3 (Caspase-3, rabbit Ab, 1:1000; Cell Signaling, USA), ERK1/2 (p44/42 MAPK (ERK1/2), rabbit Ab, 1:1000; Cell Signaling, USA) or AKT (AKT (pan) (C67E7), rabbit Ab, 1:1000; Cell Signaling, USA) at 4°C for one night on a MR-12 Rocker-Shaker (BioSan, Latvia). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:1000; Cell Signaling Technology, USA) was used as a load control for cell lysates. MVs were normalised to total protein content. After reaction with an appropriate secondary antibody (1:1000; Cell Signaling Technology, USA), signals were visualised on a ChemiDoc™ Touch Gel Imaging System (Bio-Rad Laboratories, USA) using enhanced chemiluminescence (ECL) with ECL reagents (GE Healthcare, Sweden). The intensity of bands obtained by immunoblotting was assessed using ImageLab software (Bio-Rad Laboratories, USA). Caspase activation in ECs of the EA.Hy926 cell line was assessed as per a method described previously. Caspase-3 activation was assessed as the ratio of its active fragment (p17) detected and obtained from cleavage to the endogenous level of caspase-3 inactive proenzyme (p35). Caspase-8 activation was assessed as the ratio of its active (p18) and intermediate (p43/p41) fragments to procaspase-8 (p57). The results are presented in conditional units. All experiments were repeated independently three times.

Caspase-3 and caspase-9 activity were assessed using the synthetic peptides, Ac-DEVD-pNA and Ac-LEHD-pNA, respectively, as substrates. The reaction mixture consisted of reaction buffer (20 mM HEPES, 0.1% CHAPS, 2 mM EDTA, 5 mM DTT, pH 7.4), 0.2 mM substrate and cell lysate. The total content of added protein was 60 μg. The increase in the pNA reaction product was assessed by colorimetric analysis at a wavelength of 405 nm for 150 minutes. Caspase activity was then assessed using the formula $(OD_t - OD_0)/(t*\varepsilon*c)$, where t is reaction time in minutes; OD_0 is absorption measured before adding the substrate; OD_t is absorption measured t minutes after adding the substrate; ε is the molar extinction of the product ($\varepsilon^{mM}pNA = 10.5$); c is the protein content in the sample (μg). Caspase activity was expressed in μmol pNA/min/mg of protein.

Statistical analysis was performed in Statistica 10 software (www.statsoft.com) using the nonparametric Mann–Whitney U test. Data are presented as median (upper quartile, lower quartile). Results of western blot analysis and enzyme activity assessment are presented as mean ± standard error of the mean (SEM) of at least three independent experiments. Experimental results were analysed using a t-test for independent samples. The value $p < 0.05$ was considered statistically significant.

Results

Evaluation of the protein profiles of cells of the NK-92 cell line and their MVs

Protein extraction from lysates with microelectrophoresis revealed that 12 and 5 major (> 3% of the total intensity) protein groups were released in cells of the NK-92 cell line and in their MVs, respectively, during constitutive culturing. Proteins with molecular weights of 10.5-78.0 kDa were detected in cells of the NK-92 cell line, while the range was 59.9-141.7 kDa in MVs derived from these cells. The total content of minor (< 3% of the total intensity) components was 18.3% in cells and 6.0% in MVs (Table 1, Supplementary Figure 4, p. 263).

MALDI mass spectrometry analysis of tryptic peptide mixtures showed that cells of the NK-92 cell line and their MVs contained proteins from MAP kinase group. MEKKK 1 (MAPK/ERK kinase kinase kinase 1 encoded by the MAP4K1 (mitogen-activated protein kinase kinase kinase kinase 1 isoform 1) gene) was detected in cells (SwissProt entry Q92918, molecular weight 91.2 kDa, pI 8.65, 8 tryptic peptides overlapping 2% of amino acid sequence of protein). Mnk1 (encoded by the MKNK1 (MAP kinase-interacting serine/threonine-protein kinase 1 isoform 1) gene) was detected in MVs (SwissProt entry Q9BUB5, molecular weight 51.3 kDa, pI 6.26, 6 tryptic peptides overlapping 4% of amino acid sequence).

Migratory activity of ECs of the EA.Hy926 cell line in the presence of MVs derived from unstimulated and activated NK cells of the NK-92 cell line

ECs cultured with MVs derived from cells of the NK-92 cell line (total protein content 20 μg/100 μL) had decreased migratory activity due to a lower

TABLE 1. PROTEIN PROFILE (BY MOLECULAR WEIGHT) OF CELLS OF THE NK-92 CELL LINE AND THEIR MVs DETERMINED BY CONSTITUTIVE CULTURING (M±m, n = 6)

Cells of the NK-92 cell line, n = 6				MVs derived from cells of the NK-92 cell line, n = 4		
No.	Weight, kDa	Pool, %		No.	Weight, kDa	Pool, %
1	10.50±0.09	4.60±0.60		1	59.90±0.49	62.20±6.17
2	12.40±0.05	6.20±2.23		2	85.00±0.90	7.30±1.12
3	13.60±0.08	6.00±1.48		3	95.00±0.79	6.2±0.7
4	16.40±0.38	7.00±0.89		4	118.10±4.28	4.50±0.73
5	21.20±0.33	10.10±0.92		5	141.70±1.53	13.80±2.11
6	27.90±0.27	4.80±0.59		6	minor components (from 0 to 250)	6.0 (total)
7	32.20±0.31	4.10±0.51				
8	37.90±0.35	6.00±0.47				
9	46.40±0.51	15.90±1.09				
10	55.40±0.46	3.50±0.80				
11	61.70±1.24	6.00±1.44				
12	78.00±1.39	7.50±0.64				
13	minor components (from 0 to 250)	18.3 (total)				

number of migrated ECs compared with ECs cultured without MVs (Figure 1A, B). Given these results, we selected MVs derived from cells of the NK-92 cell line with a total protein concentration of 20 μg per 100 μL of medium for further experiments.

Proliferative activity of ECs of the EA.Hy926 cell line in the presence of MVs produced by unstimulated and activated NK cells of the NK-92 cell line

Culturing EC with MVs derived from cells of the NK-92 cell line (total protein content 2 μg/100 μL) resulted in increased EC proliferative activity compared with ECs cultured in a medium without MVs. Culture of ECs in the presence of MVs with a total protein content of 10 and 20 μg/100 μL showed a dose-dependent decrease in EC proliferation compared with ECs cultured in a medium without MVs (Figure 1C).

Effect of MVs derived from cells of the NK-92 cell line on the phenotype of ECs of the Ea.Hy926 cell line

After EC incubation with MVs derived from cells of the NK-92 cell line, the number of ECs expressing VEGR1, CD34, CD31 and CD119 receptors was reduced (Figure 2). However, the intensity of VEGFR1, CD31 and CD119 expression by cells of the EA.Hy926 cell line did not change in the presence of MVs derived from cells of the NK-92 cell line, compared with the expression shown by unstimulated ECs of the Ea.Hy926 cell line (Figure 2). The decrease in number of $CD34^+$ ECs after incubation with MVs derived from cells of the NK-92 cell line was characterised by increased expression intensity of the receptor by ECs, compared with unstimulated ECs of the Ea.Hy926 cell line (Figure 2). While the number of $CD105^+$ cells of the EA.Hy926 cell line did not change after incubation with MVs derived from cells of the NK-92 cell line, the intensity of CD105 expression by cells of the Ea.Hy926 cell line reduced (Figure 2). Despite the absence of differences in the number of $CD54^+$ ECs, the intensity of CD54 expression by ECs was greater after incubation with MVs derived from cells of the NK-92 cell line, compared with unstimulated ECs (Figure 2).

After incubation of ECs of the Ea.Hy926 cell line with MVs derived from cells of the NK-92 cell line, the presence of ECs of the Ea.Hy926 cell line with the $CD45^+$ phenotype was revealed (Figure 3). The intensity of CD45 expression by ECs of the Ea.Hy926 cell line was also increased after EC incubation with MVs derived from cells of the NK-92 cell line as compared with unstimulated ECs of the Ea.Hy926 cell line (Figure 3).

Evaluation of the fluorescent tag transfer from MVs derived from cells of the NK-92 cell line to ECs of the Ea.Hy926 cell line

We established that ECs of the Ea.Hy926 cell line that were incubated with MVs derived from cells of the NK-92 cell line and pretreated with CFSE solution, dose-dependently included fluorescent CFSE (Supplementary Figure 5, p. 263).

Western blot analysis of granzyme B content showed that MVs derived from unstimulated cells of the NK-92 cell line contained granzyme B (Figure 4).

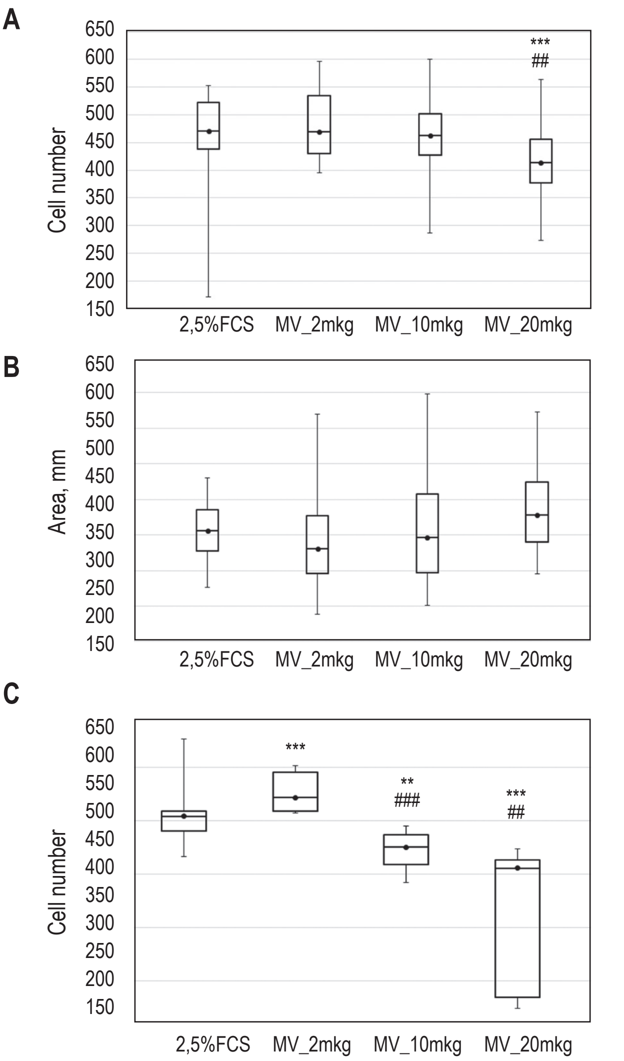

Figure 1. Effect of MVs derived from cells of the NK-92 cell line on migratory activity and proliferation of ECs of the EA.Hy926 cell line

Note. (A) Number of cells that migrated to the disrupted monolayer zone. (B) Residual area after migration of cells to the disrupted monolayer zone. (C) Number of cells that proliferated. **, $p < 0.01$; ***, $p < 0.001$ – difference compared with cells incubated without MVs (2.5% FCS); ##, $p < 0.01$; ###, $p < 0.001$ – difference compared with a lower concentration under the same conditions.

We established that granzyme B transferred from MVs to ECs after ECs of the EA.Hy926 cell line were incubated in the medium containing MVs derived from cells of the NK-92 cell line (Figure 4).

Evaluation of the effect of MVs derived from cells of the NK-92 cell line on caspase expression and activity in ECs of the Ea.Hy926 cell line

We observed a significant decrease in procaspase-8 ($p < 0.001$) and an increase in p43/41 fragment ($p < 0.001$) after EC incubation with MVs derived from cells of the NK-92 cell line, compared with unstimulated ECs. The ratio of this fragment to procaspase-8 in EC lysates after culture with MVs derived from cells of the NK-92 cell line was 6.86±3.41, which was significantly higher than with constitutively cultured ECs (0.06±0.04, $p < 0.001$). In parallel, we did not identify an increase in formation of the p18 fragment, which is the final subunit of caspase-8 cleavage and activation (Figure 5). Thus, caspase-8 activation up to the p43/41 fragment level occurs only in cells of the EA.Hy926 cell line after culture with MVs. No further activation of the p43/41 fragment up to the p18 fragment occurs. At the same time, caspase-3 activation, expressed as a ratio of active caspase-3 fragment to procaspase-3, was higher in the same ECs obtained after culture with MVs compared with unstimulated cells of the EA.Hy926 cell line ($p < 0.001$). There was also a decrease in procaspase-3 ($p < 0.01$) and an increase in active caspase-3 ($p < 0.05$) in ECs treated with MVs compared with unstimulated ECs (Figure 6).

Using the spectrophotometric method and specific substrates, we revealed increased activity of caspase-3 and caspase-9 enzymes in cells of the EA.Hy926 cell line obtained after culture with MVs derived from cells of the NK-92 cell line as compared with unstimulated ECs (Figure 7).

Western blot analysis of granzyme B content

MVs derived from cells of the NK-92 cell line contain MAP kinase of ERK1/2 protein. However, a phosphorylated form was not detected. AKT kinase was not detected in MVs derived from cells of the NK-92 cell line (Supplementary Figure 6).

Discussion

The protein profile of cells of the NK-92 cell line is represented by protein groups with fairly uniform percentage-weight compositions that do not allow isolation of any dominant groups. Protein groups with molecular weights of 21.2 kDa (10.1%) and 46.4 kDa (15.9%) can be distinguished as conditionally dominant (> 10% of the total intensity). Unlike source cells, MVs have a less representative set of protein groups by molecular weight. Further, light and medium weight fractions are not detected at all up to 59 kDa, which is the dominant fraction (62.2%). The remaining few fractions, with a pool comprising > 3% of the total intensity, aggregate to 31.8%, which is half the pool of the dominant protein group. In previous work, we used quadrupole time-of-flight (QTOF) mass spectrometry to establish that MVs from cells of the NK-92 cell line contain granzyme A, heat shock proteins, components of the ubiquitin-proteasome system, protein biosynthesis and energy metabolism enzymes, nuclear and serum proteins, and cytoskeletal proteins [35]. Our findings showed a significant difference between the proteomic profiles of cells of the NK-92 cell line and of their MVs. This indicates the need for further specification of these differences, determination of the biochemical

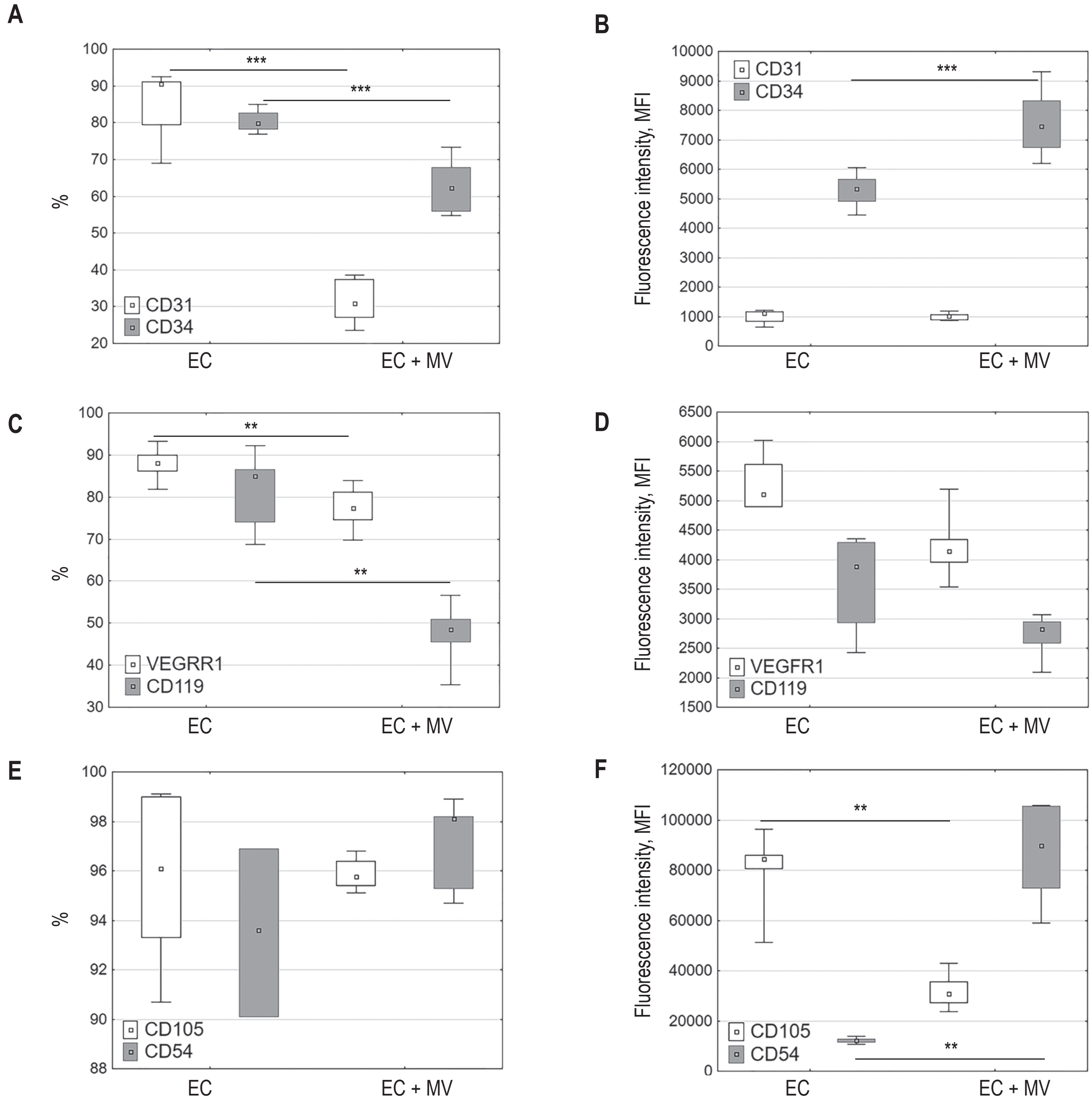

Figure 2. Expression of surface receptors by ECs after culturing with (ECs + MVs) and without (MVs) MVs derived from cells of the NK-92 cell line (Me, $Q_{0.25}$-$Q_{0.75}$)

Note. Data are presented as the relative number of ECs expressing (a) CD31, CD34, (c) VEGFR1, CD119, and (e) CD105, CD54, and on the intensity of (b) CD31, CD34, (d) VEGFR1, CD119, and (f) CD105, CD54 expression by ECs. Significance of differences compared with unstimulated ECs: **, p < 0.01; ***, p < 0.001.

nature and functional role of proteins that are MV components, and identification of target components involved in signal transmissions in the course of cell interaction.

MV membranes contain proteins that provide contact between vesicles and target cells, as well as signal transmission to target cells [11, 30]. The set of MV membrane proteins, in particular adhesion molecules and glycoproteins, as well as the degree of phosphatidylserine externalization determine the target cell pool with which these vesicles will interact [1, 11, 27]. After extracellular vesicles establish contact with a target cell, signal transmission takes place and can occur by surface protein and lipid ligand-receptor interactions, by ejecting extracellular vesicle contents into the extracellular space in the immediate vicinity of a target cell, or by fusion of extracellular vesicles with the target cell plasma membrane and release of its contents into the cytosol, and by extracellular vesicle endocytosis and subsequent fusion with endosome [61, 73]. We used flow cytometry to establish that, after incubation

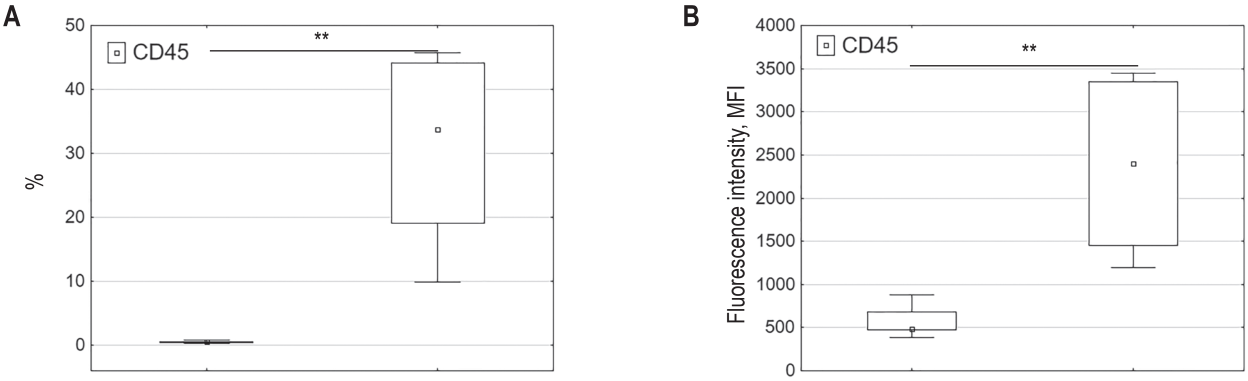

Figure 3. ECs carrying the CD45 receptor after culturing with (ECs + MVs) and without (MVs) MVs

Note. Data on the relative number of ECs with the CD45+ (a) phenotype and on the intensity of fluorescence of ECs with the CD45+ (b) phenotype are presented. Significance of differences compared with unstimulated ECs: **, p < 0.01.

of ECs of the EA.Hy926 cell line in the medium containing MVs derived from cells of the NK-92 cell line, CD45 membrane protein was transferred to ECs (Figure 3). CD45 protein is a panleukocyte marker that is not usually expressed by other cell types. Leukocyte marker transfer by MVs derived from NK cells onto the cytoplasmic membrane of ECs supports the fundamental possibility that of appearance of markers on cells that are not characteristic of this cell type. The appearance of membrane receptors that are unusual for this cell type can change cell behavior and cell response to external signals (if the cell has signal transmission paths from such receptors). We further established with flow cytometry that, after incubation of ECs of the EA.Hy926 cell line in the medium containing MVs derived from cells of the NK-92 cell line, transfer of intracellular protein labelled with fluorescent dye occurred (Supplementary Figure 5). In parallel, western blot analysis revealed the transfer of granzyme B to ECs (Figure 4). Our findings support the transfer of MV contents to ECs as occurring via fusion of the MV membrane with the EC cytoplasmic membrane. In this case, the MV membrane becomes a part of the EC membrane, and MV contents appear in EC cytoplasm. To determine the mechanisms for MV uptake by target cells, and in particular, the uptake of MVs produced by unstimulated NK cells of the NK-92 cell line by ECs of the EA.Hy926 cell line, further study of the specific MV formation is required. Also needed, is additional understanding of the functionally significant molecules contained in MVs that ensure signal transfer to target cells.

Granzyme B transmission by MVs derived from cells of the NK-92 cell line was accompanied by increased EC death. Thus, using flow cytometry and 7-AAD dye, we established that the pool of nonviable ECs was 29.9% (constitutive EC death was 6%) in the presence of MVs derived from cells of the NK-92 cell line. Being an active process, apoptosis can be triggered by external (exogenous pathway) or internal (endogenous pathway) factors. The exogenous pathway is accomplished by ligand binding to a receptor on the cell surface. These receptors include the TNFα superfamily of Fas (CD95), TRAIL-R1, TRAIL-R2, TRAIL-R3, TRAIL-R4 [70], TNF-R1 (CD120a) [76], DR5 (death receptor 5), and DR6 (death receptor 6) [3]. Ligand-receptor interactions lead to activation of an intracellular reaction cascade involving caspase-8 activating effector caspase-3, caspase-6 and caspase-7, which ensures cell death [3, 78]. The endogenous (mitochondrial, BCL-2-regulated) pathway is initiated in response to stressful situations such as DNA damage or lack of growth factors. The mitochondrial pathway of apoptosis activation is initiated by transcriptional and/or post-transcriptional upregulation of proapoptotic BH3-only members of the BCL-2 protein family. These proteins bind and inhibit BCL-2 pro-survival proteins, thereby promoting activation of BAX and BAK cell death effectors. BAX/BAK activation causes permeabilisation of the outer mitochondrial membrane followed by caspase-9 activation [5]. Caspase-9 activates effector caspase-3, caspase-6 and caspase-7, which causes cell death. We also described the involvement of mitochondrial p53 protein [68] in apoptosis induction. Protein p53 suppresses Bcl-2 and activates BAX factor. It should be pointed out that the exogenous and endogenous pathways are not always autonomous, as p53 can regulate the expression of some death receptors and the mitochondrial pathway can amplify signals associated with death receptors [3, 64].

Analysis of caspase activity in ECs after incubation with MVs derived from cells of the NK-92 cell line showed that caspase-8 activation up to the level of the p43/41 fragment occurs. No further proteolytic processing of the p43/41 fragment up to the p18 fragment occurs. Thus, contact-dependent apoptosis through Fas or TRAIL receptors remains activated and stopped at the level of the p43/41 fragment of caspase-8. The mature active caspase-8 p18 responsible for activation of subsequent effector caspases is not

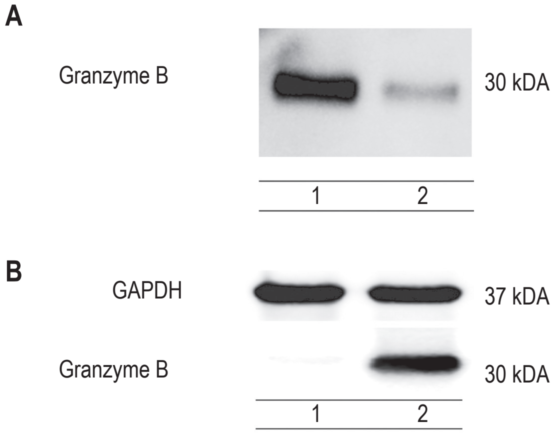

Figure 4. ECs of the EA.Hy926 cell line cultured with MVs derived from cells of the NK-92 cell line leads to granzyme B transfer from MVs to ECs

Note. (A) Representative immunoblot for granzyme B in cells of the NK-92 cell line (1) and in their MVs (2). (B) Representative immunoblot for granzyme B in unstimulated cells of the EA.Hy926 cell line (1) and after culture with MVs derived from cells of the NK-92 cell line (2).

formed. Absence of the second proteolytic stage in cleavage of the intermediate p43/41 fragment of caspase-8 may be due to increased c-FLIP$_L$ activity (cell caspase-8(FLICE)-like inhibitory protein). Caspase-8 activity in ECs depends on c-FLIP$_L$ [9]. This isoform contains a functionally inactive caspase-like domain that is sufficient to implement the first autocatalytic stage of procaspase-8 cleavage. However, FLIP$_L$ inhibits the second stage of caspase-8 activation (cleavage of the p43/41 fragment to form the p18 fragment), since it requires the presence of a catalytically active domain [37, 52]. EC treatment with sFasL can lead to increased FLIP expression and VEGF secretion, as well as promoting EC proliferation and migration [82]. A similar effect was shown with TRAIL, which at low concentrations increased c-FLIP$_L$ expression, and stimulated HUVEC proliferation and migration through implementation of the non-apoptotic functions of caspase-8 [9]. Despite this, we have established the fact of caspase-9 and effector caspase-3 activation. Our findings support the activation of caspase-3 by granzyme B via the mitochondrial pathway of apoptosis activation [44]. Earlier, we described the direct activation of caspase-3 by granzyme B [72] and established the transfer of granzyme B to ECs by MVs. This way of activation should not be ruled out.

Partial death was accompanied expectedly by a decrease in the number of ECs expressing VEGR1, CD34, CD31, and CD119 receptors, while the intensity of the expression of these molecules remained unchanged. It should be pointed out that incubation with MVs derived from cells of the NK-92 cell line resulted not only in partial EC death, but also in a change in phenotype of the ECs that remained viable. A decrease in the expression of CD105 by ECs indicates both shedding of this protein from the cell surface and a decrease in EC sensitivity to the inhibitory effect of TGF-β [38]. Change in the intensity of CD34 and CD54 expression by ECs argues for EC activation [14, 31].

During EC culturing with MVs derived from cells of the NK-92 cell line, a dose-dependent decrease in EC proliferation compared with ECs cultured in a medium without MVs was shown. Concurrently, inhibition of EC migration due to the reduced number of cells that migrated to the zone of the disrupted monolayer was established only for the maximum MV concentration (20 μg/100 μL of medium). These findings can be explained by increased EC death in the presence of MVs derived from cells of the NK-92 cell line. Despite this, as a result of culturing ECs with MVs derived from cells of the NK-92 cell line (total protein content 2 μg/100 μL of medium), an increased proliferative activity of ECs occurred compared with EC culturing in a medium without MVs. Cells of the NK-92 cell line were cultured wth IL-2. IL-2R signaling involved Lck, Jak, Fyn, Lyn, Syk, Ras, MAPK and PI3K in T cells. The MAPK and PI3K pathways participated in cell growth, differentiation and survival. ERK, a major member of the MAPK family, transduced mitogenic signals from the Ras/Raf/MEK pathway to the nucleus by activating transcription factors such as Elk-1 [32]. PI3K, which plays an important role in cell survival, induced activation of phosphatidylinositol-dependent kinase 1/2 and then activated AKT kinase [32]. AKT prevented apoptosis by disrupting the interaction between Bad and Bcl-2 or by activating the mammalian target of rapamycin (mTOR), which then phosphorylated p70 S6 kinase (S6K) leading to progression of the cell cycle. Using western blot analysis, we found ERK1/2 protein in cells of the NK-92 cell line and their MVs, which was consistent with the literature [32, 42]. We detected the presence of AKT in cells of the NK-92 cell line, which also corresponds with published works [32, 42], but we did not find it in the MVs produced by them. Extracellular signal regulated kinase 1 and 2 (ERK1/2) is involved in EC proliferation and angiogenesis [46]. Using mass spectrometry, we detected MEKKK 1 (MAPK/ERK kinase kinase kinase 1 encoded by the MAP4K1 (mitogen-activated protein kinase kinase kinase kinase 1 isoform 1) gene) in cells of the NK-92 cell line; we also found Mnk1 (MKNK1, MAP kinase-interacting serine/threonine-protein kinase 1) in MVs derived from cells of the NK-92 cell line. However, the data obtained by mass spectrometry indicated possible candidates only and requires additional verification using enzyme linked immunosorbent assay or immunoblotting. Obviously, the proteins detected in MVs are also contained in the source cells

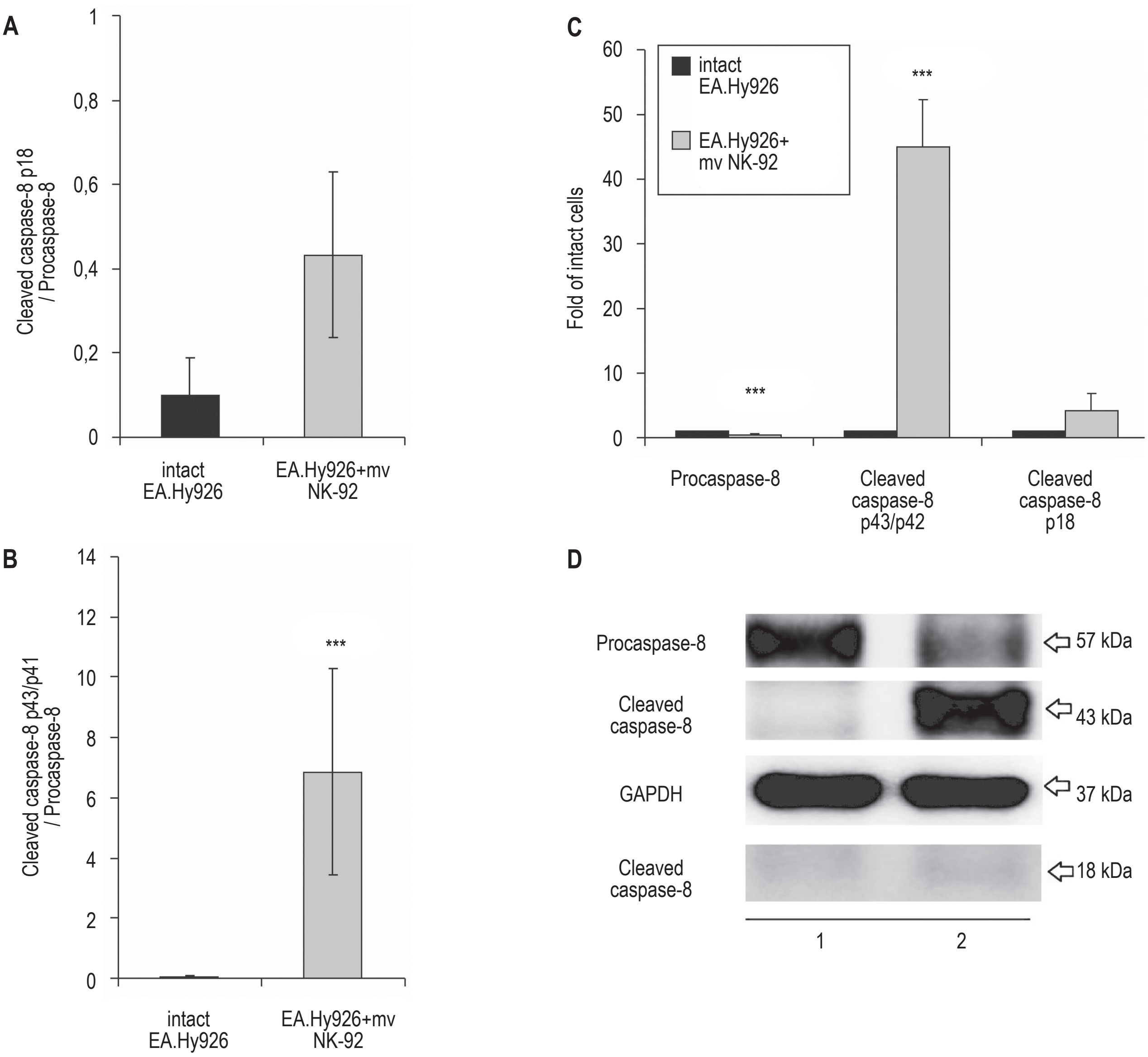

Figure 5. Caspase-8 activation (defined as the ratio of caspase-8 fragment intensity to procaspase-8) in ECs of the EA.Hy926 cell line after culture with MVs derived from cells of the NK-92 cell line

Note. (A) No difference in caspase-8 activation or procaspase-8 cleavage up to the p18 fragment of active caspase-8 was observed between the ECs after culture with MVs and unstimulated ECs. (B) EC treatment with MVs resulted in activation of caspase-8 and cleavage of procaspase-8 up to the p43/p41 fragment compared with unstimulated cells. (C) Quantitative assessment of procaspase-8, p43/p41 fragment and p18 fragment levels in ECs after culture with MVs. (D) Representative immunoblot demonstrating the effect of MVs derived from cells of the NK-92 cell line on the intensity of caspase-8 fragments (p43/p41 and p18) and procaspase-8 in ECs (1 – unstimulated ECs, 2 – ECs after culture with MVs). ***, $p < 0.001$ – difference between the indicator in ECs after their culture with MVs and the indicator in unstimulated ECs.

themselves, although our mass spectrometric analysis did not detect these. This indicated that the mass spectrometric data we obtained were selective and thus did not reflect the general pool of MAP kinase pathway enzymes in MVs derived from cells of the NK-92 cell line. It has previously been shown that MEKKK 1 and Mnk1 are involved in regulation of proliferation [28, 43, 45, 60]. Thus, transfer of ERK and possibly MNK1 (but not AKT) from MVs derived from cells of the NK-92 cell line at a concentration 2 μg/100 μL (i.e. 10 times lower than that which causes increased EC death) to ECs could lead to increased EC proliferation in our experiments.

Conclusion

Our findings indicate a significant difference in proteomic profiles of cells of the NK-92 cell line and the MVs produced by them. After contact between the MVs derived from cells of the NK-92 cell line and ECs, signal transmission takes place by means

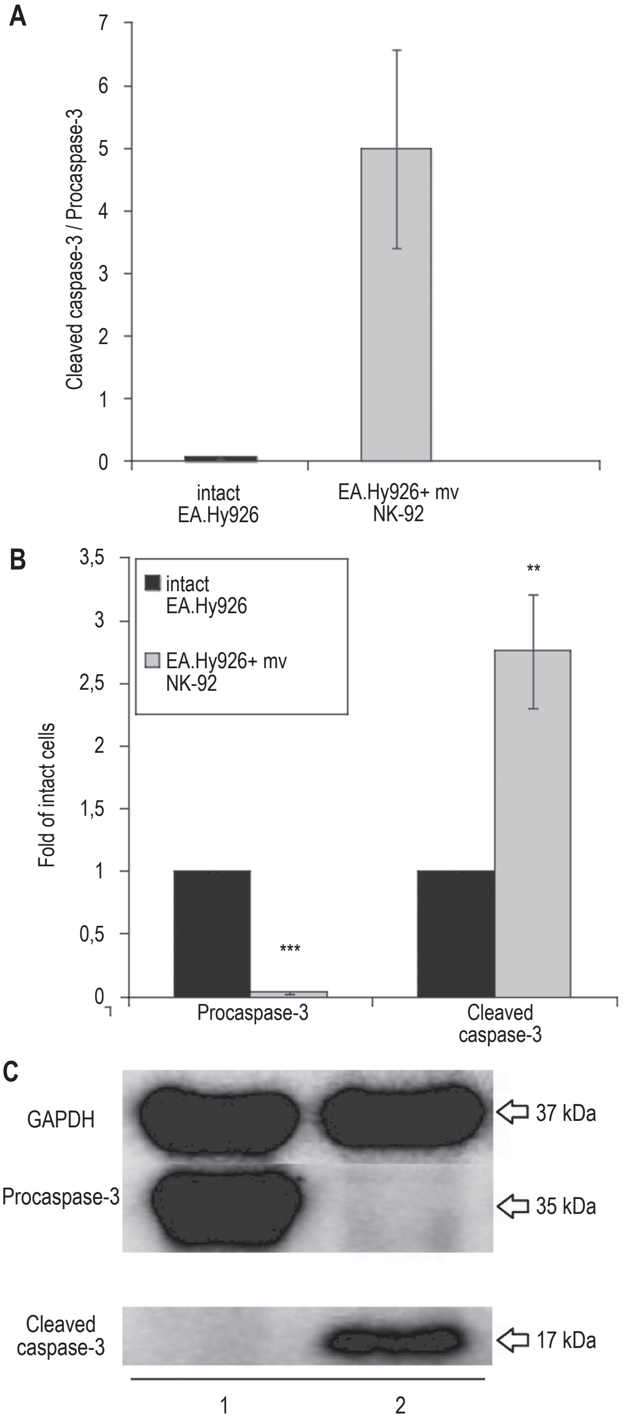

Figure 6. Caspase-3 activation (defined as the ratio of active caspase-3 to procaspase-3 fragment intensity) in ECs of the EA.Hy926 cell line after culture with MVs derived from cells of the NK-92 cell line

Note. (A) EC treatment with MVs results in activation of caspase-3 as compared with unstimulated ECs. (B) Quantitative assessment of procaspase-3 and active caspase-3 fragment levels in ECs after culture with MVs. (C) Representative immunoblot demonstrating the effect of MVs on the intensity of cleaved caspase-3 fragments and on procaspase-3 in ECs (1 – unstimulated ECs, 2 – ECs after culture with MVs). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ – difference between the indicator in ECs after culture with MVs and the indicator in unstimulated ECs.

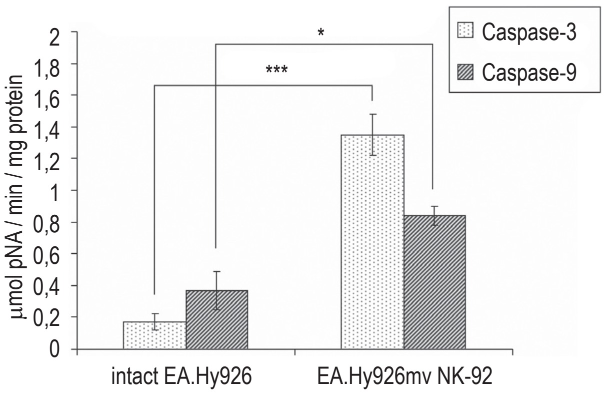

Figure 7. Effect of MVs derived from cells of the NK-92 cell line on caspase-3 and caspase-9 activity in ECs of the EA.Hy926 cell line

Note. Caspase-3 activity was assessed by spectrophotometric determination of p-nitrianiline (pNA) cleaved by caspase-3 and caspase-9 from the caspase-specific substrates, DEVD-pNA and Ac-LEHD-pNA, respectively. Activity was measured in μm of released pNA/min/mg of total cell lysate protein. *, $p < 0.05$; ***, $p < 0.001$ – difference between the indicator in ECs after culture with MVs and the indicator in unstimulated ECs.

of MV fusion with the cytoplasmic membrane and content release into the cytosol. This was shown by the appearance of the panleukocyte marker CD45 on the EC membrane, as well as by the transfer of granzyme B and intracellular protein labelled with CFSE dye. Leukocyte marker transfer by NK cell-derived MVs onto the cytoplasmic membrane of ECs indicated the fundamental possibility of appearance of receptors on cells that are not characteristic of the cell type. The appearance of such receptors on ECs can change cell behavior and its response to external signals. Incubation with MVs derived from cells of the NK-92 cell line was accompanied not only by partial death of ECs, but also by a phenotype change in the ECs that remained viable. That is, the expression of CD105 decreased, while the expression of both CD34 and CD54 increased. MVs derived from cells of the NK-92 cell line contain proteins that can decrease migration or death of a target cell (granzyme B, for example), and are also able to transmit a proliferation stimulating signal to ECs. Further study is needed to clarify whether the factors activating apoptosis, inhibiting migration, affecting phenotype and stimulating proliferation are located in the same or different MVs. Our results suggest that regulation of EC behavior (including during angiogenesis) by NK cells can also be performed by the MVs that they produce.

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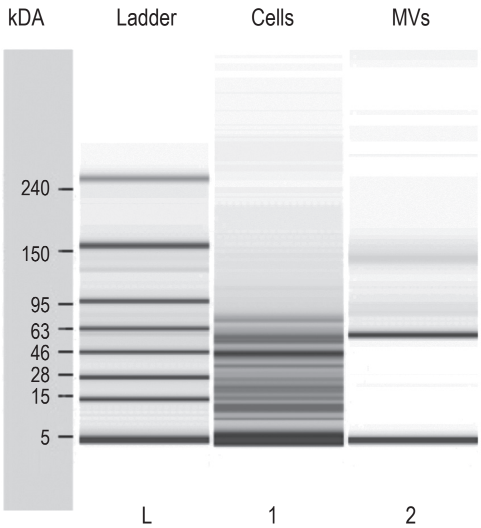
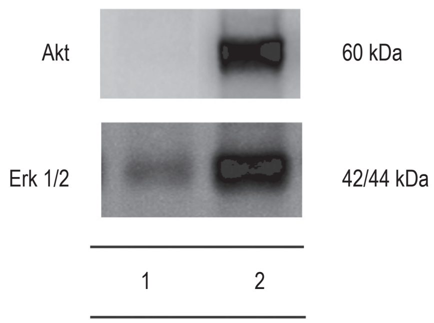
Supplementary Figure 1 – see 2nd page of cover.

Supplementary Figure 2, Supplementary Figure 3 – see 3rd page of cover.

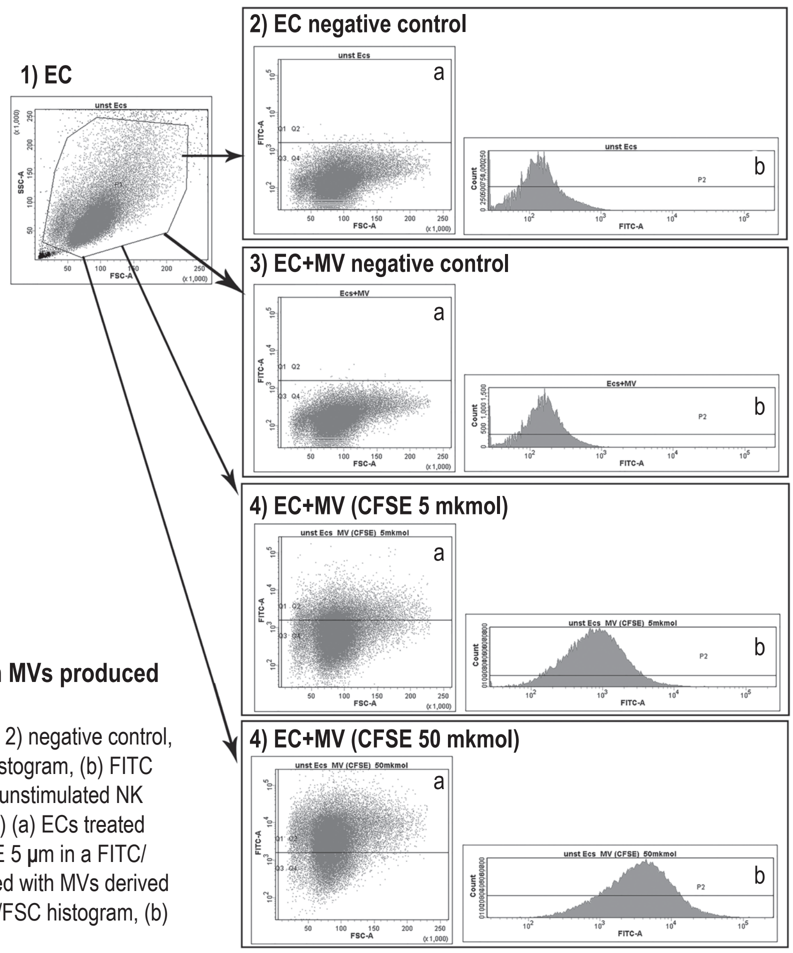


Supplementary Figure 4. Electrophoregrams of lysates of cells of the NK-92 cell line (cells) and their MVs (MVs)



Supplementary Figure 6. Representative immunoblots showing the content of ERK1/2, AKT in cells of the NK-92 cell line and their MVs

Note. 1, MVs derived from cells of the NK-92 cell line; 2, cells of the NK-92 cell line.



Supplementary Figure 5. ECs incubated with MVs produced by NK cells of the NK-92 cell line

Note. 1) distribution of ECs in a FSC/SSC histogram; 2) negative control, (a) distribution of unstimulated ECs in a FITC/FSC histogram, (b) FITC histogram; 3) (a) ECs treated with MVs derived from unstimulated NK cells in a FITC/FSC histogram, (b) FITC histogram; 4) (a) ECs treated with MVs derived from NK cells pretreated with CFSE 5 μm in a FITC/FSC histogram, (b) FITC histogram; 5) (a) ECs treated with MVs derived from NK cells pretreated with CFSE 50 μm in a FITC/FSC histogram, (b) FITC histogram.

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