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ВЛИЯНИЕ NK-КЛЕТОК НА АНГИОГЕНЕЗ В УСЛОВИЯХ КОНТАКТНОГО И ДИСТАНТНОГО СОКУЛЬТИВИРОВАНИЯ С ЭНДОТЕЛИАЛЬНЫМИ КЛЕТКАМИ И КЛЕТКАМИ ТРОФОБЛАСТА

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Резюме. Регуляция ангиогенеза в зоне маточно-плацентарного контакта определяет адекватную инвазию трофобласта, формирование и развитие плаценты, успешное протекание беременности. Наиболее значительное влияние на ангиогенез оказывают NK-клетки, макрофаги, трофобласт. На сегодняшний день довольно подробно описаны функции клеток-участников формирования плаценты как по отдельности (*in vitro*), так и в составе тканей (*in situ*). Однако до сих пор не создано моделей, отражающих взаимодействие NK-клеток, трофобласта и эндотелия в ходе ангиогенеза. До настоящего времени остается неразрешенным вопрос о вкладе каждой клеточной популяции в регуляцию не только ангиогенеза в плаценте, но и о перекрестной регуляции функций клеток-участников. Поэтому целью настоящего исследования явилось изучение контактного и дистантного влияния NK-клеток на образование капилляроподобных структур сокультурой эндотелиальных клеток и клеток трофобласта под влиянием различных цитокинов (bFGF, VEGF, PlGF, TGF-β, IL-8, IFNγ, IL-1β). Введение в сокультуру ЭК и трофобласта NK-клеток в условиях дистантного и контактного культивирования не изменяло длину капилляроподобных структур, образованных ЭК. При контактном культивировании NK-клеток с сокультурой ЭК и трофобласта в присутствии IL-1β длина капилляроподобных структур не изменялась по сравнению с культивированием в тех же условиях, но в отсутствие цитокина. При дистантном культивировании NK-клеток с сокультурой ЭК и трофобласта в присутствии IL-1β произошло увеличение длины капилляроподобных структур по сравнению с культивированием в тех же условиях, но в отсутствие цитокина. При контактном, но не дистантном, культивировании NK-клеток с сокультурой ЭК и трофобласта в присутствии VEGF длина капилляроподобных структур была больше по сравнению с культивированием в тех же условиях, но в отсутствие цитокина. В трех-

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компонентной клеточной системе провоспалительный цитокин IFNγ не оказывал эффекта в отношении ангиогенеза. При дистантном, но не контактном, культивировании NK-клеток с сокультурой ЭК и трофобласта в присутствии TGF-β длина капилляроподобных структур была меньше по сравнению с культивированием в тех же условиях, но в отсутствие цитокина. В условиях дистантного культивирования TGF-β запускает ингибирующий ангиогенез сигнал от NK-клеток. Установлено снижение длины капилляроподобных структур в условиях трехкомпонентной клеточной сокультуры в присутствии проангиогенных факторов: IL-8, PlGF (только при контактном культивировании) и bFGF (при контактном и дистантном культивировании). Таким образом, эффекты цитокинов в отношении ангиогенеза в трехкомпонентной сокультуре (NK-клетки, трофобласт, эндотелий) отличаются от установленных ранее в однокомпонентных (только эндотелий) и двухкомпонентных (сокультура эндотелия и трофобласта) клеточных моделях. Данные, полученные в настоящем исследовании, свидетельствуют о наличии в плаценте цитокиново-контактной регуляции межклеточных взаимодействий.

Ключевые слова: эндотелиальные клетки, трофобласт, NK-клетки, ангиогенез, цитокины

NATURAL KILLER CELL EFFECTS UPON ANGIOGENESIS UNDER CONDITIONS OF CONTACT-DEPENDENT AND DISTANT CO-CULTURING WITH ENDOTHELIAL AND TROPHOBLAST CELLS

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Abstract. Regulation of angiogenesis in the utero-placental bed determines adequate trophoblast invasion, placenta formation and development, as well as successful course of pregnancy. Natural killer (NK) cells, macrophages and trophoblast have the most significant effect on angiogenesis. To date, the functions of cells participating in placenta formation have been described in detail, both individually (*in vitro*) and in tissues (*in situ*). However, no models have yet been created that reflect the interactions of NK cells, trophoblast and endothelium during angiogenesis. It remains unclear, how each cell population contributes to placental angiogenesis regulation, and to the cross-regulation of participating cell functions. Therefore, the aim of this research was to study contact and distant effects of NK cells upon formation of tube-like structures through co-culture of endothelial and trophoblast cells influenced by various cytokines (bFGF, VEGF, PlGF, TGF-β, IL-8, IFNγ and IL-1β). Introduction of NK cells to the co-culture of endothelial and trophoblast cells under conditions of both contact and distance-dependent culturing did not change the length of tube-like structures formed by endothelial cells. During contact-dependent culturing of NK cells with co-culture of endothelial and trophoblast cells in presence of IL-1β, the length of tubule-like structures remained unchanged, compared with the length of tube-like structures formed under the same culturing conditions, but without the cytokine added. During distant culturing of NK cells with co-culture of endothelial and trophoblast cells in the presence of IL-1β, the length of tube-like structures increased as compared with those formed under the same culturing conditions but without the cytokine. During contact-dependent (but not distant) culturing of NK cells with the co-culture of endothelial and trophoblast cells in the presence of VEGF, the length of tube-like structures was greater than those formed under the same culturing conditions but without the cytokine. When used in a three-component cell system, the pro-inflammatory cytokine IFNγ had no effect upon angiogenesis. During distant (but not contact-dependent) culturing of NK cells with co-culture of endothelial and trophoblast cells in the presence of TGF-β, the length of tube-like structures was less than the length of tube-like structures formed under the same culturing conditions but without the cytokine. Under conditions of distant culturing, TGF-β triggered a signal in NK cells that inhibited angiogenesis. Decreased length of tube-like structures under conditions of a three-component cell co-culture in the presence of the following pro-angiogenic factors was

revealed: IL-8, PlGF (during contact-dependent culturing only) and bFGF (during both contact-dependent and distant culturing). Thus, the effects of cytokines upon angiogenesis in a three-component co-culture (NK cells, trophoblast and endothelium) differed from those revealed previously in single-component (endothelium only) and two-component (co-culture of endothelium and trophoblast) cell models. The results of these experiments indicated that regulation of placental cell interactions involved both cellular contacts and effects produced by cytokines.

Keywords: endothelial cells, trophoblast, natural killer cells, angiogenesis, cytokines

Introduction

Placenta formation is a coordinated process that requires participation of a variety of cell populations on both the fetal and maternal sides. An imbalance of cell interactions in the uteroplacental bed can lead to various obstetric complications such as preeclampsia or chronic placental insufficiency [17, 86].

Adequate development of placental vasculature plays a key role in placenta formation [15, 72]. Placenta vasculature is formed by vasculogenesis and then angiogenesis [2, 42]. VEGF, bGF, PlGF, TGF-β, IFNγ, IL-1β, IL-6 and IL-8 can be singled out from other cytokines produced in the placenta and decidua in considerable quantities. These cytokines actively affect angiogenesis. They also participate in regulation of immune cell function and in formation of immunological tolerance during pregnancy. The sources of these cytokines are both endothelial cells and the cellular microenvironment, which includes trophoblast, decidual NK cells, and decidual and placental macrophages [1, 3, 21, 54, 61].

Development of placental vasculature occurs against a background of close interaction between endothelial and trophoblast cells. In particular, this interaction occurs during transformation of the uterine spiral arteries [9, 36, 74]. Endovascular trophoblast invasion and the cytokines produced cause apoptosis of uterine spiral artery endothelial cells [35, 87]. Concurrently, trophoblast produces angiogenic factors (VEGF and MMP) and cytokines (IL-1β, IFNγ, TNFα, IL-4 and IL-10). Trophoblast affects the nature of angiogenesis in the placenta, decidua and endometrium [1]. Specific contacts are created between endothelial cells and endovascular trophoblast cells [5, 12].

Trophoblast invasion is regulated by the cellular microenvironment in which decidual NK cells play a pivotal role [49]. Decidual NK cells secrete a wide range of cytokines, chemokines and growth factors (IP-10, IL-8, VEGF, PlGF and IL-22) [85, 88], which affect the trophoblast cell phenotype [50, 68, 90]. Trophoblast cells are semiallogenic compared with the mother, thus during implantation trophoblast cells should avoid the adverse effects of maternal immune cells (decidual NK cells, in particular). This can be achieved through interactions between the inhibitory receptors on decidual NK cells (KIR2DL1/S1 and KIR2DL2/S2, NKp46, NKp30 and NKp44; LILRB1 and CD94/NKG2A [28, 57]) and molecules of the MHC class I locus on trophoblast cells (HLA-C, HLA-E and HLA-G [19]). Soluble HLA-G (sHLA-G) secreted by trophoblast cells inhibits endothelial cell proliferation and migration, as well as the angiogenesis induced by bFGF or VEGF. It does this by binding to the CD160 receptor on the surface of endothelial cells and causing apoptosis [30, 52]. sHLA-G1 has also been shown to cause apoptosis of NK cells [11, 23, 30, 89].

At pregnancy onset, the level of NK cells in the uterus increases dramatically to comprise about 70% of all endometrial leukocytes. As gestation progresses, the number of decidual NK cells decreases approaching zero in late pregnancy [13, 55, 81]. Uterine NK cells with the $CD56^{bright}CD16^{dim/-}$ phenotype are subdivided into decidual NK cells and endometrial NK cells. Like the $CD56^{dim/low}CD16^{bright}$ population of peripheral blood NK cells, they express KIR and have lytic granules [22, 34]. Uterine NK cells differ from peripheral blood NK cells due to their lower cytotoxicity and greater regulatory activity [88]. Moreover, decidual NK cells and endometrial NK cells express tetraspanin (CD9) and CD151 along with a number of immunosuppressive and angiogenic genes (expressed by no other subpopulation of peripheral blood NK cells) [34, 47, 80, 88]. Decidual NK cells secrete IL-2, IL-15, IFNγ, VEGF-A, VEGF-C, IL-8, TGF-β, PlGF, Ang1, Ang2 [43], uPA, uPAR, MMP [64] MIP1a, GM-CSF, CSF1, and other mediators [66] able to influence endothelial cells and the cellular microenvironment. Decidual NK cells are capable of cytotoxic actions via three main mechanisms: (i) exocytosis of lytic granules (contact-dependent interaction), (ii) ligand-mediated interaction with Fas and TRAIL death receptors (contact-dependent interaction) [59], and (iii) secretion of TNFα and IFNγ cytokines, and soluble forms of Fas (sCD95) receptors (distant interaction).

To date, the functions of cells participating in placental formation have been described in detail, both individually (*in vitro*) and in tissues (*in situ*). However, models have not yet been developed to reflect the interaction between NK cells, macrophages, trophoblast and endothelium during angiogenesis. Currently, the relative contribution of each cell population to the regulation of placental angiogenesis and to the cross-regulation of participating cell

functions remains unclear. In fact, all existing *in situ* models and factual descriptions are limited in this sense. Therefore, the aim of this research was to study the effect of NK cells on the formation of tube-like structures by endothelial cells when co-cultured with trophoblast cells.

Materials and Methods

Cells

Trophoblast cells of the JEG-3 line (ATCC, USA) were used. These reproduce the morphological, phenotypic and functional characteristics of the invasive trophoblast of the first trimester of pregnancy [38, 44]. The cells were cultured in DMEM supplemented with 10% inactivated fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, 0.5 mM L-glutamine, 1 ml MEM and 1 mM sodium pyruvate (Sigma-Aldrich Chem. Co., USA). The cell monolayer was disintegrated using Versene and Trypsin solutions (Biolot, Russia) mixed in a 1:1 ratio.

Endothelial cells of the EA.Hy926 cell line were kindly provided by Dr. C.J. Edgel (University of North Carolina, USA). These cells reproduce all the main characteristics of endothelial cells [27]. The cells were cultured in DMEM/F12 supplemented with 10% FCS, 100 μg/ml streptomycin, 100 U/ml penicillin (Sigma-Aldrich Chem. Co., USA), 8 mmol/L L-glutamine and HAT (Sigma, USA). Subcultivation was performed once every 3-4 days, with monolayer disintegration caused by exposure to Versene solution for 5 minutes.

Cells of the NK-92MI cell line (ATCC, USA) reproduce basic phenotypic and functional characteristics of activated NK cells [32, 46]. The cells were cultured in α-MEME containing 12.5% 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, and 0.1 mM 2-mercaptoethanol (Sigma-Aldrich Chem. Co., USA). All experiments with cell lines were carried out in an incubator, in a humid atmosphere at 37 °C under 5% CO_2. Cell viability was assessed using Trypan blue solution and was at least 96%.

Cytokines

To activate the cells, recombinant human cytokines were used: bFGF (1, 10 and 20 ng/ml), VEGF (1, 10 and 100 ng/ml), PlGF (1, 5 and 20 ng/ml), TGF-β (1, 5 and 10 ng/ml), IL-8 (1, 10 and 100 ng/ml, all from CytoLab, Israel); IFNγ (40, 400 and 1000 U/ml, Gammaferon, Ferment Scientific Production Association (NPO); Sanitas, Lithuania); IL-1β (10, 100 and 1000 U/ml, Betaleukin, Scientific Research Institute of Extremely Pure Biopreparations (NIIOChB), St. Petersburg, Russia).

To assess formation of tube-like structures, 1.5×10^5 endothelial cells of the EA.Hy926 cell line and 7.5×10^5 cells of the JEG-3 cell line, both in 250 μL of medium without FCS, were added to the wells of a 24-well plate pre-treated with Matrigel Growth Factor Reduced (Becton Dickinson, USA) [62, 69]. 5.5×10^4 cells of the NK-92MI cell line per well in 500 μL of medium without FCS were added to a number of the wells (directly to each well, contact-dependent culturing). Polycarbonate membrane inserts (1 μm pore size, BD, USA) were installed into other wells and then 5.5×10^4 cells of the NK-92MI cell line in 500 μL of culture medium without FCS were added to these inserts (distant culturing). Cytokines were added to both the wells and to the polycarbonate inserts. Culture medium (500 μL) was added to the control wells. FCS content was adjusted to 2.5% in all wells and inserts. The plate was incubated for 24 hours (37 °C, 5% CO_2). The experiments were repeated twice. Three repetitions were carried out for each position in the experiment. Using the AxioObserver.Z1 microscope and the Axio Vision computer image analysis system (Zeiss, Germany), five fields of view were taken into consideration and the length (in micrometres) of the produced tube-like structures in each well was estimated (Figure 1).

Laser scanning confocal microscopy

The relative position of endothelial cells and trophoblast cells in the absence of NK-92MI cells was assessed using the Leica TCS SP5 confocal laser scanning microscope (Germany). An oil immersion lens with a magnification of 20.0×0.7 was also used. Vital fluorescent dyes were used to stain the cells, green for endothelial cells (Calcein AM, BD, USA), and red for trophoblast cells (SNARF-1, ThermoFisher Scientific, USA), as per the manufacturer's directions. Matrigel matrix for cell culturing was preliminarily superimposed onto a cover glass (Carl Roth GmbH, Germany) placed into a well of a 24-well plate. Endothelial and trophoblast cells were then cultured on the Matrigel matrix as mentioned above. To fix the cells, we used the mounting medium with DAPI (BIOZOL, Germany). All images were obtained using identical settings of laser power and fluorochrome detection ranges. Figure 2 (see 2nd page of cover) shows images corresponding to the maximum brightness projection obtained using ImageJ software. The areas occupied by cells were calculated using the ImageJ software.

Statistical analyses were conducted using Statistica 10 software. The data are presented descriptively, with the nonparametric Mann–Whitney U-test used for between group comparisons. We estimated the length of tube-like structures formed by endothelial cells under different co-culturing conditions and with different cytokine additions. We then compared the results obtained with the control sample (endothelial cells cultured with trophoblast cells in culture medium without inducers). The level of tube-like structure

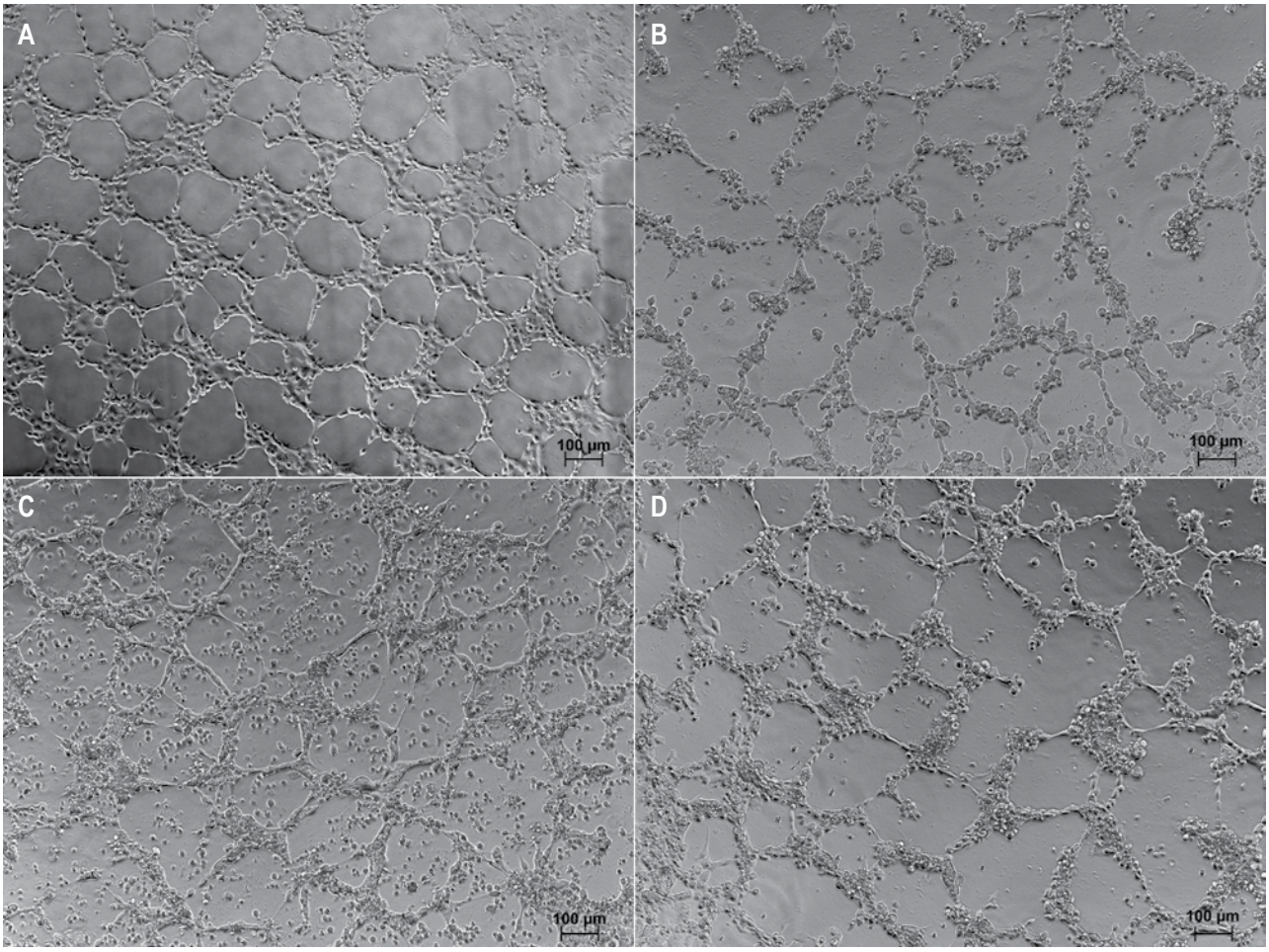

Figure 1. Tube-like structures formed by endothelial cells of the EA.Hy926 cell line in the presence of: A, 2.5% FCS (constitutive level); B, trophoblast cells of the JEG-3 cell line; C, contact-dependent culturing of endothelial cells of the EAhy926 cell line, trophoblast cells of the JEG-3 cell line and natural killers of the NK-92MI cell line; D, distant culturing of endothelial cells of the EAhy926 cell line, trophoblast cells of the JEG-3 cell line and natural killers of the NK-92 cell line. Phase contrast, ×100.

formation by endothelial cells of the EA.Hy926 cell line in the culture medium supplemented with 2.5% FCS was taken as zero.

Results

The length of tube-like structures formed by endothelial cells of the EA.Hy926 cell line was the same whether monocultured or co-cultured with cells of the JEG-3 cell line (Figure 1, Figure 3).

Scanning confocal microscopy demonstrated that when co-cultured on the Matrigel matrix, endothelial and trophoblast cells produced branched and dense cell strands (tube-like structures) of nonuniform composition (Figure 2, see 2nd page of cover). Endothelial cells (green) adjoined each other closely, exactly as trophoblast cells (violet) did. Moreover, endothelial cells contacted trophoblast cells closely to form branched cell strands. Trophoblast cells formed cell strands independently and when integrated into tube-like structures formed by endothelial cells, thus replacing or covering endothelial cells. Most of the endothelial and trophoblast cells had a round shape of the same size. In some parts of the strands, endothelial cells took a prolate shape. Using ImageJ software, we calculated the area occupied by each cell type. In Figure 2 (see 2nd page of cover), endothelial and trophoblast cells occupied 76 μm^2 and 59 μm^2, respectively. Thus, endothelial cells comprised approximately 56%, and trophoblast cells approximately 44% of a cell strand area (135 μm^2).

Introduction of NK-92MI cells into this co-culture of endothelial and trophoblast cells under conditions of both distant and contact-dependent culturing did not change the length of tube-like structures formed by endothelial cells of the EA.Hy926 cell line (Figure 1, Figure 3).

During contact-dependent culturing of endothelial cells and trophoblast cells of the JEG-3 cell line with NK cells in the presence of bFGF 10 ng/ml, the length of tube-like structures decreased compared with the

length of tube-like structures formed under the same culturing conditions but without bFGF (Figure 3). During distant culturing of endothelial cells and cells of the JEG-3 cell line with NK cells in the presence of bFGF, the length of tube-like structures decreased at all concentrations of bFGF compared with the length of tube-like structures formed under the same culturing conditions but without addition of the cytokine. It was established that the length of tube-like structures formed during distant culturing of endothelial cells and cells of the JEG-3 cell line with NK cells in the presence of bFGF 10 ng/ml was longer than the length formed under the same culturing conditions but in the presence of bFGF 1 ng/ml. It was also longer than the length of tube-like structures formed during contact-dependent culturing of endothelial cells and cells of the JEG-3 cell line with NK cells in the presence of the same cytokine concentration (Figure 3).

During contact-dependent culturing of endothelial cells and cells of the JEG-3 cell line with NK cells in the presence of VEGF, the length of tube-like structures increased at all VEGF concentrations compared with the length formed under the same culturing conditions but without VEGF (Figure 4). The length of tube-like structures formed during distant culturing of endothelial cells and cells of the JEG-3 cell line with NK cells in the presence of VEGF remained unchanged compared with the length formed under the same culturing conditions but without the growth factor.

During contact-dependent culturing of endothelial and trophoblast cells with NK cells in the presence of PlGF 1 and 20 ng/ml, the length of tube-like structures was less than the length formed under the same culturing conditions but without addition of PlGF (Figure 4). The length of tube-like structures formed during distant culturing of endothelial cells and cells of the JEG-3 cell line with NK cells in the presence of PlGF remained unchanged compared with the length formed under the same culturing conditions but without the growth factor.

During both contact-dependent and distant culturing of endothelial and trophoblast cells with NK cells in the presence of IFNγ, the length of tube-like structures did not change compared with the length formed under the same culturing conditions but without IFNγ (Figure 5).

During distant culturing of endothelial cells and trophoblast cells of the JEG-3 cell line with NK cells in the presence of IL-1β 0.1 ng/ml, the length of tube-like structures increased compared with the length formed under the same culturing conditions but without IL-1β. It was also increased compared with the length formed during contact-dependent culturing of endothelial and trophoblast cells with the same IL-1β concentration. During contact-dependent culturing of endothelial and trophoblast cells of the JEG-3 cell line in the presence of NK cells and IL-1β, the length of tube-like structures remained unchanged (Figure 5).

In the presence of IL-8 1 and 100 ng/ml, the length of tube-like structures formed during contact-dependent culturing of endothelial and trophoblast cells with NK cells decreased compared with the length of tube-like structures formed under the same culturing conditions but without IL-8. During distant culturing of endothelial and trophoblast cells with NK cells in the presence of IL-8, the length of tube-like structures did not differ from the length formed under the same culturing conditions but without the addition of IL-8 (Figure 6).

The length of tube-like structures formed during distant culturing of endothelial and trophoblast cells with NK cells in the presence of TGF-β 5 and 10 ng/ml was decreased compared with the length of tube-like structures formed under the same culturing conditions but without the cytokine. During distant culturing of endothelial and trophoblast cells with NK cells in the presence of TGF-β 5 ng/ml, the length of tube-like structures was less than the length formed during contact-dependent culturing of endothelial and trophoblast cells with NK cells in the presence of TGF-β 5 ng/ml (Figure 6).

Discussion

Angiogenesis in the placenta and in the decidua is regulated by endothelial cells, trophoblast cells, NK cells, macrophages and other microenvironment cells via contact interactions and cytokine production. Studies on the interactions between placental cells *in vitro* are difficult due to the challenges with isolating pure primary cultures of endothelial, decidual NK and trophoblast cells from the placenta of the same woman. The use of cell lines in this case is the most convenient alternative to study the interactions of cells in culture. The advantage of our approach is that the modeling of interactions between endothelial cells and microenvironment cells are under conditions that are close to those experienced *in vivo* [6]. Endothelial cells placed on a three-dimensional matrix do not proliferate, and instead quickly attach and form a network of tube-like structures [10, 33]. When cultured on the Matrigel matrix, trophoblast cells form tube-like structures and simultaneously show their endovascular phenotype [37]. Trophoblast invasion and participation in vascular remodeling in the uteroplacental bed have been studied [39, 77]. It is assumed that trophoblast cells cause apoptosis of endothelial cells and replace them, thereby contributing to formation of blood flow between mother and fetus [35, 53]. We found no literature describing the mutual arrangement of trophoblast cells and endothelial cells in placental vessels. We established in this research that trophoblast cells did

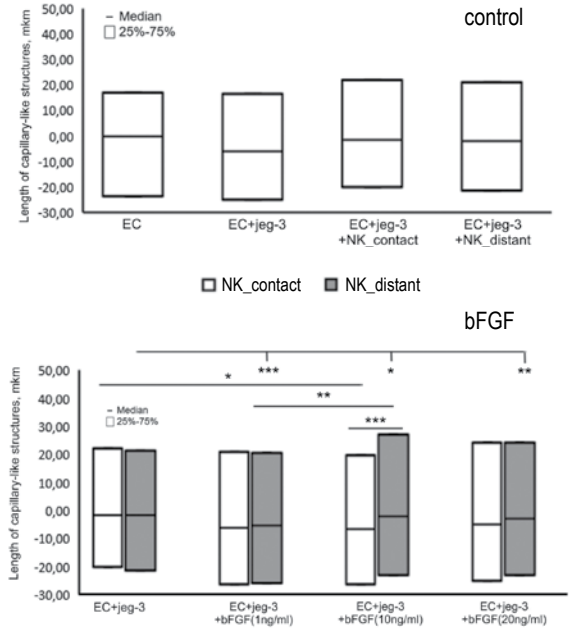

Figure 3. Formation of tube-like structures by endothelial cells of the EA.Hy926 cell line in the presence of cells of the JEG-3 cell line and bFGF during their distant or contact-dependent culturing with cells of the NK92MI cell line

Note. EC, culturing of endothelial cells (constitutive level); EC+JEG-3, culturing of endothelial cells with unstimulated cells of the JEG-3 cell line; EC+JEG-3+NK_contact, contact-dependent culturing of endothelial cells with unstimulated cells of the JEG-3 cell line and unstimulated cells of the NK92MI cell line; EC+JEG-3+NK_distant, distant culturing of endothelial cells with unstimulated cells of the JEG-3 cell line and unstimulated cells of the NK92MI cell line; EC+JEG-3+bFGF, culturing of endothelial cells with cells of the JEG-3 cell line in the presence of bFGF. NK_distant, distant culturing: a porous polycarbonate membrane separates NK cells from the co-culture of endothelial cells and cells of the JEG-3 cell line. NK_contact, contact-dependent co-culturing of cells of the NK-92MI cell line, endothelial cells and cells of the JEG-3 cell line. The significance of differences between groups: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

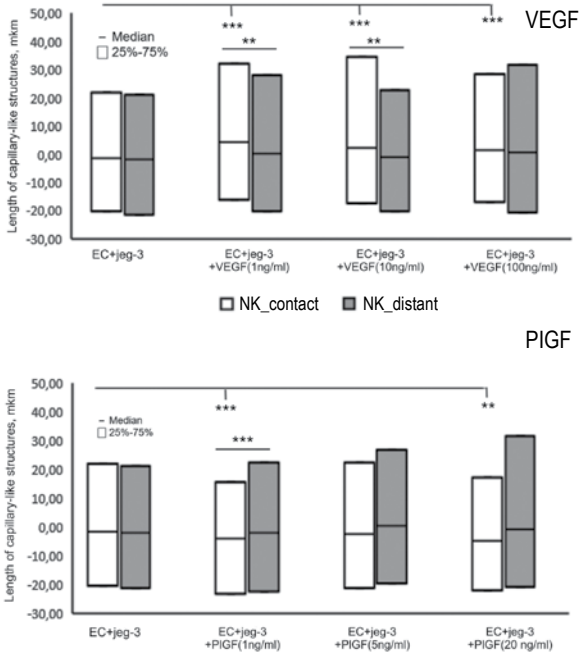

Figure 4. Formation of tube-like structures by endothelial cells of the EA.Hy926 cell line in the presence of cells of the JEG-3 cell line and VEGF or PIGF during their distant or contact-dependent culturing with cells of the NK92MI cell line

Note. EC+JEG-3, culturing of endothelial cells with cells of the JEG-3 cell line; EC+JEG-3+VEGF, culturing of endothelial cells with cells of the JEG-3 cell line in the presence of VEGF; EC+JEG-3+PIGF, culturing of endothelial cells with cells of the JEG-3 cell line in the presence of PIGF. NK_distant, distant culturing: a porous polycarbonate membrane separates NK cells from the co-culture of endothelial cells and cells of the JEG-3 cell line. NK_contact, contact-dependent co-culturing of cells of the NK-92MI cell line, endothelial cells and cells of the JEG-3 cell line. The significance of differences between groups: **, $p < 0.01$; ***, $p < 0.001$.

not change the length of tube-like structures formed by endothelial cells, which was consistent with prior work [76]. Using confocal microscopy, we found that trophoblast cells made up almost half of the tube-like structure. Previous research established that, when remodeling the uterine spiral arteries, trophoblast cells induced apoptosis of endothelial cells and replaced them by differentiating into endovascular trophoblast [35, 87]. Our model revealed that trophoblast cells cause death of some endothelial cells during tube-like structure formation by endothelial cells on the Matrigel matrix. Trophoblast cells use endothelial cells as a frame and replace them by integrating into them.

We suggested that co-culturing of three cell lines (endothelial cells, trophoblast cells and NK cells) could serve as a model of placental processes. Introduction of cells of the NK-92MI cell line into the co-culture of endothelial and trophoblast cells under conditions of both distant and contact-dependent culturing did not change the length of tube-like structures formed by endothelial cells. We found no literature indicating that NK cells acquired the phenotype and properties of decidual NK cells when cultured with trophoblast cells and/or endothelial cells on the Matrigel matrix. Studies are currently underway in this area. Cerdeira et al. showed that cytotoxic NK cells, under the action of TGF-β and methylating agents, changed their phenotype to that characteristic of decidual NK cells [18]. Macrophages and trophoblast are the main sources of TGF-β in the placenta [1, 3]. Therefore, the absence of changes in the length of tube-like structures in a three-component cell culture indirectly supports the assumption that NK cells of the NK-92MI cell line acquire regulatory functions characteristic of decidual NK cells.

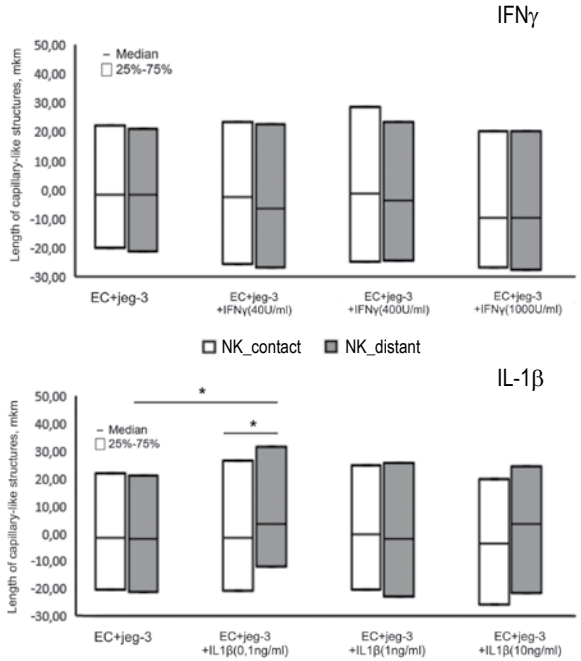

Figure 5. Formation of tube-like structures by endothelial cells of the EA.Hy926 cell line in the presence of cells of the JEG-3 cell line and IFNγ or IL-1β during their distant or contact-dependent culturing with cells of the NK92MI cell line

Note. EC+JEG-3, culturing of endothelial cells with trophoblast cells; EC+JEG-3+IFNγ, culturing of endothelial cells with cells of the JEG-3 cell line in the presence of IFNγ; EC+JEG-3+IL-1β, culturing of endothelial cells with unstimulated cells of the JEG-3 cell line in the presence of IL-1β. NK_distant, distant culturing: a porous polycarbonate membrane separates NK cells from the co-culture of endothelial cells and cells of the JEG-3 cell line. NK_contact, contact-dependent co-culturing of cells of the NK-92MI cell line, endothelial cells and cells of the JEG-3 cell line. The significance of differences between groups: *, $p < 0.05$.

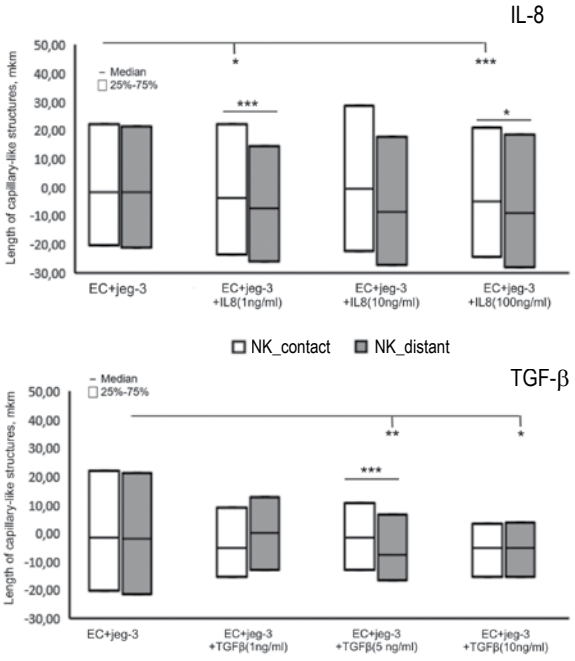

Figure 6. Formation of tube-like structures by endothelial cells of the EA.Hy926 cell line in the presence of cells of the JEG-3 cell line and IL-8 or TGF-β during their distant or contact-dependent culturing with cells of the NK92MI cell line

Note. EC+JEG-3, culturing of endothelial cells with cells of the JEG-3 cell line; EC+JEG-3+IL-8, culturing of endothelial cells with cells of the JEG-3 cell line in the presence of IL-8; EC+JEG-3+TGF-β, culturing of endothelial cells with unstimulated cells of the JEG-3 cell line in the presence of TGF-β. NK_distant, distant culturing: a porous polycarbonate membrane separates NK cells from the co-culture of endothelial cells and cells of the JEG-3 cell line. NK_contact, contact-dependent co-culturing of cells of the NK-92MI cell line, endothelial cells and cells of the JEG-3 cell line. The significance of differences between groups: *, $p < 0.05$ **, $p < 0.01$ ***, $p < 0.001$.

Stimulation of NK cells by IL-1β increases their cytotoxic function [40] due to contact interaction with a target cell. We established that, during contact-dependent culturing of NK cells with the co-culture of endothelial and trophoblast cells in the presence of IL-1β, the length of tube-like structures remained unchanged compared with the length formed under the same culturing conditions but without the cytokine. Under conditions of contact-dependent culturing, trophoblast cells expressing the HLA-C and HLA-G molecules on their surface change the functional properties of NK cells [60, 84], while suppressing their cytotoxic function. In turn, during distant culturing of NK cells with the co-culture of endothelial and trophoblast cells in the presence of IL-1β, the length of tube-like structures increased against the length formed under the same culturing conditions but without the cytokine. Previously, it was established that IL-1β stimulated angiogenesis during co-culturing of endothelial and trophoblast cells [76]. Moreover, trophoblast secretory products activated by IL-1β promote elongation of tube-like structures. Acting through the trophoblast [78], IL-1β apparently stimulated angiogenesis, primarily affecting endothelial cells as a component of the co-culture. Under conditions of distant culturing with the co-culture of endothelial and trophoblast cells in the presence of IL-1β, NK cells apparently acquire regulatory characteristics and show no cytotoxic effects.

Trophoblast [26, 75], endothelial [29, 51, 67] and NK [20, 82] cells carry receptors for VEGF on their surface and are potentially capable of responding to its effects. Moreover, all these cells are sources of VEGF in the placenta [4, 17]. The main effects of this cytokine are to increase cell viability and stimulate all stages of angiogenesis. We established that, during contact-dependent (but not distant) culturing of NK cells with the co-culture of endothelial and trophoblast cells in the presence of VEGF, the length

of tube-like structures was greater than the length of tube-like structures formed under the same culturing conditions but without VEGF. It was noted previously that VEGF dose-dependently increased the length of blood vessels formed by endothelial cells in the co-culture with trophoblast [76]. Therefore, during contact-dependent culturing, NK cells do not alter the stimulating effect of VEGF on angiogenesis in the co-culture of endothelial and trophoblast cells. In contrast, during distant culturing in the absence of contact with trophoblast cells, NK cells can inhibit the stimulating effect of VEGF on angiogenesis in the co-culture of endothelial and trophoblast cells. This may be associated with the production of TNFα by NK cells, which is capable of inhibiting angiogenesis in the absence of contact interactions between NK cells and trophoblast [31].

We established that the length of tube-like structures remained unchanged during contact-dependent and distant culturing of NK cells with the co-culture of endothelial and trophoblast cells in the presence of IFNγ. IFNγ is the most important regulator in the placenta cytokine network operating like a check and balance system. As an apoptogenic factor for endothelial cells, IFNγ can suppress blood vessel growth [7, 63]. During co-culturing of endothelial and trophoblast cells in the presence of IFNγ, stimulation of angiogenesis was observed previously [76]. IFNγ has been established to cause trophoblast apoptosis [41], which undermines the ability of trophoblast to protect endothelial cells from the cytotoxic effects of NK cells. IFNγ has also been shown to activate the cytotoxic effects of NK cells [65]. However, as noted above, trophoblast can limit the cytotoxic effects of NK cells due to its contact (expression of HLA-G) and distant influences (production of cytokines and sHLA-G). The result is that when used in a three-component cell system, the proinflammatory cytokine IFNγ has no effect on angiogenesis.

We established that, during distant (but not contact-dependent) culturing of NK cells with the co-culture of endothelial and trophoblast cells in the presence of TGF-β, the length of tube-like structures was less than the length formed under the same culturing conditions but without the cytokine. The inhibitory effect of TGF-β (a cytokine with proven stimulatory and inhibitory effects on angiogenesis [4]) on the length of tube-like structures in a three-component cell co-culture is not unexpected. TGF-β is a pleiotropic cytokine with a variety of effects on target cells depending on concentration and the cellular microenvironment [45, 70, 76]. Macrophages and trophoblast are the main sources of TGF-β in the placenta. It was established elsewhere that TGF-β promoted elongation of tube-like structures formed by endothelial cells in co-culture with trophoblast [76]. Therefore, during contact-dependent culturing of NK cells and the co-culture of endothelial and trophoblast cells, NK cells acted as an angiogenesis regulator and mitigated the TGF-β effect on length stimulation. In contrast, under distant culturing conditions, TGF-β triggered a signal in NK cells that inhibited angiogenesis. Trophoblast is apparently able to suppress the negative effect of NK cells on angiogenesis only against the background of direct contact. Increased production of TGF-β by trophoblast was revealed to occur in preeclampsia. In this case, increased production of TGF-β by trophoblast was considered to be a negative event accompanied by disordered angiogenesis in the placenta. The data obtained in our research indicated even more complicated regulation of placental cell interactions based both on cellular contacts and on effects produced by cytokines. Due to contact (via HLA-G) and cytokine (via TGF-β) signals, trophoblast is able to limit the negative effect of NK cells on the endothelium and suppress inflammation in situations of pathological processes in the placenta.

Cytokines IL-8, PlGF and bFGF are the most important pro-angiogenic factors [24, 25, 48, 56, 73] stimulating proliferation and increasing trophoblast cell viability [8]. These cytokines also activate NK cells [48, 50, 58, 79]. A decrease in the length of tube-like structures under conditions of a three-component cell co-culture in the presence of the following pro-angiogenic factors was revealed in our research: IL-8, PlGF (during contact-dependent culturing only) and bFGF (during both contact-dependent and distant culturing). Earlier work established that bFGF and IL-8 promoted elongation of tube-like structures formed by endothelial cells during co-culture with trophoblast, while PlGF promoted a decrease in length of the formed tube-like structures [76]. Thus, the observed inhibitory effect of PlGF continued to persist only under conditions of contact-dependent culturing of NK cells with the co-culture of endothelial and trophoblast cells. This effect disappeared during distant culturing. During contact-dependent and distant culturing of NK cells with the co-culture of endothelial and trophoblast cells in the presence of bFGF and IL-8, the effects of these cytokines on the co-culture changed to the opposite ones. This change occurred when NK cells were introduced into the co-culture. We did not find any literature describing the effect of PlGF, bFGF and IL-8 on the functional activity of NK cells, their cytokine production, or their interactions with endothelial and trophoblast cells. However, there are indirect data, including those presented in this paper, arguing for a change in cell behavior in multicomponent co-cultures as a result of various distant and contact interactions. For example, decidual NK cells secrete: IL-2, IL-15, IFNγ, VEGFA, VEGFC, IL-8, TGF-β, PlGF, Ang1, Ang2 [43], uPA, uPAR, MMP [64] MIP1a, GM-CSF,

CSF1 and other factors [66] that may have direct or indirect (through other cells) pro-angiogenic or anti-angiogenic effects. The pro-angiogenic properties of PlGF become apparent only when VEGF is present in the microenvironment of endothelial cells [14]. Published evidence demonstrates that sHLA-G secreted by trophoblast cells can induce apoptosis of endothelial cells by binding to the CD160 receptor expressed on the endothelial cell surface, as well as by inhibiting bFGF-induced angiogenesis [30]. The soluble form of sHLA-G secreted by trophoblast stimulates proliferation of decidual NK cells [83], while inhibiting their cytotoxic activity. It has also been established that sHLA-G suppresses the functional activity of NK cells in tumor diseases [71].

Conclusion

Cells of the NK-92MI cell line reproduce basic phenotypic and functional characteristics of activated NK cells and show a cytotoxic effect on target cells. The data obtained in this research provide indirect evidence that NK cells of the NK-92MI cell line acquire regulatory functions characteristic of decidual NK cells when cultured with the co-culture of endothelial cells and trophoblast. Under conditions of contact-dependent culturing, trophoblast expressing HLA-C and HLA-G molecules on their surface change the functional properties of NK cells [60, 84], while suppressing their cytotoxic function. Under conditions of distant culturing with the co-culture of endothelial and trophoblast cells in the presence of specific cytokines (IL-1β), NK cells also acquire regulatory characteristics and do not show inhibitory effects. In a three-component co-culture, cytokines affect all cells at once. As a result, the effects of cytokines on target cells differ from those revealed in single- and two-component cell models previously. The data obtained in this research support regulation of placental cell interactions involving both cellular contacts and effects produced by cytokines.

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