

The Regulation and Localization of Angiopoietin-1, -2, and Their Receptor Tie2 in Normal and Pathologic Human Placentae

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Abstract

Background: Angiopoietin-1 (Ang-1) and its antagonist angiopoietin-2 (Ang-2) act on the endothelial cell Tie-2 receptor to regulate vascular integrity and remodeling. The local balance of these factors and the level of other angiogenic factors determine whether blood vessels grow, are maintained or regress. Profound angiogenesis and vascular remodeling occur in the placenta and this is altered in preeclampsia, a major cause of maternal and fetal morbidity and mortality.

Materials and Methods: The mRNAs encoding Ang-1, Ang-2, and Tie-2 were detected and localized in human placentae throughout gestation. The mechanism of regulation angiopoietin mRNAs level was determined by explant culture in ambient and reduced oxygen, and in the presence of actinomycin D.

Results: In situ hybridization showed that Ang-2 mRNA was abundant in the syncytiotrophoblast in the first trimester of human pregnancy. Ang-1 mRNA could not be

detected by in situ hybridization, but was by reverse transcriptase-polymerase chain reaction (RT-PCR) and Northern blotting. Placental vascular structure is altered in preeclampsia and intrauterine growth restriction, conditions where fetoplacental oxygenation is perturbed. In villous explant cultures, a reduction in oxygen tension significantly raised the levels of Ang-2 mRNA, and this was dependent on transcription. However, similar experiments showed that the stability of the Ang-1 message was greatly reduced under these conditions. Thus, hypoxia has opposite effects on Ang-1 and Ang-2 mRNA levels. Placentae obtained from women with preeclampsia had reduced levels of Ang-2 mRNA compared to gestationally matched controls. There was no difference in the levels of Ang-1 mRNAs.

Conclusions: These data show that the relative levels of Ang-1 and Ang-2 mRNA are regulated by local oxygen tension by different mechanisms and that this may be important during normal human placentation.

Introduction

Angiogenesis is a complex process in which new capillaries are formed from preexisting vessels (1). It is regulated by angiogenic inducers and inhibitors and the interplay between these factors results in blood vessel growth and regression (2,3). In adult tissues, angiogenesis usually occurs under pathologic conditions, such as tumor growth, retinopathies, and rheumatoid arthritis (3,4). However, cyclical angiogenesis occurs in the female reproductive tract, where vessel remodeling in the ovary and endometrium takes place (5). In addition, normal placental growth requires extensive new blood vessel formation.

Vascular endothelial cell growth factor A (VEGF-A) induces angiogenesis in a variety of systems, including the chick chorioallantoic membrane (CAM) and rabbit corneal pocket. It increases

microvascular permeability (6–8) promotes endothelial cell proliferation and cell migration (6–9). Human trophoblast expresses placenta growth factor (PlGF), a member of the VEGF family of genes, but VEGF-A is expressed in villous and decidual macrophages (10). VEGF-A has two receptors, the fms-like tyrosine kinase (Flt) and the kinase insert domain-containing receptor (KDR) (11,12). Mice spongiotrophoblast cells and human first trimester trophoblasts also produce soluble Flt, which is an antagonist for VEGF-A (12–14).

In addition to the VEGF family, angiopoietin-1 (Ang-1) and -2 (Ang-2) play a part in angiogenesis and vascular remodeling. Ang-1 is a secreted factor encoding a protein of 498 amino acids. This protein contains an NH₂-terminal coiled domain and a COOH-terminal fibrinogen domain (15). In vitro, Ang-1 does not induce endothelial cell proliferation, but is chemotactic for human endothelial cells (15,16). In vivo Ang-1 promotes angiogenesis (17). Mice fetuses with null mutations in the Ang-1 gene die at day 12.5 of pregnancy and show severe vascular abnormalities characterized by disturbance in endothelial/pericyte interactions (18). The role of Ang-1 may extend beyond embryonic angiogenesis;

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it is present in normal human arteries and veins (16). Ang-2 was isolated by low-stringency screening of human and mouse cDNA libraries using a mouse Ang-1 cDNA probe (19). It contains 496 amino acids, and is 60% homologous to Ang-1 (20). Although Ang-1 and Ang-2 share a similar protein structure, their biological activities are different. Ang-2 does not stimulate endothelial cell proliferation (16), but mice fetuses overexpressing Ang-2 die at day 9.5 of gestation, when the formation of vessels is disrupted (15,19).

Both Ang-1 and Ang-2 bind to Tie2 tyrosine kinase receptor (15,19). Ang-1 induces phosphorylation of Tie2, whereas Ang-2 binds to the receptor but does not activate the receptor. Thus, Ang-2 is a competitive inhibitor of Ang-1 (19). Tie2 mRNA is confined to endothelial cells (20,21), and mice carrying the Tie2 null mutations die 1–2 days earlier than the Ang-1^{-/-} mice (22,23)

An interaction between angiopoietins and VEGF is suggested by the coordinated expression of Ang-1, Ang-2, and VEGF mRNA in the rat ovary (19). VEGF mRNA is present in the preovulatory follicle before vessel invasion and becomes abundant within the center of the developing corpus luteum. Ang-1 follows the vessel ingrowth into the early corpus luteum. However, Ang-2 mRNA is present in the late preovulatory follicle and becomes abundant in the developing corpus luteum, when new blood vessel sprouting occurs. For new vessels to form, the preexisting endothelial cells need to loosen their contacts between their supporting pericytes and one another. Ang-2, being an antagonist of Ang-1, appears to play a role in this process. Ang-2, but not VEGF, mRNA is present in granulosa cell layer in atretic follicles when vessels in the theca interna recede. Thus, the expression of Ang-2 can have opposite functions depending on whether stimulatory factors such as VEGF are present. When VEGF is present, Ang-2 promotes vascular growth, but when VEGF is absent, vascular regression occurs (19).

Ang-1 stabilizes vessels and Ang-2 antagonizes this; therefore, a key determinant for vessel fate is the ratio of these two factors. The coordinated action of factors that promote endothelial cell proliferation and migration, such as VEGF and factors (such as Ang-1, which stabilizes the newly formed vessels) is essential for the formation of mature functional vessels. Under conditions where vessel growth or remodeling is required, one might anticipate the coordinated regulation of stimulatory and inhibitory factors. Specifically, Ang-1 and Ang-2 may be reciprocally regulated. Oxygen regulates the level of mRNAs encoding a variety of metabolic and proangiogenic growth factors. We therefore investigated the localization and mechanism of regulation of these mRNAs in the human placenta. In addition, it has been suggested that placental hypoperfusion and hypoxia may lead to the pregnancy complication preeclampsia (24). Preeclampsia affects approximately

4% of all pregnancies and remains a major cause of maternal and fetal morbidity and mortality (25). We provide evidence in this study that Ang-2 mRNA levels are reduced in this condition.

In this study, using *in situ* hybridization, we demonstrate mRNA encoding Ang-2 and its receptor Tie2 in human placentae throughout gestation and confirm the presence of protein using immunocytochemistry. We show that Ang-2 mRNA levels but not Ang-1 are increased by culturing under low oxygen conditions in first and third trimester placental villi. In cultures of term villi, increased levels of Ang-2 mRNA rise due to increased transcription rather than increased stability of the mRNA. However, levels of Ang-1 mRNA decline as a consequence of reduced stability of the mRNA. The ratio of mRNAs encoding Ang-1 and Ang-2 was altered between the first and third trimesters and in response to hypoxia.

Materials and Methods

Tissue Collection and Processing

Ethical committee approval for tissue collection was obtained from the Addenbrooke's Hospital NHS Trust, Cambridge and the City Hospital, Nottingham Ethical Committee. First trimester tissues ($n = 5$) were collected at termination of pregnancy (8 weeks $n = 1$, 9 weeks $n = 3$, and 12 weeks $n = 1$). Term placentae ($n = 6$) were obtained after spontaneous vaginal deliveries from uncomplicated pregnancies. The gestational age of pregnancies was determined from the date of the last menstrual period and were within ± 7 days of the estimated gestation as judged from an ultrasound scan performed in the first trimester of pregnancy. Tissues were rinsed in phosphate-buffered saline (PBS), fixed immediately in 10% formalin for 6–8 hr, and embedded in paraffin wax. Sections (4 μm) were cut onto slides coated with 3-aminopropyltriethoxy-silane (Sigma Chemical, Poole, UK).

Nine placental biopsies were obtained from patients diagnosed with preeclampsia in the City Hospital, Nottingham, England. Nine further placental samples were obtained from normal pregnant women selected on the basis of an equivalent gestational age. The gestational ages of the gestationally matched samples are illustrated in Figure 7. Preeclampsia was defined as a blood pressure increase of 30 mmHg systolic or 15 mmHg diastolic above the values obtained before the 20th week of gestation, or an absolute blood pressure equal to or greater than 140/90 mmHg recorded on at least two occasions after the 20th weeks of gestation. All patients diagnosed with preeclampsia had proteinuria, defined as at least 2+ of protein using an albustix or at least 500 mg of protein in a 24-hr urine collection. All subjects were normotensive in the puerperium. No subjects were known to have chronic hypertension or renal disease. The control group had no

Table 1. Characteristics of the normal and preeclamptic patients

	Normal Pregnancy (n = 9)	Preeclampsia (n = 9)
Maternal age (y)	27.8 ± 1.7	26.4 ± 1.8
Maximal blood pressure (mmHg)	123/68 ± 2/1	165/108 ± 3/1
Birthweight (kg)	3.29 ± 0.3	2.42 ± 0.3
Grams of protein at 24-hr urine collection	Negative	2.85 ± 0.3

Data are presented as mean ± SEM.

hypertension or proteinuria. All subjects were non-smokers. Characteristics of normal pregnant women and women with preeclampsia are shown in Table 1.

RNA Extraction and cDNA Synthesis

RNA extraction was achieved using a modification of the protocol described by Clark et al. (26). Briefly, tissue was homogenized and the RNA purified by acid phenol extraction, followed by precipitation with isopropanol and ethanol. Complementary DNA (cDNA) was synthesized using avian myoblastosis virus reverse transcriptase enzyme (Super RT, HT Biotechnology, Ltd., Cambridge, UK) from 2 µg of total RNA with an oligo dT primer (Pharmacia Biotech, Ltd., St. Albans, Hertfordshire, UK). The RNA and primer were heated to 68°C for 3 min, and chilled on ice for 2 min. The remaining components were then added to achieve the final concentrations: 1 mM dNTPs, 25 mM Tris-HCl (pH 8.3), 5 mM KCl, 0.2 mM dithiothreitol (DTT), 0.5 mM MgCl₂, 20 U RNasin, and 20 U AMV reverse transcriptase. Synthesis of cDNA was carried out at 42°C for 1 hr. The reverse transcriptase was inactivated by heating at 80°C for 10 min (27).

Riboprobe Production

For in situ hybridization, riboprobes Ang-1 and Ang-2 were designed to avoid regions of homology between these genes. The riboprobe for Tie2 was also designed to avoid homologous regions of Tie1. The specific regions of Ang-1, Ang-2 and Tie2 were amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) from RNA extracted from term placenta.

The identity of each PCR product was confirmed by cloning into PCR-Script SK(+) (Stratagene, La Jolla, CA, USA) and sequencing by an ABI 373 fluorescent sequencer. Plasmid DNA was linearized with BamHI and EagI restriction endonucleases prior to transcription of probes. Anti-sense and sense RNA probes were transcribed with ³³P-UTP (MAXiScript in

vitro transcription kit; Ambion, AMS, Ltd., Witney, Oxon, UK). Then probes were incubated with DNase at 37°C for 10 min to remove the plasmid template, phenol extracted, and ethanol precipitated.

Ang-1 cDNA was amplified between base pairs 961 and 1421 (EMBL U83508). The primers were 5' AACCTTCAAGGCTTGGTT 3' and 5' TACTGCCTCTGACTGGT 3' with an expected product of 461 bp. The mixture was heated for 1 min at 95°C and the amplification was carried out for 35 cycles (30 sec at 95°C, 30 sec at 50°C, and 30 sec at 72°C), followed by a final extension period of 3 min at 72°C.

Ang-2 cDNA was amplified between base pairs 1047 and 1275 (EMBL AF004327), the primers were 5' AAATAGTGACTGCCACGGTG 3' and 5' ATCTCTTCTGTAGAATTAGGG 3', the resulting product being 229bp. The mixture was heated for 1 min at 95°C and the amplification was carried out for 35 cycles (30 seconds at 95°C, 30 seconds at 56°C and 30 seconds at 72°C) followed by a final extension period of 3 min at 72°C.

Tie-2 cDNA was amplified between base pairs 2047 and 2289 (EMBL L06139), primers being 5' CCTTAGTGACATTCTTCC 3' and 5' GCAAAAATGTCCACCTGG 3', with a product of 243bp. The mixture was heated for 1 min at 95°C and the amplification was carried out for 35 cycles (30 seconds at 95°C, 30 seconds at 60°C and 30 seconds at 72°C), followed by a final extension period of 3 min at 72°C.

In Situ Hybridization

The in situ hybridization protocol used in this study was based on that described by Clark et al. (26). Briefly, formalin-fixed and paraffin-embedded sections were dewaxed, pretreated with 0.2N HCl for 20 min, and proteinase K (2 µg/ml) (Sigma) for 15 min at 37°C. Slides were then immersed in a solution containing 100 mM triethanolamine (pH 8) and 0.25% acetic anhydride twice for 10 min. Slides were washed in 2× sodium chloride/sodium citrate (SSC) for 5 min, dehydrated in ethanol serials, and dried. The probes were diluted to 30,000 to 40,000 cpm/µl in hybridization buffer and denatured by boiling for 3 min. The hybridization buffer contained 0.3M NaCl, 10 mM Tris-HCl pH 6.8, 10 mM sodium phosphate pH 6.8, 5mM EDTA pH 8.0, 50% (v/v) deionized formamide, 1× Denhardt's solution (0.02% [w/v] each of bovine serum albumin [BSA], Ficoll, and polyvinyl pyrrolidone), 10% (w/v) dextran sulphate, 50 mM DTT, and yeast tRNA 1 mg/ml. The sections were incubated in a sealed container humidified with 50% formamide and 0.3M NaCl for 18 hr at 55°C.

The slides were washed in 5× SSC at 50°C for 15 min twice, followed by 2× SSC, 50% formamide at 65°C for 30 min. After washing in 2× SSC at 37°C for 5 for four times, slides were incubated with RNase A (Sigma) at a final concentration of 20 µg/ml in 1× wash solution containing 400 mM NaCl, 10 mM Tris-HCl (pH 7.5), and 5 mM EDTA. The RNase A was

removed by washing slides in $2\times$ SSC, 50% formamide at 65°C for 30 min. Slides were then washed in $2\times$ SSC and $0.2\times$ SSC at 37°C , 15 min each. Sections were dehydrated through 30%, 60%, 80%, and 95% ethanol containing 0.3 M ammonium acetate and then two final washes in 100% ethanol. Slides were coated with autoradiography emulsion (LM-1 emulsion; Amersham International PLC, Little Chalfont, UK) and exposed for 3 weeks at 4°C . Sections were developed (D19 developer; Kodak, Rochester, NY, USA), fixed (30% sodium thiosulphate), stained by hematoxylin, mounted, and photographed.

Immunocytochemistry

Anti-hAng2 is an affinity-purified goat polyclonal antibody raised against the 19 amino acid synthetic peptide mapped to the carboxyl terminus of the human molecule (Cat. No. sc-7015, Santa Cruz Biotechnology Inc., Wiltshire, UK.). Primary antibody was provided at $200\ \mu\text{g}/\text{ml}$ and used at $4\ \mu\text{g}/\text{ml}$ in $1\times$ PBS containing 10% BSA. Anti-cytokeratin antibody (Clone MNF116; Dako Ltd., High Wycombe, UK) was used in serial sections to identify extravillous trophoblast.

The control for Ang-2 staining was goat IgG, used at the same concentration as primary antibody in serial sections. In addition, the antibody was preadsorbed with a 25-fold molar excess of the peptide overnight at 4°C . To unmask the antigen for Ang-2, slides were first incubated in 6 M urea for 30 min, then treated in a pressure cooker in 0.01 M sodium citrate buffer (pH 6.0). Pretreatment for cytokeratin antibody was heat treatment only.

Serial $4\text{-}\mu\text{m}$ sections of formalin-fixed, paraffin-embedded tissue were used for immunocytochemistry. Tissue sections were deparaffinized in xylene and hydrated through ethanol serials. After pretreatment, slides were washed in PBS for 5 min ($\times 2$) and treated with 0.3% H_2O_2 in methanol for 10 min at room temperature to quench endogenous peroxidase. Sections were incubated with primary antibody at 37°C in a humidified chamber for 60 min. Slides were washed with PBS for 30 min (three changes) before application of the biotinylated secondary rabbit anti goat antibody at 1:100 dilution in PBS (Zymed Laboratories, South San Francisco, CA, USA) for 10 min at room temperature. Sections were washed in PBS three times for 30 min before application of the streptavidin conjugate horseradish peroxidase complex (Zymed Laboratories). This was followed by detection with liquid DAB-plus substrate kit (Zymed Laboratories). In most experiments, sections were counterstained using Mayer's hemalum.

First Trimester and Term Villi Primary Culture

Three first trimester villi and three term placental villi were cultured in ambient and reduced oxygen conditions. Tissues were washed in $1\times$ PBS to remove blood and then minced. Villi were cultured

in DMEM medium (Sigma) with 100 mg/l streptomycin (Gibco) and 2 mM L-glutamine (Gibco). The ambient oxygen cultures were incubated at 37°C in humidified air and 5% CO_2 for 20 hr. For reduced oxygen cultures, the tissue culture dishes ($25\ \text{cm}^2$) were sealed in a plastic container and gassed with N_2 and 5% CO_2 at 37°C for 20 hr. At the end of each incubation period, the levels of dissolved oxygen, carbon dioxide, and pH were measured using a fetal blood gas/pH monitor. The changes in oxygen and carbon dioxide concentration and pH were similar in each experiment. In normoxic culture, there was no significant decrease in oxygen tension in 20 hr. In the low oxygen cultures, oxygen tension decreased from 24.8 kPa at the start of the experiments to between 7 and 8 kPa. Carbon dioxide tension increased 0.7–0.9 kPa in normoxic culture; there was no change in the reduced oxygen medium. In ambient oxygen cultures, the pH fell from 7.4 to 7.0, and in the reduced oxygen culture, from 7.4 to 6.8. The cultured villi were collected and frozen in liquid nitrogen prior to RNA extraction.

Measurement of Ang-1 and Ang-2 mRNAs Remaining Relative to β -Actin Following Treatment With Actinomycin D

To investigate the reasons of altered Ang-1 and Ang-2 mRNA levels arising from growth under reduced oxygen, semiquantitative RT-PCR (see below) was carried out on term villi cultured in the presence of actinomycin D (final concentration $10\ \mu\text{g}/\text{ml}$) for 0, 2, 4, 6, and 8 hr. Minced term placental villi were cultured under ambient and reduced oxygen (as the same conditions as described above) for 20 hr prior to the addition of actinomycin D (Sigma). For reduced oxygen culture, villi were grown in $25\ \text{cm}^2$ flasks with a plug seal screw cap. After addition of actinomycin D, each flask was immediately gassed with nitrogen and 5% CO_2 (v/v). For normoxic conditions, the villi were incubated in air and 5% CO_2 (v/v). Tissue samples were collected at 0, 2, 4, 6, and 8 hr after the addition of actinomycin D and RNA extracted. Reverse transcription was carried out using the same amount of RNA ($2\ \mu\text{g}$) in each sample. Semiquantitative RT-PCR (see below) was performed using the same amount of cDNA in each sample. Minor differences in the cDNA were corrected by β -actin mRNA, which is unchanged under hypoxic conditions (27). The remaining Ang-1 and Ang-2 mRNA levels relative to β -actin mRNA levels were analyzed under ambient and reduced oxygen conditions. Experiments were undertaken in triplicate.

Semiquantitative RT-PCR

To assess mRNA levels of Ang-1 and Ang-2 in human first trimester villi after hypoxic culture, semiquantitative RT-PCR was carried out. Complementary DNA samples were normalized by RT-PCR with primer sets that detect β -actin. To ensure that the amplification was in the exponential phase and had

not reached the plateau, appropriate numbers of cycles for each PCR reaction was determined in pilot studies in which reactions using different numbers of amplification cycles (for Ang-1 and Ang-2) 28, 30, 32, 35, 38, and 40, and (for β -actin) 18, 20, 25, 30, were performed. The reaction products were quantified by inclusion of a small amount of ^{32}P -labeled CTP (0.01 μCi in each 20 μl reaction). Gels were dried and densitometric quantitation of the autoradiographic signals undertaken. The entire procedure was repeated three times.

Final conditions chosen for the amplification of β -actin were as follows: 95°C, 30 sec; 55°C, 30 sec; 72°C 30 sec for 20 cycles in the reaction containing 10 μM of each human actin primer (the primers were 5'CTACAATGAGCTGCGTGTGG 3', and 5'AA-GGAAGGCTGGAAGAGTGC 3', EMBL M10277), 2.5 units Taq DNA polymerase, 0.1 mM dNTPs, 0.1 mM NH_4Cl -Tris, 1.5 mM MgCl_2 , and 0.01 μCi ^{32}P -CTP (ICN) in 20 μl total volume.

PCR profile of Ang-1 was 95°C, 30 sec; 52°C, 30 sec; 72°C 30 sec for 30 cycles and that of Ang-2 was 95°C, 30 sec; 56°C, 30 sec; 72°C 30 sec for 30 cycles. The primer sets for Ang-1 and Ang-2 were as described in riboprobe production section above.

Statistical Analysis

Differences in Ang-1 and Ang-2 mRNA levels under normoxic and reduced oxygen conditions were analyzed by paired Student's *t* test. Differences were considered statistically significant at $p < 0.05$. Results are presented as mean \pm SEM. In the preeclampsia study, Wilcoxon signed ranked test was used to compare the gestationally paired samples.

Results

Localization of Ang-1, Ang-2, and Tie2 mRNAs

In first trimester villi, hybridization for mRNA encoding Ang-2 was detected in the syncytiotrophoblast with some silver grains present over the cytotrophoblast (Fig. 1A–C, E). Ang-2 mRNA localization was patchy with some villi being highly labeled, while others in the same section were negative. Even within the same villi, silver grains showed a patchy distribution (Fig. 1AC). Anticytokeratin immunohistochemistry and bright field illumination showed that the trophoblast in these regions was intact (Fig. 1DE). This was a consistent finding in all first trimester specimens analyzed. Transverse sections across small villi confirmed that the Ang-2 antisense probe hybridizes to both trophoblast layers (Fig. 1F). At term, the hybridization signal was undetectable (Fig. 1G). The specificity of the signal was confirmed by hybridization with sense probe in serial sections (Fig. 1H). Ang-1 mRNA could not be detected by in situ hybridization in human placenta throughout gestation, although it was detected by RT-PCR (data not shown).

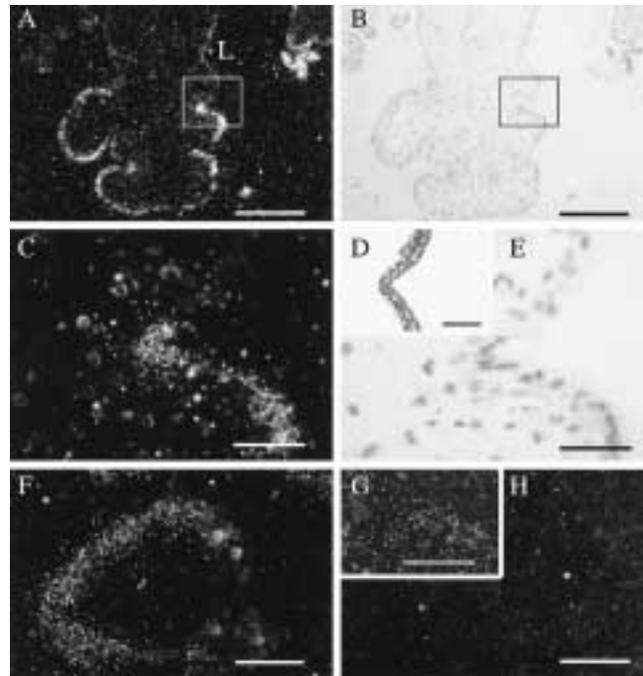


Fig. 1. In situ hybridization of Ang-2 mRNA in first trimester villi at 9 weeks gestation (A–F, H) and term placenta (G). (A) Dark field examination shows the intense silver grains over syncytiotrophoblast with some over cytotrophoblast. The distribution pattern is patchy with some trophoblast highly labeled, whereas others are negative, even in the same villus. Boxed region L indicates such a region. (B) Bright field examination of area shown in A. (C) Higher magnification view of the boxed section in A. (D) A nearby slide stained with anti-cytokeratin antibody to confirm the integrity of trophoblast layers. (E) Bright field view, at high magnification of the boxed section in B. (F) Cross section of a villus confirms that silver grains highly label syncytiotrophoblast. (G) Darkfield examination of in situ hybridization with Ang-2 antisense probe in term placenta. Only diffuse silver grains were observed. (H) Serial section hybridized with Ang-2 sense probe is negative control. Scale bar, 50 μm for A, B, H and 25 μm for C, D, E, F, G.

In the first trimester, Tie2 mRNA was detected on endothelial cells, and high levels were found in first trimester decidua (Fig. 2). At term, there was no detectable Tie2 mRNA hybridization signal. The specificity of the signal for Tie2 was also confirmed by hybridization in serial sections with the sense probe (Fig. 2F).

Localization of Ang-2 Immunoreactivity

Ang-2 immunoreactivity was detected throughout gestation and was different between first trimester and term placentae. Immunostaining was observed in first trimester syncytiotrophoblast (Fig. 3A–C). At term, anti-hAng2 only stained around endothelial cells of villous capillaries (Fig. 3D). Control sections with equivalent concentrations of goat IgG or after preabsorption with the Ang-2 peptide showed no staining (Fig. 3E).

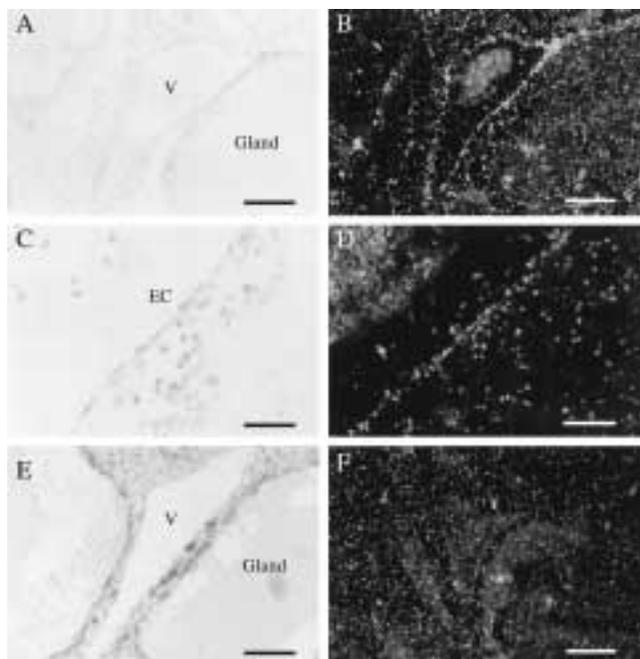


Fig. 2. In situ hybridization of Tie2 mRNA in first trimester decidua at 9 weeks gestation. (A) Bright field of in situ hybridization with Tie2 antisense probe. V indicates blood vessel. (B) Darkfield examination shows silver grains distributed over endothelial cells. Higher magnification examination shows the silver grains on the endothelial cells in (C) Bright-field and (D) Darkfield. (E) A nearby slide stained by cytokeratin antibody. Strong staining is seen in trophoblast (epithelial) cells. Endothelial cells show no staining. The specificity of hybridization was examined by hybridization of Tie2 sense probe in a serial section (F). Scale bar, 50 μm for A, B, E, F and 25 μm for C, D.

Effects of Reduced Oxygen on the Level and Ratio of Ang-1 and Ang-2 mRNA in Cultured Villi

Semiquantitative RT-PCR was used to assess the level of Ang-1 and Ang-2 mRNA levels in primary cultures of first trimester ($n = 3$) (Fig. 4A) and term villi ($n = 3$) (Fig. 4B) under both reduced oxygen and normoxic conditions. In first trimester villous cultures, culture in reduced oxygen induced a rise in the levels of Ang-1 mRNA, but this did not achieve statistical significance. Ang-2 mRNA levels increased 2.5 fold (paired Student t test, $n = 3$, $t = 4.4903$, $p = 0.046$) (Fig. 4A). In contrast, at term Ang-1 mRNA levels decreased slightly in response to low oxygen (not statistically significant), but Ang-2 mRNA levels increased approximately 4-fold (paired Student t test, $n = 3$, $t = 15.654$, $p = 0.004$) (Fig. 4B). To investigate the possible changes in the ratio of these two mRNAs the ratio Ang-1/Ang-2 was calculated. In first trimester villous cultures, there was no change in the relative amounts of Ang-1 and Ang-2 mRNA in response to reduced oxygen (Fig. 5A and the ratio under both normoxic and reduced oxygen conditions was approximately 0.5). However, the Ang-1/Ang-2 ratio in villi obtained at term and

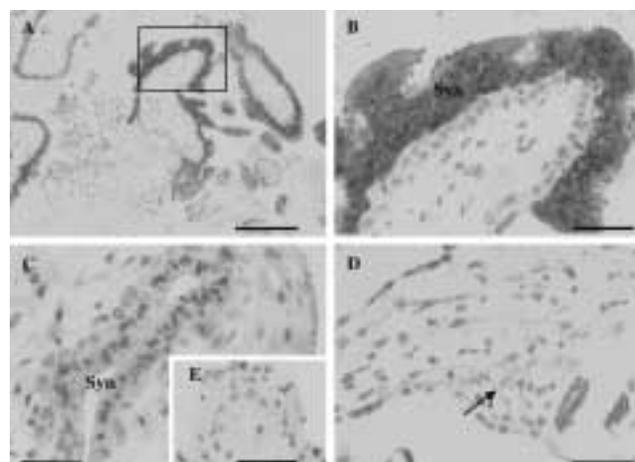


Fig. 3. Immunolocalization of Ang-2 throughout gestation. A, B, and C show sections stained with anti-Ang2 antibody; immunostaining is observed in syncytiotrophoblast layer. (D) Immunostaining with anti-hAng2 antibody of term placental villi. Staining is around the endothelium of fetal blood vessels (arrow). (E) Control in which the primary anti-hAng2 antibody was preabsorbed overnight with synthetic peptide (1:25). Scale bar, 50 μm for A and 25 μm for B-E.

cultured under normoxic conditions was approximately 2.2. However, after culture in reduced oxygen this ratio changed to approximately 0.3 (Fig. 5B, paired Student t test, $n = 3$, $t = 6.1655$, $p = 0.025$).

Ang-1 and Ang-2 mRNA Remaining Relative to β -Actin Following Treatment With Actinomycin D

To investigate the mechanisms by which reduced oxygen lead to the increase in Ang-2 mRNA level, primary cultures of term villi were treated with actinomycin D to block transcription for 0, 2, 4, 6, and 8 hr under low oxygen and normoxic conditions. Semiquantitative RT-PCR was performed to assess Ang-1 and Ang-2 mRNA levels relative to β -actin as described in Materials and Methods. This showed that Ang-1 and Ang-2 mRNA levels are regulated by different mechanisms under reduced oxygen conditions (Fig. 6). This decreased the stability of Ang-1 mRNA and levels declined so far as to be below the sensitivity of the assay after 4 h of treatment (Fig. 6A). In contrast, the decay rate of Ang-2 mRNA was the same under reduced oxygen conditions as that under normoxic conditions, indicating that the observed increase in Ang-2 mRNA level under low oxygen conditions is due to increased transcription (Fig. 6B).

Reduced Angiopoietin-2 mRNA Level in Preeclampsia

Relative Ang-1 mRNA levels were measured in the placentae of women with preeclampsia and were not found to be different from matched samples from women who remained normotensive (normal group, median = 0.4575, range 0.8020–0.1020; preeclampsia group, median = 0.2825, range 0.7250–0.0040; Wilcoxon signed rank test, $n = 9$, $p = 0.3828$)

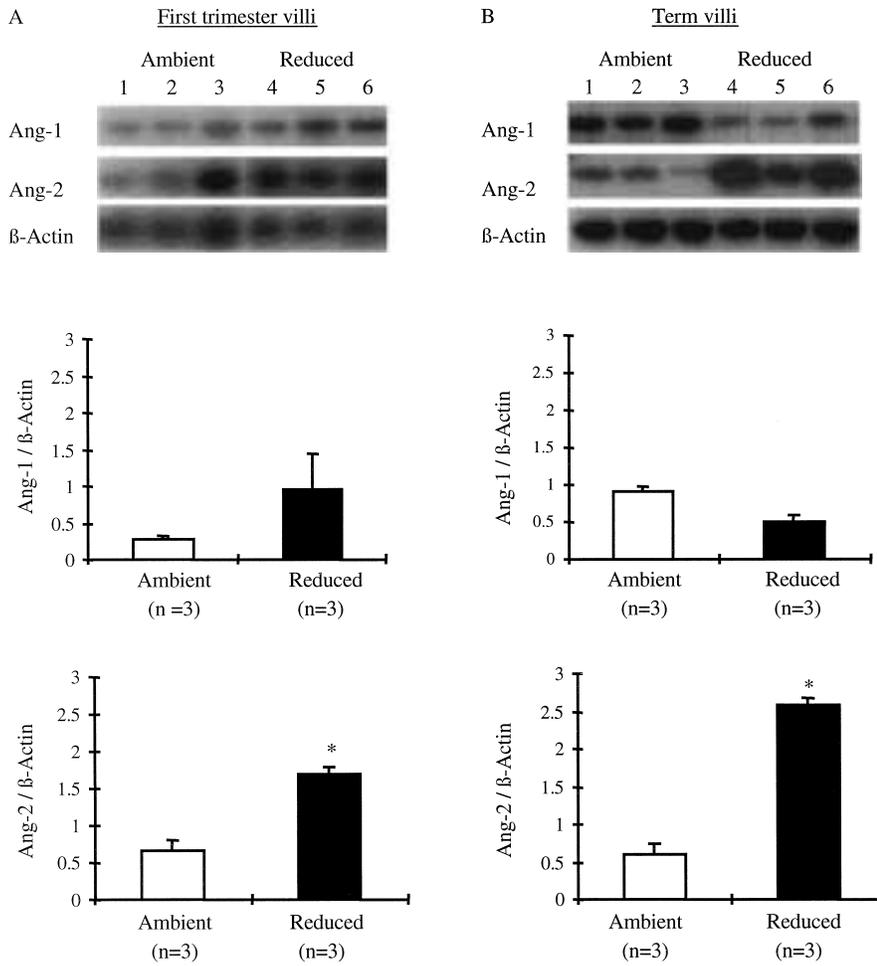


Fig. 4. Effects of reduced oxygen on Ang-1 and Ang-2 mRNA levels. Effects in the (A) first trimester ($n = 3$) and (B) term villi cultures ($n = 3$) detected by semiquantitative RT-PCR for Ang-1 and Ang-2. cDNA samples were normalized by RT-PCR with primer set detected β -actin. The proper numbers of cycle for each RT-PCR reaction were determined in a pilot study in which different numbers of amplification cycles were performed, for Ang-1 and Ang-2: 28, 30, 32, 35, 38, and 40; for β -actin: 18, 20, 25, 30. The optimal number for PCR amplification cycles was determined and chosen in the mid-exponential phase. Subsequent PCR amplification on β -actin matched samples for Ang-1 and Ang-2 were carried out. The reaction products were quantified by inclusion of a small amount of ^{32}P -labeled CTP (0.01 μCi in total 20 μl reaction) in each reaction. Autoradiography and densitometric quantitation of the signals were then carried out. The entire procedure was repeated three times. Data are presented as mean \pm SEM. Statistical comparisons were for paired data under ambient (*open column*) and reduced oxygen (*solid column*) conditions. * $p < 0.05$. Lanes 1–3, ambient oxygen; lanes 4–6, reduced oxygen.

(Fig. 7B). In contrast, relative Ang-2 mRNA levels were reduced in preeclampsia (normal group, median = 0.3280, range 0.5560–0.1630, preeclampsia group, median = 0.1870, range 0.3690–0.0020). The mRNA levels were always lower in the preeclampsia group than those in the matched control (Wilcoxon signed rank test, $n = 9$, $p = 0.008$) (Fig. 7C).

The Presence of Ang-1 and Ang-2 mRNAs in Normal and Preeclamptic Human Placenta

In addition to semiquantitative PCR, the presence of Ang-1 and Ang-2 mRNAs in normal and preeclamptic human placenta was also detected by Northern blot at 31, 34, and 38 weeks gestation (data not

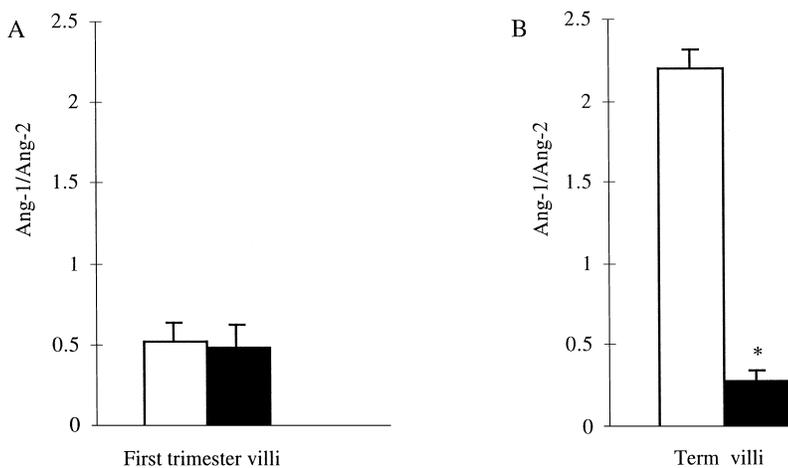


Fig. 5. The ratio of mRNAs encoding Ang-1 and Ang-2 in response to reduced oxygen in first trimester (A) and term (B) villous cultures. In the first trimester, the change of Ang-1/Ang-2 ratio was not significant in response to reduced oxygen while at term it decreased 8-fold. *Paired student t test, $n = 3$, $t = 6.1655$, $p = 0.025$. □ ambient oxygen; ■ reduced oxygen.

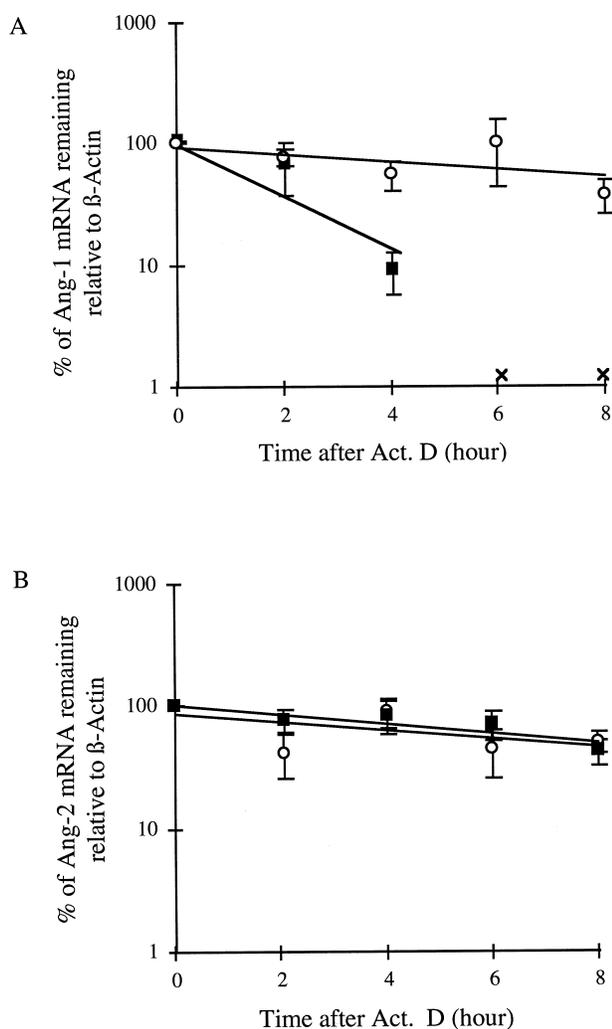


Fig. 6. Time course of Ang-1 and Ang-2 mRNA remaining in villi obtained at term relative to β -actin following treatment with actinomycin D. Villous explants were cultured with actinomycin D for 0, 2, 4, and 8 hr under ambient and reduced oxygen conditions. RNA extraction and semiquantitative RT-PCR were performed as described in the Material and Methods. The stability of Ang-1 mRNA decreased under reduced oxygen and it was below the sensitivity of this method of detection after 4 hr culture in the presence of actinomycin D. Ang-2 mRNA had a similar decay rate under ambient and reduced oxygen conditions, indicating that the increased Ang-2 mRNA detected under reduced oxygen was due to increased transcription. β -actin mRNA levels do not change under varying oxygen conditions. Data are presented as mean \pm SEM. Experiments at each time point were performed in triplicate. ○ ambient oxygen; ■ reduced oxygen; × detection below the sensitivity of this method.

shown). Due to the limited number of samples, these are not sufficient for quantification, but confirm the presence of these mRNAs by an independent method.

Discussion

Ang-1, the ligand for the Tie2 tyrosine kinase receptor, is expressed by pericytes and acts on neighboring

endothelial cells to stabilize the endothelial cell-pericyte interaction and is thought to be important for endothelial survival (18,28). Ang-2 is a natural Ang-1/Tie2 inhibitor, and disrupts the interaction between endothelial cells and the pericyte and/or matrix. In the presence of angiogenic factors, such as VEGF-A, this allows blood vessel sprouting. In the absence of VEGF blood vessel regression occurs (19,29).

The in situ hybridization studies reported here show that Ang-2 mRNA was readily detected in the syncytiotrophoblast in first trimester placenta (Fig. 1), but at term only low levels of Ang-2 mRNA were present. Fetal capillaries that closely underlie the trophoblast cells could be target cells for Ang-2 produced by trophoblast. However, for this to occur the Ang-2 would have to pass the underlying cytotrophoblast layer. This is possible as other products, for example human chorionic gonadotrophin (HCG), produced by the syncytiotrophoblast is found in the fetal circulation (30–33). The large amounts of Ang-2 immunoreactivity in syncytiotrophoblast, especially during first trimester (Fig. 3D–F), would suggest that it may be functionally important for fetal endothelial cells. The pattern of immunostaining for Ang-2 was similar to the in situ hybridization pattern (Fig. 3). We were unable to detect Ang-1 mRNA using in situ hybridization. It is probable that Ang-1 mRNA expression was below the level of detection by in situ hybridization, but was detectable by PCR, which is more sensitive. We performed immunohistochemistry for Ang-1 using a commercially available polyclonal anti sera (sc-7015, Santa Cruz Biotechnology Inc). This resulted in nonspecific staining of trophoblast.

The temporal expression of Ang-2 and its receptor Tie2 in first trimester may be important in the regulation of placental development. There is profound angiogenesis in early gestation characterized by branching angiogenesis. The control of growth and the extent of the branching are likely to depend on the interplay of local factors including Ang-1, Ang-2, VEGF, and the placentally produced sflt-1 (14). This balance may be altered in pathologic conditions (2).

The factors regulating Ang-1, Ang-2, and Tie2 expression are poorly understood. It is known that hypoxia regulates VEGF-A gene expression and may regulate Ang-1 (34). In this study, we showed that Ang-1 mRNA level was not significantly altered by reduced oxygen in cultured first trimester or term villi (Fig. 4AB). However the level of Ang-2 mRNA did alter when the PO_2 was reduced. This is in agreement with studies in bovine microvascular endothelial cells that demonstrated a 5-fold increase Ang-2 mRNA in response to hypoxia (35). It is the ratio of the angiopoietins and their interaction with other factors that is thought to regulate vessel growth and maturation. We estimated the ratio of the mRNAs encoding Ang-1 and Ang-2 by semiquantitative RT-PCR and found that in cultured villi

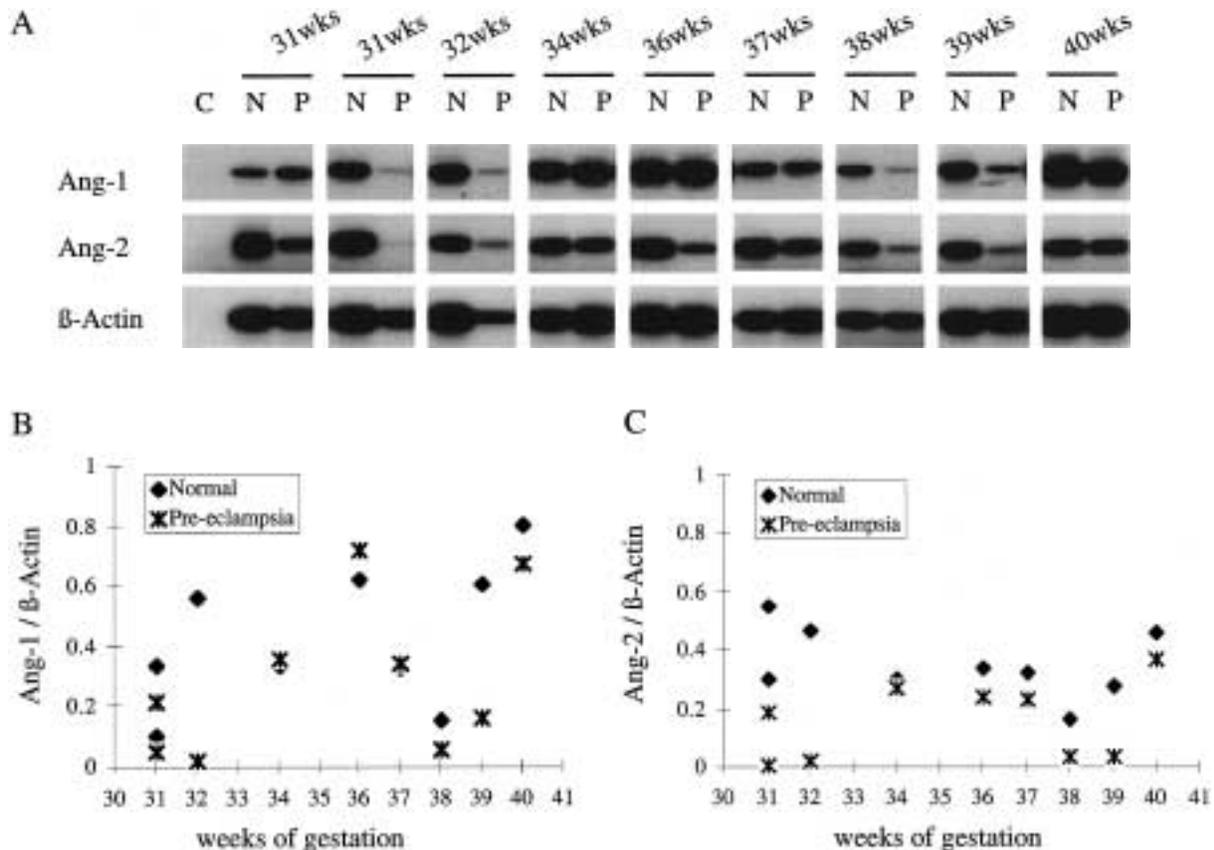


Fig. 7. Ang-1 and Ang-2 mRNA levels in placentae of woman with established preeclampsia ($n = 9$) as compared to normotensive woman matched for gestational age detected by semiquantitative RT-PCR. (A) Autoradiography. (B) Ang-1 mRNA levels had no statistical difference in preeclamptic placentae compared to matched samples from women who remained normotensive (normal group, median = 0.4575, range 0.8020–0.1020; preeclamptic group, median = 0.2825, range 0.7250–0.0040; Wilcoxon signed rank test, $n = 9$, $p = 0.3828$). (C) Ang-2 mRNA levels were reduced in preeclampsia (normal group, median = 0.3280, range 0.5560–0.1630; preeclamptic group, median = 0.1870, range 0.3690–0.0020; Wilcoxon signed rank test, $n = 9$, $p = 0.008$). The mRNA levels were always lower in the preeclampsia group than those in the matched control. ◆ normal pregnancy; ✕ preeclampsia. Gestational ages in weeks are as indicated.

it shifted in favor of Ang-1 as pregnancy progressed. In the first trimester, the ratio did not change under reduced oxygen conditions. However, there was a marked shift (in favor of Ang-2) when term villi were subjected to low oxygen conditions.

There are two probable reasons for this. First, the prevailing oxygen tension in the intervillous space during the first trimester is low (approximately 18 mmHg [36]) and at term is (90 mmHg [37]). Thus, the oxygen environment from which the tissues are obtained is very different; therefore, the cells may well respond to oxygen differently. It is known, for example, that the level of antioxidant enzymes in the villi increases as gestation progresses and indeed oxygen causes the first trimester syncytiotrophoblasts to die (36,38). Second, the extent and maturity of the blood vessels in the villous tree differs at these time points. Indeed, the proportion of villus vessels that have pericytes associated with them is higher at term than in the first trimester (E.G.Z., unpublished observation). The ratio of the

angiopoietins and their interaction with other factors to regulate vessel growth and maturation is complex it may therefore not be surprising that two tissues that, although obviously related but structurally different, have a different response to a hypoxic stimulus.

To investigate the mechanism of angiopoietin mRNA regulation, villous explants were grown under both normoxic and reduced oxygen conditions, and treated with actinomycin D to block transcription. This revealed that Ang-1 and Ang-2 mRNA levels are regulated by different mechanisms. The rate of decay of Ang-2 mRNA in the absence of transcription was unchanged by culture under reduced oxygen. Thus, the observed induced rise in Ang-2 mRNA level is not due to increased stability of mRNA, and is therefore attributable to increased transcription (Fig. 6). At present, there are no published genomic DNA sequences for Ang-2, so it remains to be determined whether the promoter contains hypoxia response elements (HREs). In contrast,

the decay rate of Ang-1 mRNA in term villi was markedly increased by growth under reduced oxygen conditions (Fig 6A).

It is well known that mRNA stability can be regulated by hypoxia; for example, both VEGF-A and erythropoietin mRNAs are stabilized under hypoxic conditions (39). However, to the best of our knowledge, this is the first study to show that reduced oxygen decreases mRNA stability. Agonist-induced destabilization of receptor transcripts has been described in the case of the human muscarinic receptor (mAChR) m1. This is due to specific sequences within the 3'-UTR and removal of such sequences abolishes the increased degradation induced by agonist (40).

We therefore conclude that both Ang-2 transcription and Ang-1 decay are increased by reduced oxygen, and this rapid change would be expected to markedly change the interaction between the pericytes and the endothelial cells. Depending on the presence or absence of angiogenic factors (such as VEGF-A), this would be expected to determine whether vessels grow or regress. Further work is necessary to determine whether a similar mechanism is operative in other cell types.

In pregnancies complicated by preeclampsia, it has generally been assumed that the placenta is hypoxic. This is thought to result from the reduced maternal blood supply to the intervillous space (41), which is secondary to the incomplete conversion of the endometrial spiral arteries by migrating extravillous trophoblast, which fails to invade the myometrial segments of these arteries (42,43). However, in the absence of direct measurements of the PO₂ in the intervillous space (IVS), this assumption is based on surrogate markers, which are thought to be influenced by the local PO₂. These analyses are further complicated by the different categories of hypoxia and the superimposition of additional factors (43-45). From a histomorphometric perspective, there is dilation of the fetal capillaries with no evidence of vessel proliferation (45-47). These changes have been suggested as secondary to reduction in the overall villous surface area rather than hypoxia per se (45).

Recently, a report was published by Dunk et al. (48), in which the localization of Ang-1 and Ang-2 in the placenta was investigated. The data presented by these authors agrees in part with the results presented here. For example, Ang-2 (both by *in situ* hybridization and immunostaining) was found in first trimester in the villus trophoblast. Dunk et al. reported *in situ* hybridization and immunolocalization for Ang-1 in the core of placental villi. In our studies, although we were able to detect Ang-1 by RT-PCR, we were unable to localize the site of expression. This apparent discrepancy may be due to the availability of antibodies and the level of sensitivity in *in situ* hybridization. However, there are major differences in the reported localization of Tie2 expressing cells. In

our studies, Tie2 mRNA was restricted to endothelial cells, and this is in agreement with the bulk of the published literature concerning this receptor system. The studies reported by Dunk et al. show that the mRNA encoding Tie2 was abundant in trophoblast. This discrepancy is difficult to explain. However, the description of the probe used by Dunk et al. is somewhat confusing. These authors describe usage of a 640-bp fragment excised by Hind III and EcoRI, which also contains a Sty I site. The published sequence for Tie2 does not contain such a fragment.

Furthermore, the *in vitro* studies using the trophoblast cell lines (ED27 and ED77), although indicating convincingly that these cells are able to respond to the angiopoietins, does not shed light on the role of these factors in placental trophoblast. The cells are now known not to be of trophoblastic origin and are derived from contaminants of the original cultures (49). Therefore, although the observations made are valid, the inference that this is how trophoblast behaves is at best suspect.

In a study by Goldman-Wohl et al. (50), Ang-2 mRNA was localized to first trimester syncytiotrophoblast and Tie-2 to the endothelial cells. This is in agreement with our findings. They did not detect Tie-2 hybridization to villus trophoblast (in contrast to Dunk et al.), but did observe hybridization to endovascular trophoblast. The studies reported here and those of Dunk et al. did not examine endovascular tissues; therefore, the role of angiopoietins directly effecting trophoblast cells is somewhat unclear.

Our study demonstrated an increase in Ang-2 mRNA by culture in reduced PO₂ and similar results have been obtained by Mandriota et al. (35) in bovine microvascular endothelial cells. Thus, if the preeclamptic placenta is associated with hypoxia, then one might anticipate an increase in placental Ang-2 mRNA level. However, our results show a reduction in Ang-2 mRNA level compared to gestationally matched controls. Similarly, we have previously shown that despite the widespread recognition that VEGF mRNA level is increased by hypoxia, the level of VEGF mRNA is decreased in the placenta of woman suffering from preeclampsia (51). These data are not consistent with the simple model of a hypoxic placenta and hypoxia-inducible genes responding to this environment. It is possible that an intrinsic defect in trophoblast differentiation in preeclampsia (52) may cause lower levels of these mRNAs or that the preeclamptic placenta truly is hypoxic, but that the mechanism for the transcriptional response is in some way deficient. The ratio of pro-/anti-angiogenic growth factors is clearly important in the development of the vasculature of the mammalian placenta, but further studies are needed to unravel this process and to determine whether it is a causative factor in the development of preeclampsia or a consequence. We have shown that the levels of mRNAs encoding Ang-1

and Ang-2 are regulated by different mechanisms, although addition studies will be necessary to determine whether this is the case in other tissues.

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