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ВЛИЯНИЕ ФАКТОРОВ, СЕКРЕТИРУЕМЫХ ПЛАЦЕНТОЙ, НА ФОРМИРОВАНИЕ СОСУДОПОДОБНЫХ СТРУКТУР ЭНДОТЕЛИАЛЬНЫМИ КЛЕТКАМИ В ПРИСУТСТВИИ КЛЕТОК ТРОФОБЛАСТА

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Резюме. Клетки трофобласта активно взаимодействуют с эндотелиальными клетками, участвуя в формировании сосудов в зоне маточно-плацентарного контакта и ремоделировании спиральных артерий матки. Продукция цитокинов клетками плаценты закономерно изменяется от первого к третьему триместру физиологической беременности; она также изменяется в зависимости от наличия акушерской патологии, например при преэклампсии. В настоящее время нет данных о влиянии как отдельных цитокинов, так и факторов, секретируемых плацентой, на взаимодействие ЭК и трофобласта. Поэтому целью настоящего исследования явилась оценка факторов, секретируемых плацентой, на формирование сосудоподобных структур эндотелиальными клетками в присутствии трофобласта.

Проводили совместное культивирование эндотелиальных клеток линии EA.Hy926 и клеток трофобласта линии JEG-3 на трехмерном коллагеновом матриксе «Matrigel» (BD, США) в присутствии факторов, секретируемых плацентой женщин с физиологической беременностью на сроке 9-11 недель (n = 15), женщин с физиологической беременностью на сроке 38-39 недель (n = 15), женщин с беременностью, осложненной преэклампсией на сроке 38-39 недель (n = 14).

Установлено, что клетки трофобласта модифицируют способность эндотелиальных клеток образовывать сосуды только в условиях физиологически протекающей беременности. При преэклампсии клетки трофобласта не способны скорректировать поведение эндотелиальных клеток и обеспечить физиологический рост сосудов.

Ключевые слова: трофобласт, эндотелий, ангиогенез, плацента, цитокины, преэклампсия

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EFFECTS OF PLACENTAL FACTORS UPON DEVELOPMENT OF TUBULAR STRUCTURES BY ENDOTHELIAL CELLS IN PRESENCE OF TROPHOBLASTIC CELLS

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Abstract. Trophoblast cells actively interact with endothelial cells participating in the process of vasculogenesis in the uterus/placenta contact area and remodeling of uterine spiral arteries. Cytokine production by the placental cells is subject to gradual changes from the 1st to 3rd trimester of physiological pregnancy. It is also changed in cases of obstetric disorders, e.g., in pre-eclampsia. At present time, there are lacking data on effects of cytokines and placenta-derived factors upon local interactions between endothelium and trophoblast cells. Hence, the aim of our study was to assess the influence of placental factors upon formation of tube-like structures by endothelial cells in presence of trophoblastic cells. We performed co-cultures of Ea.Hy926 endothelial cell line and Jeg-3 trophoblastic cells in a 3-D collagen matrix («Matrigel», BD, USA) with secretable factors from placentas of healthy pregnant women at 9-11 weeks of gestation (n = 15), healthy pregnant women at 38-39 weeks of gestation (n = 15), or the women with preeclampsia at 38-39 weeks of gestation (n = 14).

We have shown that the trophoblastic cells may modify the ability of endothelial cells to form tube-like structures only with placental factors from health pregnant women. In pre-eclampsia condition, the trophoblast cells are not able to correct the behavior of endothelial cells, and to promote physiological growth of blood vessels.

Keywords: trophoblast, endothelium, angiogenesis, placenta, cytokines, pre-eclampsia

Introduction

Appropriate fetus development during pregnancy depends on the successful formation of maternal-fetal contact, interactions between trophoblast cells and cellular microenvironment including interactions with uterine endothelial cells (EC) during spiral arteries remodeling [23], placental and decidual vessels network. Endometrium decidualization in the beginning of pregnancy is associated with invasion of trophoblast, changes in qualitative and quantitative leucocyte composition of the decidua, changes in functional state of endometrial and decidual cells, stimulation of angiogenesis and lymphangiogenesis in endometrium, decidua and placenta [18, 38], changes in cytokine and cellular microenvironment.

During invasion into the endometrium trophoblast cells differentiate into two cell types – syncytiotrophoblast covering villi outside and cytotrophoblast with proliferative activity differentiating into invasive (extravillous) trophoblast capable of invasion in interstitial direction (uterine myometrium with the formation of giant cells) and endovascular direction (uterine spiral arteries) [13]. The result of the remodeling of uterine spiral arteries is the dilated blood vessels that do not have the ability to dilate that increases the blood flow to the villi in the second trimester of pregnancy. Invasion of trophoblast cells is accompanied by the destruction of the extracellular matrix by MMP-2, MMP-3, MMP-9 and cathepsins simultaneously with the secretion of their inhibitors – TIMP-1 and TIMP-2 [13]. Lack of trophoblast cells invasion can cause complications during pregnancy (miscarriage, preeclampsia, intrauterine growth retardation [31]), the excessive invasion on the contrary may be a condition for the development of choriocarcinoma [14]. During the trophoblast invasion cells lose their ability to proliferate [35, 39]. When migrating into decidua invading cells interact with decidual stromal cells, epithelial cells and maternal immune cells. Cytokines secreted by cells of microenvironment shift the equilibrium in secretion of proteases and their inhibitors in different ways: IFNγ decreases secretion of metalloproteases, IL-12 inhibits the secretion of proteases and stimulates the secretion their inhibitors [1]. bFGF also suppresses the invasion of trophoblast by stimulation of TIMP-1 secretion and inhibition of MMP-9 secretion [33]. At the same time, HGF enhances the trophoblast invasion by stimulating the secretion of MMP [14, 37]. EGF has a similar effect by stimulating the production of MMP-2 [41] and MMP-9 [36] by trophoblast, but simultaneously EGF stimulates the production of TIMP-1 by trophoblast cells, thereby implementing the regulation by principle of negative feedback [36]. Uterine spiral arteries remodeling

comes in several stages [13, 27, 28]. Firstly, the changes based on the activation of the renin-angiotensin system of mother [43] and activation of decidual NK-cells and macrophages [40] are observed, which are accompanied by vacuolization and basophilia of EC and an increase of the lumen [19]. Further interstitial trophoblast vascular remodeling occurs accompanied by apoptosis of smooth muscle cells and deposition of fibrinoid at the site of elastic and collagen fibers due to the production of MMP-2 MMP-7 and MMP-9 by trophoblast, decidual macrophages and NK-cells [24]. Finally, it ends with vessel walls infiltration by trophoblast cells due to induction of EC apoptosis and their replacement [8]. Trophoblast acquires endovascular phenotype: expression of E-cadherin, $\alpha1\beta4$ integrin decreases, expression of VE-cadherin, VCAM-1, PECAM1, integrin $\alpha1\beta1$, $\alpha v\beta3$, $\alpha4\beta1$ increases [46]; trophoblast cells form contacts with EC with the help of VE-cadherin [11]. The invasion of trophoblast, the remodeling of spiral arteries, the interactions of trophoblast and EC are controlled by cells of microenvironment with various soluble factors secreted. Interaction of trophoblast cells and EC is amplified by the action of pro-inflammatory cytokines TNFα and IL-1β secreted by cells of microenvironment because of expression stimulation of $\alpha4\beta1$ and VCAM-1 [12]. In the presence of IFNγ or elevated concentrations of TNFα the ability of trophoblast to integrate into EC monolayer decreases [16]. VEGF family factors stimulate the expression of integrin $\alpha V\beta3$ [20] and are chemoattractants for trophoblast cells [32] that indicates their important role in the remodeling of uterine spiral arteries. VEGF-A also stimulates the proliferation and increases trophoblast viability [9], and VEGF-C reduces the cytotoxicity of NK-cells to trophoblast [26]. TGF beta, unlike the majority of the growth factors, inhibits trophoblast cells proliferation [30], stimulates their adhesion, but inhibits the invasion [45] and stimulates the secretion of VEGF by trophoblast cells [17].

Cytokine production by placental cells naturally varies from the first to the third trimester of healthy pregnancy; it also varies depending on the presence of obstetric pathology, such as preeclampsia [2, 3, 4]. There is currently no data on the impact of individual cytokines and factors secreted by placenta, on EC and trophoblast interaction. Therefore, the aim of this study was to evaluate the factors secreted by placenta on the formation of tube-like structures by endothelial cells in the presence of trophoblast.

Materials and methods

Cell lines

Cell line EA.Hy926 used in the experiment reproduces all main features of endothelial cells. Cells were cultured in culture medium DMEM/F12 with addition of 10% fetal bovine serum (FBS), 100 μg/ml of streptomycin, 100 U/ml of penicillin, 8 mmol/L of L-glutamine, HAT (Sigma, USA). Reseeding was produced one time every 3-4 days, causing the disintegration of monolayer by five-minute exposition in versene solution (Biolot, Russia). Trophoblast cell line JEG-3 reproduce all main features of the invasive type of trophoblast. Cells were cultured in culture medium DMEM, 10% FBS , 100 μg/ml streptomycin, 100 U/l penicillin, 2 mmol/L L-glutamine, 1% non-essential amino acids, 10 mM sodium pyruvate (Sigma, USA). Reseeding was produced one time in 3-4 days, causing the disintegration of monolayer by five-minute exposition in solution containing 0.135% trypsin and 0.01% EDTA (Biolot, Russia).

Obtaining placentas conditioned media

Placentas were obtained: 1) after induced abortion at normal 1st-trimester between 9-11 weeks (n = 15); 2) after caesarean-delivery at normal 3rd-trimester between 38-39 weeks (n = 15); 3) after caesarean-delivery at preeclampsial 3rd-trimester between 38-39 weeks (n = 15). The age of women varied from 18 to 37 years with an average of 31.6±4.2 years. The exclusion criteria for the pregnant women included: (i) type I diabetes mellitus, (ii) polyhydramnios, (iii) oligohydramnios, (iv) urogenital infection, (v) acute infection, or (vi) exacerbation of chronic infection, hypertension and other diseases of the circulatory system. Groups of pregnant women were matched for age, parity births and obstetric history. The diagnosis of preeclampsia in pregnant women was established on the basis of the main clinical symptoms – the presence of proteinuria, oedema, and hypertension. Fragments (weight 100±11 mg) from the central part of the placenta were cultured for 24 hours in 1 ml DMEM/F12 medium (Sigma, USA) with no added FBS. Then, the conditioned media were collected, frozen and stored at temperature -20 °C until the study.

Evaluation of the secreted placental factors influence on the formation of tube-like structures by EC in the presence of trophoblast cells

In wells of a 24-well plate pretreated with Matrigel Growth Factor Reduced matrix (BD, USA), we added 400 μl of placental conditioned DMEM/F12 medium (three wells for each sample), 25 μl of FBS (Sigma, USA). Then to each well we added EC of EA.Hy926 line at a concentration 175 000 (in 300 μl of DMEM/F12 medium) and 75 000 trophoblast cells of Jeg-3 line (in 300 μl DMEM / F12 medium) previously stained with green fluorescent vital dye CalceinAM (Sigma, USA). To control wells we added 300 μl of DMEM medium, 2,5% FBS without cells of Jeg-3 line. Then we performed the incubation for 24 hours (37 °C, 4.5% CO2). As a control (n = 15) we assessed the formation of tube-like structures by EC in the absence of trophoblasts. At that the number of

tube-like structures formed by EC of EA.Hy926 line in coculture with trophoblastic cell line JEG-3 (30±1, n = 15) was lower than in culture in the absence of JEG-3 cell line (37±1, $p < 0.001$). The length of tube-like structures formed by EC of line EA.Hy926 was similar (105.75±0.52 μm) both at monoculture and during cocultivation with JEG-3 cell line. As a level of spontaneous formation of tube-like structures we assessed tube-like structures formed in the presence of trophoblast cells in a medium containing 2.5% FBS (the spontaneous level, Figure 1A – see p. 3 of the cover). The level of tube-like structures formation by EC in the presence of trophoblast cells and IFNγ (1000 U/ml, n = 15) served as a positive control. The length of tube-like structures was longer (133.02±3.91, $p < 0.001$) than in culture in the absence of IFNγ (105.75±0.52). With AxioObserver.Z1 microscope and computer image analysis system AxioVision (Zeiss, Germany) 5 fields of view per well were taken into account. We assessed the number of formed tube-like structures and their length in micrometers. Statistical analysis was performed using computer program Statistica10. For data analysis, we used the nonparametric Mann–Whitney test.

Results and discussion

In the presence of conditioned media of placentas from women with healthy pregnancy on 9-11 weeks of gestation we observed an increase in the length of tube-like structures formed by EC of EA.Hy926 line in the presence of trophoblast cells of JEG-3 line, comparing with a spontaneous level of their formation. We also marked the reduction in the number of tube-like structures formed by EC of EA.Hy926 line in the presence of trophoblast cells of JEG-3 line (Table 1, Figure 1 – see p. 3 of the cover). In the presence of conditioned media of placentas from women with healthy pregnancy (38-39 weeks of gestation) we observed an increase in the length of tube-like structures formed by EC of EA.Hy926 line in the presence of trophoblast cells of JEG-3 line comparing with the level of their spontaneous formation. We also marked the reduction in the number of tube-like structures formed by EC of EA.Hy926 line in the presence of trophoblast cells of JEG-3 line as compared with the spontaneous level of their formation, and in comparison with the culturing in the presence of conditioned media of placentas from women in the early stages (9-11 weeks) of healthy pregnancy (Table 1, Figure 1 – see p. 3 of the cover). Thus, the effects of conditioned media of placentas from women at early and late pregnancy are different. Conditioned media of placentas from women at early pregnancy stimulate the formation of more tube-like structures comparing with conditioned media of placentas from women at late pregnancy. This fact is in favor of changing of the cytokines and growth factors balance in placenta depending on the gestation age and the predominance of non-branching angiogenesis in late healthy pregnancy and corresponds to the data present in the literature [44]. Previously in our laboratory we performed the study of cytokine secretion by placenta which showed the increased production of pro-angiogenic factors (VEGF, bFGF, MMP-2 , PDGF. Ang-2) in the first trimester of healthy pregnancy comparing with the third trimester [3]. The increased secretion of MMP-2, Ang-2, acting collaboratively with VEGF, in the first trimester of pregnancy [4, 21], stimulates extracellular matrix destruction, decreases intercellular adhesion, providing vessel destabilization, as well as stimulating the migration and proliferation of EC. Destabilizing effect is required for the formation of new vessels branches and increases the sensitivity of EC to various angiogenic factors such as VEGF, promoting stimulation of branching angiogenesis, and, consequently, meeting the needs of the growing fetus with oxygen and nutrients. At the third trimester of pregnancy there is a reduction of angiogenic factors, including Ang-2, and increasing of Ang-1 production by placental cells [4], that favors the termination of vascular network formation and provides switching of branching angiogenesis to non-branching angiogenesis.

Previously in our laboratory we conducted the experiments on evaluation of the effect of conditioned media of placentas from women with early pregnancy on the formation of tube-like structures by EC in the absence of other cell types. EC formed shorter tube-like structures compared with spontaneously formed tube-like structures, but their number was significantly higher than the number of spontaneously formed tube-like structures, indicating the physiological process of branching angiogenesis [34]. The presence of trophoblast cells surrounding EC modifies their behavior – the length of tube-like structures formed by EC increases significantly while preserving the ability to form a greater number of tube-like structures than in the presence of conditioned media of placentas from women in third trimester of healthy pregnancy. These results are consistent with the literature on switching of angiogenesis mechanisms from branching angiogenesis in the first trimester of pregnancy to non-branching angiogenesis in the third trimester of pregnancy [10]. Given that trophoblast cells are able to acquire an endothelial phenotype [11, 46], as well as incorporate into tube-like structures (Figure 1 – see p. 3 of the cover) we can expect their active participation in the development of decidua vascular network; these data are also in agreement with previously described trophoblast role in remodeling of uterine spiral arteries [8, 13, 46]. We could not find in the literature data on the nature of the interaction between EC in such co-cultivation systems, so the question of the ability of trophoblast cells to form

TABLE 1. THE EFFECT OF CONDITIONED MEDIA OF PLACENTAS FROM WOMEN ON THE TUBE-LIKE STRUCTURES FORMATION BY EC OF EA.Hy926 LINE IN THE PRESENCE OF TROPHOBLAST CELLS OF JEG-3 LINE

Cell culturing conditions	The length of tube-like structures, μm	The number of tube-like structures
DMEM/F12, 2,5% (the spontaneous level)	100.58 [83.37; 120.95]	29 [22; 37]
conditioned media of placentas from healthy pregnancy (9-11 weeks of gestation, group 1)	117.58 [95.71; 145.14]***	26 [19.5; 33]*
conditioned media of placentas from healthy pregnancy (38-39 weeks of gestation, group 2)	118.74 [96.91; 149.72]***	23 [15; 31]*** ¥
conditioned media of placentas from pregnancy with preeclampsia (38-39 weeks of gestation, group 3)	135.84 [111.94; 161.43]*** ###	18 [13; 24]*** #

Note. The significance of differences between groups: the length or the number of tube-like structures in the presence of conditioned media is different from the spontaneous level * – $p < 0.05$; *** – $p < 0.001$; the number of tube-like structures in the presence of conditioned media of placentas from women of group 2 is different from the number in group 1 – $p < 0.05$; the length or the number of tube-like structures in the presence of conditioned media of placentas from women of group 3 is different from the length or the number in group 2 # – $p < 0.05$, ### – $p < 0.001$.

tube, as it was described for the EC [7, 22], or act like pericytes remains open.

In the presence of conditioned media of placentas from women with pregnancies complicated with preeclampsia (38-39 weeks) we observed an increase in the length of tube-like structures formed by EC of EA.Hy926 line in the presence of trophoblast cells of JEG-3 line, At the same time in the presence of conditioned media of placentas from women with pregnancy complicated with preeclampsia (38-39 weeks) we observed a decrease in the number of tube-like structures formed by EC of EA.Hy926 line in the presence of trophoblast cells of JEG-3 line comparing both with the level of their spontaneous formation and with the culturing in the presence of conditioned media of placentas from women in the late stages (38-39 weeks) of healthy pregnancy (Table 1, Figure 1 – see p. 3 of the cover). Thus, the conditioned media of placentas from women with preeclampsia have pronounced stimulating effects in increasing the length of tube-like structures formed by EC along with reducing their number when cocultured with trophoblast. This fact is in favor of changing the balance of cytokines and growth factors in placenta in case of pre-eclampsia and the predominance of the processes non-branching angiogenesis at preeclampsia. Earlier we registered the similar result in experiments on assessment the effects of conditioned media of placentas from women with preeclampsia on tube-like structures formation by EC in the absence of other types of cells [34]. This fact is in favor of an significant reduction of vessels branching and the predominance of non-branching angiogenesis in preeclampsia comparing with healthy pregnancy in the absence and in the presence of trophoblast cells. These data also point to the failure of trophoblast cells to change the behavior of EC towards the physiological vascular growth when pro-inflammatory and anti-angiogenic factors prevail in the external cytokine microenvironment. Probably during pathological processes in the area of utero-placental contact endovascular trophoblast cells behave in a similar to EC way [5, 42], reducing the migration and proliferation activity [6]. In this case, we also cannot exclude an increased cytotoxic activity of trophoblast cells, which are capable of inducing apoptosis of EC [38]. Trophoblast cells express FasL and secrete its soluble form – sFasL, which interaction with Fas molecule expressed on EC [15, 25] leads to the apoptosis of EC. The development of TRAIL-dependent EC apoptosis was also shown [29]. These mechanisms may also be involved in reducing the number of tube-like structures formed by EC cell in the presence of conditioned media of women's placentas.

Conclusion

Thus, soluble factors contained in the conditioned media of placentas from women in the first and third trimester of healthy pregnancy and pregnancy complicated with preeclampsia change the characteristics of the vascular network formed by EC in the presence of trophoblast cells. Conditioned media of placentas from women in the first trimester of healthy pregnancy stimulate branching angiogenesis, while conditioned media of placentas from women in the third trimester of healthy pregnancy stimulate non-branching angiogenesis. The presence of trophoblast cells surrounding EC modifies EC behavior that is in favor of their active participation in the processes of decidual and placental vascular network formation. In case of preeclampsia trophoblast cells are not able to correct the behavior of EC and enable the physiological growth of blood vessels.

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