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ARTICLES

Extracellular Matrix-Dependent Regulation of Angiogenin Expression in Human Placenta

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Abstract Knowledge of the rapidly developing hierarchy of controls affecting vascular development in placenta is required to understand how the growth factors and their receptor-mediated signals actually produce vessels. At the cell biological level, these events clearly require stable interactions between the cells, and cells with the surrounding ECM. The objective of the study was to understand the role of integrins and ECM on the expression and secretion of angiogenin in placentas and from trophoblasts in culture. Functionally active term placental explant culture and trophoblast cultures were used to demonstrate the differential secretion profile of angiogenin and real-time quantitative RT-PCR to demonstrate the mRNA expression in the presence or absence of ECM proteins. In this study, a significant increase in expression and secretion of angiogenin occurred in the presence of vitronectin (VN) and fibronectin (FN). Using antibody-blocking experiments it was also demonstrated that the angiogenin secretion is mediated by placental integrins, $\alpha_V\beta_3$ and $\alpha_5\beta_1$. In addition, exposure to hypoxic conditions resulted in diminished angiogenin secretion in the presence of both ECMs suggesting that angiogenin expression in the presence of ECM is modulated by local O_2 concentration. In conclusion, this study provides evidence for the regulatory role of ECM and integrins on the mRNA expression and secretion of angiogenin in human placenta. ECMs may have a pivotal role in enhancing secretion of this peptide necessary for placental angiogenesis and provides the impetus as additional targets for the control of angiogenesis in pathological pregnancy. J. Cell. Biochem. 96: 36–46, 2005.

Key words: angiogenesis; hypoxia; integrins; IUGR; lightcycler

Growth of the placenta requires extensive angiogenesis for development of its vascular structure. The fetal villous angiogenesis is dependent on the proliferation of pre-existing endothelium and pericytes in early stages of pregnancy. On the other hand, when trophoblasts are penetrating the decidua, the maternal arteries also continue to elongate into the decidua resulting in extensive angiogenesis in both fetal villous tissue and in maternal deciduas [Blankenship et al., 1993]. Knowledge of this rapidly developing hierarchy of controls affecting vascular development is required to understand how the factors and their receptor-mediated signals actually produce vessels, induce branching and endothelial–accessory cell interactions and yield the array of different vessel types. At the cell biological level, these events clearly require control of cell proliferation and survival, various cell migrations and cell adhesive events, basement membrane assembly, and remodeling and stable interactions between cells and with extracellular matrix (ECM) around them. The ECM regulates these key events by means of integrins, a family of cell surface receptors that attach cells to the matrix [Hynes et al., 1999]. Signal transduction pathways from the ECM into the cell through integrins trigger changes in gene expression, regulate activities of cytoplasmic kinases, growth factor receptors, ion channels, and control the organization of the intracellular

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actin cytoskeleton [Giancotti and Ruoslahti, 1999]. The ECM is composed of structural and regulatory molecules some of which include laminin, collagen, vitronectin (VN), and fibronectin (FN). These matrix cellular proteins play a significant regulatory role in the control of cell growth and differentiation [Ruoslahti and Reed, 1994]. Indeed, a variety of physiologically important processes depend on the ability of cells to recognize and in turn respond to their immediate environment. These include angiogenesis, wound healing, bone resorption, and inflammation.

VN and FN are classical ligands for $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins respectively which have been shown to play a major role in angiogenesis [Varner et al., 1995; Suehiro et al., 1997]. VN and FN are the two major adhesive glycoproteins found in plasma and serum. These glycoproteins promote cell attachment, spreading, proliferation, migration, and differentiation in a variety of normal and neoplastic cells [Silnutzer and Barnes, 1984]. Like many other adhesion molecules, VN binds to cells through an interaction of the Arg-Gly-Asp (RGD) sequence in its cell-binding domain with VN- and FN-specific cell surface receptors, such as integrins αvβ3 and αvβ5 [Ruoslahti, 1996]. A recent study has documented the decreased adhesion of trophoblast cells to FN and VN with 1% O_2 which was reversible by re-exposure to 20% O_2. This phenomenon is probably attributed to the reduced surface expression of α_5 integrins under hypoxic conditions [Lash et al., 2001]. Adhesion of trophoblasts to VN and FN was also diminished by compounds which interfered with integrin function including EDTA and anti-integrin antibodies. This is suggestive of an important role for O_2 and ECM binding in the regulation of cellular invasion and migration, the phenomena which are essential to angiogenesis. Previously we have reported for the first time a potent angiogenic factor, angiogenin in placenta throughout gestation [Rajashekhar et al., 2003a] and hypothesized its role in placental angiogenesis. Furthermore, hypoxia was observed to elevate the expression of angiogenin in placental explants in vitro [Rajashekhar et al., 2003]. Taken together, the above studies indicate that hypoxia stimulates cellular invasiveness, at least in part through interactions with ECM. The signaling events involved in the expression and secretion of angiogenin and its possible effect in the presence of ECM molecules and integrin receptors in placenta is however, unknown at present. An understanding of the mechanisms by which the triad of integrins, growth factors, and ECM contribute to the regulation of angiogenesis could provide important clues on the regulation of blood vessel formation and regression during the course of pregnancy.

In this regard, attempts were made to understand the role of integrins and ECM on the expression and secretion of angiogenin in normal term placenta and from trophoblast cells in culture. Investigations of this nature could provide more insight on the regulatory role for these signaling molecules in angiogenesis both in terms of its underlying biology and in the etiology of uteroplacental dysfunction whereby compromised cell–cell interactions between mother and the developing fetus could lead to intrauterine growth retardation (IUGR).

MATERIALS AND METHODS

Cell Lines and Culture Conditions

NJG, a uterine choriocarcinoma trophoblastic continuous cell line [Nozawa et al., 1987], was generously supplied by HSRRB, Japan Health Sciences Foundation, Osaka, Japan. The term trophoblast cell line, A3 [Chou, 1978] was obtained from American Type Culture Collection (CRL-1584; ATCC, Manassas, VA). Cultures of both cell lines were maintained in DMEM (Invitrogen Life Technologies, Carlsbad, CA) medium supplemented with 10% FCS, 100 IU/ml of penicillin, and 100-μg/ml streptomycin (Sigma Chemical Co, St. Louis, MO). The cells were maintained as monolayer in Nunclon^{TM} 75 cm^2 cell culture flasks (Nalge Nunc Int., Rochester, NY) at 37°C in a humidified atmosphere of 5% CO_2 in air. A cervical carcinoma cell line, HeLa (CCL-2, ATCC) which served as a non-trophoblastic cell line was maintained as adherent culture in DMEM with 10% FCS and antibiotics.

Collection of Placental Specimens

Placentas were obtained immediately after delivery at term (37–41 weeks gestation) from normal healthy women who planned elective caesarean delivery ($n = 12$) after uneventful pregnancies. Tissue samples were collected in sterile plastic bags and were transported immediately to the laboratory on ice. Tissue

processing was commenced within 15 min of delivery. A small portion of the placenta was used for assessment of morphological anomalies and infection. Placental samples were used only if there was no clinical evidence of infection. Chorionic villi were dissected free from fetal membranes. Tissue either was stored in RNA-*later*™ (Ambion Inc., Austin, TX) at −80°C for RNA extraction, fixed in 4% buffered formaldehyde for histological viewing or processed immediately for explant cultures. The study was approved by National University Hospital's Institutional Review Board, and placental tissue samples were collected after obtaining informed consent from women participating in this study.

Human Chorionic Villous Explant Culture

Explant cultures were performed using the method described previously [Rajashekhar et al., 2002]. In brief, the placental tissue was placed in ice-cold PBS and was aseptically dissected to remove decidual tissue and fetal membranes. Small fragments of placental villi (300 mg wet weight) were then teased apart and placed in a six-well culture dish (Nunclon; Nalge Nunc Int) with or without ECM proteins (see below). Explants were cultured in Dulbecco's modified eagle medium (Invitrogen) containing 10% FCS (HyClone Laboratories, Inc., Logan, UT) and supplemented with 100 μg/ml streptomycin and 100 U/ml penicillin (Invitrogen) overnight at 37°C in 5% carbon dioxide in air to allow attachment. In all experiments, the explant cultures were set up in triplicate and incubated for 24 and 48 h. The morphological integrity of the placental explants as determined by histological examination of the processed tissue samples revealed that the surface structure of the villi was intact and unaffected during the culture. There was no evidence of the trophoblast and endothelial necrosis in both groups of tissues. The biochemical viability of the placental villi during the short-term explant culture experiments as assessed by measurement of β-hCG released in the culture medium (Total β-hCG assay, AXSYM, Abbott Diagnostics, Abbott Park, IL) were observed to increase in a time-dependent manner. Cultures were harvested and the conditioned media collected at the end of each incubation period were stored frozen at −80°C until analysis. Protein concentrations in the explant culture media were measured by the coomassie blue dye-binding assay (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as standard and supplemented medium as control.

ECM Coating on Cell Culture Plates (Fibronectin/Vitronectin)

Fibronectin (FN) and Vitronectin (VN) were acquired from Becton Dickinson (Bedford, MA). The ECM proteins were dissolved in calcium-free and magnesium-free PBS (Invitrogen) to the concentration of 10-μg/ml FN and 0.5-μg/ml VN. About 1 ml of the stock is used to coat each well of a 6-well culture dish in triplicate for overnight incubation at 4°C. The wells were washed thoroughly with PBS before using for the assay. For controls, the dishes were directly blocked with denatured BSA (Sigma) at 10-μg/ml concentration.

Trophoblast cells were treated with trypsin, centrifuged, and resuspended in fresh DMEM medium at a concentration of 1×10^6 cells/ml. Cell suspension was then seeded on to the culture plates precoated as indicated above at 1×10^5 cells/well. The cells were incubated for up to 48 h in culture in 5% CO_2 incubator. The medium was then aspirated and centrifuged at 500*g* for 2 min to remove non-adherent cells and stored at −80°C until analysis by ELISA. After removing the medium trophoblast cells in culture were scraped out with a rubber policeman in lysis buffer as per the manufacturer's instruction to prepare total RNA (RNeasy Mini Kit, Qiagen GmbH, Hilden, Germany).

Measurement of Angiogenin by ELISA

Concentrations of angiogenin in the explant culture media or trophoblast culture media were quantified by a sandwich enzyme immunoassay Quantikine kit (Human Angiogenin, R&D Systems Inc., Minneapolis, MN) utilizing a monoclonal antibody specific to human angiogenin as previously described [Rajashekhar et al., 2002]. The sensitivity of human angiogenin ELISA was typically less than 6.0 pg/ml with an intra-assay precision of 2.8% CV and an inter-assay precision of 7.1% CV. The range of standard curve was from 78.1 to 5,000 pg/ml. Serial dilutions of the samples yielded linear responses parallel to the standard curve. The samples were read at 450 nm with a reference wavelength of 570 nm in a 96-well automatic spectrophotometer (Bio-Tek Instruments, Inc., Winooski, VT). All samples were analyzed in the same batch of assay. Levels of angiogenin are

expressed as pg/mg of total protein from the conditioned culture media as described previously [Agarwal et al., 2001; Rajashekhar et al., 2002; Chou et al., 2003].

First Strand cDNA Synthesis

The total RNA extracted from 100 mg (wet weight) of placental samples using TRIzol method (Invitrogen) or from trophoblasts cells in culture was checked for integrity by 1% denaturing agarose gel electrophoresis and its concentration determined by measuring the absorbance ratio at 260/280 nm using a spectrophotometer (Beckman Coulter Inc., Fullerton, CA). Isolated total RNA was treated with DNase I, RNase-free (Roche Diagnostics GmbH, Mannheim, Germany) to eliminate genomic DNA contamination before using it for reverse transcription. The first strand synthesis cDNA was performed as published previously [Rajashekhar et al., 2002] using the First-Strand Synthesis System (Invitrogen) with oligo (dT) $_{12-18}$ primer and superscript reverse transcriptase enzyme. Reactions lacking the reverse transcriptase enzyme, the RT blank, were used as the negative control, which yielded no detectable products in all the experiments.

Real-Time Quantitative PCR

Real-time quantitative PCR and data analysis were carried out using a LightCycler (Roche Diagnostic GmbH), according to the manufacturer's instructions in borosilicate glass capillaries. PCR primers for the angiogenin were 5′-GTG CTG GGT CTG GGT CTG AC-3′ (sense) and 5′-GGC CTT GAT GCT GCG CTT G-3′ (anti-sense) as designed using a trial version of FastPCR software (http://www.biocenter.helsinki.fi/bi/bare-1_html/oligos.htm). This generated a 182 bp product for angiogenin (GenBank accession number NM_001145). The housekeeping gene β2-microglobulin (BMG) served as internal control which was used previously in LightCycler relative quantification on placental samples [Apodaca et al., 2000]. The PCR primers for BMG 5′-CGA GAT GTC TCG CTC CGT GG-3′ (sense) and 5′-GTC GGA TGG ATG AAA CCC AGA CAC-3′ (anti-sense) generated a 142 bp product (GenBank accession number NM_004048).

Quantitative PCR was performed in a total reaction volume of 20 μl per capillary for the LightCycler format. The reaction mixture contained 2 μl of cDNA (one tenth of the RT reaction), 2 μl LightCycler-FastStart DNA Master SYBR Green I (Roche Diagnostics), 0.5 μM of each primer, 4 mM $MgCl_2$. The amplification conditions consisted of an initial 10 min denaturation at 95°C, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 5 s, and extension at 72°C for 10 s, followed by melting curve analysis.

SYBR Green I is a DNA-binding dye that incorporates into dsDNA. It has an undetectable fluorescence when it is in its free form, but once bound to the dsDNA it starts to emit fluorescence. Since this dye can bind nonspecifically to any dsDNA [Simpson et al., 2000], we used melting curve analysis to differentiate between the products from primer-dimers. In addition, the Hot Start enzyme was used since it had been shown to reduce the primer dimer formation thereby improving the reaction efficiency. The SYBR Green I method has been successfully used in many applications with the LightCycler, including cytokine quantification [Simpson et al., 2000] and viral load detection [Schroter et al., 2001].

To compare the levels of angiogenin transcripts between the placental villi that are cultured over FN, VN, or BSA coated plates, we used comparative C_P method for relative quantification using the LightCycler Relative Quantification Software (Roche Diagnostics). Using this software employing the second derivative maximum method with arithmetic formulae were used to calculate the relative expression levels, when compared with an appropriate calibrator (a known control sample run in parallel in each batch of assay). The second derivative maximum method for quantification automatically determines the crossing points (C_P) for the individual samples. This is achieved by a software algorithm, which identifies the first turning point of the fluorescence curve, which serves as the C_P in this calculation method. The baseline adjustment, noise band or cross line settings are not required in this assay system. The amount of angiogenin transcript, normalized to the BMG endogenous housekeeping gene transcript and relative to the calibrator, is then given by $2^{-\Delta\Delta C_P}$, where $\Delta\Delta C_P = \Delta C_P$ (unknown target gene) $- \Delta C_P$ (calibrator), and ΔC_P of target or calibrator is the C_P of the target gene subtracted from the C_P of the housekeeping gene. The equation represents the expression of angiogenin relative to the expres-

sion of BMG in the unknown sample, normalized to the same expression ratio in the calibrator sample, thus providing a constant calibrator point between PCR runs. Importantly, to obtain reliable results using the $\Delta\Delta C_P$ method, the efficiency of PCR amplification for the target gene must be equal to that of housekeeping gene, which was determined by running an efficiency test with various dilutions of the template in a set of preliminary experiments.

Blockade of Angiogenin Production Using Integrin Antibodies

Integrins are known to play a major role in cellular adhesion to ECM. In this study, in order to determine whether integrin–ligand interactions are involved in angiogenin production by trophoblasts, antibodies directed towards human anti-integrin $\alpha_v\beta_3$ (Clone LM609) and anti-integrin $\alpha_5\beta_1$ (Clone JBS5; Chemicon Int., Temecula, CA) were used. The cells were preincubated with 10 μg/ml of these antibodies for 1 h at room temperature in medium, washed twice with PBS and as mentioned earlier the cells were placed onto the VN and FN-coated plates. After 24 and 48 h of incubation, the cell free supernatants were stored at -80°C for ELISA analysis. Cells preincubated without integrin antibodies or isotype matched IgG served as controls.

Effect of Hypoxia on Angiogenin Production in the Presence of ECM Proteins

To determine the role of hypoxia on angiogenin production in the presence of ECM proteins, trophoblast cells in culture were subjected to hypoxia when plated with or without ECM molecules for 24 and 48 h. The trophoblast cells were maintained in culture following the standard tissue culture conditions (5% CO_2, 95% air) as controls. The treatment group comprised trophoblast cells placed in a PRO-OX 110 (BioSpherix, NY) anaerobic culture chamber [Liu et al., 2002], which was maintained in a hypoxic environment (2% O_2, 93% N_2, 5% CO_2). The anaerobic culture chamber was placed within the host standard culture incubator (Forma Scientific, Inc, OH) thus allowing simultaneous incubation of explants and trophoblast cells in both normoxic and hypoxic conditions. The dissolved oxygen concentration within the insert chamber was monitored continuously using a digital display connected to oxygen electrode at the cell-medium interface.

These cultures were maintained in DMEM with 10% FCS and antibiotics (1×10^5 cells/well of a six-well culture dish). For hypoxic cultures, the medium was equilibrated to the desired oxygen concentration of 2% before use in experiments. This would enable a reduction of the time difference to equilibrate to the required oxygen levels in these experiments. The conditioned media were collected and the immunoreactive concentrations of angiogenin were measured by ELISA. In order to analyze mRNA expression in trophoblast cells in culture, the cells were scraped with a rubber policeman in lysis buffer and total RNA was extracted. The biochemical viability of the cultures was assessed by measurement of β-hCG released in the culture medium (total β-hCG assay, AXSYM, Abbott Diagnostics) as described previously [Rajashekhar et al., 2003a].

Statistical Analysis

Data were expressed as mean ± SEM for each group performed in triplicate for angiogenin secretion profiles and as mean ± SD for each group performed in triplicate for angiogenin mRNA expression profiles. Statistical significance was determined by Student's t test or ANOVA with the Post-hoc Scheffe's comparison test using SPSS Windows's version 11.0.1 (SPSS Inc, Chicago, IL). A probability value $P < 0.05$ was considered statistically significant.

RESULTS

Concentration of Angiogenin Released by Placental Explants in Culture in the Presence of ECM

In order to determine the levels of immunoreactive angiogenin released by placental explants cultured on ECM coated plates, this glycoprotein was quantified in the explant culture media from normal term placental tissues while the controls were plates coated with BSA. Angiogenin levels were detected in the placental conditioned medium of all samples in the three groups of tissues examined. The secretion profile of this pivotal angiogenic peptide is illustrated in Figure 1.

The mean ± SEM levels of angiogenin produced by placental villi cultured on the controls, BSA-coated plates were 101.8 ± 30.3 and

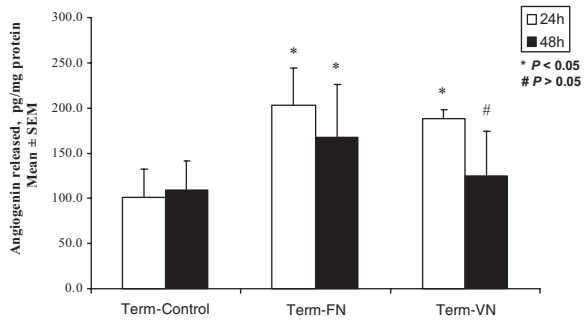

Fig. 1. Soluble levels of angiogenin in placental explants in the presence of ECM. Concentrations of human angiogenin released into the placental conditioned media of BSA coated control (term-control; n = 12), FN coated (term-FN; n = 12), and VN coated (term-VN; n = 12) cultures of term villi at 24 and 48 h of incubation. The mean concentrations of angiogenin increased significantly over their controls from FN ($P < 0.03$) and VN ($P < 0.05$) coated plates. Data represent mean ± SEM from three independent measurements performed in triplicates.

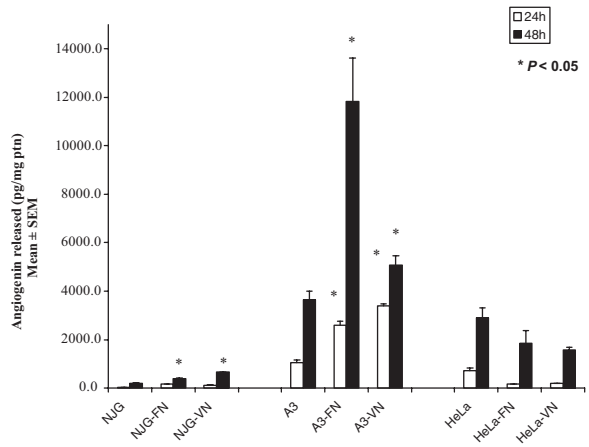

Fig. 2. Soluble levels of angiogenin in conditioned media of trophoblast cultures in the presence of ECM. Concentration of human angiogenin released into the conditioned media of BSA control (n = 4), FN (n = 4) and VN (n = 4) coated cultures of NJG; A3 (n = 4); HeLa (n = 4) at 24 and 48 h of incubation. The mean concentrations of angiogenin increased significantly over their controls from FN ($P < 0.02$) and VN ($P < 0.05$) in both NJG and A3 cell lines. However, in contrary HeLa cells showed a significant decrease in angiogenin when plated on ECM ($P < 0.05$). Data represent mean ± SEM from four independent measurements performed in triplicates.

109.2 ± 33.0-pg/mg protein, while that for FN-coated plates were 202.9 ± 40.8 and 167.7 ± 58.5-pg/mg protein and for VN-coated plates were 188.3 ± 9.1 and 125.4 ± 49.0-pg/mg protein at 24 and 48 h, respectively. Thus, angiogenin levels were significantly elevated ($P < 0.03$) in the presence of FN over that of the corresponding controls. On the other hand, although the angiogenin levels were elevated in the presence of VN over that of its corresponding controls, statistically significant levels were observed at the 24 h time point only ($P < 0.03$).

Concentration of Angiogenin Released by Trophoblasts in Culture in the Presence of ECM

Figure 2 depicts the concentration of angiogenin released by trophoblasts in culture in the presence of ECM molecules. The mean ± SEM levels of angiogenin produced by A3 cell line (normal term trophoblasts) cultured on BSA-coated plates were 1,050.5 ± 100.5 and 3,636.6 ± 358.6-pg/mg protein, while that plated on FN-coated plates were 2,588.7 ± 183.3 and 11,818.0 ± 1,809.5-pg/mg protein and on VN-coated plates were 3,387.1 ± 93.1 and 5,067.8 ± 391.8-pg/mg protein at 24 and 48 h, respectively. In a similar manner to those of placental explants, the angiogenin levels were significantly elevated ($P < 0.02$) in the presence of ECM molecules over that of the corresponding levels of trophoblasts plated on BSA-coated plates over the same time period. In addition, the choriocarcinoma cell line NJG also demonstrated an identical pattern of angiogenin secretion when plated on ECM coated wells. The mean ± SEM levels of angiogenin produced by NJG cell line plated on BSA-coated plates were 23.5 ± 2.4 and 188.2 ± 25.1-pg/mg protein, while that cultured on FN coated plates were 154.8 ± 17.0 and 390.2 ± 31.1 pg/mg protein and for VN-coated plates were 120.2 ± 29.7 and 650.5 ± 23.8 pg/mg protein at 24 and 48 h, respectively. Thus, angiogenin levels were significantly elevated ($P < 0.01$) in the presence of ECM molecules when compared with the corresponding levels of these choriocarcinoma cells cultured on BSA coated plates over the same time period. On the other hand, HeLa cell line (cervical carcinoma) demonstrated a converse pattern of angiogenin release with a significantly decreased ($P < 0.02$) secretion on ECM coated plates in culture. The mean ± SEM levels of angiogenin produced by HeLa cell line plated on BSA-coated plates were 717.4 ± 121.6 and 2,885.6 ± 425.8 pg/mg protein, while cultures on FN-coated plates were 153.8 ± 10.1 and 1,847.6 ± 524.8 pg/mg protein and for VN-coated plates were 191.2 ± 13.7 and 1,576.2 ± 92.6 pg/mg protein at 24 and 48 h, respectively.

Thus, angiogenin levels were significantly decreased ($P < 0.02$) in the presence of ECM molecules over that of the corresponding levels of cells plated on BSA coated plates over the same time period.

Integrin Mediated Secretion of Angiogenin From Trophoblasts in Culture

Pre-incubation with blocking anti-$\alpha_V\beta_3$ and anti-$\alpha_5\beta_1$ integrin antibodies significantly decreased angiogenin production in VN and FN coated plates respectively (Fig. 3). Neither the isotype matched IgG nor the cells plated on BSA coated plates were affected by this procedure. It is evident from this data that antibodies to integrins significantly reduced the secretion of angiogenin from trophoblast cells suggesting that angiogenin secretion is mediated by integrins.

Effect of Reduced Levels of Oxygen (Hypoxia) on Trophoblast Secretion of Angiogenin in the Presence of ECM

When trophoblast cells plated on FN- or VN-coated plates were subjected to hypoxia (2% O_2) for up to 48 h, angiogenin levels declined significantly ($P < 0.05$, ANOVA) over that of normoxic cultures under similar conditions (Fig. 4). The mean ± SEM levels of angiogenin produced by the A3, NJG and HeLa cell lines are summarized in Table I. It was observed that no significant differences in viability occurred among the three cell lines in culture upto 48 h

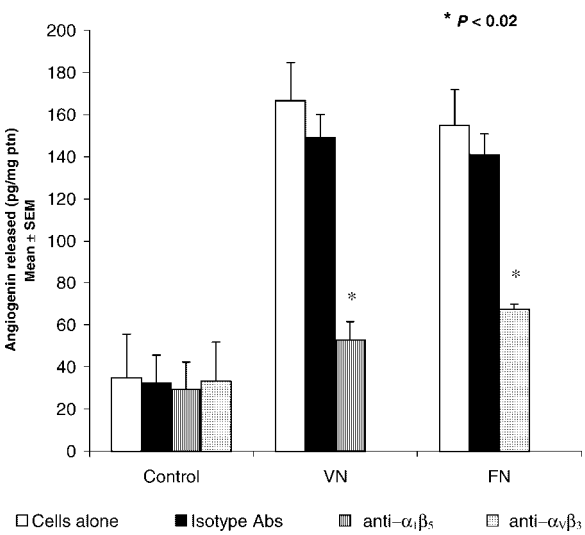

Fig. 3. Angiogenin secretion is integrin mediated. Concentration of human angiogenin released into the placental conditioned media of control (n = 12), FN (n = 12), and VN (n = 12) coated cultures of term villi at 24 h of incubation before and after pre-incubation with 10 μg/ml anti-$\alpha_V\beta_3$ and anti-$\alpha_5\beta_1$ antibodies or the respective isotype matched antibodies. The mean concentrations of angiogenin significantly decreased upon blocking integrin receptors ($P < 0.02$). Similar results were obtained at 48 h of incubation. Data represent mean ± SEM from three independent measurements performed in triplicates.

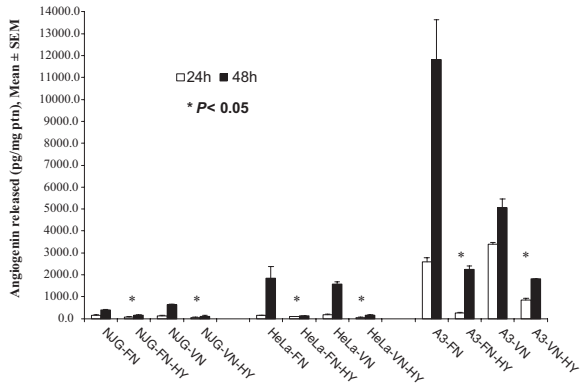

Fig. 4. Effect of hypoxia on angiogenin secretion in trophoblast cells in the presence of ECM. Concentration of human angiogenin released into the placental conditioned media of control, FN coated and VN coated cultures at 24 and 48 h of incubation before or after exposure to hypoxia (HY) from NJG, A3 and HeLa cells. The mean concentrations of angiogenin significantly decreased upon exposure to hypoxia ($P < 0.05$) in the presence of ECM. Data represent mean ± SEM from four independent measurements performed in triplicates.

TABLE I. Effect of Hypoxia on the Secretion of Angiogenin in Trophoblast Cells in the Presence of ECM

	Angiogenin-pg/mg protein (mean ± SEM)	
Treatment	24 h	48 h
NJG		
FN	154.8 ± 17.0	390.2 ± 31.1
FN-HY	77.4 ± 11.5[a]	153.0 ± 31.5[a]
VN	120.2 ± 29.7	650.5 ± 23.8
VN-HY	50.9 ± 11.8[a]	113.4 ± 40.1[a]
A3		
FN	2,588.7 ± 183.3	11,818.1 ± 1,809.5
FN-HY	261.6 ± 30.0[a]	2,231.1 ± 168.9[a]
VN	3,387.1 ± 93.1	5,067.8 ± 391.8
VN-HY	842.3 ± 101.7[a]	1,809.8 ± 17.8[a]
HeLa		
FN	153.8 ± 10.2	1,847.6 ± 524.8
FN-HY	92.2 ± 2.2[a]	128.9 ± 11.9[a]
VN	191.2 ± 13.7	1,576.2 ± 92.6
VN-HY	51.8 ± 9.9[a]	157.1 ± 35.4[a]

[a]$P < 0.05$ ANOVA.
Concentration of human angiogenin released into the conditioned media of FN (n = 4) and VN (n = 4) coated cultures of NJG; A3 (n = 4); HeLa (n = 4) at 24 and 48 h of incubation with or without hypoxia. The mean concentrations of angiogenin decreased significantly under hypoxia from ECM coated (FN-HY, VN-HY) plates in NJG, A3, and HeLa cell lines ($P < 0.05$) over their respective controls (FN, VN). Data represent mean ± SEM from four independent measurements performed in triplicates.

when subjected to hypoxic conditions as determined by trypan blue exclusion. However, there was a significant decrease in β-hCG secretion from trophoblast cultures plated on ECM coated plates compared to BSA coated plates under hypoxia (Fig. 5; $P < 0.05$).

The mRNA Expression of Angiogenin in the Presence of ECM

Human angiogenin mRNA expression detected by RT-PCR in all placental explant samples from the three groups examined is illustrated in Figure 6. The angiogenin specific 182 bp amplified PCR product was identified in all placental villi and the PCR product was observed to be identical to the published sequence, GenBank number NM_001145 [Kurachi et al., 1985].

To compare the levels of angiogenin transcripts between the placental villi that are cultured over FN, VN, or BSA coated plates, LightCycler software was used and the normalized ratio of angiogenin to BMG was considered as shown in Figure 6. Our results indicated that placental villi plated on either FN or VN showed

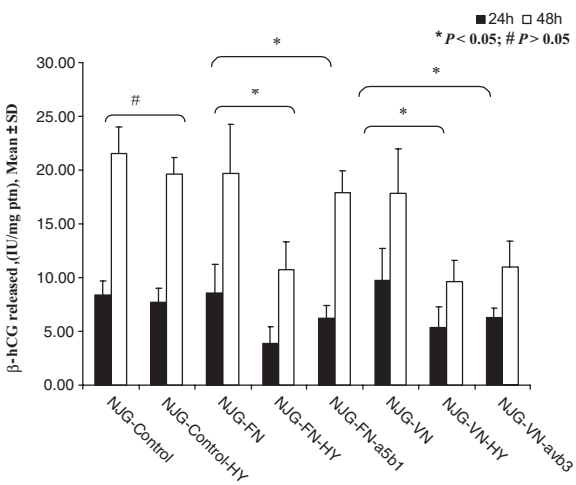

Fig. 5. Trophoblast cell viability with or without ECM. The metabolically active glycoprotein hormone of the trophoblasts, β-hCG released into the culture medium was estimated as described in Materials and Methods and the results were expressed as IU/mg protein. A significant decrease in β-hCG secretion from trophoblasts cultured on ECM coated plates (NJG-FN vs. NJG-FN-HY; NJG-VN vs. NJG-VN-HY) compared to BSA coated plates (NJG-Control vs. NJG-Control-HY) under hypoxia was observed ($P < 0.05$). The mean concentrations of β-hCG were also significantly decreased upon blocking of the integrin receptors with anti-αvβ3 and anti-α5β1 antibodies ($P < 0.04$). Similar results were also noted with the A3 cell line. Data represent mean ± SD from four independent measurements performed in triplicates.

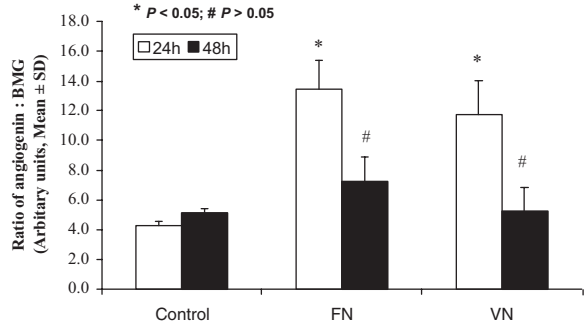

Fig. 6. Expression index for angiogenin mRNA in the presence or absence of ECM from placenta. The normalized ratio of real-time quantification of angiogenin to their internal control, BMG in the presence or absence of ECM is shown in arbitrary units. Note that angiogenin mRNA in placentas in the presence of FN and VN were significantly higher (>2-fold; $P < 0.05$) at 24 h than that of the normal term placenta under control conditions. Data represent mean ± SD from four independent measurements performed in triplicates.

approximately twofold higher levels of angiogenin mRNA when compared with that of the control BSA coated plates ($P < 0.05$) at 24 h. Although higher levels of mRNA could be discerned at 48 h, the data obtained was not statistically significant ($P > 0.05$).

In addition, trophoblast cells in culture when subjected on ECM coated plates indicated enhanced mRNA transcripts (Fig. 7). These results indicate a significant increase in angiogenin mRNA levels in both NJG and A3 cells at 48 h of incubation. In contrast, however, when

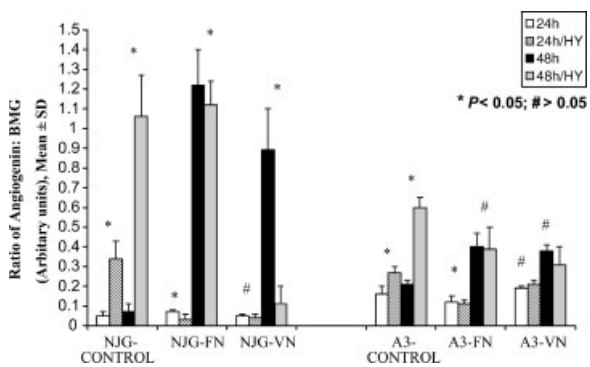

Fig. 7. Expression index for angiogenin in trophoblast cells in the presence of ECM with or without hypoxia. The normalized ratio of real-time quantification of angiogenin and the internal control, BMG in the presence or absence of ECM is shown in arbitrary units. Note that angiogenin mRNA in the presence of ECM is significantly elevated at 48 h. On the other hand, the same cells when subjected to hypoxia showed a significantly decreased (>2-fold; $P < 0.01$) angiogenin expression in the presence of ECM as opposed to control coated plates. Data represent mean ± SD from four independent measurements performed in triplicates.

these cells were subjected to hypoxia, a significant decrease in mRNA expression was observed in NJG cells compared to normoxic cultures with or without ECM ($P < 0.05$). Although lower levels of mRNA occurred at 48 h of culture with A3 cells, the data obtained was not statistically significant ($P > 0.05$).

DISCUSSION

The present study demonstrates for the first time that angiogenin expression and secretion is ECM dependent. Cells detect their extracellular milieu through interactions employing a variety of cell adhesion molecules such as integrins and syndecan molecules, and with the adjacent cells via members of the cadherin, selectin and IgCAM families. Importantly, these molecules have been discovered to participate in transduction events, either by directly eliciting signals upon engagement, or in a collaborative manner by modulating the efficiency of signaling pathways initiated by soluble factors. In this study, using placental explants and trophoblast cell lines in monolayer cultures, we have attempted to characterize signaling events associated with angiogenin.

Villous cytotrophoblasts (CTB) proliferate and differentiate, forming outgrowths that anchor villous in the presence of ECM proteins suggesting that contact between the tips of first trimester mesenchymal villi and a permissive ECM is sufficient to stimulate trophoblast columns [Aplin et al., 1999]. However, it was observed that these CTB cells of the column did not differentiate further to acquire the surface phenotype that has been reported in cells in vivo suggesting that paracrine interactions with maternal or fetal stromal, vascular, or blood-borne substances are required to complete this transition [Aplin et al., 1993]. In this regard, numerous factors have been shown to affect the motility and degradative activity such as tissue inhibitor of metalloproteinase, TGFβ1, EGF, CSF-1, IL-1, and TGF-α [Aplin, 2000]. In this study, we report angiogenin, a potent angiogenic factor that might play a vital role in this process of vascularization. The results from our experiments with placental explants suggest that in the presence of the ECM proteins, FN and VN, the expression and secretion of angiogenin amplified significantly over that of the control in vitro at 24 h. Although we are unable to explain the decrease in angiogenin secretion at 48 h time point in these viable cultures, this pleiotropic molecule possessing both intra and extracellular activities could exert autocrine negative feedback control. Moreover, the term placental cell line (A3) in monolayer culture showed similar pattern of increased angiogenin production while the cervical carcinoma cell line (HeLa) indicated a reverse trend suggesting that angiogenin production in the presence of ECM is cell type specific. Furthermore, this increased angiogenin was aptly blocked with integrin function blocking antibodies. In this regard, a previous study has shown that culture surfaces precoated with FN, but not albumin, renders trophoblast cells the ability to flatten, aggregate, and form syncytia [Kao et al., 1988]. In addition, attachment and syncytia formation has been blocked to FN surface using RGDS peptides in culture suggesting that FN mediates trophoblast attachment. Moreover, in this study a significant decrease in β-hCG secretion from trophoblast cultures plated on ECM coated plates was noted under these conditions probably indicative of failure of cells to attach. Thus, our data taken together with other reports suggest that angiogenin production is $\alpha_V\beta_3$, $\alpha_5\beta_1$ integrin, and ECM protein dependent.

Currently, little is known about regulation of signaling components of angiogenin in placental angiogenesis. The receptor for angiogenin is not yet convincingly identified. A previous study, using human aortic smooth muscle cells reported high affinity angiogenin binding complexes with apparent molecular masses of 45, 52, 70, 87, 98, 210, and about 250–260 kDa [Hatzi and Badet, 1999]. Whether these angiogenin-binding molecules are part of a complex receptor or entities bearing different functions remain to be determined. Conversely, Hu et al. [1997] have shown a 170-kDa protein as putative angiogenin receptor from subconfluent human endothelial cells. A more recent finding by the same author indicated that angiogenin can directly translocate into nucleus and bind to DNA, and thus may have a role in regulation of gene expression by direct binding to DNA [Hu et al., 2000]. Since destruction of the basement membrane is a prerequisite for endothelial cell migration during neovascularization [Folkman and Klagsbrun, 1987; Hu et al., 1994], it was suggested that angiogenin may well act as an integral part of the ECM of endothelial and fibroblast cells [Soncin, 1992], binding to it and enhancing the adhesive properties of these cells.

In accordance with these observations, our results using trophoblasts in monolayer culture indicated that a similar mechanism could be operative in trophoblast cells during placentation, thereby promoting trophoblast cell adhesion and subsequent invasion into the myometrium essential for placental angiogenesis.

While the above studies indicate that paracrine factors including angiogenin are involved in cellular invasiveness followed by proliferation, it remains unclear whether hypoxia has an effect on angiogenin expression and secretion from trophoblast cells and its interactions with the surrounding ECM. In light of the fact that integrins and the ECM system play a role in regulating angiogenin production, the present study provides a basis for the role of hypoxia and the regulatory role of oxygen concentration on cellular adhesion to these ECM proteins. The impetus for this study was indeed the dramatic changes in oxygen content of the placental environment which occurs in pathological pregnancies such as IUGR and pre-eclampsia. Thus, in order to gain a better understanding of the effects of hypoxia on in vitro production and secretion of angiogenin with or without the presence of ECM proteins in the present study, term placental explants and trophoblast cells (NJG and A3) were subjected to a culture environment of either standard culture conditions (21% O_2) or reduced oxygen content (2% O_2). In both trophoblasts and cervical cancer cell lines tested, angiogenin levels were significantly reduced under hypoxia in the presence of VN or FN. This is in contrast to a significant rise in angiogenin production under hypoxia when these cells were cultured on plastic surfaces (unpublished results). Moreover, in this study a significant decrease in β-hCG secretion from trophoblasts cultured on ECM coated plates was observed probably indicative of failure of cells to attach under hypoxic conditions. In this regard, a recent report using HTR-8/Svneo, another trophoblast cell line indicated that adhesion to VN and FN is significantly reduced under hypoxia [Lash et al., 2001]. It was also demonstrated that cell surface integrin α_5 a major receptor for FN and VN was significantly reduced under hypoxic conditions. Since hypoxia has been shown to modify the expression of several genes [Hu et al., 2003] and influence the adhesiveness of cells to the surrounding ECM [Lash et al., 2001] it could be surmised that angiogenin expression in the presence of ECM from these cells is under tight control of prevailing local O_2 concentration.

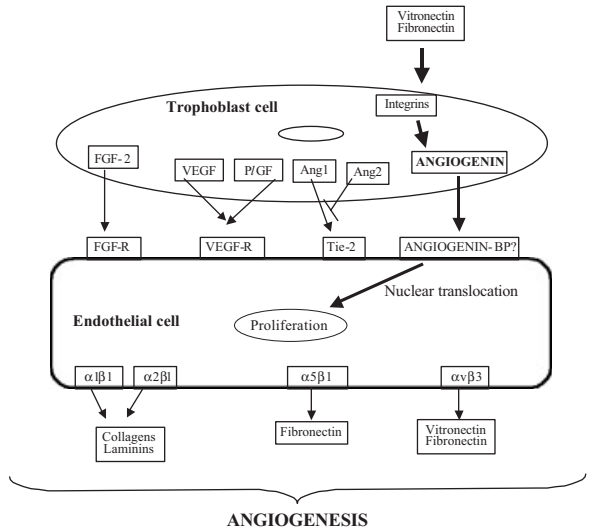

Fig. 8. Hypothetical model of angiogenin expression in trophoblast cells and its role in placental angiogenesis. In the presence of ECM, trophoblasts secrete angiogenin via integrin binding. Thus released angiogenin may bind to a hypothetical binding protein, endocytose, translocate to nucleus and potentiate endothelial proliferation. The overall outcome of these processes, probably together with other growth factors is angiogenesis.

In conclusion, the nature of extracellular matrix (ECM), oxygen concentration and the overall impact of many different paracrine factors, with either stimulatory or inhibitory effect on trophoblast/endothelial cells could be involved in placental angiogenesis (Fig. 8). Identification of growth factors, inhibitors, and receptors associated with the complex process of angiogenesis may provide potential targets for anti- or pro-angiogenic therapy. Although much work remains to be defined, the putative role for angiogenin in placenta presented in this study could provide the impetus as an additional target for regulation of angiogenesis in pathological pregnancies.

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REFERENCES

Agarwal R, Loganath A, Roy AC, Wong YC, Ng SC. 2001. Expression profiles of interleukin-15 in early and late gestational human placenta and in pre-eclamptic placenta. Mol Hum Reprod 7:97–101.

Aplin JD. 2000. The cell biological basis of human implantation. Baillieres Best Pract Res Clin Obstet Gynaecol 14:757–764.

Aplin JD, Vicovac L, Sattar A. 1993. Cell interactions in trophoblast invasion. In: Soares MJ, Handwerger S, Talamantes F, editors. Trophoblast cells—pathways for maternal–embryonic communication. New York: Springer-Verlag New York, Inc., pp 92–108.

Aplin JD, Haigh T, Jones CJ, Church HJ, Vicovac L. 1999. Development of cytotrophoblast columns from explanted first-trimester human placental villi: Role of fibronectin and integrin alpha5beta1. Biol Reprod 60:828–838.

Apodaca CC, Moore KH, Rossignol TM, Pierce B, Matej LA, Hume RF Jr, Calhoun BC. 2000. Localization of messenger ribonucleic acid for adrenomedullin and adrenomedullin receptor in the human placenta in normal pregnancies and pregnancies complicated by oligohydramnios. Am J Obstet Gynecol 183:1213–1219.

Blankenship TN, Enders AC, King BF. 1993. Trophoblastic invasion and the development of uteroplacental arteries in the macque: Immunohistochemical localization of cytokeratins, desmin, type IV collagen, laminin and fibronectin. Cell Tissue Res 271:227–236.

Chou JY. 1978. Human placental cells transformed by tsA mutants of simian virus 40: A model system for the study of placental functions. Proc Natl Acad Sci USA 75:1409–1413.

Chou CS, Zhu H, MacCalman CD, Leung PC. 2003. Regulatory effects of gonadotropin-releasing hormone (GnRH) I and GnRH II on the levels of matrix metalloproteinase (MMP)-2, MMP-9, and tissue inhibitor of metalloproteinases-1 in primary cultures of human extravillous cytotrophoblasts. J Clin Endocrinol Metab 88:4781–4790.

Folkman J, Klagsbrun M. 1987. Angiogenic factors. Science 235:442–447.

Giancotti FG, Ruoslahti E. 1999. Integrin signaling. Science 285:642–650.

Hatzi E, Badet J. 1999. Expression of receptors for human angiogenin in vascular smooth muscle cells. Eur J Biochem 260:825–832.

Hu G, Riordan JF, Vallee BL. 1994. Angiogenin promotes invasiveness of cultured endothelial cells by stimulation of cell-associated proteolytic activities. Proc Natl Acad Sci USA 91:12096–12100.

Hu GF, Riordan JF, Vallee BL. 1997. A putative angiogenin receptor in angiogenin-responsive human endothelial cells. Proc Natl Acad Sci USA 94:2204–2209.

Hu G, Xu C, Riordan JF. 2000. Human angiogenin is rapidly translocated to the nucleus of human umbilical vein endothelial cells and binds to DNA. J Cell Biochem 76:452–462.

Hu CJ, Pan Y, Simon MC. 2003. Oxygen and angiogenesis. In: Hoying JB, editor. Genetics of angiogenesis. Oxford, UK: BIOS Scientific Publishers, Ltd. pp 85–100.

Hynes RO, Bader BL, Hodivala-Dilke K. 1999. Integrins in vascular development. Braz J Med Biol Res 32:501–510.

Kao LC, Caltabiano S, Wu S, Strauss JF 3rd, Kliman HJ. 1988. The human villous cytotrophoblast: Interactions with extracellular matrix proteins, endocrine function, and cytoplasmic differentiation in the absence of syncytium formation. Dev Biol 130:693–702.

Kurachi K, Davie EW, Strydom DJ, Riordan JF, Vallee BL. 1985. Sequence of the cDNA and gene for angiogenin, a human angiogenesis factor. Biochemistry 24:5494–5499.

Lash GE, Fitzpatrick TE, Graham CH. 2001. Effect of hypoxia on cellular adhesion to vitronectin and fibronectin. Biochem Biophys Res Commun 287:622–629.

Liu H, Savaraj N, Priebe W, Lampidis TJ. 2002. Hypoxia increases tumor cell sensitivity to glycolytic inhibitors: A strategy for solid tumor therapy (Model C). Biochem Pharmacol 64:1745–1751.

Nozawa S, Udagawa Y, Sakayori M, Tsukazaki K, Takayama Y, Aoki D, Kubushiro K, Iizuka R, Inaba N. 1987. Establishment and characteristics of a new human choriocarcinoma cell line and its production of tumor markers. Nippon Sanka Fujinka Gakkai Zasshi 39:889–896.

Rajashekhar G, Loganath A, Roy AC, Wong YC. 2002. Expression and localization of angiogenin in placenta: Enhanced levels at term over first trimester villi. Mol Reprod Dev 62:159–166.

Rajashekhar G, Loganath A, Roy AC, Wong YC. 2003a. Over-expression and secretion of angiogenin in intrauterine growth retardation placenta. Mol Reprod Dev 64: 397–404.

Rajashekhar G, Loganath A, Roy AC, Mongelli JM. 2003b. Co-expression of Fas (APO-1, CD95)/Fas ligand by BeWo and NJG choriocarcinoma cell lines. Gynecol Oncol 91: 101–111.

Ruoslahti E. 1996. RGD and other recognition sequences for integrins. Ann Rev Cell Dev Biol 12:697–715.

Ruoslahti EA, Reed J. 1994. Anchorage dependence, integrins, and apoptosis. Cell 77:477–478.

Schroter M, Zollner B, Schafer P, Laufs R, Feucht HH. 2001. Quantitative detection of hepatitis C virus RNA by light cycler PCR and comparison with two different PCR assays. J Clin Microbiol 39:765–768.

Silnutzer J, Barnes DW. 1984. Human serum spreading factor (SF): Assay, preparation, and use in serum-free cell culture. In: Barnes DW, Sirbasku DA, Sato GH, editors. Vol 1. Methods for preparation of media, supplements, and substrata for serum free animal cell culture. New York: Liss, Inc., pp 245–268.

Simpson DA, Feeney S, Boyle C, Stitt AW. 2000. Retinal VEGF mRNA measured by SYBR green I fluorescence: A versatile approach to quantitative PCR. Mol Vis 6:178–183.

Soncin F. 1992. Angiogenin supports endothelial and fibroblast cell adhesion. Proc Natl Acad Sci USA 89: 2232–2236.

Suehiro K, Gailit J, Plow EF. 1997. Fibrinogen is a ligand for integrin alpha5beta1 on endothelial cells. J Biol Chem 272:5360–5366.

Varner JA, Brooks PC, Cheresh DA. 1995. REVIEW: The integrin alpha V beta 3: Angiogenesis and apoptosis. Cell Adhes Commun 3:367–374.