Endogenous tumor necrosis factor α mediates enhanced apoptosis of cultured villous trophoblasts from intrauterine growth-restricted placentae

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Abstract

Tumor necrosis factor α (TNF α) has been implicated in the abnormally high levels of trophoblast apoptosis seen in placentae from pregnancies complicated by small births. We examined the hypothesis that at physiological (35–50 mmHg) oxygen tensions, the production of TNF α stimulates the apoptosis of placental trophoblasts associated with infants that are intrauterine growth-restricted (IUGR). Highly purified cytotrophoblasts (CT) from IUGR-complicated pregnancies spontaneously underwent a higher rate of apoptosis after 24 h of culture at a normoxic (for villous CT) tension of 38 mmHg than did CT from normal placentae. Real-time PCR analysis of TNF α mRNA revealed ~threefold higher levels in IUGR trophoblasts after culturing at a pO₂ of 38 mmHg. A higher level of TNF α receptor p55 (which mediates apoptosis) was found in IUGR CT by western blot analysis at pO₂ of <10, 38, and 140 mmHg. Neutralizing antibody to TNF α significantly inhibited the apoptosis of IUGR trophoblasts cultured at 38 mmHg and addition of TNF α significantly elevated apoptosis of normal and IUGR trophoblasts but less in IUGR cells cultured at <10 mmHg. We conclude that at physiological oxygen tensions (38 mmHg), villous CT from IUGR pregnancies, when compared with uncomplicated pregnancies, undergo more TNF α -induced apoptosis both because of elevated expression of TNF α and TNF receptor p55.

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Introduction

Early pregnancy loss leading to fetal death, preterm birth of a live fetus, and birth of a small fetus (IUGR; Mintz *et al.* 1986, Rubbert *et al.* 1992, Ticconi *et al.* 2003, Wong *et al.* 2004) together constitute a significant proportion of pregnancy complications, and cause significant maternal and fetal morbidity and mortality. Alterations in placental oxygen levels are implicated in the pathophysiology of all the three disorders (Salafia *et al.* 1995, Pardi *et al.* 2002, Jauniaux *et al.* 2006). Indeed, chronic hypoxia is one of the most common insults to the conceptus and is associated with IUGR (Kingdom *et al.* 2000). Prior to ~10 weeks of gestation, the human placenta develops in a low oxygen environment but subsequent remodeling of maternal spiral arteries allows a direct flow of maternal blood from peripheral circulation (reviewed in Benirschke & Kaufmann 2000). As pregnancy advances, blood normally enters the placenta via a maternal arteriole at an oxygen tension of about 100 mmHg and leaves the

intervillous space in a maternal venule at about 40 mmHg (Soothill *et al.* 1986, Sibley *et al.* 2002). Oxygen extracted by the placenta is delivered into the fetal umbilical vein at about 27 mmHg where it supplies the oxygen demands of the fetus.

The site of gas exchange to the fetus is the villous placenta which consists of an outer layer of syncytiotrophoblast (ST), a continuous, mature and non-proliferating cell that faces the intervillous space (maternal blood) with a microvillous surface (reviewed in Benirschke & Kaufmann 2000). Directly beneath the ST are cytotrophoblasts (CT) which are cells with a single nucleus, cluster near fetal blood vessels in the third trimester and replicate and fuse with the ST to repair or expand it. The trophoblast basal lamina lies beneath the trophoblast layer and separates the trophoblast from the fetal stroma consisting of fibroblastic cells, fetal macrophages, and fetal vascular endothelium. The distance between the intervillous space and the villous blood vessels involved in gas exchange is small (<20 μ m).

Therefore, it is difficult to assign an oxygen tension in the immediate vicinity of CT, but it would be predicted to average about 30–45 mmHg for both normal and IUGR placentae (Hung *et al.* 2001).

IUGR placental villi are smaller in all cellular aspects (Mayhew *et al.* 2003), but the trophoblasts undergo an elevated frequency of apoptosis when compared with their normal counterparts (Smith *et al.* 1997). We have found that CT cultured with TNF α enhanced apoptosis (Yui *et al.* 1994a), which was stimulated via the TNF p55 receptor (Yui *et al.* 1996). Previous studies have shown that the CT isolated from IUGR pregnancies undergo elevated apoptosis rates, compared with normal CT, when cultured at the hypoxic oxygen tension of 23 mmHg (Crocker *et al.* 2003). These authors also implicated elevated TNF α as a mediator but its role was not established. However, in a study with CT isolated from normal placentae, we found that these cells were remarkably resistant to chronic hypoxia, with the lowest rate of apoptosis observed at 15 and 38 mmHg, and that apoptosis increased only when the cells were cultured in <10 mmHg (Kilani *et al.* 2003).

Since the oxygen levels that IUGR CT are exposed to *in vivo* likely are not greatly different from those of normal villous CT, we hypothesized that any abnormal apoptosis in IUGR should manifest at the relatively normoxic range of 38 mmHg. We tested this hypothesis by culturing cells at three different oxygen levels (140, 38, and <10 mmHg) measuring the frequency of apoptosis, the levels of TNF α mRNA, and the levels of TNF receptor p55 by western blot analysis.

Materials and Methods

Isolation, purification, and culture of term villous cytotrophoblasts

Placentae were obtained after normal term deliveries or term elective cesarean sections from either uncomplicated or IUGR-complicated pregnancies (we find no differences in apoptosis between vaginal- and caesarean-delivered placentae). An IUGR pregnancy was defined as a term delivery of an infant at ≤ 10 th weight centile of all deliveries in Edmonton (the inclusion criterion). Other than fetal weight ≥ 90 th centile, there were no other exclusion criteria. Mothers were not segregated by smoking, pre-eclampsia or other pregnancy complication other than weight of their baby. Four out of the nine mothers with an IUGR pregnancy also had pre-eclampsia and one of these (the only one of the nine) had absent end diastolic flow in the uterine artery. Gestational ages of normal and IUGR placentae were not statistically different (normal: $n=9$, average = 38.4 ± 0.6 weeks; IUGR: $n=9$, average = 36.2 ± 3.8 weeks; $P < 0.13$). Villous mononuclear trophoblasts were isolated by trypsin/DNase digestion of minced chorionic tissue and immunoabsorption on to Ig-coated glass bead

columns as previously described (Yui *et al.* 1994b, Kilani *et al.* 1997, Guilbert *et al.* 2002) using anti-major histocompatibility complex (MHC) class I (W6/32, Harlan Sera-Lab, Crawley Down, Sussex, England), anti-CD9 (clone 50H.19), and anti-MHC class II (clone 7H3) antibodies for immunoelimination (the latter two were prepared in our laboratory). The yield of the resulting population is approximately 67% ($n=8$) of starting unpurified cells with a range of $2-8 \times 10^8$ CT per placenta. After adhesion to tissue culture dishes and washing as described below, the resulting cultures contain fewer than five vimentin-positive (non-trophoblasts) cells/ 10^5 plated and are almost completely free of mononuclear syncytial fragments (Guilbert *et al.* 2002). The cells were cryopreserved and thawed as described previously (Yui *et al.* 1994b, Guilbert *et al.* 2002). The thawed cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM, GIBCO) supplemented with 10% fetal bovine serum (FBS, GIBCO) and antibiotics (end concentrations: penicillin 100 U/ml, streptomycin 100 μ g/ml; Sigma) at seeding densities between 5.0 and 7.5×10^5 cells/ml in 24-well plates (Corning, NY, USA). The cells were allowed to attach for 5 h, washed with warm IMDM and pre-equilibrated (at the appropriate oxygen level) 2% FBS-IMDM or medium containing TNF- α (10 ng/ml; a gift of Hoffmann LaRoche, Basel, Switzerland) or antibody to TNF- α (at final concentration of 2 μ g/ml, a dose that neutralizes 1 ng/ml TNF α (data not shown), Upstate Biotechnologies, Lake Placid, NY, USA) added. The cells were then incubated for 24 h in one of the following culture environments (all of which are fully humidified): (i) in a standard 5% CO₂ in air incubator (pO₂ 140 mmHg); (ii) in a controlled oxygen incubator (Forma Series II, Forma, Marietta, OH, USA) regulated at 5% CO₂ and 5% oxygen (pO₂ 38 mmHg) with the balance being nitrogen; and (iii) in a modular-incubator chamber (Billups-Rothenberg, DeMar, CA, USA) in which air was replaced with nitrogen containing 5% CO₂ by a time and flow (15.0 min, 200 ml/s) controlled purge (pO₂ <10 mmHg). The oxygen tension of the media in the different culture environments was measured as described previously (Kilani *et al.* 2003). All experiments were carried out independently at least twice.

Measurements of apoptosis

A total of nine different IUGR placental preparations were used in the study and compared with nine representative normal placentae. Nuclear DNA fragmentation in apoptotic cells was detected using terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin DNA-nick end labeling (TUNEL; Gavrieli *et al.* 1992) in trophoblast cultures as described previously (Yui *et al.* 1994a). The number of apoptotic and non-apoptotic nuclei was assessed by counting and averaging ten randomly selected 0.25 mm² microscopic fields in each

of the three replicate wells (magnification, $\times 200$) with a Zeiss Telaval 31 inverted microscope (Carl Zeiss, Thornwood, NY, USA). Apoptosis was also assessed by the fraction of DAPI (4,6-diamidino-2-phenylindole) bright nuclei. DAPI (150 $\mu\text{g}/\text{ml}$) was added to trophoblasts, fixed with 1% paraformaldehyde (10 min at room temperature), and then removed with two gentle washes with PBS. The cells were then visualized with an inverted phase contrast microscope (Model DS-IRB, Leica; Heerbrugg, Switzerland) equipped for epifluorescence with a 100 W high-pressure mercury lamp driven by a Ludl power source (Ludl Electronic Products; Hawthorne, NY, USA). Digital images of ten fields of each well of 24-well plates were taken with a blue filter using a SPOT digital camera (Diagnostic Instruments; St Sterling Height, Michigan, IL, USA) and the images manually evaluated for the percentage DAPI bright. The fraction of TUNEL-positive nuclei in trophoblast cultures parallels the appearance of characteristic uniform DNA degradation patterns (ladders; Yui *et al.* 1994a, Garcia-Lloret *et al.* 1996) and is identical to the fraction of compacted nuclei detected by differential propidium iodide (Yui *et al.* 1996) and DAPI (present paper) staining. Therefore, apoptosis was assessed by either TUNEL or DAPI.

Measurement of TNF α by bioassay and by quantitative (q) RT-PCR

Supernatants were saved and frozen at -20°C until analysis. Thawed supernatants were assayed for TNF- α bioactivity using recombinant human TNF- α standards in the L929-8 bioassay as described previously (Branch *et al.* 1991). The lowest level of detection was 1 pg/ml.

We carried out the TNF α mRNA analysis on samples from normal and IUGR cells (three independent experiments each) cultured for 3, 6, and 12 h (times start after the 5-h adhesion step) at three different oxygen tensions with cells from six different placentae. The results were very similar for each of the time points and only the 3-h data are presented. The primers and probe specific for human TNF α were purchased from Applied Biosystems (Foster City, California, USA). The sequences for primers and probes are confidential information, but the lot (G03991) was tested at the factory for reliability with a Stratagene Reference Set. HPLC-purified human 18S primers (forward: ctaccacatccaaggaaggc and reverse: gactcattccaattacagggc) were synthesized by Operon Biotechnologies (Huntsville, AL, USA). The cells were lysed with Trizol Reagent (GIBCO BRL Life technologies), total RNA was extracted using the protocol supplied by the manufacturer, and RNA integrity was verified by electrophoresis and visualization with ethidium bromide staining and by an average optical density (OD) 260/OD280 absorption ratio of 2.11. Total RNA (1 μg) was reverse transcribed, the reverse transcriptase was inactivated by heating at

70°C for 15 min and the resulting cDNA was used for qPCR amplification of TNF α as described previously (Arenas *et al.* 2004). The experiments were performed in triplicate for each sample. The reference gene 18S rRNA was run concurrently to normalize the results obtained. The amplification efficiency for each primer set was determined by converting the slope of the standard curve using the algorithm $E = 10^{-1/\text{slope}}$ (Pfaffl 2001). For each gene, the mean threshold cycle, corrected for the efficiency of the reaction, was expressed relative to the control sample and the mean of each treatment group was determined.

Western blot analysis

Sample protein concentrations were determined in duplicate with Micro BCA Reagent (PIERCE Chemical Company, Rockford, IL, USA) with a serum albumin standard. Sample protein (15–20 μg) was solubilized in $3\times$ sample buffer (Sigma) by boiling for 5 min and stored at -20°C until electrophoresis. SDS-PAGE was performed according to the procedure of Laemmli (1970) using 10% acrylamide (Mini-Protein II gel system, Bio-Rad Laboratories, Inc.), blotted onto nitrocellulose membranes, the blots incubated overnight at 4°C with a specific antibody (anti-TNF- α receptor p55, rabbit polyclonal, antigen purified and used at 4 $\mu\text{g}/\text{ml}$, Stressgen, Victoria, BC, Canada), exposed to X-ray film, band density after exposure digitized, and analyzed as described previously (Mackova *et al.* 2003).

Statistical analysis

The number of placentae and replicate experiments is described in individual figure legends. Differences between experimental groups were evaluated by two-way ANOVA with pairwise multiple comparison procedures (Tukey Test) and Student's *t*-test using the SigmaStat program (Jandel Scientific, San Rafael, CA, USA) as explained in the figure legends. Results are expressed as mean $\pm$ s.d. and were considered to be significant at $P < 0.05$.

Results

The frequency of apoptosis in CT isolated from IUGR compared with uncomplicated pregnancies as a function of oxygen tension

The apoptosis frequency of a preparation was assessed at oxygen tensions of 140, 38 and < 10 mmHg at the same time to minimize errors. Representative assessments of apoptosis for trophoblasts from a normal birth and from an IUGR birth are shown in Fig. 1. This pattern of apoptosis was the same in all normal preparations (high at 140 mmHg, low at 38 mmHg, and higher again at < 10 mmHg (Kilani *et al.* 2003)). The pattern was

Figure 1 Typical apoptosis frequencies of cytotrophoblasts isolated from placentae from uncomplicated (normal) and IUGR-complicated (IUGR) births. The frequencies measured on a single preparation are shown in the figure (mean $\pm$ s.d. of three wells). Apoptosis and culture procedures are detailed in the Materials and Methods.

different (lowest at 140 mmHg, higher at 38 mmHg, and slightly higher at <10 mmHg) for all IUGR preparations but with considerable scatter in absolute values (data not shown). At 140 mmHg, the average apoptosis frequency in CT from normal placentae cultured in 24-well dishes was $6.0\% \pm 3.8$ and for IUGR was $6.9\% \pm 3.3$. In order to compare the frequencies for IUGR and normal trophoblast preparations, the apoptosis frequencies of a preparation were normalized to the frequency observed at 140 mmHg. The normalized values are shown in Fig. 2

Figure 2 A comparison of the ratio of apoptosis frequencies (at all oxygen levels to the apoptosis at 140 mmHg) of trophoblasts isolated from placentae of normal (open bars) and IUGR (filled bars)-complicated pregnancies. The results are pooled data from 20 independent experiments on trophoblast preparations from nine different normal placentae ($n=9$) and 28 independent experiments on preparations from nine different IUGR placentae ($n=9$). Depicted are the mean $\pm$ s.d. of the ratios (140 mmHg/140 mmHg=1). Experimental groups labeled with different letters (a–b) are significantly different ($P<0.05$, two-way ANOVA).

with 140 mmHg shown as 1.0. The apoptosis frequencies for normal CT were always lower (0.71 ± 0.16) at 38 mmHg than at 140 mmHg, but the frequencies for IUGR CT were always higher (2.1 ± 0.36) at 38 mmHg than at 140 mmHg. When comparing the values at 38 mmHg by two-way ANOVA analysis, we found that they were statistically different ($P<0.001$). The apoptosis ratios for both normal and IUGR CT at <10 mmHg were both high and not different from each other. This comparison showed that the cells from IUGR placentae underwent a much higher ratio of apoptosis at 38 mmHg relative to 140 mmHg.

Apoptosis of trophoblasts from normal and IUGR placentae in the absence or presence of antibody to TNF α and absence or presence of added TNF α

TNF α is one of the few cytokines that increases apoptosis in trophoblasts (Yui *et al.* 1994a, Crocker *et al.* 2003, Gill & Hunt 2004). Therefore, we first examined the effect of endogenously produced TNF α on the apoptosis of cells from uncomplicated and IUGR-complicated placentae by calculating the ratio of apoptosis with to without excess TNF α antibody (Fig. 3A). The averages of ratios were near

Figure 3 The effect of neutralizing antibody to TNF α and of added TNF α on the apoptosis frequencies of normal and IUGR trophoblasts. (A) Ratio of apoptosis frequencies with to without 2 μ g/ml neutralizing antibody to TNF α added to the culture medium. The dashed line is 1.0 (no effect). The ratio of apoptosis of normal and IUGR cells was compared by Student's *t*-test at 38 mmHg. (B) Ratio of apoptosis frequencies with to without 10 ng/ml TNF α added to the culture medium. The dashed lines represent the calculated average s.d. of constitutive apoptosis (with at ratio 1.0 being no effect). The levels of apoptosis with to without TNF α were compared in those groups marked with an * by paired Student's *t*-test ($P<0.05$, data not log-transformed prior to carrying out Student's *t*-test).

unity for both uncomplicated and IUGR CT at 140 mmHg and not significantly different (a ratio of 1.0 is depicted with a dashed line). However, the ratio for IUGR CT was reduced at 38 mmHg (0.57 ± 0.15) and was significantly ($P < 0.05$) different from the ratio for normal cells (1.05 ± 0.29). At < 10 mmHg, there was no significant difference between normal and IUGR. These data show that excess neutralizing antibody to TNF α affected the apoptosis frequency only for IUGR CT at 38 mmHg.

We next cultured cells with and without added TNF α (at a near optimal dose of 10 ng/ml; Yui *et al.* 1994a) and calculated apoptosis ratios with to without TNF α . In general, the stimulation of apoptosis by exogenous TNF α decreased with decreasing oxygen tension for both normal and IUGR cells (Fig. 3B). TNF α added to the medium increased apoptosis about threefold at 140 mmHg for both normal and IUGR cells and it barely increased the apoptosis frequencies at < 10 mmHg. At 38 mmHg, TNF α increased apoptosis of IUGR cells by 1.6-fold and normal cells by greater than fourfold. However, the former increase was statistically significant ($P < 0.05$) but the latter was not, probably because the very low apoptosis of normal cells at 38 mmHg increased the scatter of the ratio (see Fig. 2).

Comparison of TNF α mRNA content of normal and IUGR placentae by qRT-PCR

The data in Fig. 3 show that endogenous TNF α accounts for a significant fraction (about 45%) of spontaneous apoptosis in IUGR cells cultured at an oxygen tension of 38 mmHg (when compared with almost nothing for normal cells). We next examined normal and IUGR cultures for levels of expression of TNF α and the TNF receptor that mediates its apoptotic effects (p55; Yui *et al.* 1996). Attempts to measure TNF α bioreactivity in the culture supernatants showed that the levels were near the level of detection (~ 1 pg/ml, data not shown). As an alternative method, we quantitatively measured TNF α mRNA levels in the cultures by qRT-PCR. Expression of 18S RNA was proportional to the amount of mRNA present (data not shown). The expression of TNF α mRNA relative to 18S RNA was measured in the samples incubated for 3 h in six-well tissue culture dishes at the three oxygen levels for both IUGR and normal samples (Fig. 4). In normal cells, TNF α mRNA levels slightly decreased with decreasing culture oxygen levels. However, TNF α mRNA levels increased in IUGR cells with decreasing culture oxygen levels. This divergence reached significance at 38 mm and < 10 mmHg ($P < 0.05$). We next examined the levels of TNF receptor p55 protein (TNF-R1) after 24 h of culture at the different oxygen levels by western blot analysis (Fig. 5). IUGR cells expressed higher levels of TNF-R1 (relative to β -actin) at all the three oxygen levels especially after culture at 38 mmHg. These data show that the TNF α expression at 38 mmHg was greater in IUGR than

Figure 4 TNF α mRNA levels (normalized to 18S RNA levels) of trophoblasts isolated from normal and IUGR pregnancies after 3 h of culture. Vertical axis, ratio of TNF α mRNA to 18S RNA levels. Horizontal axis, oxygen level of culture. Open bars, trophoblasts from normal pregnancies. Filled bars, trophoblasts from pregnancies complicated by IUGR. Depicted are the mean $\pm$ s.d. of three samples carried out from different CT purifications. Different letters by the bars (a–d) indicate statistically different groups within each time point (two-way ANOVA analysis, $P < 0.05$).

Figure 5 Protein expression of TNF receptor p55 in trophoblasts from uncomplicated and IUGR-complicated pregnancies. (A) A typical western blot analysis of TNFR1 and β -actin expression in cells from uncomplicated and IUGR-complicated pregnancies after culture at the indicated oxygen levels for 24 h. (B) The blots were scanned for both bands to obtain a ratio of TNFR1 to β -actin and this ratio was normalized to the 140 mmHg uncomplicated value. Depicted is the mean $\pm$ s.d. of the data from six placentas; three in the IUGR group and three in the normal group. Different letters by the bars (a–c) indicate statistically different groups within each time point (ANOVA analysis, $P < 0.05$).

normal cells and that, in addition, IUGR expressed more receptors.

Discussion

Based on an earlier study showing that trophoblasts from IUGR placentae had an abnormally high level of apoptosis (Smith *et al.* 1997) and that CT from normal pregnancies underwent minimal apoptosis at oxygen tensions of 15 and 38 mmHg (Kilani *et al.* 2003), we hypothesized that the trophoblasts from IUGR-complicated pregnancies would have elevated apoptosis frequencies at the normoxic levels of 38 mmHg. For CT isolated from uncomplicated pregnancies, the ratio of apoptosis at 38–140 mmHg is approximately 0.7. However, the oxygen dependency for apoptosis of CT from all nine IUGR placentae studied was strikingly different, in that the ratio of apoptosis at 38–140 mmHg was >2 . Thus, in agreement with our hypothesis, CT isolated from IUGR placentas underwent a greater level of apoptosis at a normoxic (for CT) oxygen level of 38 mmHg than did CT from normal placentas.

The placentas assessed in the present study were segregated into normal and IUGR based on fetal weight at delivery alone: those associated with fetuses in the upper 90th centile of local weight distribution were normal and those with the lower 10th centile were IUGR. Although there was a greater distribution of gestational age at delivery in the IUGR group due in part to a few early onset pre-eclampsia patients, the average ages of delivery were not significantly different. All normal and IUGR placentas were collected and CT isolated in the same manner. No other criterion was used to separate normal from IUGR; thus, the babies were probably small for a variety of reasons. However, in spite of the likely heterogeneity for IUGR baby weights, we still saw significant differences at 38 mmHg oxygen levels for apoptosis, TNF α expression, and expression of the TNF receptor p55 protein.

At the onset of culture (at 5 h), most but not all, apoptotic and dying cells were washed away (Guilbert *et al.* 2002); thus, most of the cells apoptotic at 24 h became so between 5 and 29 h. Based on our experience with TNF α as an autocrine mediator of cytomegalovirus-induced apoptosis of placental trophoblasts (Chan *et al.* 2002), we determined whether it was the causative agent. Autocrine production of TNF α was assessed by culturing the cells with excess neutralizing antibody to TNF α . At an oxygen level of 38 mmHg, an average of 45% of the spontaneous apoptosis of IUGR CT was blocked by antibody to TNF α while $<10\%$ of apoptosis was blocked by antibody in uncomplicated CT (Fig. 2). This 45% of spontaneous apoptosis for IUGR CT accounts for much of the difference between IUGR and uncomplicated CT.

The large contribution of autocrine TNF α at 38 mmHg was not manifested by measurable supernatant levels of

TNF α protein levels, possibly due to constant uptake by TNF receptors on the cells (both p55 and p75; Yui *et al.* 1996); thus, we measured TNF α mRNA expression by qRT-PCR. Determination of TNF α mRNA levels by RT-PCR has previously been used for estimating the TNF α expression by human trophoblasts (King *et al.* 1995, Bennett *et al.* 1996). Comparing expression of TNF α mRNA on cultured CT at a pO $_2$ of 38 mmHg with that at 140 mmHg, we found that cells from an uncomplicated placenta expressed significantly less ($P<0.05$, a 35% decrease) at 38 mmHg and that IUGR cells expressed more ($P<0.05$, a 55% increase) at 38 mmHg. This divergence (decreasing TNF α expression with decreasing oxygen tension for normal cells and increasing TNF α expression with decreasing oxygen tension for IUGR cells) leads to almost threefold higher levels after culture at 38 mmHg in the IUGR group. One can infer from this finding that IUGR CT produce more biologically active TNF α than CT from uncomplicated pregnancies and the cells are responding by undergoing a higher frequency of apoptosis. This inference agrees with the antibody data and supports our hypothesis that there is greater TNF α production in IUGR CT at 38 mmHg.

The regulation of TNF α expression as a function of oxygen level appears to be tissue- and disease-specific. Our findings, with highly purified CT, point to a disease-specific regulation. Hypoxia induced no increase (Hung *et al.* 2004) or a mild increase (Benyo *et al.* 1997) in TNF α secretion from placental explants, which contain other cells (placental macrophages) that express TNF α (Berkowitz *et al.* 1990). TNF α expression from mouse and human macrophages was either increased with decreasing oxygen tension (VanOtteren *et al.* 1995) or decreased (Hirani *et al.* 2001). The regulation of TNF α expression in normal mononuclear phagocytes and CT by hypoxia is complex and not completely understood. However, very clearly, expression of mRNA levels is different in CT isolated from normal and IUGR placentas.

The p55 TNF receptor mediates trophoblast apoptosis (Yui *et al.* 1996) and we found that the levels of this receptor (measured by western blot analysis) were upregulated in IUGR cells. Levels in normal trophoblasts did not vary as a function of culture oxygen tension but the levels in IUGR cells were consistently higher at all the three oxygen levels and marginally peak at 38 mmHg. We are aware that β -actin mRNA levels fluctuate somewhat with hypoxia (Zhang *et al.* 2001) and assume that the changes in protein expression will be the same in normal and IUGR cells. These results are in agreement with the observation that oxygen does not regulate the level of TNF-R1 (Hehlhans *et al.* 2001) and point to a dysregulation in IUGR CT. What the effect of increasing receptor levels in cultured CT is more difficult to predict because of the distribution of cell surface receptor levels among cells (some express none; Yui *et al.* 1996) and where the receptors are in and on the cell (inside the cell, on the surface, or on the surface but

destined to be cleaved). However, increasing cellular receptor levels will not have a deleterious effect on response and could well increase it. If p55 receptor level is a measure of capacity to respond to TNF α , we have therefore found an enhanced capacity to respond in IUGR cells, especially at 38 mmHg (Fig. 5).

Hypoxia is implicated in the pathogenesis of preterm labor, birth of a small fetus (IUGR), and early miscarriages (Salafia *et al.* 1995, Pardi *et al.* 2002, Jauniaux *et al.* 2006). TNF α upregulation is independently implicated in these pregnancy complications (Gorivodsky *et al.* 1998, Rivera *et al.* 1998, Steinborn *et al.* 1998), although there is controversy concerning miscarriages (Fidel *et al.* 1997, Lea *et al.* 1997). The relationship of TNF α expression to low oxygen, the nature and location of cells expressing TNF α , and whether these cells are hypoxic for their location in the placenta remain to be determined. However, we have found that during culture at 38 mmHg (a normoxic oxygen tension for CT), apoptosis is increased in CT from IUGR relative to CT from normal placentae and that this increase in apoptosis is, in part, due to increased TNF α . The increase in TNF α -induced apoptosis is also linked to an increase in the expression of TNF-R1. The combination could cause an increasing number of CT to undergo apoptosis in IUGR placentae and thereby contribute to placental insufficiency.

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