Immunohistochemical evaluation of apoptosis in placentae from normal and intrauterine growth-restricted pregnancies

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Summary

Objective: To investigate the extent of apoptosis within the human placenta in tissues from normotensive term pregnancies and those complicated by intrauterine growth-restriction (IUGR).

Methods: A total of 18 placentas, 10 obtained from uncomplicated term gestations and 8 from intrauterine growth restricted fetuses were included in this study. Apoptosis was identified using a terminal deoxynucleotidyl transferase-mediated deoxyuridine triophosphate nick end-labeling technique (TUNEL, Boehringer, Mannheim, Germany) in paraffin-embedded sections.

Results: Apoptosis was predominantly detected in the villous trophoblast and stromal tissue. There were no differences in the incidence of apoptosis in different parts of the same placenta. The apoptotic index in placental tissue from uncomplicated pregnancies was 0.93±0.12. Significantly more apoptotic nuclei were detected in the placental tissue from IUGR gestation (4.2±2.96, $p<0.01$).

Conclusion: These results might point toward a possible role of apoptosis in the pathophysiology of intrauterine growth-restriction.

Key words: Apoptosis; Placenta; Intrauterine growth-restriction.

Introduction

Intrauterine fetal growth-restriction (IUGR) and preterm delivery remain the most important risk factors contributing to perinatal mortality and morbidity. However, the underlying pathophysiological mechanism of IUGR still remains unknown. A number of vasoactive substances, e.g. endothelins, thromboxane and nitric oxide have been reported to be involved in the regulation of the utero-placental blood flow [1-3]. Insufficient utero-placental blood flow may result in structural changes and hypoxic-ischaemic injury of the placental vasculature leading to increased vascular resistance, which might be seen by Doppler ultrasound examination [4]. IUGR is associated with structural changes within the placental vasculature, e.g. thickened basal lamina of terminal villi and increased deposition of stromal collagen, leading to chronic fetal hypoxia [5].

Apoptosis is a term introduced by Kerr and co-workers describing a physiological form of cell death, different from necrosis [6]. Apoptosis is the most common form of eukaryotic, cell death occurring under normal physiological conditions. It is most often found during cell turnover and tissue homeostasis, embryogenesis, cytotoxic immunological reactions, development of the nervous system and endocrine-dependent tissue atrophy [7-9]. Apoptosis can also be found in cells that have been exposed to different stimuli, e.g. hypoxia, hyperthermia, ionizing and radiation. In contrast necrosis occurs when cells are exposed to extreme variance from physiological conditions, which may result in damage to the plasma membrane.

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The biochemical hallmark of apoptosis is the degradation of the genomic DNA, an irreversible event committing the cell to die. In many cell types this DNA fragmentation has been shown to result from activation of an endogenous Ca^{2+} and Mg^{2+} dependent nuclear endonuclease. This enzyme selectively cleaves DNA at sites located between nucleosomal units (linker DNA) generating mono- and oligonucleosomal DNA fragments [7]. Thus in situ 3'- end labeling of DNA might be used in detecting apoptosis.

Morphological features of cells undergoing apoptosis include chromatin aggregation, nuclear and cytoplasmic condensation, partition of cytoplasm and nucleus into membrane bound vesicles (apoptotic bodies) which contain ribosomes, mitochondria and nuclear material. In vivo these apoptotic bodies are rapidly ingested by either macrophages or adjacent epithelial cells without inflammatory response or release of noxious contents [10]. In contrast necrotic cell death is often associated with extensive tissue damage due to the breakdown of the plasma membrane and release of the cytoplasmatic contents into the extracellular fluid [6, 10]. Recent studies have shown that apoptosis occurs in decidual and trophoblast tissue during implantation in mouse embryos [11]. Increased incidence of apoptosis has been demonstrated in syncytiotrophoblast of failing first trimester pregnancies [12, 13]. In two previous studies Smith and co-workers reported on a low incidence of apoptosis in placental tissue from term gestation, with higher incidences in placental tissue of IUGR gestations [14, 15].

The aim of this immunohistochemical was to analyse the incidence of apoptosis in placental tissue from IUGR gestation and to compare its incidence to uncomplicated gestation.

Materials and Methods

The study presented has been carried out in the Department of Obstetrics and Gynecology at the University of the Saarland, Germany.

We studied placentae from pregnancies complicated by IUGR >37th week of gestation (n=8). The placentae were compared with those collected from uncomplicated term deliveries (n=10). The placental tissue was collected immediately after an uncomplicated vaginal delivery or caesarean section for obstetric reasons from the lateral and medial part of the placenta.

IUGR was diagnosed if the birth weight was <10th percentile. A birthweight chart for German speaking countries was used [16]. Cases with evidence of active or potential infective processes, e.g. urinary tract infection, preterm rupture of membranes, chorioamnionitis or preeclampsia were excluded.

Light microscopy: All tissues were formalin (3.7% buffered formaldehyde) fixed and paraffin-embedded. Each placenta was sampled at random and formed into two different paraffin-embedded blocks (medial and lateral part of the same placenta). Four sections were cut for light microscopy for each sample. These four sections were cut, two each from the two different blocks of tissue. Apoptosis was examined by light microscopy at a magnification of x 400 (x 40 objective lens and x 10 eyepiece). Apoptotic cells were easily detected because they were labeled red, compared to the non-apoptotic cells which were labeled blue. Only those stained nuclei that additionally showed morphological characteristics of apoptosis (nuclear shrinkage with chromatin condensation into apoptotic bodies, annular chromatin) were considered as positive. For each placenta 40 fields of view were examined (10 fields from each section). Microscopical analysis was performed by two independent observers. TUNEL positive cells were analyzed by an apoptotic index. Therefore the number of apoptotic cells was divided by the total number of cells counted and multiplied by 100.

Tunel staining: The technique of TUNEL (terminal deoxynucleotidyl transferase [TdT] mediated fluorescein-dUTP nick-end labeling) staining was first described by Gavrieli *et al.* [17]. In brief, DNA strand breaks occurring during the apoptotic process can be detected by enzymatic labeling of the 3'-OH DNA ends with modified nucleotides, e.g. fluorescin-dUTP. Incorporated fluorescein is detected by antifluorescein antibody Fab fragments from sheep, conjugated with alkaline phosphate. After substrate reaction, stained cells can be analyzed under light microscope.

5µm sections were cut and mounted on microscope slides pretreated with 0.01% aqueous solution of poly-L-lysine. Deparaffinization was done by transfering the slides through the following solutions: 4 times to xylene bath for 5 min, and then for 5 min to 96% ethanol, 90% ethanol, 80% ethanol and to distilled water.

After dewaxing and rehydrating the tissue sections were incubated with proteinase K (10µg/ml) for 30 min at room temperature. Then slides were rinsed in phosphate buffered saline (PBS). Then TUNEL solution (TdT and fluorescein-dUTP), (Boehringer, Mannheim, Germany) was added to cover the slides and slides were incubated in a humid atmosphere at 37°C for 60 min. After terminating the reaction by rinsing with PBS, 50µl Converter-AP (anti-fluorescein antibody, Fab fragment from sheep, conjugated with alkaline phosphatase, Boehringer, Mannheim, Germany) was added to the samples. Slides were again incubated for 30 min at 37°C in humid atmosphere and afterwards rinsed three times with PBS. For visualization of the immunocomplexed AP 50µl substrate solution (Boehringer, Mannheim, Germany) wad added. The tissue was counterstained with hematoxylin. Negative controls (without the terminal deoxynucleotidyl transferase enzyme) and a positive control (after treatment with deoxyribonulease) were performed in each experimental set up. The medians and ranges were used for nonparametric data. The significance test used was the Mann-Whitney *U* test. Data is presented as mean ± SEM. A p-value <0.05 was accepted for significant differences between the groups.

Results

Perinatal data of the two study groups are presented in Table 1. In general, few apoptotic cells were seen in term placental tissue of uncomplicated pregnancies (Figures 1 and 2, Table 2). Apoptotis was predominantly detected in the syncytiotrophoblast layer with a lower incidence in trophoblasts and stromal cells in the two study groups. Only a few extravillous trophoblasts and amnion epithelial cells underwent apoptosis. Somewhat more apoptotic nuclei were detected in the decidua capsularis. Analysis of the medial and lateral parts of placental sections did not show any significant differences concerning the incidence of apoptotis. The mode of delivery, i.e. vaginal delivery, elective caesarean section, emergency caesarean section, had no influence on the incidence of apoptotis in the examined placental tissue. By contrast a significant increase in the incidence of apoptosis was identified in placental tissue of IUGR pregnancies at term, with the highest apoptotic index in the syncytiotrophoblast layer (Table 2, p<0.01), (Figure 3). The measured incidence of apoptotic nuclei by TUNEL was slightly higher than the incidence of apoptosis assessed by light microscopy alone (see discussion).

Table 1. — *Perinatal data of the two study groups.*

	Term pregnancies means (range)	IUGR > 37 weeks means (range)
Maternal age (years)	28 (20-38)	24 (18-41)
Gestational age (weeks)	40.0 (39.0-42.0)	38.0 (37.0-39.0)
Birth weight (g)	3477 (2930-4750)	2166 (1900-2470)
pH art.	7.30 (7.20-7.48)	7.28 (7.24-7.32)
Apgar 5 min	8.6 (6-10)	8.5 (8-10)
Apgar 10 min	9.5 (8-10)	9.5 (9-10)

Abbreviations: pH art.: arterial pH, g: gramm, IUGR: intrauterine growth restriction.

Table 2. — *Incidence of apoptotic nuclei expressed as apoptotic index, (means±SEM), in the two study groups.*

	Term pregnancies	IUGR > 37 weeks	p-value
CT	1.12±0.08	3.31±1.68	p<0.01°
ST	1.46±0.13	6.90±4.43	p<0.001°
SC	0.97±0.10	2.25±2.77	p<0.05°
over all	1.12±0.31	4.21±2.96	p<0.05°
p-value	p<0.01**	p<0.01*, p<0.001**	

CT: cytotrophoblast; ST: syncytiotrophoblast; SC: stromal cell; IUGR: intrauterine growth restriction.
*: CT vs. ST, **: ST vs. SC; °: IUGR vs. uncomplicated pregnancy.

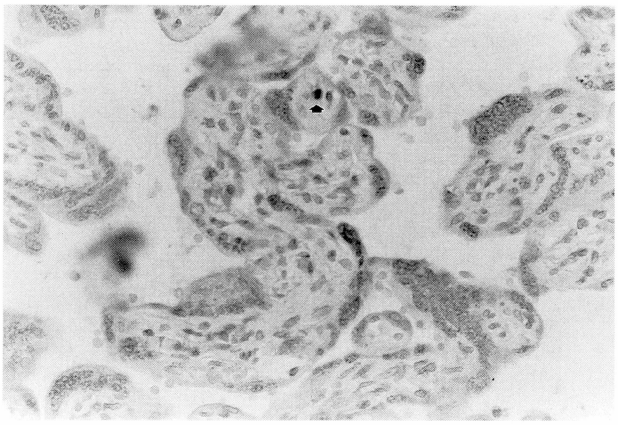

Figure 1. — Light microscopy of a 5μm section of term placenta. An apoptotic nucleus within cells of villous stroma is marked with **arrow.** Original magnification x 200.

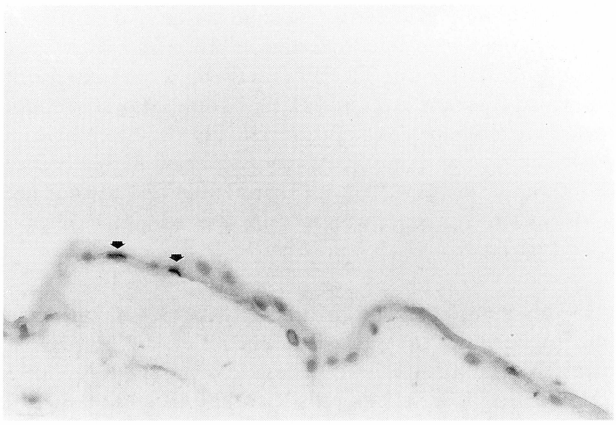

Figure 2. — Light microscopy of a 5μm section of term placenta. Apoptotic nuclei within the syncytiotrophoblast marked with **arrow.** Original magnification x 400.

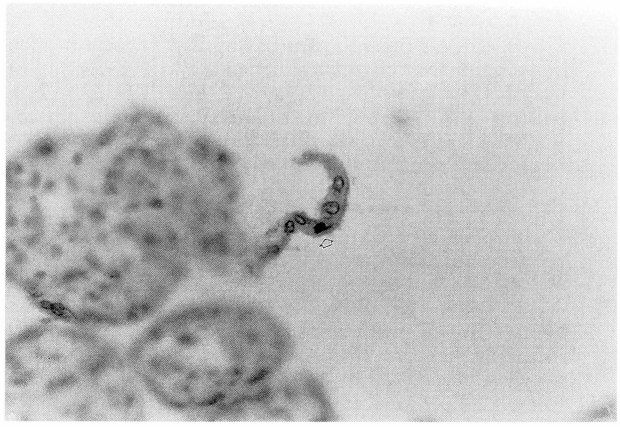

Figure 3. — Light microscopy of a 5μm section of IUGR placenta. Apoptotic nucleus within the trophoblast cell layer marked with **arrow.** Original magnification x 400.

Discussion

In the human placenta, comparable to other organs, cell development and function depends on the balance among proliferation of cells, maturation and cell death. Cell death can occur by either of two distinct mechanisms, necrosis or apoptosis. IUGR remains together with preterm birth the main risk factors for perinatal morbidity and mortality. The present study investigates the incidence of apoptosis in placental tissue in case of IUGR.

Apoptosis was first described by Kerr and co-workers as a mechanism of controlled cell death, distinct of necrosis [6]. It was suggested to be involved in cell homeostasis and physiological cell turnover not only in healthy tissues but also in malignant neoplasms [8]. The characteristic morphological changes include chromatin and cytoplasmatic condensation, and partition of cytoplasm and nucleus into membrane bound-vesicles (apoptotic bodies) which contain ribosomes, morphologically intact mitochondria and nuclear material. Apoptotic bodies are removed by various phagocytic cells without infiammatory response in the surrounding tissue. The DNA fragmentation resulting from activation of an endogenous Ca^{2+}/Mg^{2+} dependent nuclear nuclease has been described as the biochemical hallmark of apoptosis [7].

Apoptotic changes have been shown within the syncytiotrophoblast layer in the human placenta, proposing a possible role in the regulation of the maternal-fetal exchange [18]. Apoptosis has been suggested to play a role in physiologic and pathophysiologic mechanisms of the placenta, e.g. placental senescence and parturition [19, 20]. Increased incidence of apoptosis has been described in syncytiotrophoblast and decidual cells of spontaneous abortion in contrast to normal pregnancy, thus indicating a possible role of apoptosis in embryonic development [12]. In cases of complete hydatidiform mole the incidence of apoptosis was found to be higher than in normal placental tissue and p53 expression, known to be a promoter protein of apoptosis, was upregulated [13]. Additionally apoptosis was recently shown in human fetal membranes, with increasing incidence from midtrimester to term gestation [21]. In keeping with the latter report we have previously reported on a low apoptotic index (<1%) in term placental tissue of uncomplicated pregnancies, with a slightly higher apoptotic index (1-2%) in post-term gestation [20]. In our recent study the incidence of apoptosis was not related to the mode of delivery [20]. These findings support the hypothesis that apoptosis might be involved in a mechanism of placental senescence that is not associated with labor.

In the present study we demonstrate a significant increase of the apoptotic index in placental tissue of IUGR. The increase of apoptosis was most important in the syncytiotrophoblast layer and less important in cytotrophoblasts and stromal cells. Whether the observed increase in the incidence of apoptosis is a result of IUGR or is a part of the pathophysiological process leading to these complications is yet not known. These findings are in part in accordance with the report of Smith and coworkers, who found a significant increase of apoptosis in placental tissue of IUGR gestation [15]. However, Smith *et al.* reported on an incidence of only 0.14% of apopto-

sis in uncomplicated term placental tissue and 0.24% of apoptotic nuclei in IUGR gestation [14, 15]. Labeling of necrotic cells by the TUNEL method, which has been described by some authors [22, 23] could in part account for this discrepancy. Also, different TUNEL staining kits may identify apoptotic nuclei to a different extent before morphologic features can be seen. However, we did not perform electron microscopy to assess apoptosis in placental tissue.

In this study, we compared the extent of apoptosis in uncomplicated term gestations and in case of IUGR by using the TUNEL method. In conclusion our study shows an increased level of apoptosis in the placental tissue of IUGR gestation. A fact that might be partly involved in the pathophysiologic mechanisms of IUGR.

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