Effect of maternal age on incidences of apoptotic and proliferative cells in trophoblasts of full-term human placenta

Zenzo Yamada1, Masanobu Kitagawa1,3, Tamiko Takemura2 and Katsuiku Hirokawa1

1Department of Pathology and Immunology, Aging and Developmental Sciences, Division of Gerontology and Gerodontology, Graduate School, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519 and 2Department of Pathology, Japanese Red Cross Medical Center, 4-1-22 Hiroo, Shibuya-ku, Tokyo, 150-0012, Japan

3To whom correspondence should be addressed. E-mail: masa.pth2@med.tmd.ac.jp

Advanced maternal age is known to be a risk factor for various kinds of obstetric complications, including placental dysfunction. As a first step towards determining the maternal age-related changes in placental, as well as trophoblastic function, we examined the incidences of apoptotic and proliferative cells in trophoblasts of placentae from women of various ages using the TUNEL method and immunohistochemistry for Ki-67 antigen. Tissue sections were collected from the placentae of healthy mothers with normal delivery of healthy babies so that the placental cell kinetics maintaining normal pregnancy and delivery could be studied. The TUNEL-positive cells of the placenta were syncytiotrophoblasts with clustering of nuclei and the TUNEL-positive index of these cells varied from 0.28–1.2%. This index revealed a significant inverse correlation with maternal age. In contrast, the Ki-67-positive index of mononuclear trophoblasts of the placenta ranged between 1.2–2.8% and showed a positive correlation with maternal age. Many of the apoptotic cells of placental villi expressed the pro-apoptotic Bak protein, but were negative for expression of the anti-apoptotic Bcl-2 protein. These results suggest that trophoblasts have higher proliferative activity in older mothers, with a normal process of pregnancy and delivery. The Bcl-2 family proteins could be important for the regulation of trophoblastic apoptosis, although the cellular and molecular mechanisms mediating maternal age-related changes of the placenta remain to be determined.

Key words: apoptosis/Bcl-2 family/maternal age/placenta/trophoblasts

Introduction

Apoptosis is known to regulate the cell dynamics of many human reproductive tissues including the uterine epithelium (Koh et al., 1995), testis (Tapanainen et al., 1993), ovary (Rodger et al., 1995) and placental villi (Yasuda et al., 1995; Nelson, 1996; Smith et al., 1997a,b, 2000; Kokawa et al., 1998; Chan et al., 1999; Halperin et al., 2000; Levy and Nelson, 2000). Among these tissues, third trimester placental tissue has a rather frequent occurrence of apoptotic cells in the chorionic villi. The incidence of apoptosis in human placental tissue has been reported to progressively increase throughout pregnancy until close to delivery (Smith et al., 1997a, 2000; Halperin et al., 2000). This phenomenon may reflect the physiological conditions in delivery caused by a decrease in placental perfusion during uterine contraction. Under such conditions, the balance between pro- and anti-apoptotic protein expression at the placental trophoblasts would alter in a close relationship with the degree of DNA fragmentation (Cirelli et al., 1999), although the precise mechanisms of apoptosis in placental villi are still not clear.

Maternal age has an influence on the function of placenta during pregnancy and delivery (Hansen, 1986; Williams and Mittendorf, 1993). It has been well documented that risks for several kinds of antenatal, delivery and fetal complications are higher in pregnancies of older women (Fretts et al., 1995; Jolly et al., 2000). Pregnancy in older women is also associated with many confounding factors including parity, pre-existing diabetes mellitus and hypertension, and this should be taken into account if the changes associated with advanced maternal age are to be considered (Lehmann and Chism, 1987). Thus, in the present study, full-term delivered placentae were collected from healthy mothers with normal pregnancies and normal deliveries of healthy babies. Then, to investigate whether the cell dynamics of trophoblasts of placental villi are actually influenced by maternal age, apoptotic and proliferative parameters were analysed. If age-related dysfunction of villi exists and the machinery controlling cell proliferation of the villi remains normal, placental trophoblasts would need a higher proliferative activity to compensate for the lower...
functions of each cell in older mothers. As expected, there was a significant decline in the numbers of apoptotic cells and an increase in the numbers of proliferative cells in the placental trophoblasts of older mothers. The mechanisms controlling cell dynamics in the placental villi are discussed.

Materials and methods

Tissue samples

Human placental tissue samples from 34 uncomplicated term pregnancies of 37–42 weeks gestation (mean 39.2) were obtained immediately after labour and vaginal delivery. The maternal age ranged from 26–43 years of age. All women were normotensive and not hypertensive, were not taking medications and had no medical illnesses such as diabetes mellitus. All of the term infants were healthy, weighed >2500 g at birth and were free from chromosomal abnormalities. Placental samples were collected from the centre portion of the placenta near the umbilical cord where tissues were free of infarct, fibrosis and calcification. Sections were made to contain the entire wall of the placenta covering the area from the amnion to the decidua basalis. They were fixed in 10% buffered formaldehyde, embedded in paraffin, mounted on aminoethyltriethoxysilane-coated slides (MAITUSUNAMI, Tokyo, Japan) and then processed for the following analyses.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining for apoptosis

The slides were de-waxed, rehydrated and washed in phosphate-buffered saline (PBS; pH 7.4). Endogenous peroxidase was inactivated for 30 min by 3% H2O2 in distilled water. The slides were then pretreated with proteinase K (20 µg/ml) in 10 mmol/l Tris-HCl (pH 7.4) for 15 min at 25°C and washed in PBS. The sections were incubated for 60 min at 37°C with TUNEL reaction mixture [terminal deoxynucleotidyl transferase (TdT) and fluorescein-labelled deoxy-uridine triphosphate (dUTP), mixed immediately before use], washed with PBS, incubated for 30 min at 37°C with converter-POD (anti-fluorescein antibody, Fab fragment from sheep, conjugated with horseradish peroxidase; In Situ Cell Death Detection Kit, POD, Boehringer Mannheim, Germany) and washed with PBS. DNA strand breaks associated with apoptosis were detected by colour development with diaminobenzidine-hydrogen peroxide. The sections were counter-stained lightly in haematoxylin.

Analysis of the TUNEL-positive and Ki-67-positive indices

Slides were examined under a light microscope at high power magnification. Nuclei stained intense brown were regarded as a positive reaction for the TUNEL method or for immunostaining for Ki-67 antigen. More than 5000 multinucleated syncytiotrophoblasts were counted at random per section of TUNEL staining on one hand, and at least 3000 mononuclear cytotrophoblastic cells surrounding the villi were counted at random per section of Ki-67 staining on the other. The majority of mononuclear cytotrophoblastic cells were TUNEL-negative and the multinucleated syncytiotrophoblasts with clustering of nuclei were basically Ki-67-negative. The TUNEL-positive index was expressed as the ratio of positively stained nuclear clusterings to the total number of syncytiotrophoblasts with clustering of nuclei. To avoid counting the lining syncytiotrophoblasts with

![Figure 1](image1.png) Placental villi stained with TUNEL method (original magnification ×400). Note the positive staining in clustering nuclei of the syncytiotrophoblastic layer cell.

![Figure 2](image2.png) Inverse correlation between the TUNEL-positive index of placental villi and maternal age. Spearman’s correlation coefficient = –0.718 (P < 0.0001).
psuedo-clustering of nuclei caused by tangential sectioning of the top of the villi (Kaufmann et al., 1987), cells with a few accumulated but scattered nuclei were not counted, i.e. only the cells with nuclear clustering showing dense aggregation of more than five nuclei were counted as ‘syncytiotrophoblasts with clustering of nuclei’. The Ki-67-positive index was expressed as the ratio of positively stained cells surrounding the villi to the total number of lining trophoblastic cells including non-clustering syncytiotrophoblastic layer cells and cytotrophoblasts.

**Statistics**

TUNEL-positive and Ki-67-positive cell ratios were assessed in relation to the maternal age. Correlations between parameters were analysed by rank-test with Spearman’s correlation coefficient.

**Results**

**Apoptosis of trophoblasts in placental villi and maternal age**

To determine the influence of maternal age on apoptosis in the placenta, TUNEL method was performed on sections from placentae with various maternal ages. The TUNEL-positive cells located mostly to the syncytiotrophoblastic layer of the placental villi and showed clustering of nuclei (Figure 1). The TUNEL-positive index ranged from 0.28–1.2%. Placentae from young mothers revealed a more frequent occurrence of apoptosis than those from older mothers. As maternal age advanced, the TUNEL-positive index of the placenta was reduced. The TUNEL-positive index revealed a significant reverse correlation with the maternal age (Figure 2) (Spearman’s correlation coefficient, \( r = -0.718, P < 0.0001 \)).

**Proliferative cells among trophoblasts of placental villi and maternal age**

Next, to clarify the proliferative activity of placentae with various maternal ages, immunostaining for Ki-67 antigen was performed. The Ki-67 immunoreactivity was confined almost exclusively to the cytotrophoblasts, but was occasionally found in stromal cells, whereas the syncytiotrophoblasts were mostly negative. The Ki-67-positive index ranged from 1.2–2.8%. Placentae from younger mothers revealed lower frequencies of Ki-67-positive trophoblasts (Figure 3A) compared with those from older mothers (Figure 3B). The Ki-67-positive index of the placentae showed a positive correlation with maternal age (Figure 4) (Spearman’s correlation coefficient, \( r = 0.706, P < 0.0001 \)). As expected, there was a significant inverse correlation between the Ki-67-positive index and the TUNEL-positive index of the placentae (Figure 5) (Spearman’s correlation coefficient, \( r = -0.577, P < 0.001 \)).

**Localization of Bcl-2 family proteins in placental villi**

To estimate the influence of Bcl-2 family expression on placental apoptosis, the localizations of Bcl-2, Bax and Bak proteins in the placentae were examined by immunohistochemistry. The anti-apoptotic Bcl-2 protein was localized to the cytoplasm of placental trophoblasts. The majority of syncytiotrophoblastic layer cells revealed positive staining for Bcl-2, while cytotrophoblastic layer cells and a few of the syncytiotrophoblasts with clustering of nuclei were negative (Figure 6A). The intensity of the positive reaction varied from case to case, but there was no significant correlation

---

**Figure 3.** Immunohistochemical staining for Ki-67 antigen in the placental villi from pregnancies of a (A) young (27 year old) and (B) older (42 year old) mother (original magnification ×100). Note that the Ki-67-positive reaction was more frequent in cytotrophoblasts of the placenta from the older mother compared with the placenta from the younger mother. (C) Higher magnification of the villi showing Ki-67-positive reaction in the nucleus (original magnification ×400).
between the magnitude of staining for Bcl-2 protein and the TUNEL-positive index and between Bcl-2 expression and maternal age. The pro-apoptotic Bak protein was localized to the cytoplasm of syncytiotrophoblasts with nuclear clustering in part (Figure 6B). Although the reaction was very weak, a few cytotrophoblasts were also stained positively. In contrast, Bax protein-positive cells were very few and the protein was localized to the cytoplasm of syncytiotrophoblasts with nuclear clustering (Figure 6C). However, the differences in magnitude of Bak and Bax expression were not significant between placentae from younger and older mothers.

To confirm the relationship between Bcl-2 family expression and apoptosis in villi, serial sections were used for TUNEL/Bcl-2/TUNEL and Bak/TUNEL/Bcl-2 staining. As shown in Figure 7A–C, TUNEL-positive syncytiotrophoblasts were Bcl-2-negative. Both of the serial sections adjacent to the Bcl-2 staining, i.e. pre- and post-sections, revealed positive staining of the same syncytiotrophoblasts by the TUNEL method. Therefore, the TUNEL-positive syncytiotrophoblast with clustering of nuclei was really Bcl-2-negative. Figures 8A–C show the staining for Bak protein, TUNEL and Bcl-2 protein by using serial sections. The same syncytiotrophoblast with
clustering of nuclei exhibited positive staining for Bak protein and by the TUNEL method but was negative for Bcl-2 protein. These findings suggested that Bcl-2 family proteins, especially anti-apoptotic Bcl-2 and pro-apoptotic Bak, could play a role in controlling apoptosis of trophoblasts.

Discussion

Apoptosis is a normal physiological phenomenon in placenta (Smith et al., 1997b; Chan et al., 1999). Various methods such as light and electron microscopic assessment have been used in the past to demonstrate placental apoptosis. The TUNEL method is a sensitive and objective approach to determine the presence of apoptotic cells, although criticisms have been raised against the application of TUNEL-positive cell ratio of the placenta as the apoptotic cell ratio (Smith, 2000). As indicated, the rate of apoptosis can be highly variable according to the measurement system used. For example, Chan and colleagues reported the rate of apoptosis in normal placenta as ~1% of the cells in villous tissue by TUNEL method (Chan et al., 1999), whilst Smith and colleagues reported a median apoptotic rate of 0.07% by haematoxylin and eosin (HE) staining (Smith et al., 1997b) and 0.51% by the TUNEL method (Smith et al., 2000). In the present study, the overall mean value of the TUNEL-positive cell ratio (0.66%) was a little higher than that of the previous studies. This might have resulted from the different method used to calculate the ratio of apoptotic cells. We counted only the number of syncytiotrophoblasts with nuclear clustering as total cells and thus the rate became relatively high.

There has been controversy regarding the cell types exhibiting apoptosis in the placenta. Most of the previous studies (Yasuda et al., 1995; Nelson, 1996; Marana et al.,
1998; Chan et al., 1999) and the present study have revealed that apoptotic cells are located more dominantly in the syncytiotrophoblastic than in the cytotrophoblastic layer. In contrast, Kokawa and colleagues have reported that cytotrophoblasts, rather than syncytiotrophoblasts, undergo apoptosis in normal pregnancy, although apoptotic cells are more prominent in the syncytiotrophoblasts in cases of spontaneous abortion (Kokawa et al., 1998). Placental apoptosis increases significantly as pregnancy progresses, suggesting that it could play a role in the normal development and ageing of the placenta (Smith et al., 1997a, 2000; Halperin et al., 2000). However, Ishihara et al. have reported that apoptosis in the human normal placenta predominates in very early pregnancy (week 4–5) and drastically diminishes after week 5 of pregnancy (Ishihara et al., 2000). They suggested that abundant expression of Bcl-2 protein in syncytiotrophoblasts of term placenta might be responsible for the diminished apoptosis in these cells. Thus, the dynamics of apoptosis even in the normal gestational ageing of placenta is still controversial. Therefore, in the present study, we used only the full-term placenta to exclude the influence of placental ageing.

The factors responsible for regulating apoptotic cell death in the syncytiotrophoblast are unknown. Many molecules have been nominated as apoptosis-inducing factors of placenta (Levy and Nelson, 2000). To identify members of the Bcl-2 family proteins that might regulate apoptosis of trophoblasts, Ratts et al. have assessed the expression of Bcl-2, Bax and Bak proteins in the placenta from uncomplicated pregnancies (Ratts et al., 2000). The anti-apoptotic Bcl-2 protein was expressed throughout the syncytiotrophoblast and villi, while expression of the pro-apoptotic Bax protein was undetectable in the syncytiotrophoblast. Localization of Bak protein, which also has the pro-apoptotic character, was associated with apoptotic features of trophoblasts. Kim et al. have reported that the degree of Bcl-2 expression significantly decreases in placenta after the gestational period of 32 weeks, and this may be a parturition-associated biological change for inducing apoptosis in the placental villi (Kim et al., 1995). Further, an inverse relationship has been identified between apoptosis and Bcl-2 expression in the syncytiotrophoblast (Toki et al., 1999). In the present study, the apoptotic syncytiotrophoblasts with nuclear clustering exhibited a loss of Bcl-2 expression and an expression of the Bak protein, suggesting a relationship between Bcl-2 family proteins and maternal ageing.

Regarding pathological conditions of the placenta, many studies have been performed to determine apoptotic and proliferative activity of the villous tissue. For example, the incidence of apoptosis is significantly higher in placenta from pregnancies with intrauterine growth restriction compared with normal third trimester placentae (Smith et al., 1997a). In contrast, Sebire et al. have reported that the number of Ki-67-positive trophoblasts is significantly larger in a group with trisomy 18 compared with a chromosomally normal group, in spite of the fact that pregnancies with fetal trisomy 18 suffer from severe intrauterine growth restriction from the first trimester (Sebire et al., 2000). Chromosome aberrations, which are the most frequent causes of abnormal embryonic development and spontaneous abortion, may affect rates of apoptotic and proliferative cells in chorionic villi. Qumsiyeh et al. have reported that apoptotic cells are significantly higher in number in the stroma of chromosomally abnormal spontaneous abortions (Qumsiyeh et al., 2000). Further, pre-eclampsia is associated with widespread apoptosis of placental cytotrophoblasts within the uterine wall (DiFederico et al., 1999).

Risks of various placental complications have been reported to increase in pregnancies of older mothers (Jolly et al., 2000). As described above, many of the pathological abnormalities of placenta are usually accompanied by an increase of apoptosis in trophoblasts (Smith et al., 1997a; DiFederico et al., 1999; Qumsiyeh et al., 2000). However, our data show that placentae from older mothers had a lower incidence of apoptosis compared with those from younger mothers. Since we collected placentae from healthy mothers with normal pregnancies and deliveries, we could speculate that the placentae from older women represent cases where the disadvantages of age-related hypofunction have been overcome by increased proliferative and reduced apoptotic signals. Further studies would be necessary to clarify the precise mechanisms involved in the functional alteration and proliferative activity of placenta by maternal ageing.

References


Received on July 19, 2001; accepted on September 28, 2001