

## Expression of angiopoietic factors in normal and type-I diabetes human placenta: a pilot study

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### Abstract

**Objectives:** Morphological changes of blood vessel wall have been described in placenta from pregnancies complicated by diabetes mellitus type-I. **Study design:** We measured mRNA expression of vascular endothelial growth factor (VEGF), angiopoietin 1 and 2 (Ang-1 and Ang-2), their receptors VEGFR-1, VEGFR-2, Tie-2, fibroblast growth factor 2 (FGF-2), and its receptor FGF-2R in placental tissue of diabetes type-I patients, in normal term placenta, and endometrium of non-pregnant women by real time reverse transcriptase PCR. **Results:** The expression of Ang-2 and VEGFR-1 mRNAs was significantly higher in placenta ( $P \leq 0.01$ ). The expression of VEGF and VEGFR-2 mRNAs was significantly lower in normal term or diabetes type-I placenta compared to human endometrium ( $P \leq 0.01$ ). We did not detect a significant difference in the expression of Ang-1, Ang-2, Tie-2, VEGF, VEGFR-1, VEGFR-2, FGF-2, and FGF-2R in normal and diabetes type-I placenta. **Conclusion:** These data show differential expression of Ang-2, VEGFR-1, VEGF, and VEGFR-2 in placenta and endometrium. The expression of Ang-1, Ang-2, Tie-2, VEGF, VEGFR-1, VEGFR-2, FGF-2, and FGF-2R was not different in normal and type-I diabetes placenta.

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**Keywords:** Diabetes mellitus; Placenta; Angiopoietin; Vascular endothelial growth factor; Angiogenesis

### 1. Introduction

Mechanisms of blood vessel growth have been intensively studied in recent years. Vasculogenesis and angiogenesis are mechanisms cooperating to establish new blood vessels [1]. Vasculogenesis is based on mobilization of endothelial precursors from generative tissues (e.g. bone marrow), which then participate in new vessel formation in distant organs and tissues [2]. Angiogenesis is understood as local sprouting and intussusceptive growth of blood vessels [3]. In the process of vessel growth, angiopoietins 1 and 2 (Ang-1 and Ang-2), and vascular endothelial growth factor (VEGF) acting via their receptors play central role. They are responsible for vessel wall destabilization, migration and proliferation of endothelial cells and their precursors [1,2]. Diabetes mellitus type-I is associated with increased fetal and neonatal morbidity and mortality [4,5]. Impaired vessel wall structure, in terms of thinner capillary basement membrane, has been described in placenta of diabetic patients [6].

Vascular changes may lead to chronic disturbances in fetal-placental blood flow. The pathologies of placental vessels might be caused by the dysregulation of angiopoietic factors expression and function [7]. In diabetic placenta the expression of fibroblast growth factor 2 (FGF-2) was found to be increased compared to normal term placental tissue [8].

We have measured the mRNA expression of angiogenesis regulating factor VEGF, Ang-1, Ang-2, and their receptors VEGFR-1, VEGFR-2, and Tie-2 in diabetes type-I patient placenta and compared them to normal placental tissue. We determined expression of FGF-2 and its receptor FGF-2R in diabetes type-I placenta and compared them to normal placenta. We have also measured the expression of these genes in endometrium.

### 2. Methods

#### 2.1. The study subjects and sample collection

Tissue samples were collected from term placental tissue of patients with well-controlled diabetes mellitus type-I

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diagnosed in childhood and having no other complication during pregnancy detected ( $n = 4$ ), and placenta from normal term pregnancies ( $n = 4$ ). Samples were taken from three different non-necrotic locations of placenta. The expression of studied genes and control genes was not different in individual samples in each of the studied placenta. Samples of endometrial tissue were collected from non-diabetic, non-pregnant patients with hyperplastic non-malignant endometrium ( $n = 5$ ). Placental and endometrial tissues were collected to RNAlater (Ambion Inc., Austin, TX, USA) shortly after the delivery (term diabetes type-I placenta and normal term placenta) or surgical procedure (endometrium) at the Department of Gynecology and Obstetrics, First School of Medicine, Charles University, Prague, Czech Republic, after informed consent from the patient. The samples were stored at  $-20\text{ }^{\circ}\text{C}$  until RNA was isolated using RNazol B method (Tel-Test Inc. Friendswood, TX, USA).

## 2.2. Real time reverse transcriptase PCR (RT-PCR)

Reverse transcription was performed using First strand cDNA synthesis kit (Fermentas Inc., Hanover, MD, USA). The level of Tie-2, Ang-1, Ang-2, VEGF, VEGFR-1, VEGFR-2, FGF-2, and FGF-2R genes expression was evaluated against  $\beta$ -actin mRNA expression as a control gene. To quantify Tie-2, Ang-1, Ang-2, VEGF, VEGFR-1 and VEGFR-2 expression we have used Light Cycler instrument (Roche Diagnostic GmbH, Mannheim, Germany). Samples of cDNA were amplified using Light Cycler-FastStart DNA Master SYBR Green I kit (Roche Diagnostic GmbH, Mannheim, Germany). Melting curve analysis was performed to control specificity of PCR product fluorescence. Value of crossing point (CP) was determined for each gene

and sample during real time PCR. Crossing points represent cycle numbers, where fluorescence levels of all samples are the same. This specific cycle number is depending on the starting amount of target cDNA in the sample. A lower number of cycles during real time PCR mean a higher level of starting target cDNA. Value of CP is counted by Light Cycler Software version 3. PCR products were visualized on 1% agarose gel with ethidium bromide under UV light to confirm their size. RT negative samples served as a control to exclude genomic DNA amplification. Loading of cDNA was equalized according to  $\beta$ -actin expression ( $\text{CP} \pm 1$  cycle). Previously described PCR primers were used:  $\beta$ -actin-forward: gtg atg gtg ggc atg ggt ca; reverse: tta atg tca cgc acg att tcc c (510 bp) [9], Tie-2-forward: gta tgg act ctt tag ccg gct t; reverse: ttc gcc cat tct ctg gtc ac (253 bp), Ang-1-forward: aaa tgg aag gaa aac aca agg aa; reverse: atc tgc aca gtc tct aaa tgg t (266 bp) [10,11], Ang-2-forward: gac ggc tgt gat gat aga aat agg; reverse: gac tgt agt tgg atg atg tgc ttg (264 bp) [11], VEGF-forward: cga agt ggt gaa gtt cat g; reverse: ttc tgt atc agt ctt tcc tgg tga g (403 bp), VEGFR-1-forward: caa gtg gcc aga ggc atg gag tt; reverse: gat gta gtc ttt acc atc ctg (498 bp), VEGFR-2-forward: gag ggc ctc tca tgg tga ttg t; reverse: tgc cag cag tcc agc atg gtc tg (709 bp) [12], FGF-2-forward: ggc ttc ttc ctg cgc atc ca; reverse: gct ctt agc aga cat tgg aag a (354 bp), FGF-2R-forward: gac gca aca gag aaa gac ttg t; reverse: gcc agc agt ccc gca tca tca t (661 bp) [8].

## 2.3. Statistical analysis

The level of significance of real time PCR data was assessed by two sample  $t$ -test Software, (SyStat 10.0 Statistics II., SPSS Inc., IL, USA),  $P$  value of less than 0.05 was considered to be statistically significant. The results are shown as a mean  $\pm$  S.D.

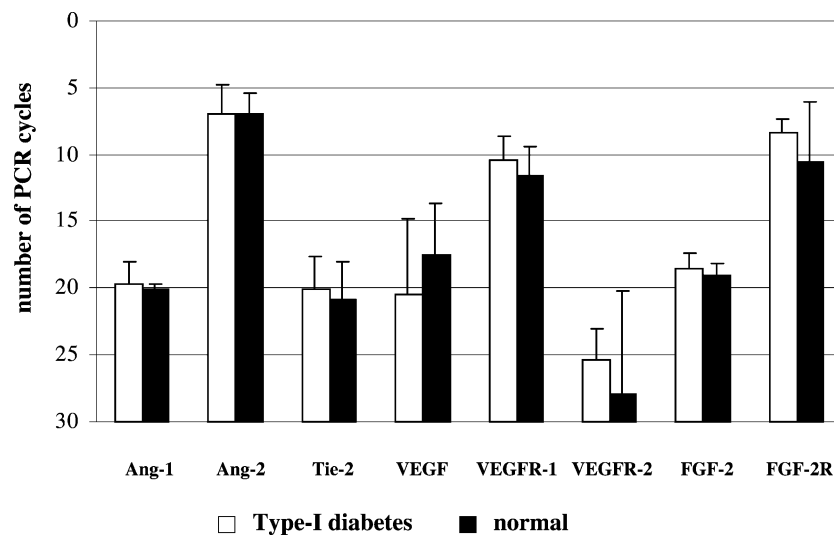


Fig. 1. Expression of Ang-1, Ang-2, Tie-2, VEGF, VEGFR-1, VEGFR-2, FGF-2, and FGF-2R mRNAs was measured in diabetes mellitus type-I (type-I diabetes) and normal term placenta by real time reverse transcriptase PCR. No statistically significant differences between diabetic and normal human placenta were detected. Correction to  $\beta$ -actin expression was used.

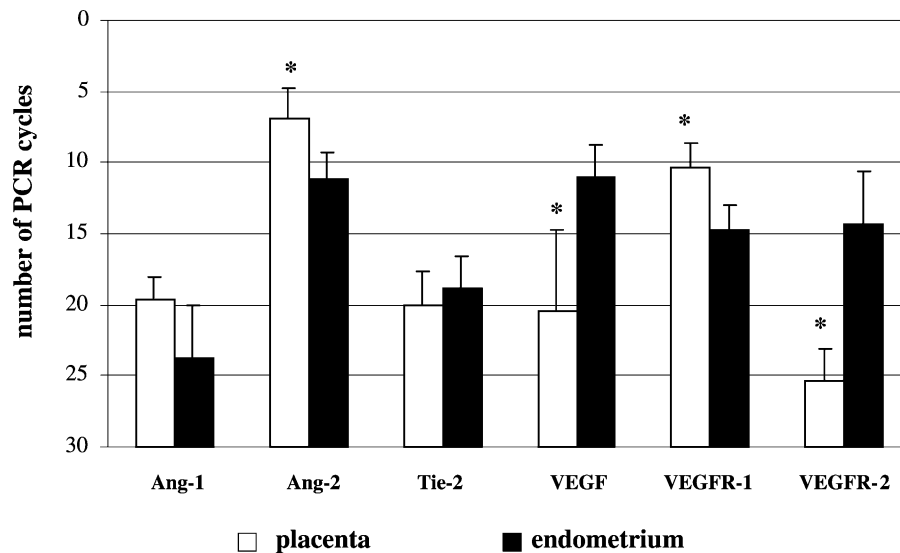


Fig. 2. Ang-1, Ang-2, Tie-2, VEGF, VEGFR-1, and VEGFR-2 mRNAs expression in human placenta was compared to endometrium. Ang-2 and VEGFR-1 mRNAs were expressed at significantly higher level in placenta ( $*P \leq 0.01$ ), no differences were detected comparing expression of Ang-1 and Tie-2 mRNA in placenta and human endometrium. Significantly lower level of VEGF and VEGFR-2 mRNAs expression in human placenta was detected, compared to human endometrium ( $*P \leq 0.01$ ). Real time reverse transcriptase PCR method and correction to  $\beta$ -actin expression was used.

### 3. Results

#### 3.1. The expression of Ang-1, Ang-2, VEGF, VEGFR-1, VEGFR-2, Tie-2, FGF-2, and FGF-2R was not significantly different in normal and diabetes type-I placenta

Using real time RT-PCR we have measured the expression of Ang-1, Ang-2, VEGF, VEGFR-1, VEGFR-2, Tie-2, FGF-2, and FGF-2R in the normal term placenta and placenta from diabetes type-I patients. We did not found a significant difference in mRNA expression of Ang-1, Ang-2, VEGF, VEGFR-1, VEGFR-2, Tie-2, FGF-2, and FGF-2R genes between the normal and diabetes type-I patient placental tissues (Fig. 1).

#### 3.2. Placenta expresses high levels of, Ang-2 and VEGFR-1 and low levels of VEGF and VEGFR-2

We compared the expression level of Ang-1, Ang-2, Tie-2, VEGF, VEGFR-1, and VEGFR-2 mRNAs in normal term placental tissue and endometrium. Ang-2 and VEGFR-1 mRNAs were significantly higher expressed in human placenta (Fig. 2) compared to endometrium. The expression of VEGF and VEGFR-2 mRNAs was significantly lower in placenta compared to endometrium. Expression of Ang-1 and Tie-2 was not different in both tissues (Fig. 2).

### 4. Discussion

Placental tissue is a site of blood vessels growth and remodeling. Placental angiogenesis is critical for normal fetal growth and development. Feto-placental blood circulation is

established early in the first trimester of gestation to allow feto-maternal oxygen and nutrients exchange. Placental blood vessels development is regulated by angiopoietic factors, which could be of both maternal and fetal origin. In recent years, several specific angiopoietic factors and their receptors were identified [13]. Pathological conditions associated with dysregulated expression of angiopoietic factors include diabetic angiopathies [14–16]. Diabetes mellitus associated angiopathies might be caused by abnormal expression of angiopoietic factors and their receptors. VEGF and angiopoietins are the essential regulatory molecules cooperating in induction of blood vessels growth and regression. Diabetic mice impaired wound healing is accompanied by persistent high expression of Ang-2 and low expression of VEGF in the tissue [17]. This is in a concordance with the theory that Ang-2 is involved in vessel regression in case of low expression of endothelial mitogenes (i.e. VEGF) in the tissue [18]. We have tested whether diabetes mellitus is associated with imbalanced expression of angiopoietins and VEGF in term placenta. We have measured expression of Ang-1, Ang-2, VEGF, VEGFR-1, VEGFR-2, and Tie-2 in term placenta of normal pregnancies and those complicated by diabetes mellitus type-I. We also compared mRNA expression of these factors in normal placental tissue and endometrial tissue to study possible role of paracrine communication between fetal and maternal tissues. From tested genes Ang-1 and Ang-2 were expressed to the highest extend in placenta. However, the expression of both Ang-1 and Ang-2 did not differ between normal and diabetes type-I term placenta. VEGF and its receptors VEGFR-1 and VEGFR-2 mRNAs were detected in term placenta, but the level of placental expression was not different in normal and diabetes type-I patients. We also did not find a difference

in expression of FGF-2 mRNA between normal and diabetes type-I placenta. These results suggest that well-controlled diabetes type-I is probably not associated with disordered vessel regression or growth in term placenta, or that there is no significant influence of compensated maternal diabetes on the expression of angiopoietic genes in placenta. This finding is in agreement with recent published study, which showed no significant differences in expression of VEGF receptors in normal and diabetic placenta on the protein level [7]. Dysregulation of angiogenesis in diabetes might be present only in non-compensated patients with severely affected metabolic status [19]. Hyper- or hypoglycemia is reported to cause dysregulation of angiopoietins expression [20–22]. Dysregulation of glycemia might play an important role in these processes.

In conclusion, our study showed that regulation of angiopoietic genes VEGF, Ang-1, Ang-2, and their receptors VEGFR-1, VEGFR-2, and Tie-2, as well as FGF-2 and FGF-2R was not different in well-controlled diabetes mellitus type-I placenta compared to normal term placenta.

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