The effects of oxygen concentration and gestational age on extravillous trophoblast outgrowth in a human first trimester villous explant model

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BACKGROUND: In the first trimester of human pregnancy, extravillous trophoblasts from placental villi invade the decidua temporarily occluding the spiral arteries, preventing maternal blood flow and creating a low-oxygen environment, which is believed to play an important role in the regulation of extravillous trophoblast outgrowth. This work aimed to quantify the effects of gestational age and oxygen concentration on extravillous trophoblast outgrowth. METHODS: A quantitative first trimester villous explant model was used to measure the frequency and area of extravillous trophoblast outgrowths from villi grown in 1.5 or 8% oxygen. RESULTS: The percentage of explants producing outgrowth declined independently of oxygen concentration as gestation increased from 8 to 12 weeks. Culture in 1.5% oxygen significantly reduced the frequency and area of outgrowths in comparison with 8% oxygen. HLA-G and α1 integrin were both expressed throughout outgrowths, with no difference in the expression between oxygen concentrations. Gestation influenced the response of explants to oxygen, with a significant differential response to oxygen concentration in placentae under 11 weeks of gestation, whereas in villi from placentae of 11 or 12 weeks, no differential response was observed. CONCLUSIONS: In the first trimester, oxygen and gestational age both regulate extravillous trophoblast outgrowth.

Key words: cytotrophoblast differentiation/extravillous trophoblast/hypoxia/placenta

Introduction

In the first trimester of human pregnancy, placental villi consist of a core of mesenchymal cells surrounded by a monolayer of mononuclear cytotrophoblast cells which either fuse to form the overlying multinucleated syncytiotrophoblast or, in anchoring villi, differentiate into extravillous trophoblasts that grow out from the villi in columns and invade into the maternal decidua (Boyd and Hamilton, 1970). As the extravillous trophoblasts in the columns move away from the anchoring villi, they also spread laterally around the implantation site and invade the maternal spiral arteries (Brosens et al., 1967; Kam et al., 1999). The invasion of the spiral arteries by extravillous trophoblasts leads to the transformation of these vessels into large-bore conduits that allow the increased maternal blood flow that is required for fetal growth. The transformation of the spiral arteries by extravillous trophoblasts is called the ‘physiological changes of pregnancy’, and inadequate physiological changes of pregnancy are found in pregnancies complicated by pre-eclampsia and intrauterine growth restriction (Brosens et al., 1967; Khong et al., 1986).

It is now apparent that from at least 5–10 weeks of gestation, extravillous trophoblasts form plugs in the maternal spiral arteries occluding the flow of maternal blood to the intervillous space (Hustin and Schaaps, 1987; Jauniaux et al., 1992, 2003; Jaffe and Woods, 1993). Thus, for the majority of the first trimester, the placenta develops in conditions of physiological hypoxia in which the local oxygen concentration is as low as 1–2% (Rodesch et al., 1992). The trophoblast plugs are believed to progressively disperse from 10 to 12 weeks of gestation, allowing maternal blood to flow into the intervillous space (Jauniaux et al., 1992, 2003; Jaffe and Woods, 1993). It is believed that the low-oxygen conditions during the first 10–12 weeks of pregnancy regulate placental development and extravillous trophoblast outgrowth (reviewed in James et al., 2005a).

In this study, we have quantified extravillous trophoblast outgrowth from large numbers of cultured first trimester villous explants to determine the effects of extravillous trophoblast outgrowth of (i) gestational age and (ii) oxygen concentration and (iii) whether these effects are interdependent.

Materials and methods

This study was approved by the Auckland Regional Ethics Committee, and all tissue samples were obtained with informed consent.
**Explant culture and image analysis**

First trimester placentae were obtained following elective surgical termination of pregnancy (TOP) and washed gently in phosphate-buffered saline (PBS, pH 7.4). The gestational age, based on crown-rump length, and fetal viability of all pregnancies before TOP were confirmed by ultrasound assessment. Villous explants were cultured as described previously (James et al., 2005b). Briefly, Matrigel (Bectin Dickinson, Sydney, Australia) was diluted to 1:10 in Dulbecco’s modified Eagle’s medium salts at 4°C (DMEM/F12) (Life Technologies, Auckland, New Zealand) and used to coat wells of sterile 96-well culture plates (Falcon, Sydney, Australia) with an extremely thin layer of Matrigel. Villous tips of ~8 mg wet weight were gently teased from the placenta, and randomly selected explants were placed in the centre of each well. The villous explants were incubated at 37°C for 5 min and then 150 μl/well of complete trophoblast medium [DMEM/F12 containing 10% fetal bovine serum (FBS), 5 ng/ml of epidermal growth factor, 5 μg/ml of insulin, 10 μg/ml of transferrin, 100 μg/ml of streptomycin, sodium selenite 20 nM, 400 U/l of hCG and 100 U/l of penicillin] was added. The plate was then centrifuged at 210 g for 5 min. At least 75 explants were prepared from each of the five placenta of culture, two-dimensional (2D) outgrowth of trophoblasts from the explants across the thin layer of Matrigel was observed by phase-contrast microscopy using an inverted microscope (Nikon EL WD 0.3990 digital camera (Nikon)). Overlapping images were aligned using Adobe Photoshop 5.0, and the areas of trophoblast outgrowth were measured by manual tracing using Image J software.

**Statistical analysis**

Data analysis was performed using Microsoft Excel 2002 and WinSTAT for Excel. The statistical significance of the relationship between gestational age and the frequency of outgrowth production was determined by analysis of variance (ANOVA). A dependent Student’s t-test was used to calculate the statistical significance of the outgrowth frequency data. The data generated from the measurement of outgrowth areas were not normally distributed; therefore, a Mann–Whitney U-test was used to calculate its statistical significance.

The differential response of explants to culture in 1.5 and 8% oxygen was defined using the formula: percentage of explants producing outgrowth in 8% oxygen – percentage of explants producing outgrowth in 1.5% oxygen. The significance of the differential response to oxygen was analysed by a t-test. The significance of this data was also determined by gestational age using a single factor ANOVA.

**In situ immunohistochemistry**

Media were removed from explants which were retained in the culture plates and washed with PBS and then fixed in 100 μl of methanol for 10 min. Wells were washed once with PBS (pH 7.4) containing 0.05% Tween-20 (PBS–TWEEN); then non-specific binding was blocked by the addition of 100 μl of 10% normal goat serum (NGS) in PBS–TWEEN (Life Technologies) for 10 min at room temperature. Wells were then washed three times with PBS–TWEEN. One hundred microlitres of 1:66 αt integrin (Sigma, Castle Hill, NSW, Australia) and 1:200 HLA-G diluted in 10% NGS in PBS-Tween was added for 1 h at room temperature (Sapphire Biosciences, Redfern, NSW, Australia). One well stained with 1:500 cytokeratin 7 was used as a positive control, and wells stained with 1:500 vimentin and 10% NGS in PBS–TWEEN containing no antibody were used as negative controls. Each antibody was used on explant cultures from 1.5 and 8% oxygen cultures from three placenta of the same gestational age, such that in total 36 outgrowths were stained for αt integrin expression and 26 outgrowths were stained for HLA-G expression. Wells were then washed three times with PBS–TWEEN, and endogenous peroxidase activity was quenched by the addition of 50 μl of 5% H2O2 in methanol for 5 min. Wells were then washed three times with PBS–TWEEN. A Zymed Histostain-Plus Kit (Zymed, San Francisco, CA, USA) containing biotinylated secondary antibody and enzyme conjugate was used according to the manufacturer’s instructions. Wells were washed three times with PBS–TWEEN, and staining was developed with 3,3′-diaminobenzidine (DAB) (0.1% w/v DAB, 0.001% H2O2 in PBS) for 20 min. Wells were then washed with de-ionized water. One hundred microlitres of haemotoxylin nuclear stain (Suripath, Australia Laboratory Services, Auckland, New Zealand) was added to each well for 4 min. Wells were washed with tap water. Outgrowth staining was observed immediately using an inverted microscope (Nikon EL WD 0.3 Phase Contrast microscope, Nikon) and digitally photographed using a Nikon Coolpix 990 digital camera (Nikon).

**Results**

**Gestational age affects the number of villi producing extravillous trophoblast outgrowth but not the size of the outgrowths**

Of the 3963 explants cultured in this study, 555 (14%) produced extravillous trophoblast outgrowths. A further 13.8% of explants floated in the culture media, and because they were not in contact with the Matrigel surface, these explants were excluded from the analysis. The percentage of explants from each placenta that produced extravillous trophoblast outgrowth declined progressively with increasing gestational age of the tissue, independent of oxygen concentration (F = 0.012 and 0.001 in 1.5 and 8% oxygen cultures, respectively) (Figure 1). However, the gestational age of the tissue did not affect the area of extravillous trophoblast outgrowths produced by individual villi.

**The effect of oxygen concentration on extravillous trophoblast outgrowth**

To determine the effect of oxygen concentration on the number of explants producing extravillous trophoblast outgrowths, we compared the percentage of explants regardless of the gestational age that produced outgrowth when cultured in either 1.5 or 8% oxygen. Significantly fewer (P = 0.0001) explants produced extravillous trophoblast outgrowths in 1.5% oxygen (14%, n = 239/1713) than in 8% oxygen (18.6%, n = 317/1702) (Figure 2).

To determine the effect of oxygen concentration on the size of extravillous trophoblast outgrowths, we compared the mean area of outgrowth from explants cultured in either 1.5 or 8% oxygen (Figure 3). The mean area of extravillous trophoblast outgrowths produced in 1.5% oxygen (0.94 mm², n = 238)
Oxygen and gestation regulate trophoblast outgrowth

The relationship between gestational age and the effect of oxygen concentration on the formation of extravillous trophoblast outgrowth

To examine whether the effect of oxygen concentration applied equally to placentae of different gestations, we examined the differential response of villous explants to oxygen concentration by determining the difference in the percentage of explants that produced outgrowths using matched cultures of explants from the same placenta. There was a significant difference ($P = 0.0005$) between the clear differential response of explants from the same placentae to oxygen concentration for placentae under 11 weeks of gestation (8.4%, $n = 15$ placentae) and the negligible differential response observed from explants from placentae of 11 weeks of gestation and greater (0.79%, $n = 10$ placentae) (Figure 5). The alteration in response to oxygen concentration with gestational age was not a gradual trend but rather appeared as a discrete change that occurred at 11 weeks of gestation (Figure 5).

Discussion

The low-oxygen environment in which placental development occurs in the first trimester of human pregnancy is believed to be important in controlling extravillous trophoblast outgrowth by directing the tightly regulated processes of trophoblast differentiation and invasion (Caniggia and Winter, 2002). In this study, we have utilized a 2D model of extravillous trophoblast outgrowth from first trimester villous explants to examine the
**Figure 4.** *In situ* immunohistochemistry of extravillous trophoblast outgrowth across the Matrigel-coated surface of a 96-well plate after 5 days of culture. Extravillous trophoblast outgrowths are counterstained with haematoxylin. HLA-G was expressed throughout the outgrowth, and no alterations in the staining pattern or intensity were observed between cultures in 1.5% (A) and 8% (B) oxygen. Likewise, α1 integrin was expressed throughout the outgrowth, and its expression was not different in cultures grown in 1.5% (C) and 8% (D) oxygen. No staining was observed with isotype-matched antibodies reactive with vimentin (E). Staining with the positive control antibody cytokeratin 7 was observed throughout the outgrowth (F). Panels A, B, E and F are of outgrowths from villous tissue of 10 weeks of gestation, whereas panels C and D are of outgrowths from villous tissue of 9 weeks of gestation.
Effects of gestation and oxygen concentration on extravillous trophoblast outgrowth from villi of 8–12 weeks of gestation.

The explant model we employ allows us to examine two separate features of extravillous trophoblast development. First, we do not select villous tips that have extravillous columns as some other researchers do (Genbacev et al., 1997; Aplin et al., 1999; Caniggia et al., 2000), but rather we dissect placenta into small explants of ∼8 mg which are randomly selected for culture. Because of the random nature of selection and the large number of explants cultured, a large proportion of the villi from each placenta are used, providing a good representation of the placenta as a whole. This technique allows us to examine the number of villous tips in a placenta that are capable of producing extravillous trophoblast outgrowth. Second, our explants are cultured on an extremely thin layer of Matrigel, which results in 2D extravillous trophoblast outgrowth that is readily and accurately quantifiable.

It is well known that the ratio of floating to anchoring villi increases as gestation progresses, particularly in the second and third trimesters, but our work shows that even during the last 5 weeks of the first trimester, the gestational age of placenta is important when considering extravillous trophoblast outgrowth and the response of explants to oxygen concentration. Independent of oxygen concentration, the percentage of randomly selected explants that produce extravillous trophoblast outgrowth declined progressively with the increasing gestational age of the tissue. It is widely believed that all villous cytotrophoblasts from first trimester placenta are bipotent and can form either syncytiotrophoblast or extravillous trophoblasts. In line with this belief, it has been suggested recently that all villi in the first trimester plaeneta are capable of forming into anchoring villi and producing extravillous trophoblasts (Baczyk et al., 2005). In contrast, as extravillous trophoblast outgrowth only occurs at the tips of the villi, it has previously been suggested that the distal tips of villi have a unique potential, which may or may not be inherent to the trophoblast at these sites (Aplin et al., 1998). Our results using very large numbers of explants expand on this suggestion by demonstrating that not all villi have the potential to become anchoring villi but rather that the numbers of villi with the potential to develop into anchoring villi decrease as the placenta expands with increasing gestational age. This study shows that as gestation progresses in the first trimester, more floating villi are formed, but these are intrinsically different to anchoring villi and as we have previously suggested, they do not have the potential to produce extravillous trophoblasts (James et al., 2005b).

In this study, villous explants produced significantly fewer extravillous trophoblast outgrowths in 1.5% oxygen than in 8% oxygen. These results are in contrast with those obtained in some other models. Others have shown that in comparison with explants cultured in 20% oxygen, first trimester villous explants of 5–8 weeks of gestation cultured under low oxygen show increased BrdU (thymidine analogue) incorporation, an increase in budding and extravillous trophoblast outgrowth from the tips of villi and an increase in the total number of cells in this outgrowth (Genbacev et al., 1997; Caniggia et al., 2000). However, the reported ‘increase’ in budding and outgrowth does not appear to have been quantified (Caniggia et al., 2000), whereas in our model, the size and frequency of extravillous trophoblast outgrowth were accurately quantified. The difference between our results and those of Caniggia et al. (2000) may also reflect the different gestational ages of the placentae studied, because this study has shown gestational age to be an important determinant of extravillous trophoblast outgrowth.
Alternatively, the differences between our results and those of Caniggia et al. (2000) used physiologically superoxic 20% oxygen (pO₂ = 140 mmHg) as their control. Once the maternal-fetal circulation is established, the blood in the intervillus space is a mixture of arterial and venous blood and blood sampled from the intervillous space at term has a pO₂ of 40 mmHg (Howard et al., 1961). Therefore, comparative concentrations of around 6–8% oxygen as used in our study may represent conditions in vivo once the maternal circulation in the intervillous space is established more accurately than 20% oxygen. Finally, the differences between our results and those of others might be explained by the small numbers of explants studied in other reports, because there tends to be considerable variation in the behaviour of individual explants. Indeed, a study by Newby et al. (2005) in which the authors were unable to observe any significant effect of oxygen on extravillous trophoblast outgrowth from villous explants highlights the difficulties of using a model in which both the nature of the primary tissue used and the experimental outcomes show a high level of variation. In this study, we have investigated a substantially larger number of explants and placentae than others to date (3693 explants), thereby allowing us to be confident that we have accurately represented villi from the whole placenta.

The decrease in extravillous trophoblast outgrowth we have shown with respect to both frequency and size in 1.5% oxygen compared with 8% oxygen could arise from four different scenarios.

(i) Only the extravillous trophoblasts proximal to villi proliferate and are able to drive outgrowth expansion (Vivovac et al., 1995; Caniggia et al., 1997; Korhonen and Virtanen, 1997). Therefore, low-oxygen conditions may inhibit the proliferation of extravillous trophoblasts in the proximal columns, thereby reducing the pool of trophoblasts able to form extravillous trophoblast outgrowth and the rate of outgrowth expansion.

(ii) It is possible that 1.5% oxygen conditions favour the proliferation of the progenitors of extravillous trophoblast in the villi over the migration of those cells into extravillous trophoblast columns.

(iii) The smaller area of outgrowth observed in 1.5% oxygen conditions may represent a greater proportion of explants that did not produce outgrowth until later in the culture period.

(iv) Culture in 1.5% oxygen may affect the viability of extravillous trophoblast progenitors or the extravillous trophoblasts themselves, thereby reducing the number of cells able to contribute to outgrowth formation and therefore the number and size of outgrowth observed.

At present, using this model, we have been unable to distinguish between these four possible explanations.

In vivo HLA-G expression is up-regulated as extravillous trophoblasts move away from the villi in proximal columns (Shorter et al., 1993; McMaster et al., 1995), and a previous study has reported the regulation of HLA-G by oxygen in the transformed trophoblast cell line HTR-8/SVneo (Kilburn et al., 2000). Therefore, we examined HLA-G expression in our model and in contrast to those previous studies observed that extravillous trophoblasts stained strongly for HLA-G throughout the cell columns in both 1.5 and 8% oxygen (Figure 4). In support of our data, more physiologically relevant experiments on outgrowth from explants and isolated first trimester trophoblasts showed no change in the expression of either the membrane-bound or the soluble isoforms of HLA-G between 20 and 2% oxygen (Genbacev et al., 1997; Nagamatsu et al., 2004). Furthermore, our own observations of placental bed biopsies show that HLA-G expression changes only in the proximal extravillous trophoblast columns (Bhalla et al., in press). Once extravillous trophoblasts enter the decidua, HLA-G is not further up-regulated nor is there an apparent difference in the level of HLA-G expression between endovascular trophoblasts which are exposed to arterial oxygen levels and extravillous trophoblasts in the surrounding decidua, which are exposed to lower oxygen levels, suggesting that HLA-G expression is not regulated by oxygen concentration (Bhalla et al., in press). Despite previous reports of oxygen-regulated expression of α₁ integrin in extravillous trophoblast outgrowths (Genbacev et al., 1997), we found that this molecule, like HLA-G, was expressed throughout the cell columns of all extravillous trophoblast outgrowths, with no difference observed between 1.5 and 8% oxygen conditions (Figure 4). Therefore, our results suggest that the expression of HLA-G and α₁ integrin is not regulated by oxygen concentration and that neither of these molecules are markers of responses to changing oxygen in trophoblasts.

It is commonly believed that the trophoblast plugs which occlude the maternal spiral arteries gradually disperse from 10 weeks of gestation, allowing maternal blood to flow into the intervillous space inducing a rise in oxygen tension across the villous surface (Jauniaux et al., 1992, 2003; Jaffe and Woods, 1993). It was interesting to note that when we examined the ability of explants to produce trophoblast outgrowth via the differential response of explants from the same placenta to 1.5 or 8% oxygen, there was an effect of oxygen concentration only for those placenta of <11 weeks. We had anticipated that there would be a gradual decline in the effect of oxygen concentration over the gestational ages we studied, because the placentae become exposed to increasing oxygen concentrations in vivo. However, our results showed a sharp change in the responsiveness to oxygen, with a clear lack of responsiveness to low oxygen in placenta of 11 weeks and greater. We interpret this result to indicate that there is a shift from a low-oxygen environment around 10 weeks of gestation such that the lack of responsiveness after this time may be either a result of the placenta preparing itself for the entry of maternal arterial blood into the intervillous space or a response to increasing oxygen concentration as the trophoblast plugs dissipate. Our data support the growing body of evidence that the trophoblast plugs dissipate exposing the placenta to maternal blood and increased oxygen concentrations from ∼10 weeks of gestation but suggest that there may be a sharp, rather than a gradual, change to arterial-like oxygen concentration in the intervillous space between 10 and 11 weeks (Jauniaux et al., 1992, 2001, 2003; Rodesch et al., 1992; Jaffe et al., 1997).

We have analysed our extravillous trophoblast outgrowth frequency data in two separate ways. These two analyses allowed us to investigate first the overall effect of gestational age on trophoblast outgrowth where we showed a progressive
decline in the ability of explants to produce outgrowth with increasing gestational age and, second, a more in-depth analysis using matched explants (differential response) allowed us to demonstrate that oxygen concentration modifies the gestational effect but only for placenta of <11 weeks of gestation with a sharp change in the responsiveness to oxygen between 10 and 11 weeks of gestation.

In conclusion, we have shown that low oxygen in the culture environment results in a decrease in the frequency and size of extravillous trophoblast outgrowths from villous explants. Furthermore, we have shown a difference in the ability of this tissue to produce extravillous trophoblast outgrowth and respond to changes in oxygen concentration with gestational age. Our results also question whether HLA-G or α1 integrin is regulated by oxygen concentration in the first trimester placenta. The data we have presented clearly show that both gestational age and oxygen play important regulatory roles in extravillous trophoblast outgrowth formation during the first trimester.

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References

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