Regulated C-C motif ligand 2 (CCL2) in luteal cells contributes to macrophage infiltration into the human corpus luteum during luteolysis

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Running title: Regulated CCL2 expression in the regressing human CL

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ABSTRACT:

Intense macrophage infiltration is observed during luteolysis in various animals including women; however, we still do not know how macrophage infiltration into the human corpus luteum (CL) during luteolysis is regulated. In this study, we examined the expression, localization, and regulation of an important chemokine for the recruitment of monocyte/macrophage lineages, C-C motif ligand 2 (CCL2), in the human CL across the luteal phase and in cultured human luteinized granulosa cells (LGCs) with special reference to the number of infiltrating macrophages and luteal cell function. CCL2 mRNA increased in the non-functional regressing CL during menstruation (P<0.01), corresponding to an elevated mRNA expression of a macrophage-derived cytokine, TNF, and an increased number of infiltrating macrophages positively stained with a macrophage marker, CD68. CCL2 protein was immunohistochemically localized to the cytoplasm of granulosa-lutein and theca-lutein cells, and CCL2 mRNA was significantly reduced by hCG both in vivo (P<0.05) and in vitro (P<0.01). CCL2 was also down-regulated by luteotrophic prostaglandin (PG) E (P<0.0001), but up-regulated by luteolytic PGF (P<0.05) in vitro. Administration of TNF significantly enhanced the CCL2 mRNA expression in cultured LGCs (P<0.01). More abundant infiltrating macrophages were found around granulosa-lutein cells lacking 3β-HSD or PGES synthase (PGES) immunostaining. CCL2 mRNA expression was negatively correlated with both HSD3B1 and PGES, suggesting that locally produced progesterone and PGE suppress macrophage infiltration into the CL. Taken together, the infiltration of macrophages in the human CL is regulated by endocrine and paracrine molecules via regulation of the CCL2 expression in luteal cells.

Key words: macrophage / CCL2 / human corpus luteum / hCG / PGE / progesterone
Introduction

The corpus luteum (CL) is a temporary endocrine gland formed from the ovarian follicle following ovulation that produces large amounts of progesterone essential for the establishment and maintenance of early pregnancy. During a non-fertile cycle in women, the CL matures structurally and functionally by the mid-luteal phase and then begins to regress in a process called luteolysis. On the other hand, if conception succeeds, the CL is rescued from luteolysis and maintains its function during early pregnancy. Although the molecular mechanisms of luteolysis and luteal rescue are not yet fully understood, human chorionic gonadotropin (hCG) secreted from the developing conceptus is known to be a key regulator of luteal rescue by preventing the CL from undergoing luteolysis (Duncan et al., 1998a).

Invasion of macrophages into the CL is a well-known phenomenon of luteal regression in a variety of mammals, and there is convincing evidence that the number of invading macrophages increases as luteolysis proceeds (Hume et al., 1984; Bagavandoss et al., 1988; Lei et al., 1991; Brännström et al., 1994; Best et al., 1996; Takaya et al., 1997; Penny et al., 1999; Komatsu et al., 2003). Infiltrating macrophages into the regressing CL is likely to be involved in phagocytosis of dying luteal cells and their remnants, and in the production of luteolytic cytokines such as tumor necrosis factor α (TNF), oxygen radicals, or substances that inhibit steroidogenesis (Pate and Keyes, 2001; Shirasuna et al., 2012). In most tissues, macrophages are recruited as monocytes from the systemic circulation and subsequently undergo a process of differentiation and activation for phagocytosis. The monocyte recruitment is facilitated by the coordinated intercellular adhesive interactions and the local elaboration of leukocyte-specific chemotactic factors. C-C motif ligand 2 (CCL2), also known as
monocyte chemoattractant protein-1 (MCP-1), is a chemokine that plays a role in the recruitment of monocytes and macrophages. An increased CCL2 expression has been shown in the regressing CL of rats (Townson et al., 1996), cows (Penny et al., 1999 Townson et al., 2002), pigs (Hosang et al., 1994), rabbits (Krusche et al., 2002), and women (Senturk et al., 1999). CCL2 has been reported to be produced by a variety of cell types including endothelial cells, fibroblasts, monocytes, and T lymphocytes (Yoshimura and Leonard, 1990). However, the production and regulation of CCL2 and cell types expressing it in the human CL remains to be elucidated.

HCG can bind to its receptor, lutrophin-choriogonadotrophic hormone receptor (LHCGR) expressed on luteal cells, and activation of this receptor promotes the production of progesterone and luteotrophic prostaglandin (PG) E. Since we previously reported that hCG prevents macrophage infiltration into the human CL (Duncan et al., 1998b), we hypothesized that hCG controls macrophage infiltration by down-regulating CCL2 expression in the human CL. However, we still do not know how CCL2 expression is regulated in the human CL. In this study, we analyzed the change in the mRNA expression of CCL2 in the human CL across the luteal phase, with reference to the number of infiltration of macrophages and the function of luteal cells, especially focused on the ability for progesterone and PGE synthesis. We also examined the regulation of CCL2 expression by endocrine and paracrine molecules which regulate luteal function, using cultured human luteinized granulosa cells (LGCs).
Materials and Methods

Ethics

All experiments using human tissues and cells were approved by the Lothian Medical Research Ethics Committee and Hokkaido University Research Ethics Committee, and all women gave informed consent.

Reagents

Reagents were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA) unless otherwise stated. Insulin-Transferrin-Selenium (ITS)-X was obtained from Life Technologies Inc. (Carlsbad, CA, USA). Matrigel is a product of BD Biosciences (San Jose, CA, USA).

Collection of the human CL

Human CLs were collected at the time of surgery from women undergoing hysterectomy for benign conditions. The menstrual stages of the CLs were determined by endometrial morphology and by the concentration of LH in daily urine samples, obtained prior to surgery as reported previously (Dickinson et al., 2008). The collected CLs were halved upon collection; one half was snap-frozen and stored at −80°C, and the other half was fixed with 10% formalin and processed into paraffin embedding. Some women were given daily doubling injections of hCG (Profasi®; Serono Laboratories, Welwyn Garden City, UK), starting at 125 IU from 7 days after the LH surge for 5–8 days until surgery. This regimen has been shown to rescue the CL and mimic the hormonal changes of early pregnancy (Dickinson et al., 2008). Only frozen tissues of the rescued CL were available from hCG-administrated women.
**Human LGC culture**

Follicular aspirates were obtained from patients undergoing assisted conception. Oocytes were removed and the remaining fluid was centrifuged at 1,800 rpm for 10 min, and cells were re-suspended in a culture medium (DMEM/Ham’s F-12 containing 2 mM L-glutamine, 10 unit/mL penicillin, 0.1 mg/mL streptomycin, 2.5 μg/mL amphotericin B, and 1× concentration of ITS-X). Cell suspension was layered over 45% Percoll in culture medium mixture, and centrifuged at 1,200 rpm for 30 min to pellet blood cells. LGCs, visible in the interface, were collected by a pipette and washed three times with Dulbecco’s phosphate buffered saline supplemented by MgCl₂ and CaCl₂.

One hundred-thousand viable cells were seeded on Matrigel-coated 24 well plates and cultured with 1 mL of the culture medium at 37°C in 5% CO₂ in air. The culture medium was changed every two days, and cells were treated on day 6 of culture for 24 hours with either hCG (100 ng/mL), PGE (10 ng/mL), TNF (10 ng/mL), PI3-kinase inhibitor (LY294002; 20 μM, Cell Signaling Technology Inc., Danvers, MA, USA), Protein kinase C (PKC) activator (Phorbol 12-myristate 13-acetate: PMA; 10 ng/mL, Merck Millipore), or cAMP/Protein kinase A (PKA) inhibitor (H89; 10 μM, Merck Millipore, Nottingham). Other cells were treated with TNF (10 ng/mL) on culture day 2 or day 10, and PGF (10 ng/mL) on culture day 10 for 24 hours. For hCG withdrawal experiment, LGCs were treated with hCG (1 ng/mL) from the next day of plating for continuous 6 days, changed to the media without hCG on day 7, and collected daily until day 11. Each experiment was performed in duplicate and repeated at least three times.
Quantitative RT-PCR (qRT-PCR)

The CLs used for a quantitative gene expression analysis were classified as early-luteal (1–5 days after the LH surge, n=4), mid-luteal (6–10 days, n=6), late-luteal (11–14 days, n=6), menstrual phase (over 14 days, n=3), and rescued by in vivo hCG injection (n=4).

Total RNA was extracted from the frozen human CLs or cultured LGCs using RNeasy Mini Kit (Qiagen Ltd., Crawley, UK) following the manufacturer’s protocol. RNA (200 ng) was used to prepare cDNA using the TaqMan Reverse Transcription regents (Applied Biosystems, Foster City, CA, USA).

Primers used for qRT-PCR analysis are listed in Table 1. Primers were pre-validated by standard PCR and by generating standard curves using qRT-PCR. Each reaction buffer contained 5.0 µL 2×PowerSYBR® Green PCR Master Mix (Applied Biosystems), 0.5 µL primer pair (5 µM), 3.5 µL of nuclease free H₂O, and 1.0 µL cDNA, and each reaction was conducted in duplicate. The qRT-PCR cycling program consisted of a denaturing step (95°C for 10 min), annealing and extension step (95°C for 15 sec and 60°C for 1 min repeated for 40 cycles), and a dissociation step (95°C, 60°C, and 95°C for 15 sec each) using a 7900 Sequence Detection System (Applied Biosystems).

The relative expression levels of each target to the housekeeping gene (glucose-6-phosphate dehydrogenase: G6PDH), previously validated using geNorm analysis (Primerdesign Ltd, Southampton, UK), were quantified using the ΔCt or ΔΔCt methods.

Immunohistochemistry

Fixed human CLs at the mid-luteal (n=4), late-luteal (n=3), and menstrual phases (n=3) were used for an immunohistochemical analysis. The tissues were dehydrated through
ethanol and embedded into paraffin according to the conventional method. Tissue
sections (5 μm thick) were de-waxed and washed twice in distilled water, then antigen
retrieval was performed for 1 minute in 0.01 M citrate buffer (pH 6.0) using a pressure
cooker. After washing twice in phosphate-buffered saline (PBS), the sections were
incubated with 3% hydrogen peroxide for 20 min followed by Avidin/Biotin blocking
solution (Vector Laboratories Inc., Burlingame, CA, USA) for 15 min each. After
pretreatment with normal goat serum for 60 min at room temperature, they were
incubated with mouse anti-human CD68 (1:100; PG-M1, Dako UK Ltd.,
Cambridgeshire, UK), mouse anti-human CCL2 (1:100; MCA2486, AbD serotec,
Raleigh, NC, USA), rabbit anti-3β-HSD (1:1,000; kind gift from Prof. Ian Mason, The
University of Edinburgh), or rabbit anti-human prostaglandin E synthase (PGES; 1:250;
160140, Cayman Chemical, Ann Arbor, MI) in goat serum at 4°C overnight. Control
sections were incubated with non-immune serum and the disappearance of
immunoreaction was confirmed. The specificity of the immunoreaction for CCL2 was
confirmed using CCL2-transfected HEK293 cells (Supplementary Figure 1).

After washing twice in PBS, the sections were incubated with biotinylated
anti-mouse or anti-rabbit IgG (1:500; Vector laboratories Inc.) for 60 min at room
temperature. The reaction sites were visualized using a Vectastain ABC Elite kit
(Vector Laboratories Inc.) for 60 min followed by ImmPACT™ DAB Peroxidase
Substrate Kit (Vector Laboratories Inc.) for 1 min. The sections were counterstained
with haematoxylin and observed under a light microscope (BX51; Olympus corporation,
Tokyo, Japan). Photographs taken from the CD68-immunostained sections were
analysed in Image J (http://imagej.nih.gov/ij/) and the number of CD68-positive cells in
the CL parenchyma was calculated.
Dual staining for macrophages and apoptotic cells

Some sections after the reaction with anti-CD68 antibody overnight were washed with PBS and incubated with AlexaFluor 594-labeled anti-mouse IgG (1:200; Life technologies Japan, Tokyo, Japan) for 2 hours room temperature. Apoptotic cells were detected in these sections using ApopTag Fluorescein in situ apoptosis detection kit (Merck Millipore, Nottingham, UK) according to the manufacturer’s direction. Stained sections were observed under a confocal laser scanning microscope (FV300; Olympus, Tokyo, Japan).

Statistical analyses

After testing for normality, all statistical analyses were performed using unpaired t-tests or one-way ANOVA with pairwise comparison using GraphPad Prism 6 software (GraphPad Software Inc., San Diego, CA, USA), and P<0.05 was regarded as significant. Values in the graphs represent mean ± SEM.
Results

Macrophage increases in number during luteal regression

Macrophages infiltrating into the human CL were identified by immunohistochemistry for a human macrophage marker, CD68. In the functional CL at the mid-luteal phase, CD68-positive infiltrating macrophages tended to localize to the border between granulosa-lutein cells and theca-lutein cells at the periphery of the CL, and scattered within the granulosa-lutein cell layer (Fig. 1A). In the CL at the late-luteal phase, abundant CD68-positive macrophages accumulated around the border between granulosa-lutein cells and theca-lutein cells, and were more dispersed within the granulosa-lutein cell layer (Fig. 1B). The regressing CL collected during menstruation contained much more CD68-positive macrophages throughout the CL parenchyma, especially in the granulosa-lutein cell layer (Fig. 1C); this increase was significant when CD68-positive cells were counted in the sections ($P<0.05$; Fig. 1D). These data would suggest stimulated macrophage chemotaxis into the CL parenchyma as luteolysis progresses.

The chemokine CCL2 increased during luteolysis

We next analyzed the localization and expression of a monocyte/macrophage chemokine, C-C motif ligand 2 (CCL2). CCL2 protein was localized to the cytoplasm of both granulosa-lutein and theca-lutein cells (Fig. 1E) and vascular endothelial cells were also weakly immunoreactive for CCL2 (arrow in Fig. 1E). In contrast, non-steroidogenic cells distributed in the parenchyma of the CL were negative for CCL2 (asterisks in Fig. 1E). The staining pattern and intensity of CCL2 protein did not alter during menstrual phase. On the other hand, the mRNA expression of CCL2 in the
human CL was particularly conspicuous in the regressing CL collected after 
menstruation had commenced (P<0.01; Fig. 1F) where abundant infiltrating 
macrophages were observed (Fig. 1C, D). Luteal rescue using exogenous hCG in vivo to 
mimic the changes of early pregnancy significantly decreased the expression of CCL2 
when compared to the late-luteal phase in the absence of hCG (P<0.05; Fig. 1G).

CCL2 is regulated by hCG and prostaglandins in luteal steroidogenic cells in vitro
Cultured human luteinized granulosa cells (LGCs) also expressed CCL2. In vitro 
treatment of hCG decreased the mRNA abundance of CCL2 (P<0.01; Fig. 2A) and the 
withdrawal of hCG promoted its expression (P<0.01; Fig. 2B). Luteotrophic PGE also 
suppressed the CCL2 expression (P<0.0001; Fig. 2C) while luteolytic PGF enhanced its 
expression in LGCs (P<0.05; Fig. 2D).

TNF expression correlates with CCL2 expression
TNF is a macrophage cytokine and its mRNA expression in the human CL peaked in the 
regressing CL collected during menstruation (P<0.01; Fig. 2E). The expression of TNF 
was highly correlated with CCL2 expression in the human CL (r=0.9774, P<0.0001; Fig. 
2F). Like CCL2, in vivo hCG treatment to rescue the CL from luteolysis reduced the 
luteal TNF expression (P<0.05; Fig. 2G). In cultured LGCs, TNF significantly increased 
the expression of CCL2 (P<0.01; Fig. 2H).

Signal transduction cascades responsible to regulate CCL2 in LGCs
HCG binds to LHCGR on luteal cells and is known to activate cAMP/PKA pathway. 
Among four types of PGE receptors (EP1-4), EP2 is predominantly expressed in LGCs
and the binding of PGE to EP2 activates cAMP/PKA pathway. The receptor for PGF acts through PKC pathway. There are two types of TNF receptors, TNFR1 and TNFR2, and the latter, which activates PI3 kinase pathway, is predominantly expressed in LGCs. To reveal the signal transduction cascades responsible to the expression of CCL2 in LGCs, we treated cells with inhibitors for PI3 kinase (LY294002) and PKA (H89) or an activators for PKC (PMA). As expected, PMA enhanced and LY294002 suppressed the expression of CCL2 in LGCs ($P<0.05$ and $P<0.0001$; Fig. 2I and J), suggesting an involvement of PKC or PI3 kinase pathways in the regulation of CCL2 expression. Unexpectedly, H89 treatment significantly reduced the expression of CCL2 in LGCs ($P<0.01$; Fig. 2K).

**Macrophage density correlates to steroidogenic cell function**

Light microscopic observation revealed that the degree of macrophage accumulation in the regressing CL was not even, showing clear regional variations (Fig. 3A). When serial sections were stained with 3β-HSD, a key enzyme for progesterone synthesis, an intimate relationship between the accumulation of infiltrating macrophages and 3β-HSD immunoreactivity in granulosa-lutein cells was observed (Fig. 3A–D). As we reported previously (Nio-Kobayashi et al., 2014), granulosa-lutein cells in the regressing human CL collected during menstruation can be classified into intact (i), pyknotic (p), and degenerating (d) cells according to the staining intensity of 3β-HSD and structural characteristics (Fig. 3B). Regions with intact granulosa-lutein cells contained few CD68-positive macrophages (“i” in Fig. 3A, B). More abundant macrophages were seen in regions with pyknotic luteal cells with condensed nuclei and fragmented cytoplasm possessing 3β-HSD immunoreactivity (“p” in Fig. 3A–D). Macrophages were more
numerous in regions with 3β-HSD-negative degenerating granulosa-lutein cells (“d” in Fig. 3A–D). Dual staining for CD68-positive macrophages and apoptotic cells clearly demonstrated an accumulation of CD68-positive infiltrating macrophages in the region occupied with pyknotic (apoptotic) and degenerating granulosa-lutein cells (“p” and “d” in Fig. 4A–F) while few CD68-positive cells were located in regions with intact granulosa-lutein cells (“i” in Fig. 4A–C).

Healthy granulosa-lutein cells actively produce a luteotrophic paracrine molecule, PGE. In other set of serial sections of the human CL at late-luteal phase, the areas with marked immunostaining for the PGE synthetic enzyme (PGES) (arrow in Fig. 5A) had low concentrations of CD68-positive macrophages (arrow in Fig. 5B), while areas of the steroidogenic cell layer with many macrophages (asterisk in Fig. 5B) displayed low levels of PGES immunoreactivity (asterisk in Fig. 5A).

The qRT-PCR analysis demonstrated that CCL2 mRNA expression in the CL tended to be inversely correlated with the expression of PGES (r=−0.3899, P=0.0892; Fig. 5C) as well as HSD3B1 (r=−0.4592, P<0.05; Fig. 5D).

TNF is not associated with a loss of steroidogenic cell function

We finally investigated whether macrophage influx was a cause of a loss of steroidogenic function in granulosa-lutein cells. The addition of 10 ng/mL TNF to LGCs on culture day 6 promoted, rather than inhibited, the expression of a key regulator of steroidogenesis, STAR (P<0.01; Fig. 6A). TNF also enhanced the expression of synthetic enzymes for luteotrophic PGE, COX2 (P<0.0001; Fig. 6B) and PGES (P<0.01; Fig. 6C), while reducing the key enzymes involved in luteolytic PGF synthesis,

Aldo-keto reductase family 1 member C1 (AKR1C1; P=0.0503; Fig. 6D) and AKR1C3
The effect of TNF on the expression of these genes did not change when cells were treated with TNF on culture day 2 or day 10 (Supplementary Figure 2), suggesting that the age of luteal cells did not influence the luteotrophic action of TNF.
This study demonstrated an increased mRNA expression of \textit{CCL2} in the regressing CL of women, associated with an increased number of infiltrating macrophages into the CL and a loss of progesterone and luteotrophic PGE synthesis in luteal cells. The number of infiltrating macrophages is abundant in regions consisting 3β-HSD- or PGES-negative granulosa luteal cells. The regional difference in the number of infiltrating macrophages in the human CL suggests an intimate correlation between luteal cell function and macrophage influx into the CL. Increased number of infiltrating macrophages in the human CL is associated with an increased mRNA expression of \textit{CCL2}, an important chemokine for the recruitment of macrophages. We also revealed here the regulatory mechanism of \textit{CCL2} expression in human luteal cells. Luteotrophic molecules, hCG and PGE, down-regulate the mRNA expression of \textit{CCL2} in luteal cells but luteolytic PGF promoted its expression. On the other hand, a macrophage cytokine, TNF, strongly enhanced the \textit{CCL2} expression in luteal cells possibly enhancing further macrophage influx into the CL. These data suggest that macrophage infiltration is one of the key events for human luteolysis, and \textit{CCL2} regulated by endocrine or paracrine molecules plays an important role in the regulation of macrophage infiltration into the human CL.

Macrophages are major immune cells in the CL and known to possess bimodal roles in the regulation of CL function (Wu \textit{et al.}, 2004). It has been reported that macrophages may facilitate the establishment of the vasculature or an increase progesterone production in the functional CL. The present study confirmed that TNF acts in a luteotrophic manner by enhancing the expression of the enzymes involved in the production of progesterone and PGE, and this action did not alter depending on the age of LGCs. In contrast, Shakil and Whitehead (1994) reported that progesterone
secretion is markedly inhibited by co-culturing granulosa cells with peritoneal macrophages. They also noted that degree of the inhibition was dependent on both the number and activation status of the co-cultured macrophages. The number of macrophages in the CL is known to be increased during luteolysis in various animals. In the present study, we confirmed an increased number of macrophages in the regressing human CL collected during menstruation, suggesting its contribution to luteolysis. Although infiltrating macrophages augmented in the CL at late-luteal phase, more marked accumulation is observed in the CL collected during menstruation. This suggests that an accumulation of macrophages is likely important for the structural luteolysis rather than the functional luteolysis. We also revealed in this study that the degree of an accumulation of infiltrating macrophages was different depending on the region in the regressing human CL: Few macrophages were observed in the region occupied with intact granulosa-lutein cells intensely expressing 3β-HSD, whereas abundant macrophages occupied the region with apoptotic or degenerating granulosa luteal cells (Fig. 4G). More abundant macrophages were observed in degenerating cell areas than in pyknotic/apoptotic cell areas, suggesting an intimate correlation of the number of infiltrating macrophages and loss of luteal cell function. The factors that regulate the number of infiltrating macrophages in the CL are therefore of particular importance in the control of luteal structure and function.

CCL2 is a chemokine that has a potent chemoattractant effect on monocytes and macrophages. An increased expression of CCL2 in the regressing CL was previously reported in various animals including women (Hosang et al., 1994; Townson et al., 1996; Penny et al., 1999; Senturk et al., 1999; Townson et al., 2002; Krusche et al., 2002). In the human CL, Senturk et al. (1999) reported that the number of macrophages
and the expression of CCL2 were highest in the CL at mid-follicular phase. This largely corresponds to our qRT-PCR analysis showing an increased CCL2 expression in the human CL collected during menstruation. Because the number of infiltrating macrophages was significantly increased in the CL at menstruation, these data strongly suggest an involvement of CCL2 in the macrophage infiltration into the CL during luteolysis.

CCL2 is produced by a variety of cell types including endothelial cells, fibroblasts, monocytes, and T lymphocytes. The cellular source of CCL2 in the CL has been reported as endothelial cells of women (Senturk et al. 1999) and cows (Townson et al. 2002), or T lymphocytes of cows (Penny et al. 1998) by immunohistochemistry. On the other hand, Hosang et al. (1994) reported the significant mRNA expression of CCL2 in porcine luteal steroidogenic cells. Using immunohistochemistry we clearly demonstrated that CCL2 protein is localized to the cytoplasm of steroidogenic cells, namely granulosa-lutein and theca lutein cells. Although a weak immunoreactivity was found in endothelial cells, non-steroidogenic cells distributed in the CL parenchyma were negative in reaction. Because our analysis of cultured human LGCs showed significant expression of CCL2 mRNA, it is reasonable to consider that luteal cells are major cellular source of CCL2 in the human CL.

The present study for the first time revealed the regulation of CCL2 mRNA expression by known endocrine and paracrine factors in the human CL and cultured human LGCs. HCG significantly decreased the mRNA expression of CCL2 in the rescued CL in vivo and in cultured LGCs in vitro. In our previous study, administration of hCG to mimic early pregnancy in women resulted in the reduction of the number of infiltrating macrophages into the CL (Duncan et al., 1998b). These findings suggest that
hCG negatively regulates macrophage infiltration via down-regulating the $CCL2$ expression in luteal cells. We clearly showed for the first time the suppressive effect of PGE on the expression of $CCL2$ in LGCs. PGE is one of the luteotrophic molecules secreted from luteal cells by stimulation of LHCGR. In contrast, PGF, a known luteolytic molecule in various animals, significantly enhanced the $CCL2$ expression in LGCs. Up-regulation of $CCL2$ expression by PGF was previously reported in the CL of sheep and cows (Tsai et al., 1997). We also showed that a macrophage cytokine, TNF, induced the $CCL2$ expression in LGCs, suggesting a possible acceleration of infiltrating macrophages by macrophages themselves. PKC and PI3 kinase pathways, possibly activated by PGF or TNF under the physiological condition, seem to be responsible to up-regulate the expression of $CCL2$ in LGCs. Although H89 is generally used as a potent PKA inhibitor, this regent is known to inhibit other signaling pathway such as Rho-associated kinase and ERK1/2 (Murray, 2008). Because ERK1/2 pathway is known to be involved in the up-regulation of CCL2, the suppressive effect of H89 on CCL2 expression may be through an inhibition of ERK1/2 pathway. Taken together, CCL2 is a possible luteolytic molecule which is negatively regulated by luteotrophic molecules but positively controlled by luteolytic molecules.

Although hCG secreted from the conceptus plays an important role in the promotion of luteal function, we still do not know how luteolysis is initiated in a non-fertile cycle. Herein we demonstrated that the number of macrophages was different depending on the region of the CL. The number of infiltrating macrophages is fewer in the regions occupied with healthy granulosa-lutein cells that actively produce progesterone and PGE, suggesting that progesterone and PGE are involved in the suppression of macrophage infiltration. This idea is supported by the previous report
showing that the progesterone negatively regulates CCL2 expression in choriodecidual cells and a breast cancer cell line T47D (Kelly et al. 1997), and the present data showing the suppressive effect of PGE on the expression of CCL2 in human LGCs. As mentioned above, PGE has a luteotrophic effect to enhance the production of progesterone and PGE secretion from LGCs. This suggests that if luteal cells can maintain to produce PGE, the expression of CCL2 in luteal cells and the following macrophage infiltration into the CL could be suppressed. The capacity of the PGE synthesis in luteal cells in response to luteotrophic stimuli may be a key function to rescue the human CL from luteolysis.

In conclusion, we revealed here a possible contribution of CCL2 to macrophage infiltration into the CL during luteolysis. The expression of CCL2 in luteal cells is suppressed by luteotrophic hCG and PGE, but enhanced by luteolytic PGF and a macrophage cytokine TNF. The ability of luteal cells to produce progesterone and PGE may be important to control the local number of infiltrating macrophages. Although our in vitro experiments may not reflect luteal cells under the structural luteolysis which was seen in vivo during menstruation, CCL2 secreted from luteal cells contributes to the regulation of macrophage infiltration into the human CL.
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Authors’ roles

Study concept and designs of the experiments: JN-K and WCD, Acquisition of data: JN-K, Analysis and interpretation of the data: JN-K, Collecting follicular fluids: MK and NS, Establishment of CCL2-transfected cells: SK, Drafting of the manuscript: JN-K, WCD, and TI.

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Conflict of interest

None declared.
References


Figure Legend

**Figure 1 Expression of CCL2 in the human CL is correlated to the number of infiltrating macrophages.** The CD68-positive infiltrating macrophages are distributed at the border of granulosa-lutein (g) and theca-lutein cells (t) and dispersed in the CL parenchyma at mid-luteal (ML) phase (A). CD68-positive macrophages tend to increase in the CL at late-luteal (LL) phase (B). Numerous CD68-positive cells are found in the CL collected during menstruation (Men), and the number of CD68-positive macrophages is significantly increased in the regressing CL at this stage (C, D). CCL2 protein is localized in the cytoplasm of granulosa-lutein and theca-lutein cells in the human CL at ML phase (E). Endothelial cells are weakly immunoreactive for CCL2 (arrows in E) while non-steroidogenic cells distributed in the CL parenchyma are negative in reaction (asterisks in the insert of E). The mRNA expression of CCL2 peaks in the CL collected during menstruation (F) and reduced by *in vivo* administration of hCG (G). EL: early-luteal phase, R: the CL rescued from luteolysis by *in vivo* administration of hCG. *P<0.05, **P<0.01.

**Figure 2 Regulation of CCL2 mRNA expression in cultured LGCs.** The mRNA expression of CCL2 is significantly decreased by the treatment of hCG *in vitro* (A). Withdrawal of hCG significantly enhances the CCL2 expression in LGCs on day 8 and 9 (B). Luteotrophic PGE decreases the expression of CCL2 (C) whereas luteolytic PGF promotes its expression (D). A macrophage cytokine, TNF, significantly increases in the CL collected during menstruation (E) and its expression is positively correlated to the expression of CCL2 in the human CL (F). The expression of TNF is reduced in the rescued CL by *in vivo* administration of hCG (G). CCL2 expression is promoted by
TNF treatment in LGCs *in vitro* (H). PKC activator (PMA) significantly enhanced the expression of *CCL2* (I) while inhibitors for PI3 kinase (LY294002: LY) and PKA (H89) suppressed its expression (J, K). Cont: control, DMSO: dimethylsulfoxide used as a vehicle for PMA, LY, and H89. EL: early-luteal, EtOH: ethanol used as a vehicle for prostaglandins, LL: late-luteal phase, Men: menstruation, ML: mid-luteal phase, R: rescued CL by *in vivo* administration of hCG. *P<0.05, **P<0.01, ****P<0.0001.

**Figure 3** Regional difference in the number of CD68-positive macrophages in the structurally regressing CL during menstruation. Granulosa-lutein cells in the CL at menstrual phase are classified into three different conditions: intact (i), pyknotic (p), and degenerating (d) by the observation of two serial sections stained with either CD68 (A, C) or 3β-HSD (B, D). Intact cell area contains few numbers of CD68-positive macrophages (A) but intensely immunostained for 3β-HSD, a key enzyme for progesterone production (B). Pyknotic cells contain intense 3β-HSD immunoreactivity (B) and there are abundant CD68-positive macrophages in this area (A). Numerous CD68-positive macrophages are found in degenerating cell area lacking in 3β-HSD immunoreactivity (A–D). C and D are closer views of the areas enclosed by squares in A and B, respectively. Arrows in C and D show the condensed nuclei of pyknotic granulosa-lutein cells. Asterisks are theca-lutein cells. Men: menstruation.

**Figure 4** Dual staining for CD68 and apoptotic cells in the regressing human CL at menstrual phase. Dual staining for CD68 (red) and apoptotic cells (green) clearly demonstrates the difference in the degree of macrophage infiltration depending on the condition of granulosa-lutein cells (A–F). There are few CD68-positive macrophages in
intact cell area (i) (A–C) while abundant macrophages are found in the area occupied
with pyknotic/apoptotic cells (p) (A–F). Marked accumulation of CD68-positive
macrophages is observed in degenerating cell area (d) where contains few number of
apoptotic cells (D–F). G is a schema showing the relationship between
immunoreactivity for 3β-HSD in granulosa-lutein cells and the number of infiltrating
macrophages. Arrowheads in A-F represent the border of the different areas. +: positive,
−: negative.

Figure 5 Relationship between the function of luteal cells and macrophage
infiltration. Two serial sections of the human CL at the late-luteal phase were
immunostained for a PGE synthase (PGES) (A) or a macrophage marker (CD68) (B).
Healthy luteal cells contained abundant immunoreactivity for PGES (arrow in A) and
there are few number of CD68-positive macrophages around them (arrow in B).
Abundant CD68-positive cells are found in the region consisted with PGES-negative
granulosa-lutein cells (asterisks in A, B). The mRNA expression of PGES (C) and
HSD3B1 (D) tends to be negatively correlated to that of CCL2 in the human CL.

Figure 6 Effect of TNF on the expression of genes related to progesterone and
prostaglandin synthesis. TNF treatment on culture day 6 enhances the expression of
STAR (A), COX2 (B), and PGES (C), key enzymes for progesterone and luteotrophic
PGE synthesis. On the other hand, TNF tends to decrease the expression of AKR1C1
(D) and significantly reduces the expression of AKR1C3 (E), which are involved in the
synthesis of luteolytic PGF. Cont: control. **P<0.01, ****P<0.0001.
### Table 1. Primers used for qRT-PCR analysis

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Figure 1 Nio-Kobayashi et al.
Figure 2 Nio-Kobayashi et al.
Figure 3 Nio-Kobayashi et al.
Figure 4 Nio-Kobayashi et al.
Figure 5 Nio-Kobayashi et al.
Figure 6 Nio-Kobayashi et al.