Transforming Growth Factor- β Expression in Human Placenta and Placental Bed in Third Trimester Normal Pregnancy, Preeclampsia, and Fetal Growth Restriction

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Normal human pregnancy depends on physiological transformation of spiral arteries by invasive trophoblasts. Preeclampsia (PE) and fetal growth restriction (FGR) are associated with impaired trophoblast invasion and spiral artery transformation. Recent studies have suggested that transforming growth factor (TGF)- β 3 is overexpressed in the placenta of PE patients and that this may be responsible for failed trophoblast invasion. There are, however, no studies on TGF- β s in the placenta in FGR or in the placental bed in PE or FGR. In this study we have used immunohistochemistry, Western blot analysis, and enzyme-linked immunosorbent assay to examine the expression of TGF- β 1, TGF- β 2, and TGF- β 3 in placenta and placental bed of pregnancies complicated by PE and FGR and matched control pregnancies. The results show that TGF- β 1, - β 2, and - β 3 are not expressed in villous trophoblasts but are present within the placenta. TGF- β 1, - β 2, and, to a much lesser extent, TGF- β 3 were present within the placental bed but only TGF- β 2 was present in extravillous trophoblast. No changes in expression of either isoform were found in placenta or placental bed in PE or FGR compared with normal pregnancy. These data are not consistent with overexpression of TGF- β 3 being responsible for failed trophoblast invasion in PE. Our findings suggest that the TGF- β s do not have a pathophysiological role in either PE or FGR. (*Am J Pathol* 2001, 159:1827-1838)

During early human pregnancy, extravillous cytotrophoblasts from anchoring villi invade the decidualized endometrium and myometrium (interstitial trophoblast) and also migrate in a retrograde direction along the spiral

arteries (endovascular trophoblast) transforming them into large diameter conduit vessels of low resistance.¹ Endovascular trophoblast invasion has been reported to occur in two waves; the first into the decidual segments of spiral arteries at 8 to 10 weeks of gestation and the second into myometrial segments at 16 to 18 weeks of gestation.¹ This physiological transformation is characterized by a gradual loss of the normal musculoelastic structure of the arterial wall and replacement by amorphous fibrinoid material in which trophoblast cells are embedded.²⁻⁷ These physiological changes are required for a successful pregnancy.

Failure of trophoblast invasion and spiral artery transformation has been documented in preeclampsia (PE), one of the leading causes of maternal death.⁸ In this syndrome reduced uteroplacental perfusion is associated with widespread endothelial dysfunction and fetal growth restriction (FGR) leading to significant maternal and perinatal morbidity. Similar spiral artery abnormalities have been reported in the placental bed of women with FGR in the absence of maternal hypertension as well as in miscarriages.^{4,9-16} Despite the importance of trophoblast invasion and vascular remodeling these processes are still not well understood. However they are thought to include changes in expression of cell adhesion molecules, matrix metalloproteinases, and their tissue inhibitors and growth factors and their receptors.^{17,18}

Transforming growth factor- β s (TGF- β s) are members of a large superfamily of cytokines including activins, inhibins, and bone morphogenic proteins.¹⁹ The family is composed of three related 25-kd homodimeric proteins TGF- β 1, - β 2, and - β 3. TGF- β exerts its biological effects through binding to cell surface receptors designated types I, II, and III. Studies have suggested that TGF- β , produced primarily by the decidua, may regulate trophoblast invasion.²⁰ Recently Caniggia and colleagues²¹ reported that TGF- β 3 was a major regulator of trophoblast invasion *in vivo* and *in vitro*. Expression of TGF- β 3 in placental villous tissue peaked at 7 to 8 weeks of gestation and was virtually undetectable by 9 weeks. The same

Accepted for publication August 3, 2001.

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Table 1. Clinical Details for Placenta Immunohistochemistry Studies

	Control (n = 18)	PE (n = 19)	FGR (n = 10)
Age (years)	30.65 ± 4.98	31.21 ± 7.5	28.8 ± 8.0
Gestational age at delivery (weeks)	36.28 ± 3.71	34.27 ± 3.72	33.67 ± 2.84
Birth weight (kg)	2.93 ± 0.86	2.16 ± 1.05*	1.4 ± 0.53*
Systolic BP (mmHg)	112.24 ± 9.27	165 ± 13.64*	124.5 ± 10.66
Diastolic BP (mmHg)	67.8 ± 7.5	109.95 ± 8.34*	73.5 ± 9.14
Maternal plasma urates (mmol/L)	—	407.26 ± 52.64	—

Values are shown as mean ± S.D.

*, $P < 0.005$ compared with control pregnant group.

group also reported that TGF- β 3 was weakly expressed in third trimester placentas but was dramatically up-regulated in placentas obtained from women with PE. It was suggested that overexpression of TGF- β 3 may account for failure of trophoblast invasion in PE.

Nothing is known about expression of TGF- β s the placental bed in PE and FGR where there is failure of normal spiral artery transformation. Thus in this article we have used immunohistochemistry, Western blotting, and enzyme-linked immunosorbent assay (ELISA) to examine the expression of TGF- β 1, - β 2, and - β 3 in placentas and placental bed biopsies from normal pregnancies and from pregnancies complicated by PE or FGR.

Materials and Methods

Study Participants

Samples were obtained from pregnant women at the Royal Victoria Infirmary, Newcastle-on-Tyne, UK. The study was approved by the Joint Ethics Committee of Newcastle and North Tyneside. Three groups of women were studied: control pregnancies with no hypertension or FGR, women with pregnancies complicated by PE, and women with pregnancies complicated by FGR in the absence of maternal hypertension. In some of the cases placentas but not placental bed biopsies were collected and *vice versa* therefore some of the clinical details differed between placental and placental bed experiments. Thus these are presented as two separate tables (Tables 1 and 2). The overall clinical details for the two groups were similar.

PE was defined as pregnancy-induced hypertension (blood pressure $\geq 140/90$ mmHg) and proteinuria (≥ 300 mg/24 hours) in women who were normotensive before

pregnancy and had no other underlying clinical problems such as renal disease. FGR was defined ultrasonically as fetal abdominal circumference <10 th centile with a decrease in abdominal circumference SD score of >1.5 SD²² and umbilical artery pulsatility index ≥ 95 th centile.²³ We have previously shown that a fall in abdominal circumference SD score of >1.5 SD is the optimal cut-off to define a group of fetuses with evidence of wasting at birth and morbidity associated with FGR.²² Birth weight centiles were obtained from charts of the Northern Region population of England;²⁴ small for gestational age was defined as a birth weight below the 10th centile.

Sample Collection

The majority of placental bed biopsies were obtained from women undergoing elective cesarean section as described previously.^{25,26} Briefly, after delivery of the infant, the position of the placenta was determined by manual palpation. Six placental bed biopsies were then taken under direct vision using biopsy forceps (Wolf, UK). In three cases placental bed biopsies were collected after vaginal delivery. These biopsies were taken under ultrasound guidance using the same biopsy forceps introduced through the cervix. Placental bed biopsies were included in this study if they contained decidual and/or myometrial spiral arteries with interstitial trophoblasts. Placental samples of ~ 1 cm³ were also collected. All samples were collected directly into liquid nitrogen-cooled isopentane and stored sealed at -70°C until required. Samples were used for subsequent immunohistochemical analysis and Western blotting experiments. Cryosections (7 μm) from each specimen were stained with hematoxylin and eosin (H&E) for histological analysis.

Table 2. Clinical Details for Placental Bed Immunohistochemistry Studies

	Control (n = 18)	PE (n = 23)	FGR (n = 9)
Age (years)	29.4 ± 6.07	29.33 ± 7.8	29.75 ± 7.9
Gestational age at delivery (weeks)	35.5 ± 3.8	33.92 ± 4.27	34.33 ± 2.39
Birth weight (kg)	2.67 ± 0.91	2.09 ± 0.97	1.38 ± 0.36*
Systolic BP (mmHg)	116.33 ± 8	157.14 ± 13.93*	119.44 ± 8.08
Diastolic BP (mmHg)	71.83 ± 7.59	110.73 ± 7.51*	73.5 ± 9.14
Maternal plasma urates (mmol/L)	—	419.05 ± 67.15	—

Values are shown as mean ± S.D.

*, $P < 0.005$ compared with control pregnant group.

Antibodies and Reagents

Desmin (NCL-DES-DERII, 1:100) and cytokeratin (NCL-LP34, 1:800) monoclonal antibodies were obtained from Novocastra, Newcastle-on-Tyne, UK. The Factor VIII monoclonal antibody was obtained from DAKO, Cambridge, UK, and used at 1:800. Rabbit polyclonal antibodies raised against TGF- β 1 (SC146), TGF- β 2 (SC90), and TGF- β 3 (SC820) were purchased from Santa Cruz Biotechnology Inc., Santa Cruz, CA. Full-length human recombinant TGF- β 1 (12.5 kd), TGF- β 2 (12.5 kd), and TGF- β 3 (12.5 kd) were obtained from Santa Cruz and used as positive controls in Western blots. All other reagents were obtained from Sigma Chemical Co., Poole, UK, unless stated otherwise.

Morphological Assessment of Spiral Arteries

After immunostaining with the above antibodies we assessed the integrity of the muscle wall of the spiral artery by the degree of medial smooth muscle remaining around the spiral artery (desmin immunostaining). Morphological assessment was based on the method described by Pijnenborg and colleagues.^{1,3} The muscle was graded as preserved, separated, disorganized, or grossly disorganized. Absent or incomplete medial changes was deemed when the smooth muscle was preserved or separated and presence of medial changes was deemed when the muscle was disorganized or grossly disorganized/absent.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Western Blots

A representative sample of 10 placentas were studied from each group. Before homogenization, a cryosection from each block was cut and stained with H&E to confirm that the specimens were placenta rather than decidua. Each frozen piece of tissue was weighed without allowing the tissue to thaw. Tissue samples were ground to a fine powder in liquid nitrogen with a mortar and pestle and added to 4 volumes of cold lysis buffer [25 mmol/L Tris, 0.25 mol/L sucrose, 1 mmol/L ethylenediaminetetraacetic acid, pH 7.6, and 50 μ g/g tissue protease inhibitor cocktail (Sigma)]. Using a Polytron homogenizer at setting 10, the sample containers were surrounded by ice and homogenized for 3×10 second intervals. The homogenate was spun at $5000 \times g$ for 10 minutes at 4°C to remove debris. The supernatant was aliquoted and stored at -70°C until required. Protein concentrations were determined by the method of Bradford²⁷ using bovine serum albumin (BSA) as a standard, and then diluted to the required concentration.

Samples were mixed 1:1 with loading buffer (1.2 ml of 1 mol/L Tris, pH 6.8, 2 ml of glycerol, 4 ml of 10% sodium-dodecyl-sulfate, 2 ml of 1 mol/L dithiothreitol, 0.8 ml of distilled water with bromophenol blue added to give a deep blue color) and boiled for 5 minutes before loading. Samples were separated on 15% sodium dodecyl sulfate-polyacrylamide-resolving gels with a 4% stacking

gel using Protean II apparatus (BioRad, Hemel Hempstead, UK) at a constant current of 30 mA. Each well was loaded with 75 μ g of protein. Low molecular weight range markers (20 to 106 kd range; BioRad Laboratories, Richmond, CA) were loaded beside the samples.

Protein was transferred overnight in buffer containing 25 mmol/L Tris, 190 mmol/L glycine, 20% methanol at a constant 30 V to BioBlot NC nitrocellulose membranes (Costar; Corning Inc., NY). Filters were blocked for 1 hour at room temperature in phosphate-buffered saline (PBS) containing 5% Marvel and 0.25% Tween-20. The antibodies (diluted 1:1000 in PBS containing 3% Marvel and 0.25% Tween-20) were added for 1 hour at room temperature. The filters were rinsed once, washed twice for 5 minutes in PBS containing 0.25% Tween-20 and then incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG (Diagnostics Scotland, Carlisle, UK) diluted 1:2000 in PBS containing 0.25% Tween-20 for 1 hour at room temperature. Blots were washed once for 5 minutes, followed by two 15-minute washes in PBS containing 0.25% Tween-20, and then one 5-minute wash in distilled water. Proteins were detected using the Amersham ECL detection system and filters were exposed to Hyperfilm ECL (Amersham, Buckinghamshire, UK).

Immunohistochemistry

Immunohistochemistry was performed using an avidin-biotin peroxidase method (Vectastain Elite rabbit kit; Vector Laboratories, Peterborough, UK). Placenta and placental bed cryosections (7 μ m) were mounted on APES-coated slides, air-dried overnight, fixed in acetone for 10 minutes at room temperature, and then wrapped in pairs and frozen at -20°C until required. Each specimen was stained with H&E for histological analysis. In addition, placental bed biopsies were immunostained for cytokeratin (1:800) to detect trophoblasts, desmin (1:100) to detect muscle, and Factor VIII (1:800) to detect endothelium. To determine TGF- β localization, sections were blocked with 1% BSA for 30 minutes followed by the kit blocker for 20 minutes. Sections then underwent a further 45-minute incubation in 0.1% phenylhydrazine to block endogenous peroxidase staining. These and all subsequent steps were performed at room temperature. Sections were then incubated for 1 hour with antibodies at a dilution of 1:200 for TGF- β 1 and 1:250 for TGF- β 2 and TGF- β 3. The remaining steps were performed according to the instructions supplied with the kit. The reaction was developed with Fast diaminobenzidine tablets. Washes between each step were performed in TBS buffer (0.15 mol/L Tris-buffered saline, pH 7.6). Sections were counterstained in Mayer's hematoxylin (BDH, Poole, UK) and mounted in DPX synthetic resin. Omission of primary antibody or substitution with nonimmune serum for the primary antibody were both included as controls. Intensity of immunostaining was scored on an arbitrary scale of 0 to +++ where 0 represents no staining, + represents weak staining, ++ represents moderate staining, and +++ represents dark staining. The scoring of the samples was performed by two separate observers

blinded to the tissue identity (FL and HS). Sections were all stained on the same day for each antibody to eliminate day to day variations in immunostaining. Because the antibodies for this study had previously been used on skin tissue,²⁸ normal human skin was used as a positive control tissue and processed as for placental samples.

ELISAs

TGF- β 2 ELISA

Placental TGF- β 2 was measured using the Promega E_{max} Immunoassay System. Assays were performed on aliquots of the homogenates prepared for Western blot analysis. The assay detects biologically active TGF- β 2 in an antibody sandwich format. Flat-bottomed 96-well plates were coated with TGF- β 2 monoclonal antibody, which binds soluble TGF- β 2 in the test sample. A second antibody to TGF- β 2 was added to complete the sandwich. After washing, an antibody-conjugate (horseradish peroxidase-TGF- β 2) was added which binds to the sandwich complex. Finally the chromogenic substrate 3,3',5,5'-tetramethyl benzidine was added. Plates were read in a Labsystems Multiscan Bichromatic plate reader connected to a PC with Genesis software. The samples were quantified against a standard curve generated with known amounts of TGF- β 2. The range of the assay is 32 to 1000 pg/ml. The specificity of the assay is <5% cross-reactivity with TGF- β 1 and TGF- β 3 at 10 ng/ml. Because some of the samples were higher than the highest standard a separate standard curve was made and the samples were reassayed so that they were measured within the linear range of the standards. For assays the samples were diluted 1:100 in the sample buffer supplied with the kit. After assay the final concentration of TGF- β 2 in the sample was calculated and expressed as pg TGF- β 2 per mg protein.

In vivo TGF- β 2 is processed from a latent form to a bioactive form. Only the bioactive form is immunoreactive with this kit. *In vitro*, the total amount of TGF- β 2 (bioactive and nonbioactive) can be determined by acid treatment of samples. However because it is the bioactive form that is most likely to influence trophoblast invasion the samples were not acid treated.

TGF- β 3 ELISA

The assay for TGF- β 3 was performed on the same samples as for TGF- β 2 using an in-house assay developed from reagents obtained from R&D Systems, Oxon, UK. The assay was modified from a protocol supplied by R&D Systems. All incubations were performed at room temperature. Plates were coated with 100 μ l of 4 μ g/ml capture antibody (anti-TGF- β 3, mAb 643) in PBS overnight. After three washes (0.05% Tween-20 in PBS, pH 7.4), plates were blocked for 1 hour with PBS containing 1% BSA and 5% sucrose. Plates were washed again and then 100 μ l of standards (human recombinant TGF- β 3 (243-83) or samples were added. Standards ranged from 2000 pg/ml to 4 pg/ml. Samples were diluted 1:2 or 1:5 in

TBS, pH 7.3, containing 0.05% Tween-20 and 0.1% BSA. After a 2-hour incubation and three washes, 100 μ l of biotinylated anti-TGF- β 3 (BAF-243) diluted 1:250 in sample diluent buffer was added for 2 hours. After three further washes, 100 μ l of 1:200 streptavidin-HRP (DY998) diluted in PBS containing 0.1% BSA was added for 20 minutes. Three more washes were performed and then 100 μ l of TMB substrate (DY999) was added for ~20 minutes or until a blue color developed. The reaction was stopped with 50 μ l of 1 mol/L H₂SO₄ and the plates were read at 450 nm with a correction wavelength of 540 nm. As for TGF- β 2 the final concentration of TGF- β 3 in the sample was calculated and expressed as pg TGF- β 3 per mg protein.

Statistical Analysis

Clinical details were compared using analysis of variance and post hoc testing was performed using the Fisher's protected least significant difference (PLSD) test. For ELISA and immunohistochemical studies, statistical comparisons were also performed using analysis of variance. Statistical differences were considered to be significant at $P < 0.05$.

Results

Western Blot Analysis

Western blot analysis was performed on a sample of 10 placental homogenates from each group (Table 1). These were randomly selected from the patient group shown in Table 1 all of which were used for subsequent immunohistochemical studies. Gestational age at delivery was comparable in the three groups. All infants in the control group had a birth weight greater than the 10th centile for gestational age. Mean birth weight was reduced in the PE group although only three infants were small for gestational age. Umbilical artery PI was abnormally elevated in four PE cases and one had reversed end-diastolic frequency. Birth weight was significantly reduced in the FGR and PE groups when compared with the control groups and the FGR group was significantly reduced when compared to the PE group; all infants in the FGR group had a birth weight below the 10th centile with six below the fifth centile. Umbilical artery PI was abnormally elevated in all of the FGR fetuses; four had absent and three had reversed end-diastolic frequencies.

Figure 1 shows the results of Western blot analysis of placental samples from each group. The number of samples necessitated running the samples on two gels. Running of gels, blotting, and hybridization were performed during the same two days to eliminate day to day variability for each antibody. The positive controls for TGF- β 1, TGF- β 2, and TGF- β 3 were clearly visible however TGF- β 1, TGF- β 2, and TGF- β 3 were not detected on any of the samples. These data suggest that either there is no TGF- β in the placenta during late pregnancy or the levels are below the sensitivity of Western blot analysis that was

Figure 1. Western blot analysis for TGF- β 1, - β 2, and - β 3 in human third trimester placentas. Each group contained 10 cases and this necessitated running three separate gels. A representative gel for TGF- β 1 (**top**), TGF- β 2 (**middle**), and TGF- β 3 (**bottom**) is shown. C, Control; P, preeclampsia; F, fetal growth restriction. Each lane was loaded with 50 μ g of protein. +, Represents recombinant TGF- β 1, 20 ng; TGF- β 2, 2 ng; TGF- β 3, 10 ng.

~0.5 ng for the positive control TGF- β 2 and >2 ng for TGF- β 1 and TGF- β 2.

Immunohistochemistry: Placentae

Normal human skin was the positive control for all three anti-TGF- β antibodies (Figure 2). TGF- β 1 was present in basal epidermis and pilosebaceous units. TGF- β 2 was detected throughout the epidermis, but strongest basally. TGF- β 2 reactivity was also seen in pilosebaceous units and, diffusely and weakly, in the dermis. Staining for TGF- β 3 was in the epidermis, predominantly in basal layers, and diffusely in the dermis. TGF- β 3 immunoreactivity was weaker than that for TGF- β 1 and TGF- β 2. These reactivity patterns are primarily in agreement with those reported by Frank and colleagues.²⁸

Omission controls were negative. Controls in which the primary antibody was replaced with 1:200 normal rabbit serum showed variable staining of Hofbauer cells in villous stroma in a minority of normal and pathological samples. This reactivity exactly mirrored that of all three anti-TGF- β antibodies, which labeled Hofbauer cells in the same samples. Immunostaining of Hofbauer cells was therefore considered to reflect Fc receptor binding in frozen sections.

The clinical details for patients used for the placenta immunohistochemistry studies are also shown in Table 1.

Figure 2. Immunohistochemical analysis of TGF- β 1 (**top**), TGF- β 2 (**middle**), and TGF- β 3 (**bottom**) antibodies on human breast skin. D, dermis; E, epidermis.

Villous syncytiotrophoblast was consistently negative for TGF- β 1, TGF- β 2, and TGF- β 3 (a few samples were +) (Figure 3; a to c and g to j). Villous cytotrophoblast, which were scanty in normal placentas and more prominent in placentas from pregnancies complicated by PE or FGR, were also negative for all three TGF- β isoforms. As discussed above, Hofbauer cells showed nonspecific reactivity for all three TGF- β isoforms in a minority of samples. TGF- β 2 also showed diffuse stromal reactivity in occasional samples (Figure 3b). Fetal vascular endothelium was negative for TGF- β 1, TGF- β 2, and TGF- β 3 (Figure 3; a to c and g to j).

Perivillous and intervillous fibrin and fibrinoid in basal plate was moderately positive (++) for TGF- β 1 (Figure 3a). Cytotrophoblast cells in cell islands and in basal plate were consistently negative for TGF- β 1 (Figure 3, d

Figure 3. Cryostat sections of placenta immunolabeled for TGF- β 1 (**a**, **d**, and **h**), TGF- β 2 (**b**, **e**, and **i**), TGF- β 3 (**c**, **f**, and **j**), and cytokeratin (**g**). **a:** Placenta from 31-week FGR pregnancy showing TGF- β 1 reactivity in intervillous fibrin (FIB) but no staining of trophoblasts. **b** and **c:** Placenta from 37-week normal pregnancy showing weak reactivity for TGF- β 2 in villous stroma (VS) but no staining of trophoblasts (**b**). There is no TGF- β 3 reactivity in trophoblasts or fibrin (FIB; **c**). **d-f:** Superficial basal plate from 30-week pregnancy complicated by PE. There is extracellular reactivity for TGF- β 1 (**d**) but trophoblast and endothelial cells are negative. There is diffuse perivascular extracellular reactivity for TGF- β 2 (**e**), and trophoblast cells are variably positive, although endothelial cells are negative. TGF- β 3 is not detected (**f**). **g-j:** Placenta (CV) and superficial basal plate from 37-week pregnancy complicated by FGR. Extravillous cytotrophoblast (CT) is positive for cytokeratin (**g**) whereas decidual cells (DEC) are negative. There is extracellular reactivity for TGF- β 1 (**h**) and to a lesser extent TGF- β 2 (**i**). EVT and decidual cells are negative for TGF- β 1 (**h**) and TGF- β 3 (**j**) but show cytoplasmic reactivity for TGF- β 2 (**i**). Original magnifications, $\times 200$. **Closed arrows**, endothelial cells; **open arrows**, trophoblasts.

and h). There was moderate to strong extracellular reactivity (++) to (+++) for TGF- β 2 in the basal plate and cell islands but, in contrast to TGF- β 1, cytotrophoblast cells at these sites were also weakly to moderately positive (+ to ++) (Figure 3, e and i). TGF- β 3 was not detected in cytotrophoblast islands, basal plate, or fibrin (Figure 3; c, f, and j).

There were no significant differences in reactivity for the three TGF- β isoforms across the gestational range studied in the uncomplicated pregnancy group. There were also no significant differences in reactivity for TGF- β 1, TGF- β 2, and TGF- β 3 between placentas from normal pregnancies and placentas from pregnancies complicated by PE or FGR.

Placental Bed Biopsies

The clinical details for patients used for the placental bed immunohistochemistry studies are shown in Table 2. Gestational age at delivery was comparable in the three groups. Umbilical artery PI was abnormally elevated in one case, one case had reversed and one case had absent reversed end-diastolic frequency. Birth weight was significantly reduced in the FGR group when compared with the control group. All infants in the FGR group had a birth weight less than the 10th centile with six below the 5th centile. Four of the infants in the PE group had birth weights <10th centile. Umbilical artery PI was abnormally elevated in all of the FGR fetuses; five had absent and one reversed end-diastolic frequencies.

In the placental bed there was focal moderate (++) extracellular reactivity for TGF- β 1 but decidual and myometrial cells were negative (Figure 3h and Figure 4, a and h). Fibrinoid around transformed spiral arteries in normal placental bed samples was moderately positive (++) (Figure 4, a and b). There was also focal extracellular reactivity (++) around nontransformed myometrial spiral arteries in PE and FGR samples (Figure 4h). All extravillous trophoblast populations in placental bed were negative for TGF- β 1 (Figure 4; a, b, and h).

Cytoplasmic TGF- β 2 was detected in decidual cells (+ to ++) and myometrial cells were weakly positive (+) (Figure 3i and Figure 4; d to f and j). There was variable weak to moderate (+ to ++) reactivity around both transformed and nontransformed spiral arteries in decidua and myometrium; TGF- β 2 immunoreactivity at this site was generally more diffuse than that for TGF- β 1 (Figure 4; d, e, and i). TGF- β 2 staining of EVT was variable, ranging from negative (1 case) to strongly positive (0 to ++++) (Figure 4; d to f, i, and j). Mononuclear interstitial trophoblast, multinucleate trophoblast giant cell, and perivascular trophoblast populations all showed this variable reactivity. All normal and pathological placental bed samples showed this variable TGF- β 2 positivity with no pattern, positive and negative cells being detected in decidua and myometrium and both adjacent to and distant from spiral arteries. The majority of transformed spiral arteries re-endothelialize in the third trimester but, where present, reactivity of endovascular trophoblasts ranged from negative to moderate [0 (2 cases) to ++].

TGF- β 3 was not detected in decidual cells, fibrinoid, or any EVT populations (Figure 3j and Figure 4c). Reactivity was confined to occasional lymphocytes in decidua and myometrium.

Placental bed samples from normal pregnancies did not show any significant variation in TGF- β immunoreactivity with gestational age. There were no significant differences in reactivity for the three TGF- β isoforms in placental bed samples from normal pregnancies compared with pregnancies complicated by PE or FGR.

ELISA

ELISAs were performed on the same placental homogenates as those used for Western blot analysis. The results for the TGF- β 2 ELISA are shown in Figure 5. TGF- β 2 was detectable in all samples. There were no significant differences between groups. These results support the immunohistochemical findings. The results for the TGF- β 3 ELISA are shown in Figure 6. Concentrations of TGF- β 3 in the placental samples were much lower than TGF- β 2. As for TGF- β 2 there were no differences in TGF- β 3 concentrations between groups as previously reported. These observations are consistent with the immunohistochemical findings.

Discussion

We believe that the present study is the most comprehensive investigation of TGF- β expression in the placenta and placental bed in normal pregnancy, PE, and FGR. Different techniques were used to determine TGF- β expression and results were generally consistent. These results suggest that third trimester villous trophoblasts do not express TGF- β 1, TGF- β 2, and TGF- β 3. Within the placental bed all EVT populations in placental bed were negative for TGF- β 1 and TGF- β 3. In contrast TGF- β 2 reactivity was found in EVT and ranged from negative to strongly positive. No changes were noted for any of the TGF- β isoforms in PE or FGR in either placenta or placental bed samples.

The majority of previous studies of TGF- β expression in the human placenta have been performed on early rather than late pregnancy and the results are inconsistent.^{29–32} Interpretation of the results is confounded by the different methods of tissue preparation, different techniques used and antibodies used, many of which are not isoform-specific. Furthermore previous studies have reported results on total TGF protein, much of which is latent and nonfunctional.¹⁹ Incorporation of ELISA immunoassay, which measures only bioactive forms, is therefore likely to yield more relevant information.

Our Western blot and immunohistochemical results suggest that trophoblasts produce little if any TGF- β 1 during late pregnancy. This is consistent with the results of Caniggia and colleagues.²¹ In contrast, Dungy and colleagues³³ reported that TGF- β 1 mRNA expression, peaked at 17 weeks of gestation and again at ~34 weeks. Immunohistochemical analysis localized TGF- β 1 to the syncytiotrophoblast. They suggested that these

Figure 4. Cryostat sections of placental bed immunostained for TGF-β1 (**a**, **b**, and **h**), TGF-β2 (**d**, **e**, and **i**), TGF-β3 (**c**), and cytokeratin (**g**; PAS counterstain). **a–f**: Placental bed sample from 37-week normal pregnancy showing transformed myometrial spiral arteries (SA). Note diffuse perivascular extracellular reactivity for TGF-β1 (**a** and **b**) and to a less extent for TGF-β2 (**d** and **e**). EVT cells are negative for TGF-β1 but variably positive for TGF-β2. Endothelial cells are negative. **f**: Variable reactivity of trophoblast giant cells for TGF-β2. **Inset**: Positive giant cells in a different area of myometrium in the same section. **g–i**: Placental bed sample from 30-week pregnancy complicated by PE showing a nontransformed spiral artery (SA). Cytokeratin-positive perivascular trophoblasts are clearly seen (**g**) but there is no endovascular or intramural trophoblasts. There is scanty extracellular reactivity for TGF-β1 (**h**) but trophoblasts are negative. Perivascular reactivity for TGF-β2 is more diffuse and trophoblast reactivity is variable. **j**: Variable TGF-β2 immunoreactivity in interstitial trophoblasts in another 30-week PE sample. Original magnifications, ×200. **Closed arrows**, endothelial cells; **open arrows**, trophoblasts.

Figure 5. ELISA for TGF- β 2 in placenta samples from control pregnancies and pregnancies complicated by PE or FGR.

peaks correlated with the completion of trophoblast invasion into the uterus and the end of placental growth. Although TGF- β 1 has been shown to inhibit trophoblast proliferation *in vitro*³⁴ trophoblasts continue to invade into the myometrium after 17 weeks of gestation. Furthermore mean placental diameter, weight, and maximal thickness increases from 150 mm, 260 g and 20 mm, respectively, at 7 months of pregnancy to 170 mm, 320 g, and 22 mm at 8 months. It further increases at 9 months to 200 mm, 400 g, and 24 mm and again to 220 mm, 470 g, and 25 mm at 10 months.³⁵ Schilling and Yeh³⁶ also reported TGF- β 1 mRNA in term placenta and immunolocalized this to the syncytiotrophoblastic layer, chorionic plate, and EVT. The immunohistochemical studies were performed on formalin-fixed material but used the same source of antibodies. The immunohistochemical studies that have suggested TGF- β expression by placental cytotropho-

blasts and syncytiotrophoblasts during the late second and third trimester have used nonisotype-specific antibodies.

TGF- β 2 is the principle TGF- β isoform produced by the third trimester placenta, although levels were too low to be detected by Western blotting. The principle site of TGF- β 2 localization seems to be the decidua, trophoblast, cell islands, and basal plate. Lysiak and colleagues²⁹ immunolocalized TGF- β 2 placentas from 23 to 28 weeks of gestation, TGF- β 2 was reported to be localized to syncytiotrophoblasts but not cytotrophoblasts, the mesenchymal core of villi, decidual cells, and decidual matrix and EVT in decidua when present. From 34 weeks of gestation on, decidual extracellular matrix staining was reduced but the other findings remained similar. Schilling and Yeh³⁶ reported similar findings for TGF- β 2 as for TGF- β 1 with intense immunostaining in the syncytiotrophoblastic layer, chorionic plate, and EVT. Minimal staining was found in decidua. We found that placental expression was not altered in PE, confirming the findings of Caniggia and colleagues²¹ who also found TGF- β 2 mRNA to be the major isoform expressed during late pregnancy. EVT populations showed immunostaining, although this was variable. We have reported similar variable staining of EVT in first and second trimester placental bed samples.³⁷

TGF- β 3 levels measured by ELISA were very low in normal third trimester placentas. Immunohistochemistry results were consistent with this and showed that no trophoblast population expressed TGF- β 3. These findings are consistent with our unpublished observations in the first and second trimester but differ from those of Schilling and Yeh.³⁶ The skin-positive controls used in the present study confirm that the antibodies detect TGF- β s in frozen tissues. We did not use formalin-fixed material because formalin-fixed material often binds antibodies nonspecifically. Recent interest has focused on the role of TGF- β 3 in trophoblast invasion following the finding by Canniggia and colleagues.²¹ We set out to extend the observations of Canniggia and colleagues²¹ by focusing on EVT in the placental bed in PE and also to extend the observations to FGR. However we were unable to confirm an up-regulation of TGF- β 3 in placental tissue in PE. Furthermore no EVT cells expressed TGF- β 3. The findings in FGR were consistent with this. We attempted to explain the discrepancies by repeating the studies using their immunohistochemical and tissue preparation methods but our findings did not alter. Thus we are unable to explain the differences. Based on our findings we conclude that altered TGF- β 3 expression is unlikely to be responsible for the failed trophoblast invasion in PE and FGR.

PE and FGR are both associated with reduced uteroplacental blood flow and both conditions are predicted by abnormal uterine artery Doppler waveforms in the second trimester.^{38–40} In PE, there is failure of normal transformation of the spiral arteries¹⁵ with <20% of myometrial vessels showing physiological change.^{17,41} Our findings are consistent with this; 73% of the myometrial vessels examined from women with PE had either completely intact or only partially disorganized muscle. Inter-

Figure 6. ELISA for TGF- β 3 in placenta samples from control pregnancies and pregnancies complicated by PE or FGR.

estingly absent physiological change in myometrial vessels is more often found in PE cases with an abnormally high uterine artery pulsatility index.^{42,43} Less is known about FGR in the absence of maternal hypertension. Several small studies have found the same morphological abnormalities in myometrial arteries in 45 to 100% of pregnancies with small for gestational age infants.^{6,44,45} Absence of physiological change in myometrial arteries is more likely in severely small infants (birth weight < 2.3rd centile), which are more likely to be growth restricted, than in those with birth weights between the 2.3rd to 10th centiles. For the present study we defined FGR according to antenatal ultrasound criteria; all fetuses were small (abdominal circumference < 10th centile) with a significant fall in abdominal circumference SD score and an abnormal umbilical artery Doppler. We have shown that these morphometric criteria are optimal at detecting wasting at birth, indicative of FGR⁴⁶ and an elevated umbilical artery is the optimal method of predicting outcome in a group of small for gestational age fetuses.⁴⁷ Using these criteria we found 45% of myometrial vessels demonstrated completely intact or partially disorganized muscle.

The environment of the early placenta is hypoxic compared to later pregnancy.⁴⁸ In early pregnancy plugs of trophoblasts block the maternal spiral arteries.⁴⁹ These are subsequently displaced and blood flow begins at ~11 weeks of pregnancy. As a result partial pressure of oxygen increases from 18 mmHg at 8 to 10 weeks of gestation to 60 mmHg at 12 to 13 weeks of gestation. There is considerable evidence to suggest that trophoblast cells are sensitive to oxygen.^{50–55} The mechanism by which oxygen concentrations are sensed is unclear but may well involve the transcription factor, hypoxia inducible factor (HIF).⁵⁶ Caniggia and colleagues⁵⁷ reported that placental HIF-1 α expression peaked at 6 to 8 weeks of gestation and then fell precipitously to ~9 weeks of gestation, paralleling the expression of TGF- β 3. In contrast Rajalumar and colleagues⁵⁸ found that HIF-1 α mRNA remained constant whereas HIF-2 α mRNA increased with gestational age. Protein levels of both isoforms decreased with gestational age. Caniggia and colleagues⁵⁹ speculated that if oxygen tension fails to increase in PE, or trophoblasts do not detect this increase, HIF-1 α and TGF- β 3 expression remain high, resulting in shallow trophoblast invasion. In support of this they have recently reported that HIF-1 α is also elevated in the placenta of women with PE.^{59,60} However these findings were not confirmed by Rajakumar and colleagues.⁶¹ Further studies are required to clarify the role of HIF in both normal and abnormal invasion.

PE is associated with failed transformation of maternal spiral arteries by EVT.⁶² Several mechanisms have been implicated.^{63,64} TGF- β 3 has been reported to be overexpressed in placentas from women with PE²¹ and this has been linked to failure of trophoblast migration. In the same study it was also shown that explants from PE placentas failed to show outgrowth or invasion. It was suggested that these data are in keeping with the reduced invasive ability of trophoblast in PE. However interpretation of these data are not straightforward be-

cause interstitial migration of EVT into the decidua and myometrium proceeds normally in PE. No such studies have been performed in FGR.

Inconsistencies in different pathological studies may, at least in part, be reflected by the type of placental pathology. Adequate transfer of oxygen to the fetus depends on both the fetoplacental and uteroplacental circulations. Three categories of fetal hypoxia have been proposed and the placental pathologies have been reviewed:^{35,51} preplacental hypoxia in which both placenta and fetus are hypoxic because of oxygen reduction in maternal blood (eg, maternal anemia and high altitude), uteroplacental hypoxia in which oxygenated blood has restricted entry to the intervillous space (eg, failed trophoblast invasion or occlusions of spiral arteries), and postplacental hypoxia in which oxygenated blood enters the intervillous space but is not extracted adequately to the fetus. Each condition results in differences in placental development. Pregnancy at high altitude results in increased capillary volume fraction and increased capillary branching. The density of cytotrophoblast increases and increased syncytial knotting occurs. Maternal anemia results in similar changes; endothelial proliferation is also increased leading to excessive branching angiogenesis. With PE at term, a typical example of uteroplacental hypoxia, the findings are more varied but most of the structural findings are similar to those found in the preplacental hypoxia group. These include syncytial knotting, increased numbers of villous cytotrophoblasts and macrophages, and increased capillary volume fraction. In this condition the restriction of oxygen entry occurs focally and is variable leading to heterogeneous changes in villous maturation. With preterm intrauterine growth restriction associated with absent and diastolic flow velocity waveform in the umbilical arteries the placenta fails adequately to transfer oxygen to the fetus. These cases are associated with reduced amount and increased proliferation of cytotrophoblasts and increased deposition of stromal extracellular matrix. Syncytial nuclei show signs of senescence.

In summary our findings suggest that TGF- β 2 is the main isoform found in third trimester placenta. In contrast to TGF- β 1 and TGF- β 3, EVT does produce TGF- β 2 but expression is very variable and not altered in PE or FGR. The absence of any changes in TGF- β 1, - β 2, or - β 3 in PE or FGR suggests that they do not play a role in the pathophysiology of these disorders.

Acknowledgments

We thank the Action Research and the British Heart Foundation for funding and Elizabeth Duffie and Barbara Innes for technical assistance.

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