Effect of angiogenesis inhibitors on oestrogen-mediated endometrial endothelial cell proliferation in the ovariectomized mouse

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It has been suggested that endometrial angiogenesis in response to the sex steroids oestrogen and progesterone is mediated at a local level via compounds such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF), acting through their respective tyrosine kinase receptors. The aim of the present study was to use SUGEN tyrosine kinase receptor angiogenic inhibitor compounds SU5416, SU5402, SU11652 and SU11685, to determine whether VEGF, FGF or PDGF play a role in mediating endometrial endothelial cell proliferation after administration of oestrogen and progesterone. Endometrial endothelial cell proliferation was induced in adult ovariectomized mice by either oestrogen alone for 24 h (E1), or a regimen using oestrogen alone, then progesterone with low dose oestrogen, followed by progesterone with high-dose oestrogen (PE) over a total of 7 days. Each angiogenesis inhibitor compound was injected daily for 4 days ($100 \text{ mg kg}^{-1} \text{ day}^{-1}$, s.c.) before

endometrial tissue collection at either the E1 or PE stage. This study also evaluated the effect of VEGF antiserum (0.2 ml, i.p.) on endothelial cell proliferation at the E1 stage. All four angiogenic inhibitor compounds significantly reduced endothelial cell proliferation activity at the E1 and PE stages. The greatest reduction in the endothelial cell proliferative index was at the E1 stage in the group treated with the VEGF receptor inhibitor SU5416 ($2.5 \pm 0.7\%$ versus $27.9 \pm 1.1\%$, $P < 0.001$), with a reduction of similar magnitude in the group treated with anti-VEGF antibody. At the PE stage, all four inhibitors significantly reduced endothelial cell proliferation to a similar extent, indicating that VEGF, FGF and PDGF are all involved. These results demonstrate that endometrial angiogenesis after acute oestrogen treatment is primarily mediated by VEGF, but that under the influence of combined oestrogen and progesterone, FGF and PDGF are also probably involved.

Introduction

Angiogenesis occurs regularly in association with cyclical changes in the adult female endometrium (Rogers and Gargett, 1999; Weston and Rogers, 2000). Investigation of hormonal regulation of endometrial angiogenesis in the ovariectomized mouse has shown that endothelial cell proliferation occurs under at least two different hormonal regimens: an early, rapid response to oestrogen alone, and a longer-term response to sequential progesterone and low-dose oestrogen followed by progesterone and high-dose oestrogen (Heryanto and Rogers, 2002). Despite limited evidence that oestrogen has direct angiogenic effects on endothelial cells (Iruela-Arispe *et al.*, 1999; Razandi *et al.*, 2000), it seems probable that

the endometrial angiogenic events described above are regulated at a local level by factors other than oestrogen and progesterone.

It has been hypothesized that numerous different angiogenic factors may be involved during endometrial angiogenesis. Among these are vascular endothelial growth factor (VEGF; Torry and Torry, 1997; Rogers and Gargett, 1999; Smith, 2001), fibroblast growth factor (FGF; Ferriani *et al.*, 1993) and platelet-derived growth factor (PDGF; Smith, 1995; Weston and Rogers, 2000). Previous workers have published evidence for regulation of these angiogenic factors by ovarian steroids. For example, VEGF mRNA expression is upregulated by oestrogen in the endometrium in rats (Cullinan-Bove and Koos, 1993; Hyder *et al.*, 1996), and transfected human endometrial cells *in vitro* (Mueller *et al.*, 2000). Although the role of progesterone in VEGF production has not been as intensively investigated as that of oestrogen, there are studies indicating that progesterone increases VEGF

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protein expression (Cullinan-Bove and Koos, 1993; Greb *et al.*, 1997; Ancelin *et al.*, 2002).

The presence of FGF in the normal human endometrium and endometriotic tissues has been demonstrated and it has been hypothesized that this growth factor plays a role in the angiogenesis of normal and ectopic endometrium (Ferriani *et al.*, 1993). Oestrogen induces FGF in the primate uterus (Samathanam *et al.*, 1998) and progesterone appears to upregulate FGF expression in the endometrium in rats (Rider and Psychoyos, 1994; Rider *et al.*, 1997). Furthermore, FGF family members play an essential role in the rodent endometrium during pregnancy (Hyder and Stancel, 1999). Taken together, these studies support an assumption that FGF plays an important role in reproductive function including angiogenesis.

Immunohistochemical studies indicate that PDGF is localized in the human endometrium and PDGF receptor- β staining intensity is increased during the proliferative phase in luminal and glandular epithelial cells (Chegini *et al.*, 1992). Furthermore, *in vitro* studies have shown that PDGF promotes endometrial epithelial cell proliferation (Munson *et al.*, 1995) and plays an important role in stromal cell proliferation during endometriosis (Surrey and Halme, 1991). The distribution pattern of PDGF has been described in the mouse endometrium, with luminal and glandular epithelia showing no staining during dioestrus and pro-oestrus, weak staining during oestrus, and strong staining at days 4 and 5 of gestation (Jaber and Kan, 1998).

Many growth factors mediate their actions through specific cell surface receptors with intrinsic protein tyrosine kinase activity (Ullrich and Schlessinger, 1990). VEGF exerts its cellular functions via two tyrosine kinase receptors, VEGF-1 (fms-like tyrosine kinase 1 (Flt-1)) and VEGF-2 (fetal liver kinase 1 (Flk-1/KDR)) (Ferrara, 1999). Similarly, the biological mechanisms of FGFs are initially mediated by their binding to FGF receptors (Moller *et al.*, 2001) with intrinsic protein tyrosine kinase activity (Mohammadi *et al.*, 1996, 1997). PDGF signals through cell surface tyrosine kinase receptors, PDGFR- α and PDGFR- β , to induce various cellular activities including proliferation (George, 2001). It has been reported that PDGFR- β is upregulated in activated endothelial cells (Plate *et al.*, 1992).

A number of angiogenesis inhibitors have recently become more widely available (Folkman, 2001), including agents that have specificity for different tyrosine kinases. SU5416 blocks signal transduction through the VEGFR-2 receptor, resulting in a reduction in endothelial cell mitogenesis (Strawn *et al.*, 1996; Fong *et al.*, 1999; Geng *et al.*, 2001). SU5402 acts as an inhibitor of FGFR-1 upon co-crystallization with the catalytic domain of FGFR-1 (Mohammadi *et al.*, 1997). The aim of the present study was to determine whether sex steroid-driven endometrial angiogenesis is mediated via VEGF, FGF or PDGF, by using inhibitors to block receptor-mediated activity for

each growth factor. Our working hypothesis was that VEGF is the primary angiogenic factor responsible for steroid-driven endometrial angiogenesis.

Materials and Methods

Animals and hormonal treatments

Adult female mice (weight 20–25 g, C57 cross CBA strain) were kept under controlled conditions (20°C, 16 h light: 8 h dark per day) with food available *ad libitum*. Induction of mouse endometrial tissue regression and hormonal treatments to induce endometrial endothelial cell proliferation were performed as reported by Heryanto and Rogers (2002). Briefly, mice were ovariectomized bilaterally under anaesthesia (Avertin, 25 mg 100 g⁻¹ body weight, injected i.p., stock solution: 5 g 2,2,2-tribromoethanol (Aldrich, Milwaukee, WI) plus 5 ml butan-2-ol (BDH Chemicals, Poole); working solution: 0.25 ml stock solution plus 10 ml 0.9% (w/v) NaCl) to induce endometrial tissue regression. After 7 days, hormonal treatments were given (s.c. in 0.1 ml peanut oil) according to the following regimen: days 1–3: 100 ng oestradiol per day; days 4–6: 10 ng oestradiol and 500 μ g progesterone per day; and day 7: 100 ng oestradiol and 500 μ g progesterone. This study was approved by the Monash Medical Centre Animal Ethics Committee A.

SUGEN angiogenesis inhibitor compounds

In the present study, four pyrrole indolinone angiogenesis inhibitor compounds, SU5416, SU5402, SU11652 and SU11685, were used. SU5416 and SU5402 have been used by many laboratories as inhibitors of Flk-1/KDR and FGFR1 kinases, respectively. Their synthesis has been described by Sun *et al.* (1998, 1999). The synthetic methods used to prepare SU11652 and SU11685 are in preparation (L. Sun, personal communication). In biochemical assays, SU11652 exhibits nM potencies for inhibition of PDGFR, Flk-1/KDR and FGFR-1 (data not shown). SU11685 exhibits comparable nM potency for inhibition of PDGFR and Flk-1/KDR, but is about tenfold less potent than SU11652 at inhibiting the kinase activity of FGFR-1 (data not shown).

Angiogenesis inhibitor treatments, blood and tissue collection

The effects of angiogenesis inhibitors SU5416, SU5402, SU11652 and SU11685 on endometrial cell proliferation were investigated on day 1 of oestrogen treatment (E1 stage) and on day 7 after progesterone and high-dose oestrogen treatment (PE stage). For the first time course experiment, groups of six mice were injected s.c. (100 mg kg⁻¹ day⁻¹) with one of the four SUGEN angiogenesis inhibitors (SU5416, SU5402, SU11652 or

SU11685), or 0.9% (w/v) NaCl as a control, commencing four days after ovariectomy. The fourth inhibitor injection was combined with oestradiol on day 1 of the hormonal regimen. For the second time course experiment, groups of six mice were injected s.c. ($100 \text{ mg kg}^{-1} \text{ day}^{-1}$) with one of the four inhibitors, or 0.9% NaCl as a control, combined with daily hormonal treatment from day 4 of the hormonal regimen to day 7. The mice were injected i.p. with bromodeoxyuridine (BrdU, 40 mg kg^{-1} body weight; Sigma, St Louis, MO) 20 h after the last angiogenesis inhibitor injection for detection of cell proliferation. The mice were anaesthetized 4 h after BrdU injection and approximately 0.5 cm of one uterine horn was dissected out and embedded in optimal cutting temperature medium (Sakura Finetek, Torrance, CA) for frozen sections. Blood samples were collected by cardiac puncture from representative animals in each experimental group, and plasma from these samples was stored at -80°C until analysed for concentrations of circulating inhibitors. Mice were perfusion fixed via a cannula inserted in the left ventricle of the heart (perfusion pressure 110–130 mm Hg) using 10% buffered formalin solution (pH 7.5) after washing out with 0.9% NaCl. Pilot studies showed that careful perfusion fixation, with a saline washout at physiological pressures, clearly opened the lumen of all identifiable endometrial capillaries. High power microscopic analysis was used to identify endothelial cells lining capillaries that were positive for BrdU. The rest of the uterus was carefully dissected out, immersion fixed with the same fixative then processed for paraffin sections.

VEGF antibody treatment

A group of six mice was treated with VEGF antiserum raised in rabbit against mouse VEGF₁₆₄ to investigate further the role of VEGF in endometrial endothelial cell proliferation at the E1 stage (Gargett et al., 1999; Rabbani and Rogers, 2001). Ovariectomized mice were injected i.p. with 0.2 ml rabbit polyclonal anti-VEGF antibody on day 6 after ovariectomy, and a second injection was given with oestradiol on day 1 of the hormonal regimen. The dose was selected on the basis of a pilot study testing 0.2–0.4 ml anti-VEGF antibody. The BrdU treatment, perfusion fixation and tissue collection were performed as described above.

Immunohistochemistry

Immunohistostaining for cell proliferation detection and capillary profile identification was as described by Heryanto and Rogers (2002). After the tissue sections ($5 \mu\text{m}$) were de-paraffinized and rehydrated, they were microwaved in sodium citrate buffer for antigen retrieval. After cooling to room temperature (20°C), the sections were treated with $0.1 \text{ mol HCl l}^{-1}$ for 45 min. Endogenous peroxidase was blocked by immersing the

sections in 3% H_2O_2 in PBS and then the sections were treated with protein blocking solution. The primary antibody, sheep anti-BrdU ($10 \mu\text{g ml}^{-1}$ in 1% BSA in PBS; BioDesign, Saco, ME) was incubated for 1 h at room temperature. After washing in PBS, the sections were incubated with biotinylated secondary antibody, donkey anti-sheep ($4 \mu\text{g ml}^{-1}$ in 1% BSA in PBS; Jackson Immuno Research Lab Inc., West Grove, PA) for 1 h at room temperature. The sections were covered with streptavidin–horseradish peroxidase followed by aminoethyl carbazole, a red chromogen, and were counterstained with Mayer's haematoxylin to visualize immunostaining. A positive control section of mouse intestine was included in each staining batch. A negative control slide was prepared by substituting non-immune sheep serum for the primary antibody. The percentage of BrdU-labelled endothelial, stromal and epithelial cells in each endometrial section was calculated. Results were based on counts per section of 300–500 luminal and glandular epithelial cells, 100–200 endothelial cells and 600–800 stromal cells.

The profiles of endometrial capillaries were identified immunohistochemically and counted to evaluate vascular density. The frozen endometrial sections were immersed in 0.3% H_2O_2 in PBS for 10 min and treated with PBA for 10 min at room temperature after fixing in cold acetone (-20°C) and air-drying. The endothelial cells were immunostained with a rat monoclonal antibody against mouse CD31 ($10 \mu\text{g ml}^{-1}$ in 1% BSA in PBS; PharMingen, Los Angeles, CA) for 1 h at room temperature. A negative control slide was prepared by substituting purified rat IgG immunoglobulin isotype standard. The sections were incubated with biotinylated secondary antibody and covered with streptavidin–horseradish peroxidase. Immunoreactivity was visualized with aminoethyl carbazole. Vascular density was quantified by counting the number of capillary profiles immunostained for CD31 per mm^2 . A ratio of vascular density to stromal cell density was calculated for each treatment based on the ratio in each control group equalling one.

Plasma sample preparation and liquid chromatograph and mass spectrometric analysis

The angiogenesis inhibitor compounds were extracted from the mouse plasma sample after mixing with four parts of the internal standard in acetonitrile ($10 \text{ ng propanolol ml}^{-1}$). The supernatants were dried after centrifugation (2750 g for 10 min) under nitrogen at 40°C and reconstituted in a 1:1 mixture of mobile phases A and B (see below).

A single calibration curve, over the range 1–2000 ng of each compound per ml, was prepared in the plasma for SU5402, SU11652 and SU11685. A separate calibration curve was prepared for SU5416 over the range 2–200 ng ml^{-1} .

The samples were fractionated on a Shimadzu liquid chromatograph 10AD (Shimadzu Biotech, CA) equipped with a Perkin-Elmer 200 auto-sampler (Perkin-Elmer, CT). For SU5402, SU11652 and SU11685, the samples were fractionated (0.8 ml min^{-1}) on a Keystone Scientific BDS Hypersil C-18 column ($50 \mu\text{m}$, $150 \text{ mm} \times 3 \text{ mm}$; Thermo Hypersil-Keystone, PA) at ambient temperature using 90% water, 10% acetonitrile and 0.7% formic acid as mobile phase A, and 10% water, 90% acetonitrile and 0.7% formic acid for mobile phase B. The liquid chromatograph gradient on injection changed from 60:40 A:B to 10:90 over the first 1.5 min. After holding at 10:90 A:B for 0.1 min, the gradient was reversed to 60:40 over 1.9 min, and held for an additional 0.1 min. Tandem mass spectrometric analysis was performed on a Sciex API 365 mass spectrometer (MDS Sciex, Ontario). The limits of quantitation for SU5402, SU11652 and SU11685 were 10, 1 and 2 ng ml^{-1} , respectively.

The samples were fractionated (0.8 ml min^{-1}) for SU5416 on a Keystone Scientific BDS Hypersil C-18 column ($50 \mu\text{m}$, $50 \text{ mm} \times 4.6 \text{ mm}$) at ambient temperature using 90% water, 10% acetonitrile 10 mmol ammonium acetate l^{-1} and 0.7% formic acid as mobile phase A, and 10% water, 90% acetonitrile, 10 mmol ammonium acetate l^{-1} and 0.7% formic acid for mobile phase B. The mobile phase on injection was held at 70:30 A:B for 1.2 min, and then changed from 70:30 A:B to 20:80 over 1 min. After holding at 20:80 A:B for 1.5 min, the gradient was reversed to 70:30 over 0.1 min, and held for an additional 1.5 min. Tandem mass spectrometric analysis was performed on a Sciex API 3000 mass spectrometer. The limit of quantitation for SU5416 was 2 ng ml^{-1} .

Statistical analyses

Values are presented as mean $\pm$ SE. The effect of treatment was evaluated by one-way ANOVA followed by Newman-Keuls multiple comparison tests. A P value < 0.05 was considered significant.

Results

Effects of angiogenesis inhibitor compounds on endometrial cell proliferation after 24 h oestrogen stimulation

BrdU immunostaining from stromal areas during the E1 stage is shown (Fig. 1). Proliferating endothelial cells show red nuclear and peri-nuclear immunostaining. Endothelial cell proliferation was increased in the control group (Fig. 1a) and showed the greatest reduction in the group treated with SU5416 (Fig. 1b). Statistical analysis demonstrated that all the angiogenesis inhibitor compounds (SU5416, SU5402, SU11652 and SU11685) significantly reduced the number of BrdU-labelled endothelial cells during the E1 stage (Fig. 2a).

Fig. 1. (a) Section of endometrium immunostained to demonstrate bromodeoxyuridine (BrdU)-labelled cells from a mouse treated with oestrogen. Many endothelial cells are labelled (arrows). (b) Section of endometrium immunostained with anti-BrdU from a mouse treated with oestrogen and SU5416. Endothelial cell labelling is significantly lower than in (a). C: capillary; LE: luminal epithelium. Scale bars represent 50 μm .

The angiogenesis inhibitor compounds also showed some effect on the luminal epithelial cells. Proliferative activity was significantly decreased in the group treated with SU5402 ($P < 0.05$) and appeared reduced in the group treated with SU5416. There was no significant change in the groups treated with SU11652 or SU11685 (Fig. 2b).

Proliferative activity in glandular epithelial and stromal cells was low and undetectable, respectively. There was no significant difference in the glandular epithelial cell-labelling index between the groups (Fig. 2c).

Effect of angiogenesis inhibitor compounds on vascular and stromal cell density after 24 h oestrogen stimulation

The effects of angiogenesis inhibitor compounds on vascular and stromal cell density at the E1 stage are summarized (Fig. 3a,b). Although there was no significant difference between these groups, there was a tendency for the number of capillaries and stromal cells per mm^2

Fig. 2. Endometrial bromodeoxyuridine (BrdU)-labelling indices (%) for (a) endothelium, (b) luminal epithelium and (c) glandular epithelium in mice treated with the angiogenesis inhibitor compounds SU5416, SU5420, SU11652 and SU11685. Columns with different superscripts are significantly different from each other ($a-c P < 0.05$; $*P < 0.01$; and $**P < 0.001$); columns with the same superscripts are not significantly different from each other. Values are mean $\pm$ SE ($n = 6$ mice per group). E1: day 1 of oestrogen treatment.

to be slightly higher in the groups treated with SU5416, SU5420 or SU11685 compared with the control group. There was no significant change in the ratio of vascular density to stromal cell density with any treatment (Fig. 3c).

Effect of angiogenesis inhibitor compounds on endometrial cell proliferation after treatment for 7 days with a combined oestrogen and progesterone protocol

All four of the angiogenesis inhibitor compounds significantly reduced endothelial cell proliferative activity

Fig. 3. Quantitative evaluation of endometrial (a) vascular and (b) stromal cell density, and (c) the ratio of vascular density to stromal cell density in mice after treatment with oestrogen and different angiogenesis inhibitors (all values have been proportionally adjusted so that the ratio of vascular to stromal cell density at E1 = 1). Values are mean $\pm$ SE ($n = 6$ mice per group). E1: day 1 of oestrogen treatment. There are no significant differences among the groups.

at the PE stage ($P < 0.01$). In contrast to the result at E1, there were no differences in the extent of reduction of the BrdU-labelling index between the four treated groups at the PE stage (Fig. 4a).

The angiogenesis inhibitor compounds significantly reduced the proliferative activity in the luminal epithelium in groups treated with SU5416, SU5420, SU11652 ($P < 0.01$) and SU11685 ($P < 0.05$) compared with the control group. There was no significant difference in the BrdU-labelling index of the luminal epithelium among the four treated groups (Fig. 4b).

Proliferative activity in the glandular epithelium was low at the PE stage in all of the groups (data not shown).

Fig. 4. Endometrial bromodeoxyuridine (BrdU)-labelling indices (%) in (a) endothelium, (b) luminal epithelium and (c) stromal cells in mice after treatment with progesterone and oestrogen plus different angiogenesis inhibitors. Columns with different superscripts are significantly different from each other (^{a,b} $P < 0.05$ and $*P < 0.01$); columns with the same superscripts are not significantly different from each other. Values are mean $\pm$ SE ($n = 6$ mice per group). PE: day of progesterone and high-dose oestrogen treatment.

Some proliferative activity occurred in stromal cells at the PE stage; however, there was no significant difference in the BrdU-labelling index among the inhibitor-treated groups (Fig. 4c).

Effect of angiogenesis inhibitor compounds on vascular and stromal cell density after treatment for 7 days with a combined oestrogen and progesterone protocol

There was no significant difference in vascular and stromal cell density between control groups and groups treated at the PE stage. There was a tendency for blood

Fig. 5. Quantitative evaluation of endometrial (a) vascular and (b) stromal cell density, and (c) the ratio of vascular density to stromal cell density in mice after treatment with different angiogenesis inhibitors (all values have been proportionally adjusted so that the ratio of vascular to stromal cell density at PE = 1). Values are mean $\pm$ SE ($n = 6$ mice per group). PE: day of progesterone and high-dose oestrogen treatment. There are no significant differences among the groups.

vessel density (BVD) to be slightly increased in groups treated with SU11652 and SU11685 (Fig. 5a). The stromal cell density increased slightly in all the treated groups compared with the control group (Fig. 5b). Apart from the group treated with SU11685, the angiogenesis inhibitor treatments tended to reduce the ratio of BVD to stromal cell density slightly at the PE stage (Fig. 5c).

Effect of VEGF antibody treatment on endothelial cell proliferation and vascular and stromal cell density

Treatment with VEGF antiserum significantly reduced endothelial cell proliferative activity compared with

Fig. 6. Endothelial bromodeoxyuridine (BrdU)-labelling indices (%) in the mouse endometrium at the E1 stage either untreated or treated with VEGF antibody or SU5416. Columns with different superscripts are significantly different from each other (** $P < 0.001$); columns with the same superscripts are not significantly different from each other. Values are mean $\pm$ SE ($n = 6$ mice per group). E1: day 1 of oestrogen treatment.

Table 1. Individual concentrations of four SUGEN angiogenesis inhibitor compounds (g ml^{-1}) in a limited number of mouse plasma samples demonstrating typical values achieved

Stage	SU5416	SU5402	SU11652	SU11685
E1	23.6	< 10	94.6	821
	80.4	< 10	45.9	2650
PE	27.4	< 10	56.4	761
	39.4			1380

E1: day 1 of oestrogen treatment; PE: day of progesterone and high-dose oestrogen treatment.

control ($P < 0.001$), resulting in activity similar to that seen in the group treated with SU5416 (Fig. 6). There was no significant effect of VEGF antiserum on BVD compared with control, as was seen with the SU5416 group (Fig. 7a). However, stromal cell density in the group treated with VEGF antiserum was significantly higher than control or the SU5416 group (Fig. 7b). As a consequence, the ratio of BVD to stromal cell density was lower in the group treated with VEGF antiserum than in the control or SU5416-treated group.

Identification of inhibitor compounds in plasma

Concentrations of each inhibitor compound as assayed in a limited number of plasma samples are presented (Table 1). There was no apparent difference in the concentration of each compound between the E1 and PE stages.

Discussion

The results from the present study provide the first mechanistic evidence identifying an individual angiogenic factor by which the sex steroid oestrogen drives endometrial angiogenesis. This study has shown

Fig. 7. Quantitative evaluation of (a) vascular and (b) stromal cell density, and (c) the ratio of vascular density to stromal cell density in the mouse endometrium at the E1 stage either untreated or treated with anti-VEGF antibody or SU5416 (all values have been proportionally adjusted so that the ratio of vascular to stromal cell density at E1 = 1). Columns with different superscripts are significantly different from each other (^{a,b} $P < 0.05$; * $P < 0.01$ and ** $P < 0.001$); columns with the same superscripts are not significantly different from each other. Values are mean $\pm$ SE ($n = 6$ mice per group). E1: day 1 of oestrogen treatment.

that SU5416, which inhibits signal transduction through VEGFR-2, and VEGF-antiserum, both almost completely eliminate endometrial endothelial cell proliferation 24 h after oestrogen treatment. These data demonstrate that the initial angiogenic activity of oestrogen on endometrium is primarily mediated via VEGF. The results of the present study do not provide any information on whether the actions of VEGF in the endometrium occur primarily through VEGFR-2, or whether activation of VEGFR-1 too plays a role.

There are several possible explanations for the observation that SU11652 and SU11685, which are more potent inhibitors of VEGFR-2 than SU5416 and VEGF-antiserum, did not completely inhibit the endothelial cell proliferative activity at the E1 stage. Sub-optimal bioavailability or distribution upon subcutaneous administration is one possible explanation. However, the plasma concentrations of SU11652 and SU11685 are equal to or greater than that of SU5416, indicating that this is not likely to be the sole explanation. Another contributing factor may be the unusual property of SU5416 to accumulate inside the endothelial cells (Mendel *et al.*, 2000). This intracellular accumulation raises the possibility that plasma concentrations of SU5416 underestimate the local concentration of the compound in the cell, thereby conferring additional apparent potency and a longer duration of action to SU5416.

Administration of each of the angiogenic inhibitor compounds over the 4 days before and during the PE stage significantly inhibited endometrial endothelial cell proliferative activity. Inhibition of the endometrial endothelial cell proliferation index at the PE stage was comparable for all four compounds, with none demonstrating the profound inhibition observed with SU5416 at E1. This observation indicates that VEGF is not the only angiogenic factor driving endothelial cell proliferation after exposure to progesterone and oestrogen. It has been reported that several growth factors including VEGF (Rabbani and Rogers, 2001), FGF (Carlone and Rider, 1993) and PDGF (Rodríguez-Martínez *et al.*, 1992) have important roles in endometrial angiogenesis during early pregnancy. It would thus not be unexpected that inhibitors of each of these angiogenic factors decrease endothelial cell proliferation at this stage. However, the apparent poor bioavailability of SU11652 and SU11685 upon subcutaneous administration makes it impossible to determine the contribution these other growth factors may be making, or if there might be additional angiogenic factors contributing to the mitogenic response of endothelial cells at the PE stage. Nevertheless, the clear difference in response to SU5416 between the E1 and PE stages demonstrates that additional factors contribute to the regulation of angiogenesis under these two different sex steroid regimens.

The present data indicate that administration of SU5402 reduces luminal epithelial cell proliferative activity at the E1 stage. It is also possible that SU5416 inhibited luminal epithelial cell proliferation at the E1 stage, although statistical analysis does not show this effect to be significant. The other inhibitor compounds, including anti-VEGF antibody (data not shown), did not have any effect in reducing luminal epithelial cell proliferation at the E1 stage.

It has been reported that basic FGF (bFGF) is localized in the basal lamina of the glandular and surface epithelial layer of the mouse endometrium (Wordinger *et al.*, 1992). Oestrogen induces luminal epithelial cell proliferation

in the ovariectomized mouse at the E1 stage (Heryanto and Rogers, 2002), and other studies have shown that oestrogen rapidly stimulates bFGF mRNA in the endometrial tissue of ovariectomized rats (Rider *et al.*, 1997). These observations may help to explain why blocking FGFR tyrosine kinase activity with SU5402 inhibits luminal epithelial proliferation at this stage.

Each of the angiogenic inhibitor compounds reduced luminal epithelial cell proliferative activity at the PE stage, as was also the case for endometrial endothelial cell proliferative activity. These results indicate that VEGF, FGF and PDGF may play a role in controlling luminal epithelial cell proliferation. Previous workers have reported a role for each of these growth factors during preparation of the luminal epithelium for implantation and placentation. bFGF increases luminal epithelial cell proliferation in early pregnant rats (Rider and Psychoyos, 1994; Srivastava *et al.*, 1998), and PDGF has an important role in luminal cell proliferative activity during early pregnancy in mice (Jaber and Kan, 1998). It has also been reported that VEGF mRNA accumulates in the luminal epithelial cells of the endometrium in mice during days 1 and 2 of pregnancy (Chakraborty *et al.*, 1995; Halder *et al.*, 2000).

The angiogenic inhibitor compounds did not significantly influence vascular and stromal cell density or the ratio of vascular density to stromal cell density at either the E1 or PE stage. Interpretation of this finding is not straightforward because of the different factors that govern endometrial BVD. It is important to remember that endometrial regression results in an increase in BVD, and that sex steroid treatment reduces absolute BVD, at least in part by increasing tissue oedema (Heryanto and Rogers, 2002). However, tissue oedema and increases in stromal cell size significantly reduce stromal cell density after oestrogen treatment, resulting in an increase in BVD when it is measured as a ratio of stromal cell density rather than per unit area. In the present study, there were no significant changes in BVD or stromal cell density at either the E1 or PE stage. This finding indicates that sex steroid effects on stromal cell growth and tissue oedema were basically unaffected by the angiogenesis inhibitors at the doses used. In contrast, the VEGF antiserum significantly increased stromal cell density, indicating that in contrast to the angiogenesis inhibitors, it was reducing endometrial vascular permeability by blocking VEGF action. The lack of any significant changes in absolute BVD throughout the inhibitor experiments indicates that this may not be a sensitive measure of angiogenesis, presumably because of the major influences that growth and regression of the surrounding tissue can have on it.

The present study found no significant effect of angiogenic inhibitor compounds on glandular epithelial or stromal cell proliferative activity. These results indicate that VEGF, FGF and PDGF are not the primary factors promoting glandular epithelial and stromal cell proliferation in the endometrium.

The results from the current study indicate that several growth factors including VEGF, FGF and PDGF contribute to endometrial endothelial cell proliferation in response to oestrogen and progesterone. Understanding of this complementarity, or redundancy, of growth factors may be more effectively achieved by studies using different combinations of inhibitor compounds.

The present study also demonstrated wide variability in plasma concentration of inhibitors, with the average concentration of SU5402 being less than 10 ng ml⁻¹, whereas the concentration of SU11685 was more than 1000 ng ml⁻¹. It has been shown that a single exposure of SU5416 has a duration of inhibition for proliferation of human umbilical vein endothelial cells of less than 24 h (Fong *et al.*, 1999). However, it appears that a once-a-day dosing regimen is efficacious for SU5416 because this compound has long-lasting effects (Mendel *et al.*, 2000). Further studies are required to determine whether different dose rates or routes of administration would result in different plasma concentrations and give altered endothelial cell proliferation results.

In conclusion, this study has demonstrated that angiogenesis inhibitor compounds reduce endothelial cell proliferation under two different sex steroid-driven episodes of endometrial angiogenesis. The response of the endometrial endothelial cells was different at each stage, indicating that the factors that regulate angiogenesis under these two different episodes are different.

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