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Effect of Cytokines on the Formation Tube-Like Structures by Endothelial Cells in the Presence of Trophoblast Cells

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Despite ample data on cytokine secretion in the uteroplacental interface, the influence of microenvironment cells, in particular, trophoblast cells on angiogenesis and the role of cytokines in this process remain poorly studied. We studied the influence of cytokines on the formation of tube-like structures by endothelial cells in the presence of trophoblast cells and showed that trophoblast cells suppressed the angiogenic potential of endothelial cells. Antiangiogenic cytokines IFN-γ, IL-10, TNF-α, and TGFβ via modulation of trophoblast cells stimulated the formation of tube-like structures by endothelial cells. In the co-culture of endothelial and trophoblast cells, the effects of cytokines changed and they gained additional regulatory functions.

Key Words: *endothelium; trophoblast; cytokines; angiogenesis; placenta*

Appropriate fetus nutrition depends on successful formation of uteroplacental interface, placental and decidual vascular network, interactions of trophoblast cells with microenvironment, including maternal immune cells located in endometrium and uterine endothelial cells (EC), during remodeling of the spiral arteries [32]. EC play an important role in placenta formation and development. Along with barrier function, EC have a variety of regulatory functions, produce a wide spectrum of bioactive substances affecting angiogenesis, hemostasis, cell adhesion, inflammatory response, and functional activity of cells of the microenvironment [18,19].

EC play a key role in the process of angiogenesis in the placenta, the key event for providing nutrients and oxygen to the fetus. Angiogenesis consisting of several consecutive stages: destruction of the extracellular matrix, migration and proliferation of EC, formation of a new capillary tube by EC, the restoration of the basement membrane [2,57]. The regulation of angiogenesis is determined by many factors: influence of cytokines and growth factors, interactions between EC mediated by adhesion molecules PECAM-1 and VE-cadherin, EC interactions with extracellular matrix proteins via integrins, production of enzymes that degrade extracellular matrix, EC sensitivity to apoptosis-inducing stimuli [2,4,57]. EC are an important source of cytokines and growth factors affecting all stages of angiogenesis. For instance, activated EC produce VEGF, IL-1, IL-6, IL-8, MCP-1, M-CSF, GM-CSF, and SDF-1. During activation, EC can produce urokinase type plasminogen activator (u-PA) and its inhibitor (PAI-1), MMP-1, MMP-2, MMP-3, and MMP-9 due to which EC can modulate the processes of extracellular matrix degradation [4].

Angiogenesis in the placenta, decidua, and endometrium is controlled by cells of the microenvironment. The key role in the regulation of angiogenesis in the placenta is played by decidual and placental macrophages that via production of cytokines (VEGF [6,20], TGFβ, M-CSF, IL-6, IL-1β [35,54], TNF-α [35], IL-8 [62,63], G-CSF [66], GM-CSF [12], IFN-γ [33], IL-10 [34], IL-15, IL-17 [60], MCP-1, and TSP-1) and directly or indirectly (through other cells of EC microenvironment) control all stages of angiogenesis.

The main cells of EC microenvironment in the placenta are trophoblast cells. For instance, a population of trophoblast cells, endovascular trophoblast,

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participates in remodeling of the uterine spiral arteries [44] by replacing the endothelial lining. This process is accompanied by expression of EC-specific adhesion molecules by endovascular cytotrophoblast cells and replacement of vascular EC by trophoblast cells via of Fas- and TRAIL-mediated induction of EC apoptosis and induction of Fas- and TRAIL-mediated apoptosis of vascular smooth muscle cells leading to dilatation of uterine vessels and increased maternal blood flow supply to the trophoblast [14]. In addition, different populations of trophoblast cells produce cytokines IL-6, IL-11, GM-CSF, IL-1β [61], IFN-γ, IL-4, TNF-α, TGFβ, IL-8, IL-10 [73], VEGF, SDF-1 [74], IGF, and RANTES responsible for autocrine and paracrine regulation of trophoblast invasion. Moreover, trophoblast is one of the main sources of enzymes MMP-2, MMP-3, MMP-9, and cathepsin [14] in placenta destructing the extracellular matrix during invasion. The production of these enzymes and cytokines can modulate functional activity of both placental and decidual EC and affect angiogenesis in the placenta and decidua and formation of contacts between endovascular trophoblast cells and EC. It is believed that cytokines secreted by EC stimulate proliferation and differentiation of trophoblast cells and regulate their invasion [22].

Thus, production of various cytokines by placental cells was studied by various methods, mainly immunohistochemistry. Proliferation, viability, migration, and invasion of trophoblast cells into the endometrium are controlled by a wide range of soluble factors secreted by microenvironmental cells, including EC. In turn, trophoblast cells produce cytokines and factors affecting EC and other microenvironmental cells. Trophoblast cells and EC interact not only via secretion of soluble factors, but also by contact interactions [44]. Despite ample data on cytokine secretion in the uteroplacental interface, the influence of microenvironmental cells, in particular, trophoblast cells on angiogenesis and the role of cytokines in this process remain poorly understood.

Therefore, the aim of the present study was to evaluate the effects of cytokines on the early stages of vessel formation by EC in the presence of trophoblast cells.

MATERIALS AND METHODS

EA.Hy926 cells used in the experiment reproduce all main features of EC [27]. Culturing was carried out in DMEM/F-12 supplemented with 10% fetal calf serum (FCS), 100 μg/ml streptomycin, 100 U/ml penicillin (Sigma), and 8 mmol/liter L-glutamine, and NAT (Sigma). The cells were subcultured every 3-4 days by 5-min treatment of the monolayer with Versene (BioloT). JEG-3 trophoblast cells reproduce all main features of invasive trophoblast [49]. The cells were cultured in DMEM containing 10% FCS, 100 μg/ml streptomycin, 100 U/ml penicillin, and 2 mmol/liter L-glutamine, and 1% non-essential amino acids, and 10 mM sodium pyruvate (Sigma) and subcultured every 3-4 days by 5-min treatment of the monolayer culture 0.135% trypsin and 0.01% EDTA (BioloT).

We used recombinant human cytokines bFGF, PlGF, TGFβ, IL-4, IL-6, IL-8, IL-10 (RD), VEGF (BD), TNF-α (Refnolin, specific activity 1U=0.06 ng), IFN-γ (Gammaferon) (Ferment, Sanitas) in various concentrations.

In wells of a 24-well plate pretreated with Matrigel Growth Factor Reduced matrix (BD) [37], 400 μl DMEM/F-12, 25 μl FCS (Sigma), and cytokines in various concentrations (3 wells for each concentration) were added. Then, 175,000 EC (EA.Hy926 cells) in 300 μl DMEM/F-12 and 75,000 JEG-3 trophoblast cells in 300 μl DMEM/F-12 previously stained with green fluorescent vital dye Calcein AM were added to each well (Sigma). The control wells contained 300 μl DMEM without JEG-3 cells. The plates were incubated for 24 h (37°C, 4.5% CO_2). The formation of tube-like structures by EC in the absence of trophoblasts served as the control (Fig. 1, *a*). Spontaneous formation of tube-like structures by EC was assessed the presence of trophoblast cells in a medium containing 2.5% FCS (Fig. 1, *b*). Formation of tube-like structures by EC in the presence of trophoblast cells and IFN-γ (1000 U/ml) served as the positive control (Fig. 1, *c*). In each well, 5 fields of view were examined under an AxioObserver Z1 microscope and the length and number of formed tube-like structures were measured using AxioVision image analysis system (Carl Zeiss).

The data were processed statistically using Statistica 10.0 software. Parametric Student's *t* test was applied for data analysis.

RESULTS

Physiological development of the placenta depends on successful cooperation of trophoblast cells and EC in the uteroplacental interface zone. Proliferation, viability, invasion, migration, and adhesion of trophoblast cells are controlled by a wide range of soluble factors secreted by microenvironmental cells, including EC. Trophoblast cells also produce cytokines and factors affecting EC, immune system cells, and other microenvironmental cells. Imbalance in the production of cytokines and other soluble factors in the placenta can cause pregnancy complications.

The interactions of EC with trophoblast cells in the placenta are traditionally studied by using im-

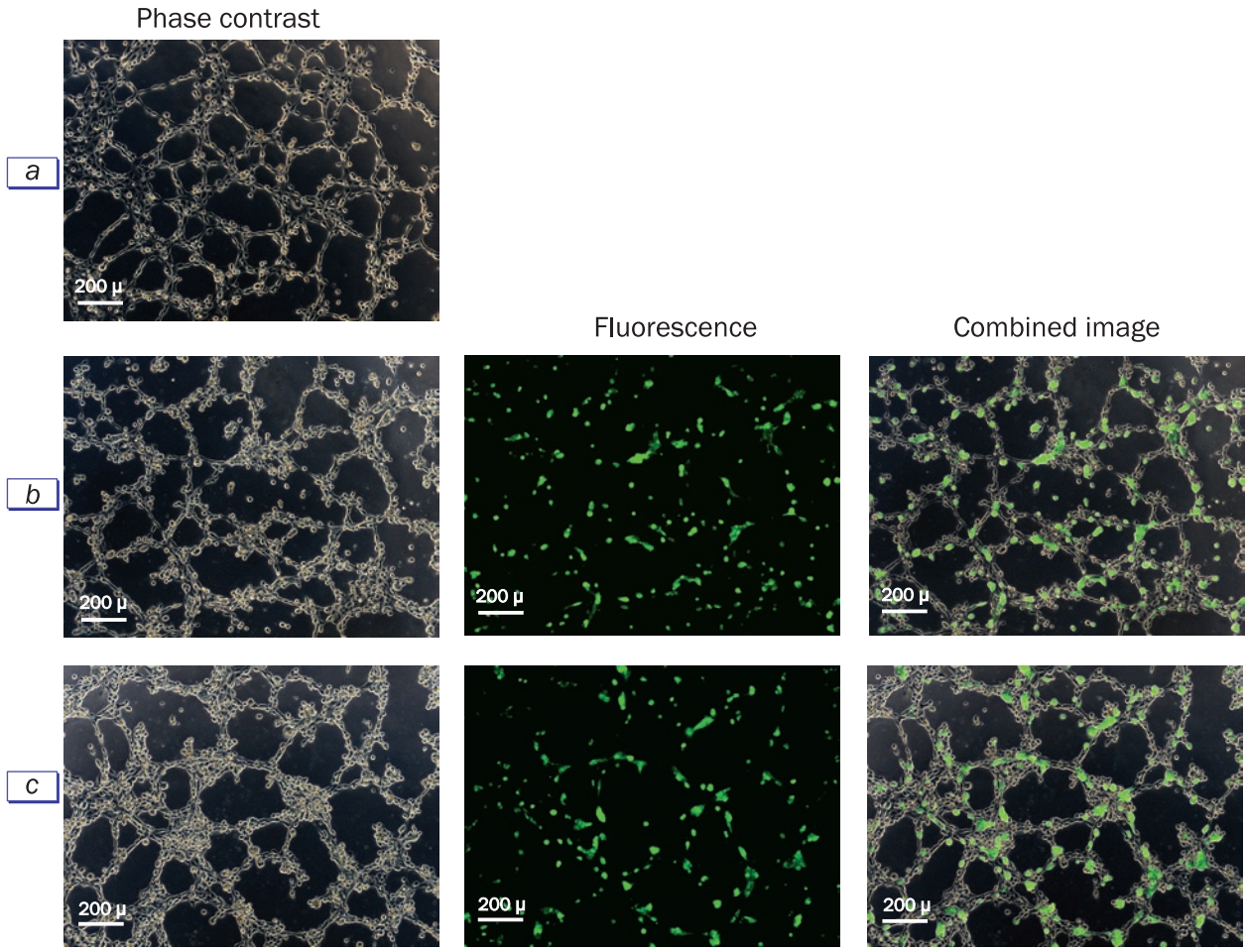

Fig. 1. Tube-like structures formed by of EA.Hy926 EC (*a*), tube-like structures formed by EA.Hy926 EC in the presence of JEG-3 trophoblast cells (stained with Calcein AM) (*b*), tube-like structures formed by EA.Hy926 EC in the presence of JEG-3 trophoblast cells (stained with Calcein AM) after addition of 1000 U/ml IFN-γ (*c*), ×100.

munohistochemical techniques. There are also other approaches to *in vitro* studies of EC—trophoblast cell interaction: integration of trophoblast cells into EC monolayer [15], perfusion of isolated uterine arteries (allows modeling of endovascular and interstitial invasion of trophoblast cells) [21], co-culture of cells on extracellular matrixes (fibronectin, Matrigel) [7,41,42,76]. The latter approach was used in the present study. Matrigel is the product of mouse sarcoma cell line Engelbreth-Holm-Swarm (EHS) and represents a mixture of extracellular matrix proteins with a high content of laminin, nidogen, and type IV collagen. It also contains minor amounts of TGFβ, EGF, IGF, bFGF, and uPA. Matrigel is used as a substrate for culturing of embryonic stem cells, a model of cancer cell invasion [48]. EC placed on a 3D matrix do not proliferate, but rapidly adhere and form a network of capillary-like structures that have inner lumen and form a basal membrane on the outer side of the vessel [31]. Later, acetylated LDL appear on EC, which is a marker of differentiated state of these cells. This was confirmed by experiments on primary EC (HUVEC) and transplantable EC cell line EA.Hy926 [8]. The advantage of this method is the possibility of modeling EC—trophoblast cell interactions under conditions approximating *in vivo* conditions, because EC cultured on Matrigel considerably differ by their morphological, phenotypic, and functional properties from EC cultured on plastic. Experiments with EC culturing on Matrigel demonstrated the influence of different cytokines, growth factors, and pharmacological agents on angiogenesis [71]. However, experimental studies with co-culturing of several cells populations (EC+monocyte-like cells [53,68], EC+trophoblast cells [26,75,76]) were performed only recently. This method allows modeling of cell—cell interaction via both secretion of growth factors and contact interactions.

Co-culturing experiments showed that the number of tube-like structures formed by EC (EA.Hy926 cells) co-cultured with trophoblast JEG-3 cells was lower (30±1) than in EC monoculture (37±1, $p<0.001$). The

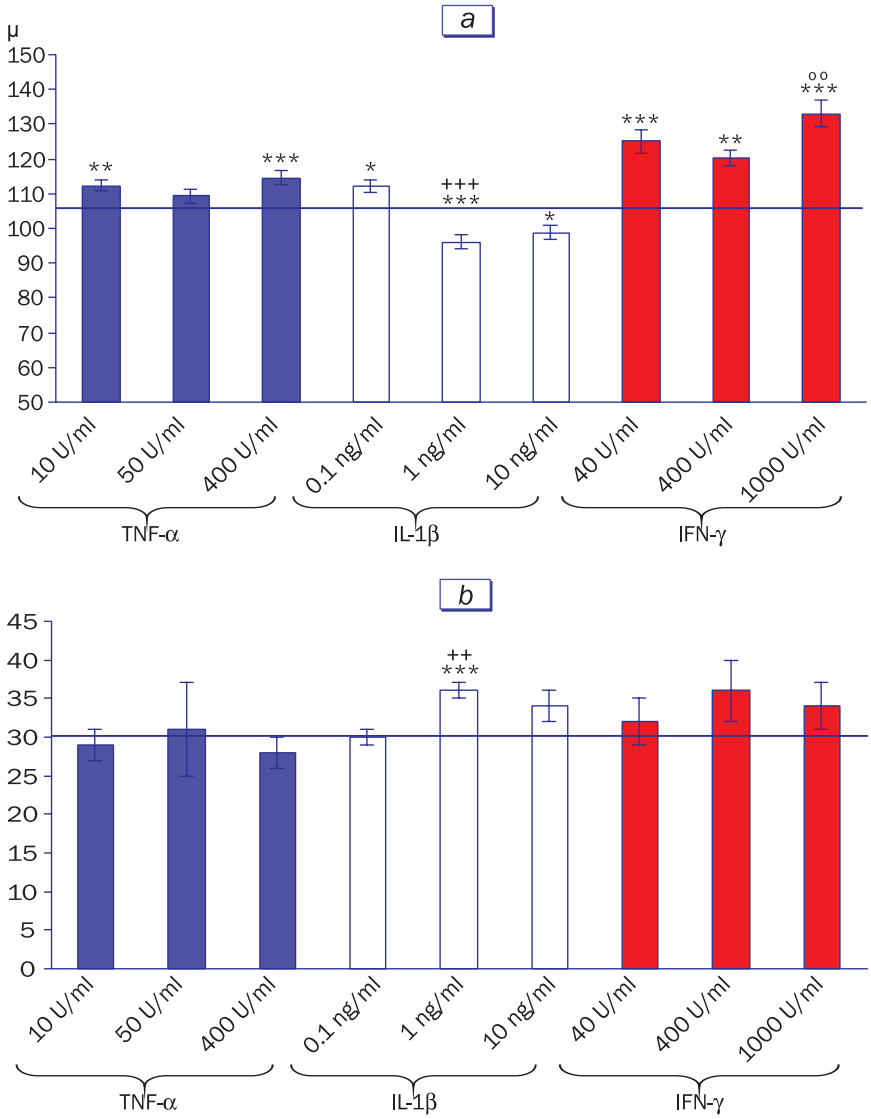

Fig. 2. Effect of proinflammatory cytokines TNF-α, IL-1β, IFN-γ on the formation of tube-like structures by EA.Hy926 EC in the presence of JEG-3 trophoblast cells. *a*) Length of tube-like structures; *b*) number of tube-like structures. Horizontal line: spontaneous level of tube-like structure formation. **p*<0.05, ***p*<0.01, ****p*<0.001 in comparison with spontaneous level; ^{++}p<0.01, ^{++}p<0.001 in comparison with 0.1 ng/ml IL-1β; ^{oo}p<0.01 in comparison with 400 U/ml IFN-γ.

length tube-like structures formed by EC (EA.Hy926 cells) in monoculture and co-culture with of JEG-3 cells was similar. There are published data on secretion of both proangiogenic (PlGF, bFGF, and VEGF) and antiangiogenic factors (sFlt1, endostatin, angiostatin, and thrombospondin) by trophoblast cells [11]. The observed decrease in the number of tube-like structures formed by EC can be related to trophoblast production of soluble factors that reduce EC viability and stimulate their apoptosis. It was shown that trophoblast can induce apoptosis by the TRAIL-dependent mechanism [45] and via interaction of Fas expressed on EC and FasL expressed on trophoblast cells [16]. Extravillous trophoblast can induce apoptosis of EC, which is necessary for remodeling of uterine spiral arteries at the expense of migration of trophoblast cells, their differentiation, and acquisition of endovascular phenotype [14].

The development of the placenta is accompanied by changes in the production of cytokines and growth factors by cells in uteroplacental contact area. It was previously found that the content of IL-10, IL-2, IL-4, IL-5, TNF-α, and IFN-γ in the placenta increased from the first to the third trimester, while the content of IL-8, VEGF, and bFGF decreased [5]. Normally, the production of pro- and antiangiogenic factors in the placenta is balanced; any shifts in this balance affects the behavior of EC and trophoblast cells and changes the profile of secretion of soluble factors and expression of surface molecules [5]. The effects of cytokines

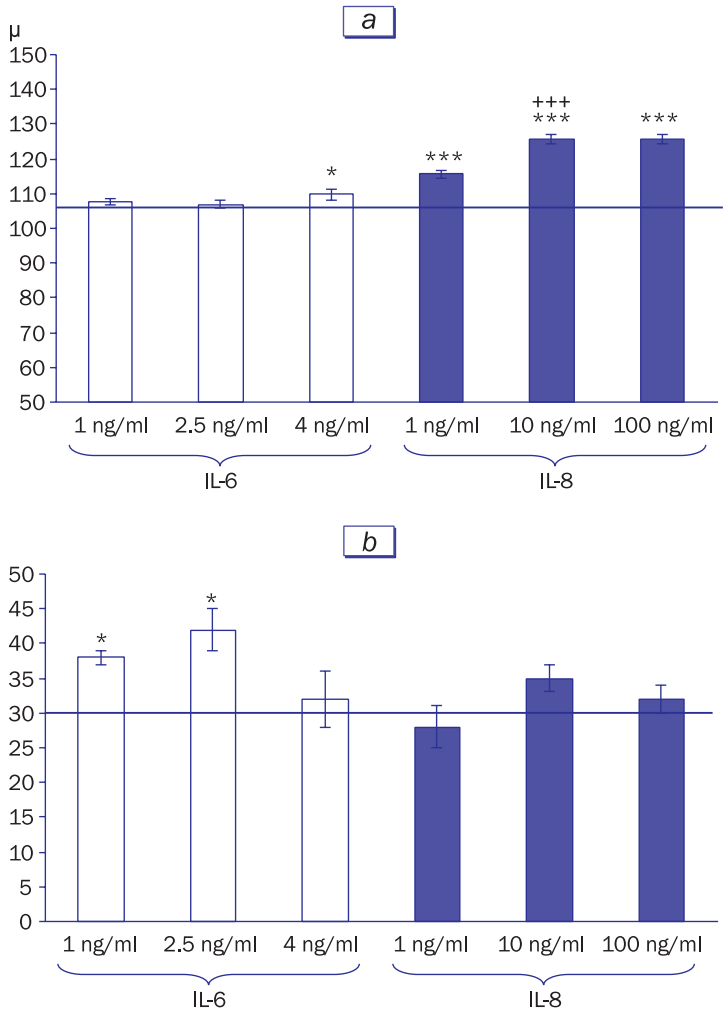

Fig. 3. Effect IL-6 and IL-8 on the formation of tube-like structures by EA.Hy926 EC in the presence of JEG-3 cells. *a*) Length of tube-like structures; *b*) number of tube-like structures. Horizontal line: spontaneous level of tube-like structure formation. *$p<0.05$, ***$p<0.001$ in comparison with spontaneous level; $^{+++}p<0.001$ in comparison with 1 ng/ml IL-8.

on the interaction between EC and trophoblast cells remain poorly studied.

The effects of TNF-α on angiogenesis and trophoblast invasion in the placenta attracts much recent attention of researchers working in the field of immunology of reproduction. It was shown that the content of TNF-α in the placentas in women with preeclampsia is higher than in healthy pregnant women [2,57]. A similar picture was observed in rats with modeled preeclampsia. Increased concentration of TNF-α in the peripheral blood of women with preeclampsia was also shown [2,76]. The regulatory role of TNF-α consists in its influence on proliferation and viability of EC and trophoblast cells and on their secretory profile. We have previously found that the length of tube-like structures formed by EC (EA.Hy926 cells) co-cultured with trophoblast JEG-3 cells increased in the presence of TNF-α in low (10 U/ml, 0.6 ng/ml) and high concentrations (400 U/ml, 24 ng/ml) in comparison with the spontaneous level of tube-like structures formation (Fig. 2). A similar result was previously obtained in EC monoculture (EA.Hy926 cells) in the presence of TNF-α in the specified concentrations. TNF-α at different concentrations produces opposite effects on angiogenesis: at low concentrations (<100 ng/ml) it stimulates this process via stimulation of EC proliferation and secretion of proangiogenic factors by EC, while at high concentrations (>1000 ng/ml) it produces a suppressive effect on angiogenesis due to stimulation of EC apoptosis [28]. There are also data of predominantly inhibitory effect of TNF-α on trophoblast cells: it suppresses migration [10,70], stimulates apoptosis by suppressing the expression of integrins [30], and inhibits trophoblast invasion into the spiral arteries of the uterus [21]. Thus, TNF-α in EC—trophoblast cell co-culture stimulates the formation of tube-like structures due to stimulating effect on EC and simultaneous inhibitory effect on trophoblast cells, due to

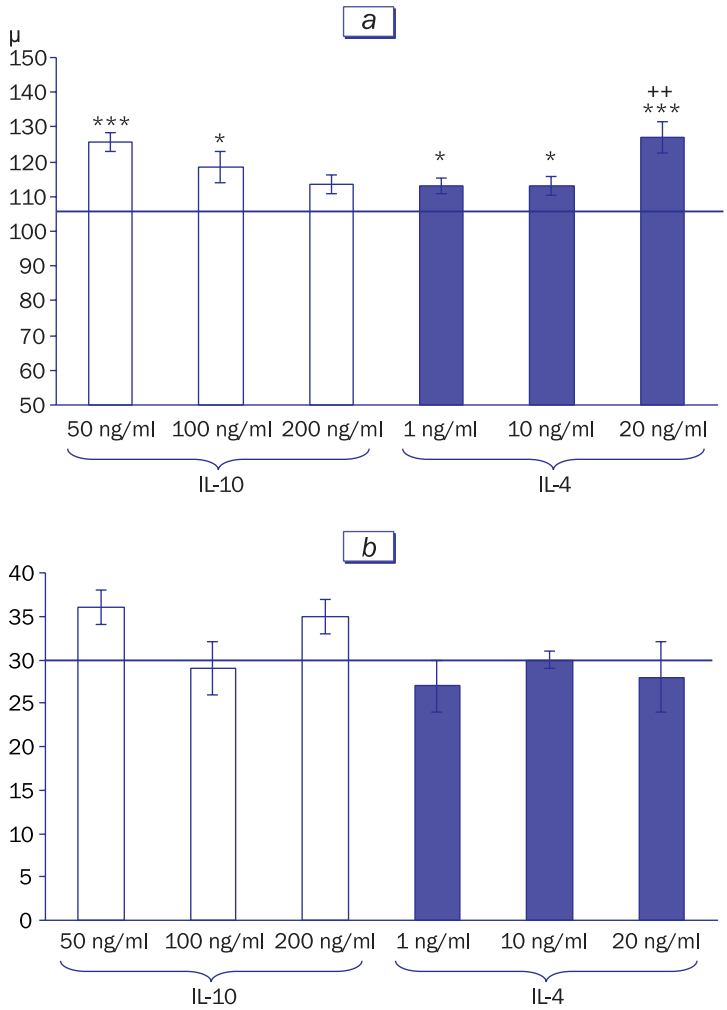

Fig. 4. Effect anti-inflammatory cytokines IL-4 and IL-10 on the formation of tube-like structures by EA.Hy926 EC in the presence of JEG-3 cells. *a*) Length of tube-like structures; *b*) number of tube-like structures. Horizontal line: spontaneous level of tube-like structure formation. $^{*}p<0.05$, $^{***}p<0.001$ in comparison with spontaneous level; $^{++}p<0.01$ in comparison with 10 ng/ml IL-4.

which the inhibitory effect of trophoblast cells on EC is compensated. In previous studies, opposite results were obtained: TNF-α in low concentrations (0.2-5 ng/ml) disturbed the formation of a common network of tube-like structures by trophoblast cells (JEG-3) and EC (human EC of microcirculatory bed of the uterus, UtMVEC) on matrix Matrigel. Under these conditions, exogenous NO abolished the inhibitory effect of TNF-α [75,76]. These differences can be attributed to individual characteristics of the used cell lines reflecting properties of microvascular (UtMVEC) or major vessel EC (EA.Hy926) [38].

In the presence of IL-1β in low concentrations (0.1 ng/ml), an increase in the length of tube-like structures was observed compared to the level of their spontaneous formation, while in the presence of IL-1β in concentrations of 1 and 10 ng/ml, the length of tube-like structures considerably decreased below the spontaneous level. IL-1β in a concentration of 1 ng/ml also increased the number of tube-like structures in comparison with the spontaneous level (Fig. 2). It should be noted that IL-1β has proangiogenic effect and stimulates the formation of tube-like structures by EC *in vitro* [72]. The proangiogenic effect of IL-1β is determined by induction of VEGF [72] and IL-8 [64] secretion by EC. Probably this effect of IL-1β on EC determines the stimulating effect on the formation of tube-like structures by EA.Hy926 EC co-cultured with JEG-3 trophoblast cells. It can be hypothesized that changes in cell behavior in co-culture in the presence of higher concentrations of IL-1β (1 and 10 ng/ml) can be caused by changes in the behavior of trophoblast cells. It was previously shown that IL-1β stimulates adhesion to EC by increasing the expression of adhesion molecules (VCAM-1 and integrin $\alpha_4\beta_1$) [13] and stimulates invasion and migration of trophoblast cells due to secretion of MMP-9 by trophoblast cells [67], which probably determined the increase in the number

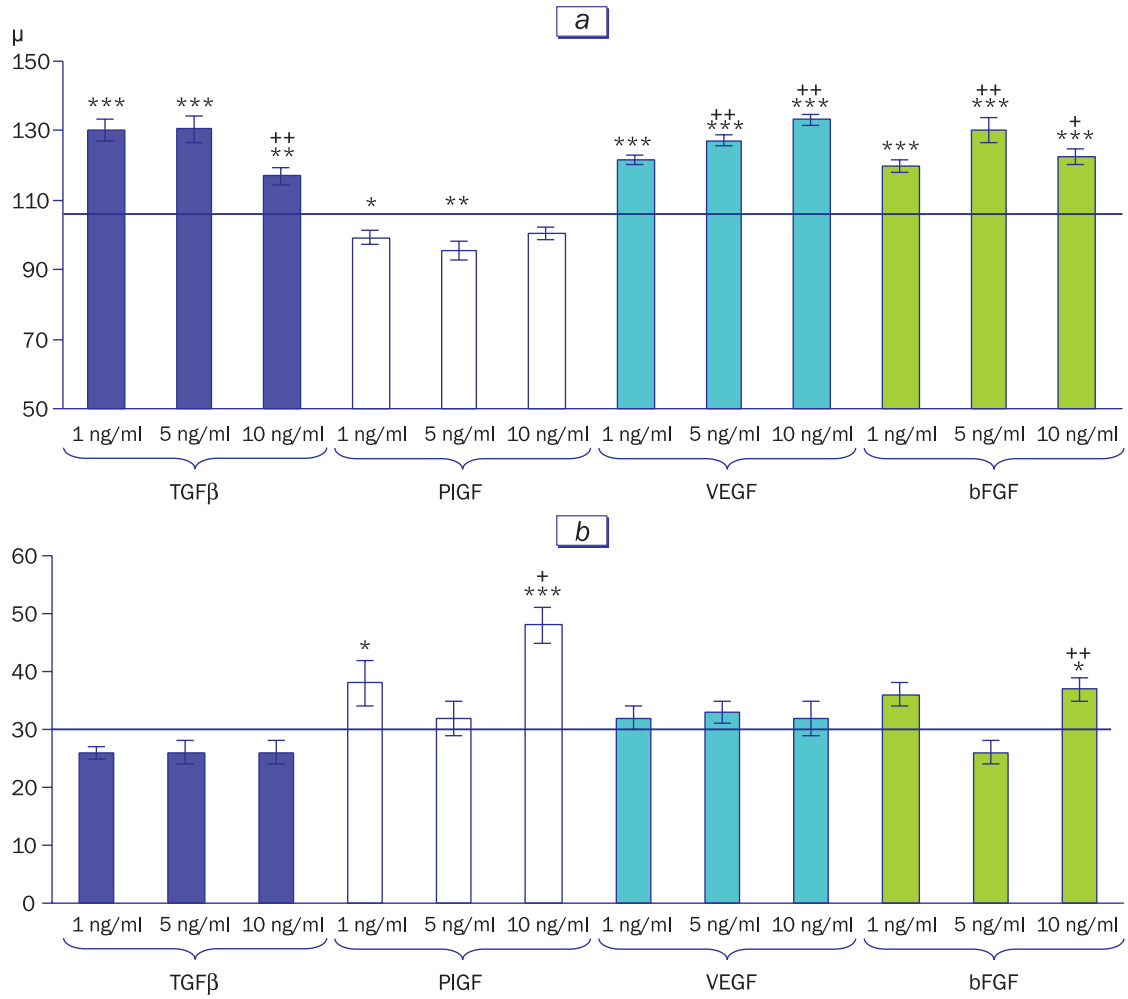

Fig. 5. Effect growth factors on the formation of tube-like structures by EA.Hy926 EC in the presence of JEG-3 cells. *a*) Length of tube-like structures; *b*) number of tube-like structures. Horizontal line: spontaneous level of tube-like structure formation. **p*<0.05, ***p*<0.01, ****p*<0.001 in comparison with spontaneous level; ^{+}p<0.05, ^{++}p<0.01 in comparison with lower concentration of the cytokine.

of tube-like structures in co-culture of trophoblast cells and EC in the presence of IL-1β.

IFN-γ dose-dependently increased the length of tube-like structures formed by EA.Hy926 EC co-cultured with trophoblast JEG-3 cells in comparison with the spontaneous level (Fig. 2). There are published reports about antiangiogenic effects of IFN-γ associated with its inhibitory influence on EC proliferation and secretion of MMP by these cells [3]. We have previously demonstrated that IFN-γ in low concentrations (40 U/ml) inhibits the formation of tube-like structures by EC, but in high concentrations (1000 U/ml) produced an opposite effect and promotes the formation of tube-like structures. Recent studies have shown that IFN-γ stimulates secretion of VEGF by EC [55], which probably determines the stimulating effect of this cytokine in high concentrations. In the co-culture of EC and trophoblast cells, IFN-γ acts on both types of cells. Previous studies showed that IFN-γ inhibited integration of trophoblast cells into EC monolayer [15] and inhibited trophoblast invasion by suppressing MMP-2 and MMP-9 secretion and stimulation of apoptosis [43,50]. The stimulating effect of IFN-γ in the co-culture can be explained by its stimulating effect on EC and inhibitory effect on trophoblast cells.

In the presence of IL-8 in all studied concentrations, the length of tube-like structures formed by EA.Hy926 EC in co-culture with JEG-3 trophoblast cells increased in comparison with its spontaneous level (Fig. 3). Chemokine IL-8 is a proangiogenic cytokine that stimulates proliferation of EC and supports their viability [57]; it stimulates EC migration and formation of blood vessels [52] due to stimulation of MMP production by these cells [51]. At the same time, trophoblast cells in the presence of IL-8 also secrete matrix metalloproteinases, which promotes remodeling of the extracellular matrix and stimulates proangiogenesis processes [39].

In the presence of IL-6 in a concentration of 4 ng/ml, an increase in the length of tubes formed by

EA.Hy926 EC in co-culture with JEG-3 trophoblast cells was shown. In the presence of 1 and 2.5 ng/ml IL-6, the number of tube-like structures increased in comparison with the level of their spontaneous formation (Fig. 3). The stimulatory effect of IL-6 could be associated with induction of autocrine secretion of VEGF by EC. There is evidence that IL-6 stimulates migration and invasion of trophoblast cells [40]. It is possible that during invasion trophoblast cells secrete MMP thus promoting remodeling of the extracellular matrix and, consequently, the formation of vessels by EC. Increased length of tube-like structures corresponds to the physiological pattern of non-branching angiogenesis, while the increase in their number corresponds to branching angiogenesis. This change in the type of angiogenesis in the presence of IL-6 in different concentrations indicates change in secretion profile of trophoblast cells. We can hypothesize that in the presence of IL-6 in low concentrations, trophoblast cells secrete greater amount of PlGF, while in high concentrations they secrete greater amount of VEGF and Ang1, but we found no published data on the effect on IL-6 on the secretory profile of trophoblast cells.

In the presence of anti-inflammatory cytokine IL-10 in low and medium concentrations (50 ng/ml and 100 ng/ml), an increase in the length of tube-like structures formed by EA.Hy926 EC in the presence of JEG-3 trophoblast cells was observed (Fig. 4). Previous studies have demonstrated that IL-10 inhibited the production of proangiogenic IL-1, IL-6, and IL-8 and MMP system of EC [65], thus producing an antiangiogenic effect. However, trophoblast cells in the presence of IL-10 secreted VEGF [69]. The stimulating effect of IL-10 in low and medium concentrations can be due to its effect on trophoblast cells, the effect of high IL-10 concentrations is leveled by inhibition of autocrine secretion of proangiogenic factors by EC.

In the presence of 4 ng/ml IL-4 in all studied concentration (Fig. 4), an increase in the length of tube-like structures formed by EA.Hy926 EC in co-culture with JEG-3 trophoblast cells was shown. Effect of IL-4 on angiogenesis is poorly studied. Previous studies showed that IL-4 stimulated the formation of tube-like structures by EC *in vitro* [29], but inhibited bFGF-induced angiogenesis *in vivo* [9]. The stimulating effect of IL-4 can be due to stimulation of autocrine secretion of IL-8 and VEGF by EC [36]. Earlier we confirmed the data on stimulating effect of IL-4 on the formation of tube-like structures by EC, but this effect was observed only at IL-4 concentration of 20 ng/ml. Strengthening of the stimulating effect of this cytokine can associated with its effect on trophoblast cells, but we failed to find published reports about the effect of IL-4 on secretory activity of the trophoblast.

In the presence of TGFβ in all studied concentrations, the length of tube-like structures formed by EA.Hy926 EC in co-culture with JEG-3 trophoblast cells increased in comparison with its spontaneous level (Fig. 5). At the same time we observed a dose-dependent decrease in the length of tube-like structures formed by EA.Hy926 EC in the presence of TGFβ in high concentration (10 ng/ml) in comparison with culturing in the presence of TGFβ in medium concentration (5 ng/ml). TGFβ is known as a cytokine with a dual effect on angiogenesis: in low concentration (0.5 ng/ml) TGFβ stimulates vascular tube formation and in high concentration (10 ng/ml) it inhibits angiogenesis by increasing the autocrine secretion of TIMP by EC [24,25,58,59] and stimulates EC apoptosis [24,25]. The stimulating effect of TGFβ, even in high concentrations, in co-culture is probably determined by its influence on the trophoblast. According to published reports, TGFβ stimulates secretion of VEGF by the trophoblast [17] and inhibits its proliferation and invasion [46,77], which can compensate the inhibitory effect of trophoblast cells on EC. Thus, less pronounced stimulatory effect of TGFβ in high concentrations in comparison with low concentrations can serve as a mechanism limiting the growth of the vascular network of the placenta.

In the presence of bFGF at all studied concentrations, we observed an increase in the length of tube-like structures formed by EA.Hy926 EC co-cultured with JEG-3 trophoblast cells (Fig. 5). This was accompanied by a dose-dependent increase in the length of tube-like structures in the presence of 10 ng/ml bFGF in comparison with 1 ng/ml bFGF and a dose-dependent decrease in the length of tube-like structures formed in the presence of 20 ng/ml bFGF in comparison with 10 ng/ml bFGF. In the presence of 20 ng/ml bFGF, an increase in number of tube-like structures formed by EA.Hy926 EC was observed in comparison with spontaneous level and lower concentration of the factor. In the presence of trophoblast cells and bFGF in high concentrations, the length of tube-like structures decreased, but their number increased; which attested to predominance of branching angiogenesis in this case in comparison with lower concentrations of the factor. This effect can be explained by enhanced secretion of TIMP-1 by trophoblast cells in the presence of bFGF with simultaneous suppression of matrix metalloproteinase secretion. Expression of bFGF in the first trimester placenta is significantly higher than during the third trimester [23]; this can be related to switching from non-branching to branching angiogenesis, which agree with our findings.

In the presence of PlGF in concentrations of 1 and 5 ng/ml, we observed a decrease of the length of tube-like structures formed by EA.Hy926 EC in

the presence of JEG-3 trophoblast cell (Fig. 5). In the presence of PlGF in concentrations of 1 and 10 ng/ml, we observed an increase in the length of tube-like structures formed by EC (Fig. The number of tube-like structures increased in the presence of 10 ng/ml PlGF in comparison with 5 ng/ml PlGF. These results indicate that PlGF in the presence of trophoblast cells stimulates branching angiogenesis, which is consistent with published reports [47]. We previously showed the absence of PlGF effect on the formation of tube-like structures by EC. At the same time, PlGF in the presence of VEGF stimulated the formation of tube-like structures and potentiated its effect on EC [56]. Trophoblast cells secrete VEGF [1]. Therefore, the increase in the number of tube-like structures observed in this study can be determined by combined influence of VEGF and PlGF on EC.

In the presence of VEGF, we observed a dose-dependent increase in the length of tube-like structures formed by EA.Hy926 EC co-cultured with JEG-3 trophoblast cells in comparison with spontaneous level (Fig. 5). These findings agree with published data. VEGF stimulates proliferation and viability of EC and produces similar effect on trophoblast cells.

Our findings suggest that in trophoblast cells in nonactivated state suppress the angiogenic potential of EC, but in the presence of various cytokines, the interaction of endothelial and trophoblast cells changes, which manifests in modulation of vascular network formation. Antiangiogenic cytokines IFN-γ, IL-10, TNF-α, and TGF-β via modulation of trophoblast cells can stimulate the formation of tube-like structures by EC. It should be noted that the functions of cytokines are changed in specific cell environment and they can acquire additional regulatory function. For instance, IFN-γ, the key proinflammatory cytokine, acts as a regulator of invasion of trophoblast cells in the placenta, and its suppressive effect on trophoblast cells serves as a feedback in the formation of vessels by EC.

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