

Expression of vascular endothelial growth factor (VEGF) receptors in rat corpus luteum: regulation by oestradiol during mid-pregnancy

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The aim of this study was to investigate the expression of vascular endothelial growth factor (VEGF) receptors, the *fms*-like tyrosine kinase (*flt-1*) and kinase insert domain-containing region (KDR), in corpora lutea obtained at different stages of the oestrous cycle and during pregnancy in rats. Immunohistochemistry revealed that both *flt-1* and KDR were localized in luteal cells in addition to vascular endothelial cells, and that the intensity of staining was stronger in pregnant rats than in cyclic rats. Rats undergoing hypophysectomy–hysterectomy on day 12 of pregnancy were treated with oestradiol until day 15 of pregnancy to determine whether oestradiol is involved in expression of *flt-1* and KDR mRNA in the corpus luteum during mid-pregnancy. The *flt-1* and KDR mRNA contents

in the corpus luteum were decreased significantly by hypophysectomy–hysterectomy, and these decreases recovered significantly after oestradiol treatment. Changes in the mass of the corpus luteum and serum progesterone concentrations paralleled the changes in expression of *flt-1* and KDR mRNA. Developmental studies indicated that *flt-1* and KDR mRNA contents in the corpus luteum were constant until day 15 of pregnancy but decreased significantly on day 21 of pregnancy. In conclusion, both *flt-1* and KDR were expressed in luteal cells in addition to vascular endothelial cells, and expression was upregulated by oestradiol during mid-pregnancy. *flt-1* and KDR may play a role in development of the corpus luteum and in production of progesterone during mid-pregnancy in rats.

Introduction

Angiogenesis plays an important role in the development of the corpus luteum and in maintenance of luteal function (Tamura and Greenwald, 1987; Gibori, 1993; Ferrara *et al.*, 1998; Fraser *et al.*, 2000; Kashida *et al.*, 2001). During mid-pregnancy in rats, the corpus luteum increases rapidly in size, mainly due to hypertrophy of luteal cells, and the number of endothelial cells in the corpus luteum and luteal blood flow also increases (Bruce *et al.*, 1984; Tamura and Greenwald, 1987; Gibori, 1993). High vascularization appears to be necessary to provide luteal cells with the large amounts of cholesterol needed for progesterone synthesis and for the delivery of progesterone to the circulation. Vascular endothelial growth factor (VEGF) is a protein that has the potential to play a dynamic role in the regulation of vascular endothelial growth, angiogenesis and vascular permeability (Ferrara and Davis-Smyth, 1997). VEGF is expressed in rat corpora lutea (Phillips *et al.*, 1990; Koos, 1995; Kashida *et al.*, 2001) and is essential for luteal angiogenesis leading to development of the corpus luteum (Ferrara *et al.*, 1998; Fraser *et al.*, 2000; Kashida *et al.*, 2001). Oestradiol is necessary for development of the corpus luteum and maintenance of luteal function during

mid-pregnancy in rats (Tamura and Greenwald, 1987; Gibori, 1993; Kashida *et al.*, 2001). Oestradiol is also responsible for the increase in angiogenesis observed in the corpus luteum during mid-pregnancy in rats (Tamura and Greenwald, 1987; Kashida *et al.*, 2001). Kashida *et al.* (2001) demonstrated that VEGF in the corpus luteum was upregulated by oestradiol and that the angiogenic effect of oestradiol was mediated by VEGF in the corpus luteum during mid-pregnancy in rats.

VEGF has two receptors, the *fms*-like tyrosine kinase (*flt-1*) and kinase insert domain-containing region (KDR), both of which are found generally on vascular endothelial cells. Both *flt-1* and KDR are expressed in luteal cells as well as in vascular endothelial cells in human corpora lutea (Otani *et al.*, 1999; Sugino *et al.*, 2000a). In addition, VEGF receptors are expressed in a variety of non-endothelial cells (Ergün *et al.*, 1997; Wen *et al.*, 1998; Krüssel *et al.*, 1999; Möller *et al.*, 2001) and are functional in these cells (Barleon *et al.*, 1996; Clauss *et al.*, 1996; Ahmed *et al.*, 1997; Brown *et al.*, 1997; Athanasiades *et al.*, 1998). However, little is known about the expression of VEGF receptors in rat corpora lutea. As luteal VEGF is upregulated by oestradiol and contributes to progesterone production (Kashida *et al.*, 2001), it would be interesting to determine whether VEGF receptors can also be upregulated by oestradiol. The aim of the present study was to examine the expression of VEGF receptors, *flt-1* and KDR in rat corpora lutea and to investigate the

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regulation of VEGF receptors by oestradiol during mid-pregnancy.

Materials and Methods

Animals

Sprague–Dawley rats (Japan SLC Inc., Hamamatsu), weighing 220–270 g, were housed at 24°C under controlled conditions (lights on from 05:00 h to 19:00 h) with free access to standard rat chow and water. Vaginal smears were obtained daily and only rats showing at least two consecutive 4 day oestrous cycles were used. Pro-oestrous rats were housed with males overnight and day 1 of pregnancy was defined as the day on which spermatozoa were found throughout a vaginal smear. The experimental protocol was reviewed and approved by the Committee for the Ethics on Animal Experiment in Yamaguchi University School of Medicine under the Law (No. 105) and Notification (No. 6) of the Government.

Immunohistochemistry

Immunohistochemistry was performed on ovaries from different stages of the oestrous cycle and on days 7, 15 and 21 of pregnancy to study the localization of flt-1 and KDR in corpora lutea. Six ovaries from three animals were used on each day. Ovaries were fixed in Bouin's solution and embedded in paraffin wax. Paraffin wax-embedded ovaries were cut into sections (4 µm in thickness). The tissue sections were deparaffinized in xylene and dehydrated in a graded series of ethanol. Immunohistochemistry for flt-1 was performed by a peroxidase–anti-peroxidase method (DAKO PAP kit; DAKO Japan Co. Ltd, Tokyo) using rabbit anti-human flt-1 polyclonal antibodies (C-17, sc-316; Santa Cruz Biotechnology, Santa Cruz, CA), which react specifically with rat flt-1. After inhibition of endogenous peroxidase activity with 0.3% (v/v) H₂O₂ for 50 min, the sections were incubated with 10% (v/v) normal swine serum for 10 min at room temperature to avoid non-specific binding. The sections were incubated with primary antibody at a dilution of 1:50 in PBS–BSA (1% w/v) overnight at 4°C. After three washes with PBS for 5 min each, the sections were incubated with swine anti-rabbit immunoglobulin for 30 min at room temperature, washed three times with PBS for 5 min each, and reacted with rabbit-PAP for 40 min at room temperature. Immunohistochemistry for KDR and for CD34, a marker of vascular endothelial cells, was performed with a streptavidin–biotin–peroxidase complex method (SAB-PO kit; Nichirei Co. Ltd, Tokyo) using mouse anti-KDR monoclonal antibodies (A-3, sc-6251; Santa Cruz Biotechnology) and mouse anti-CD34 monoclonal antibodies (Santa Cruz Biotechnology), respectively. After inhibition of endogenous peroxidase activity with 0.3% (v/v) H₂O₂ for 50 min, the sections were incubated with 10% (v/v) normal rabbit serum for 10 min at room temperature to avoid non-specific binding. The sections were incubated with the primary

antibodies at a dilution of 1:50 in PBS–BSA (1% w/v) overnight at 4°C. After three washes with PBS for 5 min each, the sections were incubated with biotinylated rabbit anti-mouse immunoglobulin for 10 min at room temperature, washed three times with PBS for 5 min each, and reacted with peroxidase-conjugated streptavidin for 5 min at room temperature. Peroxidase activity was visualized by incubating the sections with 3,3'-diaminobenzidine-4-HCl (Nacalai Tesque Co. Ltd, Tokyo) in 0.05 mol Tris–HCl buffer I⁻¹ (pH 7.6) containing 0.01% (v/v) H₂O₂ for 2 min. For controls, sections were incubated with normal rabbit serum for flt-1, or normal mouse serum for KDR and CD34, or the anti-flt-1 antibody was pre-absorbed with an excess of flt-1 blocking peptide (sc-316p; Santa Cruz Biotechnology) overnight at 4°C. Counterstaining was performed with Meyer's haematoxylin.

Experiments

A well-characterized oestradiol-treated hypophysectomized and hysterectomized pregnant rat model was used to study the involvement of oestradiol in flt-1 and KDR expression in the corpus luteum during mid-pregnancy in rats (Gibori and Keyes, 1978). Hypophysectomy and hysterectomy were performed on day 12 of pregnancy under ether anaesthesia. Hysterectomy was performed through an abdominal mid-line incision as reported by Kashida *et al.* (2001). Hypophysectomy was performed by the parapharyngeal approach as reported by Kato *et al.* (1988). Completeness of removal of the pituitary gland was assessed by the absence of the pituitary gland in the fossa at autopsy. Rats received a daily s.c. injection of either 100 µg oestradiol (Sigma Chemical Co., St Louis, MO) dissolved in 0.2 ml sesame oil or sesame oil (control) daily until the morning of day 15 of pregnancy. The ovaries were removed on day 15 of pregnancy and corpora lutea were dissected and cleaned of adhering tissue in a watch glass. Corpora lutea were weighed, frozen immediately in liquid nitrogen and stored at –80°C until RNA isolation was performed. All of the corpora lutea from each animal were used for RNA isolation and subjected to mRNA analysis as a single sample. Serum samples were stored at –20°C until oestradiol and progesterone assays were performed.

The aim of the second experiment was to study developmental changes in flt-1 and KDR expression in the corpus luteum throughout pregnancy. Corpora lutea were collected on days 3, 7, 9, 12, 15 and 21 of pregnancy and stored at –80°C until RNA isolation was performed. All of the corpora lutea from each animal were used for RNA isolation and subjected to mRNA analysis as a single sample.

Reverse transcription–polymerase chain reaction (RT–PCR)

Total RNA was isolated from corpora lutea with Isogen (Wako Pure Chemical Industries Ltd, Osaka) as directed by the manufacturer. For mRNA analysis, RT–PCR was performed as reported by Sugino *et al.* (1998a) with the oligo-

nucleotide primers for flt-1 (5'-CATGGTCAGCTGCTGGACACCGCG-3' and 5'-GACTCCCTGCATCACTAACAATAT-3') and for KDR (5'-TCAGATTACTTGCAGGGGACAGAGG-3' and 5'-GGATCACCACAGTTTTGTCTTGTT-3') designed by Wang *et al.* (1998). Direct sequence analyses of the PCR products were performed for sequence verification. Two oligonucleotide primers (5'-CTGAAGGTCAAAGGGAATGTG-3' and 5'-GGACAGAGTCTTGATGATCTC-3') were used to amplify ribosomal protein L19 as an internal control (Chan *et al.*, 1987). In brief, 3 µg total RNA were reverse-transcribed at 42°C in a reaction mixture containing 1 × PCR buffer, 2.5 mmol deoxynucleotide triphosphates l⁻¹ (Life Technologies Inc., Grand Island, NY), 5 µmol random hexamer primer l⁻¹ (Perkin-Elmer, Roche Molecular Systems Inc., Branchburg, NJ), 1.5 mmol MgCl₂ l⁻¹ and 200 U Moloney murine leukaemia virus reverse-transcriptase (Perkin-Elmer). The reverse-transcribed product was divided into two tubes for flt-1 or KDR primers and L19 primers, and PCR was performed. For PCR amplification, a mixture containing the oligonucleotide primers (50 pmol), [α -³²P]dCTP (2 µCi at 3000 Ci mmol⁻¹; Amersham, Arlington Heights, IL) and Taq DNA polymerase (2.5 U; Perkin-Elmer) was added to each reaction. Amplification was carried out for 25 cycles each consisting of 94°C for 1 min, 62°C for 1 min and 72°C for 2 min for each set of flt-1 and L19, and KDR and L19, followed by 10 min final extension at 72°C in a program temperature control system PC-800 (ASTEC, Fukuoka). The predicted sizes of the PCR products were 400 bp for flt-1, 300 bp for KDR and 194 bp for L19. A linear curve was plotted using number of cycles of amplification versus densitometric values of the PCR products, measured with a BAS 2000 (Fuji Photo Film Co., Tokyo). The optimal number of cycles for amplification that fits within the linear range was chosen for each set of primers for VEGF and L19 (data not shown). Reaction products were subjected to electrophoresis on an 8% (w/v) polyacrylamide non-denaturing gel. After autoradiography, band intensities were analysed using a bioimaging analyser BAS2000. For quantification, the densities of flt-1 and KDR were normalized to that of the internal control L19.

Hormone assay

Serum progesterone and oestradiol concentrations were determined by specific radioimmunoassay as described previously (Kato *et al.*, 1982; Sugino *et al.*, 1991). The sensitivities of the assay were 100 pg per tube for the progesterone assay and 2.7 pg per tube for the oestradiol assay. The intra- and interassay coefficients of variation were 7.0 and 14.4%, respectively, for the progesterone assay, and 10.0 and 10.4%, respectively, for the oestradiol assay.

Statistical analysis

Data were analysed by ANOVA and Duncan's new multiple range test. Differences were considered significant at $P < 0.05$.

Results

Immunostaining for flt-1, KDR and CD34 in rat ovaries is shown (Fig. 1). Immunoreactivity to flt-1 and KDR was observed in luteal cells and in vascular endothelial cells on day 15 of pregnancy (flt-1: Fig. 1a,c,f; KDR: Fig. 1b,d,g; and CD34: Fig. 1e,h). In interstitial blood vessels, flt-1 and KDR immunoreactivities were also observed in vascular smooth muscle cells (Fig. 1f,g). Immunostaining intensity was strong in luteal cells compared with that in endothelial cells. These patterns of staining were similar in the corpus luteum on days 7, 15 and 21 of pregnancy (data not shown). flt-1 immunostaining at oestrus and KDR immunostaining at dioestrus are shown (Fig. 1i,j). Compared with pregnant rats, flt-1 and KDR immunoreactivities were weak in luteal cells and vascular endothelial cells throughout the oestrous cycle. No flt-1 immunoreactivity was observed in the ovary on day 15 of pregnancy after pre-absorption of the anti-flt-1 antibody with an excess of flt-1 blocking peptide (Fig. 1k). No KDR immunoreactivity was observed in the control section (Fig. 1l).

The well-characterized oestradiol-treated hypophysectomized and hysterectomized pregnant rat model was used to study the involvement of oestradiol on flt-1 and KDR expression in the corpus luteum during mid-pregnancy in rats. In this model, hypophysectomy-hysterectomy decreased the mass of corpora lutea and serum progesterone concentrations markedly, whereas oestradiol treatment significantly ($P < 0.01$) reversed the inhibitory effects of hypophysectomy-hysterectomy, although serum oestradiol concentrations were very high (Table 1). flt-1 mRNA content in the corpus luteum was decreased significantly ($P < 0.01$) by hypophysectomy-hysterectomy, and this inhibitory effect was reversed completely by treatment with oestradiol (Fig. 2a). KDR mRNA content was also decreased significantly ($P < 0.01$) by hypophysectomy-hysterectomy, and this inhibitory effect was reversed significantly ($P < 0.01$) by the treatment with oestradiol (Fig. 2b). Changes in flt-1 and KDR mRNA contents in the corpus luteum throughout pregnancy were also examined. flt-1 and KDR mRNA contents in the corpus luteum were constant until day 15 of pregnancy but decreased significantly on day 21 of pregnancy ($P < 0.01$) (Fig. 3).

Discussion

The results of the present study demonstrate that VEGF receptors, flt-1 and KDR, are localized in luteal cells in addition to vascular endothelial cells. This finding is consistent with recent reports showing that flt-1 and KDR are expressed in luteal cells of human corpora lutea (Otani *et al.*, 1999; Sugino *et al.*, 2000a). The results of the present study also indicate that flt-1 and KDR expression are increased in corpora lutea of pregnancy and are upregulated by oestradiol in the corpus luteum during mid-pregnancy. Kashida *et al.* (2001) reported that oestradiol activates angiogenesis and vascular permeability via VEGF

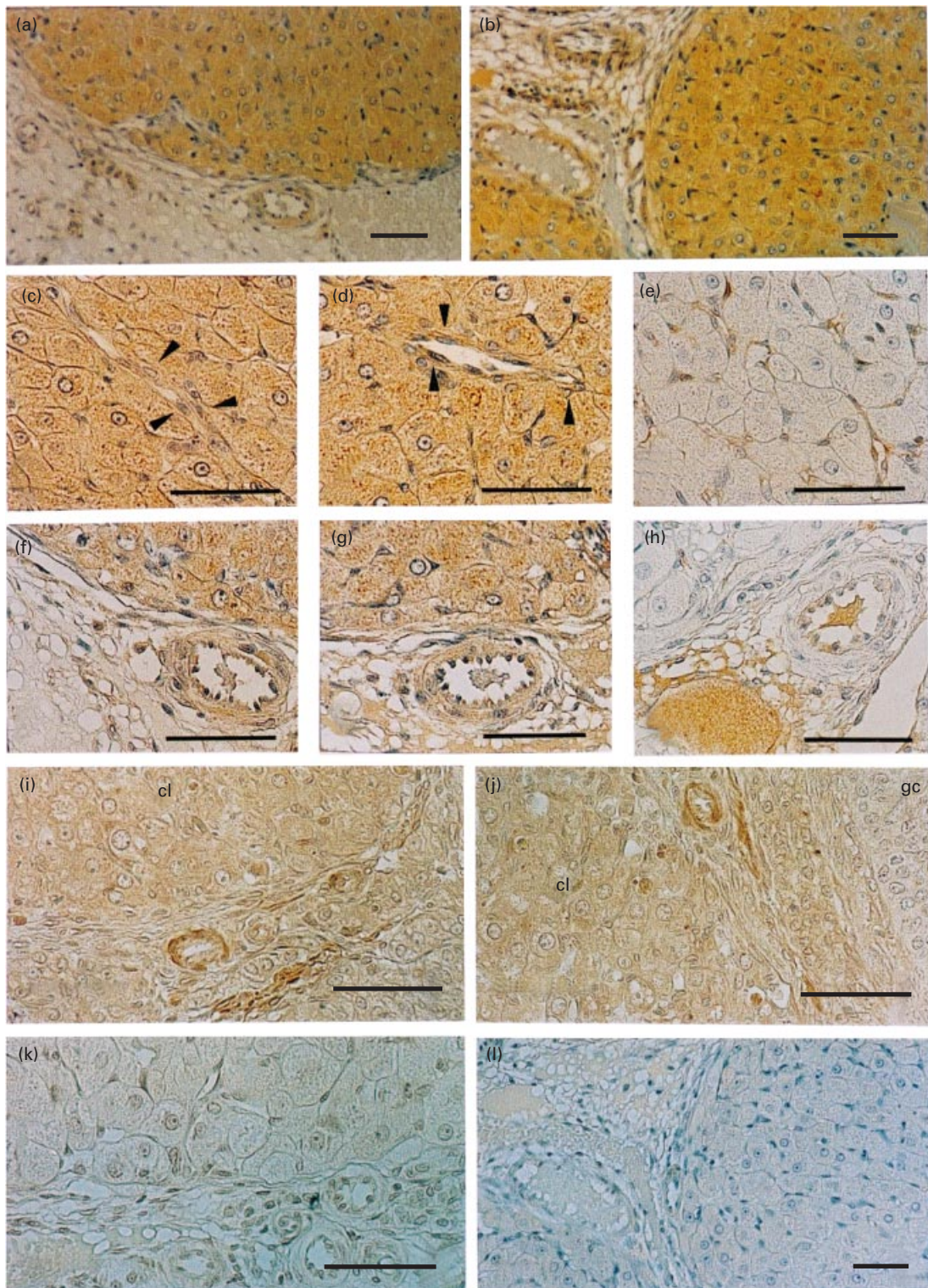


Fig. 1. Immunohistochemical staining for *fms*-like tyrosine kinase (*flt-1*), kinase insert domain-containing region (*KDR*) and *CD34* in rat corpus luteum. (a) *flt-1* and (b) *KDR* immunoreactivities were observed in luteal cells and interstitial blood vessels in the ovary on day 15 of pregnancy. At a higher magnification, (c) *flt-1* and (d) *KDR* immunoreactivities were observed in the cytoplasm of luteal cells and in vascular endothelial cells indicated by arrowheads in the corpus luteum. In interstitial blood vessels, (f) *flt-1* and (g) *KDR* immunoreactivities were observed in endothelial cells and smooth muscle cells. *CD34* immunoreactivities were localized in vascular endothelial cells (e,h). During the oestrous cycle, (i) *flt-1* and (j) *KDR* immunoreactivities were faint in luteal cells

Table 1. Mass of the corpus luteum, and serum progesterone and oestradiol concentrations in hypophysectomized–hysterectomized rats treated with or without oestradiol

Treatment	<i>n</i>	Mass of corpus luteum (mg)	Serum progesterone (ng ml ⁻¹)	Serum oestradiol (pg ml ⁻¹)
Control (day 15 of pregnancy)	5	4.1 ± 0.1	132.8 ± 9.1	66.4 ± 7.4
Hypophysectomy–hysterectomy + oil	8	2.5 ± 0.1 ^a	22.5 ± 1.2 ^a	24.4 ± 4.1
Hypophysectomy–hysterectomy + 100 µg oestradiol	7	3.9 ± 0.1	94.6 ± 6.1 ^b	2244 ± 489 ^b

Control: intact rats on day 15 of pregnancy.

Hypophysectomy–hysterectomy were performed on day 12 of pregnancy and oestradiol or sesame oil (control) was administered once a day until day 15 of pregnancy.

Values are mean ± SEM.

^a*P* < 0.01 versus day 15 control or oestradiol-treated rats. ^b*P* < 0.01 versus day 15 control.

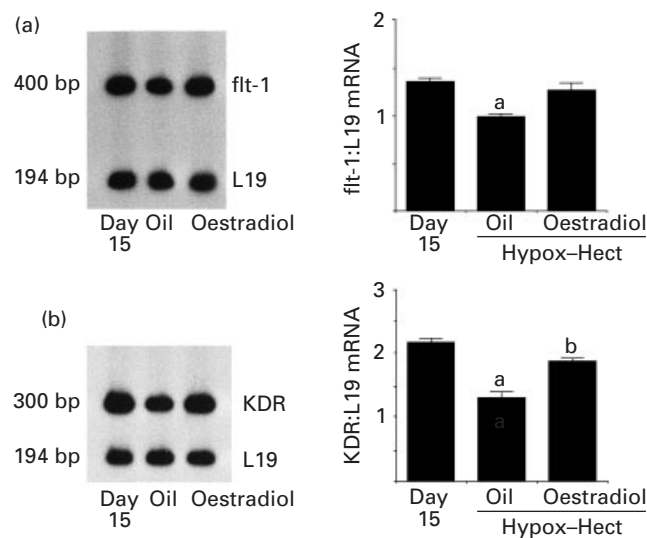


Fig. 2. Effects of oestradiol on mRNA content of (a) fms-like tyrosine kinase (flt-1) and (b) kinase insert domain-containing region (KDR) in the corpus luteum during mid-pregnancy. Hypophysectomy and hysterectomy (Hypox–Hect) were performed on day 12 of pregnancy and oestradiol (100 µg, *n* = 7) or sesame oil (*n* = 8) was injected daily until day 15 of pregnancy. Intact rats on day 15 of pregnancy (*n* = 5) were used as a control. The flt-1 and KDR mRNA contents in the corpus luteum were determined by RT–PCR. The intensity of the signals of flt-1 or KDR was normalized to that of the internal control L19. The quantification data (the ratio of flt-1 or KDR to L19) represent the mean ± SEM. ^a*P* < 0.01 versus day 15 and oestradiol. ^b*P* < 0.05 versus day 15.

in rat corpora lutea and contributes to the increases in both the size of the corpus luteum and production of progesterone during mid-pregnancy. The results of the present study indicate that oestradiol can activate VEGF and

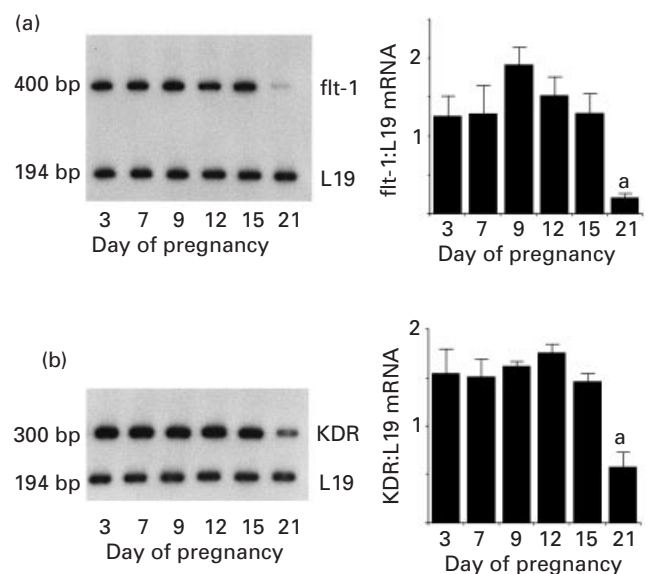


Fig. 3. Developmental changes in content of (a) fms-like tyrosine kinase (flt-1) and (b) kinase insert domain-containing region (KDR) mRNA in the corpus luteum during pregnancy. Corpora lutea were obtained on days 3 (*n* = 3), 7 (*n* = 3), 9 (*n* = 4), 12 (*n* = 5), 15 (*n* = 5) and 21 (*n* = 5) of pregnancy. Total RNA was isolated and subjected to RT–PCR. The quantification data (the ratio of flt-1 or KDR to L19) represent the mean ± SEM. ^a*P* < 0.01 versus the other days.

its receptor system in rat corpora lutea during mid-pregnancy. This finding is in good agreement with the knowledge that gonadotrophic hormones stimulate the VEGF system, which contributes to the development of corpora lutea (Otani *et al.*, 1999; Berisha *et al.*, 2000; Sugino *et al.*, 2000a; Fraser and Lunn, 2001). In the present study, flt-1 and KDR were also localized in vascular smooth

(cl) compared with those in interstitial blood vessels. No KDR immunoreactivity was observed in granulosa cells (labelled 'gc'). (k) No flt-1 immunoreactivity was observed in the ovary on day 15 of pregnancy after preabsorption of the anti-flt-1 antibody with an excess of flt-1 blocking peptide. (l) No KDR immunoreactivity was observed in the control section. Scale bars represent 50 µm.

muscle cells. As Brown *et al.* (1997) reported that uterine smooth muscle cells express flt-1 and KDR and proliferate in response to VEGF, it is possible that the VEGF system may be involved in the proliferation of vascular smooth muscle cells.

The present study raises the possibility that VEGF may exert a paracrine–autocrine role in regulation of luteal function in rats, as VEGF receptors are expressed in luteal cells. This possibility has also been suggested for human corpora lutea (Otani *et al.*, 1999; Sugino *et al.*, 2000a). Expression of VEGF receptors has been noted in a variety of non-endothelial cells (Ergün *et al.*, 1997; Wen *et al.*, 1998; Krüssel *et al.*, 1999; Möller *et al.*, 2001). In addition, flt-1 and KDR can act as functional receptors for VEGF in non-endothelial cells (Barleon *et al.*, 1996; Clauss *et al.*, 1996; Ahmed *et al.*, 1997; Brown *et al.*, 1997; Athanassiades *et al.*, 1998). It is of special interest to note that VEGF can act as a survival factor and inhibit apoptosis (Spyridopoulos *et al.*, 1997; Gerber *et al.*, 1998). Apoptosis may play an important role in the determination of the lifespan of luteal cells (Juengel *et al.*, 1993; Sugino *et al.*, 1996, 1999a, 2000b; McCormack *et al.*, 1998; Roughton *et al.*, 1999). Sugino *et al.* (2000a) reported that expression of flt-1 and KDR in human luteal cells was high during the mid-luteal phase and in early pregnancy. In addition, in the present study, the constant high expression of flt-1 and KDR until mid-pregnancy and upregulation of flt-1 and KDR by oestradiol may indicate that flt-1 and KDR are involved in the regulation of luteal lifespan. This possibility may be supported by the finding that flt-1 and KDR mRNA contents were low in the corpus luteum on day 21 of pregnancy, when it is undergoing luteolysis. This low expression of VEGF receptor mRNA may be due to the decrease in the stimulatory effect of oestradiol or to a cytokine such as tumour necrosis factor α (TNF- α), the concentration of which increases in the corpus luteum during regression (Bagavandoss *et al.*, 1990; Shaw and Britt, 1995) and can inhibit expression of VEGF receptor (Patterson *et al.*, 1996). Although there appears to be a discrepancy between low mRNA content and intense immunostaining on day 21 of pregnancy, this may be due to the different sensitivities of RT-PCR and immunohistochemistry.

As the content of VEGF receptor mRNA was increased in the corpus luteum by oestradiol during mid-pregnancy, the change in VEGF receptor expression in the corpus luteum throughout pregnancy was examined. However, until day 15 of pregnancy, no correlation was found between expression of VEGF receptor mRNA and the developmental changes that occur in the corpus luteum. It is unclear why the mRNA contents of flt-1 and KDR in the corpus luteum were constant until day 15 of pregnancy although oestradiol concentrations in the corpus luteum increase in the second half of pregnancy (Taya and Greenwald, 1981) and expression of flt-1 and KDR mRNA is upregulated by oestradiol. It is possible that other factors in addition to oestradiol are involved in the regulation of expression of flt-1 and KDR mRNAs in the corpus luteum during mid-

pregnancy. In fact, the effects demonstrated by the experimental animal model or the *in vitro* model are not always consistent with the developmental changes that occur during pregnancy, which is indicative of the involvement of several factors (Parmer *et al.*, 1991; Sugino *et al.*, 1998b, 1999b; Telleria *et al.*, 1998).

In conclusion, the results of the present study demonstrate that VEGF receptors, flt-1 and KDR, are expressed in luteal cells in addition to vascular endothelial cells and are upregulated by oestradiol in the corpus luteum during mid-pregnancy. These results indicate that flt-1 and KDR may play a role in the regulation of luteal function.

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