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ORIGINAL ARTICLE

Placental gene expression of transforming growth factor beta 1 (TGF-β1) in small for gestational age newborns

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Abstract

Objective: The gene expression of transforming growth factor beta-1 (TGF-β1) in human placental samples obtained from pregnancies with small for gestational age fetuses (SGA) was compared to those of normal pregnancies.

Methods: In 2011 placental samples from 101 pregnancies with SGA and from 140 normal pregnancies were obtained for analysis of TGF-β1 gene expression. Several clinical parameters were also assessed for correlation between genetic and clinical parameters.

Results: There were no significant differences in gene activity of the TGF-β1 gene between the SGA versus normal pregnancy groups ($Ln2^{\alpha}$: 0.16; $p = 0.07$). Within the SGA group, no fetal gender-dependent differences were seen in TGF-β1 gene expression ($Ln2^{\alpha}$: −0.11; $p = 0.05$). Similarly, no significant differences in gene activity were observed by the degree of severity of SGA as assessed by percentile fetal birth-weight ($Ln2^{\alpha}$: 0.32; $p = 0.06$).

Conclusion: We found no change in gene expression of TGF-β1 in placental samples obtained from SGA pregnancies versus normal pregnancy suggesting an absence of a direct role of the TGF-β1 gene in the development of SGA. However, the absence of increased gene expression of TGF-β1 in SGA can be conceptualized as a failure to mount a compensatory response in the SGA environment.

Keywords

Gene expression, intrauterine growth restriction, placenta, small for gestational age, TGF-β1, transforming growth factor beta-1

History

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Introduction

With respect to size, the fetus may be normally grown, small for gestational age or large for gestational age. The term *small for gestational age* (SGA) has been widely used to categorize a newborn, whose birth-weight is below the 10th percentile for its gestational age. Other often used terms have included fetal growth retardation or intrauterine growth restriction. Intrauterine growth restriction (IUGR) is defined below 10 percentile birth-weight per fetal gender and gestational age [1]. (As the diagnosis of IUGR could be based only on serial ultrasound examinations, which are typically not possible in the pregnancy care, we tried to prefer the expression small for gestational age to characterize our examined group, though it can be supposed that these newborns were restricted in their intrauterine development). IUGR is a multifactorial disorder. Although placental dysfunction is the most common etiology, it may also develop due to a variety of fetal developmental disorders or intrauterine infection.

Actually only a few informations are available about the molecular mechanisms resulting a small for gestational age fetus. However, several growth factors have been implicated and an important role for multiple growth factor-related gene families and other specific genes have been firmly established [2,3]. During gestation plasma levels of several growth factors were found to rise in the maternal blood. These growth factors are thought to participate in the regulation of fetal growth and help maintain normal placental function. Among these factors, insulin-like growth factor 1 and 2 (IGF-1, IGF-2) are implicated in the development of both SGA and preterm delivery [4,5]. Vascular endothelial growth factor (VEGF-A) is thought to be instrumental in stimulating angiogenesis while one of the important functions of epidermal growth factor (EGF) is to promote placental growth [6,7].

The active biological form of transforming growth factor beta is a 25 kD homodimer. It has three isoforms including β1 (19q13.1), β2 (1q41) and β3 (14q24). All isoforms show a high degree of sequential homology amounting to approximately 80% [8].

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The biological function of TGF-β is rather complex. The main function seems to be in the regulation of cellular growth and development, occurring in all cell types. The role of TGF-β is especially prominent in the differentiation of endothelial cells. Furthermore, TGF-β promotes vascular development in multiple tissue types. It is also instrumental in the maintenance of endothelial integrity. Decreased function of TGF-β may lead to decreased blood supply, increased fragility of the vascular wall and bleeding [8,9].

TGF-β plays an active role in several physiological processes associated with normal female sexual function. Secretion of growth factors belonging to the TGF-beta family may be enhanced at several time points during the menstrual cycle. Under physiological circumstances, TGF-β1 and TGF-β3 are both secreted in both the epithelial and stromal cells of the endometrium, whereas TGF-β2 is predominantly produced by the stromal cells. Together with activin (2q14.2), also secreted by the endometrium, isoforms of TGF-β are also thought to participate in the preparation of the endometrium before implantation [10,11]. It is interesting to note the opposite effects of activin and TGF-β during the process of decidualization and in the regulation of placental endocrine function. While the main effect of TGF-β is inhibitory, activin exerts a stimulatory effect during both processes [11–13].

During the final phase of gestation TGF-β1 is present in multiple cell types including syncytiotrophoblast, cells of the chorionic plate and extravillous trophoblasts. In trophoblasts, TGF-β1 has a stimulatory effect on the production of multiple substances in the extracellular matrix. This effect seems to be indispensible for physiological placental function and thus for normal fetal intrauterine development [14,15].

The potential role for TGF-β1 in the development of SGA pregnancies is not well elucidated. Most studies performed *in vitro* seem to suggest that while in the initial phases of gestation TGF-β1 stimulates fetal growth, this effect gradually fades and becomes absent during the later part of gestation [16,17]. Accordingly, in pregnancies eventually destined to turn into SGA pregnancies, maternal serum level of TGF-β1 rises during the first trimester [18,19]. It is assumed that this may represent a compensatory mechanism by which pathological mechanisms eventually resulting in SGA are partially compensated for a time.

In the minority of cases of IUGR where preeclampsia is also present a potential role for TGF-β1 was assessed using genetic polymorphism analysis. It was found that c.869T > C polymorphism was significantly associated both with lone preeclampsia and with IUGR-associated preeclampsia [19,20].

In the present study, our primary objective was to characterize gene expression patterns of TGF-β1 in human placental samples from SGA pregnancies and analyze changes in gene activity using placental samples from normal pregnancy as control group. Our hypothesis was that if a significant change in gene expression is found in SGA, this may suggest a compensatory role for TGF-β1 in this condition in an attempt to restore placental growth. As a secondary objective, we assessed the possible effect of fetal gender on placental expression of TGF-β1 in order to better understand gender-specific regulation of intrauterine fetal development in SGA pregnancies. We also analyzed the possible correlation between the degree of intrauterine growth restriction and placental TGF-β1 expression by dividing the SGA group into two categories based on a more severe (fetal birth-weight 0–5 percentile) or less severe (fetal birth-weight 5–10 percentile) degree of growth restriction. We also assessed several clinical parameters to assess any association with changes in gene expression.

Materials and methods

Patients, clinical characteristics

During the period between January 1, 2011 and January 1, 2012, we obtained placental samples postpartum for genetic studies from 101 pregnant mothers treated in our clinic at Semmelweis University, Budapest for SGA. SGA was defined as fetal birth-weight below 10 percentile per fetal gender and gestational age. In order to confirm the diagnosis, we measured fetal abdominal circumference (AC) and compared it to standard size AC for gestational age. SGA cases were considered to be due to placental dysfunction whenever intrauterine infections, chromosomal abnormalities, other fetal developmental disorders, maternal nutritional deficiencies, multiple pregnancy or other placental pathology could be excluded [21]. Cases within the SGA group were subdivided into two categories based on percentile scores: those with fetal birth-weight falling between 0–5 percentile were considered severely growth restricted, whereas those with 5–10 percentile birth-weight were defined as moderately growth restricted. During the same period 140 placental samples were taken postpartum from normal pregnancies to serve as the control group.

We extracted several clinical parameters for analysis from both group of patients. These included maternal age, paternal age, obstetric history, genetic background, general medical history, maternal birth-weight, gestational age at delivery, presence of gestational diabetes or other gestational pathology, fetal birth-weight, Apgar score, gestational weight gain and gestational increase in Body Mass Index (BMI). We made no distinction among cases with different delivery methods; both groups included cases with both vaginal delivery and cesarean section. Consent for study participation and genetic testing were obtained in each case according to local ethic committee standards, records on file.

Placental sampling, RNA preparation, cDNA synthesis

Placental sample sizes were approximately $2 \times 2 \times 2$ cm ($8\,cm^3$) in all cases. Samples were immediately frozen and stored at $-70\,^{\circ}C$ until genetic testing. Subsequently, whole RNA content was extracted from the placental samples using the Quick RNA microprep kit (Zymo Research, Irvine, CA). Total RNA concentration was then determined by NanoDrop spectrophotometer (NanoDrop, Rockland, DE). Reverse transcription (RT) was performed in 20 µl end volume adding 5 µg whole RNS, 75 pmol random hexamer primer, 10 mM dNTP (Invitrogen, Foster City, CA), 20 U M-MuLV Reverse Transciptase enzyme (MBI Fermentas) and $1 \times$-es buffer (MBI Fermentas, Pittsburgh, PA). The reaction mix was then incubated at $42\,^{\circ}C$ for two hours and the enzyme was subsequently inactivated at $70\,^{\circ}C$ for 15 min.

Table 1. Primers and sequences used in real-time PCR.

Gene name and code	Forward primer	Reverse primer
TGF-β1 (NM_000660)	5′-CCCAGCATCTGCAAAGCTC-3′	5′-GTCAATGTACAGCTGCCGCA-3′
β-Actin (M10277)	5′-GGCACCCAGCACAATGAAG-3′	5′-GCCGATCCACACGGAGTACT-3′

Real time PCR

The reverse transcription reaction mix was diluted three-fold using nuclease-free water as solvent. For the real time PCR procedure 1 µl diluted cDNA (~15 ng RNA equivalent) and 1 × SYBR Green Master Mix (Applied Biosystems) was used. Primers were constructed using the Primer Express Software (Applied Biosystems, Foster City, CA). For primer sequences see Table 1.

Real time PCR was performed with 1 µl cDNA, 1 pmol, gene-specific Forward and Reverse primer and 1 × SYBR Green PCR Master mix in 20 µl end volume. All real time PCR reactions were performed using the same MX3000 Real-time PCR (Stratagen, Santa Clara, CA) equipment programmed as: 40 cycles, denaturing at 95 °C for 15 s, primer insertion at 60 °C, elongation and detecting for 60 s. The relative expression of the tested gene was expressed using human β-actin gene as standard.

Statistical analysis

A two-sample t test was used to compare relative TGF-β1 gene expression between groups (confidence interval, CI 95%). For the purpose of determining degree of freedom, the Welch-Satterhwaite correction was performed. Relative gene expression was expressed using three categories as follows: (1) over-expression if calculated Ln value was >1, $p<0.05$; (2) under-expression if calculated Ln value was <-1, $p<0.05$; (3) no change in expression if calculated Ln value was <1, >-1, $p<0.05$. For all statistical procedures, evaluation was performed using the GraphPad Prism 3.0 (GraphPad Software Inc, La Jolla, CA) software.

Clinical and demographic data was analyzed using SPSS software (SPSS, Budapest, Hungary). When multiple independent variables were present either logistic regression (with dichotomous outcome variable) or ANOVA or linear regression models were used as appropriate. Level of significance was set at $p<0.05$ in all cases.

Results

During the study period lasting one calendar year, a total of 101 placental samples from SGA pregnancies were obtained for analysis of TGF-β1 gene expression. The total number of normal pregnancy cases representing the control group amounted to 140 (Table 2). There was no significant difference between the two groups in TGF-β1 gene expression ($Ln2^{\alpha}$: 0.16; $p=0.07$).

Within the SGA group no fetal gender-related differences were seen in placental expression of the TGF-β1 gene ($Ln2^{\alpha}$: −0.11; $p=0.05$; Table 3).

Similarly, no significant differences could be detected within the SGA group by growth restriction severity categories. In more severe growth restriction, represented by the subgroup of cases with fetal birth-weight falling between 0–5 percentile, TGF-β1 gene activity was not significantly different from the subgroup of cases with fetal birth-weight between 5–10 percentile ($Ln2^{\alpha}$: 0.32; $p=0.06$; Table 4).

The main clinical parameters of the SGA and control groups are summarized in Table 5.

Table 2. Comparison of placental gene expression of TGF-β1 in the SGA versus normal pregnancy groups.

Gene name	α value ± SE(α)	Ln 2^{α}	p	Change in gene expression
TGF-β1	0.24 ± 1.02	0.16	0.07	No change

$n_{normal}=140$; $n_{IUGR}=101$; $\alpha=\Delta Ct_{normal}-\Delta Ct_{IUGR}$, Control gene: β-actin.

Table 3. Placental gene expression of TGF-β1 in SGA pregnancy-the influence of fetal gender; gene expression in male fetal gender is shown compared to gene expression in female gender used as control.

Gene name	α value ± SE(α)$^{(C)}$	Ln 2^{α}	p	Change in gene expression
TGF-β1	−0.17 ± 0.93	−0.11	0.05	No change

$n_{female}=64$; $n_{male}=37$; $\alpha=\Delta Ct_{female}-\Delta Ct_{male}$; Control gene: β-actin.

Table 4. Placental gene expression of TGF-β1 in SGA-influence of severity of growth restriction; gene expression in more severe growth restriction (fetal birth-weigh 0–5 percentile) versus less severe growth restriction (fetal birth-weigh 5–10 percentile).

Gene name	α value ± SE(α)	Ln 2^{α}	p	Change in gene expression
TGF-β1	0.47 ± 0.88	0.32	0.06	No change

$\alpha=\Delta Ct_A-\Delta Ct_B$; $\Delta Ct_A=Ct_{TGF\beta1}-Ct_{control\ gene}$ (5–10 percentile IUGR); $\Delta Ct_B=Ct_{TGF\beta1}-Ct_{control\ gene}$ (0–5 percentile IUGR); ($n_A=61$, $n_B=40$), Control gene: β-actin.

Table 5. Main clinical parameters of the examined groups.

	SGA cases	Control cases	p
Maternal age (median value) (year)	30.8 ± 4.3	31.4 ± 3.1	>0.05
Male:female ratio	0.58	1.09	<0.05
Gestational weight gain (kg)	14.8	10.9	<0.05
Maternal BMI increase in pregnancy	5.3	4.1	<0.05

Discussion

Morbidity and mortality in SGA is significantly higher than in normal pregnancy. Early detection, timely diagnosis and development of preventive strategies could contribute to the eventual improvement in postnatal prognosis in SGA. However, the multifactorial nature of intrauterine growth restriction makes it sometimes difficult to pinpoint the responsible pathomechanism rendering such clinical goals more difficult to achieve.

TGF-β1 along with several other growth factors belonging to the TGF-beta family participates in the preparation of the endometrium for implantation. It appears therefore that even at this early stage of gestation this group of growth factors has an important physiological role. As growth factors belonging to the TGF-beta family are known to promote endothelial cell differentiation, their primary function appears to be in promoting vascular development and in the maintenance of adequate blood supply to the endometrium. This effect is indispensible for normal fetal, placental and uterine development. During the later stages of gestation, the stimulatory effect of TGFβ-1 on fetal growth becomes less pronounced. On the other hand, its effect on elements of the extracellular matrix in the placenta becomes gradually more marked. This mechanism is necessary for the maintenance of physiological placental function and for proper fetal development at this stage [14,15].

In the present study, we could not detect a significant change in placental TGF-β1 gene expression in SGA compared to normal pregnancy. This finding accords with previous observations reported by other investigators, though most of their studies had a much lower number of subjects. Taken together these results suggest that the primary contribution of TGF-β1 is in the early rather than the later stages of gestation when it promotes placental and fetal growth [22–26].

In contrast to our results, Todros et al. found increased placental expression of TGF-β1 in SGA pregnancies maintained throughout the whole period of gestation. They interpreted this finding stating that increased TGF-β1 activity in SGA represents a compensatory mechanism by which vascular smooth muscle proliferation is stimulated to restore adequate placental blood supply in this condition [27].

Several reports documented increased placental TGF-β1 gene expression in normal pregnancy, i.e. in the absence of SGA. Dungy and coworkers found that peak expression occurred during gestational weeks 17 and 34 [28].

In our study population, most cases of SGA were due to placental dysfunction. The precise etiology of placental dysfunction could not be established for certainty in the majority of these subjects. However, impaired placental blood supply was a common denominator. It is well known that TGF-β1 –among other actions– promotes vascular development in placental tissue by stimulating endothelial growth. It appears that TGF-β1 is an important player in maintaining adequate placental blood supply. However, while this effect of TGF-β1 manifests predominantly in the earlier phases of gestation, the time period crucial for determining SGA outcomes is in the third trimester. As demonstrated by our findings, the compensatory effect of TGF-β1 is not present at this late period. We conclude that it is this time discrepancy that makes TGF-β1 less important in influencing SGA outcomes. Any compensatory effect for impaired placental blood supply in SGA appears to be independent of TGF-β1. At the same time the fact that TGF-β1 is not under = expressed in SGA pregnancies makes a primary role for TGF-β1 in the pathomechanism of SGA less likely.

We could not detect a fetal gender-dependent difference in TGF-β1 gene expression in our SGA population. We conclude that fetal gender is unlikely to influence placental TGF-β1 gene expression in SGA. This tends to confirm previous assertions on the lack of association between fetal gender and TGF-β1 expression made by several authors.

Additionally, we found no correlation between TGF-β1 gene expression and the degree of intrauterine growth restriction as characterized by fetal birth-weight categories. It seems likely that factors other than TGF-β1 determine the severity of SGA.

In summary, we found no difference in placental gene expression of TGF-β1 between the SGA and normal pregnancy groups. This finding suggests that the stimulatory effect of TGF-β1 on vascular endothelial cell proliferation [29] observed in the early phase of gestation is no longer operational in later stages of pregnancy when SGA tends to develop. In the third trimester, the critical time period determining SGA-related outcomes, TGF-β1 does not seem to mount a compensatory mechanism restoring placental blood supply. We therefore conclude that TGF-β1 does not play a direct role in the development of SGA. Neither fetal gender, nor the degree of growth restriction is associated with placental TGF-β1 expression in SGA pregnancies.

Declaration of interest

The authors report no declaration of interest.

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