Mid-luteal angiogenesis and function in the primate is dependent on vascular endothelial growth factor

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Abstract

Vascular endothelial growth factor (VEGF) is essential for the angiogenesis required for the formation of the corpus luteum; however, its role in ongoing luteal angiogenesis and in the maintenance of the established vascular network is unknown. The aim of this study was to determine whether VEGF inhibition could intervene in ongoing luteal angiogenesis using immunoneutralisation of VEGF starting in the mid-luteal phase. In addition, the effects on endothelial cell survival and the recruitment of peri-endothelial support cells were examined. Treatment with a monoclonal antibody to VEGF, or mouse gamma globulin for control animals, commenced on day 7 after ovulation and continued for 3 days. Bromodeoxyuridine (BrdU), used to label proliferating cells to obtain a proliferation index, was administered one hour before collecting ovaries from control and treated animals. Ovarian sections were stained using antibodies to BrdU, the endothelial cell marker, CD31, the pericyte marker, alpha-smooth muscle actin, and 3′ end DNA fragments as a marker for apoptosis. VEGF immunoneutralisation significantly suppressed endothelial cell proliferation and the area occupied by endothelial cells while increasing pericyte coverage and the incidence of endothelial cell apoptosis. Luteal function was markedly compromised by anti-VEGF treatment as judged by a 50% reduction in plasma progesterone concentration. It is concluded that ongoing angiogenesis in the mid-luteal phase is primarily driven by VEGF, and that a proportion of endothelial cells of the mid-luteal phase vasculature are dependent on VEGF support.

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Introduction

Formation of the primate corpus luteum (CL) is accompanied by prolific angiogenesis in the early luteal phase, and throughout the life span of the CL angiogenesis continues at a lower rate until the late luteal phase when endothelial cell proliferation decreases further (Jablonka-Shariff *et al.* 1993, Rodger *et al.* 1997, Dickson & Fraser 2000). The angiogenic process is regulated by a number of growth factors, degradation of the extracellular matrix and cell–cell interactions. One growth factor primarily involved is vascular endothelial growth factor (VEGF).

The importance of VEGF in the onset of luteal angiogenesis has been demonstrated by specific VEGF neutralisation in the rat (Ferrara *et al.* 1998) and the marmoset monkey (Fraser *et al.* 2000) which resulted in suppression of endothelial cell proliferation, restricted development of the microvascular tree and decreased progesterone production. These studies have solely addressed *prevention* of angiogenesis by targeting the early luteal phase just before the onset of angiogenesis (see Fig. 1) (Dickson & Fraser 2000, Fraser *et al.* 2000). Surprisingly, while immunoneutralisation of VEGF for the first 3 days of the luteal phase markedly suppressed angiogenesis, extending treatment to 10 days post ovulation failed to affect mid-luteal cell proliferation (Fraser *et al.* 2000). It is possible that endothelial cell proliferation was unaffected at this stage as a result of (1) induction of a compensatory effect from other growth factors after chronic inhibition of VEGF, (2) an immune response against the mouse monoclonal antibody or (3) the possibility that VEGF was not involved in stimulating endothelial cell proliferation during this period. In the light of this observation the current study investigated the role of VEGF specifically during the mid-luteal phase to determine whether VEGF inhibition would *intervene* in ongoing luteal angiogenesis.

First, using quantitative immunocytochemistry we established that VEGF was present in high amounts during the mid-luteal phase. We then investigated whether it was possible to intervene in the already established angiogenic process by administering anti-VEGF treatment for 3 days in the mid-luteal phase and examining the effects on angiogenesis and luteal function.

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Previous studies on the effects of withdrawal of VEGF in other angiogenic-dependent systems report the requirement of VEGF for the survival of immature endothelial cells. For example, in human prostate cancer the loss of VEGF as a result of androgen ablation therapy leads to selective apoptosis of endothelial cells in vessels devoid of periendothelial support cells (Benjamin *et al.* 1999). To investigate whether endothelial cells of the primate CL may be susceptible to withdrawal of VEGF, we determined the incidence of apoptosis after anti-VEGF treatment. Finally, to assess the relationship of periendothelial support cells to endothelial cell survival, the area of pericyte coverage in control CL and after anti-VEGF treatment was quantified.

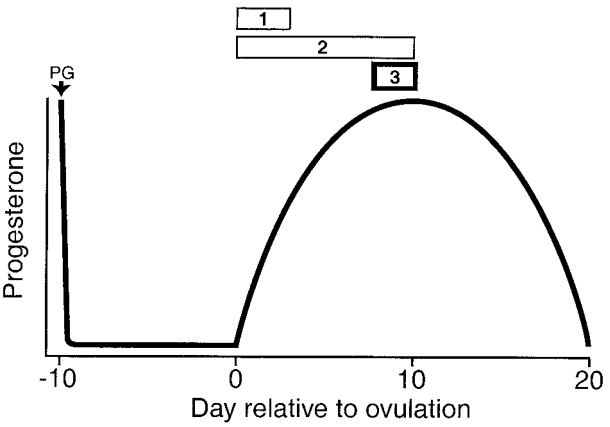

Figure 1 Schematic diagram showing the different treatment regimes (1, 2 and 3) with VEGF monoclonal antibody designed to determine whether inhibition of angiogenic factors can *prevent* angiogenesis (regimes 1 and 2) or intervene once the process is underway (regime 3). The approach used in the current study was to *intervene* in the already established angiogenic process by administering a 3-day treatment with VEGF monoclonal antibody in the mid-luteal phase according to regime 3. PG, prostaglandin $F_{2\alpha}$ analogue.

Materials and Methods

Animals and treatments

Marmoset monkeys (*Calithrix jacchus*) were housed as described previously (Fraser *et al.* 1999*a*) and procedures were carried out in accordance with the Animals (Scientific Procedures) Act 1986. Blood samples were collected by femoral venepuncture three times per week without anaesthesia. Ovulatory cycles were monitored by radioimmunoassay of plasma progesterone as previously described (Smith *et al.* 1990). The day of ovulation (day 0 of the luteal phase) was taken as the day on which progesterone concentration rose above 30 nmol/l when followed by a sustained increase, characteristic of the luteal phase.

Ovaries collected from animals in the early (luteal days 2–4), mid- (days 8–10) and late (days 16–20) luteal phase of the ovulatory cycle ($n=4$–5 animals per group) for previous studies (Fraser *et al.* 1999*a*,*b*, Dickson & Fraser 2000) were used for immunocytochemical localisation and quantification of VEGF protein.

The mid-luteal phase was targeted for anti-VEGF treatment to determine whether luteal angiogenesis could be prevented at the stage when high levels of VEGF are present, as seen by VEGF immunolocalisation throughout the luteal phase of the marmoset ovulatory cycle.

Animals were given 1 μg prostaglandin $F_{2\alpha}$ analogue (Planate; Coopers Animal Health Ltd, Crewe, Cheshire, UK) i.m. in the mid- to late luteal phase of the pre-treatment cycle to induce luteolysis and to synchronise subsequent ovulation which was presumed to occur 10 days after prostaglandin treatment (luteal day 0) (Fig. 1) (Summers *et al.* 1985). Blood samples were collected every second day, then daily from the first day of treatment. The properties of the VEGF monoclonal antibody have been described previously (Fraser *et al.* 2000). Four animals were subsequently treated with 2 mg VEGF monoclonal antibody on luteal day 7 and 1 mg on luteal days 8 and 9, and four control animals were given equivalent doses of mouse gamma globulin (Fig. 1). Ovaries were collected on day 10 as previously described by Fraser *et al.* (1998), 1 h after administration of 20 mg bromodeoxyuridine (BrdU; Boehringer Mannheim, Lewes, East Sussex, UK) dissolved in 500 μl physiological saline, to label proliferating cells in the S phase of the cell cycle. Ovaries were fixed immediately in 4% paraformaldehyde in 0·01 M PBS (phosphate-buffered saline, pH 7·4, containing 2·7 mM KCl, 0·137 M NaCl) for paraffin-embedding.

Immunocytochemistry

Paraffin-embedded ovarian sections (5 μm) were mounted onto TESPA- (Sigma, Poole, Dorset, UK) coated glass slides and dried at 50 °C overnight. To carry out immunocytochemistry for VEGF, sections were dewaxed in Histoclear (National Diagnostics, Aylesbury, Bucks, UK), rehydrated in descending concentrations of industrial methylated spirits and washed in distilled water. VEGF antigen was retrieved by pressure cooking slides on full power in 3 M glycine, 0·1% EDTA buffer, pH 3·5, for 5 min. The slides remained in hot buffer for a further 20 min and were washed in TBS (0·05 M Tris–buffered saline, pH 7·4, containing 50 mM Tris–HCl, 150 mM NaCl). Endogenous peroxidase activity was quenched with a 30-min incubation in 3% hydrogen peroxide in methanol at room temperature. The following procedures, with the exception of immunostaining development, were carried out in Sequenza racks (Shandon Scientific Ltd, Runcorn, Cheshire, UK). To block endogenous biotin, sections were incubated in avidin (Vector Laboratories, Burlingame, CA, USA) at a concentration of 8 drops/ml normal swine serum (NSS, 1:5 dilution in TBS+0·25 g bovine serum albumin), followed by a 20-min incubation

in biotin (Vector Laboratories) in TBS (8 drops/ml). Rabbit polyclonal VEGF antibody (2 μg/ml pre-diluted in NSS, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added and sections were incubated at 4 °C overnight. Negative controls were incubated in primary antibody pre-absorbed with VEGF peptide in 1:5 ratio of VEGF antibody:blocking peptide (Santa Cruz). Immunolocalisation was undertaken using the rabbit EnVision kit (Dako Ltd, Cambridge, Cambs, UK) according to the guidelines of the manufacturer.

BrdU immunocytochemistry was carried out using a monoclonal antibody (Boehringer Mannheim) as previously described (Fraser *et al.* 2000). Immunocytochemistry for the endothelial cell marker, CD31 (Dako Ltd), and the pericyte marker, alpha-smooth muscle actin (SMA; Dako Ltd), were performed using monoclonal antibodies (20·5 μg/ml in TBS and 4·3 μg/ml in TBS respectively) and the same method as for BrdU immunostaining.

Apoptosis was detected by 3′ end labelling as previously described in the marmoset CL (Young *et al.* 1997), with modifications according to Sharpe *et al.* (1998). An additional proteinase K digestion step was performed after dewaxing and rehydrating the sections. Slides were incubated for 6 min at room temperature in 20 μg/ml proteinase K in buffer containing 0·05 M Tris, pH 8, 0·05 M EDTA, pH 8, in distilled H_2O. After blocking of endogenous peroxidase activity, it was necessary to block endogenous biotin using the avidin-biotin block in PBS as above.

Analysis

A BrdU proliferation index (PI) was obtained as previously described (Dickson & Fraser 2000). Endothelial cell and pericyte areas were quantified in at least 6 randomly chosen areas of $5{\cdot}3 \times 10^4$ μm^2 (approximately two thirds of each CL) as described by Fraser *et al.* (2000) for the endothelial cell marker, Factor VIII. The area of VEGF immunostaining was measured using Photoshop version 5·0 according to the method of Otani *et al.* (1999). Statistical analysis of VEGF immunostaining throughout

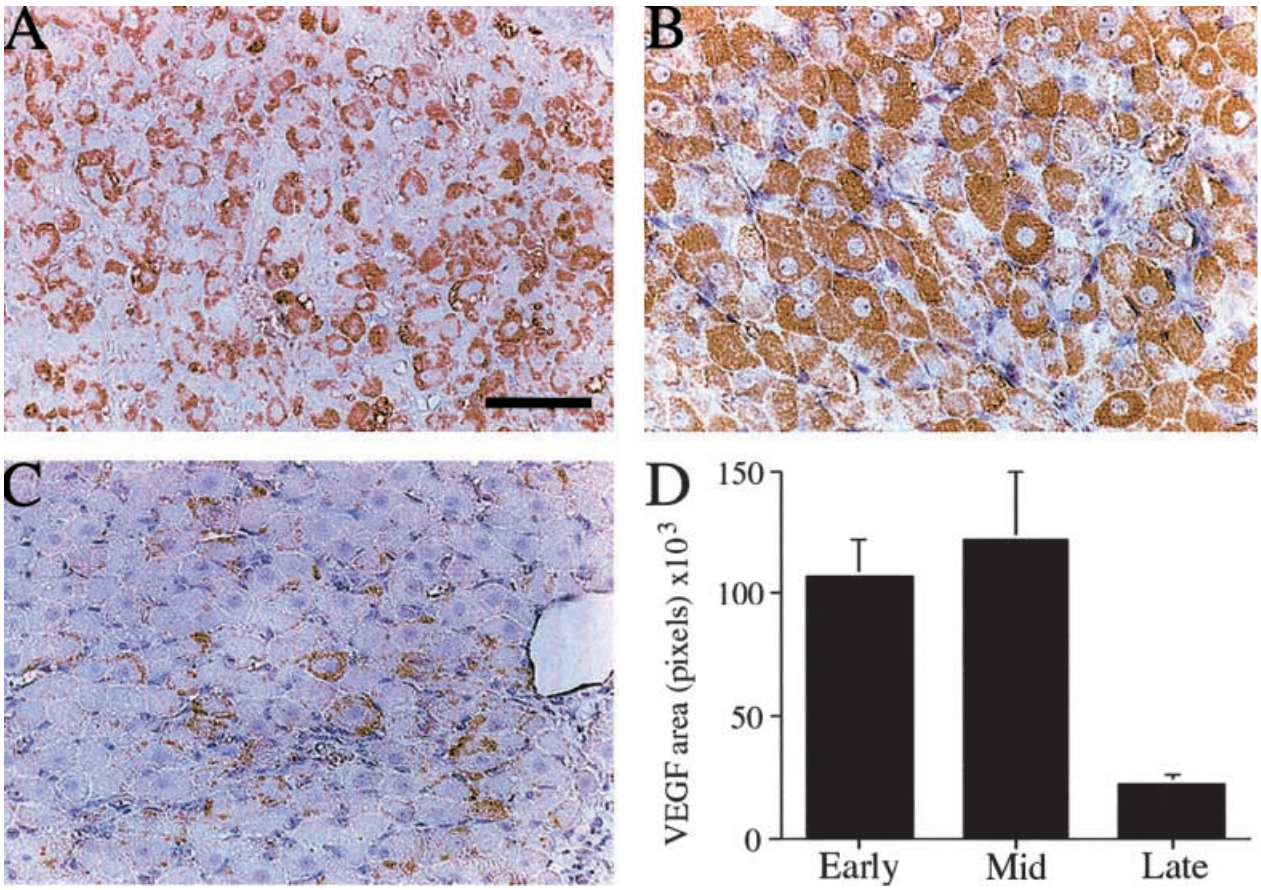

Figure 2 Photomicrographs of marmoset corpora lutea showing VEGF localisation (brown staining cytoplasm) in (A) an early luteal phase corpus luteum, (B) a mid-luteal phase corpus luteum and (C) a late luteal phase corpus luteum. Bar represents 50 μm. (D) Quantification of the area of VEGF immunostaining in the marmoset corpus luteum throughout the luteal phase of the ovulatory cycle. Early and mid-luteal values are significantly higher ($P=0{\cdot}04$ and $P=0{\cdot}02$ respectively) than the late luteal value. Data are means ± S.E.M.

the ovulatory cycle was carried out using a factorial one-way analysis of variance (ANOVA) with Fisher's PLSD (protected least significant difference) post-hoc test at 5% significance. The effects of anti-VEGF treatment on PI, endothelial cell and pericyte areas, apoptotic index and VEGF immunostaining, as compared with controls, were determined using separate two-tailed, unpaired *t*-tests, with 95% confidence intervals. Serum progesterone concentrations were analysed using a repeated measures ANOVA with Fisher's PLSD post-hoc test at 5% significance. All tests were performed using Statview version 4·0.

Results

VEGF immunocytochemistry throughout the cycle

Figure 2 illustrates the localisation and quantification of VEGF immunostaining in the CL during the early, mid- and late luteal phase. Early luteal sections show intense, punctate VEGF staining in the cytoplasm of lutein cells (Fig. 2A) which becomes more uniform and widespread in the mid-luteal phase CL (Fig. 2B). Staining was absent from recognisable endothelial cells. In the late luteal phase CL, VEGF staining was markedly reduced (Fig. 2C). Quantification confirmed a high area of staining in the early and mid-luteal phase which declined markedly in the late luteal phase CL, as compared with early and mid-luteal levels ($P=0·04$ and $P=0·02$ respectively) (Fig. 2D).

BrdU, CD31 and SMA immunocytochemistry

Figure 3 shows comparisons of BrdU incorporation, CD31 and SMA immunostaining, and their quantification in mid-luteal control and anti-VEGF-treated CL. Moderate BrdU incorporation into endothelial cells was observed in control CL (Fig. 3A) but this was significantly lowered by anti-VEGF treatment (Fig. 3B). This was confirmed by comparing the PI from control and treated groups ($P<0·01$) (Fig. 3C). In the control CL, CD31 immunostaining confirmed the establishment of the microvascular tree demonstrated by numerous blood vessels and capillary endothelial cells being observed in association with each lutein cell (Fig. 3D). In the treated animals, the number of capillaries appeared reduced (Fig. 3E) and quantitative analysis revealed a significant ($P=0·02$) decrease in endothelial cell area after treatment (Fig. 3F). The converse was apparent for SMA immunostaining. In control sections, pericytes were mostly found aggregated in rings surrounding luminal vessels (Fig. 3G), whereas after anti-VEGF treatment single immunopositive cells were also found distributed through the CL (Fig. 3H). Quantification of pericyte area in control sections and after anti-VEGF treatment is shown in Fig. 3I ($P<0·01$).

Apoptotic cell death

Figure 4 demonstrates 3′ end labelling in mid-luteal control and treated sections. One positive apoptotic endothelial cell associated with a blood vessel can be seen in Fig. 4A. The incidence of apoptotic nuclei increased with anti-VEGF treatment as seen in Fig. 4B which shows two positive nuclei probably of endothelial cell origin. Positive apoptotic nuclei remained sparsely distributed, even though anti-VEGF treatment significantly ($P=0·03$) increased the occurrence of apoptosis (Fig. 4C). The incidence of apoptotic cells was measured per CL section as the size of the control and treated CL did not differ significantly. The regular shape of the steroidogenic lutein cells in control CL was retained in the treated CL.

Plasma progesterone concentration

A sustained elevation of plasma progesterone was observed prior to treatment in all animals. After administration of anti-VEGF treatment starting on luteal day 7 there was a marked reduction in plasma progesterone concentrations which had fallen by over 50% by day 10 (Fig. 5) ($P=0·01$).

Discussion

This study has demonstrated for the first time that VEGF is essential for luteal angiogenesis even when the process is already established. Furthermore, inhibition of VEGF at this time suppresses the function of the CL. We have also demonstrated that VEGF appears to have a role in maintenance of the vascular network in the mid-luteal CL as shown by an increase in 3′ end labelling after VEGF withdrawal.

Our findings also show that the expression of VEGF is high not only in the early luteal period of intense angiogenesis, but also in the less prolific mid-luteal phase CL, and is only down regulated after initiation of luteolysis. This agrees with VEGF measurements throughout the life span of the CL in the bovine (Goede *et al.* 1998) and the human CL (Otani *et al.* 1999). In the present study, the deleterious effect of acute removal of VEGF on mid-luteal angiogenesis shows that ongoing mid-luteal angiogenesis is driven primarily by this growth factor. This indicates that the failure to observe an effect on mid-luteal endothelial cell proliferation after chronic withdrawal of VEGF treatment from the time of ovulation (Fraser *et al.* 2000) is a consequence of induction of other growth factors to compensate for the inhibition of VEGF or inactivation of the neutralising antibody by an immune response.

The high mid-luteal expression of VEGF could also reflect some of the non-angiogenic functions of VEGF, such as the regulation of vascular permeability or the mediation of endothelial cell survival as suggested by Goede *et al.* (1998). By the mid-luteal phase, the vasculature is becoming highly developed and progesterone is being maximally secreted. If the effect of anti-VEGF was solely to suppress angiogenesis it might be anticipated that progesterone would be maintained at pretreatment levels,

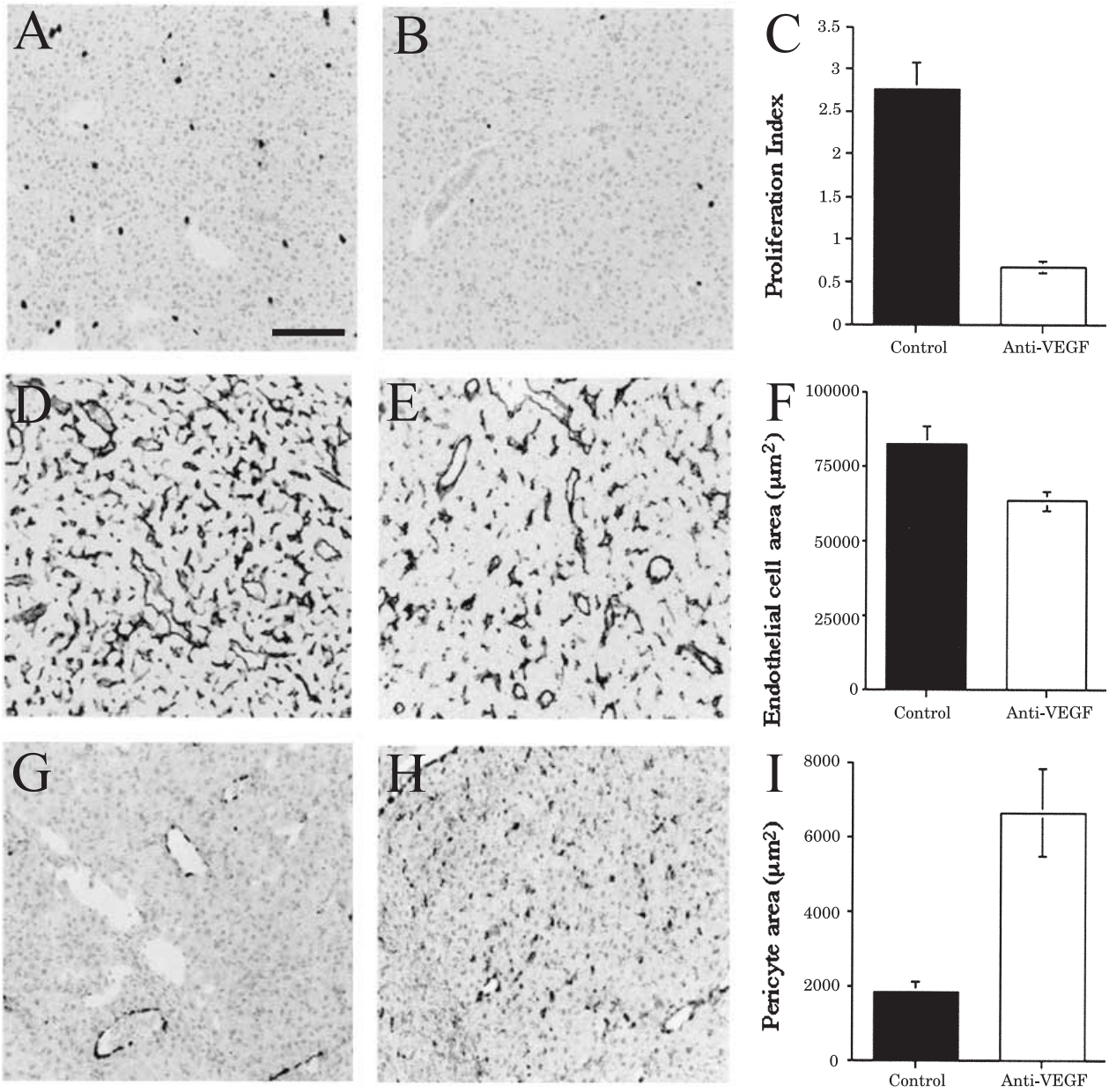

Figure 3 Low power photomicrographs of marmoset corpora lutea showing the general distribution of endothelial cell BrdU incorporation (dark-staining nuclei) in a control section (A) and its reduced incorporation after anti-VEGF treatment (B). (C) The proliferation index in corpora lutea from mid-luteal phase controls (solid bar) and anti-VEGF-treated (open bar) marmoset corpora lutea. Values from treated animals were significantly lower ($P<0{\cdot}01$) than those from controls. (D) CD31 localisation (dark staining) in the endothelial cells of the vascular network in a mid-luteal phase control corpus luteum. (E) Reduced CD31 staining after anti-VEGF treatment. (F) Quantification of CD31 immunostaining in control (solid bar) and anti-VEGF-treated (open bar) marmoset corpora lutea. Values from treated animals were significantly lower ($P=0{\cdot}02$) than those from controls. (G) Smooth muscle actin localisation in pericytes in a control section, and (H) increased smooth muscle actin immunostaining after anti-VEGF treatment. Note that the dark stained cells in control and treated sections are characteristic of perivascular cells, based on the small size and elongated shape; none appears to be of lutein cell origin. (I) Quantification of alpha-smooth muscle staining in control (solid bar) and anti-VEGF-treated (open bar); values from treated animals were significantly ($P=<0{\cdot}01$) higher than those from controls. Data are means ± S.E.M. Bar represents 100 μm.

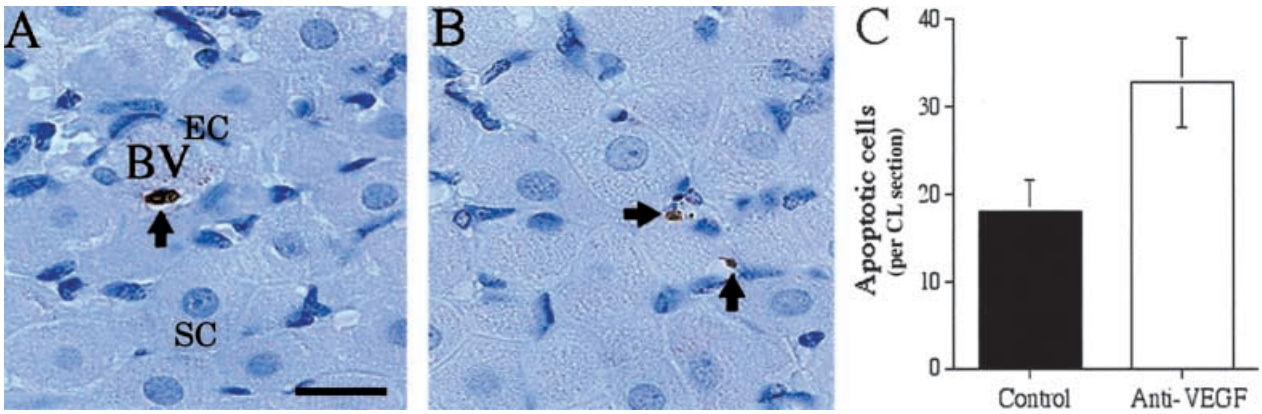

Figure 4 High power photomicrographs of 3′ end labelled apoptotic cells (brown staining) in (A) a mid-luteal phase control marmoset corpus luteum and (B) after anti-VEGF treatment. Note the presence of endothelial cells (EC) surrounding a blood vessel (BV) in the control section, and smaller dark haematoxylin-stained nuclei representing endothelial cells and periendothelial support cells in both control and treated CL. Arrows point to positive apoptotic nuclei assumed to be endothelial cells by their small, elongated form. The morphology of steroidogenic cells (SC) appears unaffected by treatment. (C) Quantification of positive cells in control (solid bar) and treated sections (open bar) shows increased presence of positive nuclei in anti-VEGF-treated sections. Note that values are per corpus luteum section so the incidence of apoptosis even after treatment is very low. Data are means ± S.E.M. Bar represents 20 μm.

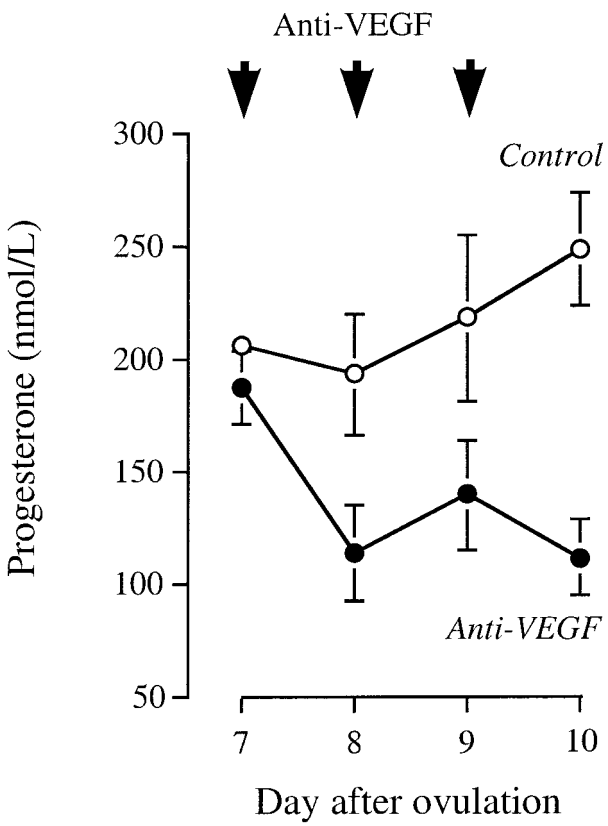

Figure 5 Plasma progesterone concentrations in control (○) and anti-VEGF-treated (●) marmosets. Treatment started on day 7 after ovulation and is associated with a significant suppression (P=0·01). Data are means ± S.E.M.

at least during the first 1–2 days of treatment. The rapid decline in plasma progesterone concentration seen on the first day of this acute mid-luteal phase anti-VEGF treatment, indicates that in addition to the regulation of ongoing mid-luteal angiogenesis, VEGF also modulates vascular permeability in the CL. An effect on CL vascular permeability could deprive the lutein cells of both the necessary precursors for progesterone production and the efficient release of their products into the bloodstream, which would result in marked reductions in plasma progesterone values.

After anti-VEGF treatment, morphological changes in lutein steroidogenic cells were not apparent. This contrasts with observations on the characteristic effects of withdrawal of the trophic factor, luteinising hormone (LH), which have been described recently (Fraser *et al.* 1999*b*, Dickson & Fraser 2000), suggesting that anti-VEGF treatment does not markedly interfere with pituitary LH secretion.

In this study, we have also observed an increase in pericyte coverage as a result of VEGF withdrawal. Pericytes are believed to be involved in the remodelling process which occurs during blood vessel maturation (reviewed by Darland & D'Amore 1999), a process also thought to take place in the CL (Tsukada *et al.* 1996, Goede *et al.* 1998). Benjamin *et al.* (1999) demonstrated that in both xenografted tumour and primary human tumours, VEGF withdrawal resulted in specific obliteration of immature vessels and that in human prostate cancer loss of VEGF led to selective apoptosis of endothelial cells devoid of periendothelial cells. Mature vessels with associated pericytes are believed to be VEGF

independent (Benjamin *et al.* 1998). It is possible that, in the current study, the increase in apparent apoptosis in endothelial cells after anti-VEGF treatment is a consequence of a lack of VEGF support to susceptible, immature vessels with no associated pericytes. It could follow, therefore, that endothelial cells of more mature vessels with associated pericytes would be VEGF independent and not susceptible to anti-VEGF treatment. Our finding that mid-luteal VEGF withdrawal led to an increase in endothelial cell apoptosis, the occurrence of which was rare, in the presence of extended pericyte coverage suggests that it is the endothelial cells associated with the few remaining immature capillaries which were susceptible to loss of VEGF support. This, in turn, indicates that the reduction in endothelial cell area after anti-VEGF treatment may be a consequence not only of a decreased angiogenic rate, but also of increased endothelial cell death.

Factors involved in the recruitment of pericytes include platelet-derived growth factor-B (reviewed in Darland & D'Amore 1999) and angiopoietin-1 (Davis *et al.* 1996, Maisonpierre *et al.* 1997, Koblizek *et al.* 1998), while transforming growth factor β is thought to regulate pericyte differentiation (Hirschi *et al.* 1999). It remains to be determined whether factors such as angiopoietin-1 may be activated after VEGF inhibition to act as a survival mechanism to 'rescue' existing vessels from the absence of the immature endothelial cell survival factor, VEGF.

In conclusion, this study demonstrates that VEGF is a primary factor controlling angiogenesis and luteal function in the mid-luteal phase. The rapid decline in plasma progesterone after VEGF inhibition suggests that another function of VEGF at this stage may be in the maintenance of vascular permeability. In addition, the increase in endothelial cell apoptosis indicates a role for VEGF in the survival of a proportion of endothelial cells, perhaps those without associated periendothelial support cells. However, further investigation into the role of VEGF in pericyte recruitment is required to further elucidate the processes of blood vessel maturation in the CL. Since the mid-luteal phase is a crucial period in the establishment of early pregnancy and a time at which survival of the luteal vasculature may be important in the 'rescue' of the CL, manipulation of VEGF may have clinical importance with respect to, on the one hand, treatment of early pregnancy loss, and, on the other hand, interruption of pregnancy.

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