University of Pécs
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Doctoral (PhD) dissertation

The etiologic role of apoptosis in the formation of such pathological states which are common in obstetrics and gynaecology; its connection to angiogenetic factors in developing intrauterine growth restriction

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Abstract

The etiologic role of apoptosis in the formation of such pathological states which are common in obstetrics and gynaecology; its connection to angiogenetic factors in developing intrauterine growth restriction

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Programmed cell death (apoptosis) plays a basic role in the development of the placenta and in the regulation process of its aging, in which pro-, (promoting programmed cell death) and antiapoptotic (inhibiting programmed cell death) genes take apart. Apoptotic genes are present in the placenta during the pregnancy, they are traceable from it. Apoptosis plays an important role in the etiology of obstetrical and gynaecologic diseases. From these diseases we examined the uterine leiomyoma, the premature birth, and the intrauterine growth retardation (IUGR). In the latter case we examined some genes regulating angiogenesis and its connections to genes taking apart in apoptosis.

As placental dysfunction is commonly identified in the development of premature birth and intrauterine growth restriction, so regarding the examination of predisposing genes and group of genes, the placenta and the sample obtained from it may count with importance.

From among the pro-, and antiapoptotic genes regulating apoptosis, BAX gene from the former group, and BCL2 from the latter group is the most important. The collective and complex examination of these genes can give a favourable account on the etiologic effect of apoptosis in premature birth. In connection with the regulation of angiogenesis we examined the VGFA, endoglin and PLGF genes.

The placental activity of the vascular endothelial growth factor A and endoglin genes are increased in case of pregnancies where intrauterine growth retardation was diagnosed compared to pregnancies with eutrophic foetal growth. The starting point of the phenomenon is an increase in the activity of placental endoglin, which because of its antiangiogenic effect leads to the formation of placental dysfunction and chronic foetal hypoxia. Due to the latter one the placental vascular endothelial growth factor A, as a sort of compensation, shows

increased activity. Through stimulating angiogenesis, it promotes the correction of blood circulation conditions.

In intrauterine growth restriction a decrease in the inhibition of apoptosis leads to a balance change of programmed cell death. In case of infants suffering from intrauterine growth restriction, decrease in the apoptotic inhibition evolved due to the under expression of the BLC2 gene, while the expression of the proapoptotic BAX gene does not change. To the intensified placental activity of the antiangiogenic endoglin gene accompanies the increased expression of the angiogenic VEGFA gene. The collective examination of the biological system of the apoptosis and angiogenetic phenomenon shows that the change of the balance of apoptosis is less compensable to the system where the mother, the foetus and the placenta take place, than the dysfunction of the angiogenic system.

The proapoptotic BAX gene increased activity was shown in the myometrial tissue samples of premature infants, however in the expression of antiapoptotic BCL2 gene significant change was not detected. It is probable that premature rupture of the membrane (the starting event of the premature birth) is a consequence of the functioning of the metalloproteinase enzymes, which activates due to the BAX gene.

Developing uterine leiomyoma disease, for which formation an apoptotic imbalance is responsible, the over expression of an antiapoptotic gene (BCL2) plays a role, instead of the under expression of proapoptotic gene. The positive family history for uterine leiomyoma did not significantly influence the myometrial tissue expression of either examined apoptotic gene. In conclusion, the apoptosis regulating genes play a smaller role in the genetic background of the disease.

INTRODUCTION

It is becoming a more general phenomenon for women having a child later in their life. So, while it has predominantly social causes, a lots of biological consequences (genetic and obstetric) has to be taken into account and professionals has to observe these pregnancies with increased attention.

Older maternal age raises the chance for the formation of several obstetrical and gynaecological diseases. Moreover it increases the risk of genetic disorders and the occurrence of foetal chromosome aberrations in a statistically significant rate during pregnancy.

The chance of having uterine leiomyoma (benign tumour of the smooth muscle of the uterus) associated to pregnancies is higher in case of women around or after the age of 40. This disease can have a major effect on the formation of a pregnancy and carrying it to term.

The older the maternal age is, the more common an obstetric pathological disease is formed, such as intrauterine growth restriction (IUGR), diseases in connection with high blood pressure during pregnancy or premature birth.

On this basis, my research work is directed into the examination of the genetic background of two major obstetrical (the intrauterine growth restriction, premature birth) and one gynaecologic (uterine leiomyoma) disease. I wanted to interpret the results of my research in respect to the most important clinicodemographic data.

In case of the intrauterine growth restriction I aimed to clarify the pathogenic background behind the imbalance of apoptosis and angiogenesis, while in case of premature birth I focused my examinations on the occurent etiologic factors of apoptosis.

Regarding the uterine leiomyoma, I tried to focus on the genetic regulation of the outstanding phenomenon of apoptosis and its possible changes concerning the tumour formation. My research work confirmed many changes in gene expression values and I tried my best to interpret it on the basis of the available clinical information. I hope my research work can contribute to understand the complex background of the most common obstetrical and gynaecologic diseases and it can give new ideas to assign further examinational directions.

AIMS

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The aims of my study are the following:

Intrauterine growth restriction

The genes studied during the gene expression examinations performed are the followings:

- BAX
- BCL2
- VEGFA (vascular endothelial growth factor A)
- Endoglin
- PLGF (placental growth factor)

1. Is there a connection between intrauterine growth restriction and the placental expression of the genes examined?
2. Is there a correlation between the severity rate of the intrauterine growth restriction and the placental activity of the genes examined?
3. Is the sex of the foetus affects the placental expression of the genes studied?
4. Is there a connection between the gestation age progression and the activity of the vascular endothelial growth factor A (VEGFA) in case of intrauterine retardation?

5. Is there a connection between the angiogenetic and apoptotic activity in case of intrauterine growth restriction?

Premature birth

The genes studied during the gene expression examinations performed are the followings:

- BAX
- BCL2

6. What is the rate of placental gene activity of the genes studied on placental samples from premature birth and childbirth resulting in a mature infant?
7. Is there a connection between the placental activity of the genes examined and the sex of the foetus?
8. Is there a correlation between the placental activity of the genes examined and gestational age during childbirth?

Uterine leiomyoma

The genes studied during the gene expression examinations performed are the followings:

- BAX
- BCL2

9. How does the expression activity of the examined genes change in uterine leiomyoma tissue samples compared to the expression activity measured in normal myometrial samples serves as control?
10. Can the difference in gene expression activity be confirmed between myometrium samples from patients with a positive anamnesis of uterine leiomyoma and control samples, in case of the studied genes?
11. Are the number of myoma nodules affects the gene expression activity of the myometrial tissue in case of the studied genes?
12. Are the gene expression activity of the myometrial tissue can be affected by the length of the breastfeeding period(s) in case of former pregnancy(ies), before the diagnosis of the uterine leiomyoma was set?

PATIENT SAMPLE AND METHODS

In connection with our examinations regarding intrauterine growth retardation we compared the gene expression activity of placental tissue samples from 101 new born babies suffering from IUGR with 140 eutrophic new born babies. The babies were born at the Department of Obstetrics and Gynaecology II of the Semmelweis University, between the period of 1st of January 2010 and 1st of January 2011.
In cases when tissue samples were taken, other several clinical information were also collected:

- age of the mother and the father
- obstetrical, genetic and general medical history
- gestational age at the time of delivery
- sex of the new born
- weight gain and BMI changes of the mother during pregnancy
- smoking during pregnancy
- weight of the pregnant women at her birth
- type of delivery
- the possibility of an imminent intrauterine asphyxia
- weight of the new born after birth
- Apgar-score after birth

To settle the diagnosis of IUGR, we defined the standard 10 percentile value as a borderline, which was based on the estimated weight of the foetus considering the sex and the gestational age. We classified the new born babies suffering from IUGR into to two groups, according to the severity rate of this state, which was 0-5 and 5-10 percentile and the basis of the classification was their weight values.
(In medical literature, both the 3 and 5 percentile threshold limit in sever intrauterine retardation occurs.)

In each cases examined, the type of delivery was decided by the clinical information, during sample processions, classification was not made based on the type of delivery.

During sampling from the placenta, we took a 2x2x2 cm ($8cm^3$) big tissue sample in each case, and samples were stored on -70 degrees until the gene expression examination was done.

In connection with our examinations regarding **premature birth,** patient sample was taken from the Department of Obstetrics and Gynaecology II of the

Semmelweis University between the period of 1st of January 2010 and 1st of January 2011. After the birth of a premature infant we took tissue samples from the placenta for the purpose of gene expression examinations, a total of 104 cases. Other several clinical and demographic data were also collected, which helped us to interpret the results of our gene expression examinations. We included those cases into our study, when the pregnancy has ended before the 37th gestation week, and/or the infant's weight at time of delivery was less than 2500 gramme. Beyond the cases of induced labour we ruled out the pregnancies when multiple gestation, developmental disorder, abnormal placental implantation and adherence or the congenital developmental disorder of the genitals occurred.
During the procession of the placental tissue samples obtained, the type of delivery was not relevant.

The following clinical information were collected:

- age of the mother and the father
- obstetrical, genetic and general medical history
- weight of the mother at the time she was born
- gestational age at the time of delivery
- sex of the foetus
- weight gain and BMI changes of the mother during pregnancy
- vaginal Streptococcus-B infection in the third trimester
- smoking during pregnancy
- disorder of carbohydrate metabolism during pregnancy
- other diseases in connection with pregnancy
- weight of the new born after birth
- Apgar-score after birth

In connection with our examinations regarding **uterine leiomyoma** patient sample was taken from the Department of Obstetrics and Gynaecology II of the Semmelweis University, between the period of 1st of May 2010 and 31st of October 2011.
We compared the gene expression results of tissue samples from 101 patients operated on uterine leiomyoma and the gene expression results of control cases. There were 110 control cases, where the indication of the hysterectomy was not based on oncological diagnosis. The preoperative diagnosis of uterine leiomyoma was settled by bimanual and ultrasound examinations. During the interpretation of the gene expression examination results we didn't make a difference based on the

type of the surgery done (vaginal or abdominal hysterectomy, myomectomy). The histological examination performed on the surgical dissection confirmed the preoperative diagnosis, and during the gene expression examinations we only took into consideration the cases, when the pre-, and the postoperative diagnosis were equivalent. In case of the control cases we followed the same concept.

Together with the gene expression, the following clinical information were collected:

- age
- uterine leiomyoma in medical history
- date of the first menstruation
- number of pregnancies, number of deliveries and its types
- summary of breastfeeding period (s)
- failed pregnancies (spontaneous abortion, missed abortion, intrauterine death)
- use of oral contraceptives and its length (summary)
- the content of ultrasound examination reports before surgery
- number, size and location of the myoma nodules
- method of the surgery done
- result of the histological examination
- the summarized length of previous pregnancies (We calculated on an average gestational week of 37/pregnancy. We didn't take it into consideration the pregnancies ended up with spontaneous or missed abortion as being too short in time.)

In case of myomectomy, we took a 1x1x1 cm ($1cm^3$) big tissue sample from the tumour, which was stored on -70 degrees until the genetic examination was done. If more myoma nodules were removed, than samples were taken from every nodules, and took the average of their gene expression examination results as final value. In the case of a hysterectomy, if it was possible, the tissue sample was taken from the myoma nodule directly. Inasmuch as the leiomyoma was not well separated from its surrounding, then a volume of an approximately 6-8 cm^3 tissue from the base of the uterus was removed. As control samples, we used 2x2x2 cm tissues resected from the base of the uterus, too.
The examinations were done in possession of a valid ethical permission for research. The patient gave their consent for the examinations after they were informed in detail and filled and signed the informed consent form.

RESULTS

1. Results of gene expression examinations in IUGR

1.1 In foetus suffering from intrauterine growth restriction, decrease in apoptosis inhibition occurs because of the under expression of the BCL2 gene. The expression of the proapoptotic BAX gene, which takes part in regulating the apoptosis, was unchanged. The placental activity of the antiangiogenic endoglin gene has increased and in parallel the angiogenic VEGFA gene's placental activity has become more expressed.

1. chart Placental activity of the apoptotic and angiogenesis regulating gene regarding intrauterine growth retardation

Name of the Gene	$\Delta Ct_{eutrophic} \pm SE^{(A)}$	$\Delta Ct_{IUGR} \pm SE^{(B)}$	α-value ± SE(α)$^{(C)}$	Ln 2^{α}	Change in gene expression
BAX	3.18±0.63	4.04±0.67	–0.86±0.39	0.13	no change in expression
BCL2	4.48±0.82	6.32±0.86	–1.84±0.81	–1.83	under expression
VEGFA	3.24±0.72	1.27±0.7	1.97±0.41	1.36	over expression
VEGFA*	4.02±0.68	1.76±0.81	2.26±0.77	1.56	over expression
endoglin	5.02±0.63	2.57±0.59	2.45±0.73	1.69	over expression
PLGF	3.68±0.76	2.34±0.56	1.34±0.78	0.92	no change in expression

A: ΔCteutrophic = Ctexamined gene– Ctβ-actin; *A: ΔCtmatured = Ctexamined gene – CGADPH
B: ΔCtIUGR = Ctexamined gene– Ctβ-actin; *B: ΔCtIUGR = Ctexamined gene – CtGADPH
C: α = ΔCteutrophic – ΔCtIUGR
eutróf = 140; nIUGR = 101
$p<0.05$: significant difference
*Control gene: GAPDH

1.2 No changes in the expression of the BAX-, and BCL2 genes were detected regarding severe and mild stage of intrauterine retardation.

1.3 In cases of severe stage of intrauterine retardation in new born babies (0-5 percentile weight range), the gene expression of the VEGFA gene in placental samples didn't show significant difference in comparison to the ones suffering from the mild stage of the disease (5-10 percentile weight range).

1.4 In case of severe and mild stage of intrauterine retardation, the gene expression activity of the endoglin gene didn't show significant difference in placental tissue samples obtained from new born babies.

1.5 In cases of severe stage of intrauterine retardation in new born babies (0-5 percentile), the gene expression of the PLGF gene in placental samples showed significant decrease in comparison to the ones suffering from the mild stage of the disease (5-10 percentile).

1.6 There were no sign of change in placental BAX-, and BCL2 gene expression activity regarding new born girls or boys suffering from intrauterine growth retardation.

1.7 There were no difference in sex of the new born babies suffering from IUGR regarding placental VEGFA gene expression activity.

1.8 There were no difference in endoglin gene expression activity regarding new born girls and boys suffering from IUGR.

1.9 In pregnancies when intrauterine retardation were diagnosed, the placental gene expression of the PLGF gene didn't showed significant difference regarding new born girls or boys.

1.10 In cases of intrauterine retardation developing before the 33rd week, the placental VEGFA gene expression showed significant over expression in comparison to the gene expression values of the eutrophic control cases. It was the same for the pregnancies when IUGR developed between the 33rd and 37th week and after 37th week.

2. Results of gene expression examinations in premature birth

2.1 Regarding the placental expression of the proapoptotic BAX gene and antiapoptotic BCL2 gene, the activity of the BCL2 gene compared to the gene expression of matured infants showed no significant change. However the over expression of the BAX gene were showable.

2.2 There were no significant difference in the placental gene expression of the BAX and BCL2 genes in premature girl or boy infants.

2.3 The values from placental gene expression of the BAX and BCL2 genes regarding premature deliveries and control deliveries showed diverse results. The expression of the BCL2 gene between the 24^{th} and 28^{th}, 28^{th} and 32^{nd}, and 32^{nd} and 36^{th} didn't show any difference. However the BAX gene over expression was experienced in deliveries between 28^{th} and 32^{nd}, and 32^{nd} and 36^{th}, while its activity didn't changed between the 24^{th} and 28^{th} gestational period.

3. Results of gene expression examinations in uterine leiomyoma

3.1 The expression of antiapoptotic BCL2 gene, in accordance to both control gene, showed a significant elevation compared to the gene expression of control group cases. The expression of proapoptotic BAX gene didn't show any significant difference.

3.2 Nor the expression of proapoptotic BAX gene, neither the antiapoptotic BCL2 gene were not influenced by positive medical history regarding uterine leiomyoma.

3.3 The number of myoma nodules didn't influence significantly the expression of BAX gene compared to the control cases, but the activity of BCL2 gene showed significant connection to the number of nodules. The more the nodule was, the most the expression of the gene increased ($p<0.05$).

3.4 The length of the breast feeding period in medical history didn't influenced significantly the gene expression activity of the BCL2 and BAX genes.

CONCLUSIONS

1. In case of intrauterine growth restriction, the under expression of the antiapoptotic BCL2 gene in the placental tissue was detected. The placental activity of the proapoptotic BAX gene didn't show any significant difference compared to the eutrophic control pregnancies. The conclusion of our result are the following: in intrauterine growth restriction a decrease in the inhibition of apoptosis lead to a balance change of programmed cell death.

   In the placental tissue samples of infants suffering from IUGR, the VEGFA and endoglin genes showed significant over expression compared to control cases. According to my hypothesis the increased placental activity of the antiangiogenic endoglin cause vascular dysfunction in the placenta, that leads to the formation of permanent hypoxia. This hypoxic state stimulates the increased placental activity of VEGFA, which gene through its angiogenic effect aims to correct the vascular background of the circulation.

   In placental tissue samples of infants suffering from IUGR, the activity of PLGF-gene didn't show significant difference compared to samples of eutrophic infants. It is probable that the PLGF gene doesn't show any relationship to vascular dysfunction which is in the background of intrauterine growth retardation however it shows promising predictive effect in connection with hypertensive diseases during pregnancy.

2. The severity rate of intrauterine growth restriction didn't have a significant effect on the placental activity of the examined BCL2 and BAX genes.

   The severity rate of intrauterine growth retardation didn't influence the placental activity of the VEGFA gene significantly.

   The severity rate of intrauterine growth restriction didn't have a significant connection with the placental activity of the endoglin gene.

   In severe cases of intrauterine growth restriction, the placental activity of PLGF gene decreased compared to the less severe cases, so the angiogenic activity of the gene decrease in cases of IUGR when foetal weight is between 0-5 percentile.

3. The sex of the infants didn't have any significant effect on the placental activity of the BAX or the BCL2 genes in case of IUGR.

   There were no significant difference between the gene expression activity values of the placental VEGFA gene of new born girls and boys suffering from IUGR.

   The sex of the infants didn't have any significant effect on the placental activity of the endoglin gene.

   The sex of the new born babies suffering from IUGR didn't show to have any significant effect on the placental expression of PLGF gene.

4. In all pregnancies with intrauterine growth restriction, irrespectively of gestational age, the placental VEGFA gene showed significant over expression values.

5. In case of infants suffering from intrauterine growth restriction, decrease in the apoptotic inhibition evolved due to the under expression of the BLC2 gene, while the expression of the proapoptotic BAX gene doesn't change. To the intensified placental activity of the antiangiogenic endoglin gene accompanies the increased expression of the angiogenic VEGFA gene. The collective examination of the biological system of the apoptosis and angiogenetic phenomenon shows that the change of the balance of apoptosis is less compensable to the system where the mother, the foetus and the placenta take place, than the dysfunction of the angiogenic system.

6. On the placental tissue samples of the premature infants, the increased activity of the proapoptotic BAX gene could be seen, while change in the expression of the BCL2 gene (inhibiting the apoptosis) was not detected. It is probable that premature rupture of the membrane (the starting event of the premature birth) is a consequence of the functioning of the metalloproteinase enzymes, which activates due to the BAX gene.

7. The placental expression activity of the BAX and BCL2 genes didn't show significant connection with the sex of the premature infants.

8. Gestational age didn't take significant effect on the placental activity of the antiapoptotic BCL2 gene. At the same time the proapoptotic BAX

gene showed significant over expression in premature deliveries between 28th and 32nd, and 32nd and 36th, while its activity didn't changed in cases of deliveries which ended between the 24th and 28th gestational period. According to this, apoptosis plays a role only in premature deliveries after the 28th gestational week.

9. The BCL 2 gene's activity in the myometrial tissue showed significant elevation compared to the control group. Developing uterine leiomyoma disease, for which formation an apoptotic imbalance is responsible, the over expression of an antiapoptotic gene (BCL2) plays a role, instead of the uder expression of proapoptotic gene.

10. The positive family history for uterine leiomyoma didn't influence significantly the myometrial tissue expression of either examined apoptotic gene. In conclusion, the apoptosis regulating genes play a smaller role in the genetic background of the disease.

11. Between the number of myoma nodules and the myometrial tissue expression activity of the BCL2 gene showed significant connection, which was not able to be confirmed in case of the proapoptotic BAX gene. According to this, in the formation of uterine leiomyoma the BCL2 gene plays a role in disease development, since the fact of tumour formation and the number of myoma nodules show a relationship with the tissue activity of the gene.

12. Although the length of breast feeding period(s) didn't take an effect on the examined apoptotic genes' function, in case of shorter periods of breast feeding in medical history, the antiapoptotic effect (BCL2 gene activity) showed to be more expressed (however not in a significant way).

LIST OF OWN PUBLICATIONS

PUBLICATIONS IN CONNECTION WITH THE THEME OF THE DISSERTATION

I Szentpéteri, A Rab, L Kornya, **L Kovács**, JG Joó
Gene expression patterns of vascular endothelial growth factor (VEGF-A) in human placenta from pregnancies with intrauterine growth restriction
JOURNAL OF MATERNAL-FETAL & NEONATAL MEDICINE 26:(10) pp. 984-989. (2013)
IF: 1,208

I Szentpéteri, A Rab, L Kornya, P **Kovács**, R Brubel, J G Joó
Placental gene expression patterns of endoglin (CD105) in intrauterine growth restriction
JOURNAL OF MATERNAL-FETAL & NEONATAL MEDICINE 27:(4) pp. 350-354. (2014)
IF: 1,367

P Kovács, JG Joó, V Tamás, D Burik-Hajas, Zs Molnár, J Bódis J, L Kornya
The role of apoptosis in the complex pathogenesis of the most common obstetrics and gynaecology diseases
PHYSIOLOGY INTERNATIONAL 107 pp. 106-119. (2020)
IF: 0,73

P Kovács, A Rab, I Szentpéteri, JG Joó, L Kornya
Placental gene activity of significant angiogenetic factors in the background of intrauterine growth restriction.
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