Leukemia Inhibitory Factor (LIF) and Vascular Endothelial Growth Factor (VEGF) diminish intensity of apoptosis in hypoxic human trophoblast: first-trimester versus term trophoblast cultures.

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Abstract: Leukemia Inhibitory Factor (LIF) and Vascular Endothelial Growth Factor (VEGF) influence trophoblast invasiveness and differentiation. Hypoxia disturbs these processes and induces apoptosis. Sensitivity of the human trophoblast to apoptotic stimuli is changing throughout pregnancy. We examined whether LIF- and/or VEGF-dependent changes in functional status of the cultured trophoblast cells influence apoptotic activity in hypoxic conditions. Placental samples were obtained after abortions in the first trimester (Group I) and after normal term pregnancies (Group II). The cytotrophoblast cells were isolated using trypsin-deoxyribonuclease digestion, followed by a Percoll gradient centrifugation. Established cultures within both groups were divided into 3 subgroups: LIF- (10ng/mL), VEGF- (100ng/mL) and LIF+VEGF-treated, cultured under hypoxia (2% O₂) for 48 hours. The cultures were terminated and quantitative determination of apoptosis was performed by ELISA, using cytokeratin-18 as an apoptosis marker. LIF, VEGF and LIF+VEGF produced decrease in intensity of hypoxia-related apoptosis. This anti-apoptotic influence was stronger in the first-trimester trophoblast. The differences in mean reduction of apoptosis intensity between Group I and II amounted to 76.09, 43.72 and 68.45 % (LIF-, VEGF- and LIF+VEGF-treated cultures, respectively; p<0.05). LIF- and VEGF-related effects on trophoblast cells may protect against hypoxia and consequently apoptosis, especially in the first-trimester of pregnancy.

Key-Words: Leukemia inhibitory factor, vascular endothelial growth factor, apoptosis, hypoxia, trophoblast

1 Introduction
Normal placentation and development of the placental vascular network are crucial for adequate blood supply to the fetus [1,2]. In situ formation of blood vessels from progenitor endothelial cells (angioblasts) and development of new blood vessels from pre-existing vasculature play an essential role in embryonic development and normal growth of placental tissue [3]. Major placental angiogenic factors include the vascular endothelial growth factor (VEGF) gene family of cytokines, which may be up-regulated by decreased oxygen tension [4]. Hypoxia within the placental tissue may lead to various complications of pregnancy, including preeclampsia and intrauterine growth retardation [5]. Extravillous trophoblast cells resemble malignancies with their invasive and destructive features, except that they are sequentially restricted to the first trimester of pregnancy. Trophoblast cells from the term placenta have reduced invasive capacity [6]. As the properties of human trophoblast cells change over the course of pregnancy (e.g., susceptibility to hypoxia and hypoxia-related apoptotic triggers), it has been suggested that the hypoxic environment may be to some extent physiologic for the first trimester trophoblastic cells, but abnormal in the later trimesters [7,8].

Placental villous hypoxia reduces trophoblast differentiation and increases apoptosis in the trophoblast cells [9,10]. As one of the main forms of programmed cell death in multicellular organisms, apoptosis plays an important role in the physiology and pathophysiology of the human trophoblast [11]. This very complex process is a component of normal development and differentiation within placental tissue and includes a cascade of events involving the activation of many genes and the production of various protein-based compounds [12,13]. However, alteration of the precise balance between cellular proliferation, differentiation and death is considered a crucial component in cytotrophoblast-related placental dysfunction [11,14,15]. There are many factors that may affect apoptosis, including leukemia inhibitory factor (LIF) and VEGFs [16,17]. LIF, an interleukin 6
class pleiotropic cytokine, binds to the specific LIF receptor (LIFR-α), which forms a heterodimer with a specific subunit (GP130 signal transducing subunit) common to all members of that family of receptors [16]. Although the inhibition of myeloid leukemic cell lines through the induction of differentiation is a well known ability of LIF (defining its name), this cytokine exerts different biological actions in various tissue systems. Data about the role of LIF in reproduction are still accumulating. Song & Lim demonstrated that LIF is crucial for the successful implantation of embryos in mice [18].

Both LIF and VEGF influence trophoblast invasiveness and differentiation through the inhibition of apoptosis, acting via the Janus kinase (JAK)/Signal Transducers and Activators of Transcription (STATs) pathway [19]. STAT3 and STAT5 induce growth (including tumor progression) through the cell cycle, prevent apoptosis, and upregulate oncogenes such as c-myc and bcl-X [20]. LIF, a member of the IL-6 family of cytokines, signals via STAT3. VEGF uses STAT3 and STAT5 for antiapoptotic signaling, impinging on the transcription of FAS, a well-known proapoptotic gene [16,21]. VEGF activates STATs via VEGF receptor-2 (KDR) [22]. It has been recently suggested that the VEGF/JAK2/STAT5 signaling axis may partially mediate tolerance to cell hypoxia [21]. In independent studies, the expression of LIF and VEGF have been confirmed in both human cytotrophoblast cells and in vitro differentiated syncytiotrophoblast cells [16,23].

2 Aim of the study
The aim of this study was to compare first trimester and third trimester human trophoblast cells to ascertain whether LIF- and/or VEGF-dependent changes in the functional status of cultured trophoblast cells influence apoptotic activity under hypoxic conditions.

3 Materials and methods
This study was conducted in compliance with international and local laws of human experimentation and the project was approved by the local ethics committee. All subjects provided written ethical consent to the collection and use of their tissues, according to the standards published by British Medical Research Council as “Human tissue and use of biological samples: operational and ethical guidance 2001 (includes Addendum to MRC 2001 guidance following the Human Tissue Act 2004)”.

3.1 Placental sample collection
First trimester placental tissue samples (approx. 1.5 x 1.5 x 1.0 cm) were obtained within 60 min after spontaneous missed abortions of unknown origin (without any recognized underlying disease, excluding cases with evidence of an inflammatory process) between the tenth and the twelfth week of gestation (Group I; N=16). Time of onset of abortion was determined by absence of the fetal heart rate (FHR) in repeated ultrasonographic examination. Third trimester placental tissue samples were obtained after normal term pregnancies (Group II; N=16). From the maternal surface of each placenta, three samples were excised in a standardized manner; therefore 48 trophoblast cultures were developed.

3.2 Trophoblast cultures
The cytotrophoblast cells were isolated using our modification of the method given previously by Kliman et al. [24]. This method has been described in detail elsewhere [25,26]. Briefly, placental samples were rinsed with saline to remove blood cells. Minced villous tissue was digested with 0.125% trypsin and 0.2 mg/mL deoxyribonuclease I for 30 min at 37 degrees C. Following enzymatic digestion, the resultant cell suspension was filtered and fractionated by centrifugation through a 5-70% Percoll gradient (1200 rpm for 20 min). The cell layers that accumulated between 40.50% (density 1.048-1.060 g/mL) were collected, washed and resuspended (5 x 10^5 cells/ml) in Ham’s F12/Dulbeco’s modified Eagle’s medium (1:1) with 15% fetal bovine serum. This procedure yields a highly purified preparation (approx. 95% pure) of cytotrophoblast cells with >90% viability. After 5 days, the cultures within both groups were divided into three subgroups: LIF- (10ng/mL), VEGF- (100ng/mL) and LIF+VEGF-treated, then cultured under hypoxic conditions (2% O2). After 48 hours, the cultures were terminated and apoptosis intensity measurements were performed. Respective normoxic and hypoxic controls were also established.

3.3 Assessment of apoptosis intensity
Quantitative determination of apoptosis was performed using an immunoenzymatic assay with antibodies against a neo-epitope of cytokeratin 18 (an apoptosis marker) [27]. M30-Apoptosense ELISA Kit (Peviva AB, Sweden) was used for specific and sensitive detection of the caspase-cleaved intermediate filament protein cytokeratin-18 (CK18Asp396-N: NE; M30 neo-epitope). In this method, the well-established M30 CytoDEATH monoclonal antibody serves as a validated marker of apoptosis that does not react with viable or necrotic cells. Apoptosis detection with the
M30 CytoDEATH assay has been described in several publications as a superior quantitative technique when compared to TUNEL or to detection of gammaH2AX phosphorylation [28].

3.4 Statistical analysis
The results are reported as mean percentage values ±SEM of compared control values (taken as 100% for Group II). The differences in mean percentages of apoptotic intensity were examined using Mann-Whitney’s U test and deemed statistically significant if p < 0.05.

4. Results
The results are presented in Fig. 1. In both groups, administration of LIF, VEGF and LIF+VEGF produced a significant (p <0.05) decrease in hypoxia-related apoptosis. During hypoxia, this anti-apoptotic influence was stronger in the first-trimester trophoblasts. As shown in Fig. 1A, the differences in the mean reduction of apoptosis intensity between Group I and Group II amounted to 76.09%, 43.72% and 68.45% (LIF-, VEGF- and LIF+VEGF-treated cultures, respectively; p <0.05). The results obtained in LIF+VEGF treated, the first-trimester trophoblast hypoxic subgroup (not substantially different from both, LIF- and VEGF- treated the respective subgroups) may suggest that LIF and VEGF at least partially compete for the same signaling pathway.

In hypoxia control cultures without exogenous LIF or VEGF, the differences in apoptosis intensity between first-trimester trophoblasts and term trophoblasts were not significant (see Fig. 1A Control). In normoxia, the addition of LIF, VEGF or LIF+VEGF to the culture media also diminished the mean intensity of apoptosis as compared to controls (see Fig. 1B), but this effect was similar in both studied groups (p >0.05). Apoptotic intensity in normoxic controls (Fig. 1B Control) was significantly lower than in the hypoxic controls. The concentration of plasma cytokeratin 18 fragments in the culture supernatants ranged from 122 U/L to 321 U/L (normoxic versus hypoxic controls, respectively).

5. Discussion
Apoptosis occurs in most tissues as an important process related to normal development and differentiation [29]. Within trophoblast cells of the human placenta, disturbances of the ratios between cellular growth/differentiation and programmed cell death may produce abnormal placental function [11,14,15]. Hypoxia is known as a deteriorating factor in most tissues, acting as an inducer of apoptosis [15]. On the other hand, properties of trophoblast cells are very unique, because early placental development occurs in an environment of relative oxygen deficiency [3,7]. The relative resistance of early trophoblast cells (transforming into the syncytiotrophoblast phenotype) to acute and extreme hypoxia may be a defense mechanism by which this crucial cell layer preserves critical functions even in the presence of extreme environmental stress. Hypoxia promotes angiogenesis, increases local VEGF concentration and up-regulates the expression of type 1 and 2 VEGF receptors [7,9,25].

It is very likely that sensitivity of the human trophoblast to apoptosis changes throughout normal pregnancy, but may be disturbed in abnormal hypoxic conditions.

In many obstetric pathologies characterized by placental underperfusion and villous hypoxia e.g., pregnancy induced hypertension, anemia, intrauterine growth retardation (IUGR), diabetic pregnancy, cigarette smoking and living in a high altitude, increased apoptosis has been reported [8,14,30]. On the other hand, Khaliq et al.[31] suggested “placental hyperoxygenia” instead of “placental hypoxia” as the crucial factor involved in the pathophysiology of IUGR. Interestingly, both hypoxia and hyperoxia promote programmed cell death [9,32]. Because of their clinical importance, the molecular and cellular mechanisms of the trophoblast cells responses to hypoxia have been studied extensively in recent years [7,9,11]. Oxygen deficiency below a certain level is known to hinder differentiation in cultured trophoblasts, while enhancing the expression of p53 and Bax, two proapoptotic proteins that predominate in cytotrophoblast [9,11,33]. The differentiated trophoblast phenotype (i.e., syncytiotrophoblast) is more resistant to hypoxia-related apoptosis than the less differentiated cytotrophoblast phenotype. Levy et al.[34] found that programmed cell death occurred primarily in the first 24 hours of culture, when most of the cells are undifferentiated cytotrophoblasts [35]. Thus, we hypothesized that the greater resistance of the first trimester trophoblasts to apoptotic stimuli under hypoxia may be related to their ability to differentiate more quickly than the third trimester cultured trophoblast, governed by LIF and VEGF. Further experiments are needed to test this hypothesis. James et al. [36] reported recently that both the week of gestation (i.e., gestational age of the trophoblast) and oxygen concentration play important regulatory roles in extravillous trophoblast outgrowth formation during the first trimester. In our study, we analyzed the
Figure 1.
Influence of LIF, VEGF and LIF+VEGF on the mean reduction/change of apoptosis intensity in hypoxia and normoxia (A. & B., respectively): first-trimester (Group I) versus term (Group II) trophoblast cultured in vitro. The mean intensity of apoptosis for term trophoblast was taken as 100%.
influence of hypoxia on trophoblastic cells derived from two more distant time points in pregnancy. To the best of our knowledge, this is the first paper to analyze the importance of gestational age of human trophoblasts with respect to LIF and VEGF antiapoptotic activities.

It is important to note that we cultured highly purified trophoblasts, avoiding the influence of other cells and related factors normally present in placental trophoblast surroundings. For example, in vivo the role of placental mast cells (MC) in trophoblast differentiation and apoptosis should be taken into consideration. Human placenta is a relatively rich source of MC, containing both VEGF and LIF as well as many other substances in their vesicular structures [37,38,39]. Moreover, we reported recently that histamine, a major MC amine mediator, is involved in the human trophoblast differentiation, increasing this process by the stimulation of αvβ3 integrins through the histamine H1 receptor [40]. Previously, we found that MC numbers and the concentration of histamine are different in human placental tissue obtained after normal versus complicated pregnancies [41,42]. This may be particularly important considering that hypoxia promotes degranulation of MC and thus the release of many (both pro- and antiapoptotic) mediators [43].

Interestingly, in another study of human term trophoblasts co-cultured with MC, we observed that MC-derived VEGF diminished the expression of the VEGF type 2 receptor (KDR) during hypoxia [25]. Thus, under certain conditions, significant alteration of the VEGF/JAK2/STAT5 axis may reduce the hypoxic tolerance level of trophoblast cells, leading to increased apoptosis [21].

We realize that our study has some limitations in that our first trimester human trophoblasts were obtained after spontaneous abortions. The results pertaining to the first trimester trophoblasts should be confirmed in further studies conducted with first trimester trophoblast cultures established after elective surgical termination of pregnancy. In conclusion, LIF- and VEGF-related increases in the differentiation and invasiveness of trophoblast cells may protect the cells during hypoxia and consequently reduce apoptosis intensity, especially during the first trimester of pregnancy.

References:
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