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Original paper

Angiogenin distribution in human term placenta, and expression by cultured trophoblastic cells

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Note: This study is dedicated to the memory of Dr Christiane Dorey.

Abstract

Human angiogenin is a 14-kDa secreted protein with angiogenic and ribonucleolytic activities. Angiogenin is associated with tumour development but is also present in normal biological fluids and tissues. To further address the physiological role of angiogenin, we studied its expression *in situ* and *in vitro*, using the human term placenta as a model of physiological angiogenesis. Angiogenin was immunodetected by light and transmission electron microscopy, and its cellular distribution was established by double immunolabelling with cell markers including von Willebrand factor, platelet/endothelial cell adhesion molecule-1 (PECAM-1), CD34, Tie-2, vascular endothelial cadherin (VE-cadherin), vascular endothelial growth factor receptor-2 (VEGF-R2), erythropoeitin receptor (Epo-R), alpha-smooth muscle actin, CD45, cytokeratin 7, and Ki-67. Angiogenin immunoreactivity was detected in villous and extravillous trophoblasts, the trophoblast basement membrane, the endothelial basal lamina, foetal blood vessels, foetal and maternal red blood cells, and amnionic cells. Its expression was confirmed by *in situ* hybridisation with a digoxygenin-labelled cDNA probe and reverse transcriptase-polymerase chain reaction amplification. Villous cytotrophoblasts, isolated and differentiated *in vitro* into a functional syncytiotrophoblast, expressed and secreted angiogenin. Given its known biological activities *in vitro* and its observed pattern of expression, these data suggest that, in human placenta, angiogenin has a role not only in angiogenesis but also in vascular and tissue homeostasis, maternal immune tolerance of the foetus, and host defences.

Key words: angiogenesis, angiogenin, placenta, placental ribonuclease inhibitor, trophoblast

Abbreviations: PRI, Placental Ribonuclease Inhibitor; RNase, Ribonuclease

Introduction

Angiogenin is one of the most potent inducers of neovascularization in experimental models *in vivo* ([1,2], for reviews). It was the first angiogenic protein to be isolated from conditioned medium of human tumour cells, being characterised by its capacity to induce neovascularization [3]. Angiogenin is associated with tumour development, but is also present in normal human tissues and fluids such as plasma [4], amnionic fluid [5] and follicular fluid [6]. Angiogenin expression is developmentally regulated in rats and humans [7,8], predominating in the adult liver of both species [7,9]. Angiogenin is a 14-kDa protein showing 35% amino acid sequence identity with human pancreatic ribonuclease (RNase 1) but only weak ribonucleolytic activity. As pancreatic ribonuclease is unable to induce angiogenesis, this structural similarity has served to study angiogenin's structure/function relationships relative to bovine pancreatic ribonuclease (RNase A). An intact catalytic site and cell-binding domain are required for angiogenin to induce neovascularization ([10], for review). Here we used the human placenta as a model to further decipher the physiological roles of angiogenin.

The term angiogenesis was coined by Arthur T. Hertig in 1935 to describe the formation of new blood vessels in the placenta [11]. Being readily available, the human placenta is an excellent model of both physiological and pathological angiogenesis [12]. The placenta assumes several roles essential for successful pregnancy: it is an exchanger between the foetal and maternal blood circulation and also an endocrine tissue ([13], for review), and it provides local immune protection for the foetus. The human placenta, composed of both zygote-derived and maternal cells, develops from the blastocyst trophectoderm and from the maternal endometrium. The foetal circulation extends through the placental villous tree, bathing in maternal blood that enters the intervillous space *via* utero-placental arteries. Villi are covered by an epithelium-like multinucleated surface layer (syncytiotrophoblast) that arises by fusion of its underlying epithelial stem cells (cytotrophoblasts). A subset of chorionic villi anchor the

placenta to the uterine wall. At their base, proliferating extravillous cytotrophoblasts aggregate in columns. During the first and second trimesters, waves of highly invasive extravillous cytotrophoblasts stop proliferating and invade the uterine interstitium. Thus, the placenta is also a valuable model of pseudomalignant development [14].

We examined the distribution and cellular sources of angiogenin in human term placenta. Placental structures were analysed from the chorionic plate and umbilical cord down to the basal plate in contact with maternal tissues. In order to identify cells immunopositive for angiogenin, we used markers for trophoblast, vascular endothelial and smooth muscle cells, haematopoietic cells, angiogenic status, and proliferation. Angiogenin expression in primary cultures of isolated villous trophoblasts was also studied. The cellular distribution of angiogenin was analysed on the basis of its known biological activities *in vitro*.

Materials and methods

Reagents

DNase I, ovalbumin and Triton X-100 were from Sigma Chemical Co. (St Louis, Missouri). Tween 20 was from Merck (Darmstadt, Germany). Percoll was from Amersham Pharmacia (Uppsala, Sweden). Culture media and Hanks buffered saline solution (HBSS) were from Gibco Laboratories (Grand Island, New York). Trypsin was from Difco Laboratories (Detroit, Michigan). Foetal bovine serum (FBS), 10x HBSS, penicillin and streptomycin were from Biological Industries (Kibbutz Beit Haemek, Israel). Sera were heat-inactivated before use. Paraformaldehyde was from Electron Microscopy Sciences (Fort Washington, Pennsylvania). Polyclonal rabbit anti-angiogenin antibodies were prepared as described below. Monoclonal mouse anti-angiogenin IgM (clone MANG-1) was from Bachem (San Carlos, California). Monoclonal anti-vascular endothelial growth factor receptor-2 (VEGF-R2) IgG1 (clone KDR-2), and anti-alpha smooth muscle actin IgG2a (clone 1A4) were from Sigma (St Louis, Missouri). Monoclonal anti-pan cytokeratin IgG1 (clone MNF116), anti-cytokeratin 7 (clone OV-TL 12/30) and anti-platelet/endothelial cell adhesion molecule-1 (PECAM-1, CD31; clone JC/70A) were from Dako (Glostrup, Denmark). Monoclonal anti-*von Willebrand* factor (vWF) IgG1 was from Roche (Roche Diagnostics, Meylan, France). Monoclonal anti-vascular endothelial cadherin (VE-cadherin) IgG1 (clone TEA1/31), anti-CD34 (clone Qbend 10) and anti-Ki-67 (clone MIB-1) were from Immunotech (Marseille, France). Rabbit anti-erythropoietin receptor (Epo-R) and anti-tyrosine kinase with immunoglobulin and epidermal growth factor homology domains-2 (Tie-2) IgG were from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibodies conjugated to either FITC (donkey anti-mouse IgG, goat anti-mouse IgM, goat anti-rabbit IgG) or Texas Red (donkey anti-rabbit IgG, goat anti-mouse IgG), donkey normal serum and human IgG were from Jackson Immunoresearch (West Grove, Pennsylvania). Rhodamine-labelled goat anti-rabbit IgG was from Sigma. Chemicals

for SDS-polyacrylamide gel electrophoresis and molecular mass markers were from BioRad (Hercules, California). All chemicals were of analytical grade.

Angiogenin and rabbit anti-angiogenin IgG

Human recombinant angiogenin was produced as previously described [15]. Rabbit polyclonal antibodies were raised against angiogenin. Three female New Zealand rabbits (2-2.5 kg) were immunised subcutaneously with 200 µg of Met-(-1) angiogenin emulsified in complete Freund's adjuvant (Difco Laboratories, Detroit, Michigan); 21, 43 and 65 days following the initial injection, animals were boosted with respectively 200, 133 and 133 µg of angiogenin in incomplete Freund's adjuvant. Blood was collected two weeks later. The polyclonal antibody to angiogenin was purified by chromatography on a G-protein-Sepharose column. Specific anti-angiogenin IgG was purified by affinity chromatography on angiogenin immobilised in CH-Sepharose 4B gel (Pharmacia, Uppsala, Sweden); unretained IgG was collected and used as negative control. The specificity of the anti-angiogenin antibodies from each rabbit was checked by enzyme-linked immunoassay (ELISA), western immunoblotting and immunodiffusion before pooling. Affinity-purified antibody was conjugated to horseradish peroxidase for ELISA.

Tissue collection

This study was approved by our local ethics committee. Ten placentas were obtained, after elective caesarean section or normal delivery, from healthy mothers with uncomplicated pregnancies delivered near term (Hôpital Saint-Vincent de Paul, Hôpital Tenon, UCLA Hospital). For immunofluorescence experiments and *in situ* hybridisation, fragments of placenta were embedded in Tissue-Tek OCT Compound (Sakura Finetek Europe, Zoeterwoude, The Netherlands), frozen in isopentane cooled with liquid nitrogen, and stored

at -80°C until cryostat section. For electron microscopy, 1-mm^3 samples were fixed with 4% paraformaldehyde in 0.15 M sodium cacodylate buffer pH 7.4, for 45 min at 4°C, then washed once with the same buffer for 20 min; the remaining free aldehyde groups were blocked by adding 10 mM glycine, pH 7.2. After dehydration in increasing concentrations of ethanol (33%, 50%, 75% and 100%) followed by washes in ethanol/resin baths with increasing resin concentrations (LR-White, Electron Microscopy Sciences, Fort Washington, Pennsylvania), the samples were kept overnight at 4°C in 100% resin then transferred to size 3 BEEM capsules and polymerised at 58°C for 18 hours under vacuum. Sections 80 nm thick were obtained using an RMC MT-6000 XL ultra-microtome.

Trophoblast isolation and primary culture

Cytotrophoblasts were isolated from human term placentas by using the method of Kliman *et al.* [16] essentially as described elsewhere [17]. Briefly, following trypsin digestion of placental villi with microscopic monitoring, the digestion product was layered over a 70 to 10% discontinuous Percoll gradient prepared in HBSS without Ca^{++}, Mg^{++}, and $NaHCO_3$. The steps were 70, 60, 55, 50, 45, 40, 35, 30, 20 and 10% Percoll (vol/vol). After centrifugation at 1200 *g* for 20 min at room temperature, the middle layer containing mononuclear cells (corresponding to the 35% step) was collected and washed once in HBSS by centrifugation at 200 *g* for 10 min at room temperature. The purity of the trophoblast preparation was checked by immunostaining with anti-cytokeratin antibody. Cells were seeded in DMEM/20% FBS, 100 units/ml penicillin and 100 µg/ml streptomycin (4 ml/4 million cells/20-cm^2 culture dish), and maintained at 37°C in humidified 5% CO_2/95% air. The medium was changed daily for three days. The collected medium was centrifuged, frozen in liquid nitrogen and stored at -20°C until use. In parallel experiments, cells were collected for protein extraction or were fixed for immunocytochemistry. Experiments (n = 10) were performed in triplicate.

In situ hybridisation on frozen placenta sections

Angiogenin cDNA (BBG28, R&D systems, Abingdon, UK) was labelled by incorporation of digoxigenin-labelled-dUTP by random priming with the DIG High Prime Labelling and Detection Kit I (Roche Diagnostics, Meylan, France) according to the manufacturer's recommendations.

Seven-micrometer-thick frozen sections of human placenta were mounted on Polysine^{TM} slides (Menzel-Gläser, Braunschweig, Germany), dried and fixed with 4% paraformaldehyde for 30 min at 4°C. The sections were then dehydrated with graded ethanol solutions (30%, 2 x 50%, 70%, 2 x 100%), rapidly air-dried and stored at -80°C. The sections were rehydrated with graded ethanol solutions (100%, 70%, 2 x 50%), digested with 1 µg/ml Proteinase K (Sigma) in 20 mM Tris-HCl, 1 mM EDTA, pH 7.6 for 15 min at 37°C, and again fixed with 4% paraformaldehyde for 20 min. The sections were prehybridised at 40°C for 4 hours in hybridisation buffer containing 5 x standard saline citrate (SSC, Gibco), 0.1% N-lauroylsarcosine (Sigma), 20% blocking solution from the Boehringer kit, 50% deionised formamide (Fluka chimie, Buchs, Switzerland), and 0.02% SDS (Bioprobe, Montreuil, France). Hybridisation was performed overnight at 40°C in the same buffer containing 0.25 ng/ml labelled cDNA. After hybridisation, sections were washed twice in 2 x SSC for 5 min and twice in 1 x SSC for 15 min, at 40°C. After treating the sections with blocking buffer for 45 min, alkaline phosphatase-conjugated anti-digoxigenin Fab (1:600) was added for 2 hours. Bound antibody was revealed overnight by adding 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro-blue tetrazolium (NBT) in the presence of 2 mM levamisole to inhibit endogenous alkaline phosphatase activity. The sections were then counterstained with Mayer's hematoxylin and mounted in Glycergel (Dako SA, Trappes, France). Negative

controls were prepared either by pretreating the sections with 100 µg/ml RNase A for 1 h at 37°C or by using a non specific digoxygenin-labelled DNA probe (pBR 328).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from frozen placental tissues with TRIZOL® reagent (Invitrogen SARL, Cergy Pontoise, France). Total RNA was isolated from cultured cells as described in the Atlas Pure Total RNA Labeling System user manual from Clontech Laboratories (Palo Alto, California). The total RNA concentration was determined at 260 nm and its integrity was checked by 1% agarose gel electrophoresis in the presence of ethidium bromide with UV visualisation.

First-strand complementary DNA was synthesised from 2 µg of total RNA using 0.5 µg oligo(dT)$_{15}$ as primer (Promega Co., Lyon, France) and SuperScript™ II RNase H reverse transcriptase (Invitrogen SARL, Cergy Pontoise, France) in a 20-µl reaction volume as per the manufacturer's instructions. The synthesised cDNA was then subjected to polymerase chain reaction amplification (50 µl reaction volume) using Taq DNA polymerase from Invitrogen Co. (Platinium® PCR SuperMix) in a programmable thermal cycler (Perkin-Elmer Applied Biosystems, Foster City, California) with a cycling regime of denaturation at 94°C for 1 min, primer annealing at 56°C for 1 min and primer extension at 72°C for 1 min. The cycles were preceded by a 10-min denaturation step at 94°C and ended with a final extension step at 72°C for 10 min. Half the RT reaction mix was used for amplification (35 cycles) with angiogenin-specific primers, and one-quarter of the RT reaction mix was used for amplification (30 cycles) with ß-actin-specific primers. The gene-specific primers were as follows: human angiogenin ([18], Genbank accession # M11567) 5'-CAT CAT GAG GAG ACG GGG-3' (sense, bp 1964-1981) and 5'-TCC AAG TGG ACA GGT AAG CC-3' (antisense, bp

2227>2208); ß-actin (after [19], Genbank accession # M10277) 5'-ACA ATG AGC TGC GTG TGG CT-3' (sense, bp 1496-1515) and 5'-CTC CTT AAT GTC ACG CAC GAT TTC-3' (anti-sense, bp 2307>2284). The predicted sizes of angiogenin and ß-actin PCR products were 264 and 371 bp, respectively. RT-PCR negative controls lacked total RNA. Poly A^+ RNA from human liver (Clontech laboratories, Inc.) was used as a positive control for angiogenin expression. Absence of contaminating DNA was confirmed by the presence of a single RT-PCR product for ß-actin, the primers being located in different exons. The PCR products from angiogenin cDNA amplification were subjected to 2% agarose gel electrophoresis, extracted with JETsorb (GENOMED GmbH, Bad Oeynhausen, Germany), cloned in the pCR®II-TOPO® plasmid vector (Invitrogen SARL) and sequenced using the ABI PRISM dRhodamine Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer Applied Biosystem, Foster City, California).

Immunolocalisation of angiogenin in human placenta

Indirect immunofluorescence staining

Ten-micrometer-thick cryosections were mounted on Superfrost Plus slides, air-dried, rinsed twice with PBS, and fixed. For angiogenin immunolocalisation, sections were fixed with 4% paraformaldehyde in PBS for 20 min at 4°C. Remaining free aldehyde groups were blocked by adding 50 mM NH_4Cl for 10 min. After washing twice in PBS, tissues were permeabilized by adding 0.5% Triton X-100 in PBS for 5 min. Following two washes with PBS, saturation was achieved with blocking buffer (PBS, 10% donkey or goat serum according to the secondary antibody used, 50 µg/ml human IgG), for 30 min at room temperature. Human IgG was added to decrease the background signal by saturating highly expressed Fc receptors which bind rabbit IgG. Angiogenin was immunodetected with either angiogenin-specific rabbit IgG (6 µg/ml) or anti-angiogenin mouse IgM (6 µg/ml) in blocking buffer overnight at

4°C. After washing 6 times with PBS for 2 min, sections were incubated with blocking buffer for 30 min and bound antibody was revealed by 2-h incubation at room temperature with Texas Red-conjugated donkey anti-rabbit IgG, FITC-conjugated goat anti-mouse IgM diluted 1:400 in blocking buffer, or Rhodamine-labelled anti-rabbit IgG diluted 1:1000. Finally, the sections were washed six times with PBS and once with distilled water. Nuclei were counterstained by adding Vectashield mounting medium with Dapi (Vector Laboratories, Burlingame, California). Negative controls were generated by omitting the primary antibody or by substituting the same concentration of non specific isotypic immunoglobulin. In order to characterise the structures stained for angiogenin, the following antibodies were added to the incubation medium: anti-cytokeratin 7 (2 µg/ml), anti-alpha smooth muscle actin (dilution: 1/300), anti-vWF (4 µg/ml), anti-VE-cadherin (4 µg/ml), anti-CD31 (4 µg/ml), anti-CD34 (4 µg/ml), anti-Tie-2 (6 µg/ml), anti-VEGF-R2 (6 µg/ml), anti-Epo-R (6 µg/ml), anti-CD45 (6 µg/ml) or anti-Ki-67 (4 µg/ml). Bound antibodies were revealed with FITC-, Rhodamine- or Texas Red-conjugated secondary antibodies, the choice of the fluorochrome depending on that used for angiogenin detection. The slides were examined using an Olympus phase-contrast microscope with a fluorescence attachment (BX-60). Images were captured with a Hamamatsu C4742-95 CCD camera and VisionExplorer® VA software (Graftek, Montelimar, France).

Electron microscopy

Fixed 80-nm sections mounted on Formar and carbon-covered nickel grids (150 mesh, Ted-Pella Inc., Redding, California) were washed twice with 50 mM Hepes, 0.9% NaCl, pH 7.4 (Hepes/NaCl), incubated in blocking buffer (50 mM Hepes, 0.9% NaCl, pH 7.4, 7% goat serum, 0.5% ovalbumin) for 30 min and reacted with anti-angiogenin IgG (100 µg/ml) overnight at 4°C. The sections were then washed 5 times with Hepes/NaCl (3 min each) and incubated with blocking buffer for another 30 min. Gold-conjugated goat anti-rabbit IgG (colloidal particle diameter 10 nm, Ted-Pella) diluted 1/50 in blocking buffer was added for

1.5 h at room temperature. Sections were washed 5 times with Hepes/NaCl and three times with water, then counterstained with 2% (w/v) uranyl acetate in water for 30 min. A JEOL 1200-EX electron microscope operating at 80 kV was used.

Immunofluorescence staining of cultured trophoblasts

After two washes with PBS, trophoblasts were fixed with cold absolute methanol for 10 min at -20°C. Following three washes with PBS, saturation was achieved with PBS, 10% donkey serum, 0.5% ovalbumin (blocking buffer) for 30 min at room temperature. Cells were then exposed to 6 µg/ml affinity-purified anti-angiogenin rabbit IgG in blocking buffer overnight at 4°C. After six washes with PBS for 2 min, the cells were incubated with blocking buffer for 30 min. The bound antibody was revealed by 2-h incubation at room temperature with Texas Red-conjugated donkey anti-rabbit IgG diluted 1:400 in blocking buffer. Finally, the cells were washed six times with PBS and once with distilled water, then mounted in a mix of 50% Vectashield/50% Vectashield with 1.5 µg/ml Dapi. In control experiments the primary antibody was either omitted or replaced by non specific rabbit IgG. The purity of the primary culture was checked by double staining with 0.9 µg/ml mouse anti-pan cytokeratin IgG. Antibodies bound to the cells were revealed with FITC-conjugated donkey anti-mouse IgG.

Western blot

Term placentas obtained after caesarean delivery were homogenised in an Ultra-Turrax in PBS containing protease inhibitors (2 mM EDTA, 10 µM leupeptin, 10 µM pepstatin and 1 mM phenylmethanesulfonyl fluoride), for 3 min in pulse mode (0.15 sec per second) at 90 W and 4°C, and then centrifuged at 39 200 *g* for 15 min at 4°C. The supernatant was clarified by ultracentrifugation at 400 000 *g* for 30 min at 4°C. Protein content was determined using the BioRad Assay. Tissue extracts were diluted in sample buffer (1x: 1% SDS, 5% glycerol, 0.14

M ßmercaptoethanol, 0.001% bromophenol blue in 50 mM Tris-HCl pH 6.8), boiled for 5 min and briefly microcentrifuged; then, aliquots were separated by SDS-polyacrylamide gel electrophoresis in 17% resolving gel. Recombinant angiogenin (60 ng) was used as control. Protein was transferred to a 0.1-µm nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany) using a semi-dry apparatus (BioRad) for one hour at 25 V at room temperature. The blot was saturated with PBS supplemented with 4% fat-free milk overnight at 4°C, then exposed to 7 µg/ml affinity-purified rabbit anti-angiogenin IgG for 4 h in the same buffer at room temperature. After six washes for 3 min, bound antibodies were detected with 1/30 000 peroxidase-conjugated goat anti-rabbit IgG for 1 h, followed by phototope western detection system (Supersignal, Pierce, Rockford, Illinois). The membrane was then exposed to X-OMat film (Eastman Kodak, Rochester, New York) for signal detection.

Enzyme immunoassay

The angiogenin enzyme immunoassay was finalised in our laboratory using a rabbit polyclonal anti-angiogenin antibody. The detection limit was 15-30 pg/ml and the ED50 was 116-280 pg/ml. Microtiter plates (High Molecular Weight, Immulon® 3; Dynatech, Chantilly, France) were coated with 1.5 µg/ml anti-angiogenin IgG diluted in PBS (50 µl) overnight, at 4°C. After three washes with PBS, 0.5‰ Tween 20 (PBS/Tween), plates were blocked with PBS, 0.5‰ Tween 20, 0.1% gelatine for 1.5 h at room temperature. Solutions (50 µl) containing either standard or samples buffered with 20 mM MOPS, pH 7.2, and diluted if necessary, were distributed and incubated overnight at 4°C in a moist chamber. The wells were then washed three times with PBS/Tween, and 50 µl of peroxidase-conjugated affinity-purified anti-angiogenin IgG (5 µg/ml in PBS/Tween containing 1% ovalbumin) was added for 2 h at 37°C. After three washes with PBS/Tween, bound antibody was revealed with dihydrochloride-O-phenylene diamine and H_2O_2. Absorbance was determined at 490 nm

using a microplate reader (Dynatech MR 5000). Normal human plasma was used as positive control.

The human chorionic gonadotropin (hCG) concentration was determined in culture medium by using an enzyme-linked fluorescence assay (Vidas System, Biomérieux, Marcy l'Etoile, France) with a detection limit of 2 mU/ml.

Results

Angiogenin immunolocalisation in human term placenta

In the umbilical cord, the umbilical vein and the two umbilical arteries were strongly labelled at the level of the endothelium and the smooth muscle tunic (figure 1.A). Many cells in Wharton's jelly were also labelled (not shown). Amnionic cells were immunopositive for angiogenin (not shown).

On cross-sections of the chorionic plate, the amnionic epithelium, in contact with amnionic fluid, showed cytoplasmic labelling, particularly around the nucleus, possibly corresponding to the nuclear envelope or the peri-nuclear endoplasmic reticulum (figure 1.B). The foetal vessels were also labelled (not shown).

In chorionic villi, strong angiogenin labelling was observed in the villous trophoblast (figure 2A), which was also positive for cytokeratin 7 (not shown) as already reported by Rajashekhar *et al.* [20]. The knots of syncytium, characterised by aggregated nuclei surrounded by cytoplasm devoid of most organelles, showed weaker labelling than the rest of the trophoblastic layer (not shown). In many villi, the labelling appeared stronger on the basal side of the trophoblast layer, underlying the trophoblast basement membrane (figure 2.A.a). Proliferating cytotrophoblasts, labelled for Ki-67 antigen, showed similar angiogenin labelling to other trophoblastic cells (not shown). A similar pattern was observed with the two antibodies used (figure 2.A. a and c), but the intensity of the main underlined structures was different according to the antibody. The polyclonal antibody strongly stained the trophoblast layer and its basal lamina, whereas the monoclonal antibody highlighted vessels in the stroma. The specificity of staining was confirmed by the negativity of control sections treated with immune serum unretained on the angiogenin-affinity column (figure 2.A.b), or non specific isotypic mouse IgM (figure 2.A.d), or the second antibody alone (not shown). Western blot

analysis of placental extracts revealed a major band at 14 kDa, as expected, and a higher molecular weight component at 30 kDa, possibly corresponding to angiogenin dimers (figure 2.B).

Strong labelling was observed in the stromal core of the villi. In stem villi, large foetal vessels were easily identified: endothelial and vascular smooth muscle cells were labelled for angiogenin. In intermediate and terminal villi, where small vessels and capillaries are found, characterisation was performed by using double indirect immunolocalisation (figure 3). Angiogenin immunoreactivity co-localised with alpha-smooth muscle actin immunoreactivity (figure 3.A) and with endothelial markers (figure 3.B-E: VE-cadherin, vWF, VEGF-R2 and Tie-2, and CD31 (not shown)) indicating that the structures labelled for angiogenin were foetal capillaries and venules. Angiogenin labelling appeared more basal than VEGF-R2 and VE-cadherin labelling on endothelium (figure 3.B, D). We observed remarkable co-localisation of Tie-2 and angiogenin immunoreactivity in the foetal endothelium (figure 3.E). The transmembrane sialoglycoprotein CD34 is expressed by haematopoietic progenitors, umbilical artery and vein endothelium, and small-vessel endothelial cells [21]. In the heart of the chorionic villi, some CD34-positive cells corresponding to microvessel endothelial cells were also angiogenin-positive (not shown). Some proliferating endothelial cells, labelled for Ki-67 antigen, did not show noteworthy labelling for angiogenin (not shown). Leukocyte common antigen (CD45), a marker of bone marrow-derived cells, has been reported to be present on Hofbauer cells in chorionic villi [22]. No CD45-immunopositive cells labelled positively for angiogenin, indicating that foetal macrophages do not express detectable amounts of angiogenin (not shown). Angiogenin and erythropoietin receptor labelling showed a similar pattern (figure 3.F), with stronger labelling at the basal level of the trophoblast layer and in endothelial cells. Thus, the trophoblast layer and foetal vessels were immunopositive for angiogenin. It should be noted that the intensity of labelling was heterogeneous, and that

not all villi were labelled. There was no correlation between the intensity of labelling and the position of the villus in the villous tree.

In order to determine the subcellular localisation of angiogenin, transmission electron microscopy was performed using the polyclonal antibody and a second antibody conjugated to colloidal gold. The cellular distribution was similar to that observed by immunofluorescence staining. In chorionic villi, syncytiotrophoblast and endothelial cells showed cytoplasmic labelling (figure 4). In the syncytiotrophoblast, microvilli, cytoskeletal filaments, reticulum and mitochondria were labelled. Angiogenin was immunodetected in nucleus of endothelial, cytotrophoblast and syncytiotrophoblast cells as well as in the nuclear envelope, in keeping with the presence of a nuclear targeting signal in angiogenin [23,24]. Angiogenin was also immunodetected in the basal lamina of villous trophoblast and foetal endothelial cells. Furthermore, maternal and foetal red blood cells showed strong angiogenin labelling (figure 4.A, B). Negative controls omitting the primary antibody or using the immune serum fraction not adsorbed by immobilised angiogenin (figure 4.C) were negative.

The cell layer lining the basal plate, which is in contact with the maternal blood circulating in the intervillous space, is composed of two cell types: the syncytiotrophoblast (often degenerative) and endothelial cells (believed to be of maternal origin). This cell layer was strongly stained with the monoclonal anti-angiogenin antibody (figure 5.A). On adjacent sections, this cell layer was shown to correspond to endothelial cells, being negative for cytokeratin 7 (figure 5.B) and positive for CD31 (figure 5.C), CD34, VE-cadherin and vWF (not shown). Two kinds of foetal cells are found in the heart of the basal plate: the extra-villous cytotrophoblast, with an invasive phenotype, and multinucleated giant bed cells. The latter cells are formed by fusion of invading extra-villous cytotrophoblasts [25]. The extravillous cytotrophoblasts, stained for cytokeratin 7 (figure 5.B), were strongly labelled for angiogenin (figure 5.A) and Epo-R (not shown). On adjacent sections, they were negative for CD31 (figure 5.C), CD34, CD45, Ki-67, Tie-2 and VE-cadherin (not shown). Giant cells were

cytokeratin 7-positive and Epo-R positive (not shown), and strongly labelled for angiogenin (figure 5.D). Some fibroblast-like cells stained positively for angiogenin (figure 5.D) and negatively for cytokeratin 7 (not shown) were maternal components such as decidual cells, stromal endometrial cells, fibroblast-like cells and macrophages.

Taken together, these results indicate that angiogenin is present in the human placenta, in vascular, trophoblastic and amnionic epithelial cells.

Angiogenin mRNA expression in the human term placenta

As angiogenin has a signal peptide for secretion and is present in blood [4] and amnionic fluid [5], we examined the origin of the detected protein by using *in situ* hybridisation. Angiogenin mRNA was detected in foetal vessels and syncytiotrophoblast. A stronger signal was observed in knots, in contrast to the weak angiogenin protein staining (figure 6.A.a). No signal was observed on ribonuclease-pretreated frozen sections (figure 6.A.b) or on sections hybridised with the same amount of a non specific DNA probe (figure 6.A.c). Although the protein was detected, no transcripts were found on frozen sections of placentas from spontaneous deliveries. This might result from the molecular events involved in the labour process and in the delivery of the placenta. Northern blotting revealed no angiogenin transcripts in caesarean placental RNA, as previously reported by Futami *et al.* [9], with liver RNA as positive control (not shown). The presence of angiogenin transcripts was subsequently shown by RT-PCR (figure 6.B).

Angiogenin secretion by isolated villous cytotrophoblasts

Cultured explants of human placental villi released angiogenin into the medium (not shown) as already reported by Rajashekhar *et al.* [20]. As our explant cultures were enriched in

trophoblastic cells, we studied angiogenin expression by isolated cytotrophoblasts from term placenta. Highly purified trophoblast cultures were obtained, as shown by cytokeratin immunoreactivity specific for trophoblastic cells in chorionic villi (figure 8.A.d-f). As described by Kliman, cytotrophoblasts did not proliferate in culture but differentiated to form syncytiotrophoblasts [16]. Angiogenin was immunodetected in medium conditioned by trophoblastic cells on the first day of culture, on the second day when cells were aggregated, and also when cytotrophoblasts were differentiated (figure 7.A) and formed a fully functional syncytium (as shown by the levels of hCG, figure 7.B). Angiogenin immunoreactivity was detected by immunofluorescence throughout the differentiation process (figure 8.A.a-c). Labelling was cytoplasmic and underlined sites of focal adhesion in small syncytia and in isolated cytotrophoblasts (figure 8.B). No signal was observed in cells treated either with non specific rabbit IgG (figure 8.A. g-i) or with the second antibody alone (not shown). By RT-PCR, angiogenin transcripts were detected in freshly isolated villous cytotrophoblasts as well as in functional syncytiotrophoblast obtained *in vitro* (figure 6.B). Together, these data indicate that the villous trophoblast expresses and secretes angiogenin *in vitro*.

Discussion

During human placental development, vessel formation occurs both by vasculogenesis and by angiogenesis [26]. Angiogenesis occurs by elongation in the term placenta [27]. Angiogenin, one of the most potent angiogenic factors *in vivo*, is present throughout the foetal vascular tree. Its expression by endothelial and vascular smooth muscle cells *in vivo* corroborates its expression pattern observed *in vitro* [28]. As *in vitro* experiments have shown that these cells are also angiogenin target cells, angiogenin might have an autocrine and/or paracrine action on the two vascular components. Indeed, angiogenin binds to these cells *via* high-affinity binding sites [15,29] and induces intracellular events such as diacylglycerol formation, and induction of phospholipase A_2 in endothelial cells [30] and phospholipase C in vascular smooth muscle cells [31]. Consequently, angiogenin stimulates cell-associated proteolytic activity [32] and proliferation [33,34] in endothelial cells, and causes either growth inhibition or proliferation in vascular smooth muscle cell cultures, depending on their phenotype (as modulated by the culture conditions) [15,35,36]. Although angiogenin expresses *in vitro* most of its properties more weakly than other angiogenic factors such as fibroblast growth factor-1 or 2 (FGF) and VEGF, its potent angiogenic action is observed *in vivo* in various models of angiogenesis [3,37,38]. The results of these previous studies, together with the pattern of angiogenin expression in the human placenta, suggest that its action might be relevant to placental angiogenesis and/or vascular homeostasis.

Angiogenin expression is not restricted to foetal vascular cells. Angiogenin is located in mature foetal and maternal erythrocytes, as shown in figure 4. It has also been detected in foetal nucleated red blood cells during the first trimester (unpublished data). Its potential function is unclear, but erythroblasts have been reported to be a source of angiogenic factors such as VEGF and placental growth factor (PlGF) [39].

Angiogenin is expressed by all trophoblastic cells. The villous trophoblastic layer ensures nutrient, gas-exchange and endocrine functions, and produces angiogenin. The physiological role of angiogenin expression might be related to endothelial-like functions assumed by the syncytiotrophoblast in direct contact with maternal blood. Angiogenin is not relative to the proliferation state of endothelial and villous trophoblastic cells. However, its expression in placental explant cultures increases with development [20]. For successful pregnancy, both the vascular and the trophoblastic components of the villi must develop in a harmonious and co-ordinated manner to form a functional area of exchange between the maternal and foetal blood. Angiogenin expressed by the two components might play a role in this morphogenesis.

Angiogenin binds to the trophoblast basement membrane, as shown by the strong labelling observed on the basal site of the trophoblast layer by immunofluorescence and immunogold electron microscopy. Angiogenin is a heparin-binding protein [40] and could thus bind to the basal lamina through heparan sulphate, a component of the trophoblast basement membrane (together with collagen IV and laminin) [13]. The basal lamina of the placental foetal endothelium is also labelled, as observed by electron microscopy (not shown), in agreement with *in vitro* experiments showing that angiogenin binds to the extracellular matrix of endothelial cells [29]. The extracellular matrix ([41], for review) could create localised available angiogenin that would be released on cell activation.

Stronger immunoreactivity was observed in the extravillous cytotrophoblast and giant bed cells than in villous trophoblasts. Trophoblastic invasion of maternal tissues, considered to be a pseudo-tumoral process, is tightly controlled [42]. Angiogenin might play a role in cell migration, as it has been shown to mediate *in vitro* endothelial and tumour cell adhesion, an important step in the invasion process [43,44]. In addition, angiogenin has been shown to inhibit the degranulation of polymorphonuclear leukocytes [45] and to be immunosuppressive *in vitro* [46], and could thus participate in maternal immune tolerance towards the foetus (as a semi-allograft).

Angiogenin is present in amnionic fluid [5]. The strong staining observed in amnionic cells (figure 1) suggests a possible contribution of these cells to the angiogenin level in amnionic fluid. Increased angiogenin concentrations in amnionic fluid have been associated with preterm delivery [5]. The aetiology of preterm delivery is multifactorial, but maternal placental vascular disorders and inflammatory reactions accompanied by leukocytic infiltration and/or infection are major risk factors [47]. Among the inflammatory cytokines, interleukin-6 (IL-6) is induced in amnionic fluid by labour and infection [47]. Higher concentrations of IL-6 and angiogenin have been described in characterised microbial infection [48]. Angiogenin has been shown to be regulated as an acute phase protein *in vivo* [49] and to be induced by IL-6 *in vitro* [50]. In the context of host defence, angiogenin could act directly on bacterial and fungal pathogens as an endogenous antimicrobial protein, as suggested by its microbicidal activity [51].

In conclusion, this work shows that angiogenin is expressed by key placental cells, namely vascular, trophoblastic and amnionic cells. The *in situ* cellular distribution of angiogenin, in light of its known biological activities *in vitro*, offers new insights into the roles that angiogenin might play in the human placenta. Indeed, this potent angiogenic factor could act in concert with other peptide growth factors -- stimulators or regulators of angiogenesis -- expressed in the human placenta, such as FGF-2, HGF, PlGF, VEGF, angiopoietin-1 (Ang-1) and Ang-2, platelet-derived growth factor-B (PDGF-B) and transforming growth factor-ß (TGF-ß) ([12], for review). Based on the other known biological activities of angiogenin and its pattern of expression, these data suggest that its functions in the placenta are not limited to angiogenesis but may also include vascular and tissue homeostasis, maternal immune tolerance of the foetus, and host defences.

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FIGURE LEGENDS

Figure 1. Immunodetection of angiogenin in human term umbilical vein (A) and amnionic cells of the chorionic plate (B). Frozen sections were labelled by indirect immunofluorescence using a polyclonal antibody specific for angiogenin as described in Materials and Methods. (A) Endothelial cells (arrow head) and media vascular smooth muscle cells are immunoreactive for angiogenin. VL: vein lumen; M: media. (B) The amnionic epithelium (AE) showed strong angiogenin immunoreactivity, with intense perinuclear labelling (arrow). AC: amnionic cavity. Bar, 30 µm.

Figure 2. (A) Detection of angiogenin immunoreactivity in human term placenta villi. Frozen sections of human placenta were reacted with either polyclonal antibody purified by affinity chromatography on immobilised angiogenin (a) or monoclonal anti-angiogenin (c). Bound antibodies were detected with rhodamine-conjugated goat anti-rabbit IgG (a) or FITC-conjugated goat anti-mouse IgM (c). (a) and (c) show fluorescence within chorionic villi of the syncytiotrophoblast layer (ST), the basal membrane (BM), and foetal vessels (FV). There was no immunoreactivity in sections treated with either immune rabbit IgG not retained on the angiogenin affinity column (b) or isotypic mouse IgM (d). IS = intervillous space. Bar, 40 µm. (B) Western blot analysis of two term placenta protein extracts: lanes 2 and 3 (30 µg total protein) were blotted with the specific polyclonal anti-angiogenin antibody. In both extracts, a major 14-kDa protein, comparable to that of recombinant angiogenin (60 ng, lane 1), was detected. The weak 30-kDa band might correspond to angiogenin dimers.

Figure 3. Characterisation by double-labelling of angiogenin-labelled cells in the stroma of chorionic villi. Frozen sections of human chorionic villi were reacted with a mix of two

primary antibodies. The bound antibodies were detected with fluorescent secondary antibodies. Anti-angiogenin was either a polyclonal antibody purified by affinity chromatography on immobilised angiogenin (A, B, C, D, in red) or a monoclonal antibody (E, F, in green). The other antibody was anti-alpha smooth muscle actin (A, in green), anti-VE-cadherin (B, in green), anti-*vWF* (C, in green), anti-VEGF-R2 (D, in green), anti-Tie-2 (E, in red) or anti-Epo-R (F, in red). Nuclei were counterstained with Dapi, in blue. Bar, 50 µm.

Figure 4. Immunodetection of angiogenin in term placental villi by electron microscopy. Resin-embedded sections were incubated with anti-angiogenin IgG and the bound antibodies were detected with gold-conjugated goat anti-rabbit IgG (10 nm diameter). A variety of fixation methods were used. The preservation of angiogenin epitope was only achieved in 4% paraformaldehyde, but this resulted in some loss of ultrastructure. Labelling is highlighted by arrows. (A) In chorionic villi, labelling was observed in the syncytiotrophoblast (ST), cytotrophoblast (CT), basal membrane (BM), endothelial cells (EC) and foetal erythrocytes (FE). In the syncytiotrophoblast, angiogenin immunoreactivity was detected in the nucleus (n), the nuclear envelope (ne), the endoplasmic reticulum (er), mitochondria (m), and syncytial microvilli (mv). Labelling was also associated with some cytoskeletal filaments (c). Bar, 400 nm. (B) Maternal erythrocytes (ME) were strongly labelled for angiogenin. Bar, 500 nm. **(**C) No immunoreactivity was detected in sections reacted with non immune rabbit IgG. Bar, 500 nm.

Figure 5. Angiogenin immunoreactivity in the basal plate. Frozen sections were reacted with monoclonal anti-angiogenin antibody and the bound antibodies were revealed with an FITC-conjugated goat anti-mouse IgM (in green). Nuclei were counterstained with Dapi (in blue). (A) The cell layer lining the basal plate in contact with the intervillous space was strongly

stained for angiogenin (arrow), as were extravillous trophoblasts (cubic arrow) in a deeper decidual layer. (B) and (C) On adjacent cryosections, the intervillous surface of the basal plate in contact with maternal blood was CK7-negative (B) and CD31-positive (C**)** indicating the presence of endothelial cells (arrow) (also positive for CD34, VE-cadherin and vWF, not shown). Extravillous trophoblasts (cubic arrow) strongly stained for angiogenin were CK7-positive (B), but CD31-negative (C). (D) Giant bed cells are formed by fusion of invading extravillous cytotrophoblasts. Those multinucleated cells were also strongly labelled (arrowhead). Some fibroblast-like cells (incurved arrowhead) labelled for angiogenin could not be fully characterised with the panel of antibodies used. IS: intervillous space; BP: basal plate. Bar, 100 µm.

Figure 6. Expression of angiogenin transcripts. (A) *In situ* hybridisation of angiogenin mRNA in term placental villi. The frozen placental sections (7 µm) were hybridised with a digoxygenin-labelled cDNA probe. Probe binding was detected with an anti-digoxigenin antibody coupled to alkaline phosphatase, and visualised with a colorimetric alkaline phosphatase substrate (NBT/BCIP). Counter-staining used Mayer's hematoxylin. (a) Angiogenin mRNA was detected in the syncytiotrophoblast (arrow), foetal vessels (arrow head) and mesenchymal cells (cubic arrow). (b) Control: no signal was detected in RNase-pretreated frozen sections. (c) Control: no signal was detected with a non specific digoxygenin-labelled DNA probe. *, foetal vessel; IS, intervillous space; TB, trophoblastic layer. Bar, 30 µm. (B) Reverse transcriptase-polymerase chain reaction (RT-PCR) detection of a 264-bp fragment of angiogenin transcripts visualised on 2% agarose gel with ethidium bromide. Lanes: 1, human liver RNA (positive control); 2, human term placenta RNA; 3, RNA from cytotrophoblasts isolated from human term placenta, prior to culture (CTo); 4, RNA from 72-h cultured cytotrophoblast differentiated *in vitro* into a syncytiotrophoblast (ST). PCR for ß-actin transcripts yielded a 371-bp product.

Figure 7. Release of angiogenin by cultured isolated villous cytotrophoblasts. Conditioned media were collected as indicated and assayed for angiogenin by using a sandwich ELISA, and for hCG by using an automated enzyme-linked fluorescent assay. Results are means ± SD of triplicate determinations in representative experiments. (A) Angiogenin was released during *in vitro* differentiation of cytotrophoblasts into syncytiotrophoblast. (B) hCG was detectable on day 2 and maximally expressed on day 3, indicating a functional syncytium.

Figure 8. Immunodetection of angiogenin during differentiation of human villous cytotrophoblasts *in vitro*. The cells were fixed with methanol and then reacted with a mix of polyclonal anti-angiogenin and monoclonal anti-pan cytokeratin (CK). The bound antibodies were revealed with Texas-Red-conjugated donkey anti-rabbit IgG (in red) and FITC-conjugated donkey anti-mouse IgG (in green). Nuclei were counterstained with Dapi. (A) Angiogenin immunoreactivity (a, b, c) was observed at all stages of syncytium formation, with a cytoplasmic pattern. On these representative fields, all but two of the cells were cytokeratin-positive (d, e, f), indicating the high purity of the isolated trophoblasts. Controls with non specific rabbit IgG (g, h, i) and isotypic mouse IgG (not shown) were negative. Bar, 40 µm. (B) Focus on a small syncytium (2 nuclei) on day 2: angiogenin labelling was punctuate and especially abundant around the nuclei and close to the cell membrane. Focal adhesion sites (arrow) and membrane limits of the syncytium (arrowhead) were strongly labelled. Bar, 10 µm.