Gene expression profiles in the bovine corpus luteum (CL) during the estrous cycle and pregnancy: Possible roles of chemokines in regulating CL function during pregnancy

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Abstract. To determine functional differences between the corpus luteum (CL) of the estrous cycle and pregnancy in cows, gene expression profiles were compared using a 15 K bovine oligo DNA microarray. In the pregnant CL at days 20–25, 40–45 and 150–160, the expressions of 138, 265 and 455 genes differed by a factor of > 2-fold (P < 0.05) from their expressions in the cyclic CL (days 10–12 of the estrous cycle). Messenger RNA expressions of chemokines (eotaxin, lymphotactin and ENA-78) and their receptors (CCR3, XCR1 and CXCR2) were validated by quantitative real-time PCR. Transcripts of eotaxin were more abundant in the CL at days 40–45 and 150–160 of pregnancy than in the cyclic CL (P < 0.01). In contrast, the mRNA expressions of lymphotactin, ENA-78 and XCR1 were lower in the CL of pregnancy (P < 0.05). Messenger RNAs of CCR3 and CXCR2 were similarly detected both in the cyclic and pregnant CL. Tissue protein levels of eotaxin were significantly higher in the CL at days 150–160 of pregnancy than in the CL at other stages, whereas the lymphotactin protein levels in the CL at days 20–25 of pregnancy were lower (P < 0.05). Immunohistochemical staining showed that CCR3 was expressed in the luteal cells and that XCR1 was expressed in both the luteal cells and endothelial cells. Collectively, the different gene expression profiles may contribute to functional differences between the cyclic and pregnant CL, and chemokines including eotaxin and lymphotactin may regulate CL function during pregnancy in cows.

Key words: Chemokines, Corpus luteum, Cow, Microarray analysis, Pregnancy

The corpus luteum (CL) is a transient ovarian organ established by cells of the follicle following ovulation. Progesterone (P4), the primary product of the CL, is required for the establishment and maintenance of pregnancy. If pregnancy does not occur, the CL degenerates. On the other hand, when pregnancy is established, the CL lifespan is prolonged, and the CL continues to produce P4 during the gestation period in cows [1]. The bovine CL also produces other intraluteal factors, including prostaglandins (PG), growth factors and cytokines [1]. In all mammals, the CL is requisite during the early phase of pregnancy. The CL is required for the entire gestation period in some species (e.g., cows, pigs, goats and dogs) [2], whereas the CL is not required for the entire gestation period in other species (e.g., primates, sheep) because luteal P4 secretion can be replaced by placental P4 secretion [3]. These findings suggest that the physiological roles and functions of the pregnant CL differ depending on the species and that normal CL function is crucial to maintenance of pregnancy in cows.

The structural and functional properties of the CL of the estrous cycle and pregnancy are similar [4, 5], but several differences have been demonstrated in cows [6, 7], sheep [8, 9] and pigs [10]. In sheep, the response of small luteal cells to luteinizing hormone was weaker at days 40–50 than at day 25 of pregnancy [9, 11]. The volume densities of steroidogenic small and large luteal cells gradually increase, peaking between days 60 and 142 of pregnancy in sheep [8, 9]. The gene expressions of PGE and PGF synthases are higher in the pregnant CL than in the cyclic CL in pigs [12]. In the cow, the CL seems to have additional functions during pregnancy, such as production of relaxin [13]. In addition, the mRNA and protein expressions of oxytocin are much lower in the pregnant CL than in the cyclic CL [14–16].

Although regulation of CL function throughout the estrous cycle has been intensively studied, studies of CL function during the entire gestation period are limited. Understanding the role of CL function during pregnancy might help to determine a way to improve reproductive efficiencies and reduce the number of defective fetuses. Therefore, in the present study, we looked for differences in global gene expression patterns in the CL of pregnant and nonpregnant stages in cows using a custom-made 15 K bovine oligo DNA microarray. Since the expressions of some chemokines including eotaxin, lymphotactin and ENA-78 drastically changed between the
pregnant and nonpregnant CL, gene and protein expressions of these chemokines and their receptors were also evaluated.

**Materials and Methods**

**Collection of bovine corpora lutea**

Bovine ovaries containing corpora lutea were obtained from Japanese Black cows at the National Institute of Agrobiological Sciences ranch within 10–30 min of exsanguination. Tissue samples were collected from cows on days 10–12 of the estrous cycle (cyclic) and on days 20–25, 40–45 and 150–160 of gestation (n = 4 animals/stage). The day of artificial insemination was designated as day 1. The CLs were immediately separated from the ovaries and then cut into small pieces (< 0.5 cm³). These CL pieces were submerged in RNA later (Qiagen GmbH, Hilden, Germany) or liquid nitrogen and stored at −80°C until use. All procedures for animal experiments were carried out in accordance with guidelines approved by the Animal Ethics Committee of the National Institute of Agrobiological Sciences for the use of animals.

**Microarray analysis**

A custom-made 15 K bovine oligo DNA microarray (GPL9284) was used for the microarray analysis, which was performed as described previously [17]. After verifying the quality of the RNA with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and an Experion RNA StdSens kit (700-7104JA, Bio-Rad Laboratories, Hercules, CA, USA), we performed one-color microarray analysis. RNA integrity was confirmed; all samples had an A260/280 ratio greater than 1.8 and an RNA integrity number greater than 8.5. The oligo microarray produced by Agilent Technologies (Palo Alto, CA, USA) was used in this study. Thirty-mer nucleotide probes for the customized microarray were synthesized on a glass slide. cDNA synthesis, Cy3-labeled cRNA preparation, hybridization and the washing and scanning of the array slides were performed according to the Agilent one-color microarray-based gene expression analysis protocol. Briefly, 400 ng of total RNA from each sample were reverse transcribed into cDNA using a Quick Amp Labeling Kit (Agilent Technologies) with an oligo dT-based primer, and then Cy3-labelled cRNA was prepared by *in vitro* transcription. Labeled cRNA was purified with an RNeasy Mini Kit, and the concentration and Cy3 dye incorporation (pmol Cy3/µg cRNA) were measured with a spectrophotometer. Labeled cRNA (600 ng) was fragmented and hybridized using a Gene Expression Hybridization Kit (Agilent Technologies), according to the manufacturer’s instructions. The arrays were washed using a Gene Expression Wash Pack Kit and scanned using an Agilent Microarray Scanner. Feature Extraction ver. 9.5 was used for image analysis and data extraction. The microarray data from each sample were imported into GeneSpring 12 (Agilent Technologies) for further data characterization. The GEO accession numbers are as follows: platform, GPL9284; samples, GSM1446266 to GSM1446281; series, GSE59770.

**Real-time PCR**

Total RNA isolation and subsequent reverse transcription and real-time PCR steps were carried out as previously described [18]. The primers encoding the bovine sequences were chosen using an online software package (http://primer3.ut.ee/) and synthesized as listed in Table 1. The primer length (20 bp) and GC contents of each primer (50 to 60%) were selected to avoid primer dimer formation.

Gene expression was measured by real-time PCR using an Mx3000P Real-Time PCR System (Agilent Technologies) and Quantifast SYBR Green PCR kit (204054, Qiagen) starting with 200 ng of reverse-transcribed total RNA. The expression of 18S ribosomal RNA (RN18S1) was used as an internal control. The PCR was performed under the following conditions: (first step) 95°C for 5 min; 40 cycles of 95°C for 15 sec, 60°C for 30 sec and (second step) 95°C for 60 sec; and then 60°C for 30 sec. The reaction was then held at 25°C. Each PCR was followed by obtaining melting curves to ensure amplification of a single product. As standard curves, serial dilutions of appropriate cDNA were used for gene quantification. The obtained data were normalized on the basis of RN18S1 mRNA content. Previous studies from our laboratory and by other investigators have validated the use of RN18S1 as a normalizing standard while assessing gene expression at different luteal developmental stages in cows [19, 20]. Use of the Mx3000P Real-Time PCR System at elevated temperatures resulted in reliable and sensitive quantification of the RT-PCR products with high linearity (Pearson correlation coefficient r > 0.94). To exclude any contaminating genomic DNA, all experiments included controls lacking the reverse transcription enzyme. As a negative control, water was used instead of RNA for PCR to exclude any contamination from buffers and tubes.

**Immunohistochemistry**

Immunohistochemistry for CCR3 and XCR1 in the bovine CL on days 150–160 of pregnancy was performed using the automated Ventana HX System Discovery with a DabMapKit (Roche Diagnostics GmbH, Mannheim, Germany) as described previously in detail by our laboratory [21]. The CL samples for immunohistochemistry were fixed with 10% neutral formalin for 24 h. The 5 µm-thick sections from paraffin embedded luteal tissue were incubated at room temperature with a rabbit polyclonal anti-human CCR3 antibody (251536, Abbiotec LLC, San Diego, CA, USA) or rabbit polyclonal anti-human XCR1 antibody (LS-A159, LifeSpan BioSciences, Seattle, WA, USA) diluted 1:100 each in Discovery Ab diluents (Roche) for 2 h. Nucleotide and amino acid sequence homologies of human CCR3 with bovine CCR3 are 80% and 74%, and those of human XCR1 with bovine XCR1 are 85 and 78%, respectively. The signals were detected using anti-rabbit IgG-Biotin conjugate (Sigma-Aldrich, St Louis, MO, USA) diluted 1:100 for 1 h and then counterstained with hematoxylin. Negative controls were performed using normal rabbit IgG (NBP2-24891, Novus Biologicals, LLC, Littleton, CO, USA) diluted at concentrations equivalent to the primary antibodies. The sections were observed with a Leica DMRE HC microscope (Leica Microsystems, Tokyo, Japan) and a Nikon Digital Sight DS-Fi1-L2 (Nikon Instech, Tokyo, Japan).

**Collection of supernatants derived from homogenized luteal tissues**

Luteal tissue (0.4–1.0 g dry wt) was transferred into 4–10 ml phosphate buffer (pH 7.2; 160-14481; Wako Pure Chemical Industries, Osaka, Japan) containing a proteinase inhibitor tablet (cOmplete Ultra Tablet, EDTA-free, Roche) and was homogenized in an ice
Statistical analyses

At 23,500 × g for 45 min at 4 °C. The supernatant were collected and stored at –80 °C.

ELISA

The eotaxin and lymphotactin concentrations in the luteal tissues were determined using commercial bovine ELISA kits (eotaxin [CCL11]; E0026b, Wuhan EIAab Science, Wuhan, P.R. China, lymphotactin [XCL1]; E11L0269, BlueGene Biotech, Shanghai, P.R. China). The ranges of the standard curves were 0.078–5.0 ng/ml for eotaxin and 0.5–10 ng/ml for lymphotactin, respectively. The intra-assay coefficients of variation were 7.2% for eotaxin and 8.6% for lymphotactin. The samples for the ELISA were diluted 5-fold with assay buffer.

Statistical analyses

Microarray data were analyzed statistically with the Student’s t-test and summarized using GeneSpring 12 (Agilent Technologies). All extracted microarray data were analyzed with one-way ANOVA followed by the Turkey-Kramer multiple comparison test using the JMP 7 software (SAS Institute, Cary, NC, USA). The correlations between the flow cytometric collection and gradient collection were determined by the Pearson product–moment correlation coefficient using the JMP software, and the correlation coefficients were calculated as r-values. Experimental data for real-time PCR and ELISA are presented as the mean ± SEM. All data were analyzed for normality and homogeneity of variance by the Bartlett’s test. The statistical significance of differences in the abundance of mRNA expressions and in protein concentrations was assessed by one-way ANOVA with KaleidaGraph 3.6 (Synergy Software, Reading, PA, USA). When ANOVA showed a significant effect of stage or treated group, the stages or groups were compared by the Bonferroni post hoc multiple comparison test. A P-value < 0.05 was considered statistically significant.

Results

Gene expression profiles in the bovine CL during pregnancy

The expressions of many genes in the pregnant CL differed by a factor of > 2-fold (P < 0.05) from their expressions in the cyclic CL. These included 138 genes during days 20–25, 265 genes during days 40–45 and 455 genes during days 150–160 of pregnancy (Table 2). Some of the genes, including chemokines, growth factor-related proteins and extracellular matrix proteins, were differentially expressed between the pregnant CL and cyclic CL. Remarkably, the expression of eotaxin was 2991-fold higher at 150–160 days of pregnancy and 223-fold higher at 40–45 days of pregnancy than in the cyclic stage. Fibroblast growth factor (FGF)-1 and insulin-like growth factor binding proteins (IGFBP) 2 and 6 were more strongly expressed in the pregnant CL than in the cyclic CL, while IGFBP5 was more weakly expressed. Both collagen type I-IX and fibronectin type I mRNAs were more weakly expressed in the CL at days 150–160 of pregnancy than in the other stages. The gene expressions of PGE receptor type 3 (PGER3), annexin A8-like 1, proenkephalin, haptoglobin, major histocompatibility complex (MHC) class 1A and oxytocin in the pregnant CL differed by more than 10-fold from those in the cyclic CL (Table 2).

Messenger RNA expressions of several chemokines (including eotaxin, lymphotactin and ENA-78) and their receptors (CCR3, XCR1 and CXCR2) were checked by real-time PCR (Fig. 1). Transcripts of eotaxin appeared to be more abundant in the CL at days 40–45 and 150–160 of pregnancy than in the cyclic stage (Fig. 1A; P < 0.01 or lower). In contrast, the expressions of both lymphotactin and ENA-78 mRNA were lower during pregnancy than in the estrous cycle (Fig. 1B–1C; P < 0.05). Messenger RNAs of CCR3, XCR1 and CXCR2 were detected in the bovine CL of both the estrous cycle and pregnancy. Although the expressions of CCR3 and CXCR2 were not significantly different, XCR1 mRNA expression was significantly lower in the pregnant CL than in the cyclic CL (Fig. 1D–1F; P < 0.05).

Table 1. Primers used in real-time PCR

| Gene          | Primer | Sequence (5’-3’) | GenBank accession no. | Product size (bp) |
|---------------|--------|------------------|-----------------------|-------------------|
| **Eotaxin (CCL11)** | Forward | TCACGAGCAGCAAATGTCTCT | BC114735 | 101 |
|               | Reverse | CATGCCATTTCTGGACCCACT |           | 112 |
| **Lymphotactin (XCL1)** | Forward | CAGCTCTCTAATCTGACAAGCT | NM_175716 | 111 |
|               | Reverse | CTTCACCTCCCAACACCTTCC |           | 112 |
| **ENA-78 (CXCL5)** | Forward | TGGACCCAGAAGCTCTCTTGTG | BC142108 | 118 |
|               | Reverse | TGCTGAAAGAACTGGCAACTT |           | 112 |
| **CCR3 (CCL11-R)** | Forward | AACCCTGCGCATTTCTGAGCT | NM_001194960 | 108 |
|               | Reverse | TGGACGATGCGAAGATACCG |           | 108 |
| **XCR1 (XCL1-R)** | Forward | CCTCAACCTCTGCTCCTCAG | NM_001194965 | 107 |
|               | Reverse | TTAGGGACCTTTGACAGGGAG |           | 107 |
| **CXCR2 (CXCL5-R)** | Forward | GGTATCCTTGTGCTGCGTG | NM_001101285 | 141 |
|               | Reverse | GACAGGTCTCGGCAATCACA |           | 141 |
| **RN18S1** | Forward | TCGCGGAAGAAGTTAAAGTG | DQ222453 | 141 |
|               | Reverse | AAACGGCTACCACATCAAG |           | 141 |
Table 2. Fold change in mRNA levels of selected genes in the CL of pregnancy vs. the CL of the estrous cycle (10–12 days)

| Gene                        | Change | Days of pregnancy (number of genes that changed by > 2-fold) |
|-----------------------------|--------|------------------------------------------------------------|
|                             |        | 20–25 (138) 40–45 (265) 150–160 (455)                     |
| (Chemokines)                |        |                                                           |
| Eotaxin                     | Up     | 223 2991                                                  |
| Lymphotactin                | Down   | 7.82 10.6 16.8                                           |
| ENA-78                      | Down   | 6.65 6.51                                                 |
| (Growth factors related)    |        |                                                           |
| FGF-1                       | Up     | 2.15 2.34 2.20                                           |
| IGFBP2                      | Up     | 29.3                                                      |
| IGFBP6                      | Up     | 2.41 2.84 3.75                                           |
| IGFBP5                      | Down   | 3.03 4.18 3.46                                           |
| (Extracellular matrix)      |        |                                                           |
| Collagen Type I             | Down   | 3.10                                                      |
| Collagen Type II            | Down   | 2.58                                                      |
| Collagen Type III           | Down   | 2.19                                                      |
| Collagen Type IV            | Down   | 2.09                                                      |
| Collagen Type VI            | Down   | 3.85                                                      |
| Collagen Type XI            | Down   | 2.37                                                      |
| Fibronectin Type I          | Down   | 3.87                                                      |
| (Others: >10-fold)          |        |                                                           |
| PGER3                       | Up     | 34.7 87.0                                                 |
| Annexin A8-like 1           | Up     | 10.2 66.4                                                 |
| Proenkephalin               | Up     | 63.8                                                      |
| Haptoglobin                 | Up     | 8.14 6.88 11.4                                           |
| MHC Class IA                | Up     | 15.8                                                      |
| Oxytocin                    | Down   | 16.5 250                                                  |

**Protein levels of chemokines and their receptors in the bovine CL during pregnancy**

Commercial ELISA kits revealed significantly more bovine eotaxin at days 150–160 of pregnancy (P < 0.01 or lower) and significantly less bovine lymphotactin at days 20–25 of pregnancy than in the cyclic stage (Fig. 2A; P < 0.01). The protein expressions in the pregnant CL (Fig. 2A) tended to follow the mRNA expressions (Fig. 1A–1B). Immunohistochemical staining showed that CCR3 was expressed in the luteal cells and that XCR1 was expressed in both the luteal cells and endothelial cells of the bovine CL (Fig. 2B).

**Discussion**

The preceding results demonstrate that the number of genes whose expressions changed in the CL during the transition from the estrous cycle to pregnancy gradually increased with the stage of gestation. This suggests that the function of the pregnant CL is different from that of the cyclic CL. In particular, the gene and protein expressions of eotaxin were much higher in the CL of the mid-pregnant stage than in the CL of the estrous cycle, whereas those of lymphotactin were lower in the early pregnant CL (Figs. 1 and 2A). The binding sites of eotaxin and lymphotactin were distributed in the luteal cells or endothelial cells in the bovine CL in this study (Fig. 2B). These observations suggest that eotaxin and lymphotactin play a role in regulating bovine CL function.

Immune cells have powerful local effects both on luteolysis and on prolonging the functional life span of the CL through their secretion of cytokines and chemokines during pregnancy [22, 23]. Chemokines are small peptides that cause leukocytes to adhere to the endothelium and emigrate into tissues [24]. Chemokines are classified into four families (CC, CXC, CX3C and C) based on the number and localization of conserved cysteine residues [25]. For example, CC chemokines possess four cysteine residues in which the first two are adjacent. CC chemokines form a rather heterogeneous family containing two major subfamilies, the monocyte chemoattractant protein (MCP) subfamily and macrophage inflammatory protein (MIP) subfamily, in humans [26]. Eotaxin is a CC chemokine (named CCL11), and lymphotactin is a C chemokine (named XCL1). Chemokine receptors are also categorized into four families according to their corresponding ligand families, including CXCR (bind CXC chemokines), CCR (bind CC chemokines), XCR (bind C chemokines) and CX3CR (bind CX3C chemokines) [27]. One notable characteristic of chemokine receptors is that many different ligands bind the same receptor and many ligands bind multiple receptors. Eotaxin, lymphotactin and ENA-78, which is a CXC chemokine, bind CCR3, XCR1 and CXCR2, respectively [28].

A representative chemokine of CC chemokines is MCP-1 (named CCL2), a potent chemotactic molecule that induces the recruitment
of monocytes and T-lymphocytes. MCP-1 is currently the most studied chemokine involved in luteal regression. Expression of MCP-1 increases near the time of luteolysis in many species [19, 29]. Expression of MCP-1 is induced by PGF2α [30] and can attract and activate monocytes/macrophages [31]. However, little information is available about how other chemokines including eotaxin, lymphotactin and ENA-78 regulate bovine CL function. Eotaxin mRNA signals are weak in the CL of the estrous cycle in cows [32] and pigs [33], suggesting that eotaxin does not have a physiological role in the CL. Eotaxin mRNA levels are also low in human granulosa cells [34] and in rat ovaries [35]. Our finding of low levels of eotaxin expression in the cyclic CL is consistent with these findings. However, since the gene and protein expressions of eotaxin were much higher in the pregnant CL than in the cyclic CL, eotaxin may play a role in regulating luteal function during pregnancy, especially at the mid-pregnant stage. On the other hand, lymphotactin mRNA expression was significantly lower in the CL during the gestation period than in the CL during the estrous cycle (Table 2, Fig. 1B), but its protein level in the CL at days 40–45 and 150–160 of pregnancy was comparable to that of the cyclic CL (Fig. 2A). We could not find an appropriate explanation for this phenomenon. The discrepancy between the expression of lymphotactin mRNA and the concentrations of lymphotactin in the pregnant CL might be due to posttranslational processing that involves transcription, translation, protein storage and ultimately secretion. Otherwise, another explanation for the discrepancy might be that it is due to an increase in the accumulation of free lymphotactin, which could not bind to its receptors, in the mid-pregnant CL. Although the source cells of eotaxin and lymphotactin in the CL are still unclear, endothelial cells and immune cells might be the sources, as they are sources of MCP-1 and other chemokines [28]. Our finding that binding sites of eotaxin and lymphotactin are present in the CL (Figs. 1D–1E and 2B) suggests that these chemokines and their receptors are involved in immune regulation and recruitment of leukocytes or endocrine regulation in the bovine CL during pregnancy.

The microarray analysis demonstrated that the gene expressions of FGF-1 and IGFBPs in the pregnant CL were different from their expressions in the cyclic CL (Table 2). The function of IGF is modulated by IGFBP. Growth factors including FGF, IGF and vascular endothelial growth factor (VEGF) are potent regulators of luteal function in cows [36]. Growth factors regulate both the
formation and the regression of the CL in many species [37, 38], but they may have other functions in the pregnant CL that have not yet been identified. Our results show that collagen and fibronectin genes in the CL are expressed more weakly at days 150–160 of pregnancy than in the estrous cycle (Table 2). Furthermore, the gene expressions of six intraluteal factors including PGER3, proenkcephalin and oxytocin in the pregnant CL differed by more than 10-fold from those in the cyclic CL. Further studies are needed to determine how these substances affect the functional and morphological properties of the CL during pregnancy in cows.

This study investigated gene expression profiles of the bovine CL from days 20 to 160 of pregnancy. Recent studies clearly demonstrated that the expression of interferon-stimulated gene 15 (ISG15) is upregulated by interferon-t (IFNT) in the bovine [39, 40] and ovine [41] CL before day 20 of pregnancy. IFNT, a pregnancy recognition hormone in ruminants, is produced by mononuclear trophoectoderm cells of conceptuses at a critical time to prevent regression of CL function. The concentration of bioactive IFNT was found to be higher in uterine venous blood than in uterine arterial blood on day 15 of pregnancy in ewes [41]. This suggests that IFNT could move into the ovarian artery from the uterine vein by a countercurrent mechanism and act on the CL function on that day. Bovine IFNT, which is secreted between days 12 and 38 of pregnancy, also prevents secretion of luteolytic pulses of PGF2α by uterine epithelia and blocks which is secreted between days 12 and 38 of pregnancy, also prevents

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