Effects of Tumor Necrosis Factor α and Interferon γ on the Viability and mRNA Expression of TNF Receptor Type I in Endothelial Cells from the Bovine Corpus Luteum

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Abstract. The corpus luteum (CL) is mainly composed of luteal steroidogenic cells (LSCs) and luteal endothelial cells (LECs). Cell death of LSCs and LECs is essential for structural luteolysis. Therefore, it is important to understand the mechanisms regulating cell death in both types of luteal cells. We previously reported that a treatment combining tumor necrosis factor α (TNF) and interferon γ (IFNG) induced cell death in LSCs and that LECs express TNF receptor type I (TNFRI). To investigate the mechanism of cell death in LECs, in the present study we determined the effects of the same cytokines on cell viability and TNFRI mRNA expression in cultured LECs. To induce cell death in LECs, LECs were treated with TNF or IFNG (0, 0.05, 0.5, 1.0, 2.5 nM) and a combination of TNF (0.5 nM) and IFNG (0.5 nM) for 24 h. The viability of LECs was reduced by a single treatment with TNF or IFNG dose-dependently (P<0.05). Cell viability was further decreased by treatment with a combination of TNF and IFNG (P<0.05). Cells with DNA fragmentation (TUNEL-positive cells) were observed after the treatment with TNF and IFNG. Semi-quantitative RT-PCR analysis revealed that treatment with IFNG alone or in combination with TNF increased the expression of TNFRI mRNA compared with the control (P<0.05). In summary, TNF and IFNG increased cell death in cultured bovine LECs. The upregulation of TNFRI mRNA expression by IFNG suggests that TNF and IFNG synergistically affect the death of LECs resulting in acute luteolysis.

Key words: Bovine, Cell death, Corpus luteum, Luteal endothelial cell, Luteolysis

The corpus luteum (CL) is a transient organ that forms from the wall of a Graafian follicle following ovulation and secretes progesterone (P4) [1]. It reaches structural and functional maturity by the mid luteal phase and then begins to regress after Day 17 post-ovulation of a non-fertile cycle. In cows, luteal regression is characterized by a reduction in P4 production (functional luteolysis) and tissue degeneration by apoptosis (structural luteolysis) [2, 3].

Development of the bovine CL is associated with intensive angiogenesis [4], so that the mature CL becomes one of the most highly vascularized organs in the body [5–7], and vascular endothelial cells account for up to 50% of the total cells of the mid CL [8, 9]. Recently, capillaries without smooth muscle and a few blood vessels with smooth muscle were found in the center of the mid bovine CL [10], and we reported that the capillaries disappeared, but not the large blood vessels during luteolysis [11]. Luteal endothelial cells (LECs) are the first cells to undergo programmed cell death (apoptosis) during luteal regression, resulting in the loss of capillaries [12, 13].

The number of leukocytes in the bovine CL (e.g., T lymphocytes, macrophages) increases at the time of luteolysis [14], and leukocytes are known to produce a variety of cytokines, including tumor necrosis factor α (TNF) and interferon γ (IFNG). A combination of IFNG and TNF has been shown to induce DNA fragmentation and reduce the viability in bovine luteal steroidogenic cells (LSCs), which suggests that TNF and IFNG are responsible for apoptotic cell death in the CL during structural luteolysis [15]. LECs express TNF receptor type I (TNFRI) [16], which is involved in most of the TNF effects. TNF induces programmed cell death in LECs [17]. IFNG induces TNFR expression in extra-ovarian cells in humans [18], rats [19] and mice [20]. Based on these findings, we hypothesized that TNF and IFNG induce apoptosis in LECs synergistically by regulating TNFRI expression. To test this hypothesis, we investigated the effects of TNF and IFNG on cell death and TNFRI mRNA expression in bovine LECs.

Materials and Methods

Bovine LEC isolation and cell culture

LECs were isolated from the CL at the mid-luteal phase (days 8–12 of the estrous cycle) [21, 22] using magnetic beads as described previously [23, 24] and validated in our laboratory [24, 25]. Briefly, magnetic tosylactivated beads (Dynabeads M-450, 140.04; Dynal ASA, Oslo, Norway) were coated with 0.15 mg/ml lectin from Bandeiraea simplicifolia (BS-1; L2380; Sigma-Aldrich, St. Louis, MO, USA), which specifically binds the glycoproteins expressed by bovine ECs [23].

A mixed population of luteal cells obtained after tissue dispersion and CL perfusion was suspended in PBS with 0.1% BSA (w/v), mixed with beads (5 beads for each endothelial cell) at a con-
concentration of $4 \times 10^4$ beads/ml, and incubated for 20 min at 4°C on a rocking platform. More than 80% of the cells in the cell suspension were LECs. The BS-1 positive cells were washed with PBS containing 0.1% BSA and concentrated using a magnet until the supernatant was free of BS-1 negative cells. The BS-1 positive cells were subsequently eluted by 0.1 M fucose (F2252; Sigma-Aldrich) solution in PBS.

LEC s were seeded (1 $\times 10^4$ cells/cm²) in 75-cm² culture flasks (658175; Greiner Bio-One GmbH, Frickenhausen, Germany) precoated with 0.01% rat tail collagen for 2 h at room temperature. The cells were cultured in EC growth medium (MV 2; C22121; PromoCell, Heidelberg, Germany). The MV 2 was diluted 1:9 in culture medium consisting of 1:1 DMEM/F-12 (D/F; D8900; Sigma-Aldrich) supplemented with 10% (v/v) calf serum (C6278; Sigma-Aldrich) and 2 μg/ml amphotericin B (A9528; Sigma-Aldrich) until the cell cultures formed small colonies. The cells were cultured at 37°C in a humidified atmosphere of 5% CO₂ in air. The medium was changed every 2 days. Only colonies with a homogeneous cell population were gently scraped off using an Eppendorf pipette tip, removed with a pipette and transferred into 15 ml conical tubes (188271; Greiner Bio-One). The selected cells were centrifuged with 10 ml D/F at 1,000 rpm and 4°C for 10 min. The cells were cultured in collagen-coated 25-cm² culture flasks (690175; Greiner Bio-One). The cultures and passages were repeated until a homogeneous population of pure ECs was obtained. The ECs isolated in this study were confirmed to have originated from bovine CLs by immunocytochemical staining for rabbit anti-human von Willebrand factor (factor-VIII, F3520; Sigma-Aldrich) and CD31 expression as previously reported [23, 25].

The LECs were removed from the 25-cm² flasks after 5 min incubation with 0.02% trypsin (T4799; Sigma-Aldrich) solution and then cultured until the cells reached confluence. For each passage, the cell suspension was split into three portions. One portion was seeded in 75-cm² culture (1 $\times 10^6$ viable cells/ml; 3860-096; IWAKI) for determination of the dose-dependent effect of TNF or IFNG and a combination of TNF (0.5 nM) and IFNG on LEC viability. The second portion was seeded in 96-well plates (1 $\times 10^5$ ml/; 662160; Greiner Bio-One) for 24 h of culture, the viability of the cells was determined by a Dojindo Cell Counting Kit including WST-1 (Dojindo, Kumamoto, Japan, No. 345-06463) as described previously [15, 26]. Briefly, WST-1, a kind of MTT (3-(4, 5-dimethyl-2-thiazoyl)-2, 5-diphenyl-2H-tetrazolium bromide), is a yellow tetrazolium salt that is reduced to formazan by live cells containing active mitochondria. The culture medium was replaced with 100 ml D/F medium without phenol red, and a 10-ml aliquot (0.3% WST-1, 0.2 mM 1-methoxy PMS in PBS, pH 7.4) was added to each well. The cells were then incubated for 4 h at 37°C. The absorbance was read at 450 nm using a microplate reader (Model 450; Bio-Rad, Hercules, CA, USA). In this assay, data were expressed as percentages of the appropriate control values.

**TUNEL and propidium iodide labeling**

The dispersed LECs were seeded at 1.0 $\times 10^6$ viable cells in 1 ml on glass slides in six-well cluster dishes (Sumitomo Bakelite, Tokyo, Japan, No. MS-80060). After the cells became confluent, the medium was replaced with fresh medium. The cells were then exposed to TNF and IFNG (0.5 nM) for 24 h. After 24 h of culture, the cells were washed twice in 1 ml PBS (Seikagaku, Tokyo, Japan, No.05193). The cells were fixed for 1 h at room temperature in PBS containing 4% paraformaldehyde and then washed twice in PBS before permeabilization with 0.5% Triton X-100 (Bio-Rad) in PBS for 20 min. Cells were then briefly washed twice in PBS. The cells were incubated in 30 ml of fluorescein-conjugated dUTP and terminal deoxynucleotidyl transferase (TUNEL; MBL, Nagoya, Japan, No. 8445) for 1 h at 37°C in a dark, moist chamber. After the TUNEL reaction, the cells were washed twice in PBS and once in PBS containing 0.002% propidium iodide (PI; Sigma, No. P4170). Then, the cells were washed three times in PBS and stored in the dark at 4°C. The cells were observed under fluorescent illumination using a 470-nm excitation filter and a 515-nm absorption filter for fluorescein isothiocyanate (FITC) and a 545-nm excitation filter and a 610-nm absorption filter for PI.

**Expression of TNFRI mRNA**

Total RNA was prepared from cultured LECs using Isogen (Nippon Gene, Toyama, Japan) according to the manufacturer’s directions. One microgram of each total RNA was reverse-transcribed using a First-strand cDNA Synthesis Kit (Pharmacia Biotech, Tokyo, Japan, No. 27-9261-01), and one-tenth of the reaction mixture was used in each polymerase chain reaction (PCR) using specific primers for bovine TNFRI and β-actin (ACTB), respectively.

The reverse transcription (RT)-PCRs were carried out with the housekeeping genes, ACTB, as an internal standard. The sequence of the TNFRI primers, 5'-AGAAGCTTCACTGGGAGCAAGCC-3' (5' primer, 20 mer) and 5'-CTCCTACGGAACATTCTGTTG-3' (3' primer, 20 mer), were synthesized according to bovine TNFRI cDNA (GenBank accession number U90937), and these primers generated a specific 304-base pair (bp) product from all cell types. The primers for ACTB were 5'-GAAGAGTCTGTCACCAAC-3' (5' primer, 18 mer) and 5'-AGAAGCTCACAGGGACACG-3' (3' primer, 19 mer). These primers generated a specific 189-bp product from all cell types. Each PCR yielded only a single amplification product. The PCR products were carried out using an Ampl-
iTaq Gold DNA Polymerase (Perkin Elmer, Foster City, CA, No. N888-0240) and a thermal cycler (Takara TP240, Tokyo, Japan). The conditions for the PCRs were as follows: after activation of the DNA polymerase by incubation for 7 min at 94°C, 28 (TNFRI) or 26 (ACTB) cycles of reactions including denaturation for 1 min at 94°C, annealing for 1 min at 60°C and extension for 2 min at 72°C were performed, followed by an additional extension for 5 min at 72°C. The PCR amplification was calibrated to determine the optimal number of cycles that would allow detection of the appropriate mRNA transcripts while still keeping amplification of these genes in the log phase. A two-fifths aliquot of each reaction mixture was electrophoresed on a 1.5% agarose gel containing ethidium bromide with a known standard (100-bp Ladder; New England BioLabs, Beverly, MA, USA, No. N3231S) and photographed under ultraviolet illumination. The band intensities were analyzed by computerized densitometry using NIH Image (National Institutes of Health, Bethesda, MD, USA). This method allowed only a relative quantification.

**Effect of cytokines on TNFRI mRNA expression in cultured bovine LECs**

The dispersed LECs were seeded at 1.0 × 10^6 viable cells in 1 ml, in 24-well culture dishes (Costar, Cambridge, MA, USA, No. 3524). After the cells became confluent, the medium was replaced with fresh medium. The cells were then exposed to 0.5 nM recombinant human TNF and/or 0.5 nM recombinant bovine IFNG for 24 h. After the 24 h of culture, total RNA was prepared from the cells.

**Statistical analysis**

All experimental data are shown as means ± SEM. The statistical significances of the viability of LECs and the expression of TNFRI mRNA were assessed by ANOVA followed by a Fisher protected least significant difference procedure (PLSD) as a multiple comparison test.

**Results**

**Dose-dependent effect of TNF and IFNG on cell viability in LECs**

A single treatment of LECs with TNF (0.05, 0.5, 1.0 and 2.5 nM) or IFNG (0.05, 0.5, 1.0 and 2.5 nM) reduced cell viability dose-dependently. Cell viability was decreased to approximately 70% by a single treatment with 0.5 nM TNF or 0.5 nM IFNG compared with the control (Figs. 1 and 2; P<0.05). Cell viability was further decreased by the combination of TNF (0.5 nM) and IFNG (0.5 nM) for 24 h (Fig. 3; P<0.05).

**Effects of TNF and/or IFNG on DNA fragmentation and TNFRI mRNA expression**

Staining with PI showed fewer nuclei after treatment with TNF and IFNG than in the control (Fig. 4A and B). Although there were no TUNEL-positive cells in the control, some TUNEL-positive cells appeared after treatment with TNF and IFNG (Fig. 4C and D). ACTB was used to normalize TNFRI mRNA expression. The expression of TNFRI mRNA significantly increased in LECs treated with IFNG alone or IFNG in combination with TNF (Fig. 5; P<0.05).

**Discussion**

Luteolysis is essential to reset the ovarian cycle so that the animal returns to estrus and has another opportunity to become pregnant. We recently reported that blood capillaries disappeared in the regressing bovine CL, although blood vessels with smooth muscle were maintained [11]. The loss of capillaries has been...
thought to be due to programmed cell death (apoptosis) of LECs [12, 13]. Since one of the most important roles of capillaries is the supply of oxygen to cells [26], the loss of microcapillaries may cause the CL micro-environment to be in a low oxygen (hypoxia) condition. Hypoxia has been shown to reduce P4 production and cell viability in cultured LSCs [27]. Thus, the loss of luteal blood capillaries by death of LECs should be essential for luteolysis. In the present study, TNF and IFNG increased cell death in cultured LECs. Since TUNEL-positive cells were observed after cytokine treatment, the cell death induced by these cytokines in the present study seems to be apoptosis. These results suggest that TNF and IFNG induce cell death not only in LSCs [28] but also LECs and that the latter leads to loss of luteal blood capillaries.

In the present study, cell death was clearly induced in LECs by a single treatment of TNF with a concentration of more than 0.05 nM for 24 h. However, in our previous study, TNF (2.9 nM) did not induce cell death even if LSCs were exposed to TNF for 48 h [29]. The fact that a single treatment with a lower concentration of TNF induced cell death in LECs but not in LSCs indicates that LECs are more sensitive to TNF than LSCs and suggests that there are different mechanisms of TNF action on cell death between LECs and LSCs. The different sensitivities of each cell type to TNF may be due to the difference in secretory products between LECs and LSCs. Progesterone secreted by LSCs has been shown to inhibit cell death in both LECs [17] and LSCs [29]. On the other hand, endothelin-1 (ET-1), a vasoactive peptide secreted by LECs, induces luteolysis in the bovine CL [30, 31]. Furthermore, since TNF stimulates ET-1 secretion in cultured LECs [16], the cell death of LECs induced by cytokines in the present study may be partly due to the effect of ET-1 stimulated by TNF.

LEC have been reported to produce TNF in response to IFNG stimulation [32]. In the present study, TNF and IFNG had a synergistic effect on death of LECs. This effect may be due to an
increase of TNF production by LECs stimulated by IFNG. However, there was no significant difference in cell viability of LECs between treatment with 1.0 nM TNF and 2.5 nM TNF in the present study. Thus, there seem to be other factors induced by IFNG to stimulate TNF-induced cell death further.

There are two types of TNFR, type I and type II (TNFRII), and they have different physiological roles. TNFRI is involved in most TNF effects such as proliferation and injury of tumor cells and antiviral activity, although TNFRII has weaker affinity for TNF than TNFRII [33]. TNFRI is expressed in bovine LECs [16] and in bovine LSCs [17]. IFNG has been found to induce expression of TNFR and Fas, which is a member of the TNFR family, in some types of cell [18–20, 28]. Therefore, we hypothesized in the present study that IFNG upregulates TNFRI expression, resulting in an increased effect of TNF on cell death. As expected, expression of TNFRI mRNA was induced by treatment with IFNG. This result suggests that IFNG is an important modulator of TNFR and that the synergistic effect of the cytokines on the death of LECs is due to increased expression of TNFRI induced by IFNG. These findings suggest that TNF and IFNG induce the loss of luteal blood capillaries resulting in acute luteolysis. Because IFNG alone also reduced the viability of LECs dose-dependently, LECs seem to have an IFNG receptor. However, the IFNG receptor and its regulation have not been reported in LECs. Further studies are needed to determine the relevance of IFNG receptor regulation in LECs and cell death.

The overall results suggest that TNF and IFNG play important roles in the loss of luteal cell capillaries during luteolysis by inducing cell death of LECs and that these cytokines synergistically affect the death of LECs resulting in acute luteolysis.

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References