Role of vascular endothelial growth factor in endometrial vascular events before implantation in rats

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Two endometrial vascular events, increased permeability and endothelial cell proliferation, characterize the early stages of embryo implantation in rats. Vascular endothelial growth factor (VEGF), a heparin-binding homodimeric glycoprotein, is expressed in the uterus at the time of implantation. The aim of this study was to investigate the effects of systemic administration of blocking antibody to VEGF on endometrial vascular permeability and endothelial cell proliferation around the time of embryo implantation in rats. Rats were injected i.p. with 1.0, 0.8 or 0.6 ml of anti-VEGF antibody or control serum on day 3 of pregnancy and Evans blue dye was administered i.v. on day 5 of pregnancy. The number of implantation sites (blue bands along the uterus) was counted and endothelial cell proliferation was identified using double staining immunohistochemistry. The number of blue bands was significantly lower ($P < 0.05$) after injection of 1.0 ml blocking antibody (1.8 ± 1.56) compared with that in control rats (11.6 ± 1.97). The number of blue bands was significantly different among rats injected with 1.0 (1.8 ± 1.56), 0.8 (6.0 ± 3.67)

or 0.6 (10.7 ± 0.33) ml anti-VEGF antibody, indicating a concentration effect of anti-VEGF antibody. No significant differences in endothelial cell proliferation at embryo implantation sites were observed after injection with anti-VEGF antibody or control serum. At intersites (uterine zones between the blue bands), injection of 0.6 ml anti-VEGF antibody caused a significant reduction ($P < 0.05$) in the percentage of vessels with proliferating endothelial cells ($7.25 \pm 2.39\%$), with a non-significant reduction using 1.0 or 0.8 ml anti-VEGF antibody. No significant differences in microvascular density at either embryo implantation sites or intersites were observed between rats injected with anti-VEGF antibody or control serum. In conclusion, VEGF is the main factor responsible for increased endometrial vascular permeability at implantation. It may regulate endometrial endothelial cell proliferation before implantation and at intersites, thus indicating that factors or mechanisms other than VEGF may regulate endothelial cell proliferation at the site of implantation.

Introduction

Two endometrial vascular events characterize the early stages of embryo implantation in rats. The first of these is a localized increase in endometrial vascular permeability, which is one of the earliest indicators of the impending implantation site. This increase in endometrial vascular permeability has been observed in many species including rats (Psychoyos, 1960; Rogers *et al.*, 1983), guinea-pigs (Deansley, 1967) and sheep (Boshier, 1970). The increase in endometrial vascular permeability can be shown using dyes such as Evans blue, trypan blue or pontamine sky blue, which bind to plasma proteins, especially albumin, and leak into the surrounding tissues when given intravenously (Psychoyos, 1960; Rogers *et al.*, 1982). As a result, blue uterine bands can be observed around the uterus at each implantation site.

The second endometrial vascular event associated with

embryo implantation is increased endothelial cell proliferation. Macpherson and Rogers (1993) measured endothelial cell proliferation around the time of embryo implantation in rat endometrium. Macpherson and Rogers (1993) showed that endothelial cell proliferation was increased from day 3 of pregnancy and increased throughout the entire endometrium up to day 5 of pregnancy. Endothelial cell proliferation increased at embryo sites only from day 6 onwards and decreased to basal values at intersites, which are defined as the areas between the implantation sites. These results were interpreted as showing that endothelial cell proliferation is controlled maternally throughout the whole endometrium before implantation and is mediated locally by the embryo after implantation.

There have been limited studies to date investigating the factors or mechanisms responsible for increased endometrial vascular permeability and endothelial cell proliferation at the time of implantation, despite the reported presence of a number of vascular permeability-inducing and angiogenic agents in rat or mouse endometrium. These agents include prostaglandins (Kennedy, 1980), basic fibroblast growth factor (bFGF) (Carlone and Rider, 1992, 1993), transforming

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growth factor β (TGF- β) (Das *et al.*, 1992), tumour necrosis factor α (TNF- α) (De *et al.*, 1993) and epidermal growth factor (EGF) (Huet-Hudson *et al.*, 1990). There is little direct evidence that any of these agents mediate the vascular events at implantation directly.

Vascular endothelial growth factor, VEGF A (40–45 kDa), forms a family of heparin-binding homodimeric glycoproteins. There are at least five members of the family, which arise from alternatively spliced mRNA products from a single gene (Tischer *et al.*, 1991). The human isoforms have 121, 145, 165, 189 and 206 amino acids. Three isoforms, 120, 164 and 188, have been identified in mice; isoform 164 is the predominant isoform in mouse uterus (Halder *et al.*, 2000).

VEGF mRNA is expressed in different compartments of rat endometrium throughout the oestrous cycle (Karuri *et al.*, 1998) and in mouse uterus at the time of implantation (Chakraborty *et al.*, 1995; Halder *et al.*, 2000). These studies indicate that VEGF may play an important role during implantation by stimulating endothelial cell proliferation and increasing vascular permeability. However, no studies to date have provided functional evidence for a direct role for VEGF in regulating peri-implantation endometrial vascular permeability or endothelial cell proliferation. Thus, the aim of the present study was to investigate the effect of systemic administration of blocking antibody to VEGF on endometrial vascular permeability and endothelial cell proliferation around the time of implantation in rats. The hypothesis that acutely blocking the action of endogenous endometrial VEGF will reduce the implantation-associated increase in vascular permeability and endothelial cell proliferation was examined.

Materials and Methods

Experimental animals

Female Sprague–Dawley rats aged 3–6 months were purchased on day 1 of pregnancy, which was defined as the presence of spermatozoa in a vaginal smear. Rats were housed in a 12 h light:12 h dark regimen, with light commencing at 08:00 h. The study received ethical approval from the Monash Medical Centre Animal Ethics Committee A.

Antibody treatment

Purified recombinant mouse VEGF₁₆₄ (a gift from S. Stacker, Ludwig Institute, Melbourne) was used to immunize rabbits to produce polyclonal anti-VEGF antibodies using standard methods. The titre and specificity of the anti-VEGF response caused by this antibody had been established previously by inhibition of VEGF-mediated proliferation of VEGF receptor 2/Epo receptor bioassay cells and western blotting, respectively (Gargett *et al.*, 1999). Rats ($n = 12$) were injected i.p. with 1.0 ($n = 5$), 0.8 ($n = 4$) or 0.6 ($n = 3$) ml anti-VEGF antibody on day 3 of pregnancy between 16:00 h and 17:00 h. Controls ($n = 11$) were injected i.p. with 1.0 ($n = 5$), 0.8 ($n = 4$) or 0.6 ($n = 2$) ml normal rabbit

serum. This time and these doses were chosen after a limited pilot study testing 0.2–1.0 ml anti-VEGF antibody on different days of pregnancy.

Tissue collection

On day 5 of pregnancy, the rats were anaesthetized with Ketamil (0.1 mg kg⁻¹ body weight, i.m.; Troy Laboratories Pty Ltd, Smithfield) and Rompun (0.1–3.0 mg kg⁻¹ body weight; Bayer Australia Ltd, Pymble) between 18:00 h and 19:00 h. The rats were then injected i.v. with 1 ml of 2% (w/v) Evans blue in 0.9% (w/v) saline (Sigma Chemical Co., St Louis, MO). This dye was allowed to circulate for 15 min before the rats were killed by carbon dioxide inhalation. As a result of the leakage of Evans blue dye from vasculature into the extracellular space at points of increased vascular permeability, blue uterine bands were visible along the uterus at each implantation site. The spaces between the blue bands were termed intersites. The number of blue bands was counted and recorded. Each uterus was cut transversely into tissue blocks containing sites of embryo implantation (as defined by blue bands) and intersites.

Tissue preparation

Tissues were fixed in 10% (v/v) buffered formalin for 4 h and then stored in PBS at 4°C until they were embedded in paraffin wax using a histokinnette (Type E.7326; British American Optical Co. Pty Ltd, Slough).

Two blocks for implantation sites and two blocks for intersites were sectioned for each rat. Sections (5 μ m thickness) were cut using a standard microtome (Reichert-Jung Supercut 2050) and mounted onto slides coated with 3-aminopropyltriethoxysilane (APES; Sigma) to ensure tissue adhesion. Sections were dried and incubated at 37°C overnight.

Immunohistochemistry

A standard streptavidin–biotin immunohistochemical method using a double staining technique as described by Macpherson and Rogers (1994) was used for identifying proliferating endothelial cells. The reagents were from Zymed Laboratories Inc. (San Francisco, CA) unless otherwise stated. Sections were dewaxed and hydrated using safsolve (Ajax Chemicals, Auburn) and reducing concentrations of alcohol, and were then washed in distilled water for 5 min. After washing with PBS (three times for 2 min each), endogenous peroxidase activity was quenched using 3% H₂O₂ in methanol (10 min). PCNA (Clone PC10; Novacastra, New-castle), a monoclonal antibody against proliferating cell nuclear antigen, was used to identify all proliferating cells. Anti-PCNA antibody was diluted 1:50 in 1% (w/v) BSA–PBS and sections were incubated for 60 min at 37°C followed by biotinylated secondary anti-mouse IgG, streptavidin horseradish peroxidase and 3 amino 9-ethylcarbazole (AEC) chromogen. This procedure gives red nuclear staining to PCNA-positive cells.

Lectin BS-1 from *Griffonia (Bandeiraea) simplicifolia* (Sigma) was used to identify endothelial cells. This lectin binds selectively to the sugars α -methyl-D-galactopyranosyl and *N*-acetylgalactosamine and has been used previously as an endothelial cell marker in rats (Hansen-Smith *et al.*, 1988). After trypsin (Sigma) digestion for 10 min at 37°C (0.1% (w/v) in 0.1% (w/v) PBS), sections were incubated with 50 μ g lectin BS-1 in 1% (w/v) BSA-PBS for 30 min at room temperature, followed by streptavidin-alkaline phosphatase (AP) conjugate and AP blue chromogen.

Negative controls were performed with 10 μ g mouse IgG2a (in 1% (w/v) BSA-PBS instead of PCNA antibody) and 0.01 mol methyl α -D-galactopyranoside l^{-1} (Sigma) mixed with the lectin.

Separate sections were also stained using 25 μ g lectin ml^{-1} with the AEC chromogen (red) and haematoxylin (stains all cell nuclei blue) for an estimation of total numbers of blood vessels.

Quantification

The number of blue bands along both horns of each uterus was counted and means were calculated for all animals in each group. One section was taken from each tissue block. Areas of highest stromal cell proliferation, based on PCNA immunostaining, were chosen and three fields were captured using a digital video camera (Fujix; Fuji, Tokyo). The light intensity setting on the microscope and the parameters of hue, intensity and saturation on the camera were set the same for viewing of all sections. The total numbers of proliferating cells as well as the number of proliferating endothelial cells were counted in each field of view using Analytical Imaging Station™ software (Image Research Inc., Ontario). The percentage of proliferating endothelial cells was calculated by dividing the number of proliferating endothelial cells by the total number of proliferating cells to determine whether anti-VEGF antibody treatment specifically inhibited endothelial cell proliferation compared with proliferation in other endometrial cell types.

Blood vessels from the same area were counted using a Zeiss Axioskop microscope and the images were projected to a Sony PVM1440QM video monitor using a Sony CCDIRIS video camera. Vessel counts per field were converted to vessels mm^{-2} and then the mean number of vessels mm^{-2} was calculated.

Statistical analysis was undertaken using the Kruskal-Wallis (a non-parametric test), chi-squared and two-sample Wilcoxon rank sum (Mann-Whitney) tests from the SPSS statistical package. Differences between injection of 1.0, 0.8 and 0.6 ml blocking antibody and normal rabbit serum were tested for. Values are mean $\pm$ SEM. Differences were considered significant at $P < 0.05$.

Results

Significantly fewer ($P < 0.05$) blue bands were observed after injection of 1.0 ml anti-VEGF antibody (1.8 ± 1.56)

Fig. 1. Comparison of numbers of uterine blue bands in rats injected with 1.0, 0.8 or 0.6 ml anti-vascular endothelial growth factor (VEGF) antibody (■; $n = 5, 4$ or 3 , respectively) or normal rabbit serum (□; $n = 5, 4$ or 2 , respectively). Values are mean $\pm$ SEM. *The number of blue bands is significantly lower in rats that received 1.0 ml antibody compared with rats that received 1.0 ml normal rabbit serum ($P < 0.05$). There was also a significant difference in the number of blue bands among rats that received 1.0, 0.8 and 0.6 ml anti-VEGF antibody, indicating that there is probably a dose-dependent effect.

compared with the number in control rats (11.6 ± 1.97). Only one of five rats injected with 1.0 ml blocking antibody had blue uterine bands, with two of four and three of three rats having blue bands for the 0.8 and 0.6 ml anti-VEGF antibody treatments, respectively. There was also a significant difference in the number of blue bands ($P < 0.05$; Kruskal-Wallis and chi-squared tests) among the three antibody doses of 1.0 (1.8 ± 1.56), 0.8 (6.0 ± 3.67) and 0.6 (10.7 ± 0.33) ml, indicating a concentration effect of anti-VEGF antibody. This difference was not evident among the three groups injected with normal rabbit serum (Fig. 1).

As a general observation, endometrial cellular proliferative activity, including endothelial cell proliferation, was higher in the stromal compartment around the lumen than in the areas closer to the myometrium.

Analysis of implantation site and intersite endothelial cell proliferation was not possible for the four rats in the 1.0 ml blocking antibody group and two rats in the 0.8 ml anti-VEGF antibody group that did not have blue bands. Endothelial cell proliferation in embryo implantation sites of the remaining rats injected with 1.0, 0.8 and 0.6 ml anti-VEGF antibody was not significantly different from that in rats treated with normal rabbit serum (Fig. 2).

Endothelial cell proliferation was consistently lower at intersites in all blocking antibody-treated groups compared with normal rabbit serum controls. However, due to the small number of animals available for analysis in the 1.0 ml

Fig. 2. Endothelial cell proliferation at embryo sites of rats injected with 1.0, 0.8 or 0.6 ml anti-vascular endothelial growth factor (VEGF) antibody (■; $n = 1, 2$ or 3 , respectively) or normal rabbit serum (□; $n = 5, 4$ or 2 , respectively). Rats with no blue uterine bands were excluded from the 1.0 ml and 0.8 ml anti-VEGF antibody groups. Values are mean $\pm$ SEM. No significant differences were detected between the anti-VEGF antibody and normal rabbit serum groups.

Fig. 3. Endothelial cell proliferation at intersites of rats injected with 1.0, 0.8 or 0.6 ml anti-vascular endothelial growth factor (VEGF) antibody (■; $n = 1, 2$ or 3 , respectively) or normal rabbit serum (□; $n = 5, 4$ or 2 , respectively). Rats with no blue uterine bands were excluded from the 1.0 ml and 0.8 ml anti-VEGF antibody groups. Values are mean $\pm$ SEM. *Endothelial cell proliferation was significantly lower in the 0.6 ml anti-VEGF antibody group than in the corresponding normal rabbit serum group ($P < 0.05$).

and 0.8 ml anti-VEGF antibody groups, a significant reduction in endothelial cell proliferation was seen only in the 0.6 ml anti-VEGF antibody group (Fig. 3).

In the four 1.0 ml anti-VEGF antibody and two 0.8 ml anti-VEGF antibody uteri with no blue bands, mean endothelial cell proliferation rates were $10.0 \pm 3.65\%$ and $10.0 \pm 5.23\%$, respectively (Fig. 4). These data are shown with endothelial cell proliferation data at intersites from rats treated with 1.0 or 0.8 ml normal rabbit serum as a comparison (Fig. 4).

The results for microvascular density at embryo sites for rats injected with 1.0 ($n = 1$), 0.8 ($n = 2$) or 0.6 ($n = 3$) ml anti-VEGF antibody were 356.93 ± 49.02 , 384.75 ± 35.66 and 442.4 ± 17.64 vessel profiles mm^{-2} , respectively. The data for rats injected with 1.0 ($n = 5$), 0.8 ($n = 4$) or 0.6 ($n = 2$) ml normal rabbit serum were 359.55 ± 22.28 , 482.76 ± 61.49 and 516.67 ± 50.56 vessel profiles mm^{-2} , respectively. No significant differences in microvascular density at embryo sites were detected between anti-VEGF antibody and normal rabbit serum treatments.

Similarly, no significant differences in microvascular density were detected between rats treated with anti-VEGF antibody or normal rabbit serum at intersites. The results for microvascular density at intersites after treatment with 1.0 ($n = 1$), 0.8 ($n = 2$) and 0.6 ($n = 3$) ml blocking antibody were 350 ± 49.02 , 384.61 ± 71.32 and 419.95 ± 40.64 vessel profiles mm^{-2} , respectively. The results for microvascular density at intersites after treatment with 1.0 ($n = 5$), 0.8 ($n = 4$) or 0.6 ($n = 2$) ml normal rabbit serum were

405.05 ± 31.18 , 447.25 ± 82.33 and 417.82 ± 28.89 vessel profiles mm^{-2} , respectively.

Discussion

The major finding of this study was that anti-VEGF antibody reduced the endometrial vascular permeability response to implanting embryos in a dose-dependent manner. One millilitre of anti-VEGF antibody decreased the vascular permeability such that blue bands did not appear in four of five rats. As the volume of antibody was decreased, there was a gradual increase in permeability that was manifested by more blue bands appearing. This is the first clear evidence that VEGF (known formerly as vascular permeability factor (VPF) due to strong vascular permeability-inducing action) is the factor responsible for implantation-associated increases in vascular permeability in rat endometrium. This is a very convincing demonstration of the role of VEGF in increasing vascular permeability and is in accordance with other studies indicating this role of VEGF in other vascular beds (humans, pigs and rabbits: Collins *et al.*, 1993; Hippenstiel *et al.*, 1998). Although it seems most likely that the VEGF antibody is acting directly to block increases in vascular permeability, it is also possible that it could have a direct effect on embryo survival, which in turn would reduce the number of implantation sites.

The endothelial cell proliferation data from the present study are less clear than the vascular permeability result. There was relatively high endothelial cell proliferation around day 5 embryo sites, with no apparent effect of anti-

VEGF antibody. However, it should be noted that most rats (4/5) receiving 1.0 ml anti-VEGF antibody had no blue bands and, thus, the animals with the highest doses of antibody were excluded from the embryo site groups for evaluation of endothelial cell proliferation and vascular density. Earlier workers have hypothesized that embryo-initiated angiogenic mechanisms take over from maternal mechanisms at the time of implantation in rats. Macpherson and Rogers (1993) proposed that proliferative activity, including endothelial cell proliferation, increases in the endometrial stroma on day 3 of pregnancy and continues to increase on days 4 and 5. In the present study, high proliferative activity, including endothelial cell proliferation, was detected in the control (normal rabbit serum-treated) stroma on day 5 of pregnancy, which is in accordance with the results of Macpherson and Rogers (1993).

Possible explanations for the lack of difference in endothelial cell proliferation between embryo sites treated with anti-VEGF antibody and normal rabbit serum are that the embryo-controlled angiogenic mechanisms do not require VEGF or that local production of VEGF is sufficient to negate the effect of the anti-VEGF antibody concentration administered in the present study.

Another explanation for the lack of difference in endothelial cell proliferation between embryo sites treated with anti-VEGF antibody versus normal rabbit serum is that angiogenesis can be driven by multiple mechanisms with some considerable degree of redundancy between them. Thus, blocking of VEGF alone may not be sufficient to prevent endometrial angiogenesis, as other mechanisms may compensate for the reduced VEGF activity.

In contrast to endothelial cell proliferation rates at embryo sites, there is evidence that anti-VEGF antibody causes a reduction in endothelial cell proliferation at intersites. This result supports the idea that maternal regulation of endometrial angiogenesis before implantation is different from that which occurs once the embryo has implanted, and is occurring, at least in part, via VEGF.

The uteri that were treated with 1.0 and 0.8 ml antibody with no blue bands were compared with control normal rabbit serum-treated intersites and there is evidence that endothelial cell proliferation rates may be different between the two groups. Without blue bands to act as a guide, it was not possible to determine whether embryo sites and intersites existed, and thus it is unclear which control normal rabbit serum-treated group these results should be compared with.

The vascular density data from the present study show no differences in anti-VEGF antibody versus normal rabbit serum for either embryo implantation sites or intersites. The most likely explanation for this is that the dose and duration of application of anti-VEGF antibody were too limited to cause a significant effect. As it is clear from the endothelial cell proliferation data that angiogenesis is still occurring, with only intersites showing evidence for a reduction in endothelial cell proliferation rates, it seems unlikely that vascular density will be affected. Our data support the

Fig. 4. Endothelial cell proliferation in the uteri of rats injected with 1.0 (■; $n = 4$) or 0.8 (□; $n = 2$) ml anti-vascular endothelial growth factor (VEGF) antibody that subsequently had no blue bands compared with the 1.0 (■; $n = 5$) and 0.8 (□; $n = 4$) ml normal rabbit serum intersite results. Values are mean $\pm$ SEM. *A significant difference in endothelial cell proliferation was detected between the 0.8 ml anti-VEGF antibody group and the normal rabbit serum intersite group ($P < 0.05$).

finding of Macpherson and Rogers (1995) that oedema caused by the localized increase in vascular permeability at implantation sites reduces vascular density.

Rat endometrium produces a low concentration of endothelial cell migratory factor on days 3–4 of pregnancy, and this signal increases on day 5 and then decreases on days 6 and 7 of pregnancy (Abberton and Rogers, 1995). The data from the present study indicate that VEGF is present and active in rat endometrium on day 5 of pregnancy, thus raising the possibility that one candidate for this endothelial cell migratory factor could be VEGF, which has potent endothelial cell migration stimulatory activity.

In conclusion, these results indicate that VEGF is probably the main factor responsible for increased endometrial vascular permeability at implantation and that VEGF may also play a role in regulating endometrial endothelial cell proliferation before implantation and at intersites. In contrast, these data indicate that factors or mechanisms other than VEGF may regulate endothelial cell proliferation at the implantation site.

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