CHANGES IN THE FUNCTION AND DENSITY OF LUTEAL VASCULATURE THROUGHOUT THE ESTROUS CYCLE AND ITS REGULATORY MECHANISM IN CATTLE

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PREFACE

The experiments described in this dissertation were carried out at the Graduate School of Natural Science and Technology (Doctor Course), Okayama University, Japan, from April 2009 to September 2011, under the supervision of Professor Kiyoshi OKUDA.

This dissertation has not been submitted previously in whole or in part to a council, university or any other professional institution for a degree, diploma or other professional qualifications.

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CONTENTS

LIST OF FIGURES AND TABLES

ABSTRACT

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The corpus luteum (CL) formed from the wall of an ovulated follicle is one of the most highly vascularized organs in the body and luteal blood vessels seem to be essential for the CL function during the estrous cycle and to initiate the luteolytic process. However, the changes of blood vessels in the CL throughout the estrous cycle and during luteolysis induced by prostaglandin F2α (PGF) administration remain unclear.

In cows, luteal regression is characterized by a reduction in progesterone (P4) production (functional luteolysis) and tissue degeneration by apoptosis (structural luteolysis). Although cytokines such as tumor necrosis factor α (TNF) and interferon γ (IFNG) are well known to induce apoptosis in luteal steroidogenic cells, their effects on apoptosis in bovine luteal endothelial cells (LECs) are not well understood.

In the present study, to clarify the physiological relevance of luteal blood vessels in the regulation of luteal function during the estrous cycle and specifically during luteal regression, I investigated (1) the changes in the density and number of luteal blood vessels, with and without smooth muscle, throughout the estrous cycle and after administration of a PGF analogue in cattle, and (2) the effects of TNF and IFNG on apoptosis in LECs.

(1) CLs at the early (Days 2-3 post-ovulation), developing (Days 5-7), mid- (Days 8-12), late (Days 15-17), and regressed (Days 19-21) stages were collected. In addition, CLs were collected by colpotomy from 12 cows (Day 10 after ovulation), i.e. non-treated (n=3, 0 h, control) at 0.5 (n=3), 2 (n=3) and 12 h (n=3) after a luteolytic dose of PGF. Immunohistochemical staining with von Willebrand Factor (specific for endothelial cells) revealed that the density of luteal blood vessels was significantly higher at the developing and the late luteal stages ($P<0.05$) than at the other stages, whereas the number of larger blood vessels (stained with α-smooth muscle actin) was higher at the late and regressed luteal stages ($P<0.05$) than at the other stages. Furthermore, both the density of blood vessels and the number of blood vessels with smooth muscle were significantly higher in the CL obtained at 2 h and 12 h after PGF

administration ($P<0.05$) than in those without PGF treatment. These results demonstrate that the capillaries disappeared earlier than the large blood vessels during structural luteolysis, and suggest that (i) the number of blood vessels with smooth muscle per unit area in the regressing CL increased as a result of losing steroidogenic cells and capillaries, and (ii) the loss of capillaries in the CL results in a reduced supply of nutrients and oxygen to luteal cells followed by cell death.

(2) To investigate the mechanism of cell death in LECs, I examined the cell viability and mRNA expression of *TNFRI, FAS, BAX, BCL2, CASP8, CASP9* and *CASP3* in LECs cultured for 24 h 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). 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. Real-time RT-PCR analysis revealed that treatment with IFNG alone or in combination with TNF, but not treatment with TNF alone, increased the expression of *TNFRI* and *CASP3* mRNA compared with the control ($P<0.05$). In summary, TNF and IFNG increased cell death in cultured bovine LECs. The different effects between TNF and IFNG on the expression of apoptosis signals suggest that TNF and IFNG independently activate different pathways for inducing apoptosis, and that they synergistically contribute to luteolysis by accelerating the disappearance of capillaries.

CHAPTER 1

GENERAL MATERIALS AND METODOLOGY

Chemicals

Recombinant human tumor necrosis factor α (TNF) was kindly donated by Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan) and recombinant bovine interferon γ (IFNG) was kindly donated by Dr. S. Inumaru, NIAH (Ibaraki, Japan). Dalbecca modified Eagle medium (DMEM; No. 1152) and a culture medium DMEM & Ham's F-12 (1:1 [v/v]; No. D8900) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Calf serum (CS; No. 16170-078) and gentamicin (No. 15750-060) were purchased from Gibco BRL (Grand Island, NY, USA). Collagenase (No. 45-0041-97) was purchased from Worthington Biochemical Corporation (Lakewood, NJ, USA). Deoxyribonuclease 1 (DNase 1; NO. 63-0016-00) was purchased from Biozyme Laboratories (Blaenavon, UK).

Animal tissue collection

For determination of the vascular changes throughout the estrous cycle, ovaries with CL of Holstein cows were obtained from a local abattoir in accordance with protocols approved by the local institutional animal care and use committee. Ovaries were obtained within 30 min after exsanguination and were transported to the laboratory within 1-1.5 h on ice. Luteal stages were classified as being early (Days 2–3 after ovulation), developing (Days 5-7), mid (Days 8–12), late (Days 15–17), and regressed (Days 19–21) by macroscopic observation of the ovary and uterus as described previously [1, 2].

Animal procedures were approved by the local institutional animal care and use committee in Olsztyn, Poland (Agreement No.5/2007, 6/2007 and 88/2007). Healthy, normally cycling Polish Holstein Black and White cows were used for collecting the CL. The estrus of the cows was synchronized by two time injections of an analogue PGF2α (dinoprost, Dinolytic; Upjohn – Pharmacia N.V.S.A., Belgium) with an 11-day interval. The ovulation was determined by a veterinarian via *per rectum* USG examination using DRAMINSKI ANIMAL profit Scanner (Draminski Electronics in Agriculture, Olsztyn, Poland, www.draminski.com). To determine the effect of PGF2α on luteal vasculature, the ovaries were collected from 12 cows through colpotomy using Hauptner's effeninator (Hauptner & Herberholz GmbH & Co. KG, Solingen, Germany) on Day 10 post-ovulation, i.e. non-treated (0 h, control, n=3) and at 0.5 (n=3), 2 (n=3) and

12 h (n=3) after injecting a luteolytic dose of PGF2α analogue (cloprostenol; Bioestrophan, Biowet, Gorzow Wielkopowski, Poland).

CLs were enucleated from the ovary and dissected free of connective tissue. Tissue samples were fixed in 10% (v/v) neutral phosphate buffer formalin (pH 7.0) for 24 h at room temperature and then embedded in paraffine wax. Serial section of 4 μm were mounted on to glass microscope silanized slides (Dako).

Immunohistochemistry

Immunohistochemical staining was done by the avidin-biotin peroxidase complex (ABC) method [1]. The sections were dehydrated in a graded series of ethanol, cleared in xylene, embedded in paraffin, and treated with 0.3% H_2O_2 in methanol for 10 min at room temperature to inactivate endogenous peroxidase. The slides were then washed in 0.01 M PBS (pH 7.4). After treatment with normal goat serum (2%) for 30 min at room temperature, the sections were incubated with a polyclonal von Willebrand factor antibody (vWF: diluted 1 : 1000; 228160; Lab Vision Corporation, California, USA), which is a marker of endothelial cells, and a monoclonal α-smooth muscle actin antibody (ASM-1: 65001; PROGEN Biotechnik GmbH, Heidelberg, Germany), which is a marker of smooth muscle, overnight at 4℃. As a negative control, the sections were incubated with goat anti-rabbit IgG overnight at 4℃. After incubation, the sections were washed in PBS, incubated for 30 min at room temperature with biotinylated goat anti-rabbit IgG (diluted 1 : 200; Vector Laboratories Inc, Burlingame, CA, USA) for vWF and negative control and with biotinylated goat anti-mouse IgG (diluted 1 : 200; Vector Laboratories Inc, Burlingame, CA, USA) for ASM-1, and then washed in PBS. Horseradish peroxidase (HRP)-conjugated ABC (Vector Laboratories, Inc., Burlingame, CA) combined with secondary antibody was applied to tissue slides at room temperature for 30 min. The binding sites were visualized with 0.02% 3,3'-diaminobenzidine tetrahydrochloride (DAB) in 50 mM Tris-HCl (pH 7.4) containing 0.02% H_2O_2. After immunohistochemical staining, the sections were lightly counterstained with Mayer's hematoxylin. The sections were washed in DW, dehydrated in graded series of ethanol, and cleared in xylene and coverslipped.

Preparation of Luteal Endothelial Cells

For cell culture, corpus luteum tissue were obtained at the mid luteal stage (Days 8-12). Luteal endothelial cells (LECs) were isolated from the CL at the mid-luteal phase (days 8-12 of the estrous cycle) [2, 3] using magnetic beads as described previously [4, 5] and validated in our laboratory [5, 6]. 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 [4].

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 concentration of 4×10^8 beads/ml, and incubated for 20 min at 4 C on a rocking platform. More 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.

Culture of LECs

The LECs were removed from the 25-cm^2 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-cm^2 culture (1×10^6/ml) flasks for the next passage. The second portion was seeded in 96-well plates (1×10^5/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 (0.5 nM) for 24 h on LEC cell viability.

Reverse Transcription and Real-time PCR

Total RNA was extracted from endometrial tissue and cells using TRIZOL reagent (Invitrogen; 15596-026) according to the manufacturer's directions. One μg of each total RNA was reverse transcribed using a ThermoScript™ RT-PCR System (Invitrogen; 11146-016), and 10% of the reaction mixture was used in each PCR using specific primers for TNF receptor I (TNFRI), FAS, Caspase-3, Caspase-8, Caspase-9, BCL2 and BAX from the bovine sequence (Table 1). Quantification of mRNA expression was determined by QuantiTect™ SYBR Green PCR system (Qiagen GmbH, Hilden, Germany) starting with 2 ng of reverse-transcribed total RNA as described previously [7]. Briefly, *ACTB* mRNA expression was used as an internal control. For quantification of the mRNA expression levels [8], and PCR was performed under the following conditions: 95 C for 15 min, followed by 55 cycles of 94 C for 15 sec, 55 C for 20 sec and 72 C for 15 sec. Use of the QuantiTect™ SYBR Green PCR system at elevated temperatures resulted in reliable and sensitive quantification of the RT-PCR products with high linearity (Pearson correlation coefficient: $r>0.99$).

CHAPTER 2

CHANGES IN THE VASCULATURE OF BOVINE CORPUS LUTEUM DURING THE ESTROUS CYCLE AND PROSTAGLANDIN F2α-INDUCED LUTEOLYSIS.

Introduction

The corpus luteum (CL) is a transient organ that forms from the wall of Graffian follicle following ovulation and secretes progesterone (P4) [9]. It reaches structural and functional maturity by the mid luteal phase and then begins to regress after Day 17 post-ovulation of the nonfertile cycle. Luteal regression in cows is known to be induced by uterine PGF2α until the CL is completely degraded. In cows, luteal regression is characterized by a reduction in P4 production (functional luteolysis) and tissue degeneration by apoptosis (structural luteolysis) [10, 11].

The development of the bovine CL is known to be associated with an intensive angiogenic process. The intensity of this angiogenic process in the developing CL is similar to the intensities of angiogenesis in the fastest growing tumors [12], so that, the mature CL becomes one of the most highly vascularized organs in the body [13-15] and vascular endothelial cells account for up to 50% of the total cells of the mid CL [16, 17]. Furthermore, luteal endothelial cells (LEC) have been known to secrete some vasoactive subsances that directly regulate P4 secretion within the CL [18-20]. Therefore, in order to know the mechanisms regulating the function of bovine CL, it is important to investigate the changes in the luteal vasculature.

Recently, capillaries and a few blood vessels with smooth muscle were found in the center of the mid bovine CL [21]. These two types of blood vessels differ not only structurally but also functionally. Whereas micro capillary blood vessels, which consists of a single layer of LEC attached to a basement membrane, permit exchanges of materials between the blood and the surrounding luteal cells, large blood vessels with a smooth muscle layer are specialized in the transport of hormones, nutrients and metabolites. The changes of both types of blood vessels in the CL throughout the estrous cycle remain unclear.

Administration of a luteolytic dose of PGF2α induces an acute increase in the blood flow followed by luteal regression in the CL of Days 6-17 after ovulation but not in the early CL (Days 1-5 after ovulation) [22, 23]. This vascular change observed after PGF2α injection was more evident in the surface than in the center of the mid CL [24].

In addition, endothelial cells are the first type of cells to undergo apoptosis during luteolysis [25, 26]. However, our recent *in vitro* studies demonstrated that the steroidogenic cells from bovine mature CL don't undergo apoptosis after treatment of PGF2α in the absence of endothelial cells [27], suggesting that the luteal vasculature plays some important roles during luteolysis, and that the luteolytic action of PGF2α is mediated by endothelial cells. However, there is little information about the changes in luteal vasculature after administration of PGF2α in cattle.

The aim of the present study was to investigate the changes in the density and number of blood vessels, with and without smooth muscle, throughout the estrous cycle and after administration of PGF2α in the bovine CL.

Materials and Methods

Quantification methods

To calculate the density of blood vessels (%) and the number of blood vessels with smooth muscle, sections of the center of the CL without connective tissue were selected randomly and examined at ×400 magnification.

An eyepiece grid covered each area (approximately 0.13 mm^2) on the sections. Each crosshair on the grid that coincided with positive immunostaining or a capillary lumen was counted. The percentage of blood vessels in the CL was calculated as X/Y x 100, where X is the total number of points that coincided with positive immunostaining or a capillary lumen and Y is the total number of points on a unit area [28, 29]. The density of the microvessels was expressed as a percentage mean ± SEM. The number of ASM-1-positive lumina was counted as the number of blood vessels with a smooth muscle layer.

Results

Changes in the density of luteal blood vessels

Some large blood vessels were observed between immature luteal cells at the early luteal stage (Figure 1A). The developing luteal stage was characterized by a dense network system of blood vessels (Figure 1B). The density of the blood vessels decreased at the mid luteal stage, and then increased at the late luteal stage (Figures 1C, D). Several large blood vessels were observed in the regressed CL (Figure 1E).

The network of clear blood vessels was less developed in the CL without PGF2α administration (control) than in CL collected at 0.5 h, 2 h and 12 h after PGF2α

administration (Figures 2B, C, D).

The densities of blood vessels at the developing and the late luteal stages were significantly higher than those at the other luteal stages. Interestingly, the density of luteal blood vessels at 0.5 h and 2 h after PGF2α administration was higher than in CL without PGF2α treatment (control) (Figure 3).

Changes in the number of luteal blood vessels with smooth muscle

The number of blood vessels with smooth muscle in the center of the CL between the early and the mid luteal stages was significantly different ($P<0.05$; Figures 4A, B, C). However, the number of blood vessels with smooth muscle significantly increased ($P<0.05$) at the late and regressed luteal stages (Figures 4D, E, 6A).

The number of blood vessels with smooth muscle did not change significantly at 0.5 h after PGF2α administration (Figure 5B), but increased at 2 h ($P<0.05$) and further increased at 12 h ($P<0.05$) after PGF2α administration (Figures 5C, D, 6B). A clear increase in the number of large blood vessels with smooth muscle was observed at 12 h after PGF2α (Figure 5D).

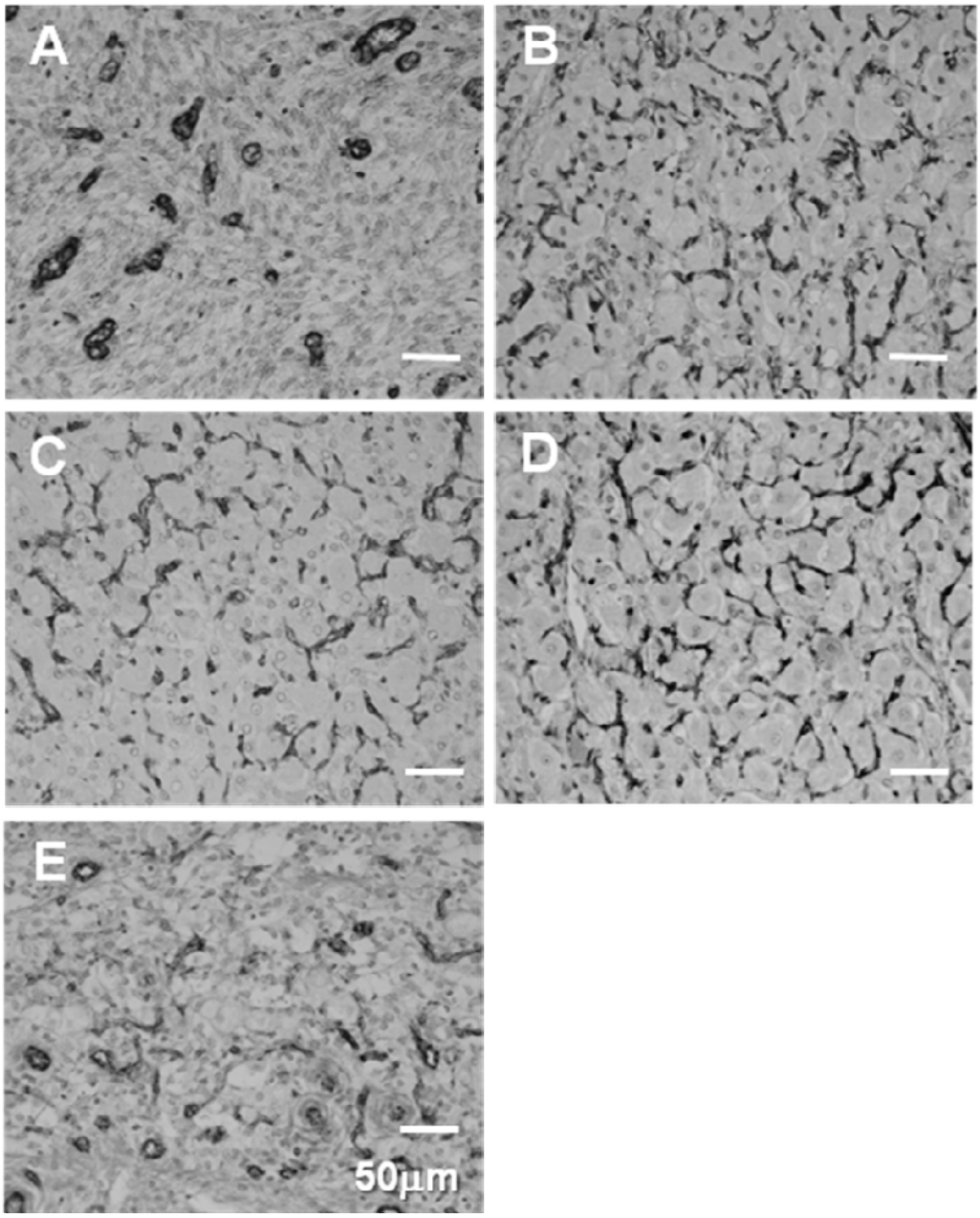


Fig. 1

Representative image of immunohistochemical staining for von Willebrand factor (vWF) in the center of the bovine CL throughout the estrous cycle (early, A; developing, B; mid, C; late, D; and regressed, E luteal stages). Scale bars represent 50 μm.

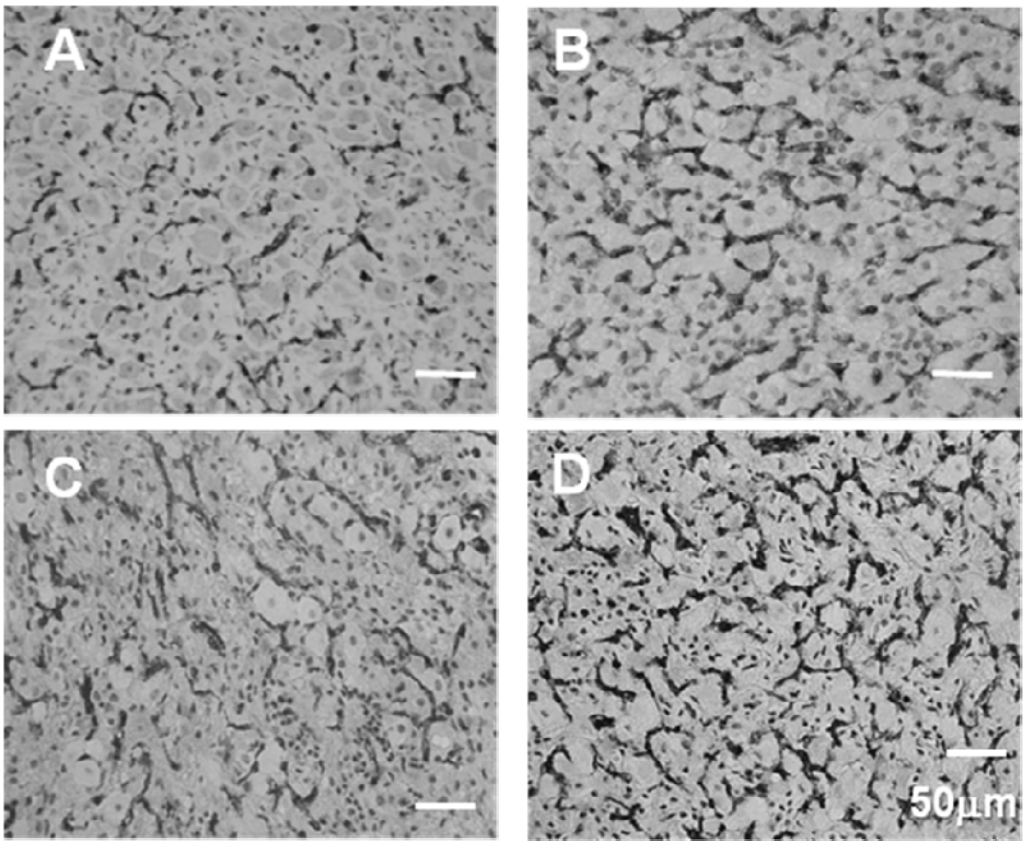


Fig. 2

Representative image of immunohistochemical staining for von Willebrand factor (vWF) just before PGF2α injection (0 h; control: A) and then at 0.5 h (B), 2 h (C) and 12h (D) after the injection at the mid luteal stage. Scale bars represent 50 μm.

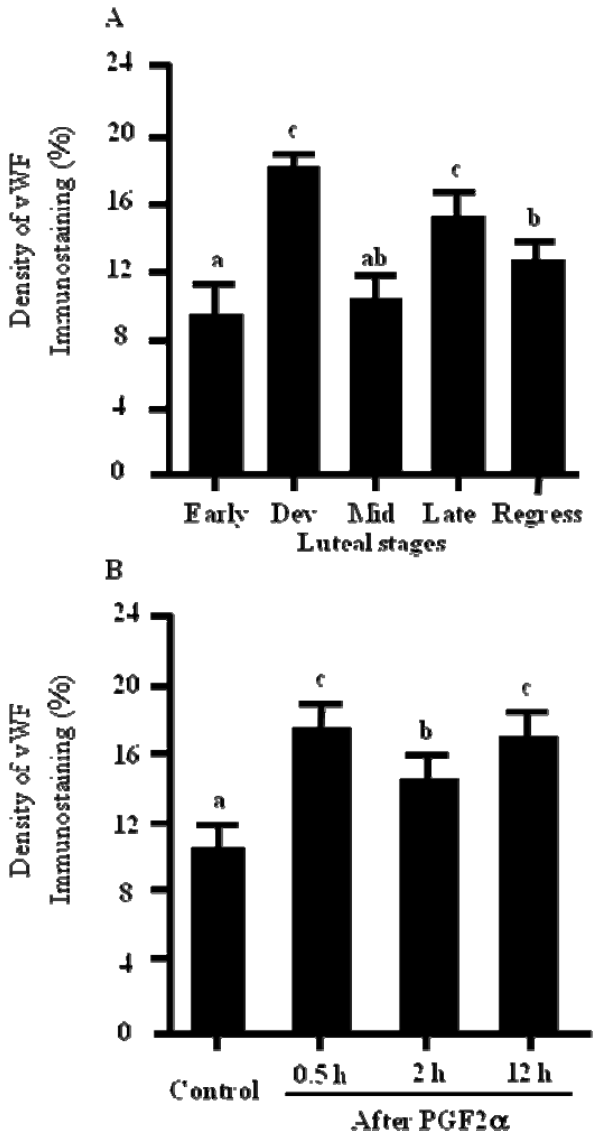


Fig. 3

Changes in the density of blood vessels in the center of the bovine CL during the estrous cycle (A), and after administration of PGF2α (B). The tissue sections were the same as those used in the immunohistochemical study of vWF shown in Fig. 1 and Fig. 2. The density of blood vessels was determined by counting the points on the vWF-positive vessels and dividing by the total number of points per unit area in the histological section at ×400. Counting was done on three randomly chosen areas (microscopic field).

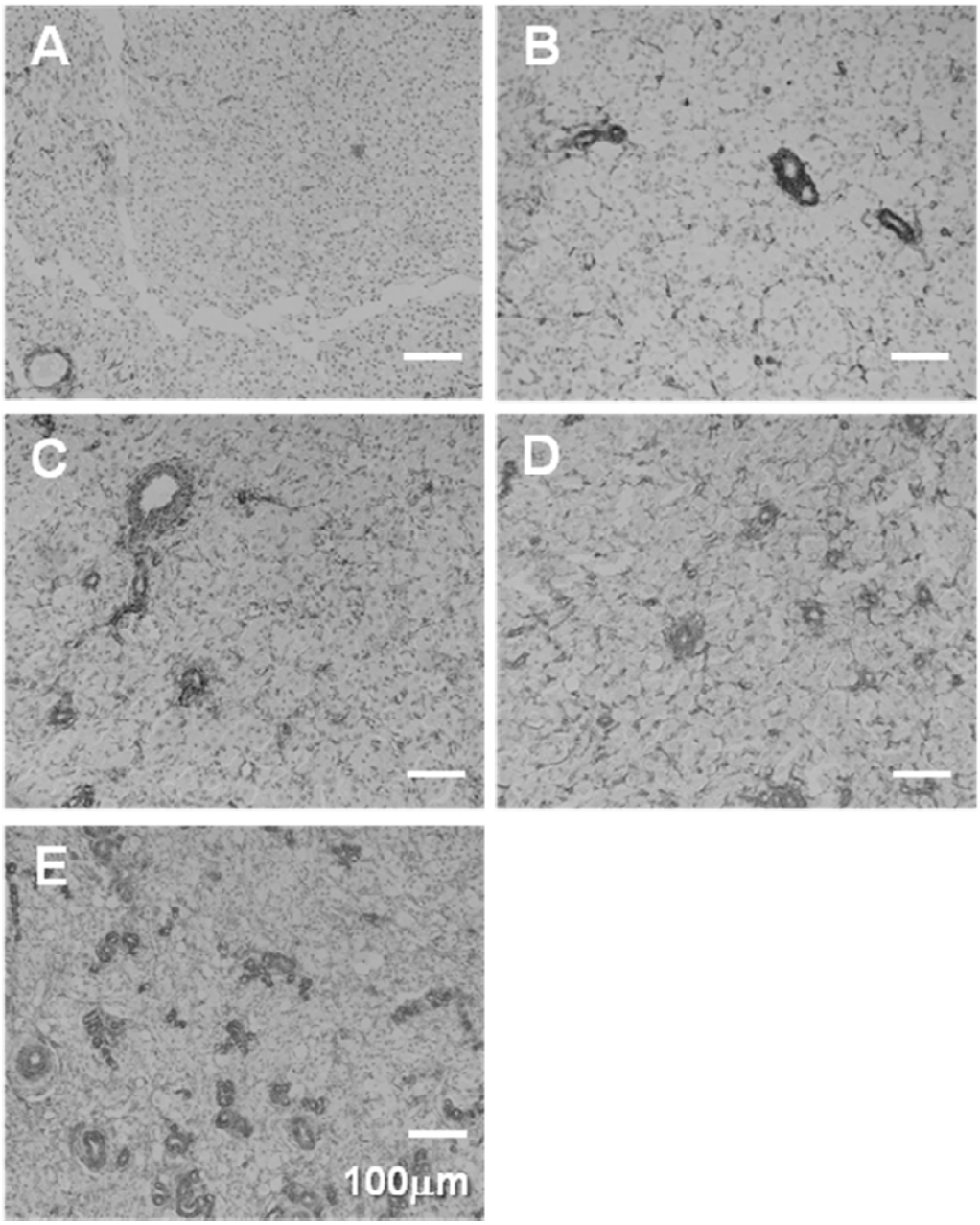


Fig. 4

Representative image of immunohistochemical staining for α-smooth muscle actin (ASM-1) in the center of the bovine CL throughout the estrous cycle (early, A; developing, B; mid, C; late, D; and regressed, E luteal stages). Scale bars represent 100 μm.

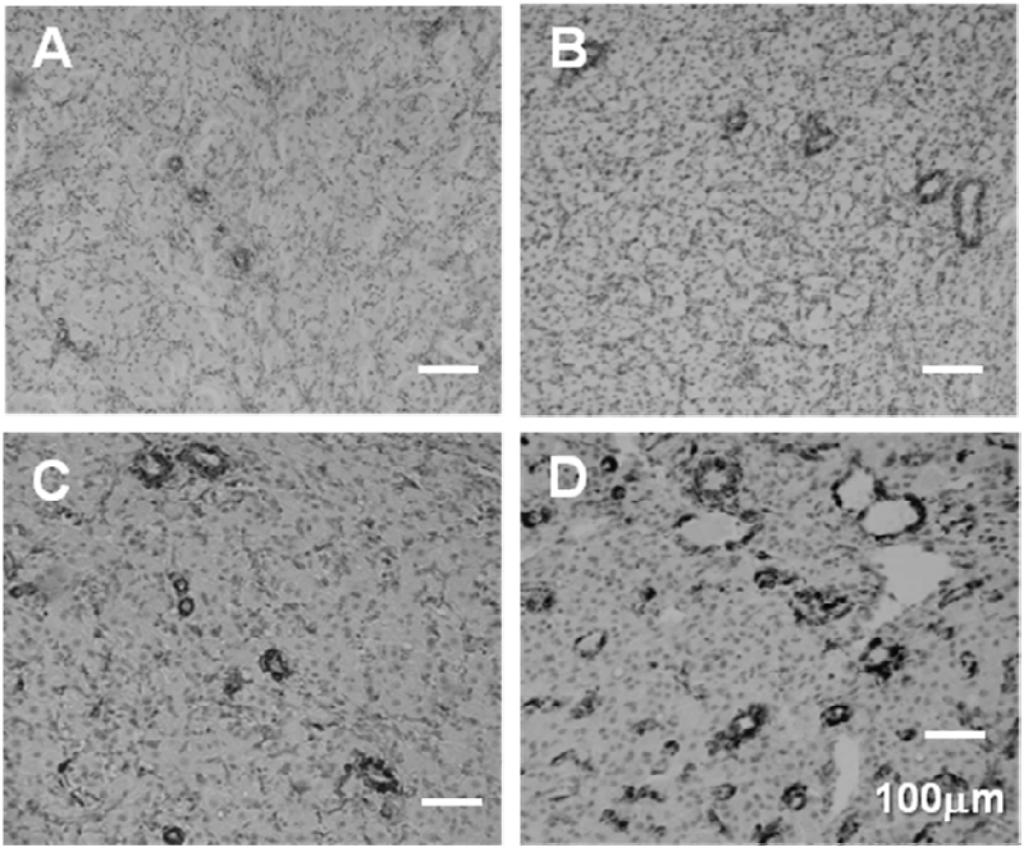


Fig. 5

Representative image of Immunohistochemical staining for α-smooth muscle actin (ASM-1) just before PGF2α injection (control: A) and then at 0.5 h (B), 2 h (C) and 12 h (D) after the PGF2α injection at the mid luteal stage. Scale bars represent 100 μm.

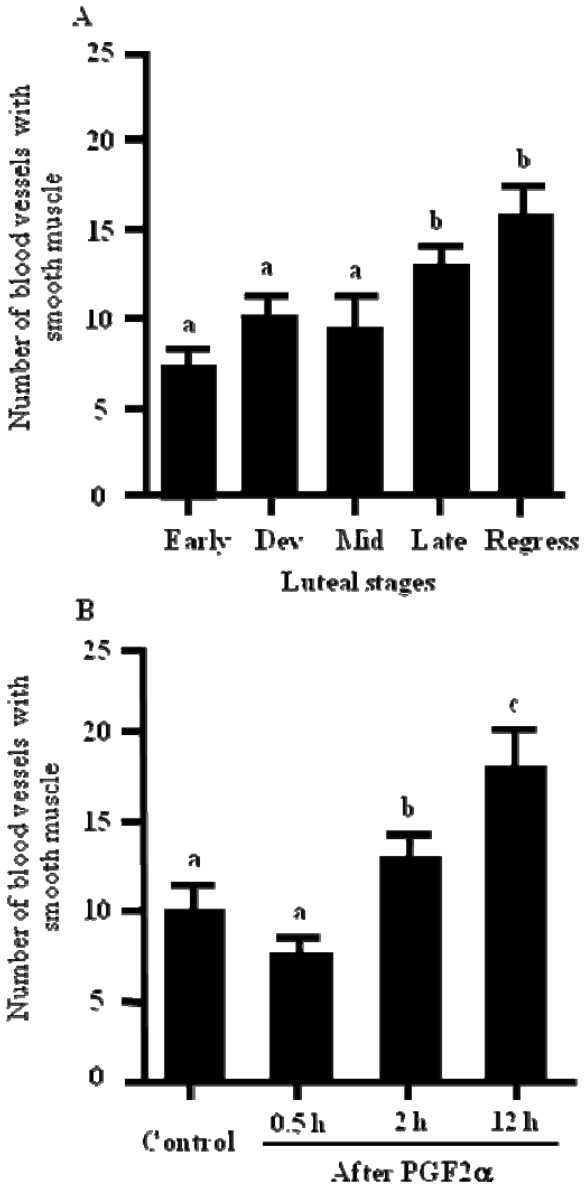


Fig. 6

Changes in the number of blood vessels with smooth muscle in the center of the CL during the estrous cycle (A), and after administration of PGF2α on Day 10 post-ovulation (B). The tissue sections were the same as those used in the immunohistochemical study of ASM-1 shown in Fig. 4 and Fig. 5. The number of blood vessels was determined by counting the number of ASM-1-positive vessels per unit area in the histological section at ×400 magnification. Counting was done on three randomly chosen areas (microscopic fields). Different letters indicate significant differences ($P<0.05$) as assessed by ANOVA followed by a Fisher's protected least significant difference procedure (PLSD) as a multiple comparison test.

Discussion

Angiogenesis has been shown to be necessary for establishing CL and maintaining its function [16]. The CL is one of the most highly vascularized organs in the body [17]. Although the changes in the density and localization of endothelial cells were reported in the bovine CL throughout estrous cycle [30], the changes in luteal microvasculature and large blood vessels with smooth muscle have not been studied [22]. In the present study, the density of blood vessels at the developing and the late luteal stages was higher than at the other stages. Interestingly, vascular density decreased from the developing to the mid luteal stages and then increased at the late luteal stage. The decrease in the density of blood vessels at the mid luteal stage was associated with the maximum volume of the CL and the full growth of luteal steroidogenic cells. Moreover, the number of blood vessels with smooth muscle layer per unit area increased at the late and the regressed luteal stages. In contrast, the density of blood vessels decreased at the regressed luteal stage. Previous studies demonstrated that the number of luteal endothelial cells positively stained with angiopoietin-2 (ANPT-2) antibody increased in the bovine CL at the regressed stage [31], and that infusion of ANPT-2 (100 ng/ml) acutely inhibited P4 release from the late CL [32]. The above findings suggest that a decrease in the density of blood vessels and P4 production in the regressing CL occurs due to a destabilization of capillary blood vessels induced by ANTP-2.

Injecting a cow with PGF2α at the mid luteal phase decreases P4 production and then induces luteal cell death (apoptosis) [33-35]. However, *in vitro* studies demonstrated that PGF2α increases P4 production and cell viability in cultured bovine luteal steroidogenic cells [23, 27]. Interestingly, a decrease in P4 release was observed when bovine luteal steroidogenic cells were co-cultured with endothelial cells and exposed to PGF2α [36]. These findings suggest that vascular components (luteal endothelial cells) and some of their secretory products are crucial for the luteolytic action of PGF2α. In the present study, the density of blood vessels at 0.5 h and 12 h after PGF2α administration was significantly higher than in the untreated CL (control). Injecting cows with a luteolytic dose of PGF2α has been demonstrated to induce an acute increase in blood flow from 0.5 h to 2 h in the periphery of the mature CL, but not in the early CL [22, 24]. Recently, we have demonstrated that PGF2α administration increased the levels of oxygen (pO2) and NO in the ovarian venous blood between 0.5 h and 2 h post-treatment [37]. These findings suggest that the increase of blood vessel density at 0.5 h after PGF2α administration is caused by a vasodilatation, which is

associated with increase in blood supply not only in the periphery but also in the center of the CL.

PGF2α receptors are presented in bovine LEC [38], and PGF2α has been demonstrated to increase nitric oxide (NO) production in endothelial cells [4], which induces luteal cells apoptosis in human [39] and cow [40]. These findings suggest that vascular components including LEC play an important role for luteolysis. Therefore, it is important to determine the changes in the density and number of blood vessels. The increase in the density of blood vessels at 0.5 h and 12 h after PGF2α administration seems to be temporarily associated with the initiation of functional and structural luteolysis, respectively. The present study showed an increase not only in the density of blood vessels but also the number of blood vessels with smooth muscle layer at 12 h after PGF2α administration. These results suggest that the blood vessels with smooth muscle are maintained during functional and structural regression of CL. The number of blood vessels with smooth muscle per unit area might increase as the result of losing steroidogenic cells and capillary endothelial cells in the regressing CL, whereas the density of total endothelial cells decreased.

The overall results demonstrate that capillaries lacking in smooth muscle disappear earlier than do blood vessels with a smooth muscle layer during structural luteolysis, and suggest that the loss of capillaries results in a reduced supply of nutrients to luteal cells followed by luteal cell death.

Summary

To investigate the possible role of the vasculature in the local regulation of corpus luteum (CL) function, we determined the densities of capillaries and large blood vessels in the center of the bovine CL during the estrous cycle and following PGF2α-induced luteolysis. The CLs at the early (Days 2-3 post-ovulation), developing (Days 5-7), mid- (Days 8-12), late (Days 15-17), and regressed (Days 19-21) stages were collected. In addition, the CLs were collected by transvaginal ovariectomy from 12 cows (Day 10 after ovulation), i.e. non-treated (n=3, 0 h, control) at 0.5 (n=3), 2 (n=3) and 12 h (n=3) after a luteolytic dose of prostaglandin (PG) F2α. Immunohistochemical staining with von Willebrand Factor (specific for endothelial cells that are found in both types of blood vessels) revealed that the density of luteal blood vessels was significantly higher at the developing and the late luteal stages ($P<0.05$) than at the other stages, whereas the number of larger blood vessels (those stained with α-smooth muscle actin) was higher at the late and regressed luteal stages ($P<0.05$) than at the other stages. Furthermore, both the density of blood vessels and the number of blood vessels with smooth muscle were significantly higher in the CL obtained at 2 h and 12 h after PGF2α administration ($P<0.05$) than in those without PGF2α treatment. These results suggest that the number of blood vessels with smooth muscle per unit area in the regressing CL increased as the result of losing steroidogenic cells and capillary endothelial cells, whereas the density of total endothelial cells decreased. The overall results demonstrate that the capillaries disappeared earlier than the large blood vessels during structural luteolysis, and suggest that the loss of capillaries in CL results in a reduced supply of nutrients and oxygen to luteal cells followed by cell death.

CHAPTER 3

EFFECTS OF TUMOR NECROSIS FACTOR α (TNF) AND INTERFERON γ ON THE VIABILITY AND mRNA EXPRESSION OF TNF APOPTOTIC PATHWAY IN ENDOTHELIAL CELLS FROM THE BOVINE CORPUS LUTEUM

Introduction

The corpus luteum (CL) is a transient organ that forms from the wall of a Graafian follicle following ovulation and secretes progesterone (P4) [12]. 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) [11, 34].

Development of the bovine CL is associated with intensive angiogenesis [30], so that the mature CL becomes one of the most highly vascularized organs in the body [13-15], and vascular endothelial cells account for up to 50% of the total cells of the mid CL [16, 17]. Recently, capillaries without smooth muscle and a few blood vessels with smooth muscle were found in the center of the mid bovine CL [21], and we reported that the capillaries disappeared, but not the large blood vessels during luteolysis [41]. Luteal endothelial cells (LECs) are the first cells to undergo programmed cell death (apoptosis) during luteal regression, resulting in the loss of capillaries [25, 26].

The number of leukocytes in the bovine CL (e.g., T lymphocytes, macrophages) increases at the time of luteolysis [42], and leukocytes are known to produce a variety of cytokines, including TNF and 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 [43].

Apoptosis is characterized as a fragmentation of DNA into oligonucleosomal units [44]. Three major pathways have been identified during apoptosis as follows: the death receptor pathway, mitochondrial pathway and nucleus pathway [45]. Many cytokine membrane receptors [44, 45] and regulator proteins [46] are involved in apoptosis. FAS, a member of death receptor, engages its ligand (FAS ligand) to induce apoptosis [47]. Members of the BCL protein family regulate the mitochondrial pathway

of apoptosis [46]. The ratio of proapoptotic BAX to antiapoptotic BCL2 protein determines the induction of apoptosis [46]. Caspases (CASPs) are another type of regulatory protein that participates in transmission of the apoptosis signal from the cytoplasm to the nucleus [48]

LECs express TNF receptor type I (TNFRI) [49], which is involved in most of the TNF effects. IFNG induces TNFR expression in extra-ovarian cells in humans [50], rats [51] and mice [52]. Based on these findings, we hypothesized that TNF and IFNG induce apoptosis in LECs synergistically by regulating intracellular apoptosis-related factors expression. To test this hypothesis, we investigated the effects of TNF and IFNG on cell death and mRNA expression of death receptors, CASPs and BCL2 family in bovine LECs.

Materials and Methods

Cell viability

LECs were exposed to various concentrations of TNF (0.05, 0.5, 1.0, 2.5 nM) in combination with IFNG (0.05, 0.5, 1.0, 2.5 nM) for 24 h. After treatment with cytokines, 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 [44]. Briefly, WST-1, a kind of MTT (3-(4, 5-dimethyl-2 thiazolyl)-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 1.0×10^6 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 Corporation, 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 reagents; 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 0.0002% propidium iodide (PI; Sigma, No. P4170). Then, the cells were washed three times in PBS and stored in the dark at 48 C. The cells were observed under florescent 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.

Effect of cytokines on 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 and used to determine expression of mRNA by real-time RT-RCR.

Table 1

Gene	Forward and reverse Primers	Accession No.	Product
TNFRI	F 5'-CCCGACCTTCAACTGGTAAA-3'	U90937	117bp
	R 5'-AGCGACATGCTAAGACGACA-3'		
FAS	F 5'-TCCAGATCTCACGCAAACAG-3'	NM_174662	150 bp
	R 5'-CAGTTGCCTCCCTTCATCAT-3'		
CASP8	F 5'-TGTCACAATCGCTTCCAGAG-3'	DQ319070	119 bp
	R 5'-CCGAGGTTTGCTTGTCATTC-3'		
CASP9	F 5'-CAGACTTGGGCCTTCTTGAG-3	XM_002694144	132bp
	R 5'-GCCCTGAAAGCAAGAAACAG-3'		
BCL2	F 5'-CATCGTGGCCTTCTTTGAGT-3'	U92434	111 bp
	R 5'-CGGTTCAGGTACTCGGTCAT-3'		
BAX	F 5'-AACATGGAGCTGCAGAGGAT-3'	U92569	104 bp
	R 5'-CAGTTGAAGTTGCCGTCAGA-3'		
CASP3	F 5'-AAGCCATGGTGAAGAAGGAA-3'	XM_582296	134 bp
	R 5'-GGCAGGCCTGAATAATGAAA-3'		
ACTB	F 5'-CAGCAAGCAGGAGTACGATG-3'	AY141970	137 bp
	R 5'-AGCCATGCCAATCTCATCTC-3'		

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. 7, 8; $P<0.05$). Cell viability was further decreased by the combination of TNF (0.5 nM) and IFNG (0.5 nM) for 24 h (Fig. 9; $P<0.05$).

Effects of TNF and/or IFNG on DNA fragmentation and mRNA expression of apoptotic pathway

Staining with PI showed fewer nuclei after treatment with TNF and IFNG than in the control (Fig. 10A, B). Although there were no TUNEL-positive cells in the control, some TUNEL-positive cells appeared after treatment with TNF and IFNG (Fig. 10C, D). ACTB was used to normalize each mRNA expression. The expression of *TNFRI* mRNA significantly increased in LECs treated with IFNG alone or IFNG in combination with TNF (Fig. 11A; $P<0.05$). The expression of *FAS* mRNA significantly increased in LECs treated with IFNG in combination with TNF but not treated with TNF or IFNG alone (Fig. 11A; $P<0.05$). The mRNA expression of *BAX* and *BCL2* didn't change (Fig. 12). Although the mRNA expression of *CASP8* and *CASP9* didn't change, that of *CASP3* significantly increased in LECs treated with IFNG alone or IFNG in combination with TNF compared with control (Fig. 13).

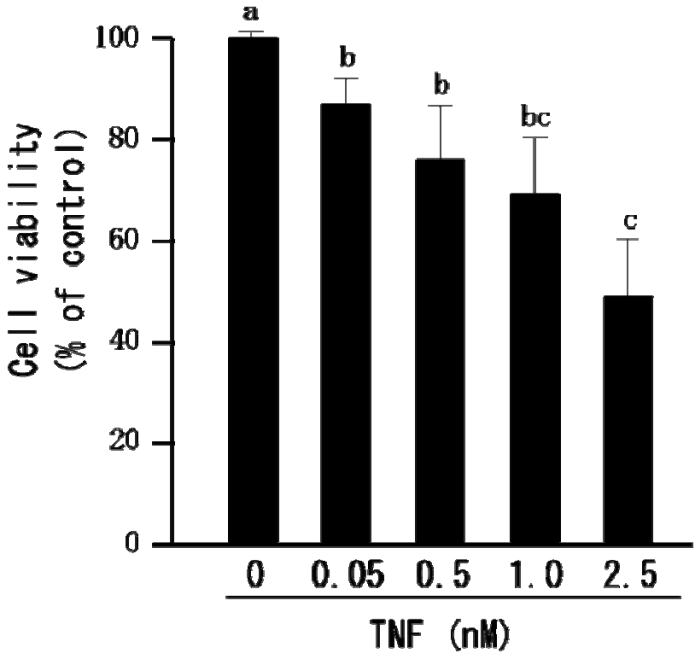


Fig. 7

Effect of TNF on cell viability. Luteal endothelial cells were exposed to TNF (0, 0.05, 0.5, 1.0, 2.5 nM) for 24 h. After 24 h of culture, cell viability was determined by optical density at 450 nm in a WST-1 assay. All values are expressed as percentages of the control and represent means ± SEM of three separate experiments. Different letters indicate significant differences ($P<0.05$) as determined by ANOVA followed by a Fisher PLSD as a multiple comparison test.

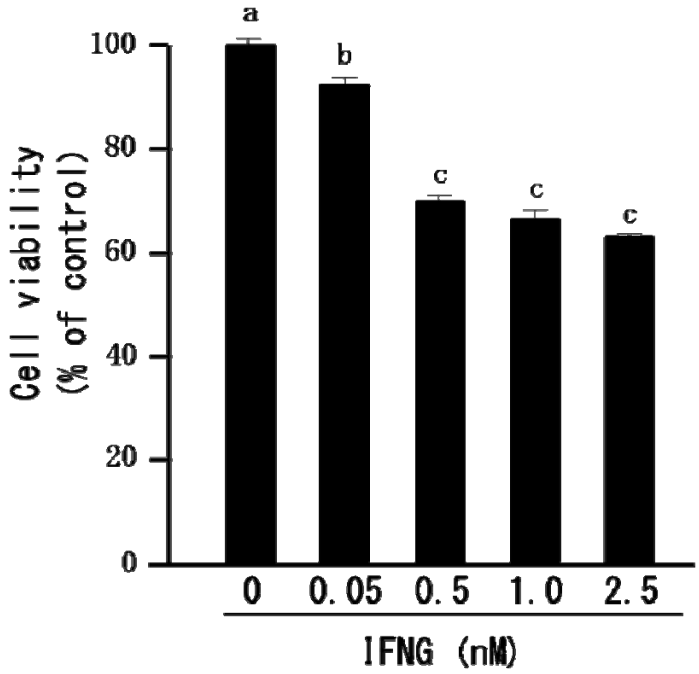


Fig. 8

Effect of IFNG on cell viability. Luteal endothelial cells were exposed to IFNG (0, 0.05, 0.5, 1.0, 2.5 nM) for 24 h. After 24 h of culture, cell viability was determined by optical density at 450 nm in a WST-1 assay. All values are expressed as percentages of the control and represent means ± SEM of three separate experiments. Different letters indicate significant differences ($P<0.05$) as determined by ANOVA followed by a Fisher PLSD as a multiple comparison test.

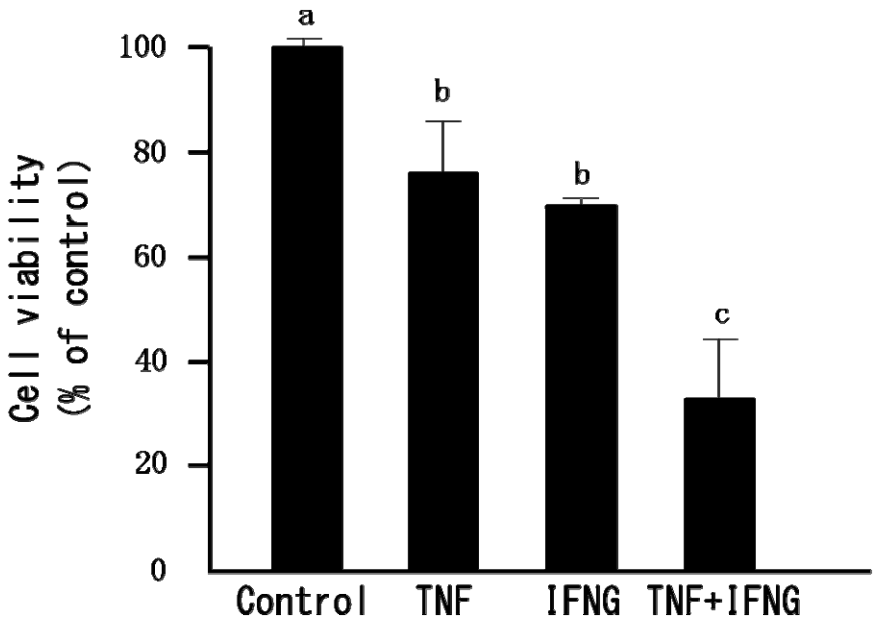


Fig. 9

Effect of TNF and IFNG on cell viability. LEC were exposed to 0.5 nM of both TNF and IFNG for 24 h. After 24 h of culture, cell viability was determined by optical density at 450 nm in a WST-1 assay. All values are expressed as percentages of the control and represent means ± SEM of three separate experiments. Different letters indicate significant differences ($P<0.05$) as determined by ANOVA followed by a Fisher PLSD as a multiple comparison test.

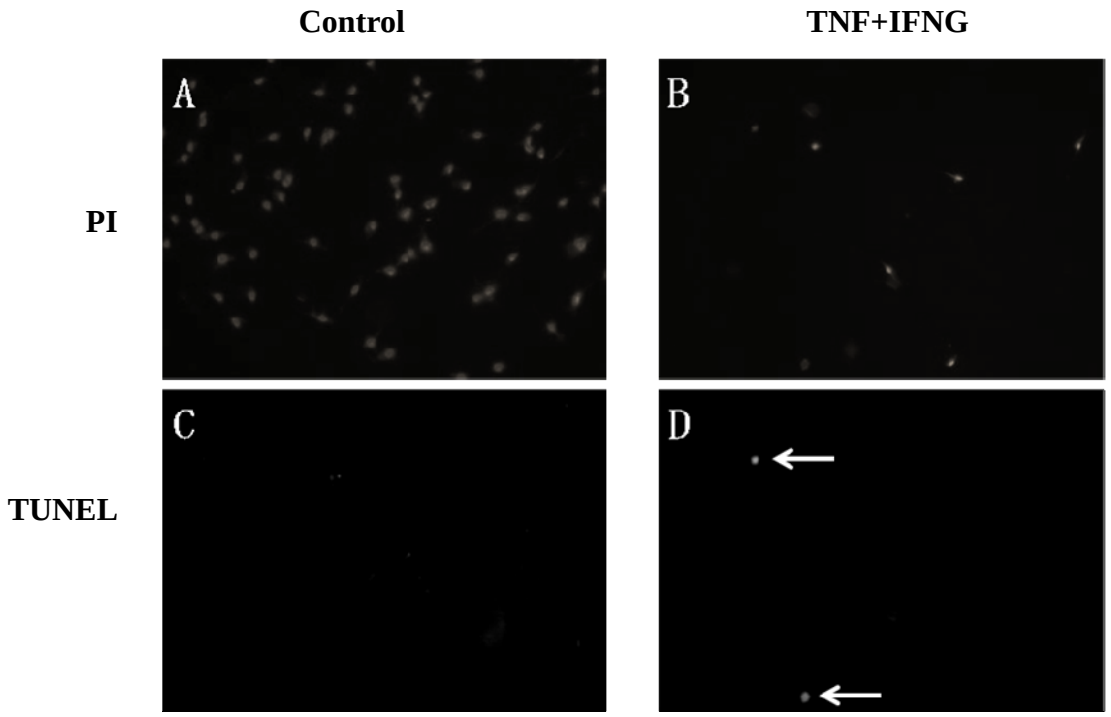
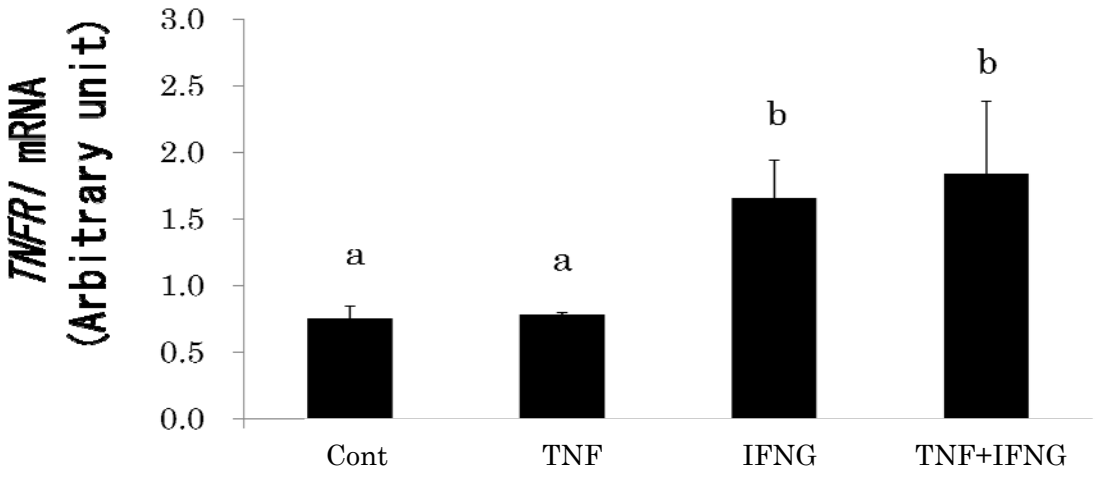


Fig. 10

Detection of DNA fragmentation in cultured bovine LEC treated with TNF and IFNG. The cells were cultured with a combination of TNF (0.5 nM) and IFNG (0.5 nM) for 24 h; B and D or control cells A and C. After 24 h of culture, the cells were stained with PI (A, B) and FITC-conjugated dUTP (C, D; TUNEL assay) and were visualized by fluorescence microscopy. Magnification, x100. Arrows point to TUNEL-positive cells. This procedure was repeated three times.

A



B

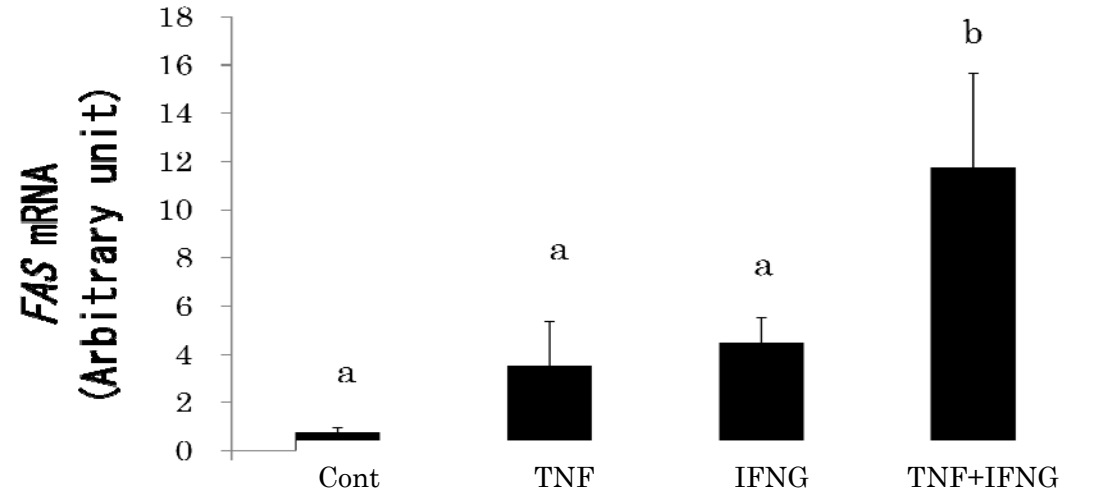
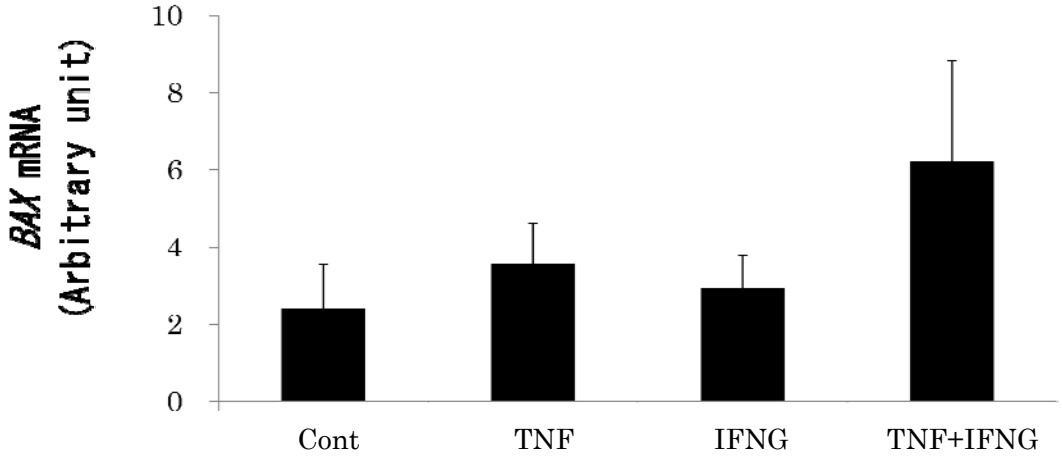


Fig. 11

Effects of TNF and/or IFNG on the expression of *TNFRI* (A) and *FAS* (B) mRNA in cultured bovine LECs obtained from the mid luteal stage of the estrous cycle (n = 3). The levels of *TNFRI* and *FAS* mRNA in the cells are relative to the amounts of *ACTB* mRNA. All values are means ± SEM of the densitometric analysis of each mRNA levels in the cells (relative to *ACTB* mRNA levels). Different letters indicate significant differences ($P<0.05$) as determined by ANOVA followed by the Fisher PLSD as a multiple comparison test.

A



B

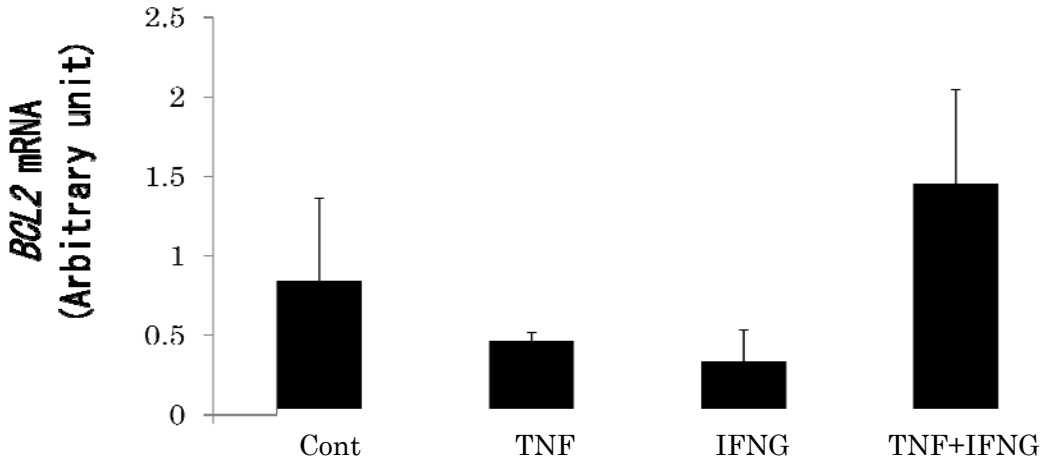


Fig. 12

Effects of TNF and/or IFNG on the expression of *BAX* (A) and *BCL2* (B) mRNA in cultured bovine LECs obtained from the mid luteal stage of the estrous cycle (n = 3). The levels of each mRNA in the cells are relative to the amounts of *ACTB* mRNA. All values are means ± SEM of the densitometric analysis of each mRNA levels in the cells (relative to *ACTB* mRNA levels). Different letters indicate significant differences ($P<0.05$) as determined by ANOVA followed by the Fisher PLSD as a multiple comparison test.

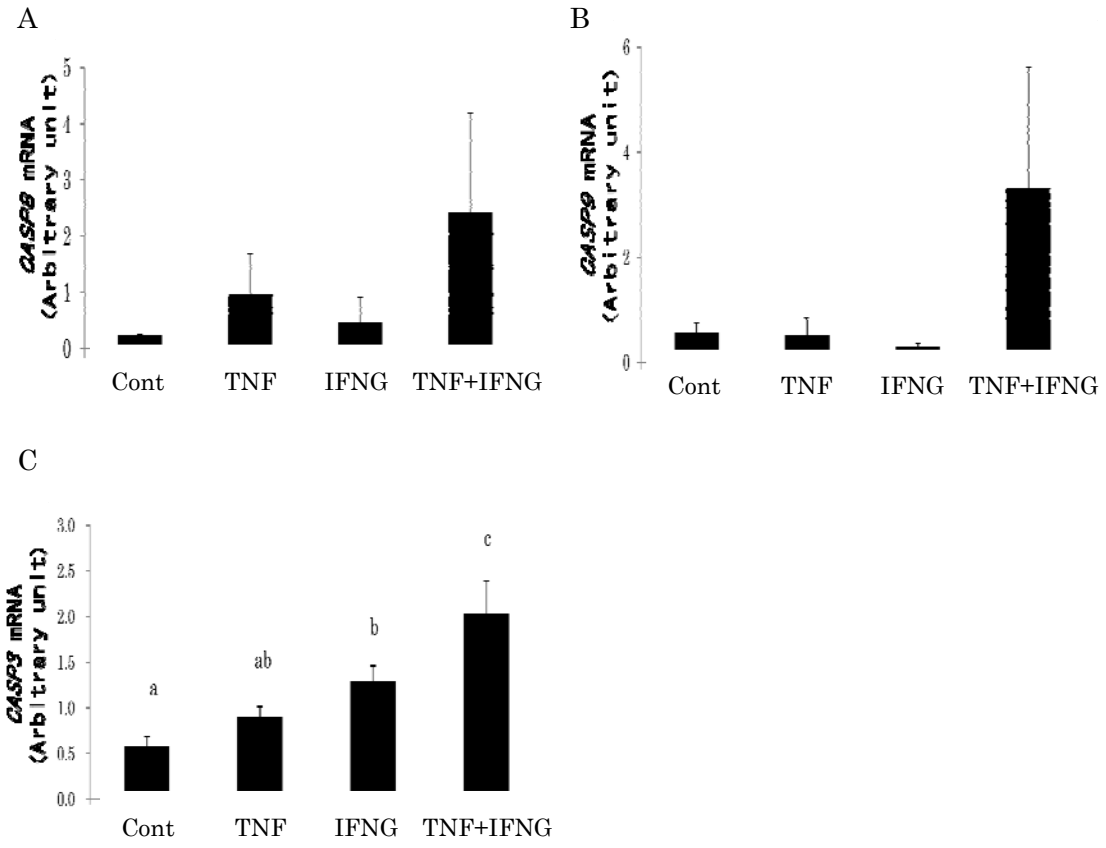


Fig. 13

Effects of TNF and/or IFNG on the expression of *CASP8* (A), *CASP9* (B) and *CASP3* (C) mRNA in cultured bovine LECs obtained from the mid luteal stage of the estrous cycle (n = 3). The levels of each mRNA in the cells are relative to the amounts of *ACTB* mRNA. All values are means ± SEM of the densitometric analysis of each mRNA levels in the cells (relative to *ACTB* mRNA levels). Different letters indicate significant differences ($P<0.05$) as determined by ANOVA followed by the Fisher PLSD as a multiple comparison test.

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 [41]. The loss of capillaries has been thought to be due to programmed cell death (apoptosis) of LECs [25, 26]. Since one of the most important roles of capillaries is the supply of oxygen to cells [53], 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 [54]. 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 [56] 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 [56]. 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 [57] and LSCs [48]. On the other hand, endothelin-1 (ET-1), a vasoactive peptide secreted by LECs, induces luteolysis in the bovine CL [58, 59]. Furthermore, since TNF stimulates ET-1 secretion in cultured LECs [47], 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.

In the present study, although TNF reduced the viability, it didn’t affect mRNA expression of apoptotic signal factors in LECs. These findings suggest that TNF induces caspase-independent cell death in LECs. In fact, there are some reports about caspase-independent programmed cell death [60-62]. TNF has been reported to induce cell death, but not apoptosis, in bovine mammary endothelial cells [63]. Thus, cell death induced by TNF in LECs may not be apoptosis. Further studies are needed to clarify the mechanism of cell death pathway activated by TNF in LECs.

LECs have been reported to produce TNF in response to IFNG stimulation [64]. 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 anti-virus activity, although TNFRI has weaker affinity for TNF than TNFRII [65]. TNFRI is expressed in bovine LECs [49] and in bovine LSCs [57]. IFNG has been found to induce expression of TNFR and Fas, which is a member of the TNFR family, in some types of cell [50-52, 56]. 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 TNFRI 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 each TNF and IFNG activate independent pathway and they synergistically affect the death of LECs resulting in acute luteolysis.

Summary

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 mRNA expression of TNFRI, FAS, BAX, BCL2, CASP8, CASP9 and CASP3 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, but not treatment with TNF alone, increased the expression of *TNFRI and CASP3* mRNA compared with the control ($P<0.001$). In summary, TNF and IFNG increased cell death in cultured bovine LECs. The different effect between TNF and IFNG on the expression of apoptotic pathway suggests that each TNF and IFNG activate independent pathway and they synergistically affect the death of LECs resulting in acute luteolysis.

CONCLUSION

The present study investigated the changes in the density and number of blood vessels, with and without smooth muscle in bovine CL, and the regulatory mechanisms of apoptosis in bovine LECs. The first series of experiments capillaries lacking of smooth muscle disappear earlier than do blood vessels with a smooth muscle layer during structural luteolysis suggesting that the loss of capillaries results in a reduced supply of nutrients to luteal cells followed by luteal cell death. The second series of experiments showed that TNF and IFNG synergistically stimulated apoptosis in LEC and that TNF and IFNG reduced the viability and induced DNA fragmentation in LECs

Overall results suggest that TNF and IFNG independently activate different pathways for inducing apoptosis, and that they synergistically contribute to luteolysis by accelerating the disappearance of capillaries.

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ABSTRACT IN JAPANESE

ウシ黄体機能の周期的変化と黄体内血管分布変化ならびに

その制御機構に関する研究

法上　拓生

黄体は、妊娠の成立維持に必須のプロジェステロン (P4) を分泌する排卵後に形成される一過性の内分泌器官である。黄体の形成時には、低酸素環境を反映し著しい血管新生の起こることが知られており、成熟した黄体は血管が非常に密な組織となる。黄体に見られる血管は、血管内皮細胞 (luteal endothelial cell: LEC) 単層で構成される毛細血管および平滑筋を有する血管の 2 種類に大きく分類される。黄体において毛細血管は血液の通り道であるだけでなく、黄体細胞への酸素や栄養の供給を司っており、黄体機能の発現ならびに黄体細胞の生存に重要な役割を果たす。さらに LEC は、P4 分泌をはじめとする黄体細胞の機能に重要な役割を果たす多くの生理活性物質を分泌することから、黄体内における LEC ならびに血管の分布変化は黄体の機能変化に密接に関与すると考えられる。

毛細血管は LEC 単層で構成されていることから、黄体内における毛細血管の分布調節機構を調べる上で LEC の生死を調節する機構を明らかにすることは重要である。黄体期後期以降の黄体に流入する免疫細胞由来の腫瘍壊死因子 (TNF) およびインターフェロン γ (IFNG) は、黄体細胞のアポトーシスを誘導する。また、LEC に TNF receptor type I (TNFRI) の発現することが知られていることから、これらのサイトカインが LEC アポトーシスを誘導する可能性がある。しかし、LEC アポトーシスに及ぼす TNF および IFNG の影響は明らかにされていない。

本研究では、ウシ黄体機能調節機構を解明する研究の一環として、(1) 発情周期を通じた黄体組織ならびに黄体中期のウシに黄体退行因子であるプロスタグランジン F2α (PGF) を投与後の黄体組織における 2 種類の血管の分布変化、(2) LEC アポトーシスに及ぼす TNF および IFNG の影響を調べた。

(1) 発情周期各期 (黄体初期、黄体形成期、黄体中期、黄体後期および黄体退行期) のウシ黄体よりパラフィン切片を作成し、LEC の分布を知る目的で von Willebrand factor 抗体を用いて免疫組織染色を行った。染色後、結合組織を含まない中心部を 400 倍で撮影し、得られた組織像に 450 の交差点を有する格子を重ね、陽性を示す部分と重なる交差点の割合を算出することで血管分布を算

定・評価した。また、α-smooth muscle actin の局在を血管平滑筋の指標として調べ、血管平滑筋を伴う血管の数を算出した。次に黄体期中期に PGF を投与した雌ウシから投与、0.5, 2, 12 時間後に黄体を摘出し、上と同様に血管の分布を調べた。

単位面積あたりに占める血管の割合を数値化した結果、形成期および後期において他の周期と比較して高く ($P<0.05$)、また血管平滑筋を有する血管の数は後期および退行期に有意に多かった ($P<0.05$)。PGF 投与後の黄体組織内に占める血管の割合ならびに平滑筋を有する血管の数はともに時間依存的に増加した。これらの結果より、後期から退行期にかけて黄体内の血管占有率が減少する一方で、平滑筋を有する血管の数が増加したことから、黄体機能の衰退に伴い毛細血管が減少していることが示された。黄体の退行する時期と毛細血管の消失する時期が一致することから、毛細血管の消失が黄体退行の促進に関与することが示唆された。

(2) ウシ中期黄体より単離した LEC を播種し、コンフルエントになるまで培養後以下の実験に用いた。TNF または IFNG をそれぞれ 1, 10, 20, 50 ng/ml 添加し、24 時間後 MTT-assay により細胞生存率を調べた。さらに TNF と IFNG を組み合わせて添加し、細胞生存率を調べた。次に TNF (10 ng/ml) および IFNG (10 ng/ml) を単独または組み合わせて添加し、24 時間培養後 real-time RT-PCR により *TNFR1, FAS, BAX, BCL2, CASP8, CASP9* および *CASP3* mRNA 発現を調べた。

いずれのサイトカインを添加した区においても濃度依存的に LEC の細胞生存率が低下した ($P<0.05$)。また、TNF および IFNG を組み合わせて添加した区においてそれぞれのサイトカインを単独で添加した区と比較して細胞生存率が有意に低下した ($P<0.05$)。IFNG を単独または TNF と組み合わせて添加した区において control 区ならびに TNF 単独添加区と比較して *TNFR1* および *CASP3* mRNA 発現が有意に増加した ($P<0.05$)。以上の結果より LEC において TNF および IFNG はそれぞれ異なる経路を介してアポトーシスを誘導することにより、相乗的な作用を示し、血管消失の一因となることが示唆された。

本研究の結果から黄体期後期以降の黄体内において、TNF および IFNG が相乗的に LEC のアポトーシスを誘導することが示され、TNF および IFNG によって誘導された LEC のアポトーシスにより黄体内の毛細血管が消失し、黄体退行が促進される可能性が考えられる。