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Expression of genes involved in inflammation and growth – does sampling site in human full-term placenta matter?

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Abstract

Objective: To investigate the placental gene expression of substances in the inflammatory cascade and growth factors at nine different well-defined sampling sites in full-term placentas from 12 normal weight healthy non-smoking women with an uncomplicated singleton pregnancy.

Methods: All placentas (six girls and six boys) were delivered vaginally. Quantitative real-time polymerase chain reaction was used to analyze toll receptor-2 and -4, interleukin-6 and -8, tumor necrosis factor- α , leptin, ghrelin, insulin-like growth factor-1 and -2, hepatocyte growth factor, hepatocyte growth factor receptor and insulin receptor (IR).

Results: The leptin gene and the IR gene showed higher expression in lateral regions near the chorionic plate compared to central regions near the basal plate ($P = 0.028$ and $P = 0.041$, respectively).

Conclusion: Our results suggest that the sampling site may influence the gene expression for leptin and IR in placental tissue obtained from full-term normal pregnancies. We speculate that this may be due to differences in placental structure and perfusion and may be important when future studies are designed.

Introduction

The placenta plays a central role in maternal adaptation to pregnancy and in fetal development and growth [1]. Substances produced by the placenta influence the placenta itself, the pregnant woman and the fetus. Such substances include inflammatory markers and growth factors [2, 3]. Toll receptor-2 and -4 (TLR-2 and -4), interleukin-6 and -8 (IL-6 and IL-8), and tumor necrosis factor- α (TNF- α) produced by the placenta decrease maternal insulin sensitivity among other things, which is crucial for the flow of nutrients to the fetus [3, 4]. The placental expression of the leptin, insulin-like growth factor-1 and -2 (*IGF-1* and *IGF-2*), insulin receptor (*IR*), hepatocyte growth factor (*HGF*) and hepatocyte growth factor receptor (*HGFR*) genes have significant impact on placental function and the developing fetus [5–7]. In addition, ghrelin produced by the placenta seems to be involved in the course of pregnancy [8]. Thus, the study of inflammatory and growth-promoting substances in the placenta is essential for our understanding of pregnancy-induced maternal adaptation, fetal growth and development, and pregnancy complications. However, the placental sampling site may influence the results of such studies.

The placenta contains several cell types, which are not uniformly distributed, and they express genes differently [9]. For example, inflammatory substances and growth-promoting factors are expressed by trophoblasts covering the villus tree, endothelial cells and placental macrophages found in the villus core [3, 6]. Furthermore, some genes, such as *leptin* may be expressed simultaneously in all these cell types, whereas other genes, for example, *TNF- α* , may be expressed in only one of the cell types at the time, to generate pleiotropic biological effects [3]. Earlier studies evaluating the importance of the sampling site in the placenta have shown divergent results. The expression of genes encoding human chorionic gonadotropin, human placental lactogen, placental growth

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hormone, genes related to hypoxia and genes with aberrant expression in intrauterine growth restriction (IUGR), and pre-eclampsia (PE) were all found to be dependent on the sampling site [10–13]. However, the expression of genes involved in local hemostasis and angiogenesis were not [14]. To the best of our knowledge, the impact of the sampling site on the placental expression of genes encoding inflammatory or growth factors has not been explored. This study aimed at investigating this issue in full-term placentas from healthy women with uncomplicated pregnancies. To minimize the possible influence of the fetal sex [15], we included an equal number of placentas from girls and boys. Our hypothesis was that the gene expression of inflammatory substances or growth factors differ by sampling site.

Materials and methods

Subjects

We included 12 women in this study. For estimation of the sample size needed, we reviewed previous publications on the subject [10, 11]. One study found differences in gene expression according to sampling site using 10 placentas [10] and another using six placentas [11]. The women in this study gave birth between May 2013 and April 2015, all with Nordic ethnicity. To be included in the study, the woman had to be healthy, have normal weight [body mass index (BMI) 18.5–25 kg/m²] and gain weight during the pregnancy within the recommendation of the Institute of Medicine (11–16 kg) [16]. Furthermore, the pregnancy had to be uncomplicated and singleton. The delivery had to be vaginal as placental gene expression seems to be affected by delivery mood [15, 17]. It also had to be uneventful and spontaneous at full term (38–42 weeks of gestation). The newborn baby had to be healthy with an Apgar score above 7 at 5 min of age and without any signs of malformation. The infant's birth weight was not restricted in the study. The newborns' birth weight and length z-scores were calculated using a Swedish reference population [18]. We included an equal number of female and male placentas. Women who smoked during the pregnancy were not eligible for the study. Written informed consent was obtained from all participating women, and the study was approved by the Ethical Review Board in Uppsala, Sweden (2012/531).

Placental tissue sample collection

Only placentas with a centrally inserted umbilical cord (i.e. located within the inner third of the placental disk radius) and placentas without any macroscopic abnormalities were included in the study. The same person (MA) collected all samples. She was present at the delivery of all children included in the study to avoid delayed collection of tissue samples as this may impact gene expression [15]. Thus, all samples were taken immediately after delivery. The samples were taken from nine well-defined sites in each placenta as described

Figure 1: Locations of sampling sites 1A–3C within the placenta. A = mean of 1A, 2A, 3A; B = mean of 1B, 2B, 3B; C = mean of 1C, 2C, 3C. 1 = mean of 1C, 1B, 1A; 2 = mean of 2C, 2B, 2A; 3 = mean of 3C, 3B, 3A. Original artwork by Wyatt et al. [11] reproduced with permission.

previously by Wyatt et al. [11] (Figure 1). To obtain these samples, a triangular segment of each placenta was excised with its base located 2 cm from the lateral edge and its apex near the cord insertion. The decidua layer along with the basal plate, as well as the chorionic surface and membranes, were removed. The samples, approximately 0.5 g each, were rinsed in saline (Gibco, Life Technologies, Stockholm, Sweden) to remove blood and stored at –80°C in RNAlater (Ambion, Stockholm, Sweden) until analysis.

RNA extraction and cDNA synthesis

The procedures for RNA preparation and cDNA synthesis have been described previously [19]. Fifteen to 20 mg of placental tissue was homogenized in batches using TissueLyser LT (Qiagen, Nordic, Sollentuna, Sweden) for 4 min at 30 Hz and then placed in –80°C until RNA isolation with Allprep DNA/RNA/protein Mini Kit, (Qiagen) was completed according to the manufacturer's instructions. The RNA concentrations were measured by a Thermo Scientific NanoDrop 2000 (Fisher Scientific, Göteborg, Sweden). cDNA was synthesized from 100 ng of RNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. For each sample, four cDNA reactions were performed. They were pooled and stored at –20°C until further analysis.

Quantitative real-time PCR and choice of reference gene

Quantitative real-time PCR (qPCR) was performed using a 7900HT Fast Real-Time polymerase chain reaction (PCR) System (Applied Biosystems, Foster City, CA, USA). The probes used and their assay IDs are presented in Table 1. The PCR reagents and the well plates were obtained from Applied Biosystems. Samples from the same placenta were run in duplicate with a TaqMan® Fast Universal PCR Master Mix (2×, no AmpErase® UNG (Applied Biosystems™) in 384-well plates. Crossing threshold (Ct) values were calculated by 7900HT Fast Real-Time PCR System software using the second-derivative maximum method. Transcription of reference genes may differ between clinical groups [21]. Cytochrome c-1 (*CYC1*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (*YWHAZ*), and topoisomerase 1 (*TOP1*) were selected as possible reference genes. The expression stability value (Table 1) was calculated using the

Table 1: The probes used together with assay IDs of the genes of interest and the stability values of the reference genes.

	Stability value ^a	Assay ID
Inflammatory and growth factor genes		
Toll-like receptor 2 (<i>TLR-2</i>)		Hs00152932_m1
Toll-like receptor 4 (<i>TLR-4</i>)		Hs01060206_m1
Interleukin 6 (<i>IL-6</i>)		Hs00985639_m1
Interleukin 8 (<i>IL-8</i>)		Hs00174103_m1
Tumor necrosis factor alpha (<i>TNFα</i>)		Hs00174128_m1
Leptin		Hs00174877_m1
Ghrelin		Hs01074053_m1
Insulin-like growth factor 1 (<i>IGF-1</i>)		Hs01547656_m1
Insulin-like growth factor 2 (<i>IGF-2</i>)		Hs00171254_m1
Hepatocyte growth factor (<i>HGF</i>)		Hs00300159_m1
Hepatocyte growth factor receptor (<i>HGFR</i>)		Hs01565584_m1
Insulin receptor (<i>IR</i>)		Hs00961554_m1
Reference genes		
Cytochrome c-1 (<i>CYC1</i>)	0.004	Hs00357718_g1
Glyceraldehyde-3-phosphate dehydrogenase (<i>GAPDH</i>)	0.006	Hs99999905_m1
Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (<i>YWHAZ</i>)	0.014	Hs03044281_g1
Topoisomerase 1 (<i>TOP1</i>)	0.015	Hs00243257_m1

^aAccording to NormFinder [20]. The top-ranked genes are those with the lowest values.

“NormFinder” algorithm [20], and *CYC1* was chosen for normalization of the genes of interest as it showed the best stability value in our samples. The ΔCt method was used to normalize the expression level of the gene of interest to the reference gene *CYC1* [22]. The $\Delta\Delta\text{Ct}$ method was used to determine fold change in gene expression normalized to *CYC1* and relative to a control site [22]. Ct values reported as >35 or as “not detected” were considered to be negative, according to the manufacturer’s recommendation.

Statistical analysis

Data are given as the mean $\pm$ standard error of the mean (SEM) or as proportions (%). To reduce the number of comparisons needed, the nine sampling sites were collected into three variables (r_1 , r_2 and r_3). This accomplished in two steps. First, the mean values of the expression at the horizontal and the vertical axes were calculated. This gave rise to three horizontal mean values (sites A, B and C) and three vertical mean values (sites 1, 2 and 3), where site A represents the mean basal expression, site B represents the mean middle horizontal expression and site C represents the mean chorionic expression. Site 1 represents the mean central expression, site 2 represents the mean middle vertical expression, and site 3 represents the mean lateral expression (see Figure 1). Three transverse values also exist: the expression at sites 1A, 2B and 3C (see Figure 1). Second, the r value was calculated using Pearson’s correlation test. r_1 is the correlation between the values at sites 1, 2 and 3 (vertical location). r_2 is the correlation between the values at sites A, B and C (horizontal location) and r_3 is the correlation between the expression value at 1A, 2B and 3C (transverse location). This was performed for every normalized gene of interest in each placenta. Finally, r_1 was compared with r_2 , r_2 was compared with r_3 , and r_1 was compared with r_3 using a Wilcoxon signed ranks test. Statistical analyses were performed with SPSS version 22 (IBM Corporation, Armonk, NY, USA) and the level of statistical significance was set at a two-sided $P < 0.05$.

Results

Clinical characteristics

The clinical characteristics of the study subjects, divided into female and male newborns, are summarized in Table 2. The mean birth weight was approximately 200 g higher in girls than in boys, and a trend towards higher placental weight was seen in the female group of children.

Table 2: Clinical characteristics of women and their newborns divided into female and male infants.

	Female infant (n = 6)	Male infant (n = 6)	P-value
Women			
Maternal age, year	32.5 $\pm$ 1.7	30.0 $\pm$ 2.2	0.393
Early pregnancy BMI (kg/m ²) ^a	21.3 $\pm$ 0.5	20.7 $\pm$ 0.5	0.440
Gestational weight gain, kg	13.2 $\pm$ 0.8	13.2 $\pm$ 0.8	1.000
Multiparous, %	100	67	0.455
Infants			
Gestational age, week	40.4 $\pm$ 0.3	40.0 $\pm$ 0.4	0.467
Birth weight, g	3,615 $\pm$ 84	3,414 $\pm$ 168	0.310
Birth length, cm	49.7 $\pm$ 0.4	50.3 $\pm$ 0.8	0.496
Birth weight z-score	0.3 $\pm$ 0.2	-0.3 $\pm$ 0.3	0.107
Birth length z-score	-0.2 $\pm$ 0.2	-0.2 $\pm$ 0.4	0.972
Placental weight, g	596 $\pm$ 33	519 $\pm$ 16	0.062

Data are means $\pm$ SEM or proportions (%). P-value was calculated by independent-samples T -test or chi-square test. Statistical significance was set at $P < 0.05$. ^aEarly pregnancy weight was measured, and BMI was calculated in gestational weeks 6–10.

However, these differences did not reach statistical significance. Neither birth weight nor birth weight z-score correlated with placental weight ($r=0.063$, $P=0.845$ and $r=0.093$, $P=0.778$, respectively).

The gene expression of inflammatory substances and growth factors according to sample site

The expression of all genes was successfully analyzed and no Ct values exceeded 35. The coefficients of variation

(CVs) were below 11% for all transcripts. High CVs were obtained for *leptin* expression, with a mean CV of 7%. The mean CVs for the other transcripts were 2% or below. The expression of the *leptin* and the *IR* gene were high at sites 2C and 3C and differed significantly by sampling site (for *leptin*: r_1 vs. r_2 , $P=0.028$, and for *IR*: r_2 vs. r_3 , $P=0.041$). The comparison between r_2 and r_3 almost reached statistical significance for *leptin* ($P=0.050$) (Figure 2). In one of the placentas, the *leptin* expression was very high at site 2C (Figure 3). However, the difference between r_1 and r_2 in *leptin* expression was still statistically significant when that outlier was excluded from the calculations

Figure 2: The gene expression of inflammatory and growth mediators at nine different placental sampling sites (1A–3C according to Figure 1, $n=12$).

The expression at each site is given as the mean fold change ($\pm$ SEM) in relation to the expression at site 1A. r_1 , the trend of gene expression from the placental medial to lateral sites; r_2 , the trend of gene expression from the placental basal to chorionic sites; r_3 , the trend of placental transversal gene expression at the sampling sites 1A, 2B, and 3C. The comparisons between the trends (r -values) were performed with the Wilcoxon signed ranks test. * $P < 0.05$.

Figure 2: (continued)

($P = 0.028$). Furthermore, the difference between r_2 and r_3 in *leptin* expression reached statistical significance when the outlier was omitted ($P = 0.019$).

When separating data by sex, gene expression of *leptin* showed significant difference between r_1 and r_2 only in

female placentas ($P = 0.028$). When the outlier with high leptin expression at site 2C was excluded, a significant difference in *leptin* expression was seen also between r_2 and r_3 in female placentas ($P = 0.046$), but not in male placentas. Gene expression of *IR* showed a trend towards different

Figure 3: Heatmaps visualizing the expression of *leptin* and *IR* genes at nine sampling sites (1A–3C) in 12 different placentas. Rows, different placentas. Columns, different sites. F, female, M, male. Birth weights in grams. Birth lengths in centimetres. Placental weights in grams. Colour: red, higher gene expression, green, lower gene expression. Gene expression related to the reference gene cytochrome c-1 (*CYC1*).

expression when comparing r_2 vs. r_3 in male placentas ($P=0.075$). No significant difference in gene expression of *IR* between r_1 , r_2 or r_3 was seen in female placentas.

The expression of the other genes did not differ significantly by site (Figure 2). The heat maps of the expression profiles of *leptin* and *IR* showed that in addition to

differing expression by site, low or high expression generally occurred in different placentas (Figure 3).

Discussion

In this study, we compared the expression of 12 genes involved in inflammation or growth at nine different locations in placentas from uncomplicated singleton pregnancies delivered vaginally at full term. We chose to include an equal number of placentas from each sex. We found significant differences by sampling sites in the expression of two of the studied genes (*leptin* and *IR*), but not in the other genes. Both the *leptin* gene and the *IR* gene showed higher expression in the lateral regions near the chorionic plate compared to the central regions near the basal plate. The lateral-chorionic region is associated with impaired perfusion compared with the centro-basal region [11], and this may explain the increased expression of *leptin* and the *IR* found here since both *leptin* and *IR* are up-regulated by hypoxia [23, 24]. Increased *IR* expression by low-oxygen tension has been shown in preterm placental mesenchymal stem cells [24] indicating that the *IR* expression may be affected by oxygenation. *IR* is found mainly in endothelial cells in the placenta at full term [25]. Here, fetal insulin regulates insulin-dependent processes in the placenta, such as angiogenesis [6]. Increased insulin signaling increases the amount of nitric oxide [26], which in turn may increase the expression of the vascular endothelial growth factor (*VEGF*) gene [6]. *VEGF* is a well-known initiator of angiogenesis, and increased expression of its gene has been reported in the lateral-chorionic site of the placenta compared to the centro-basal site [11]. Thus, our finding of increased expression of the *IR* gene in the lateral-chorionic site of the placenta may reflect the poorer oxygenation in that part of the placenta and a means to manage it. *Leptin* is an important placental hormone with multiple functions regulating placental growth and development [5]. It is known to stimulate the synthesis of proteins, hormones and cytokines [27] and to inhibit apoptosis in placental cells by downregulating p53 during stress [28]. Perhaps the increased gene expression of *leptin* at the lateral-chorionic region found in this study is a result of impaired perfusion and reflects a regulatory attempt to reduce apoptosis in this region of the placenta. Our finding of regional differences in *leptin* expression agrees with an earlier report investigating children born appropriate-for-gestational age (AGA) or afflicted by IUGR [12]. That study found a difference in *leptin* expression between central and lateral regions in the AGA placentas

[12]. However, the AGA children in that study had a wider gestational age range than the children in our study, and some of the mothers were smokers [12]. Our findings indicate that the placental expression of *leptin* may be dependent on the sampling site even in non-smoking women with an uncomplicated full-term pregnancy.

A limitation of this study is the small sample size. We cannot exclude that more statistically significant differences between sampling sites may have been found if we had examined more placentas. The strengths of this study are that the placentas were strictly selected to exclude as many confounding factors as possible and that all samples were taken immediately after delivery by the same person. Furthermore, we attempted to avoid too many comparisons by using only three variables representing the horizontal, vertical and transverse gene expression of the nine sampling sites. Likewise, we examined four reference genes and chose the one most appropriate according to the stability values in our samples for normalization of the expression of the genes of interest.

The differences in expression we found between sampling sites were modest. They were all below 3-fold, which is consistent with the results of Wyatt et al. [11]. Furthermore, in 10 of our 12 investigated genes, no significant differences by sampling site were found. However, for two of our studied genes, the sampling site influenced the expression. This finding should be confirmed in future studies, and their causes should be explored. Special attention should be paid to the sampling procedure in future placental research as the placenta is a heterogeneous organ. Larger sample sets may overcome differences in placental structures between different sites, but we suggest that the lateral-chorionic part of the placenta should be avoided in sampling, when examining gene expression susceptible to impaired perfusion and oxygenation.

We conclude that sampling site may influence the placental expression of the *leptin* and *IR* genes, but not the expression of the other studied genes involved in the inflammatory cascade or growth in normal full-term pregnancy.

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