Accentuated placental apoptosis as shown by TUNEL reaction in pregnancies complicated by intrauterine growth restriction and pregnancy induced hypertension

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Abstract
Apoptosis is a physiologic, programmed, active and suicidal cell death. Apoptosis acts in opposition to mitosis and both processes decide about the number of cell in each population. Objective: The purpose of this work was to estimate the level of placental apoptosis in pregnancy induced hypertension and intrauterine growth restriction. Material and method: Study groups consisted of 23 pregnant women with pregnancy induced hypertension and 21 patients with pregnancies complicated by intrauterine growth restriction. The control group consisted of 26 pregnant women with uncomplicated pregnancies and without any clinical symptoms of intrauterine infection. From the placental tissue 191 immunohistochemical slides in TUNEL staining were prepared. (59 slides in control group, 69 in PIH group and 63 in IUGR group). The analysis of all slides were performed by the same observer in light microscopy. Results: The highest percentage of TUNEL positive cells in histochemical slides was detected in endothelial cells of vessels. It was the highest in the group of patients with intrauterine growth restriction coexisting with pregnancy induced hypertension. Conclusions: Apoptosis is present in all types of placental cells in both complicated and uncomplicated pregnancies (pregnancy induced hypertension and intrauterine growth restriction). The highest percentage of apoptotic cells is located in endothelium and is associated with mentioned complications of pregnancy. Increased apoptosis is probably associated with insufficient invasion of trophoblast and decreased concentration of oxygen in placenta.

Key words: TUNEL reaction, apoptosis, intrauterine growth restriction, pregnancy induced hypertension

Introduction
Apoptosis is a physiologic, programmed, active and suicidal cell death. Apoptosis acts in opposition to mitosis and both processes decide about the number of cells in each population [1]. It is the reason why apoptosis is so vital to the development, growth, embryogenesis and involution of different organs. It is necessary to highlight the key role of apoptosis in elimination of injured, precancer cells and cells which underwent mutations. Cells with damaged DNA strands are directed to phase G1 of the cell cycle and arrested until the genetic lesion is repaired; otherwise, the program of self-destruction is started [2-4].

In early pregnancy placental villi are developing surrounded by maternal blood, finally becoming branched structures which form terminal villi. They consists of stroma, with fetal blood vessels, layer of cytotrophoblast cells, which are covered by syncytiotum and syncytiotrophoblast cells [5]. Syncytiotrophoblast forms barrier between fetal and maternal circulatory systems, and plays a crucial part in function of placenta. The process of cytotrophoblast turnover into syncytium is associated with presence of caspase 8 [5]. The intensity of placental villi apoptosis, which is a physiologic process, changes throughout the pregnancy and it is less intensive in the first trimester and increases with advancing gestational age becoming the most intensive in the third trimester, especially after 40th gestational week. Increased placental apoptosis has been detected in following pathologies: recurrent early pregnancy loss, preeclampsia, IUGR and gestational trophoblastic diseases. As a consequence of increased placental apoptosis take place a process of liberation of syncytiotrophoblast microparticles and is found in maternal circulation from early stages of pregnancy [5, 6]. The amount of liberated placental material is increased in following complications of pregnancy: pregnancy induced hypertension and IUGR. The presence of STBM

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Material and methods

The two study groups consisted of 23 pregnant women with pregnancy induced hypertension (PIH) and 21 patients with pregnancies complicated by intrauterine growth restriction (IUGR). The clinical characteristics of the study groups are presented in the table 1. The control group consisted of 26 pregnant women with uncomplicated pregnancies and without clinical symptoms of infection. As a control for TUNEL reaction negative control of placental villous sample was used prepared according to the protocol delivered by the ROCHE company for the negative control. A variable number of tissue samples were taken from the central part of each placenta, after which H & E staining was initially performed. This was followed by TUNEL staining (191 TUNEL slides were analyzed: 59 slides in control group, 69 in PIH group and 63 in IUGR group). Microscopic analysis using light microscopy was performed by the same observer.

Placental samples

Immediately after delivery, tissue samples were obtained to approximate 3 cm$^3$ of tissue from the central part of the placenta. One portion was stored in 10% buffered formalin for 48 hours and subsequently embedded in paraffin.

TUNEL (TdT-mediated dUTP-X nick end labeling) method

Tissue samples were checked for DNA fragmentation, as shown by TUNEL positive cells. H&E staining was performed to facilitate visualization of morphologic villous structure. Before the TUNEL staining was used, the samples were washed in PBS and incubated for one hour at room temperature in blocking solution, after which the samples were again washed in PBS and placed in permeabilizing solution for 2 min in 4°C.

Table 1. Clinical characteristics

<table>
<thead>
<tr>
<th>Clinical characteristic</th>
<th>Control (n = 26)</th>
<th>Pregnancy induced hypertension (n = 23) PIH</th>
<th>Intrauterine growth restriction (n = 11) IUGR</th>
<th>Intrauterine growth restriction with pregnancy induced hypertension (n = 10) IUGR-PIH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age ± SD</td>
<td>28.7 ± 5.8</td>
<td>30.1 ± 6.4</td>
<td>28.4 ± 6.2</td>
<td>27.5 ± 5.4</td>
</tr>
<tr>
<td>Mean gestational age (gestational weeks ± SD)</td>
<td>38.3 ± 1.4</td>
<td>36.6 ± 3.1</td>
<td>34.5 ± 2.7</td>
<td>34.1 ± 2.5</td>
</tr>
<tr>
<td>First pregnancy (%)</td>
<td>45</td>
<td>50</td>
<td>59</td>
<td>50</td>
</tr>
<tr>
<td>Multipara (%)</td>
<td>55</td>
<td>50</td>
<td>41</td>
<td>50</td>
</tr>
<tr>
<td>Cesarean section (%)</td>
<td>38</td>
<td>70</td>
<td>94</td>
<td>100</td>
</tr>
<tr>
<td>Others obstetrical operations (%)</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Physiological deliveries (%)</td>
<td>58</td>
<td>30</td>
<td>6</td>
<td>0</td>
</tr>
</tbody>
</table>
To distinguish 3-OH groups which appear after the DNA double strand is broken, 25 µl of TUNEL (ROCHE) reagent mixture is applied to the slides and left for 1 hour at 37°C. Then 25 µl of POD (peroxidase) is added and the slides are incubated for 30 min. at 37°C. Next, 50 µl DAB (diaminobenzydyne) is added and the slides are left for 10 minutes at room temperature. They are then washed in PBS and stained by 0,1% H&E for 3 minutes at room temperature. At the end, the cells with undamaged DNA are stained in blue, whereas TUNEL positive cells are stained in brown.

The slides were analyzed with light microscopy (Je- naval-Zeiss, Germany) a 600× microscopic magnification and 1600x computer magnification.

Statistical analysis

For the data analysis Kruskal-Wallis test was used. For slides analysis Image J software was used. GraphPad software (InStat, USA) and Statistica for Widnows (Stat-Sof) were used for statistical analysis.

Results

Examples of histopathologic analysis in studied groups (controls, IUGR, PIH) are shown in figure 1-3.

Table 2 shows the percentage of TUNEL positive cells among all cells in the field of vision. Significant difference in the percentage of TUNEL positive cells was observed for trophoblast between pregnancies complicated by PIH and IUGR.

The highest percentage of TUNEL positive cells was detected in endothelial cells of blood vessels. It was the highest in patients with IUGR and when this complication coexisted with PIH. In this latter group of patients the highest percentage of TUNEL positive cells were present in all types of examined cells. The difference between percentage of TUNEL positive cells in the patients with PIH alone and the patients with PIH and IUGR was significant (p 0,05) [table 2].

Discussion

The process of apoptosis is observed in all elements of villous structure in both uncomplicated and complicated pregnancies, thereby influencing function and activity of the placenta [12, 13]. Our experimental findings mirror those of Smith et al., but also show that this process is accentuated in pregnancies complicated by PIH and IUGR [15].

The characteristic morphologic qualities of the cells which undergo apoptosis is the most reliable evidence for its presence. The most characteristic quality of apoptosis using light microscopy is chromatin condensation, which leads to production of multiple dense apoptotic bodies [14, 15].

One of the unsolved questions in the pathogenesis of preeclampsia is the association between placental ischemia and endothelial cell dysfunction. Systemic endothelial damage occur often in preeclampsia [15-17].
Table 2. The percentage of TUNEL positive cells among all cells in the field of vision (a), stroma cells (s), endothelial vessels cells (e), trophoblast cells (t), (mean value ± SD)

<table>
<thead>
<tr>
<th>Type of cells</th>
<th>Control (n = 26)</th>
<th>Pregnancy induced hypertension (n = 23) PIH</th>
<th>Intrauterine growth restriction (n = 11) IUGR</th>
<th>Intrauterine growth restriction with pregnancy induced hypertension (n = 10) IUGR-PIH</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>35,1 ± 7,9</td>
<td>36,7 ± 9,6</td>
<td>35,8 ± 10,2</td>
<td>39,9 ± 11,4</td>
</tr>
<tr>
<td>s</td>
<td>48,3 ± 9,1</td>
<td>49,0 ± 10,3</td>
<td>49,7 ± 11,6</td>
<td>50,0 ± 11,5</td>
</tr>
<tr>
<td>e</td>
<td>58,0 ± 9,6</td>
<td>57,2 ± 10,6</td>
<td>58,1 ± 9,3</td>
<td>60,5 ± 9,1</td>
</tr>
<tr>
<td>t</td>
<td>17,4 ± 7,8</td>
<td>17,0 ± 7,9</td>
<td>18,1 ± 7,7</td>
<td>23,1 ± 9,1*</td>
</tr>
</tbody>
</table>

*p < 0,05 between PIH and IUGR-PIH

One of the theories for this presented by Redman and others, is that systemic endothelial damage is caused by deposition of syncytiotrophoblast microvillus membrane particles in endothelium [16, 17]. These microelements can be detected in the plasma of normal pregnancies but the amount of them is increased in women with preeclampsia. Increased syncytiotrophoblast release in preeclampsia is caused by more intensive apoptosis in trophoblast. It is proposed, that apoptosis plays an important role in the exchange of old syncytium and in its replacement by a new trophoblast cells. Apoptotic nuclei are found in syncytial knots and probably they appear there as a consequence of shedding of syncytial fragments into the maternal circulation [17-19]. This process is observed in overabundance in the syncytium of placentas from pregnancies complicated by preeclampsia and intrauterine growth restriction. When the maternal immune and circulatory systems are unable to compensate for this overabundance, generalized and systemic endothelial damage usually occurs [17].

Another interesting hypothesis is that elevated secretion of TNF-α induces increase of apoptosis in endothelial cells [6, 21, 22]. The next evidence for apoptosis-induced trophoblast microparticles release is the increased level of free DNA in the circulatory system of pregnant women with preeclampsia. In 1997 Smith et al detected the presence of apoptosis in placentas of patients with IUGR [15]. This group was the first that estimated the coefficient of placental apoptosis in uncomplicated pregnancies. They reported presence of apoptosis in all types of placental cells. More than 50% of apoptotic cells were detected in trophoblast cells.

In our study we examined placentas from clinical complications associated with placental hypoxia and oxidative stress. Our results highlight the link between poor placentation, oxidative stress and liberation of apoptotic material into the maternal circulation. In the two stage model of development of preeclampsia oxidative stress is the primary placental problem causing preeclampsia. Oxidative stress is one of many forms of cellular stress [20, 23, 24]. Reactive oxygen species form kind of response stress pathways. An initial short lasting reaction is causing longer duration homeostasis response, acting as long as oxygen stress is present [25-28]. The development of the cellular stress is associated with protein production response, which takes place in endoplasmic reticulum. Often the need for specific proteins production in oxidative stress exceeds capacity of the endoplasmic reticulum what leads into deeper homeostasis disturbances. In pregnancy induced hypertension there are many indicators of syncytiotrophoblast stress, one of them are STBM [5, 17]. Their origin is uncertain. It is proposed that they represent a apoptotic or pre-apoptotic phase preceding the execution, irreversible phase, when the cell is directed to programmed death. In our study we examined the issue of placental apoptosis, which is not simple. Several authors described, increased number of microcellular material in syncytiotrophoblast in preeclampsia [16, 17]. From the other point of view there is a new evidence, that final phases of apoptosis in a syncytium can cause its destruction, because the process is not restricted as it is in mononuclear cells. If syncytial knots are the form of preapoptotic material, they may be a form of arrested apoptosis, what is associated with syncytiotrophoblast oxidative stress and generalized response for its presence [12, 28, 29].

Our study confirms the presence of apoptosis in all types of villous cells, but more so in placentas of patients with IUGR and PIH. Whereas the detection of apoptotic cells in normal pregnancies highlights the natural origin of apoptosis, it is important to recognize that the higher percentage of apoptotic cells were detected among patients with complicated pregnancies.

For this research project the approval from Ethics Committee, from Poznan University of Medical Sciences was given.

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Author's contribution to the manuscript:
Mateusz Madejczyk: project development, data collection, manuscript writing, Joanna Dorszewska: data collection, data analysis. Anna Dera: manuscript editing, Magdalena Frydrychowicz: data collection, Grzegorz H. Bręborowicz: data analysis.

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