Immunohistochemical detection of apoptosis in the human term and post-term placenta

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Summary

Purpose: To study the incidence of apoptosis in human term and post-term placenta and to determine its presence in different areas of placentas of uncomplicated pregnancies.

Methods: A total of 15 placentas, 8 obtained from spontaneous deliveries and elective caesarean sections at term (37-41 weeks of pregnancy) and 7 from spontaneous deliveries and elective caesarean sections post-term (>41 weeks of pregnancy) were included in this study. Apoptosis was identified by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling method (TUNEL, Boehringer, Mannheim, Germany) in paraffin-embedded sections.

Results: Apoptosis was predominantly detected in trophoblast and stromal tissue. There were no significant differences in the incidence of apoptosis in different parts of placental tissue. A significant increase of apoptosis was seen in both trophoblast and stromal cells of post-term placentas ($p<0.05$; $p<0.005$).

Conclusion: Apoptosis could be detected in the human term and post-term placenta, with increasing incidence in post-term placental tissue, suggesting a possible role of apoptosis in the mechanism of parturition and placental senescence.

Key words: Placenta; Apoptosis; Pregnancy; Trophoblast.

Introduction

Apoptosis is a term introduced by Kerr and co-workers describing a physiological form of cell death, different from necrosis [1]. The greek term apoptosis is used to describe the "dropping off" or "falling off" of petals from flowers or leaves from trees. Apoptosis is the most common form of eukaryotic cell death. Apoptosis is a mode of cell death that occurs 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 [2, 3, 4]. Apoptosis can also be found in cells that have been exposed to different stimuli, e.g. hyperthermia, ionizing radiation and hypoxia. In contrast necrosis occurs when cells are exposed to extreme variance from physiological conditions, which may result in damage to the plasma membrane.

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 [5].

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 [6]. 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 [1, 6].

Apoptosis within placental tissue has been described before [7-10]. Apoptosis has been proposed as a mechanism in parturition and placental senescence, which is of particular interest to the perinatologist [8].

The aim of this immunohistochemical study was to quantify the incidence of apoptosis in human term and post-term placenta and to investigate its incidence in different parts of placental tissue by use of TUNEL staining.

Materials and Methods

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

Normal term and post-term placental tissue of singleton pregnancies free of visible infarct, calcification or heamatoma was collected immediately after uncomplicated vaginal delivery or elective caesarean section from the lateral and medial part of the placenta. All women were normotensive, denied smoking or alcohol abuse and had no medical illness. Neither placentas of growth retarded infants with a birth weight <10th percentile for gestational age nor cases of prolonged rupture of the membranes were included. The characteristics of the included cases are shown in Table 1.

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

	Term pregnancies means (range)	Post-term pregnancies means (range)
Age (years)	28 (20-38)	28 (23-33)
Gestational age (weeks)	40.0 (39.0-40.6)	41.5 (41.1-42.0)
Birth weight (g)	3241 (2,930-3,700)	3714 (2,960-4,750)
pH	7.33 (7.20-7.48)	7.28 (7.21-7.40)
Apgar, 5 mins	8.8 (8-10)	8.5 (6-10)
Apgar, 10 mins	9.6 (9-10)	9.4 (8-10)

Table 2. — *Incidence of apoptotic cells (means ± SEM) in term (n=8) and post-term (n=7) pregnancies*

	Incidence of apoptotic cells (%) Term pregnancy	Post-term pregnancy	p-value
Trophoblast cells (n=100)	1.12±0.13	1.46±0.12	p<0.05
Stromal cells (n=100)	0.74±0.10	1.20±0.12	p<0.005
Trophoblast cells + stromal cells (n=100)	0.93±0.12	1.33±0.11	p<0.005

Table 3. — *Comparison of observed incidence of apoptosis in trophoblast and stromal cells of term (n=8) and post-term (n=7) pregnancies*

	Incidence of apoptotic cells (%) Trophoblast cells (n=100)	Stromal cells (n=100)	p-value
Term placenta	1.12±0.13	0.74±0.10	p<0.001
Post-term placenta	1.46±0.12	1.20±0.12	n.s.

The technique of TUNEL (terminal deoxynucleotidyl transferase [TdT] mediated fluorescein-dUTP nick end labeling) staining was described first by Gavrieli *et al.* 1992 [11]. 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. fluorescein-dUTP. Incorporated fluorescein is detected by anti-fluorescein antibody Fab fragments from sheep, conjugated with alkaline phosphatase. After substrate reaction, stained cells can be analyzed under light microscope.

Placental tissue was fixed in 3.7% buffered formaldehyde, and embedded in paraffin. 5µm sections were cut and mounted on microscope slides pretreated with 0.01% aqueous solution of poly-L-Lysine. Deparaffinization was done by transferring 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). 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 a humid atmosphere and afterwards rinsed three times with PBS. For visualization of the immunocomplexed AP 50µl substrate solution (Boehringer, Mannheim, Germany) was added. The tissue was counterstained with hematoxylin. Negative controls (without TdT) were performed in each experimental set up. 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. For all samples, cells in five fields of view were counted. The number of apoptotic trophoblast and stromal cells was expressed as a percentage of 100 trophoblast and 100 stromal cells counted in the slide.

Medians and ranges were used for nonparametric data. The significance tests used were the Mann-Whitney *U* test and the Student's *t*-test. Data is presented as mean ± SEM. A p value <0.05 was accepted for significant differences between the groups.

Results

The characteristics of the cases enrolled in this study are presented in Table 1. Apoptotic cells could be detected in human term and post-term placental tissue of uncomplicated pregnancies by the TUNEL method. The majority of apoptotic cells were trophoblast or stromal cells. Only in a few endothelial cells was apoptosis detected. Figure 1 and Figure 2 are light microscope photographs showing a trophoblast nucleus (Figure 1) and a nucleus within villous stroma (Figure 2) that have undergone apoptosis. Analysis of the medial and lateral parts of term and post-term placental tissue did not show any significant differences in the incidence of apoptosis, neither in trophoblast cells, nor in stromal cells. The mode of delivery (uncomplicated vaginal delivery or elective caesarean section) had no significant influence on the incidence of apoptosis in placental tissue (data not shown). By contrast, a significant increase in the incidence of apoptosis in both, trophoblast and stromal cells, was demonstrated in the post-term placenta as compared to the term placenta (p<0.05; p<0.005), (Table 2). The incidence of apoptosis in trophoblast cells was significantly higher than in stromal cells in term but not in post-term placental tissue (p<0.001), (Table 3). However, the overall incidence of apoptotic cells in the term and post-term placenta was low (0.93% ± 0.12% vs 1.33% ± 0.11%, p<0.005).

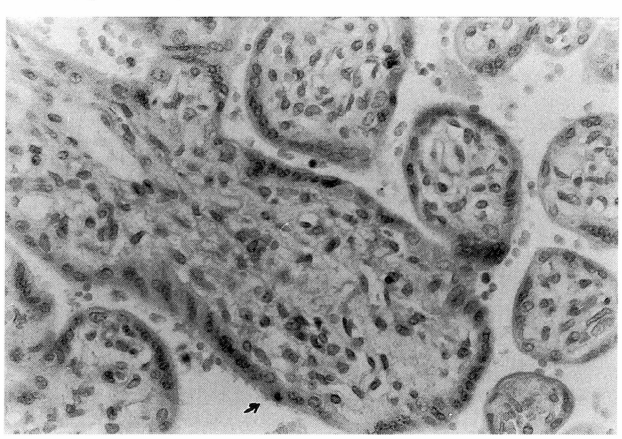

Figure 1. — Light microscopy of a 5µg section of term placenta. There is one apoptotic nucleus within trophoblast cells (*arrow*). Original magnification x 400.

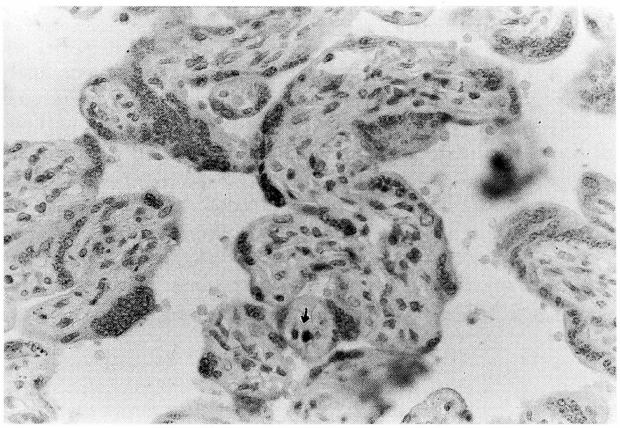

Figure 2. — Light microscopy of a 5µg section of term placenta. An apoptotic nucleus within cells of villous stroma is marked with *arrow*. Original magnification x 400.

Discussion

Apoptosis was first described by Kerr *et al.* as a mechanism of controlled cell death, distinct of necrosis [1]. It was suggested to be involved in cell homeostasis and physiological cell turnover not only in healthy tissues but also in malignant neoplasms [2]. 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 inflammatory response in the surrounding tissue. The DNA fragmentation resulting from activation of an endogenous Ca^{2+}/Mg^{2+} dependent nuclear nuclease was described as the biochemical hallmark of apoptosis [5, 12].

Recent studies have demonstrated that apoptosis occurs in human placental tissue [7, 8, 9]. Nelson *et al.* have shown apoptotic changes within the syncytiotrophoblast layer in the human placenta, suggesting a possible role in the regulation of the maternal-fetal exchange [10]. Apoptosis has been suggested to play a role in physiologic and pathophysiologic mechanisms of the placenta, e.g. placental senescence and parturition [8].

The present study investigated the incidence of apoptosis in different parts of human placenta and compared the incidence of apoptosis in trophoblast and stromal cells in the human term and post-term placenta. As mentioned in the results section, we could detect apoptotic cells predominantly within trophoblast and stromal cells, with only few apoptotic endothelial cells. These findings are in keeping with the report of Smith *et al.* who identified a majority of apoptotic cells in the third trimester placenta as trophoblast or stromal cells and with the results of Mayhew *et al.* who reported on a proportion of 40% trophoblast cells, 47% stromal cells and 13% endothelial cells in the human placenta at term [7, 13, 14].

Our study did not show any significant differences in the incidence of apoptosis in different parts of the same placenta, neither in term nor in post-term placental tissue, which is in good accordance to the results of Smith *et al.* [7].

There is an increasing body of evidence, that apoptosis might play an important role during implantation, early pregnancy and placental senescence. Kokawa *et al.* demonstrated in their study, that apoptosis occurs in trophoblasts and decidua during the first trimester of pregnancy and that its incidence is increased in cases of miscarriage [15]. Lea *et al.* examined the immunostaining of bcl-2, a proto-oncogene believed to inhibit apoptosis, of biopsies from first trimester failing pregnancies. Immunostaining of bcl-2 was less intensive in failing pregnancies [16]. Sakuragi *et al.* described that bcl-2 expression is most prominent in normal placental tissue and that there might be an inverse relation between bcl-2 and p53 expression, a proto-oncogene believed to promote apoptosis in trophoblast cells [17]. Kim *et al.* reported a decrease in the expression of bcl-2 within placental tissue in the third trimester [9].

In view of the facts mentioned-above, our finding of a significantly higher incidence of apoptosis in the post-term placenta compared to the term placenta is in good accordance. However, we did not examine bcl-2 expression in placental tissue.

Identification of apoptosis by use of morphologic criteria alone might be difficult. Therefore we used TUNEL staining which is believed to be a very sensitive and specific method for the detection of apoptosis. However, Yasuda *et al.* reported the possible labeling of necrotic or ischemic placental tissue by the TUNEL method [18]. As Gold *et al.* pointed out in their study, in early stages of cell death in vitro, cells undergoing apoptosis are preferentially labeled by the TUNEL method, whereas necrotic cells were identified by nick translation (enzymatic labeling of DNA breaks by use of DNA polymerase) [19]. In the study presented necrotic cells were identified by their characteristic morphological features by light microscopy.

In the present study we demonstrated a significant increase of apoptosis in trophoblast and stromal cells of post-term placental tissue in comparison to term placental tissue, proposing a possible role of apoptosis in the mechanism involved in parturition and placental senescence. Furthermore there were no significant differences in the incidence of apoptosis in different areas of the same placenta.

Further studies are being pursued by our group to elucidate the regulation of apoptosis in placental tissue by oncogenes, different growth factors and cytokines expressed in the human placenta, such as bcl-2, bax, transforming growth factor-ß, epidermal growth factor, tumour necrosis factor, interleukins and insulin-like growth factors.

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