

Placental Villous Trophoblast: the Altered Balance Between Proliferation and Apoptosis Triggers Pre-eclampsia

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During the morula stage of human embryo development segregation of the first two cell lineages takes place: the trophoblast and the embryoblast. For the development of a healthy baby, the embryonic tissues and cells need to show high rates of proliferation and differentiation, as well as high rates of apoptosis. Only the concerted action of all three processes leads to a proper development of all tissues and organs and is crucial for morphogenesis in general.

This is also true for the extraembryonic tissues such as the trophoblast, which gives rise to the placenta and provides the epithelial cover of the placental villous trees. This villous trophoblast comes into direct contact with maternal blood and similar to stratified epithelia displays a continuous turnover of its layers. The villous trophoblast displays proliferation and differentiation of its precursor cells, termed villous cytotrophoblast. Their final differentiation event is syncytial fusion with the overlying multinucleated layer, the syncytiotrophoblast. Here a second differentiation stage takes place, with a final apoptotic shedding event, releasing apoptotic syncytial knots into the maternal circulation.

*As a normal constituent of trophoblast turnover apoptosis and the release of apoptotic material does not induce an inflammatory response of the mother. The pregnancy pathology pre-eclampsia is characterised by an altered balance between proliferation and apoptosis of villous trophoblast resulting in a dysregulated release of material from the syncytiotrophoblast into maternal blood. Beside the normal apoptotic release there seems to be an increasing release by necrosis, and due to ongoing apoptosis within the syncytiotrophoblast, the necrotic release of apoptotic material leads to aponecrotic shedding. Cell-free components of the syncytiotrophoblast may now be able to damage the maternal endothelium and hence trigger pre-eclampsia. **J Reproduktionsmed Endokrinol 2006; 3 (2): 103–8.***

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Human Development and the Onset of Apoptosis

During human development particularly after the onset of the embryonic genome apoptotic cells can be observed. Hence, TUNEL positive nuclei in *in vitro* embryos first appear at the morula stage [1]; and at the blastocyst stage embryoblast and trophoblast are evenly affected by apoptotic events (7–8 % TUNEL positive; [1]).

During the preimplantation period there is a clear alteration of transcription levels. During oogenesis expression is high, while it decreases down to baseline during the 4- and 8-cell stages. Upon onset of the embryonic genome, RNA expression rises again to reach maximum levels at the blastocyst stage. This has been shown for several genes that peak at different times during development [2]. Down-regulated transcription levels following fertilisation and prior to onset of the embryonic genome have also been reported for genes involved in regulating apoptosis, such as proteins of the Bcl-2 family [3]. Pro-apoptotic genes such as RB1 and Bad reach maximum levels after compaction, the time when first apoptotic cells can be detected [2, 4]. At the protein level it became obvious that post translational regulation is important for the active control of apoptosis rather than transcription of mRNA [3]. Also on the protein level, active caspase proteins are associated with apoptotic nuclei only at the morula stage or later, i. e. after establishment of the embryonic genome [4].

With onset of the embryonic genome and the first segregation of cell lines, the trophoblast further expands and starts to develop the placenta. This organ will grow for the next nine months and thus respective stem cells of the

trophoblast need to be maintained over this period of time. But what is known about trophoblast stem cells?

Mouse Trophoblast Stem Cells

In mice the presence of specific transcription factors is decisive for the differentiation of trophoblast stem cells into different phenotypes: Hand1 furthers the differentiation of trophoblast giant cells, Gcm1 is crucial for differentiation into syncytiotrophoblast, and Mash2 is in charge of the differentiation into spongiotrophoblast [5].

Murine trophoblast stem cells express fibroblast growth factor receptor 2 (FGFR2), and one of its ligands, fibroblast growth factor 4 (FGF4) maintains the proliferative capacity of these cells [6]. There is no evidence so far that the steadily proliferating trophoblast cells are the real stem cells, but Kunath et al. [7] have shown that the trophoblast stem cell lineage in mice is maintained by signals such as FGF4, which are derived from the epiblast. *In vitro* the withdrawal of FGF4 leads to the preferential differentiation of murine trophoblast cells along the invasive pathway [6].

Hughes et al. [8] showed that the combination of FGFR2 and FGF4 is able to maintain the proliferation as well as stem cell character of trophoblast cells. Furthermore, the ability of FGF4 to maintain the proliferative capacity of trophoblast stem cells was overwhelmed by transfection of the cells with Stra13 or Hand1, genes involved in driving differentiation of trophoblast cells [8]. After transfection these cells stop proliferation and start differentiation after leaving the cell cycle.

Human Trophoblast Stem Cells

Despite the huge and quickly increasing knowledge of the factors involved in the maintenance of murine trophoblast stem cells, the exact localisation, and fate of human trophoblast stem cells is mostly unclear. Also the

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knowledge on transcriptional and post-translational regulation of trophoblast differentiation is slow in growth.

Also in the human blastocyst there are trophoblast cells that express FGFR2 [7]. During later development some villous trophoblast cells within the villous trees seem to remain in a stem cell phenotype and maintain their proliferative capacity again by the action of FGF4 on FGFR2 [9], similar to what has been found in the mouse [7].

Also the differentiation of mononucleated cytotrophoblasts into the syncytiotrophoblast depends on the expression of the same transcription factor, GCM1 [10]. GCM1 is up-regulated in those mononucleated cytotrophoblasts that morphologically display a high degree of differentiation and will syncytially fuse shortly after with the syncytiotrophoblast. This takes place throughout pregnancy to maintain the syncytiotrophoblast and to transfer new proteins and RNA into this highly differentiated layer that has lost its proliferative capacity.

It needs to be elucidated which additional transcription factors together with ligands and their receptors are needed to drive differentiation of human trophoblast cells.

Turnover of Villous Trophoblast

Villous trophoblast represents the epithelial covering of the placental villous trees. Since the trophoblast has differentiated prior to the development of the three layered embryo, it is not recognised as a classic epithelium because it is not derived from the ectoderm or endoderm. However, it shares all the typical features of other stratified epithelia such as:

- A basement membrane to separate it from the underlying connective tissues;
- Proliferating precursor cells resting on the basement membrane;
- Differentiation along a gradient from the basement membrane to the surface;
- Release of old and apoptotic material from the surface into the lumen.

Villous trophoblast is the fetal surface of the chorionic villi that comes into direct contact with maternal blood and that separates maternal blood from fetal placental tissues. It comprises a basal layer of mononucleated cells termed cytotrophoblast that include a subset of stem cells that still needs to be defined and characterised. The second and outer layer is a multinucleated layer without any lateral cell borders, the syncytiotrophoblast, which is generated and maintained by syncytial fusion of cytotrophoblasts with this layer. Due to its high degree of differentiation the syncytiotrophoblast has lost its proliferative capacity [11, 12]. Hence throughout pregnancy functional and morphological maintenance of the syncytiotrophoblast completely depends on continuous fusion of cytotrophoblasts with this layer. The continuous input of material via syncytial fusion into the syncytiotrophoblast is counter balanced by a continuous extrusion of apoptotic material into the maternal circulation [13, 14]. This material is packed into membrane sealed structures, the syncytial knots [12] (Fig. 1A).

A detailed observation on the turnover of villous trophoblast involves:

- Cytotrophoblast progenitor cells proliferate.

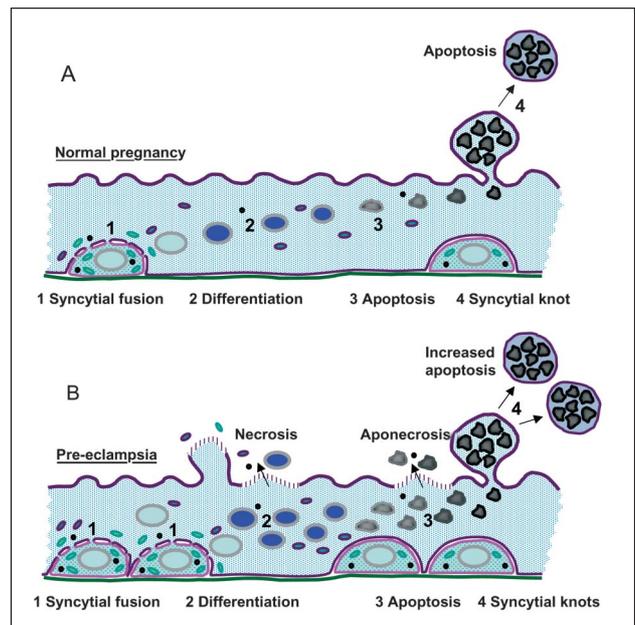


Figure 1. Schematic representation of trophoblast turnover. **(A)** Normal pregnancy: The final event of cytotrophoblast differentiation, syncytial fusion, results in incorporation of fresh organelles and other cellular material into the syncytium (1). Within the multinucleated syncytiotrophoblast differentiation (2) and subsequently late apoptosis (3) take place. Finally, the late apoptotic material is packed into protrusions of the apical plasma membrane, syncytial knots. These knots are released into the maternal circulation as tightly sealed corpuscles (4). **(B)** Pre-eclampsia: Enhanced proliferation and syncytial fusion (1) may overwhelm the capacity of the syncytiotrophoblast in terms of differentiation (2) and apoptotic release (3). This may result in a necrotic breakdown of specific sites of the syncytiotrophoblast. If at these sites apoptosis has not yet started, pure necrosis can be observed; if however apoptosis has already lead to first cleavage of proteins, apoptotic material will be necrotically released (aponecrosis). At the same time the syncytiotrophoblast tries to counter balance for the increased input by increasing the release of apoptotic syncytial knots (4). Modified from [15].

- Daughter cells leave the cell cycle and start to differentiate.
- Differentiation of cytotrophoblast finally leads to syncytial fusion of this cell with the overlying syncytiotrophoblast [10].
- Further differentiation within the syncytiotrophoblast results in the production of hormones, transporters, and other proteins crucial for maintaining pregnancy.
- Finally nuclei show signs of aging and late apoptosis, and are packed into syncytial knots [16].
- Syncytial knots are released from the syncytiotrophoblast and enter the maternal blood stream [17].
- Syncytial knots are engulfed and cleared in the first capillary system behind the placenta, i. e. the lung [13].

Fusing mononucleated cytotrophoblasts integrate their cytoplasmic content into the syncytiotrophoblast and thus maintain this layer, which has not only lost its generative potential but has also down-regulated RNA expression (transcription). Richart [18] used ^3H -thymidine incorporation to show that DNA synthesis does not take place in nuclei of the syncytiotrophoblast. Others have extended these experiments and have shown that incorporation of ^3H -uridine to display RNA expression is again restricted to the cytotrophoblast layer of villous trophoblast [19, 20]. In their *in vitro* experiments these authors were not able to detect any measurable levels of RNA incorporation within the syncytiotrophoblast.

It is simple to explain the biological necessity of a continuous fusion and integration of new material throughout pregnancy by the termination of DNA synthesis as well as the predominant down-regulation of RNA expression within the syncytial layer of villous trophoblast. Hence fusion of cytotrophoblasts with the syncytiotrophoblast provides the continuous supply of new mRNA, proteins, and organelles to maintain syncytial function [12] and differentiation [14].

Apoptosis as a Means to Safely Remove Old Trophoblast Material

The process of apoptosis itself was originally described more than thirty years ago [21]. However, use of the term apoptosis in research of human placenta started only twelve years ago when Sakuragi et al. [22] used *in situ* nick end labelling of fragmented DNA to detect apoptosis in human trophoblast. Only within the last ten years major advances have been made in the field of placental apoptosis. In particular villous trophoblast has been studied extensively since an increasing body of evidence shows that in villous trophoblast – similar to other systems – the process of apoptosis is tightly linked to the process of differentiation.

Apoptosis is a programme that is activated in all stratified epithelia. As soon as the cells lose their contact to the basement membrane, an apoptotic process called *anoikis* [23] is activated leading to the apoptotic death of a cell. The villous trophoblast of a young placenta during the first trimester displays two layers: a complete layer of cytotrophoblasts attached to the basement membrane and a second multinucleated layer, the syncytiotrophoblast. It could be speculated that the trophoblast undergoes a process similar to *anoikis* since just prior to syncytial fusion (which in the first trimester is accompanied by loss of contact to the basement membrane) early stages of the apoptosis programme originate in these cells [16, 20, 24].

In the syncytiotrophoblast late and end stages of the apoptosis cascade can be detected. Activity of execution caspases [16, 20], degradation of specific target proteins of caspases such as lamins and cytokeratins [16, 25, 26], and degradation of DNA (TUNEL positivity) have all been described for specific sites of the syncytiotrophoblast. During the final event of apoptosis within the syncytiotrophoblast late apoptotic material, mostly nuclei, is packed into protrusions of the apical membrane, the syncytial knots [16, 27, 28] (Fig. 1A).

These structures are mostly found at tips of villi and contain multiple nuclei that display clear morphological features of late apoptosis such as shrinkage, chromatin condensation, and annular distribution of chromatin. The syncytial knots are the sites of extrusion of late apoptotic material [16, 27, 28] and hence can be taken as structures that correlate with apoptotic bodies of mononucleated cells. In a mononucleated cell late apoptosis results in the formation of apoptotic bodies, fragments of the cell that contain smaller fragments of the nucleus. In a multinucleated system like the syncytiotrophoblast the 'apoptotic bodies' are much larger and do not contain fragments of a single nucleus but rather multiple late apoptotic nuclei. These syncytial knots together with the apoptotic nuclei can be recognised as fragments of the

syncytium. Within a multinucleated syncytium the formation of syncytial knots containing several late apoptotic nuclei may represent the formation of apoptotic bodies found in late apoptotic mononucleated cells.

The syncytial knots are finally released and shed from the apical syncytiotrophoblast membrane. They enter the maternal blood stream as membrane sealed structures that do not release any content. The multinucleated syncytial knots can be detected in uterine vein blood of healthy pregnant women [17] as well as in the capillary bed of the maternal lung [13, 29]. They are engulfed in the first capillary system behind the placenta, i. e. the lung [13, 30] and thus their numbers are clearly reduced to nearly undetectable levels in peripheral blood [17, 31].

The calculation of the turnover of villous trophoblast over gestation has resulted in an estimate on the amount of apoptotically shed material into the maternal circulation. In the third trimester of pregnancy several grams of material are shed as apoptotic syncytial knots into the maternal circulation per day [12, 14, 16]. It is critical for the health of the mother to have the late apoptotic trophoblast material being shed as membrane sealed syncytial knots. One important feature of this apoptotic material is that it does not induce an inflammatory response of the mother – and indeed there is minimal activation of the maternal immune system during normal pregnancy [32].

Trophoblast Turnover in Pre-eclampsia

The turnover of villous trophoblast is altered in pregnancy pathologies such as intra-uterine growth restriction (IUGR) and pre-eclampsia. As has been described already 15 years ago, there is an increase in the rate of proliferation of villous trophoblast in pre-eclampsia [33]. Several studies have shown that there is also an increase in the rate of apoptosis in IUGR [34–37] as well as in pre-eclampsia [36, 38, 39].

In extension of such studies, we correlated the pO_2 of the uterine vein with the rates of trophoblast proliferation and apoptosis within the respective delivered placentas in controls, early-onset IUGR and early-onset IUGR further complicated with pre-eclampsia [40, 41]. The oxygen concentrations within the blood of uterine veins behind the placenta were measured just prior to delivery during caesarean section (I. Cetin, Milano, and T. Todros, Torino; [41]). For the cases with pre-eclampsia the correlation of the pO_2 with the rates of proliferation and apoptosis revealed the following [40, 41]:

- In general the pO_2 within the uterine vein behind the placenta of pre-eclamptic cases is shifted to higher values compared to normal pregnancies.
- At a given pO_2 the cytotrophoblasts from a pre-eclamptic placenta show a higher rate of apoptosis compared to cytotrophoblasts from a normal placenta.
 - Cytotrophoblasts from normal placentas do not show changes in the apoptosis rate within the range of oxygen tensions measured in these cases.
 - Cytotrophoblasts from pre-eclamptic placentas do not show changes in the apoptosis rate within the range of oxygen tensions measured in these cases.
- At a given pO_2 the cytotrophoblasts from a pre-eclamptic placenta show a higher rate of proliferation compared to cytotrophoblasts from a normal placenta.

- Cytotrophoblasts from normal placentas do not show changes in the proliferation rate within the range of oxygen tensions measured in these cases.
- Cytotrophoblasts from pre-eclamptic placentas show a clear dependency of their proliferation rate on the oxygen tensions measured in the uterine veins: the higher the oxygen tension the lower the proliferation rate.

The following scenarios for villous trophoblast in pre-eclampsia can be deduced from these data (Fig. 1B):

1. In pre-eclampsia not only apoptosis is increased but since the rate of proliferation is enhanced as well, it can be assumed that the whole turnover of villous trophoblast from proliferation via fusion to apoptosis is accelerated.
2. The increased rate of proliferation is linked to a higher rate of syncytial fusion since a multi-layering of cytotrophoblasts is not a feature of pre-eclampsia.
3. More proliferation and subsequently more syncytial fusion of cytotrophoblasts with the overlaying syncytiotrophoblast introduce a greater amount of material into the syncytiotrophoblast as compared to the normal situation.
4. Increased apoptosis of the syncytiotrophoblast may be a simple mechanism to counter balance the increased input via syncytial fusion and hence may not necessarily represent a pathological process *per se* but rather an adaptive process of this layer.

The question that remains open in the above mentioned scenarios is whether the syncytiotrophoblast is able to compensate for the enormous load of new material introduced into this layer. An estimation of the duration of differentiation within the two layers of villous trophoblast clearly uncovered differences between the two layers. Kaufmann and Huppertz calculated the differentiation times and found that from proliferation to fusion it takes only a few days (cytotrophoblast), while from fusion to extrusion via syncytial knots it takes several weeks (syncytiotrophoblast) [12, 16].

In a disturbed pre-eclamptic placenta the oxygen tension within the intervillous space may show temporal as well as spatial variations [42, 43]. Since the villous trophoblast of such a pre-eclamptic placenta changes its rate of proliferation due to changes of oxygen, this will result in an up and down of proliferation, syncytial fusion and thus input into the syncytiotrophoblast.

It is now tempting to speculate that the syncytiotrophoblast is unable to perfect a new physiological balance between input and output due to all these variations and due to its slow machinery of differentiation. It may well be that if in a given time frame there is too much input to be compensated by apoptotic release, then the apoptotic machinery of the syncytiotrophoblast is overwhelmed and material may be released from the syncytiotrophoblast in a non-apoptotic and more chaotic manner. The accelerated input may not permit sufficient time for the slowly reacting syncytiotrophoblast to complete the apoptotic cascade before releasing the material. In this situation a necrotic breakdown of sites of the syncytiotrophoblast would be the consequence.

If material is shed and apoptosis has not occurred, then necrotic release takes place. At sites where apoptosis be-

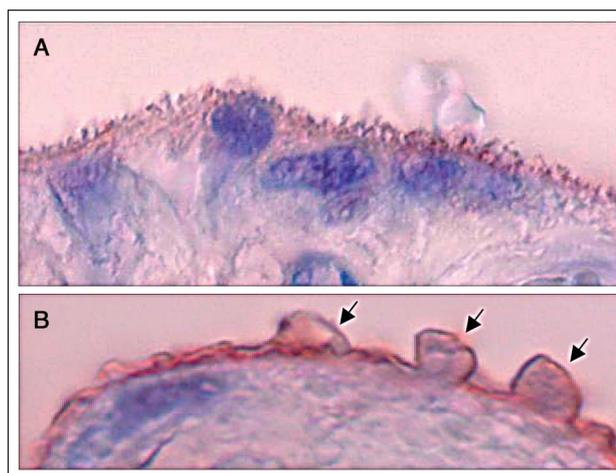


Figure 2. Representative images of chorionic villi from term placentas derived from a normal pregnancy (A) and a pregnancy complicated by late-onset pre-eclampsia (B). In both images staining was performed using a primary antibody against placental-protein 13 (PP13), which is present in the apical membrane of the syncytiotrophoblast. In (A) the normal villous brush border can be seen, which is stained for PP13. In (B) the alterations of the brush border membrane are obvious. Protrusions on the cellular level can be seen (arrows) that may be released into the maternal circulation. Magnification $\times 900$.

gun but not yet reached its final event, necrotic damage of the syncytiotrophoblast will lead to the cell-free release of apoptotic material – this is what we call aponecrotic release [44]. We have described this type of release as ‘aponecrosis’ following the proposal of a chimerical type of cell death, which describes a truncated form of apoptosis with an incomplete execution that is followed by degeneration via necrosis [45].

Conclusions

The release of apoptotic material by extrusion of syncytial knots into the maternal circulation does not incite the inflammatory system of the mother. Hence an increased number of released apoptotic structures in IUGR and pre-eclampsia does not alter the inflammatory status of the pregnant mother in both pathologies.

On the other hand, the enhanced necrotic release of intact or apoptotic subcellular fragments (aponecrosis) into the maternal circulation during pre-eclampsia could well explain the clinical manifestations of this syndrome such as:

- Local placental damage (excessive deposition of fibrin-type fibrinoid on the surface of placental villi) [46];
- Activation and damage of maternal endothelial cells [47–49];
- Activation of the inflammatory system of the mother [50, 51].

Also the presence of subcellular membrane fragments of the syncytiotrophoblast (Fig. 2) and fetal cell-free molecules in the peripheral blood of pre-eclamptic mothers [31] can be made clear. It is more than a simple up-regulation of apoptosis that is necessary to explain the classical features of the clinical syndrome of pre-eclampsia. Therefore, the present data can be explained by the release of material via necrotic and aponecrotic mechanisms, which are not present in IUGR placentas [52].

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