Molecular mechanisms influencing glucocorticoid and estrogen activity during the pregnancy

Ph.D. thesis

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INTRODUCTION

Steroids take part in several complex processes such as sexual differentiation and regulation of metabolism. Glucocorticoids are a branch of steroid hormones which are responsible for stress reaction. They play an important role in glucose, protein, fat, calcium and bone metabolism, immune function, growth and regulation of behaviour as well. The action of these lipophilic hormones is mainly mediated by the glucocorticoid receptors (GR), which belongs to the group of nuclear receptors. The binding of steroid hormone to the GR in the cytosol results in dimerization, activation and translocation to the nucleus, where its binding to different transcription factors influences the transcription of target genes. GR expression is present in different fetal tissues at the 40th gestational day. Since 1970 the beneficial effect of glucocorticoids on the fetal lung maturation has been known. All of these point to a significant role of glucocorticoids during fetal life.

Despite extensive research efforts, the cause of preterm delivery, preeclampsia and its severe variant, the HELLP syndrome (haemolysis, elevated liver enzymes and low platelet counts) remains an unsolved problem in obstetrics. Preeclampsia is a pregnancy-specific multisystem disorder, which can be characterized by hypertension, proteinuria and edema occurring after the 20th week of pregnancy. The cause of preeclampsia and HELLP syndrome is unknown, although there have been several experimental and clinical studies proposing some main pathogenetic factors which may play a role in the development of the disease. The generalized endothelial dysfunction with an increased level of free radicals and pathological maternal-fetal immune adaptation during early pregnancy can be influenced by glucocorticoids.

The activation of the fetal hypothalamic-pituitary-adrenal axis is supposed to play a central role in the initiation of the parturition. In lambs, lesions in the basal region of the fetal hypothalamus, fetal hypophysectomy or bilateral adrenalectomy in utero lead to prolonged pregnancy, whereas infusion of adrenocorticotropic hormone or glucocorticoid hormone in the fetal lamb results in premature parturition.

Polymorphisms of the GR gene have been studied in several diseases and pathologic states. These studies show that the increased or decreased glucocorticoid sensitivity caused by polymorphisms of the GR gene can predispose to the development of certain diseases. GR gene polymorphisms have not been investigated in preterm neonates, healthy and pathologic pregnancies. In the first part of my research I examined the clinical association between these conditions and the three most common polymorphisms of the GR gene.

The second part of my research includes studies on serum androgen and estrogen levels from the 7^{th} gestation week until delivery in a pregnant women suffering from hyperandrogenism. I wanted to explore whether hormonal parameters reflecting placental aromatase activity could predict the effect of maternal hyperandrogenism on the foetus. After delivery I studied *in vitro* the role of the placental aromatase activity in preventing androgen exposure of the foetus.

OBJECTIVES

Polymorphisms of the GR gene have been studied in several diseases and pathologic status. The results of these studies showed that associations could exist between the increased or decreased glucocorticoid sensitivity caused by certain polymorphisms of the GR and body composition, obesity, pathologic metabolic parameters and/or risk for certain diseases.

Polymorphisms of the GR gene have not been studied among preterm neonates, healthy and pathologic pregnancies. During my PhD studies I performed studies aiming to explore the following objectives

1) To compare the allelic frequencies of the BclI, N363S and ER22/23EK polymorphisms of the GR gene in preterm neonates born at 28-35 weeks of gestation and those found in healthy unrelated Hungarian adults in order to examine whether any associations could exist among the three polymorphisms of the GR gene, clinical parameters and perinatal morbidities of preterm neonates. In addition, I wanted to study possible associations between GR gene polymorphisms of preterm neonates and the effect of the glucocorticoid treatment of the mother for threatening preterm birth on clinical parameters and perinatal morbidities of preterm neonates.

2) To compare the allelic frequency of the BclI, N363S and ER22/23EK polymorphisms of the GR gene in pregnant women suffering from severe preeclampsia or HELLP syndrome and those detected in healthy unrelated Hungarian adults. To analyze whether any association could exist between the three polymorphisms of the GR gene and clinical parameters indicating seriousness of the disease. To find any association between the evolutionary pattern of the polymorphic BclI site in different species and susceptibility to preeclampsia that is specific to human.

3) To explore whether the BclI, N363S and ER22/23EK polymorphisms of the GR gene could have an impact on

weight gain and change in BMI in women during uncomplicated pregnancies. To compare weight gain and BMI changes during pregnancy among carriers and non-carriers of these polymorphisms.

4) To explore whether maternal hormonal parameters such as markers of placental aromatase activity could predict the effect of maternal hyperandrogenism on the foetus in case of severe maternal hyperandrogenism during the whole pregnancy. To confirm using *in vitro* studies on placental tissues obtained after delivery the protective effect of placental aromatase activity against androgen exposure of the foetus.

PATIENTS AND METHODS

4.1. Patient groups

4.1.1. Preterm neonates

A total of 125 randomly selected preterm neonates (62 boys and 63 girls) born at 28-35 weeks' gestation (median: 31 weeks) were enrolled. Gestational age was estimated using the last menstrual period confirmed by ultrasonography before the 20th weeks of pregnancy. The birth weight was adjusted to the gestational age according to customised centile.

Based on maternal glucocorticoid treatment, the study population was divided into two groups; the first group included neonates whose mothers were treated with 24 mg dexamethasone at least once 24 hours before delivery (n=68), whereas the second group involved neonates without maternal dexamethasone treatment (n=57). The following perinatal complications have been encountered: necrotizing enterocolitis (NEC), intraventricular haemorrhagia (IVH), patent ductus arteriosus (PDA), respiratory distress syndrome (RDS), bronchopulmonary dysplasia (BPD)

and sepsis. The diagnosis of NEC was based on clinical and radiologic symptoms. IVH was diagnosed by the neurosonograms. PDA was established on echocardiography and clinical signs after the 5th postnatal days. RDS was defined clinically by the need for respiratory support and oxygen in the presence of radiographic chest findings. BPD was diagnosed as an oxygen dependency at 32 weeks or longer according to gestational age. Neonatal sepsis was defined by positive blood or cerebrospinal fluid cultures and/or clinical evidence of sepsis.

4.1.2. Patients with preeclampsia and HELLP syndrome

The study included 150 pregnant women with severe preeclampsia, including 17 women with complete HELLP syndrome. In each case the cause of the preeclampsia or HELLP syndrome was idiopathic. The past medical history of all patients was unremarkable. Gestational age was estimated using the last menstrual period confirmed by ultrasonography before the 20^{th} weeks of pregnancy. The diagnosis of severe preeclampsia was established according to the American College of Obstetricians and Gynecologists (ACOG) recommendation (≥160 mmHg systolic or ≥110 mmHg diastolic blood pressures occurring after the 20^{th} weeks of gestation in women, whose blood pressure was previously normal, or proteinuria with protein excretion of 5 g or more in 24-h urine specimen). The complete HELLP syndrome was diagnosed according to the Mississippi classification (platelet counts ≤150.000/µl, aspartate transaminase, AST or alanine transaminase, ALT ≥40 U/L, and lactate dehydrogenase, LDH ≥600 U/L) [10]. The platelet counts, serum enzymes and urinary protein excretions were measured using fully automated analyzers.

4.1.3. Healthy pregnant women

The study included 300 healthy pregnant women as a control group, and as an independent study for the analysis of associations between GR gene polymorphisms and clinical parameters. Gestational age and birth weight were estimated as described earlier. Maternal weight and BMI were recorded at the first prenatal visit and before delivery.

4.2. Molecular genetic methods

4.2.1. DNA isolation

Total genomic DNA was isolated from peripheral blood lymphocytes of pregnant women, and from placental blood samples dried blood samples obtained from neonates for diagnostic and scientific purposes. Total genomic DNA was isolated from peripheral blood lymphocytes using a QIAamp DNA Blood Mini Kit. The blood spots samples were denatured by heat inactivation. The DNA samples were stored at -70 C-on until using.

4.2.2. Determination of the BclI, N363S and ER22/23EK polymorphisms of the GR gene

The BclI and N363S polymorphisms were determined by allele-specific polymerase chain reactions, as previously described by our work group. The ER22/23EK polymorphism was detected by restriction fragment length analysis following polymerase chain reaction amplification of the corresponding region of the GR gene. Heterozygote and homozygote genotypes were distinguished by DNA sequencing.

4.3. Statistical analysis

In the group of preterm neonates the birth weight was adjusted to the gestational age according to customised centile. The BMI was calculated from the weight (kg)/height (m^2) formula. The distribution of BclI

genotypes was in Hardy-Weinberg equilibrium in each group. Hardy-Weinberg equilibrium was not calculated for the N363S and ER22/23EK polymorphisms because of the low frequency of the polymorphic genotypes.

The frequencies of genotypes were compared in patients groups by □2 test analysis. Clinicopathologic variables were tested with T probe or Mann-Whitney U-test for quantitative data and with contingency table□2 test for qualitative data. A value of $p<0.05$ was considered to be statistically significant. The associations between genotype distributions and clinical characteristics including perinatal morbidities were evaluated using the one-way analysis of variance (ANOVA) procedure and contingency table□2 test or Fischer exact t-test. A value of $p<0.05$ was considered to be statistically significant. Statistical power analysis was performed using a web-based software package SPSS.

4.4. Studies on hyperandrogenism during pregnancy

4.4.1. Steroid hormone measurement

Steroid hormone levels were measured at the Clinical Endocrinology Laboratory of the 2nd Department of Medicine, Semmelweis University using radioimmunoassay methods.

4.4.2. Determination of placental aromatase activity

Microsomal fractions were prepared from placental tissues of a pregnant women suffering from severe hyperandrogenism during pregnancy. Aromatase activity was determined using the NADPH-regenerating system. The amount of estradiol formed from 1.5 μM added testosterone was determined by chromatography/flame ionization detection after 5 min. incubation at 37C.

RESULTS

5.1. Preterm neonates

5.1.1.Clinical findings

The gestational age, birth weight and gestational age-adjusted birth weight (illustrated as the number and percent of neonates with age-adjusted birth weight below –1, between –1 and +1, and above +1 standard deviations) were similar in neonates with and without maternal dexamethasone treatment. However, the Apgar scores at 1 and 5 minutes were significantly (p=0,002 and p=0,024, respectively) higher in neonates whose mothers received dexamethasone treatment. The prevalence of perinatal morbidities, such as NEC, IVH, PDA, and BPD was also lower in neonates with maternal dexamethasone treatment, but the difference between the two groups was not statistically significant. The frequencies of RDS and sepsis were similar in the two groups of neonates.

5.1.2. Carrier and allelic frequencies of the Bcl1, N363S and ER22/23EK polymorphisms of the GR gene

There were no significant differences in carrier and allelic frequencies of the BclI, N363S and ER22/23EK polymorphisms between preterm neonates and healthy controls consisting of 160 healthy unrelated Hungarian adults. The carrier and allelic frequencies of these three polymorphisms were also similar in the two groups of preterm infants.

5.1.3. Association between GR gene polymorphisms and clinical findings including perinatal morbidities

A significant association was detected between the carrier status of the BclI polymorphism and the gestational age-adjusted birth weight. The frequency

of the polymorphic BclI allele was significantly higher in neonates whose gestational age-adjusted birth weight was above +1 standard deviation compared to those who had a gestational age-adjusted birth weight in a range between –1 and +1, or below –1 standard deviation. This association was documented in the whole group ($p=0.004$, with ANOVA), and it was independent from the maternal prenatal dexamethasone treatment ($p=0.487$, with ANOVA).

By contrast, the N363S and ER22/23EK polymorphisms failed to show any associations with gestational age-adjusted birth weight or other clinical parameters.

There were no associations between any of the three GR polymorphisms and perinatal morbidities in either group of neonates.

5.2. Patients with preeclampsia and HELLP syndrome

5.2.1. Demographic data of women with pathologic and healthy pregnancies

As compared to healthy pregnant women, patients with pathologic pregnancies including those with severe preeclampsia and HELLP syndrome had significantly lower age at the time of delivery and a significantly higher BMI both at the beginning of pregnancy and at the time before delivery. The gestational age at the time of delivery and the birth weight were significantly lower in women with pathologic pregnancies than in healthy pregnant women. When the two groups of women with pathologic pregnancies were compared, women with HELLP syndrome had significantly lower BMI both at the beginning of pregnancy and at the time before delivery as well as lower increases of body weight and BMI during pregnancy than those with severe preeclampsia.

The blood pressure of women with pathologic pregnancies at the time of the diagnosis of severe preeclampsia or HELLP syndrome was markedly increased (systolic blood pressure, median, 179.7, range 150-240 mmHg, diastolic blood pressure, 111.2, range, 90-170 mmHg). The platelet counts, AST, ALT, γ-GT, LDH and ALP expressed as mean±SD or median (range) were 210±75 per µl, 27 (9-399) U/L, 20.5 (6-355) U/L, 23.8±18.8 U/L, 431 (170-1250) U/L and 297 (135-764) U/L in women with severe preeclampsia, respectively, and 92.5±39.2 per µl, 179 (40-744) U/L, 159 (34-583) U/L, 27 (9-199) U/L, 981 (721-3620) U/L and 337.5 (212-938) U/L in women with HELLP syndrome, respectively. All laboratory data of healthy pregnant women were in the normal range.

5.2.2. Carrier and allelic frequencies of the BclI, N363S and ER22/23EK polymorphisms of the GR gene in women with pathologic and healthy pregnancies

There were no significant differences in carrier and allelic frequencies of the BclI, N363S and ER22/23EK polymorphisms between women with pathologic pregnancies including both severe preeclampsia and HELLP syndrome and those with healthy pregnancies. However, when women with severe preeclampsia and those with HELLP syndrome were separately analysed, the BclI polymorphism proved to be significantly overrepresented in women with HELLP syndrome as compared to those with severe preeclampsia ($p=0.013$; Odds ratio, 2.56, 1.26 to 5.23) and women with healthy pregnancies ($p=0.004$; Odds ratio, 2.89, 1.45 to 5.74).

5.2.3. Associations between polymorphisms of the GR gene and clinical data and laboratory findings in women with pathologic pregnancies

Women with pathologic pregnancies including both severe preeclampsia and HELLP syndrome who were carriers of the BclI polymorphism had significantly higher AST (p=0.003), LDH (p=0.014) and alkaline phosphatase (ALP) (p=0.032) levels compared to non-carriers. The ALT was also higher and the PLT was lower in BclI polymorphism carriers compared to non-carriers, but the difference was not statistically significant (p=0.057 and p=0.050, respectively). In addition, women with pathologic pregnancies carrying the BclI polymorphism had significantly higher (p=0.006) systolic (median, 180; range, 150-240 mmHg) but not diastolic blood pressure (median, 110 range 90-170 mmHg) compared to non-carriers (systolic, median, 170, range, 150-230; diastolic, median, 110, range, 90-130 mmHg). The N363S and ER22/23EK polymorphisms failed to show any associations with clinical or laboratory parameters.

5.2.4. Alignment analysis of DNA sequences of the BclI site in vertebral species

Because preeclampsia and HELLP syndrome develop exclusively in human, we performed an alignment analysis of DNA sequences of the BclI site in 7 evolutionary distant species including Bos taurus (ENSBTAG00000019472), Canis familiaris (ENSCAFG00000006293), Homo sapiens (ENSG00000113580), Macaca mulatta (ENSMMUG00000000421), Mus musculus (ENSMUSG00000024431), Pan troglodytes (ENSPTRG00000017363) and Rattus norvegicus (ENSRNOG00000014096). None of the species other than human had the polymorphic G allele in DNA sequences corresponding to the BclI site.

5.3. Association between GR gene polymorphisms and weight gain during pregnancy in healthy pregnant women

In pregnant women the allelic frequencies of the BclI, N363S and ER22/23EK polymorphisms were 0.31, 0.05 and 0.023, respectively. Similar frequencies of these polymorphic alleles in healthy Hungarian population have already been reported (0.36,
0.03, and 0.015, respectively). In these pregnant women there were no associations between any of the three polymorphisms and body weight or BMI at the 1st trimester of pregnancy or before delivery. Also, the gestational age at the time of delivery and the birth weight of infants were also similar in carriers and non-carriers of the BclI, N363S and R22/23EK polymorphic alleles of the GR gene. However, we found that the final weight gain of pregnancy was significantly lower in heterozygous carriers of the ER22/EK23 polymorphism (median, 11.0 kg; range, 2–30 kg) compared with that found in non-carriers (median, 13.0; range, 2–38) (p=0.044). The increase of BMI during pregnancy also showed similar significant differences (p=0.044) between heterozygous ER22/23EK carriers (median, 3.9 kg/m2; range, 0.7–10.9 kg/m2) and non-carriers (median, 4.8 kg/m2; range, 0.7–14.8 kg/m2). In addition, the final weight gain corrected for the birth weight of newborns (weight of newborns subtracted from the weight of mothers before delivery) was significantly lower in heterozygous carriers of the ER22/23EK polymorphism (median, 7.1 kg; range, -2.1 to 26.2 kg) compared with that found in non-carriers (median, 9.8; range, -1.85 to 34.1 kg) (p=0.048). Statistical power analysis showed that the allele frequency was appropriate for this study (statistical power was 100%, b value 0, a confidential level 5%).

5.4. Hyperandrogenismus during pregnancy

A 33-year-old primagravida at the 7th week of pregnancy was referred because of increased facial hair. Her past medical history revealed that after

normal puberty, her menarche occurred at the age of 14 years. She underwent a bilateral ovarian fenestration for PCO syndrome at the age of 20 years, and then she was treated with oral contraceptives until the age of 33 years. One month after discontinuation of the pill she became pregnant.

At presentation she had symptoms of moderate hyperandrogenism (Ferriman-Gallwey score, 12/36). Her serum testosterone level was nearly two times higher than the upper limit of normal (8.3 nmol/l; normal pregnancy range, 3.5-4.8 nmol/l). At the 11th week of pregnancy the hormone measurements revealed serum testosterone of 15.2 nmol/l, dihydrotestosterone of 1.9 nmol/l, androstenedione of 85.7 nmol/l, dehydroepiandrosterone of 3.5 nmol/l, dehydroepiandrosterone-sulfate of 3.3 umol/l, and estradiol of 8358 pmol/l. Abdominal and pelvic ultrasound exams performed repeatedly during pregnancy showed no evidence of adrenal or ovarian masses or other structural abnormalities. There was a moderate increase of hair growth on her face and abdomen, but the pregnancy was otherwise normal. Serum hormone levels, regularly measured during pregnancy showed marked increases of testosterone, androstendione and estradiol, and some increase of sex hormone-binding globulin (SHBG), while dehydroepiandrosterone and dehydroepiandrosterone-sulfate remained in the normal range. Serum beta-hCG was slightly increased. Repeat fetal ultrasound examination indicated normal female external genitalia. The woman decided to keep the pregnancy independently of the risk of virilisation of the foetus, and she refused fetal karyotype exam.

At the 39 weeks of pregnancy she delivered a female newborn with normal female external genitalia. Umbilical cord hormone levels were normal, except a modest increase of serum testosterone. Placental aromatase activity, measured by conversion of testosterone to estradiol in microsomal

preparations was normal as compared to that found in placental tissues obtained from an uncomplicated pregnancy. At the age of six weeks serum androgen concentrations of the infant were normal and bone age was not accelerated. One week after delivery androgen levels of the mother decreased dramatically, but they were slightly above the upper limit of normal. Three months after delivery all hormone values of the mother returned to normal and symptoms of hyperandrogenism were also slightly improved.

After delivery the aromatase activity was similar in placental microsomal preparations obtained from the patient and from a woman with uncomplicated pregnancy (*in vitro* incubation 1.1 and 1.2 nmol estradiol/mg protein/min, respectively).

CONCLUSIONS

1. When studying the three frequent functional polymorphisms of the GR gene in preterm neonates, healthy and pathologic pregnancies I have established the following clinical associations.

   There were no significant differences in carrier and allelic frequencies of the BclI, N363S and ER22/23EK polymorphisms between preterm neonates and healthy unrelated Hungarian adults. A significant association was detected between the carrier status of the BclI polymorphism and the higher gestational age-adjusted birth weight. By contrast, the N363S and ER22/23EK polymorphisms of the GR gene failed to show any associations with anthropometric parameters or other clinical findings in preterm neonates. Based on our findings the BclI polymorphism of the GR gene

is a potentially important genetic modifier of age-adjusted birth weight, but it does not influence the outcome of maternal dexamethasone treatment on perinatal morbidity of preterm neonates.

2. There were no significant differences in carrier and allelic frequencies of the BclI, N363S and ER22/23EK polymorphisms between women with pathologic pregnancies including both severe preeclampsia and HELLP syndrome and those with healthy pregnancies. However, when women with severe preeclampsia and those with HELLP syndrome were separately analysed, the BclI polymorphism proved to be significantly overrepresented in women with HELLP syndrome as compared to those with severe preeclampsia. Women with pathologic pregnancies including both severe preeclampsia and HELLP syndrome who were carriers of the BclI polymorphism had significantly higher AST, LDH and ALP levels compared to non-carriers. The ALT was also higher and the PLT was lower in BclI polymorphism carriers compared to non-carriers, but the difference was not statistically significant. Our alignment analysis of DNA sequences of the BclI site in 7 evolutionarily distant species including human showed that none of the examined species other than human had the polymorphic BclI allele in DNA sequences corresponding to the BclI site. Because preeclampsia develops exclusively in human, it is tempting to propose that the BclI polymorphism of the GR gene may play a role in the susceptibility of HELLP syndrome. The N363S

and ER22/23EK polymorphisms failed to show any associations with clinical or laboratory parameters.

3. Our study showed that the weight gain and the increase of BMI was significantly lower in ER22/23EK carrier women with uncomplicated pregnancy. In addition, the final weight gain corrected for the birth weight of newborns was also significantly lower in heterozygous carriers than in non-carriers. These results confirmed and completed earlier observations showing that the ER22/23EK has beneficial effect on body composition. These findings suggest that the ER22/23EK polymorphism exerts a protective effect against an excessive weight gain during pregnancy. The N363S and BclI polymorphisms had not impact on weight gain in women with uncomplicated pregnancy.
4. Despite markedly elevated androgen levels present from as early as the 7th week of pregnancy until delivery in a woman with hyperandrogenism, the absence of virilisation was documented in the female foetus. By monitoring of hormone levels during the whole pregnancy and by measurement of the *in vitro* aromatase activity of placental tissues obtained after delivery I confirmed that normal aromatase activity is appropriately effective in preventing androgen exposure of the foetus during pregnancy.

LIST OF PUBLICATIONS

Publications directly related to the PhD thesis

Original articles:

1. **Bertalan R**, Patócs A, Nagy B, Derzsy Z, Gullai N, Szappanos Á, Rigó J Jr, Rácz K. (2009) Overrepresentation of BclI polymorphism of the glucocorticoid receptor gene in pregnant women with HELLP syndrome. Clin Chim Acta, 405:148-52. **IF: 2.96 (2008)**
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