IGF2, IGF binding protein 1, and matrix metalloproteinases 2 and 9 in implantation-stage endometrium following immunoneutralization of vascular endothelial growth factor in the rhesus monkey

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Abstract

Blastocyst implantation in the rhesus monkey is inhibited by administration of antibody against vascular endothelial growth factor (VEGF) A during peri-implantation period with no change in the circulatory concentrations of estradiol, progesterone, and VEGF. In this study, we have investigated the effect of administration of a MAB to VEGFA on days 5 and 10 after ovulation upon the mRNA expression, immunopositive protein expression, and immunohistological localization of IGF2, IGF binding protein 1 (IGFBP1) and matrix metalloproteinases (MMPs) 2 and 9 in the implantation-stage endometrium collected on day 13 after ovulation from fecund cycles of rhesus monkeys. The comparison between isotype-matched IgG (control; $n=8$)- and VEGF antibody (VEGF Mab; $n=8$)-treated animals revealed higher ($P<0.05$) IGF2 in lacunar and villous syncytiotrophoblasts, trophoblast cell columns, migrating extravillous trophoblast cells, and endovascular trophoblast cells in control animals, but with no change in the various cell types of maternal endometrium between the two groups. No change in IGFBP1 expression in the endometrium was observed between the two groups. MMPs 2 and 9 were detected in syncytiotrophoblast in lacunae and villi, trophoblast cell columns, and extravillous trophoblast cells in control samples. MMP9 transcript expression in maternal endometrium and its immunopositivity in endometrial stroma and trophoblast cells were lower ($P<0.05$) with no change in MMP2 level in VEGF Mab-exposed samples compared with those in control samples. A functional network involving VEGF, IGF2, and MMP9 in early placental trophoblast cells and maternal endometrium appears to be important for normal placentation.

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Introduction

Blastocyst implantation in the rhesus monkey is initiated around days 7–9 after ovum fertilization. During early stage of implantation, syncytiotrophoblast cells intrude between epithelial cells to penetrate through the basal lamina, rapidly expand in the lateral plane of the uterine endometrium, and tap subepithelial capillaries to establish the early stage of placentation on days 12–13 after fertilization in the rhesus monkey (Enders 1995, Sengupta & Ghosh 2002). An obligatory component of the above-mentioned implantation-associated endometrial responses is a marked change in the vascular component under the action of vascular endothelial growth factor (VEGF; Ghosh *et al.* 2000, Wulff *et al.* 2002). Timed systemic administration of a neutralizing MAB directed against VEGFA resulted in pregnancy failure, and it was associated with shallow trophoblastic invasion, however, with no change in the concentrations of estradiol-17 β (E_2), progesterone, and free VEGF in the peripheral circulation (Sengupta *et al.* 2007).

Cytotrophoblast differentiation along the invasive pathway is a complex multistep process. As the cells lose their proliferative ability, they invade the uterine wall by expressing specific matrix metalloproteinases (MMPs; Xu *et al.* 2001, Staun-Ram *et al.* 2004). It has been demonstrated that VEGF and MMPs 2 and 9 may comprise a functional network regulating the invasive phenotype in a variety of cell types including trophoblast cells (Anteby *et al.* 2004, Hollborn *et al.* 2007). Also, it appears that a critical balance of insulin-like growth factors (IGFs) and their binding proteins between trophoblast cells and decidual cells may regulate the invasive property of trophoblast cells (Dhara *et al.* 2001, Irwin *et al.* 2001, Nayak & Giudice 2003). On the other hand, our previous report suggested that insufficient trophoblast invasion was the likely major causative factor in pregnancy failure in rhesus monkeys subjected to anti-VEGF antibody treatment (Sengupta *et al.* 2007). Taken together, in this study, we have investigated the effect of administration of a MAB to VEGFA during days

5–10 after ovulation upon IGF2, IGF binding protein 1 (IGFBP1), MMP2, and MMP9 in the implantation-stage endometrium of the rhesus monkey.

Results

Tables 1 and 2 show the scores of immunohistochemical (IHC) staining for different candidate proteins in day 13 lacunar-stage primary implantation sites of rhesus monkeys treated with or without VEGF Mab.

The feto–maternal interface in samples collected from both control (Fig. 1A)- and VEGF Mab (Fig. 1B)-treated animals showed immunopositivity for IGF2 in plaque, glandular, and stromal decidual cells with no marked difference between the two groups (Table 1). No overall change in the transcript and protein expressions for IGF2 was observed between the two groups in the endometrium, except a marginal attenuation of its expression in VEGF Mab-treated endometrium compared with control-treated implantation-stage endometrium (Tables 1 and 3). As shown in Fig. 1, at sites of embryo implantation, IGF2 was detected in trophoblast cells

Table 1 Rank scores of immunopositive levels of insulin-like growth factor 2 (IGF2) and insulin-like growth factor binding protein 1 (IGFBP1) in conceptus and endometrium at primary implantation sites collected on day 13 after ovulation from rhesus monkeys injected s.c. with isotype-matched mouse IgG ($n=8$) or monoclonal mouse antibody against VEGFA (VEGF Mab, $n=8$) on days 5 and 10 after ovulation.

Cell type	IGF2		IGFBP1	
	IgG	VEGF Mab	IgG	VEGF Mab
Conceptus				
Cytotrophoblast cells within chorionic membrane	0 (0–2)	2 (1–3)	ND	ND
Cytotrophoblast cells (lacunar and villous)	2 (1–3)*	0 (0–1)	ND	ND
Syncytiotrophoblast cells (lacunar and villous)	3 (2–4)*	0 (0–1)	ND	ND
Non-polarized cytotrophoblast cells in cell columns	2 (1–3)*	0 (0–1)	ND	ND
Syncytiotrophoblast cells lining cell columns	2 (1–3)*	0 (0–1)	ND	ND
Endovascular trophoblast cells	2 (1–3)*	0 (0–1)	ND	ND
Extravillous trophoblast cells	2 (1–3)*	0 (0–1)	ND	ND
Heterokaryons	3 (2–4)	3 (1–3)	1 (0–2)	1 (0–3)
Endometrium				
Epithelium				
Luminal	2 (1–3)	2 (1–3)	1 (0–3)	1 (0–3)
Glandular	2 (2–4)	2 (2–4)	2 (1–4)	2 (1–3)
Plaque	2 (2–4)	2 (2–4)	ND	ND
Stromal/decidual cells	2 (2–4)	1 (1–3)	2 (1–3)	2 (1–3)
Endothelial cells	0 (0–1)	0 (0–1)	ND	ND

ND, not detected. Scoring was done using a standardized 5-scale system: 0 (<5%), 1 (5–25%), 2 (26–50%), 3 (51–75%), and 4 (76% and more). * $P<0.05$.

Table 2 Rank scores of immunopositive levels of matrix metalloproteinases (MMPs) 2 and 9 in conceptus and endometrium at primary implantation sites collected on day 13 after ovulation from rhesus monkeys injected s.c. with isotype-matched mouse IgG ($n=8$) or monoclonal mouse antibody against VEGFA (VEGF Mab, $n=8$) on days 5 and 10 after ovulation.

Cell type	MMP2		MMP9	
	IgG	VEGF Mab	IgG	VEGF Mab
Conceptus				
Cytotrophoblast cells within chorionic membrane	ND	ND	1 (0–2)	2 (0–3)
Cytotrophoblast cells (lacunar and villous)	1 (0–2)	0 (0–1)	3 (2–4)*	0 (0–1)
Syncytiotrophoblast cells (lacunar and villous)	0 (0–1)	0 (0–1)	3 (2–4)*	0 (0–1)
Non-polarized cytotrophoblast cells in cell columns	0 (0–1)	0 (0–1)	2 (1–3)*	0 (0–1)
Syncytiotrophoblast cells lining cell columns	0 (0–1)	0 (0–1)	3 (2–4)*	0 (0–1)
Endovascular trophoblast cells	0 (0–1)	0 (0–1)	2 (1–3)*	0 (0–1)
Extravillous trophoblast cells	1 (0–1)	0 (0–1)	3 (2–4)*	0 (0–1)
Heterokaryons	1 (0–1)	1 (0–1)	3 (2–4)	2 (1–3)
Endometrium				
Epithelium				
Luminal	2 (1–3)	1 (0–2)	2 (1–3)	1 (1–3)
Glandular	1 (0–2)	1 (1–2)	2 (2–4)	2 (2–3)
Plaque	2 (1–3)	1 (1–3)	2 (1–3)	2 (0–4)
Stromal/decidual cells	1 (0–2)	1 (0–2)	3 (2–4)*	1 (0–3)
Endothelial cells	1 (1–2)	1 (0–1)	2 (2–3)	1 (1–3)

ND, not detected. Scoring was done using a standardized 5-scale system: 0 (<5%), 1 (5–25%), 2 (26–50%), 3 (51–75%), and 4 (76% and more). * $P<0.05$.

lining lacunae and early villi, in trophoblast cell columns, in migrating extravillous trophoblast and in endovascular trophoblast cells in control animals (Fig. 1A) confirming our earlier report (Dhara *et al.* 2001). However, samples from VEGF Mab-treated animals showed a significantly ($P<0.05$) lower expression of IGF2 in trophoblast cells (Fig. 1B). Although IGF2 was detected in trophoblast cells lying within the chorionic membrane and in heterokaryons formed by symplasma of lining uterine epithelial cells and trophoblast cells at the implantation site in VEGF Mab-exposed samples, trophoblast cells at invasive front exhibited lower ($P<0.05$) immunostaining for IGF2 (Table 1; Fig. 1B). The profiles of IGFBP1 were similar in decidualized stromal cells surrounding trophoblast cells and plaque cells and within the chorionic plate, and in glandular epithelium between samples collected from control (Fig. 1C)- and VEGF Mab (Fig. 1D)-treated animals (Table 1). The results of estimations of RNA and protein for IGFBP1 in the endometrium of both groups (Table 3) substantiated the IHC observation.

Table 2 shows that a substantial level of MMP9 was detected in trophoblast cells lining lacunae and villi, in

Table 3 mRNA and protein expression of insulin-like growth factor 2 (IGF2), insulin-like growth factor binding protein 1 (IGFBP1), matrix metalloproteinases (MMPs) 2 and 9 in the implantation-stage endometrium collected on day 13 after ovulation from rhesus monkeys injected s.c. with isotype-matched mouse IgG ($n=8$) or monoclonal mouse antibody against VEGFA (VEGF Mab, $n=8$) on days 5 and 10 after ovulation.

Name	IgG		VEGF Mab	
	RNA ^a	Protein ^b	RNA ^a	Protein ^b
IGF2	4.9±0.2	3.9±0.2	4.1±0.4	3.6±0.1
IGFBP1	4.7±0.3	4.1±0.3	4.8±0.4	4.0±0.2
MMP2	6.0±0.1	4.2±0.2	5.8±0.2	4.1±0.3
MMP9	2.7±0.4	4.2±0.1	1.8±0.3*	3.9±0.3

* $P<0.05$.

^aLog of copy number per μg RNA. ^bIntegrated optical density normalized by that of β -actin per 25 μg protein.

trophoblast cell columns, and also in an invasive population of extravillous trophoblast cells within the endometrial bed at the feto–maternal interface in control samples (Fig. 2B), but these cells showed only marginal level of MMP2 (Fig. 2A). MMP9 (Fig. 2C) was found to be lower ($P<0.05$) in trophoblast cells with no change in MMP2 expression level (Fig. 2D) in VEGF Mab-exposed samples compared with that in control samples (Table 2). Plaque cells and heterokaryons as well as cells in epithelial and vascular compartments at sites adjoining implantation sites were positive for MMP2 (Fig. 2A and D) and markedly positive for MMP9 (Fig. 2B and C) in both groups with no marked difference between the two groups (Table 1). No marked change was observed in MMP2 transcript and protein expression in maternal endometrium between control-treated and VEGF Mab-treated groups (Tables 2 and 3). However, implantation-stage maternal endometrium showed higher MMP9 expression ($P<0.05$) in stromal compartment of control samples (Fig. 2H) compared with that in VEGF Mab-exposed samples (Fig. 2F and I) in immunohistochemical analysis, as well as, in the estimation of RNA, but not in the estimation of protein for MMP9 in the endometrium of both groups (Tables 2 and 3). MMP9-positive endovascular trophoblast cells (Fig. 2E and H) were clearly detected in IgG-exposed control animals, while VEGF Mab-exposed samples failed to reveal MMP9-positive endovascular trophoblast cells. MMP9 level in glandular epithelium was similar between control (Fig. 2K)– and VEGF Mab (Fig. 2L)–treated samples (Table 2).

Discussion

The process of trophoblastic invasion involves remodeling of uteroplacental blood vessels and extracellular matrix (Cohen & Bischof 2007). As pointed out in the following section, three candidates appear to be important players in the process.

Figure 1 Immunohistochemical localization of insulin-like growth factor 2 (IGF2; A, B, and E) and insulin-like growth factor binding protein 1 (IGFBP1; C, D, and F) in primary implantation sites obtained on day 13 after ovulation from animals treated with control isotype-matched IgG (A, C, E, and F) shown as (C) or anti-VEGF Mab (B and D) shown as (V). Higher degree of immunopositive staining for IGF2 is observed in cytotrophoblast and syncytiotrophoblast lining lacunae and villi, in cytotrophoblast cell columns, in migrating extravillous trophoblast cells (arrows) in control samples (A) compared with that in anti-VEGF Mab-treated samples (B). Gland cells and decidual cells (arrows) surrounding plaque cells, migrating front of extravillous trophoblast cells, and adjacent to blood vessels show clear immunopositivity for IGFBP1 in both control (C)– and anti-VEGF Mab-treated (D) samples. No staining is observed in control immunohistochemistry for IGF2 and IGFBP1 in primary implantation site samples with medium containing pre-neutralized primary antibody against IGF2 (E) and medium containing pre-neutralized primary antibody against IGFBP1 (F) respectively. bv, blood vessel; cc, cytotrophoblast cell column; ct, cytotrophoblast; dc, decidual cell; evt, extravillous trophoblast cell; gl, gland cell; l, lacunae; pl, plaque cells; st, syncytiotrophoblast. Bar=40 μm (F), 50 μm (A, B, and E), and 60 μm (C and D).

Figure 2 Immunohistochemical localization of matrix metalloproteinase 2 (MMP2; A, D, and G) and matrix metalloproteinase 9 (MMP9; B, C, E, F, H–J, K, and L) in primary implantation sites obtained on day 13 after ovulation from animals treated with control isotype-matched IgG (A, B, E, G, H, J, and K) shown as (C) or anti-VEGF Mab (C, D, F, I, and L) shown as (V). Low level of MMP2 is observed in cytotrophoblast cells of cell columns and in migrating extravillous trophoblast cells (arrow) in control animal (A) and in plaque cells of VEGF Mab-treated animal (D). Marked MMP9 is observed in cytotrophoblast cells of cell columns, syncytiotrophoblast cells lining lacunae, migrating extravillous trophoblast cells (arrow) (B), trophoblast cell (arrow) invading into blood vessel (E), endovascular cytotrophoblast cells (H), and glandular epithelial cells (K). MMP9 is observed in plaque cells and decidual cells adjoining blood vessel (C, F, and I), glandular epithelium (L), while cytotrophoblast cells within chorionic cavity show variable and cytotrophoblast cells at feto–maternal junction (arrows) show negative staining for MMP9 in VEGF Mab-treated sample (C). Heterokaryons that are fused maternal epithelial cells and cytotrophoblast cells found at the outer margin of feto–maternal interface show immunopositivity for MMP9 (C). No staining is observed in control immunohistochemistry for MMP2 and MMP9 with medium containing pre-neutralized primary antibody for MMP2 (G) and medium containing pre-neutralized primary antibody against MMP9 (J) respectively. bv, blood vessel; cc, cytotrophoblast cell column; cv, chorionic cavity; ct, cytotrophoblast; dc, decidual cell; end, endothelial cells; evct, endovascular cytotrophoblast cell; gl, gland cell; hk, heterokaryon; l, lacunae; pl, plaque cells; spart, spiral arteriole; st, syncytiotrophoblast. Bar = 45 µm (G and J), 60 µm (B, E, F, K, and L), and 70 µm (A, C, D, H, and I).

- (1) VEGF: there is an up-regulated expression of VEGF and its receptors during the early stages of gestation (Ghosh *et al.* 2000, Schiessl *et al.* 2009) that directly or indirectly results in endometrial perfusion, trophoblast proliferation, and trophoblast invasion (Staun-Ram & Shalev 2005). These findings taken together with the observation that VEGF and its receptors are down-regulated in placental samples of pre-eclampsia (Zhou *et al.* 2002, Jarvenpää *et al.* 2009) suggest that a defect in angiogenic function may result in defective trophoblastic invasion and placentation process (Jauniaux *et al.* 2003, Prefumo *et al.* 2004, Ferretti *et al.* 2007). The suggestion is corroborated by the observation that timed systemic administration of VEGF Mab in the peri-implantation period in the rhesus monkey resulted in compromised expression of VEGF and its receptors along with shallow invasion and pregnancy failure in the rhesus monkey (Sengupta *et al.* 2007). The results of this study also substantiate the above observation.
- (2) IGF2: of the two major IGF types, IGF2 in lamellar syncytiotrophoblast cells specifically functions in their transition from proliferating to differentiated state, potentiating trophoblast migration within the chorionic plate (Dhara *et al.* 2001, Nayak & Giudice 2003). On the maternal side, uterine decidua expresses high levels of basement

membrane-type extracellular matrix and secretes IGFBP1 (Ghosh *et al.* 2004). The results of this study also substantiate the above observation. Interestingly, IGFBP1 expression in maternal endometrium exhibits temporal and spatial patterns specific to conceptus-derived signals (Ghosh *et al.* 2004). The function of IGF2–IGFBP1 at the feto–maternal interface may thus involve a delicate balance between invasion and its suppression toward normal placentation (Irwin *et al.* 2001, Forbes & Westwood 2008). In this study, we observed that administration of anti-VEGF antibody that had resulted in implantation failure (Sengupta *et al.* 2007) was associated with down-regulation of IGF2 in the trophoblast and with its marginal decreased expression in protein and transcript levels along with no change in IGFBP1 level in the maternal endometrium.

- (3) MMPs: substantial evidence suggests that gelatinase-type MMPs, especially MMP2 (gelatinase A) and MMP9 (gelatinase B), play a crucial role in the process of trophoblast invasion, and their inadequacy may result in defective placentation (Nagase & Woessner 1999, Bischof & Irminger-Finger 2005). It now appears that MMP9 expression/activation is critical for trophoblast invasion and its expression is markedly reduced with no change in MMP2 expression in the pre-eclamptic placentas (Ferretti *et al.* 2007, Shokry *et al.* 2009). In this study, we also observed that administration of anti-VEGF antibody that had resulted in implantation failure (Sengupta *et al.* 2007) was associated with down-regulation of MMP9 in trophoblast cells along with decrease in its transcript copy number in stromal compartment of the maternal endometrium without any change in MMP2 expression.

Thus, we observed in this study that failed angiogenic function in early placenta following administration of anti-VEGF antibody resulted in shallow invasion associated with an attenuation of IGF2 and MMP9 in trophoblast cells without influencing IGFBP1 in maternal endometrium and MMP2 in placental trophoblast cells. This is consistent with the notion that inadequate angiogenic function may result in defective trophoblastic invasion (Jauniaux *et al.* 2003, Prefumo *et al.* 2004, Egbor *et al.* 2006). It also appears that a functional network involving VEGF–IGF2–MMP9 is operative in the physiological process of trophoblast invasion in the rhesus monkey. As discussed below, several lines of indirect evidence indeed indicate that a putative functional network involving VEGF–IGF2–MMPs in trophoblast cells is important in the process of placentation.

The angiogenic switch under hypoxic stress generally involves both major gelatinases (MMP2 and MMP9); MMP9 may influence synthesis and secretion of VEGF in

non-trophoblast cells (Hollborn *et al.* 2007). Also pro-angiogenic and pro-metastatic molecules such as VEGF and MMP9 have common promoter regions containing binding sites for nuclear transcription factors, NF- κ B and AP-1, that regulate proliferation, apoptosis, angiogenesis, and invasion (Uzzo *et al.* 2006, Cohen *et al.* 2008). MMP9 may also exert a modulator activity in the VEGF-induced regulation of trophoblast invasion and migratory functions (Lash *et al.* 1999, 2003, Anteby *et al.* 2004). The reduced expression of MMP9 has been linked with insufficient invasion of trophoblast in pre-eclamptic placentas (Shokry *et al.* 2009). Taken together and based on the results of this study, a central role of MMP9 and VEGF in trophoblast invasion and vascular remodeling in the placentation process can be suggested (Tsatsaris *et al.* 2003, Prefumo *et al.* 2004, Papageorghiou *et al.* 2008).

IGF2 regulates the role of syncytiotrophoblast cells in placental functions of nutrient and oxygen transport, and it potentiates the migratory functions of trophoblast cells in the chorionic plate (Irving & Lala 1995, Dhara *et al.* 2001). IGF2 signaling has been found to up-regulate VEGF function in part by increasing hypoxia-inducing factor (Kwon *et al.* 2004, Kim & Kim 2005). Interestingly, we did not observe any change in endometrial IGFBP1 between anti-VEGF antibody-treated and control samples, despite lower expression of IGF2 in trophoblast cells in anti-VEGF antibody-treated samples. It appears possible that placental alkaline phosphatase might influence the degree of phosphorylation of IGFBP1 and thereby could affect the IGF-binding affinity of IGFBP1, for example non-phosphorylated IGFBP1 isoform shows reduced affinity for IGF1 (Westwood *et al.* 1994, Yu *et al.* 1998). Although we observed a marked reduction in placental immunopositive alkaline phosphatase expression in anti-VEGF antibody-exposed samples compared with control samples (data not shown), it is likely that non-phosphorylated IGFBP1 does not show marked difference in the specific binding affinity for IGF2 compared with phosphorylated IGFBP1 (Coppock *et al.* 2004). On the other hand, it is rather likely that reduced MMP9 in maternal endometrial decidual cells following anti-VEGF antibody treatment in this study might have resulted in a higher relative availability of phosphorylated IGFBP1 at feto–maternal interface resulting in an attenuated IGF2 activity (Forbes & Westwood 2008).

Collectively, it appears that hypoxia-sensitive extravillous trophoblast cells may show positive cooperation involving VEGF, IGF2, and MMP9 in invasive cytotrophoblast cells within a hypoxic milieu, thereby modulating early gestational trophoblast invasive functions in normal placentas. Insufficiency in the functional networks of VEGF–IGF2–MMPs as triggered by anti-VEGF antibody may result in defective placentation and pregnancy failure.

Materials and Methods

Animals

Proven fertile adult female rhesus monkeys (age 5–7 years; $n=18$) showing two consecutive normal menstrual cycles of 26–30 days were placed for mating during cycle days 8–16 with proven fertile adult male monkeys (age 6–8 years; $n=10$). Peripheral blood samples were collected for hormone assays, and vaginal smears were checked daily for the presence of spermatozoa and/or sperm plug. Ovulation was detected from the peripheral steroid hormonal profile for E_2 and progesterone (P) as detected using rapid assay (Sengupta *et al.* 2007). The assumed day of ovulation was taken 24 h after peak rise of E_2 in peripheral circulation and with detectable rise in serum P level (Ghosh & Sengupta 1993). Vaginal smears were microscopically scored on a grade scoring system recommended by the World Health Organization (WHO 2003). Of the 18 animals, two failed to show ovulation, and the remaining 16 animals that showed positive ovulation and sufficient sperm profiles were randomly assigned into two groups: control ($n=8$) animals that received 10 mg isotype-matched mouse IgG, and experimental ($n=8$) animals that received 10 mg monoclonal mouse antibody against VEGFA (VEGF Mab) between 1000 and 1030 h of days 5 and 10 after ovulation. The IgGs were prepared in 2 ml sterile PBS, pH 7.2 and injected s.c. as reported elsewhere (Sengupta *et al.* 2007). On day 13 after ovulation, all animals were laparotomized under ketamine anesthesia (12 mg/kg body weight, Parke Davis, Mumbai, India) for performing hysterectomy under aseptic conditions to collect implantation-stage maternal endometrium (Ghosh *et al.* 1999, Sengupta *et al.* 2003). The Primate Research Facility of the All India Institute of Medical Sciences provided routine care and management of the monkeys used in this study. The study was conducted with the approval of the ethics committee for the Use of Non-Human Primates for Biomedical Research, All India Institute of Medical Sciences, New Delhi. The source materials employed in this study were obtained from the animals that were used in a previous study (Sengupta *et al.* 2007).

Tissue processing

Uterine corpus was carefully removed and washed in ice-cold PBS (pH 7.2) to remove adherent blood and examined under stereozoom microscope to identify the primary implantation site. Implantation sites were sliced out and immediately fixed in cold neutral phosphate-buffered paraformaldehyde (4%, w/v). Samples were embedded in paraffin wax, and 5 μ m paraffin sections were processed for routine histology and immunohistochemistry (Sengupta *et al.* 2003, Ghosh *et al.* 2004). The details of immunohistochemistry are described below. Endometrial areas adjacent to the implantation site were sliced out from all samples and directly used for protein and RNA extraction as described below.

Immunohistochemistry

IHC staining for IGF2, IGFBP1, MMP2 and MMP9 in fetal and maternal compartments were performed in three randomly selected section specimens from individual samples (control

treated, $n=8$; VEGF Mab treated: $n=8$) using optimized methodology as described previously (Dhara *et al.* 2001, Kar *et al.* 2007). Briefly, 5 μ m deparaffinized sections were subjected to blockade for endogenous peroxidase activity and non-specific binding followed by incubation with primary antibodies overnight at 4 °C and then with biotinylated secondary antibody. Dilutions of stock primary antibodies for incubation were pre-calibrated based on 3–5-point titration and the information provided by the manufacturer. The sources of primary antibodies and the dilutions at which they were used in this study are shown in Table 4. Final visualization was achieved using the ABC peroxidase Elite kits (Vector Laboratories, Burlingame, CA, USA) and freshly prepared 3,3'-diaminobenzidine hydrochloride with hydrogen peroxide (Sigma Chemical Co.). The color characteristics of immunopositive staining ranged between brown and rust color. The nuclei were counterstained with Gill's hematoxylin. Specificity of the antibody binding was assessed by omitting primary antibodies, immunoadsorption of primary antibodies with target peptides, replacing primary antibodies with unrelated IgG from same species and other species, omitting secondary antibodies, and replacing secondary antibodies with unrelated IgGs from same or other species in parallel sections from each sample (control IgG, $n=8$; VEGF Mab, $n=8$). Labeled and unlabeled IgGs, non-immune sera, and other supplies were purchased from Vector Laboratories and Sigma Chemical Co.

Microscopic image analysis

The images were viewed and documented using a Leica DMRD microscope (Leica Microsystems, Wetzlar GmbH, Germany) and were analyzed by a computer-assisted subjected image analysis system (Leica QWIN DC 200, Cambridge, UK). The criteria for identification for different cell types have been described elsewhere (Enders 1993, Dhara *et al.* 2001). Multinuclear heterokaryons were identified at the periphery of the primary placenta with histology atypical to trophoblast and luminal epithelium as described by Enders (1993). It was, however, not certain whether the marginal ring of

Table 4 Characteristics of primary antibodies used in immunohistochemical (IHC) and immunoblot (WB) analyses of implantation-stage endometrium following maternal inhibition of vascular endothelial growth factor.

Antigen	Specification of antisera (Catalog number)	Dilution (μ g/ml)		Source
		IHC	WB	
β -actin	Sheep IgG (AF 4000)	–	2.0	R&D Systems ^a
IGF2	Goat IgG (AF292)	5.0	2.0	R&D Systems ^a
IGFBP1	Mouse IgG	0.5	0.2	S C Bell ^b
MMP2	Goat IgG (AF902)	5.0	2.0	R&D Systems ^a
MMP9	Mouse IgG (MAB911)	5.0	2.0	R&D Systems ^a

^aMinneapolis, MN, USA. ^bGift from Prof. S C Bell, Department of Obstetrics and Gynaecology, Leicester Warwick Medical School, University of Leicester, UK.

multinucleate masses at the implantation site was the product of trophoblast–epithelial fusion (Enders 1995). The cells were detected using an interactive planimeter analyzer only in cases where discernability was distinct. The immunopositive cells were identified by detecting positive profiles in digitized images. Following image grabbing and segmentation, digitalized images were subjected to image analysis for estimation of immunopositive area fraction based on manual outlining method and using an optimized gray level threshold yielding normal distribution of image pixels throughout the entire gray scale and shading correction. Pixel calibration was done against the standard provided by the manufacturer. All possible non-overlapping fields in a given section, at least three different sections for each parameter and for each sample, were evaluated. The results of immunopositive area fractions in percentage in different compartments were transformed into rank scores using a standardized 5-scale scoring system: 0 (<5%), 1 (5–25%), 2 (26–50%), 3 (51–75%), and 4 (76% and more) as described elsewhere (Kar *et al.* 2007).

RNA extraction

The methodological details of RNA extraction have been given elsewhere (Ghosh *et al.* 2009). Briefly, total RNA was extracted using TRIzol (Invitrogen) and cleaned up with DNase I (Sigma Chemical Co.) and subjected to re-extraction when it was necessary. The yield and purity of the extracted RNA were checked using standard protocols of absorbance ratio between 260 and 280 nm, and 1% agarose gel electrophoresis (Farrell 1998a). Furthermore, RIN score of individual samples was determined using Agilent 2100 Bioanalyzer, RNA 6000 Nano LabChip kit, and Agilent 2100 Expert Software (Agilent Technologies, Inc., Santa Clara, CA, USA) (Schroeder *et al.* 2006), and all samples had RIN score >8.0.

Quantification of target gene expression by real-time RT-PCR

The relative expression of candidate genes in reference to β -actin (*ACTB*) as the endogenous control in samples collected from all control IgG- and VEGF Mab-treated animals were performed and compared using SYBR Green chemistry-based real-time RT-PCR (Farrell 1998b) on a Bio-Rad platform (iCycler iQ™ Real-time PCR detection system, Bio-Rad). Primers for specific genes were designed on Beacon Designer software7 (Labware Scientific, Inc., Milipitas, CA, USA) using NCBI GenBank reference for the rhesus monkey and obtained from Qiagen. Details of primers for target genes are given in Table 5. Two-step RT-PCR for each target RNA (5 μ g) was done using optimized kits (QuantiTech Reverse Transcription kit for cDNA synthesis and QuantiFast SYBR Green PCR kit) from Qiagen. The calculation of absolute quantification of target transcripts was performed using an optimized method described by Bustin (2002) using standards obtained from Clontech (Takara Bio, Inc., Shiga, Japan) and pooled RNA as internal control, and expressed as log of copy number per μ g RNA.

Table 5 Primer sequences used in real-time PCR.

Gene name (Gene ID)	Primer sequence (5' to 3')	<i>T_m</i>
<i>ACTB</i>	GTGCGTGACATTAAGGAG (s)	49.7
(NM_001033084)	AGGAAGGAAGGTTGGAAG (as)	49.5
<i>IGF2</i>	CAGCAATCGGAAGTGAGC (s)	52.7
(XM_002799440)	TGGAGATGGAACCTGATGG (as)	52.7
<i>IGFBP1</i>	AACCAAGGCACAGGAGACATC (s)	57.5
(XM_001085935)	GTAGACGCACCAGCAGAGC (as)	57.6
<i>MMP2</i>	TGGTCCGTGTGAAGTATG (s)	50.3
(XM_001087335)	AAGTTGTAGGTGGTAGAGC (as)	50.5
<i>MMP9</i>	TGACAGAGACAAGAAGTG (s)	47.5
(XM_001104871)	AGTGAATCGGTACATAGG (as)	46.7

a, antisense; s, sense.

Immunoblots

Profiles of the candidate proteins in the endometrial homogenates of all samples (control treated, $n=8$; VEGF Mab treated: $n=8$) were assessed in samples of 25 μ g protein content along with pre-stained molecular weight markers based on SDS-PAGE/western immunoblotting method on nitrocellulose membrane using electrophoresis and transblot equipment, and chemicals obtained from Bio-Rad as described elsewhere (Asubel *et al.* 1994, Lalitkumar *et al.* 2005). Final visualization was achieved by using Vectastain ABC immunoperoxidase kits (Vector Laboratories). Respective primary antibody and secondary antibody controls were run simultaneously to examine the specificity of procedure. The molecular weights and semi-quantitative densitometric analysis of bands were determined using a densitometric equipment (Pharos FX Plus Molecular Imager) and an optimized densitometric (PD Quest Advanced) analysis software from Bio-Rad. The integrated measures of optical densities for individual antigens were calculated from log of transmittance for each of the target antigen and normalized with that of β -actin.

Statistical analysis

Statistical analyses of data for rank scores were done using non-parametric equivalents of ANOVA test followed by multiple comparison test based on rank sums Wilcoxon's test, and RNA and protein expression data were analyzed using Welch *T*-test (SPSS17 software; Chicago, IL, USA). The probability level of $P=0.05$ was taken as the limit of significance.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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