Dimethylarginine dimethylaminohydrolase (DDAH) regulates trophoblast invasion and motility through effects on nitric oxide

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BACKGROUND: Invasion of trophoblast into the uterine environment is crucial for establishing a successful pregnancy. Physiological production of nitric oxide (NO) by extravillous trophoblasts results in significant pro-invasive effects. NO synthesis is competitively inhibited by methylated arginine analogues such as asymmetric dimethylarginine (ADMA) but not the enantiomer symmetric dimethylarginine (SDMA). Within cells, the concentration of ADMA is regulated by the activity of the enzyme dimethylarginine dimethylaminohydrolase (DDAH). The aim of this study was to examine DDAH expression and function in trophoblasts. METHODS AND RESULTS: DDAH-1 and DDAH-2 messenger RNA and protein were demonstrated in first trimester placental tissue, primary extravillous trophoblasts and extravillous trophoblast-derived cell lines. DDAH activity was demonstrated in both cells and tissue. Overexpression of DDAH-1 in trophoblasts led to a number of significant changes, including an 8-fold increase in enzymatic activity, a 59% decrease in production of ADMA (but not SDMA), a 1.9-fold increase in NO and a 1.6-fold increase in cyclic guanosine monophosphate (cGMP) production. Functional assays showed that increased DDAH activity led to significantly increased cell motility and invasion in response to hepatocyte growth factor (HGF).

CONCLUSIONS: DDAH may play an important role in the regulation of extravillous trophoblast function via its effects on ADMA and NO production.

Key words: ADMA/asymmetric dimethylarginine/placenta/pregnancy

Introduction

During the process of placentation, fetal cytotrophoblasts proliferate to form aggregates known as columns that anchor peripheral villi to the maternal decidual surface. From these columns, extravillous trophoblasts invade the uterine wall and remodel the decidual and myometrial segments of the maternal spiral arteries by displacing smooth muscle and endothelial cells. The narrow, high-resistance arteries become dilated, forming low-resistance vessels to maximize blood flow to the maternal–fetal interface, a vital contribution to maintaining a normal pregnancy.

To achieve controlled remodelling without loss of integrity of the uterine wall, there must be stringent regulation of trophoblast invasion. In normal pregnancy, extravillous trophoblasts invade the decidual segments of the spiral arteries in the first trimester, and invasion continues into the myometrial segments in the second trimester. In pregnancies complicated by pre-eclampsia and intrauterine growth restriction, deficient invasion into the myometrial spiral arteries is observed (Brosens et al., 1970, 1977; Meekins et al., 1994; Brosens et al., 2002; Kadyrov et al., 2003), resulting in diminished utero-placental blood flow and a risk of fetal oxygen and nutrient deprivation.

The regulation of cellular invasion is complex and little understood but is likely to involve the interplay between a number of growth factor and cytokine-stimulated signalling pathways. Of particular interest is the role of hepatocyte growth factor (HGF), because homozygous HGF knock-out mice produce embryos with small placentae and a lack of trophoblast invasion, eventually proving lethal. We and others have shown that HGF regulates cellular processes, including motility, invasion and cell survival in a variety of cell types, including extravillous trophoblasts (Cartwright et al., 1999; Kauma et al., 1999; Cartwright et al., 2002; Purdie et al., 2002; Dash et al., 2003a,b). Our previous studies have identified that many of the effects of HGF on extravillous trophoblasts are mediated through the production of the signalling molecule nitric oxide (NO) (Dash et al., 2005).

NO is involved in a diverse range of physiological processes and is implicated in the maternal adaptation to pregnancy. It acts as a vasodilator and has regulatory functions in the implantation of the blastocyst (Gagioti et al., 2000),
that both iNOS and eNOS are expressed by trophoblasts. Ariel et al. (2000; Hambartsoumian et al. 2001) have shown that trophoblasts express eNOS and iNOS both in situ and in vitro (Tsatsaris et al., 2002). Significantly, Tsatsaris et al. have demonstrated that first trimester extravillous trophoblasts express eNOS and iNOS both in vitro and in vivo (Tsatsaris et al., 2002). Given the importance of NO as a signalling molecule in pregnancy and the range of cells that will be generating NO, regulation of its production is likely to be of great significance in the placental environment.

The synthesis of NO within cells can be regulated in a number of ways: NOS gene expression, enzymatic activation or changes in substrate or cofactor availability. The synthesis of NO is competitively inhibited by methylated analogues of arginine, such as asymmetric dimethylarginine (ADMA). ADMA is synthesized by most cells by the post-translational methylation of arginine residues in proteins and liberated upon their hydrolysis. Free ADMA is found in plasma and urine. There is evidence that elevated levels of ADMA are important in a number of conditions, including diabetes, atherosclerosis and renal failure. Circulating ADMA falls early during normal pregnancy but is elevated in pregnancies complicated by pre-eclampsia (Fickling et al., 1993; Holden et al., 1998; Savвидou et al., 2003).

Within cells, the concentration of ADMA and mono-methyl-arginine (L-NMMA), but not the enantiomer symmetric dimethylarginine (SDMA), is regulated by the activity of the cytoplasmic enzyme dimethylarginine dimethylaminohydrolase (DDAH). We and others have proposed that the intracellular concentration of ADMA, modulated by changes in DDAH activity, is an important means of regulating NO synthesis. Inhibition of DDAH activity results in the accumulation of ADMA, which, in turn, will reduce the production of NO by inhibiting NOS (MacAllister et al., 1996; Fickling et al., 1999; Dayoub et al., 2003).

Regulation of the local ADMA/NO environment in the placenta may have profound effects on cellular functions. It was therefore our aim to investigate whether alteration in intracellular DDAH activity could provide a mechanism for the control of trophoblast invasion.

Materials and methods

Trophoblast cell culture

SGHPL-4 cells, derived from primary human first trimester extravillous trophoblasts, are well characterized, confirming the retention of normal extravillous trophoblast features, including expression of HLA-G, cytokeratin-7, CD9 and human placental lactogen. They have been used extensively as a model of extravillous trophoblasts (Choy and Manyonda, 1998; Cartwright et al., 1999; Choy et al., 2000; Shiverick et al., 2001; Cartwright et al., 2002; Cartwright and Balarajah, 2005). SGHPL-4 cells were cultured in Hams F10 media supplemented with 2 mM l-glutamine, 100 units/ml penicillin, 0.1 mg/ml streptomycin and 10% (v/v) fetal calf serum (FCS). First trimester placenta were obtained at therapeutic termination of apparently healthy pregnancies. Ethical approval from Wandsworth Local Research Ethics Committee was in place and informed consent was obtained. Chorionic villi were plated onto collagen gels in a serum-free mixture (50:50 of Dulbecco’s modified Eagle’s medium (DMEM) and Hams F12 supplemented with penicillin (100 units/ml) and streptomycin (0.1 mg/ml). Extravillous trophoblasts proliferate, differentiate and migrate out of the villi at high purity over several days (Aplin et al., 1999).

Whole-mount staining of placental explant cultures for cytokeratin-7

The chorionic villous explants were kept in culture for 7 days to enable extravillous trophoblast outgrowth. The cultures were washed twice with phosphate-buffered saline (PBS) and fixed in methanol for 30 min. Following three further washes with PBS, parts of the explants not participating in anchoring to the gel were removed under a dissecting microscope. The cultures were incubated for 16 h at 4°C in 4% (w/v) bovine serum albumin (BSA) in PBS. The culture was incubated with anti-cytokeratin-7 (OV-TL 12/30, Dako, Ely, UK) or mouse isotype control immunoglobulin (Ig) at 5 μg/ml for 45 min at room temperature. Following three washes in PBS for 30 min, biotinylated goat anti-mouse antibodies (Vector Laboratories, Burlingame, CA) were added at 7.5 μg/ml for 45 min. After three further washes, cultures were incubated with fluorescein-streptavidin (Vector Laboratories) at 15 μg/ml for 15 min, washed extensively and observed by fluorescence microscopy.

PCR for DDAH-1 and DDAH-2

The chorionic villous explants were kept in culture for 7 days to enable extravillous trophoblast outgrowth. The medium was removed and the cultures were stored in RNAlater (Ambion, Huntingdon, UK) at 4°C. The villous tissue was removed under a dissecting microscope. The extravillous trophoblasts and surrounding collagen were then carefully removed, and the cells from four to six outgrowths/patients (approximately 10⁵ cells in total) were added to 1 ml of cell lysis buffer preheated to 45°C (MicroFastTrak Kit, Invitrogen, Paisley, UK). The messenger RNA (mRNA) was isolated from the lysate according to the manufacturer’s instructions. Complementary DNA (cDNA) was synthesized using the First Strand Synthesis kit (GE Healthcare, Chalfont St Giles, UK) according to the manufacturer’s instructions. The conditions for the PCR were as follows: denaturation, 95°C for 1 min, annealing, 55°C for 2 min, elongation, 72°C for 2 min, 30 cycles. The isomorph specific primers used in the reaction were as follows: DDAH-1 (forward) 5’-TCCACCTACTCCTGCCACCC-3’, DDAH-1 (reverse) 5’-GTTGATCGCTTCCTGAACAT-3’, producing a PCR product of 175 bp; DDAH-2 (forward) 5’-GGTGTCTGGGAGGTAAAATCG-3’, DDAH-2 (reverse) 5’-CTCGGTTCCTCCTCTCTATT-3’, producing a product of 230 bp.

Overexpression of DDAH-1 in trophoblasts

SGHPL-4 cells were transfected to overexpress DDAH-1 using the full-length rat DDAH-1 cDNA cloned into pcDNA/hygro (Kostourou et al., 2002). Medium was replaced with 3 ml culture medium containing 10 μg DNA/9 cm plate and 10 μg/ml poly-L-ornithine (Sigma-Aldrich, Dorset, UK, 15 000 MW). Plates were incubated for 6 h at 37°C with gentle mixing every 1.5 h. The medium was aspirated and replaced with 30% (v/v) dimethylsulphoxide in culture medium for 3
min at room temperature. The plates were then washed with medium, and 10 ml fresh medium was added. After 48 h, cells were selected in medium supplemented in 200 μg/ml hygromycin B. The concentration of hygromycin B used was previously determined as the lowest concentration that caused death of non-transfected SGHPL-4 cells within 7 days. The overexpressing cell line represents a mixed population of transfected cells because colonies were not selected.

**Western blot analysis of DDAH expression**

Expression of DDAH-1 and DDAH-2 was detected in homogenized human placental tissue, SGHPL-4 cells and DDAH-1-overexpressing SGHPL-4 cells. Cells were grown to confluence in Hams F10 media supplemented with 10% (v/v) FCS before being trypsinized. First trimester placental tissue was snap frozen in liquid nitrogen before homogenization in a pestle and mortar on dry ice. Tissue (1 g) and approximately 3.5 × 10⁶ cells were harvested at 4°C in 1 ml RIPA buffer [1x PBS, 1% (v/v) Nonidet P-40 (NP-40), 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulphate (SDS), 0.1 mg/ml phenylmethylsulphonyl fluoride (PMSF), 30 μl/ml aprotinin and 1 mM sodium orthovanadate], and the lysate was passed through a 21-gauge needle to shear the DNA. Total cell lysates were incubated on ice for 1 h after a further addition of PMSF (10 μl of a 10 mg/ml solution) before centrifugation at 15 000 g for 20 min at 4°C. The amount of solubilized protein was determined by Bradford assay (BioRad, Hemel Hempstead, UK). Protein was separated by SDS-polyacrylamide gel electrophoresis and transferred onto Hybond P membrane (GE Healthcare, Chalfont St Giles, UK). Blots were blocked for 1 h at room temperature in Blocking buffer [10 mM Tris, pH 8, 150 mM NaCl, 0.05% (v/v) Tween 20, 2% (v/v) BSA] and incubated for 16 h at 4°C with rabbit polyclonal anti-human DDAH-1 antibody (Arrigoni et al., 2003), diluted 1 : 1000 in blocking buffer or rabbit polyclonal anti-human DDAH-2 (SGH87Ab), diluted 1 : 500 in blocking buffer. After stringent washing, the membrane was incubated with goat anti-rabbit IgG (whole molecule) peroxidase conjugate (A6154, Sigma) diluted 1 : 12 000 in blocking buffer. Detection of membrane-bound antibodies was carried out according to the manufacturer’s instructions using a chemiluminescence kit and Hyperfilm ECL Plus (GE Healthcare, Chalfont St Giles, UK).

**DDAH activity assay**

Whole cells

DDAH metabolizes L-NMMA and ADMA to citrulline and mono- and di-methylarginine. SGHPL-4 cells were seeded at 2 × 10⁵ cells/well in a 24-well plate and incubated at 37°C in 5% CO₂ for 16 h to allow adherence. Cells were washed three times in HEPES-buffered Krebs (131 mM NaCl, 5.5 mM KCl, 2.5 mM CaCl₂, 1 mM MgCl₂, 25 mM NaHCO₃, 1 mM NaH₂PO₄, 20 mM HEPES and 5.5 mM glucose) warmed to 37°C. To determine DDAH activity, 250 μl of ¹⁴C-labelled L-NMMA (0.04 μCi/ml, specific activity 56 μCi/mM, radio-labelled at the C5 position, final concentration 50 μM) ± 0.1 mM ADMA (Sigma) or SDMA (Calbiochem, San Diego, CA) in Krebs solution was added to triplicate wells, and plates were incubated at 37°C in 5% CO₂ for 1 h. Medium was removed and cells were washed three times in ice cold HEPES-buffered Krebs solution and lysed in 0.5% (v/v) NP-40. The addition of 1 ml 50% (v/v) activated DOWEX 50X8-400 ion-exchange resin (Sigma-Aldrich, Dorset, UK) removed remaining unconverted ¹⁴C-labelled l-NMMA after a 3 min centrifugation at 12 000 g. The formation of ¹⁴C-labelled citrulline was determined as previously described (MacAllister et al., 1996). DDAH activity was represented as disintegrations per minute (dpm) and compared with that of ADMA- and SDMA-treated cells and also as the total counts attributed solely to cellular DDAH.

**Homogenized tissue**

First trimester placental tissue was snap frozen in liquid nitrogen before grinding in a pestle and mortar on dry ice. The tissue was mechanically homogenized in phosphate buffer (0.1 M NaH₂PO₄.H₂O, adjusted to pH 6.5 with 5 M NaOH). ¹⁴C-labelled L-NMMA (50 μl, final concentration 250 μM) was added to 400 μl of homogenized tissue and incubated at 37°C in 5% CO₂ for 1 h. Samples were centrifuged for 3 min at 12 000 g after the addition of 1 ml of 50% (v/v) activated DOWEX. The ¹⁴C content of 400 μl of the supernatant was measured, and results were expressed as the total activity.

**Measurement of dimethylarginines**

SGHPL-4 cells were cultured for 48 h to confluence in Hams F10 with 10% (v/v) FCS, and assessment of the concentration of ADMA and SDMA in culture medium was performed by high-performance liquid chromatography (HPLC) analysis as previously described (Holden et al., 1998).

**Measurement of cyclic guanosine monophosphate and nitrate/nitrite**

SGHPL-4 cells were seeded at 7.5 × 10⁴ cells/well in six-well tissue culture plates and incubated in Hams F10 plus 10% (v/v) FCS for 3 days until confluent. The medium was replaced with Hams F10 plus 0.5% (v/v) FCS for 16 h. The cells were then incubated with 3-isobutyl-1-methylxanthine (IBMX; 0.3 mM) to inhibit cyclic guanosine monophosphate (cGMP) phosphodiesterases for 24 h, before the media was collected. The cells were washed with PBS and lysed in 0.1 M NaOH, and the protein concentration was determined by Bradford assay (BioRad). cGMP levels in the culture media were determined in duplicate using a cGMP (low pH) Immunoassay kit (R&D Systems, Abingdon, UK) according to the manufacturer’s instructions. Nitrite and nitrate are formed from NO upon release from cells. Nitrate and nitrite within the media samples were determined by converting nitrate to nitrite and measuring total nitrite using the Nitrate/Nitrite Fluorometric Assay Kit (Cayman Chemicals, Ann Arbor, Michigan).

**Cell motility studies**

SGHPL-4 cells were seeded into six-well plates (2–13 × 10³ cells/well) in Hams F10 supplemented with 10% (v/v) FCS and incubated at 37°C in 5% CO₂ overnight to allow cell adherence. Cells were incubated for a further 24 h in 0.5% (v/v) FCS-supplemented Hams F10. Motility was determined in the presence or absence of HGF (R&D Systems; 10 ng/ml) as previously described (Cartwright et al., 1999). Motility studies employed time-lapse microscopy using an Olympus IX70 inverted microscope with motorized stage connected to a Hamamatsu C4742-95 CCD camera and an enclosed, heated chamber at 37°C in an atmosphere of 5% CO₂ in air (Solent Scientific, SEGENSWORTH, UK). Images were taken every 15 min over a 6 h period, and the distance each cell moved was analysed using Image Pro Plus software (Media Cybernetics, Silver Spring, MD, USA). Triplicate experiments were performed, and at least 10 cells per field of view were analysed.

**Cell invasion studies**

Invasion studies were carried out using a modification of the method described by Cartwright et al. (1999). SGHPL-4 cells and cells over-expressing DDAH-1 were grown to confluence in Hams F10 media supplemented with 10% (v/v) FCS. The cells were removed from the culture dish using trypsin/EDTA and diluted to 3 × 10⁴ cells/ml. In a flat-bottomed container, collagen-coated micro-carrier beads [prepared according to the manufacturer’s instructions (Sigma)] were added at approximately 1500 beads per 5 × 10⁶ cells and incubated at 37°C for 1 h to allow the cells to adhere to the beads. The bead–cell
complexes were washed twice in PBS to remove traces of FCS and allowed to settle. Sterile fibrin gels [2.5 mg/ml bovine fibrinogen (Sigma), 200 U/ml aprotinin (Trasylol, Bayer Plc, Newbury, UK) and 0.5 U/ml thrombin (Sigma) in calcium- and magnesium-free PBS] were prepared by mixing with the bead–cell complex and adding 1 ml/35 mm plate. Gels were allowed to set and incubated in media containing 0.5% (v/v) FCS at 37°C for 24 h. Both cell types were treated with HGF (10 or 50 ng/ml) diluted in media containing 0.5% (v/v) FCS and were incubated at 37°C in 5% CO₂ for 24 h. Images of at least 20 beads per dish were captured using a JVC TK-C1360E CCD camera connected to an Olympus IX50 inverted microscope, and the number of invasive processes was determined using Image Pro Plus software. Experiments were performed in triplicate and repeated on three separate occasions.

Statistical analysis
Statistical analyses were carried out using GraphPad Prism (GraphPad Software, San Diego, CA). Results were expressed as mean ± SEM and analysed using the Students t-test, with statistical significance assumed at the 0.05 level. Experimenter analysed the invasion and motility studies blind to the treatments used.

Results
Expression and activity of DDAH in human placental tissue and trophoblasts
Western blot analysis of homogenized first trimester placental tissue confirmed the presence of DDAH-1 and DDAH-2 (Figure 1A and B), and RT–PCR demonstrated expression of mRNA for DDAH-1 and DDAH-2 in primary first trimester extravillous trophoblast cells (Figure 1D). The trophoblastic nature of the migrating cells used for the isolation of RNA was confirmed by staining for cytokeratin-7 (Figure 1C). The available antibodies for DDAH-1 and DDAH-2 were not suitable for immunohistochemical analysis of primary extravillous trophoblasts from explant cultures.

The metabolic conversion of l-NMMA to l-citrulline by DDAH was measured in homogenized placental tissue using 14C-labelled l-NMMA, and mean total activity was found to be 6.76 × 10⁵ ± 1.56 × 10⁵ dpm/mg protein (±SEM, n = 20), showing that the placenta expresses active DDAH.

The SGHPL-4 trophoblast cell line was transfected to over-express DDAH-1. Western blot analysis showed a similar level of expression of DDAH-1 in the wild-type SGHPL-4 cells and the homogenized placenta, but a marked increase in DDAH-1 expression in the overexpressing cells (Figure 1A). DDAH-2 was also expressed by SGHPL-4 cells (Figure 1B).

DDAH activity was determined by the conversion of 14C radio-labelled l-NMMA to l-citrulline. DDAH catabolizes both l-NMMA and ADMA, the endogenous inhibitors of NO. The addition of ADMA to each cell type competes with l-NMMA, effectively reducing the production of radio-labelled l-citrulline by 65–78%. SDMA, an analogue of ADMA, which is not metabolized by DDAH, had a small negative effect on radio-labelled l-citrulline production (3–18%, attributed to competition with l-NMMA for transport into the cell) when compared with the control. Consistent with the high level of protein expression, SGHPL-4 DDAH-1-overexpressing cells displayed an average 8.1-fold increase in enzymatic activity attributed directly to DDAH (the SDMA reduction subtracted from the ADMA reduction) when compared with control cells (P = 0.0013), as determined by the conversion of 14C radio-labelled l-NMMA to l-citrulline (Figure 2).
Overexpression of DDAH-1 decreases ADMA production

The concentration of ADMA and its non-functional analogue SDMA were measured by HPLC analysis (Figure 3). The level of ADMA in the culture media from the SGHPL-4 DDAH-1-overexpressing cells was 59% lower ($P = 0.009$) when compared with the wild-type SGHPL-4 cells, but no difference in SDMA concentration was detected ($P = 0.9$).

Overexpression of DDAH-1 increases NO and cGMP production

NO production results in an accumulation of nitrate/nitrite in the media, which can be measured by reducing nitrate to nitrite and measuring the total nitrite concentration (Figure 4A). The level of nitrite in the culture medium from DDAH-1-overexpressing SGHPL-4 cells was 1.9-fold higher than that detected from the wild-type SGHPL-4 cells ($P = 0.0018$). Many of the actions of NO are mediated through the production of cGMP via the activation of soluble guanylate cyclase. NO can act in an autocrine manner to activate guanylate cyclase. Measurement of cGMP in the culture media showed that overexpression of DDAH-1 led to 1.6-fold increase in cGMP production by SGHPL-4 cells (Figure 4B, $P = 0.034$).

DDAH-1 increases HGF-stimulated trophoblast cell motility

HGF increased cell motility in both wild-type and DDAH-overexpressing SGHPL-4 cells as determined by time-lapse microscopy (Figure 5). However, HGF had a greater stimulatory effect on the DDAH-1-overexpressing cells (a 1.7-fold stimulation, $P < 0.0001$) than the wild-type SGHPL-4 cells (a 1.3-fold stimulation, $P = 0.004$).

DDAH-1 increases HGF-stimulated trophoblast cell invasion

The functional role of DDAH-1 was studied using invasion assays. Wild-type and DDAH-1-overexpressing SGHPL-4 cells were grown on micro-carrier beads embedded in fibrin gels and treated with 0, 10 or 50 ng/ml HGF. Cells first extend processes and eventually detach from the bead to migrate into
DDAH regulates trophoblast invasion

the fibrin gel. The number of invasive processes, which can be used as an index of invasive activity, was analysed after 24 h (Figure 6). The addition of HGF resulted in a significant increase in the number of processes extended in both cell types, with SGHPL-4 cells showing a 1.7-fold increase on stimulation with 10 ng/ml HGF ($P < 0.0001$) and a 1.33-fold increase on stimulation with 50 ng/ml HGF ($P = 0.0001$). DDAH-1-overexpressing trophoblasts showed a 3-fold stimulation with 10 ng/ml HGF ($P < 0.0001$) and a 2.8-fold stimulation with 50 ng/ml HGF ($P < 0.0001$). Overexpression of DDAH-1 significantly stimulated the invasion of SGHPL-4 cells into the

Figure 4. The effect of dimethylarginine dimethylaminohydrolase (DDAH)-1 on nitric oxide and cyclic guanosine monophosphate (cGMP) production by SGHPL-4 cells. (A) Nitrate/nitrite concentration in the culture media of SGHPL-4 cells was measured by fluorimetric assay. Results are shown as pmol nitrite per μg protein and are given as mean ± SEM of three experiments, each performed in duplicate ($**P = 0.0018$, compared with SGHPL-4 cells). (B) cGMP was measured in the culture media of SGHPL-4 cells by immunoassay. Results are shown as pmols cGMP per mg protein and are given as mean ± SEM of three experiments, each performed in duplicate ($*P = 0.034$, compared with SGHPL-4 cells).

Figure 5. The effect of dimethylarginine dimethylaminohydrolase (DDAH)-1 on trophoblast motility. Cells were incubated in the presence and absence of hepatocyte growth factor (HGF, 10 ng/ml) and images captured every 15 min over a 6 h period. Cells were chosen at random and the distance moved was quantified using Image Pro Plus software. Results are expressed as a percentage of control with mean ± SEM of $n = 60$ cells from three experiments, $**P < 0.005$. $***P < 0.0001$.

Figure 6. The effect of dimethylarginine dimethylaminohydrolase (DDAH)-1 on trophoblast invasion. The number of invasive processes formed by SGHPL-4 and SGHPL-4 DDAH-1-overexpressing cells in response to stimulation with 10 ng/ml hepatocyte growth factor (HGF) and 50 ng/ml HGF. Results are shown as a percentage of control with mean ± SEM from three experiments. $***P < 0.0001$. 
fibrin gel compared with the non-transfected cells at both 10 and 50 ng/ml (P < 0.0001).

Discussion

It is likely that NO signalling has multiple roles in the placenta and that these will vary depending on gestational age and the rate of production. Trophoblast and other placental cells express NOS. We and others have demonstrated that NO plays a central role in regulating key trophoblast functions which are crucial for the establishment of a successful pregnancy, including invasion and survival (Ni et al., 1997; Lyall et al., 1998; Cartwright et al., 1999; Rossmanith et al., 1999; Sanyal et al., 2000; Dash et al., 2003a,b, 2005). Inhibition of NOS activity leads to an inhibition of HGF-stimulated trophoblast invasion and motility (Cartwright et al., 1999, 2002; Tse et al., 2002).

Trophoblasts also produce ADMA, the endogenous inhibitor of NOS (Cartwright et al., 1999). ADMA can inhibit all isoforms of NOS, and ADMA made in one cell can inhibit NO synthesis in adjacent cells (Fickling et al., 1999), making it a versatile regulator of NO production. This is particularly important in the placenta where a number of cell types capable of generating NO will be present. Cells actively transport ADMA and the intracellular concentrations may rise to 5-fold higher than in the surrounding medium, sufficient to inhibit NO synthesis (Bogle et al., 1995). Many studies have proposed that varying the intracellular concentration of ADMA by changing DDAH activity is a means of regulating NO synthesis (MacAllister et al., 1996; Fickling et al., 1999). This has been supported by studies showing that DDAH-1 overexpression increases NOS activity both in vitro and in vivo (Dayoub et al., 2003).

It has previously been shown that mRNA for DDAH, the enzyme that metabolizes ADMA, is highly expressed in placental tissue at term (Leiper et al., 1999). Two isoforms of DDAH have been identified and both are widely expressed (Leiper et al., 1999). Here we have demonstrated the expression of both DDAH-1 and DDAH-2 isoforms in placental tissue and an extravillous trophoblast-derived cell line, and DDAH-1 and DDAH-2 mRNA expression by extravillous trophoblasts derived from first trimester chorionic villous explants.

Because NO is a known regulator of key trophoblast functions and the means of regulating its synthesis (ADMA production and DDAH expression) are present in trophoblasts and the placenta, we hypothesized that such regulation may affect trophoblast invasion. We have shown that overexpression of DDAH-1 in trophoblasts results in decreased ADMA production and increased NO and cGMP production. Consistent with our hypothesis, increased trophoblast cell motility and invasion were seen in response to HGF stimulation. It could be envisaged that in normal placentation, one of the important effects of growth factors such as HGF would be to induce production of NO and thereby increase trophoblast invasion. The levels of ADMA could be controlled by the expression of active DDAH ensuring low levels of methylarginines throughout the placenta under normal conditions. Interestingly, the administration of NOS inhibitors to pregnant rats results in features similar to pre-eclampsia, including a reduction in placental size and utero-placental blood flow, both of which can be reversed by the administration of L-arginine, suggesting a specific NO-mediated effect (Buhimschi et al., 1995). In pre-eclampsia inhibition of NO synthesis due to increased ADMA may predispose trophoblasts towards decreased invasion, resulting in a failure to remodel uterine vessels.

Significantly, the effects of DDAH overexpression on trophoblast invasion were more marked than the effects on trophoblast motility. Because cell motility is only one component of the invasive process, this may suggest that there are effects on other factors such as matrix metalloproteinases (MMPs). This will be interesting to study further because NO has been shown to increase MMP activity in a number of cell types (Tsuruda et al., 2004) including trophoblast (Novaro et al., 2001).

Another possible action of increased DDAH expression may be through effects on the production of growth factors, such as vascular endothelial growth factor (VEGF), because increased DDAH expression has been shown to increase tumour and endothelial cell VEGF production (Kostourou et al., 2002; Smith et al., 2003). VEGF has significant effects on trophoblast motility (Lash et al., 1999).

In conclusion, we have shown that DDAH expression regulates ADMA, NO and cGMP in trophoblasts, resulting in significant pro-invasive effects. During pregnancy, decreased expression or activity of DDAH could increase the concentration of ADMA and inhibit NO synthesis, leading to a decrease in motility and invasion. Trophoblastic colonization of the uterine wall and vascular transformation occur progressively during the first 4.5 months of pregnancy. Relatively small changes in the kinetics of invasion may lead to incomplete transformation of the spiral arteries as seen, for example, in pre-eclampsia. Thus, defective expression or activity of DDAH could contribute to the disease. In a recent study, it was suggested that polymorphisms in the DDAH-1 gene might modify susceptibility to pre-eclampsia (Akbar et al., 2005). Although the influence of these polymorphisms on function is unknown, the expression and activity of DDAH and indeed the role of the different isoforms in pre-eclampsia are thus highlighted as intriguing areas for future investigation.

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References


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