In Vitro Expression of Vascular Endothelial Growth Factor and Its Receptors by Placental Macrophages

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The expression of VEGF and membrane-bound and soluble forms of the VEGF-R1 receptor in cultured placental macrophages (trimesters I and III of pregnancy) was studied by flow cytometry, cytometric bead array, and ELISA. Nearly all population of placental macrophages (98%) was capable of producing VEGF during the early and late gestational periods. However, the expression of cellular VEGF-R1 varied from 3.4 to 92%. VEGF secretion was relatively low in the first and third trimesters (0.5 and 1.1 pg/10^5 cells, respectively). Cultured placental macrophages produced soluble receptor sVEGF-R1 in the first and third trimesters (86.4 and 36.4 pg/10^5 cells, respectively). Stimulation with LPS was followed by a 4-fold increase in sVEGF-R1 secretion. Our results indicate that placental macrophages are involved in the autocrine and paracrine regulation in chorionic villi. The data suggest that these cells have a physiological and pathogenetic role in gestation.

Key Words: *placental macrophages; VEGF; VEGF-R1; sVEGF-R1*

Placental macrophages (Kashchenko–Hofbauer cells) perform a variety of functions. Apart from the anti-infectious defense, placental macrophages play a role in immunoglobulin transport, binding of immune complexes [13], and immunomodulation [9]. These cells are probably involved in the processes not directly related to immunity, *e.g.*, transport of water and electrolytes in the system of placental villi [6], morphogenesis and remodeling of placental tissue [3], and modulation of trophoblast functions [5].

In situ studies revealed that macrophages play a role in the formation of the placental vascular bed and functional regulation of placental vessels [2,7,12]. Macrophages were shown to express vascular endothelial growth factor (VEGF), which plays a central role in the regulation of placental vasculogenesis and angiogenesis [2,7].

Vasculogenesis and angiogenesis are essential for normal development and functioning of the placenta. Impairment of these processes contributes to serious obstetric diseases, including placental insufficiency and gestosis [1]. Therefore, elucidation of cellular and molecular mechanisms for local regulation of blood vessels is an urgent problem. Previous studies showed that angiogenic activity of placental macrophages should be evaluated *in vitro* [16].

Here we performed a quantitative study of the expression of VEGF and two forms of the VEGF-R1 receptor (membrane-bound and soluble) in the isolated population of placental macrophages during the early and late gestational periods.

MATERIALS AND METHODS

Villous chorion tissue (first trimester, 8-12 weeks) was obtained in therapeutic abortion. The third trimester placental tissue (37-40 weeks) was isolated during cesarean section.

Villous chorion tissue was washed with Hank's solution (Biolot) and cut with scissors into fragments (1-3 mm^2). The dissociated tissue was placed in an enzymatic mixture containing 0.2% collagenase from

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Kamchatka crab hepatopancreas (Pacific Ocean Institute of Bioorganic Chemistry), 60 U/ml DNase (Sigma), 4 ml FBS (Bioclear), and 15 mM HEPES in DMEM/F-12 medium (Biolot). Incubation was performed at 37°C and constant agitation for 1.5 h. The tissue was precipitated. The supernatant was collected. Freshly prepared enzyme mixture of the same composition was added to the non-dissociated tissue. The procedure of dissociation was repeated at 37°C for 1.5 h. Both portions of the supernatant with dissociated cells were filtered through a nylon filter (pore size 70 μ, BD Biosciences). The cells were precipitated by centrifugation at 200*g* for 5 min. Precipitated cells were washed 3 times with Hank's solution and 5% FBS (200*g*, 5 min) and resuspended in DMEM/F-12 medium containing 15 mM HEPES. Aliquots of the cell suspension (5 ml) were put into centrifuge tubes and centrifuged in a Histopaque-1077 density gradient (2 ml, 1.077 g/ml, Sigma) at 400*g* for 30 min. The cell layer was isolated from the phase boundary and washed 3 times with Hanks' solution. The cells were resuspended in DMEM/F-12 medium containing 10% FBS and 50 μg/ml gentamicin (Sigma).

The cell suspension was placed into wells of 6-well plates ($1.5\text{-}2\times10^6$ cells) or cover glasses (18×18 mm, 2×10^5 cells). They were incubated at 37°C and 5% CO_2 for a night to achieve the adhesion of cells. Nonadherent cells were removed by thorough agitation and 3-fold washing with a culture medium.

The cultures on cover glasses were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS, Biolot) and cold methanol. The cells were stained by the method of immunocytochemistry with monoclonal antibodies against CD68 (PG-M1 clone) and cytokeratin 7. We also used an EnVision visualization system. All reagents were manufactured by DakoCytomation.

Freshly prepared culture medium (2 ml) was added to cells in 6-well plates. Incubation was performed at 37°C and 5% CO_2 for 23 h. The conditioned medium was collected after incubation, centrifuged, divided into aliquots, and stored at -70°C. Freshly prepared culture medium (2 ml) was added to the cells; intracellular transport inhibitor (0.1% Golgi Plug, BD Biosciences) was added to some wells. LPS (1 μg/ml, Sigma) was used for stimulation. The cells were incubated at 37°C and 5% CO_2 for 4.5 h, isolated with versene (Biolot), resuspended in PBS, and used to study the intracellular and surface antigens.

The cells were fixed and permeabilized with Cytofix/Cytoperm kit (BD Biosciences) for the detection of intracellular antigens. The cells were fixed 4% paraformaldehyde in PBS for the detection of surface antigens. Intracellular VEGF and surface receptors for VEGF were detected with the corresponding monoclonal antibodies (R&D Systems). FITC-labeled monoclonal antibodies against CD68 (KP1 clone, DakoCytomation) and CD14 (BD Biosciences) were used for the identification of macrophages. The samples were analyzed on a FACScan flow cytometer (Becton Dickinson). The gating area was highlighted on the point chart for event distribution to exclude the non-cellular debris and events with high values of forward and lateral scattering.

Secretory VEGF in the conditioned medium was assayed with Cytometric Bead Array (BD Biosciences kit) on a FACSCanto II flow cytometer (Becton Dickinson).

The soluble receptor VEGF-R1/Flt-1 (sVEGF-R1) in the conditioned medium was studied by ELISA with R&D Systems kit.

The results were analyzed by GraphPad Prism 4.0 software (Graph Pad Software Inc.) with nonparametric Mann–Whitney test.

RESULTS

Immunocytochemical study showed that the cultures obtained in the first trimester contained 87.3±4.1% macrophages ($CD68^+$ cells) and 12.9±2.3% trophoblast cells (cytokeratin 7^+ cells). The ratio of macrophages and trophoblast cells in third trimester cultures was 93.5±3.4 and 2.3±1.5%, respectively.

Flow cytofluorometry showed that the ratio of $CD68^+$ cells in the gating area is 97.7±1.5 and 98.2±1% (cultures of the first and third trimesters, respectively). Placental macrophages were able of synthesizing VEGF at the early and late stages of gestation. Intracellular VEGF was present in 97.7 and 98% cells (median values) of the first and third trimesters of pregnancy, respectively. *In vitro* stimulation with LPS had no effect on the ratio of VEGF-producing cells in third trimester cultures (median value 99%; Fig. 1).

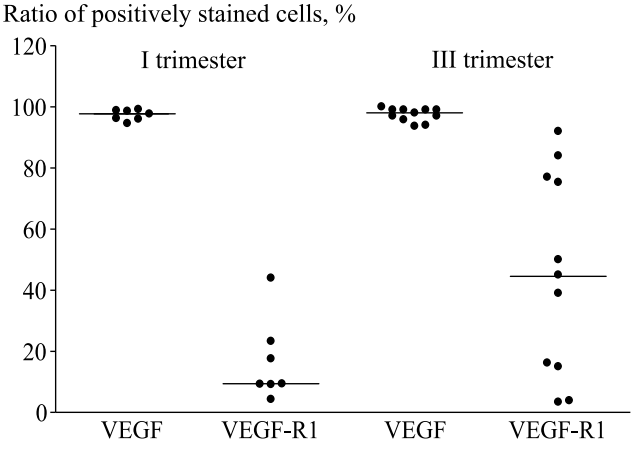

Fig. 1. Expression of VEGF and VEGF-R1 by cultured placental macrophages in the first (*n*=7) and third trimesters (*n*=11). Horizontal lines, median values.

Cytokine expression in all samples was characterized by high homogeneity (*i.e.*, varied in a low range of 94-100%).

As differentiated from a uniform expression of this cytokine, expression of the cellular receptor VEGF-R1 was characterized by high variability. The ratio of VEGF-R1$^+$ cells in cultures of the first and third trimesters was 4-44% (median value 9.4%) and 3.4-92% (median value 44.5%; Fig. 1), respectively.

VEGF content in the macrophage conditioned medium was close to the sensitivity limit of the method. Hence, studying the secretory activity of macrophages was difficult. However, we were successful in estimating the secretion of this cytokine in cultures of the first and third trimesters (0.48 and 1.14 pg/10^5 cells, respectively; Fig. 2). Cytokine secretion in third trimester cultures was shown to decrease under the influence of LPS. However, these changes were not statistically significant.

Secretion of the soluble receptor sVEGF-R1 was much higher than that of the ligand (86.4 and 36.4 pg/10^5 cells in cultures of the first and third trimesters, respectively; Fig. 3). As differentiation form the cytokine, its soluble receptor responded to stimulation with LPS by a 4-fold increase in the secretion (median value 157.1 pg/10^5 cells; Fig. 3).

These data are consistent with and complement the results of *in situ* studies. Placental macrophages produce VEGF and have a regulatory effect on endothelial cells during pregnancy. These influences are manifested in the regulation of angiogenesis and modulation of properties (permeability and tone) of placental vessels.

Studying the intracellular distribution of VEGF revealed that nearly all population of placental macrophages produces this cytokine at the early and late stages of pregnancy. As differentiation form the pattern of production of other cytokines by the same cells (functional heterogeneity) [10], an extremely low variability of the number of VEGF-producing cells illustrates the biological significance of this function of macrophages in the overall period of gestation. The same conclusion was made on the basis of other investigations. Previous studies showed that the expression of VEGF decreases in trophoblast structures, but increases in placental macrophages with the progression of pregnancy [7].

Our experiments showed that nearly all CD68$^+$ cells contain intracellular VEGF. We expected that these cells would be characterized by high level of VEGF secretion. However, the concentration of an extracellular cytokine in the medium conditioned by placental macrophages was extremely low and hardly detectable in our study. These features were probably related to the formation of cytokine complexes with the soluble receptor, which prevents binding to specific antibodies for the detection of VEGF. However, it can be concluded that placental macrophages secrete this important angiogenic factor at various stages of gestation. Therefore, these cells have a modulatory effect on functions of endothelial cells that form the vessels of placental villi. Besides the angiogenic effects, macrophage-derived VEGF probably modulate trophoblast functions *in vivo* (similarly to *in vitro* conditions) [8]. Hence, the ability of placental macrophages to produce and secrete VEGF contributes to their regulatory effect on the main components of placental villi (*e.g.*, trophoblast structures and blood vessels).

Apart from the paracrine regulation of other cells, the autocrine regulation can occur. This conclusion is confirmed by the presence of VEGF-R1 receptors on the surface of macrophages. Macrophages respond to

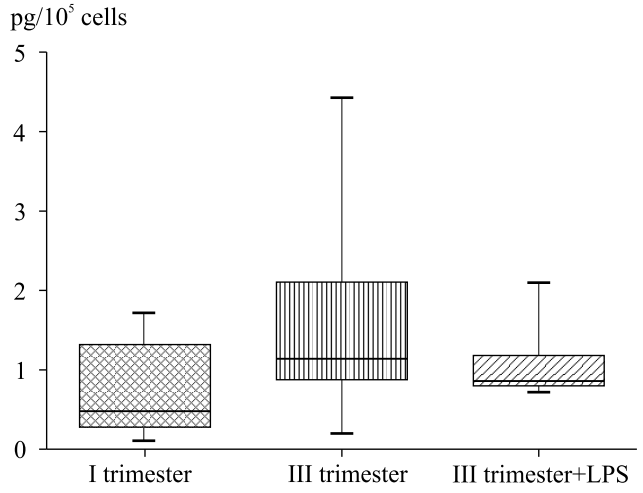

Fig. 2. VEGF content in the medium conditioned by placental macrophages of the first (basal secretion, *n*=7) and third trimesters (basal secretion, *n*=17; LPS-stimulated secretion, *n*=8) after 23-h culturing.

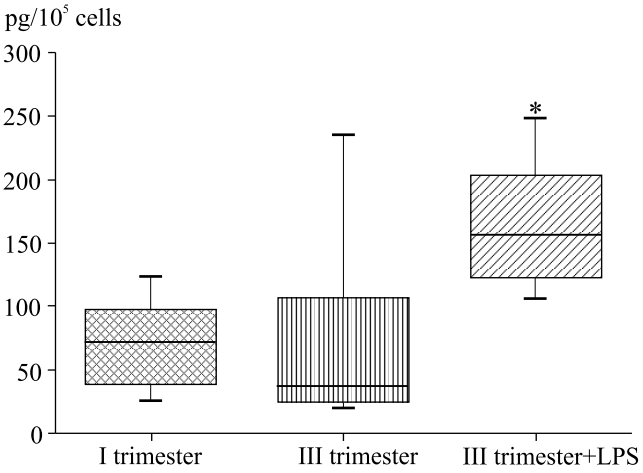

Fig. 3. sVEGF-R1 content in the medium conditioned by placental macrophages of the first (basal secretion, *n*=9) and third trimesters (basal secretion, *n*=14; LPS-stimulated secretion, *n*=9) after 23-h culturing. *p=0.0074 compared to the basal secretion.

VEGF by variations in the functional activity, which is mediated by the VEGF-R1 receptor [11]. Since these receptors bind not only VEGF, but also placental growth factor (PIGF), the functions of placental macrophages can be regulated by both factors. The heterogeneity of placental macrophages in VEGF-R1 expression probably reflects a different level of their activation. The factors that cause this activation should be evaluated in further studies.

The expression of a free form of the VEGF-R1 receptor (sVEGF-R1) is of particular interest. This receptor serves as an antagonist of VEGF and counteracts its effects. Our experiments showed that placental macrophages can secrete this factor in the concentration which can be easily detected. To our knowledge, it is the first report regarding the expression of sVEGF-R1 by macrophages of the fetal placenta.

It is postulated that sVEGF-R1 has at least three physiological effects, including the antiangiogenic (inhibition of the proangiogenic signal mediated by the interaction of VEGF with the VEGF-R2 receptor on the endothelial cell surface), anti-edematous (reduction of vEGF-mediated vascular permeability), and anti-inflammatory effects (decrease in the activation and migration of monocytes/macrophages mediated by the interaction of VEGF with VEGF-R1) [15]. The increased production of sVEGF-R1 under the influence of bacterial endotoxin can be considered as a compensatory reaction, which prevents the excess inflammatory process.

Overproduction of sVEGF-R1 in the placenta under hypoxic conditions serves as one of the key factors in the pathogenesis of gestosis [15]. Much attention is paid to the secretion of this factor by macrophages, which are highly sensitive to various exogenous signals. A multifold increase in the secretion during *in vitro* stimulation by LPS suggests that macrophages contribute to the elevation of placental sVEGF-R1 content during gestosis. This conclusion is derived from a similarity of intracellular mechanisms for the activation of macrophages during LPS treatment and hypoxia [4].

The antagonistic effects of sVEGF-R1 on VEGF are realized not only via binding and decrease in the extracellular cytokine concentration, but also due to the formation of heterodimers with the membrane-bound VEGF receptor that results in the inactivation of this structure [15]. Our results indicate that placental macrophages express all components for the realization of both mechanisms (VEGF, membrane-bound form of the VEGF-R1 receptor, and secretory form of the sVEGF-R1 receptor). It provides not only the paracrine regulation of functional activity of the endothelium and trophoblast, but also the autocrine regulation of the proper activity. These influences have various and even opposite effects (*e.g.*, proangiogenic and antiangiogenic effects), which depends on the spatiotemporal features of production and quantitative ratio of both factors.

We conclude that placental macrophages express a variety of molecules. This feature allows macrophages to perform not only the VEGF-mediated paracrine and autocrine regulation of main cells in placental villi, but also to realize a fine and complex regulation of intercellular signaling with the involvement of cytokines of the VEGF family. These data contributes to the understanding of the role of placental macrophages in physiological and pathological processes during gestation.

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