Changes in vascular leakage and expression of angiopoietins in the corpus luteum during pregnancy in rats

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Abstract

The present study investigates changes in blood vessel stability and its regulation in the corpus luteum (CL) during pregnancy in the rat. First, blood vessel stability in the CL was evaluated during pregnancy based on vascular leakage, which was quantified by the Evans blue assay. Vascular leakage was highest on day 3, thereafter decreased until day 15 and increased again on day 21. Secondly, to study the regulation of vascular leakage, the expression of angiopoietins was examined in the CL during pregnancy. Angiopoietin-1 (Ang-1) effects maturation and stabilization of newly formed blood vessels, while Ang-2 produces the opposite effect by allowing vascular remodeling. An immunohistochemical study showed both Ang-1 and Ang-2 expression in luteal cells. mRNA and protein levels of Ang-1 were significantly higher on days 12 and 15 than those on days 3 and 21, whereas there was no significant change in Ang-2 expression. Since estradiol contributes to CL development during mid-pregnancy, we finally studied whether estradiol regulates vascular leakage and angiopoietin expression. Rats undergoing hypophysectomy and hysterectomy (hypox-lect) on day 12 were treated with estradiol until day 15. Vascular leakage was increased and Ang-1 expression was decreased by hypox-lect, and these effects were completely reversed by estradiol treatment. In conclusion, blood vessel stability in the CL is likely to be associated with CL development and CL regression, and may be regulated by angiopoietins. Estradiol contributes to blood vessel stabilization in the CL during mid-pregnancy, which is associated with an increase in Ang-1 expression.

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Introduction

Angiogenesis has been recognized to play important roles in the development of the corpus luteum (CL) and maintenance of CL function (Tamura & Greenwald 1987, Smith *et al.* 1994, Ferrara *et al.* 1998, Fraser *et al.* 2000, Sugino *et al.* 2000a, Kashida *et al.* 2001, Bowen-Shauver & Gibori 2004, Pauli *et al.* 2005). Previous studies have demonstrated that vascular endothelial growth factor (VEGF) stimulates angiogenesis in the CL and contributes to CL development and progesterone production (Ferrara *et al.* 1998, Fraser *et al.* 2000, Sugino *et al.* 2000a, Kashida *et al.* 2001, Pauli *et al.* 2005). In addition, studies on the mechanism of angiogenesis have focused on the role of other growth factors, the angiopoietins, which function in concert with VEGF for formation, stabilization and regression of blood vessels (Suri *et al.* 1996, Hanahan 1997, Maisonpierre *et al.* 1997, Darland & D'Amore 1999, Yancopoulos *et al.* 2000). It is well known that angiopoietins are critical for sprouting, stabilization and regression of blood vessels. Angiopoietin-1 (Ang-1) acts

on vascular endothelial cells through a tyrosine kinase receptor (Tie-2) and is involved in blood vessel stabilization (Suri *et al.* 1996). On the other hand, Ang-2, which antagonizes the action of Ang-1 and Tie-2, plays a role in the destabilization of existing blood vessels (Maisonpierre *et al.* 1997). Thus, it has been generally thought that in the presence of VEGF, Ang-2 can promote vessel sprouting by blocking the Ang-1 signal, whereas in the absence of VEGF, Ang-2 inhibition of the Ang-1 signal can induce blood vessel regression (Hanahan 1997).

The CL is essential for the maintenance of pregnancy throughout the entire pregnancy in rats. To maintain progesterone production for successful pregnancy, not only high vascularization but also stabilization of blood vessels in the CL is necessary to provide luteal cells with the large amounts of cholesterol needed for progesterone synthesis and to deliver progesterone to the circulation. Therefore, it seems that blood vessels in the CL need to stabilize or mature to serve as functional vessels (Jain & Booth 2003). However, little is known regarding the change in blood

vessel stability and its regulation in the CL. Therefore, in the present study, we have evaluated blood vessel stability in the CL by quantifying vascular leakage, and furthermore the involvement of angiopoietins in the regulation of blood vessel stability has been examined in the CL during pregnancy in rats.

Materials and Methods

Animal models and tissue preparation

Sprague–Dawley rats (Japan SLC Inc., Hamamatsu, Japan), weighing 180–240 g, were housed at 24 °C under controlled conditions (lights on from 0500 to 1900 h) with free access to standard rat chow and water. Vaginal smears were obtained daily, and only those rats showing at least two consecutive 4 day estrous cycles were used. Proestrous rats were housed with males overnight, and day 1 of pregnancy was defined as the day on which sperm were found through a vaginal smear. The experimental protocol was reviewed and approved by the Committee for the Ethics on Animal Experiments in Yamaguchi University School of Medicine under the Law (no. 105) and Notification (no. 6) of the Government.

The first experiment was planned to examine blood vessel stability in the CL during pregnancy. Blood vessel stability was evaluated on days 3, 7, 9, 12, 15 and 21 of pregnancy, based on vascular leakage, quantified by the Evans blue dye assay as described below.

The second experiment was set up to determine the levels of Ang-1, Ang-2 and Tie-2 in the CL during pregnancy. Rats were laparotomized under ether anesthesia on days 3, 7, 9, 12, 15 and 21 of pregnancy. The ovaries were perfused with saline via the portal vein during draining of the inferior vena cava to remove the blood, and then removed as reported previously (Sugino *et al.* 1993a). CL were dissected and cleaned of adhering tissue in a watch glass. Only newly formed CL were used in the present study. CL were immediately frozen in liquid nitrogen, and stored at –80 °C until RNA isolation for RT-PCR and protein isolation for Western blot analysis.

The third experiment was performed to study the involvement of estradiol in vascular leakage, and Ang-1 and Ang-2 expression in the CL during mid-pregnancy. For this purpose, a well-characterized estradiol-treated hypophysectomized and hysterectomized rat model was used. This model was reported by Gibori & Keyes (1978) and has been widely used to study the role of intraluteal estrogen in the regulation of rat CL function. Hypophysectomy and hysterectomy were performed on day 12 of pregnancy, and rats were injected s.c. with either 100 µg 17β-estradiol (Sigma Chemical Co., St Louis, MO, USA) dissolved in 0.2 ml sesame oil or sesame oil (control) daily until the morning of day 15 of pregnancy, as reported previously (Kashida *et al.* 2001). Rats were used for the measurement of vascular leakage and Ang-1 and Ang-2 expression in the CL.

Autofluorescence imaging of Evans blue dye

Evans blue dye is widely used to measure vascular protein leakage (Udaka *et al.* 1970, Saria & Lundberg 1983, Murphy & Lever 2001, Hamer *et al.* 2002). Increased vascular leakage causes the leakage of albumin out of blood vessels. Since Evans blue dye binds to albumin in the circulation, vascular leakage can be evaluated by measuring the Evans blue dye that has exuded from blood vessels. First, we observed exudation of the Evans blue dye in the CL by autofluorescence imaging of Evans blue dye as reported previously (Murphy & Lever 2001). On days 3 and 15 of pregnancy, Evans blue dye (Sigma; 30 mg/kg) was injected via a femoral vein. The ovaries were perfused with saline to remove blood 30 min after injection and then taken out. The ovary was mounted in Tissue-Tek compound and cooled by liquid nitrogen. Frozen sections (6 µm) were cut at –21 °C on a Leica cryostat, dipped in cold acetone (–20 °C) for 1 min and then air-dried at room temperature. The sections were then dipped into xylene and mounted with a glass cover-slip. Alternate sections were stained with hematoxylin (Wako Pure Chemical Industries Ltd, Osaka, Japan) and eosin (Merck, Darmstadt, Germany) (HE), and mounted with Entellan neu (Merck). The HE-stained sections were viewed with bright-field light microscopy, while the unstained frozen sections were viewed by fluorescence microscopy with the use of a green wave-length filter set.

Quantification of vascular leakage

On days 3, 7, 9, 12, 15 and 21 of pregnancy, Evans blue dye (30 mg/kg) was injected via a femoral vein. The ovaries were perfused with saline to remove blood 30 min after injection and then taken out. CL were dissected, cleaned of adhering tissue and weighed. Evans blue dye exuded in the CL was extracted by incubating four to six CL in 0.15 ml formamide for 24 h at 65 °C, and dye concentrations were measured by absorption at 620 nm and determined by a standard curve as reported previously (Udaka *et al.* 1970, Saria & Lundberg 1983). The amount of Evans blue dye was expressed as ng of Evans blue dye per mg wet weight of the CL. The optimal number of CL for extraction was chosen since a linear Evans blue dye concentration was obtained between three and six CL in 0.15 ml formamide. The intra- and interassay coefficients of variation in this assay were 2.2 and 2.4% respectively.

To estimate the number of blood vessels in the CL, after Evans blue dye extraction they were embedded in paraffin and tissue sections were made. Blood vessels were identified with vascular endothelial cells in the HE-stained histological sections as shown in Fig. 2A, and the number of blood vessels was counted within a microscopic field at ×400 (Fig. 2A). Counting was done on five randomly chosen fields from two or three CL of several sections. More than one field was evaluated per CL. The mean value was used as a number of blood vessels in a unit area. Since the size of luteal cells changes during pregnancy, the

luteal cell size influences the proportion of the blood vessels per unit area of the histological section. In fact, the marked impact of luteal cell size on the proportion per unit area of blood vessels has been pointed out (Wulff *et al.* 2001, Sugino *et al.* 2005). For example, in early pregnancy, luteal cells are relatively small so that in a given unit area the number of blood vessels is relatively high. In contrast, in mid-pregnancy, the luteal cell size is relatively large so that in a given unit area the number of blood vessels is relatively low. Therefore, to adjust for this effect, the number of blood vessels in a unit area was multiplied by the CL wet weight in order to estimate the number of blood vessels in each CL. This value reflects the number of blood vessels in the whole CL and was used as a vascular index in the present study. Vascular leakage was determined by dividing the amount of Evans blue dye per CL by the vascular index, which indicates the leakage of Evans blue dye per blood vessel in the CL.

Immunohistochemistry

For immunohistochemistry, ovaries were fixed in Bouin's solution and then embedded in paraffin. Paraffin-embedded ovaries were sectioned at 4 μ m. The tissue sections were deparaffinized in xylene and dehydrated in a graded series of ethanol. Immunohistochemistry for Ang-1 and Ang-2 was performed with a peroxidase–anti-peroxidase (PAP) method (Dako PAP kit; Dako Japan, Co. Ltd, Tokyo, Japan) using goat polyclonal Ang-1 and Ang-2 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). To retrieve antigen, the sections were heated three times in a microwave oven for 5 min each at 600 W in 0.01 M citrate buffer, pH 6.0. After inhibition of endogenous peroxidase activity with 0.3% (v/v) H_2O_2 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 then 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 rabbit anti-goat immunoglobulin for 30 min at room temperature, washed three times with PBS for 5 min each, and reacted with goat PAP for 40 min at room temperature. Peroxidase activity was visualized by incubating the sections with 3,3'-diaminobenzidine tetrahydrochloride (Nacalai Tesque Co. Ltd, Tokyo, Japan) in 0.05 M Tris–HCl buffer (pH 7.6) containing 0.01% (v/v) H_2O_2 for 5 min. Control sections were incubated with normal goat serum. Counterstaining was performed with Mayer's hematoxylin.

RT-PCR

Total RNA was isolated from CL with Isogen (Wako) by the method provided by the manufacturer. For mRNA analysis, RT-PCR was performed as reported previously (Sugino *et al.* 1998) with the oligonucleotide primers for Ang-1 (5'-GTGGCTGGAAAACTTGAGA-3' and

5'-TGGATTCAAGACGGGATGT-3'), for Ang-2 (5'-GACC-AGTGGGCATCGCTACG-3' and 5'-CTGGTTGGCTGATG-CTACTG-3') and for Tie-2 (5'-TGCCACCATCACTCAATACC-3' and 5'-AAACGCCAATAGCACGGTGA-3') designed on the basis of the rat Ang-1, Ang-2 and Tie-2 cDNA sequences (Sato *et al.* 2001). Two oligonucleotide primers (5'-CTGAAGGTCAAAGGGAATGTG-3' and 5'-GGACAGAGT-CTTGATGATCTC-3') were also used to amplify ribosomal protein L19 as an internal control (Chan *et al.* 1987). In brief, 3 μ g total RNA were reverse transcribed at 42 °C in a reaction mixture (single strength PCR buffer, 2.5 mM deoxynucleotide triphosphates, 5 μ M random hexamer, 1.5 mM MgCl_2 , and 200 U Moloney murine leukemia virus reverse-transcriptase (Perkin-Elmer, Roche Molecular Systems Inc., Branchburg, NJ, USA)). The RT product was divided into two equal aliquots (one tube was for L19 primers), and PCR was performed. For PCR amplification, a mixture containing the oligonucleotide primers (50 pmol), [α - 32 P]dCTP (2 μ Ci at 3000 Ci/mol; Amersham, Arlington Heights, IL, USA) and Taq DNA polymerase (2.5 U; Perkin-Elmer) was added to each reaction. Amplification was carried out for 30 cycles consisting of 94 °C (1 min), 60 °C (1 min) and 72 °C (1 min) for Ang-1, Ang-2 and Tie-2, followed by 10 min of final extension at 72 °C in a programmed temperature-control system PC-800 (ASTEC, Fukuoka, Japan). The predicted sizes of the PCR-amplified products were 201 bp for Ang-1, 170 bp for Ang-2, 214 bp for Tie-2 and 194 bp for L19. A liner curve was plotted using number of cycles for amplification vs densitometric values of the PCR products, measured with an FLA2000 (Fuji Photo Film Co., Tokyo, Japan). The optimal number of cycles for amplification that fit within the linear ranges were chosen for each sets of primers for Ang-1, Ang-2, Tie-2 and L19 (data not shown). Reaction products were subjected to electrophoresis on an 8% (v/v) polyacrylamide non-denaturing gel. After autoradiography, band intensities were analyzed using a bioimaging Analyzer FLA2000. For quantification, the densities of Ang-1, Ang-2 and Tie-2 were normalized to that of the internal control L19.

Western blot analysis

CL were homogenized with PBS containing a protease inhibitor cocktail tablet (Complete Mini; Roche Diagnostics, Mannheim, Germany) and centrifuged at 800 g for 10 min at 4 °C. The supernatant was used for Western blot analysis as reported previously (Sugino *et al.* 2000b). In brief, 50 μ g of protein of the supernatant, determined by the Lowry *et al.* (1951) method, were loaded in each sample and separated by SDS-PAGE in 7.5% (v/v) gels under reduced conditions. The proteins on the gel were electrophoretically transferred to nitrocellulose membranes and reacted with the rabbit polyclonal Ang-1 antibody (Santa Cruz Biotechnology) or rabbit anti-mouse Ang-2 antibody (Alpha Diagnostic International, San Antonio, TX, USA) at a dilution of 1:50 with 0.5% (w/v)

skimmed milk in Tris-buffered saline (pH 7.5). The membranes were then immersed in the reaction buffer containing PAP conjugated swine anti-goat immunoglobulin (1:3000). The reacted band was developed on a film with an ECL kit (Amersham Pharmacia Biotech, Bucks, UK). To reuse of the blot, the membranes were stripped in Restore Western Blot Stripping Buffer (Pierce, Rockford, IL, USA) and reacted with mouse monoclonal β -tubulin antibody (Sigma) at a dilution of 1:500 with 0.5% (w/v) skimmed milk in Tris-buffered saline (pH 7.5). β -Tubulin is a ribosomal protein and was used as an internal control. The membranes were immersed in the reaction buffer containing PAP conjugated rabbit anti-mouse immunoglobulin (1:3000). The reacted band was developed on a film with the ECL kit.

Progesterone assay

Progesterone concentrations in the serum were determined by a specific RIA as reported previously (Kato *et al.* 1982). The sensitivity of the assay was 100 pg/ml, and the intra- and interassay coefficients of variation were 7.0 and 14.4% respectively.

Statistical analysis

Data were analyzed by ANOVA and Duncan's new multiple range test. Differences were considered to be significant if $P < 0.05$.

Results

To evaluate the stability of blood vessels in the CL, we tried to evaluate vascular leakage in the CL. For this purpose, the Evans blue dye assay was used in this study. We first observed Evans blue dye that had exuded from blood vessels in the CL by fluorescence imaging. Figure 1 shows fluorescence images of Evans blue dye in the CL on day 3 (Fig. 1A) and on day 15 of pregnancy (Fig. 1C). The orange color indicates the Evans blue dye that has exuded from blood vessels, which was seen in the CL on day 3 of pregnancy, with intense staining in the center (Fig. 1A). In contrast, there was a faint staining in the CL on day 15 of pregnancy (Fig. 1C). This finding suggests that vascular leakage in the CL was more apparent on day 3 compared with on day 15 of pregnancy. Although there may be a fluorescence that biological tissues naturally have, so-called autofluorescence, there was a faint staining in the CL on day 15 of pregnancy, suggesting that autofluorescence in the CL tissue can be regarded as negligible.

Secondly, we quantified the vascular leakage by measuring the amount of Evans blue dye exuded in the CL. To evaluate the leakage per blood vessel, the amount of Evans blue dye in the CL was divided by the number of blood vessels in the CL, the vascular index. The vascular index gradually increased until day 12, rapidly increased from day 12 to day 15, and decreased on day 21 of

Figure 1 Autofluorescence image of Evans blue dye in the CL on day 3 (A) and on day 15 of pregnancy (C). HE staining is also shown on day 3 (B) and on day 15 of pregnancy (D). The orange color in the CL indicates the Evans blue dye that has exuded from blood vessels in the CL, which was apparent on day 3 (A) compared with day 15 of pregnancy (C). Bar = 100 μ m.

pregnancy (Fig. 2B). As shown in Fig. 3, vascular leakage was highest on day 3 and thereafter decreased until days 12 and 15, which are lowest during pregnancy (Fig. 3). However, vascular leakage increased on day 21 of pregnancy again (Fig. 3). These findings suggest that the blood vessel is not yet stabilized on day 3 and stabilized during mid-pregnancy, but destabilized on day 21 of pregnancy.

Figure 4 shows the immunostaining for Ang-1 (Fig. 4A) and Ang-2 (Fig. 4B) in the CL on day 15 of pregnancy. Both Ang-1 and Ang-2 were localized in luteal cells, with no visible difference between Ang-1 and Ang-2, and there

Figure 2 Changes in the vascular index in the CL during pregnancy. Blood vessels were identified with vascular endothelial cells (arrow heads) in the HE-stained histological sections on day 15 (A), and the number of blood vessels was counted within a microscopic field at $\times 400$ on day 3 ($n = 7$), day 7 ($n = 8$), day 9 ($n = 8$), day 12 ($n = 6$), day 15 ($n = 6$) and day 21 ($n = 7$) of pregnancy. Counting was done on five randomly chosen fields. The mean value was used as the number of blood vessels in a unit area. Since the size of the CL changes during pregnancy, the number of blood vessels in a unit area was multiplied by the CL wet weight in order to estimate the number of blood vessels in each CL. This value reflects the number of blood vessels in the whole CL and was used as the vascular index (B). Values are means $\pm$ S.E.M. of the number of animals. Different letters indicate significant differences ($P < 0.01$ for a–b, c–d, d–e, and d–e; $P < 0.05$ for b–c) between groups. Bar = 25 μ m.

Figure 3 Changes in vascular leakage in the CL during pregnancy. Vascular leakage was quantified by the Evans blue dye assay in the same animals as shown in the legend to Fig. 2. Values are means $\pm$ S.E.M. of the number of animals shown in the legend to Fig. 2. Different letters indicate significant differences ($P < 0.01$ for a–b and c–d; $P < 0.05$ for a–d and b–c) between groups.

was no difference in immunointensities between day 3, day 15 and day 21 of pregnancy (data not shown).

Ang-1 mRNA expression was high in the CL on days 12 and 15 of pregnancy, but decreased on day 21 of pregnancy, which was the same level as on day 3 of pregnancy (Fig. 5A). However, there was no significant change in Ang-2 mRNA levels (Fig. 5B) and Tie-2 mRNA levels (data not shown).

Figure 6 shows Western blot analysis of Ang-1 and Ang-2 in the CL on days 3, 15 and 21 of pregnancy. Ang-1 protein expression was barely detectable on day 3 of pregnancy, but it was highly expressed on day 15 and also stronger compared with that on day 21 of pregnancy (Fig. 6A). There was no significant change in Ang-2 protein expression between days 3, 15 and 21 of pregnancy (Fig. 6B).

It is well known that estradiol is necessary for the development of the CL and the maintenance of CL function during mid-pregnancy in rats (Gibori *et al.* 1977, Bowen-Shaver & Gibori 2004). Furthermore, we have

Figure 4 Immunohistochemical staining for Ang-1 (A) and Ang-2 (B) in the CL on day 15 of pregnancy. (C) Negative control. Bar = 50 μ m.

demonstrated that estradiol stimulates angiogenesis via VEGF in the rat CL between day 12 and day 15 of pregnancy (Kashida *et al.* 2001). Therefore, we studied whether estradiol is involved in the decreased vascular leakage and high Ang-1 expression between day 12 and day 15 of pregnancy. For this purpose, vascular leakage and protein expression of Ang-1 and Ang-2 were examined in the CL of hypophysectomized–hysterectomized rats treated with estradiol. In this rat model, hypophysectomy–hysterectomy remarkably decreased the CL weight and serum progesterone concentrations, while estradiol treatment completely reversed those inhibitory effects of hypophysectomy–hysterectomy (Fig. 7A and B). Vascular leakage was significantly increased by hypophysectomy–hysterectomy, and this effect was significantly reversed by estradiol treatment (Fig. 7C). Furthermore, Ang-1 protein expression was decreased by hypophysectomy–hysterectomy and this effect was reversed by estradiol treatment (Fig. 7D), whereas there was no significant change in Ang-2 protein expression by hypophysectomy–hysterectomy or estradiol treatment (Fig. 7E).

Discussion

This is the first report showing the change in vascular leakage in the rat CL throughout pregnancy. In the present study, blood vessel stability was evaluated by quantifying vascular leakage in the CL using the Evans blue dye assay, which has been widely used to study vascular protein leakage (Udaka *et al.* 1970, Saria & Lundberg 1983, Murphy & Lever 2001, Hamer *et al.* 2002). Vascular leakage was highest on day 3, then decreased until days 12 and 15, which were lowest during pregnancy, and increased on day 21 again, suggesting that blood vessels in the CL are not yet stabilized in the early luteal phase, but stabilized during mid-pregnancy (mid-luteal phase), and destabilized during the regression phase. It has been reported that Ang-1 is involved in blood vessel stabilization, whereas Ang-2, which antagonizes Ang-1 action, plays a role in destabilization of blood vessels (Suri *et al.* 1996, Hanahan 1997, Maisonpierre *et al.* 1997, Darland & D'Amore 1999, Yancopoulos *et al.* 2000). Therefore, the change in vascular leakage may be explained by the present result that Ang-1 expression is high in the CL during mid-pregnancy compared with the early luteal phase and regression phase.

It is likely that the changes in vascular leakage and angiopoietins are related to angiogenesis in the CL. The number of blood vessels in the CL gradually increase until mid-pregnancy, as shown in the present result (Fig. 2B), which is consistent with the report by Tamura & Greenwald (1987). Since it is reported that Ang-2 is involved in destabilization of blood vessels and promotes vessel sprouting in the presence of VEGF, angiogenic changes seen in the early luteal phase may be induced by the Ang-2 action because of low Ang-1 expression, although

Figure 5 Changes in mRNA levels of Ang-1 (A) and Ang-2 (B) in the CL during pregnancy. Total RNA was isolated and subjected to RT-PCR. Depicted are representative autoradiographs of day 3 ($n = 8$), day 7 ($n = 8$), day 9 ($n = 7$), day 12 ($n = 6$), day 15 ($n = 6$) and day 21 ($n = 7$) of pregnancy. The intensity of the signals of Ang-1 or Ang-2 was normalized to that of the internal control L19. The quantification data (the ratio of Ang-1 or Ang-2 to L19) represent the means $\pm$ S.E.M. of animals. Different letters indicate significant differences ($P < 0.05$) between groups.

blood vessels are still immature in the CL of the early luteal phase.

It has been reported that angiogenesis in the CL is activated and parallel to the rapid growth of the CL during mid-pregnancy (Tamura & Greenwald 1987, Kashida *et al.* 2001). The present data have also shown an increase in the vascular index in the CL during mid-pregnancy. On the other hand, the present study showed high Ang-1 expression and decreased vascular leakage between day 12 and day 15 of pregnancy, suggesting that blood vessel stability is parallel to the angiogenesis in the CL in this period. The present data, therefore, seem inconsistent with the story that angiogenesis is induced by VEGF when blood vessels are destabilized by Ang-2 action. However, we hypothesize that angiogenesis may occur accompanied by blood vessel stabilization in the CL during mid-pregnancy. Recently, Wulff *et al.* (2001) and our group (Sugino *et al.* 2005) reported the same hypothesis, in which they suggested increased angiogenesis together with blood vessel stabilization in the human CL of pregnancy because significant increases in the number of endothelial cells and perivascular cells (pericytes) were found in the CL rescued by human chorionic gonadotropin. In fact, overexpression of Ang-1 has been shown to produce highly branched and numerous leakage-resistant blood vessels in the skin of transgenic mice (Suri *et al.* 1998, Thurston *et al.* 1999). Mice lacking Tie-2 receptor or Ang-1 showed that endothelial cells are present in normal numbers and are assembled into tubes, but the blood vessels are immature, lacking branching networks and proper organization into large and small blood vessels (Sato *et al.* 1995, Suri *et al.* 1996). Furthermore, recent

reports have shown that co-administration of Ang-1 and VEGF increases angiogenesis and reduces vascular leakage in the ischemic myocardium (Siddiqui *et al.* 2003), which is a rational approach for creating more stable vessels for functional improvement (Zhu *et al.* 2002, Yamauchi *et al.* 2003). These findings, overall, strongly suggest that both Ang-1 and VEGF are necessary for the formation of stabilized mature blood vessel networks. Thus, we presume that mature blood vessels are actively formed in the CL during mid-pregnancy in rats.

It is well known that estradiol is necessary for development of the CL and maintenance of CL function during mid-pregnancy in rats (Gibori *et al.* 1977, Bowen-Shauver & Gibori 2004). The present study has demonstrated that estradiol decreases vascular leakage with an increase in Ang-1 expression in the CL during mid-pregnancy. Since estradiol stimulates angiogenesis via VEGF in the rat CL during mid-pregnancy (Kashida *et al.* 2001), estradiol contributes to both blood vessel stabilization and angiogenesis in the CL during mid-pregnancy. This finding again supports our hypothesis that angiogenesis may occur accompanied by blood vessel stabilization in the CL during mid-pregnancy. The mechanism by which estradiol modulates Ang-1 expression is unclear at present. There is no estrogen response element in the rat Ang-1 promoter region (gene accession No. AB080023), and there is a report that estradiol decreased Ang-1 expression in non-reproductive tissues in rats, in contrast to the present result (Ye *et al.* 2002). These findings suggest the possibility that estradiol modulates Ang-1 expression through some mediators.

Figure 6 Western blot analyses for Ang-1 (A), Ang-2 (B) and β -tubulin (C) in the CL on days 3, 15 and 21 of pregnancy. Equal amounts of protein were separated by SDS-PAGE, transferred to nitrocellulose membrane, and analyzed by Western blotting. Depicted are representative immunoblots of days 3, 15 and 21 of pregnancy ($n = 4$).

Also, the present data may seem to be inconsistent with the report that estrogen increases microvascular permeability via VEGF in the rat uterus, which is observed especially at implantation (Rockwell *et al.* 2002). Blood vessel stabilization is modulated by Ang-1 and it would be important to note that Ang-1 expression is still low in the mouse uterus at implantation (Matsumoto *et al.* 2002). As described above, it is likely that the regulation of Ang-1 expression by estrogen in the CL is different from that in other tissues. These findings may suggest that the estrogen-induced microvascular permeability is dependent on the tissue.

It is of interest to note that vascular leakage is lowest when progesterone production is most activated. This result suggests that maintenance of CL function may involve stabilization of blood vessels in the CL. Vascular networks in the CL need to not only supply nutrients or substrates to luteal cells for progesterone synthesis but also deliver progesterone into the circulation. It is presumed that blood vessels in the CL during mid-pregnancy

are mature enough to serve as functional vessels for progesterone production. It has been reported that VEGF leads to immature, leaky and hemorrhagic blood vessels, which are non-functioning. However, Ang-1 leads to leakage-resistant blood vessels (Yancopoulos *et al.* 2000). Therefore, Ang-1 may contribute to CL function by stabilizing blood vessels in the presence of VEGF. In addition, it is of interest to note that vascular leakage reduced concomitantly with the prolongation of the life span of the CL; from day 3 of pregnancy, at which the CL is rescued by the pituitary prolactin, and from day 12 of pregnancy, at which the CL is rescued by placental lactogens. Further studies are needed regarding the correlation between CL function and blood vessel stability.

For blood vessel stabilization, Ang-1 has been reported to recruit pericytes (Hanahan 1997, Darland & D'Amore 1999, Yancopoulos *et al.* 2000). It is controversial at present whether pericytes exist in the rat CL (Tsukada *et al.* 1996, Arfuso & Meyer 2003, Pauli *et al.* 2005). There are some reports showing the presence of pericytes in the rat CL, although they are immature and few (Tsukada *et al.* 1996, Arfuso & Meyer 2003). However, Ang-1 can directly act on endothelial cells for vascular network stabilization (Papapetropoulos *et al.* 1999, Carlson *et al.* 2001). Ang-1 plays a crucial role in mediating interactions between endothelial cells, the surrounding matrix and pericytes (Suri *et al.* 1996, Hanahan 1997). Although it is unclear how much pericytes are involved in blood vessel stabilization in the rat CL, it is likely that direct action of Ang-1 on endothelial cells, at least in part, contributes to blood vessel stabilization.

Increased vascular leakage in the CL on day 21 of pregnancy suggests that blood vessels are destabilized in the CL during the regression phase. It has been reported that deletion of endothelial cells or detachment of endothelial cells from the basement membrane is involved in blood vessel regression during the CL regression (Azmi & O'Shea 1984, Modlich *et al.* 1996, Goede *et al.* 1998). In fact, the number of blood vessels decreased in the CL on day 21 of pregnancy in the present study. It has been reported that destabilization of blood vessels caused by Ang-2 in the absence of VEGF induces endothelial cell death, probably by apoptosis (Hanahan 1997). This may apply to the present result, because we reported that VEGF action is lacking due to the remarkably low expression of VEGF receptors in the rat CL on day 21 of pregnancy (Sugino *et al.* 2001). Although mechanisms of CL regression have been a matter of concern and still seem complex (Sugino *et al.* 1993b, 1996, 1997, 1999, 2000b, Kato *et al.* 1997, Takiguchi *et al.* 2000, 2004, Sugino 2005), destabilization of blood vessels and blood vessel regression may be the first event in the CL undergoing functional luteolysis in pregnant rats (Plendl 2000).

In conclusion, the present study, for the first time, showed changes in vascular leakage and angiopoietins in the rat CL throughout pregnancy. It is likely that angiopoietins are involved in the regulation of blood vessel

Figure 7 Effects of hypophysectomy-hysterectomy (hypox-heck) and estradiol treatment on CL weights (A), serum progesterone concentrations (B), vascular leakage (C), and protein expression of Ang-1 (D), Ang-2 (E) and β -tubulin (F) in the CL. Hypox-heck was performed on day 12 of pregnancy, and then estradiol (E2; 100 μ g, $n = 9$) or sesame oil (oil, $n = 10$) was injected daily until day 15 of pregnancy. Intact rats on day 15 of pregnancy (D15, $n = 8$) were used as a control. Values are means $\pm$ S.E.M. of the number of animals. Different letters indicate significant differences ($P < 0.01$) between groups.

stability in the CL. Especially, estradiol contributes to blood vessel stabilization with an increase in Ang-1 expression in addition to stimulating angiogenesis via VEGF in the CL during mid-pregnancy, which may play important roles in CL development and maintenance of CL function.

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