دراسة دور بروتين الـcFLIP كعامل مضاد للموت الخلوي في الجسم الأصفر عند الأبقار

The Role of cFLIP as Anti-Apoptotic Protein in the Bovine Corpus Luteum

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المُلَخَّص

يعد موت الخلايا المبرمج آلية أساسية في التحلل اللوتيئيني للجسم الأصفر. إن زيادة المعرفة في العوامل المساعدة و المضادة لموت الخلايا في الجسم الأصفر يمكن أن تساعد في تطوير استراتجية جديدة في التحريض على التحلل اللوتيئيني للجسم الأصفر. هدفت هذه الدراسة لمعرفة دور cFLIP، كبروتين مضاد للموت الخلوي، في الحياة الوظيفة للجسم الأصفر عند الأبقار. تمَّ جمع عينات من الجسم الأصفر البقري في مراحل مختلفة من الدورة التناسلية لدراستها باستعمال المناعة الكيميائية النسيجية. كما تمت معالجة مزارع خلوية للخلايا اللوتيئينية بـIFNγ/TNFα لإحداث موت صناعي في الخلايا اللوتيئينية. تركز وجود cFLIP في سيتوبلازما الخلايا اللوتيئينية وغير اللوتيئينية بما فيها البطانية الوعائية و المناعية في المراحل مختلفة من الدورة التناسلية. لوحظت زيادة في كثافة الصبغة المناعية في المراحل المبكرة المتطورة و المتوسطة من الدورة التناسلية، في حين كان هناك انخفاض ملحوظ في المرحلة المتأخرة و المتنكسة ($P<0.05$). أظهرت خلايا شبيهة بالخلايا البالعة في المرحلة المتنكسة كثافة في الصبغة المناعية. يؤدي إحداث الموت الصناعي باستعمال IFNγ/TNFα الى انخفاض نسبة الخلايا الإيجابية لـ cFLIP و ازدياد الخلايا الميتة والإيجابية لإختبار TUNEL. بالمقارنة مع الشاهد ($P<0.01$). تبين من خلال هذه النتائج ان نسبة cFLIP تنخفض أثناء التحلل اللوتيئيني للجسم الأصفر ما يشير الى وجود دور انعاشي لـ cFLIP في الحياة الوظيفة للجسم الأصفر عند الأبقار في المراحل التطور و النضوج و يمنع الموت الخلوي ويحمي الجسم الأصفر من التحلل.

الكلمات المفتاحية: corpus luteum، الجسم الأصفر، الموت الخلوي.

Abstract

Apoptosis, programmed cell death, is a key mechanism in luteal regression. Improving our understanding of pro- and anti-apoptotic factors during luteolysis can advance our knowledge and may lead to a new strategy

in manipulating luteolysis. This study aimed to elucidate the role of cellular Fas like interleukin converting enzyme (FLICE) inhibitory protein {cFLIP} an anti-apoptotic protein, in the bovine corpus luteum function. Corpora lutea were collected at different stages throughout the estrous cycle for immunolocalization of cFLIP. Additionally, cultured mid luteal cells were treated with IFNγ/TNFα (tumor necrosis factor alpha/ interferon gamma) to induce apoptosis and examine cFLIP expression. Immunostaining was found predominantly in the cytoplasm of luteal and non-luteal cells, including endothelial and immune cells. The density of cFLIP immunostaining was high during the early, developing and mid luteal stages and then decreased significantly ($P<0.05$) at the late and regressed luteal stages. Macrophage like cells were the only type of cells immunostained in the regressing CL. However, cultured mid luteal cells showed higher percentage of cFLIP positive cells with lower percentage of TUNEL (terminal deoxynucleotidyl transferase- mediated dUTP nick-end labelling) positive cells compared with treated luteal cells with cytokines ($P<0.01$). The above results suggested regulation of cFLIP during structural luteal regression and cFLIP plays a crucial role in the bovine CL.

Keywords: Apoptosis, cFLIP, Bovine, Corpus Luteum.

Introduction

Apoptosis is a crucial mechanism in the ovarian function through its significant contribution to cell deletion during follicular atresia (Tilly *et al.,*1996) and luteal regression (Juengel *et al.,*1993). It is generally accepted that apoptosis is the main type of cell death during structural luteolysis in many species (Davis and Reuda, 2002). Apoptosis is a highly regulated process and various factors are known to be involved in apoptosis in the corpus luteum (CL), such as hormones (progesterone), cytokines such as tumor necrosis factor alpha (TNFα) interferons (INFγ) , interleukins (IL), nitric oxide (NO) and others (Bcl2 family, Fas and Fas ligand system, caspase family and reactive oxygen species) (review see, Sugino and Okuda 2007). Better understanding of pro- and anti-apoptotic factors can advance our knowledge and may improve manipulation techniques of luteolysis which is a key element in oestrous cycle control in clinical bovine reproduction. Since the role of some gene-encoded proteins have been demonstrated to orchestrate the apoptotic cascade process during luteolysis such as, members of Bcl2 family, Fas and Fas ligand system and members of caspase family (Sugino and Okuda 2007), little information is available on the role of anti-apoptotic factor, cellular cellular Fas like interleukin converting enzyme (FLICE), inhibitory protein {cFLIP} in the bovine CL.

cFLIP is an important regulator of death receptor-mediated apoptosis (Irmler *et al.,*1997). At mRNA level, cFLIP exists as multiple splice variants, but at protein level two endogenous forms, cFLIP long ($cFLIP_L$) and c-FLIP short ($cFLIP_S$) have been detected (Djerbi *et al.,*2001). $cFLIP_S$ consists of two DEDs (death effect domains) and is highly homologous to the N-terminus of caspase-8. While $cFLIP_L$ is similar to the short form but lacks enzymatic activity (Thome *et al.,*1997), both $cFLIP_{L/S}$ can inhibit apoptosis mediated by TNF receptor gene superfamily members such as Fas, tumour necrosis factor receptor-1 (TNFR-1), by interacting with Fas-

associated death domain (FADD) and procaspase-8. Fas-induced death involves clustering of Fas at the cell membrane and recruitment of the FADD to the trimerized intracellular death domain of the receptor (Chinnaiyan *et al.,* 1995). FADD recruits procaspase-8 leading to the formation of death-inducing signaling complex (DISC) (Boldin *et al.,* 1996). After the formation of the DISC, activated caspase 8 activates caspase 3 directly or indirectly by the truncation of Bid. The truncated form of Bid (tBid) translocates to mitochondria leading to cytochrome C release and activation of caspase 9 (Boldin *et al.,* 1996). C-FLIP$_{S/L}$ inhibits caspase 8 activation by interfering with the recruitment and the processing of pro-caspase 8 within the DISC and blocking its function (Krueger *et al.,* 2001; Thome and Tschopp, 2001).

Both intrinsic and extrinsic apoptotic pathways have been demonstrated during luteolysis in many species including cattle (Sugino and Okuda 2007; Yadav et al 2005). Moreover, the activation of mitochondrial apoptotic pathway cascade preceded the activation of Fas-mediated apoptosis in the bovine CL (Yadav *et al.,* 2005). Since cFLIP suppresses extrinsic apoptotic pathway induced by TNF receptor gene superfamily members, we hypothesized, that cFLIP plays a role in the apoptotic cascade during luteolysis in cattle. This study was designed to elucidate the role of cFLIP in the bovine CL function by examining mRNA and protein expression and immunolocalization of cFLIP in the bovine CL throughout the oestrous cycle in luteal tissue and in cultured luteal cells treated with TNFa/IFNg.

Materials and Methods

Collection of bovine CL:

Forty Ovaries were collected from Holstein cows at a local slaughterhouse within 10–20 min after exsanguination in Okayama city. The stage of the estrous cycle was defined as described in previous study (Miyamoto et al 2000). Ovaries with CL were classified as early (Days 2–3 after ovulation, n=3), developing (Days 5–6, n=3), mid (Days 8–12, n=3), late (Days 15–17, n=3), and regressed (Days 19–21, n=3) luteal stages. After determination of these stages, CL tissues were immediately separated from the ovaries. For immunohistochemistry pieces of CL tissue were fixed in 10% (v/v) neutral formalin (pH 7.4) for 20-24h and then embedded in paraffin. For cell culture 25 ovaries with CL in the mid luteal stage were submerged in ice-cold physiological saline and transported to the laboratory.

Cell isolation and apoptosis induction:

Only those CLs classified in the mid luteal stage were collected for the cell culture. Luteal tissue was enzymatically dissociated and luteal cells were cultured as described previously (Okuda et al 1992). Dissociated luteal cells from CLs were pooled. The luteal cells were suspended in a culture medium, DMEM, and Ham's F-12 medium [D/F; 1:1 (v/v); Sigma-Aldrich, MO, USA, D8900] containing 5% fetal calf serum (Life Technologies, NY, USA, 16170-078) and 20 μg/ml gentamicin (Sigma, G1397). Cell viability was greater than 85% as assessed by trypan blue exclusion. The cells in the cell suspension consisted of about

70% small luteal cells, 20% large luteal cells, 10% endothelial cells or fibrocytes, and no erythrocytes. The dispersed luteal cells were seeded at 2.0 x 10^5 viable cells in 1 ml, cultured on sterilized coverslips in 6 well microplates for immunofluorescence then incubated in a humidified atmosphere of 5% CO_2 in air at 38°C in a N_2-O_2-CO_2-regulated incubator (ESPEC CORP., Osaka, Japan, BNP-110). After 12 h of culture, the medium was replaced with fresh medium containing 0.1% BSA (Sigma, A7979), 5 ng/ml sodium selenite and 5 μg/ml transferrin, and apoptosis was induced by 2.9 nM TNFα (kindly donated by Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) and 2.5 nM IFNγ (kindly donated by Dr. S. Inumaru, NIAH, Ibaraki, Japan). After 48 h culture, total RNA was prepared from the cells and the coverslips subjected to cFLIP and TUNEL immunofluorescence.

Immunohistochemistry:

Sections of 4 μm thick were mounted on glass slides, sections were immersed in antigen retrieval buffer (0.01M citrate acid, pH 6) at 95 °C for 10 min, then the sections were incubated with rabbit anti-human cFLIPS/L antibody (Santa Cruz Biotechnology, sc-8347) diluted 1:100 with PBS containing 1% (w/v) BSA overnight at 4 °C, while negative control sections were incubated with PBS. Sections were incubated with biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA, 1:200) for 30 min, then incubated for 30 min at room temperature with VectaStain avidin-biotin-peroxidase complex (Vector Laboratories, PK-6100) according to the manufacturer's instructions. The sections were visualized with 0.05% (w/v) 3,3'-diaminobenzidine (Dojindo, Kumamoto, Japan, 7411-49-6) in 0.01M PBS, pH 7.4, and 0.01% (v/v) H2O2. To estimate the density of staining, 3 animals from each luteal stage and 3 sections from each animal were examined at light microscope. 5 digital micrographs were taking randomly from each section at 400x magnification. The density of the immunostaining was quantified as described in (Lehr et al., 1997) using Photoshop program software. The system was optimised for each individual section based on the density of the stain. The data were expressed as arbitrarily unit mean ± SEM.

Double immunofluorescence TUNEL (terminal deoxynucleotidyl transferase- mediated dUTP nick-end labelling) and cFLIP:

Cultured cells on coverslips were washed 3 times in PBS then fixed with 4% paraformaldehyde at 4°C for 10 min followed by 3 times wash in PBS. The coverslips were treated with blocking buffer 1% BSA in PBS for 30 min at room temperature, subsequently incubated with rabbit anti-human cFLIPS/L antibody diluted 1:100 with PBS containing 1% BSA overnight at 4°C and followed by three times washing in PBS. Coverslips were incubated with secondary antibody (donkey anti-rabbit Cy3 labeled, Jackson ImmunoResearch, PA, USA, 711-165-152) diluted 1:400 in blocking buffer for 1 h at room temperature, then washing three times in PBS. For double staining, apoptotic cells were detected using MEBSTAIN Apoptosis Kit Direct (MBL International Corporation, MA USA, 8445) according to manufactures instructions. Briefly, coverslips were incubated with TdT buffer II for 5-10 min at room temperature, then

incubated with (mixture of 45 µl of TdT buffer II, 2.5 µl of FITC-dUTP, and 2.5 µl of TdT) for 60 min at 37ºC, followed by immersion in TB buffer for 15 min and washed 3 times in PBS. The coverslips were mounted with VECTASHIELD mounting medium DAPI (Vector Laboratories, H-1500) on slide glass and were examined at x400 magnification using fluorescence microscope. The number of positive cells for TUNEL and cFLIP were counted in 20 random microscopic fields. The percentage of immuno-positive cells for TUNEL and cFLIP was calculated as follows: number of positive cells/total number of the cells X100, for each individual microscopic field and expressed as mean ± SEM (Alziabi *et al.,* 2002).

Statistical analysis:

All experimental data are shown as the mean ± SEM. Statistical significance of differences immunostaining density or percentage of cells in cultured cells between control and treated groups were assessed by ANOVA followed by the Fisher protected least-significant difference procedure as a multiple comparison test using Minitab statistical package.

Results

Immunohistochemistry:

Positive immunostaining for cFLIP was found predominantly in the cytoplasm of luteal cells and non-luteal cells, including endothelial and immune cells. No positive staining was observed in negative control sections (Fig. 1A).

Luteal and non luteal cells were immunostained during the early (Fig. 1B) and developing (Fig. 1C) luteal stages.

At the mid luteal stage more intense immunostaining was seen (Fig. 1D).

Pale immunostaining was found in few luteal cells during the late luteal stage (Fig. 1E); by the regressed stage most of the luteal and endothelial showed no immunostaining. However, immune cells, probably, macrophage like cells were immunostained (Fig. 1F).

The density of cFLIP immunostaining was high during the early, developing and mid luteal stages and then decreased significantly ($p<0.01$) by the late and regressed stages compared with the early, developing and mid luteal stages (Fig. 2).

Double immunofluorescence of TUNEL and $cFLIP_{L/S}$ in cultured luteal cells:

Untreated cultured luteal cells showed immunofluorescence for cFLIP (Fig 3A), with few cells were positive for TUNEL (Fig. 3B), while luteal cells treated with TNFα/IFNγ were positive for cFLIP (Fig. 3C).

The majority of the cells showed positive immunostaining for TUNEL (Fig. 3D).

However, cultured mid luteal cells showed higher percentage of cFLIP positive cells with lower percentage of TUNEL positive cells compared with treated luteal cells with cytokines ($P<0.01$) (Fig. 3 E-F).

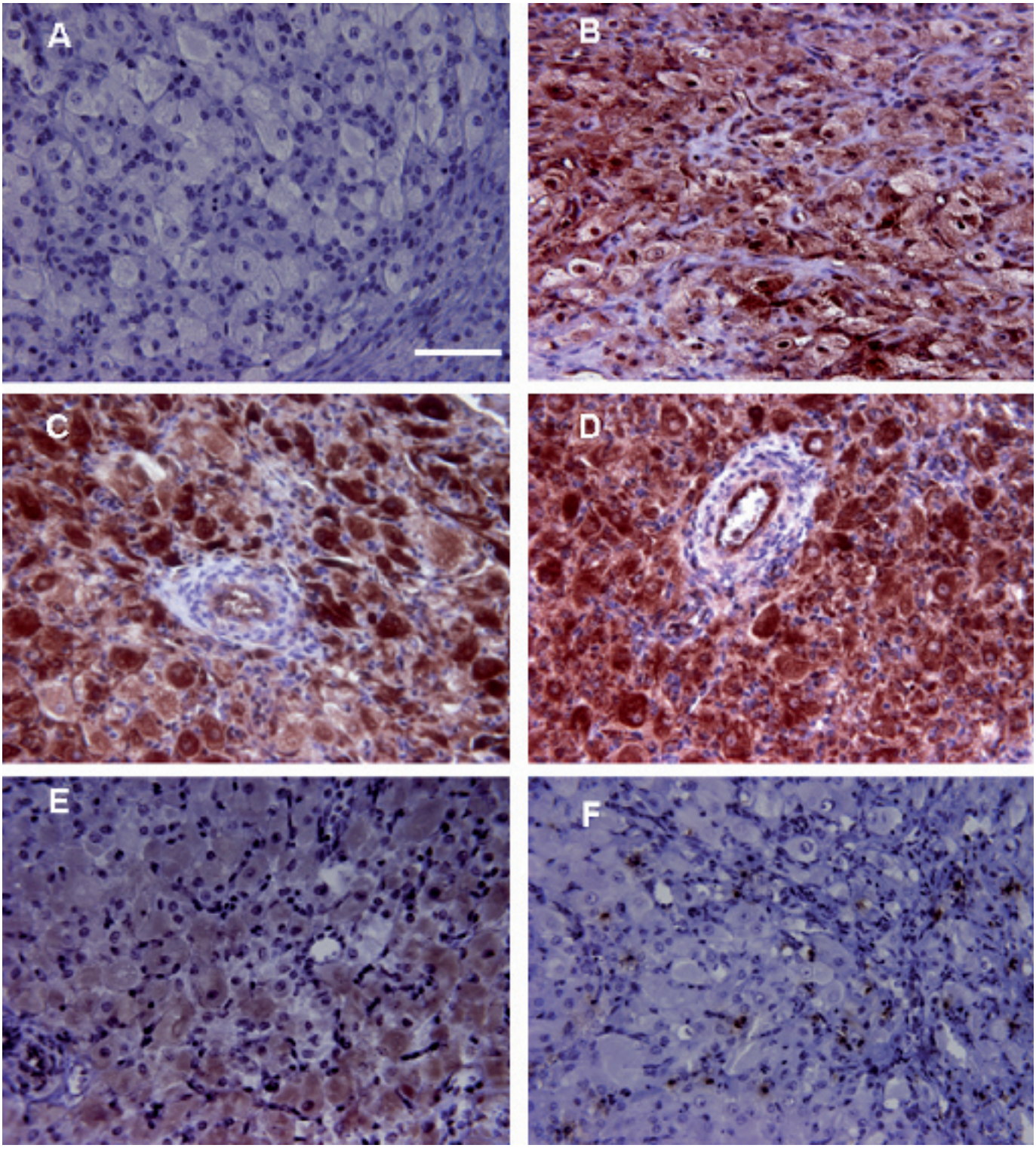

Fig.1. Immunolocalization of cFLIP throughout the luteal stages. The immunostaining were predominantly found in the cytoplasm of luteal cells and non-luteal cells, including endothelial and immune cells. A) negative control showed no immunostaining. B) luteal cells showed cytoplasmic immunostaining during early luteal phase. C-D) luteal cells and blood vessels showed intense immunoreactivity during developing and mid luteal phase respectively. E) pale immunostaining were seen during late luteal stage. F) luteal cells and blood vessels showed no immunostaining, however, macrophage like cells show positive immunostaining. Scale bar = 50 µm.

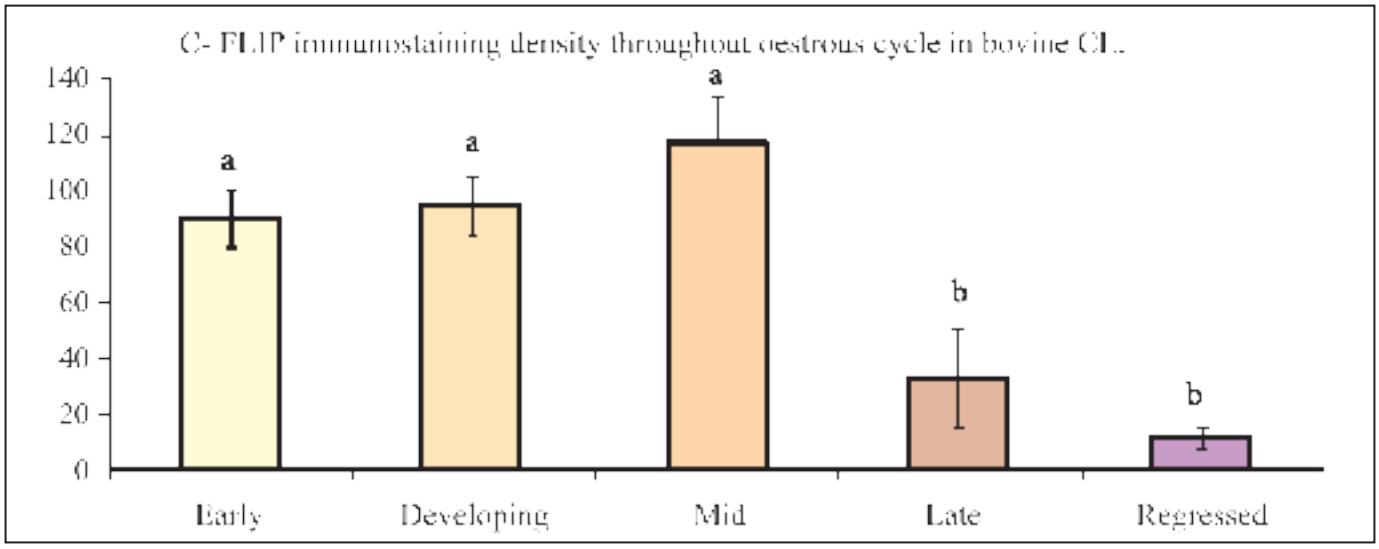

Fig. 2. Quantitative analysis of cFLIP immunostaining density throughout the luteal stages. 15 micrographs of cFLIP immunostaining form each stage were quantified as reported by [21] using Photoshop software. All data are expressed as mean ± SEM, different letters indicate significant difference ($P < 0.05$) within each luteal stages.

Discussion

The present study investigated the expression and localization of cFLIP in the bovine CL and showed a down-regulation of cFLIP during structural luteolysis. Apoptosis mediated by death receptors of TNF superfamily can be blocked by several mechanisms, including the production of soluble Fas and the over-expression of inhibitory proteins such as cFLIP (Irmler *et al.,*1997). cFLIP is more efficient than other anti-apoptotic genes such as, TRAF1, TRAF-2, cIAP-1, and cIAP-2, in preventing cell death induced by death receptors (Wang et al 1998).

Fas-mediated apoptosis system was demonstrated in the human CL (Quirk *et al.,*1995)], rat (Kuranaga et al 1999, 2000), mouse (Komatsu et al 2003) and bovine (Taniguchi *et al.,*2002, Yadav *et al.,*2005). Fas was found to be elevated during luteal regression in bovine (Taniguchi et al 2002; Yadav et al 2005) and rat (Roughton et al 1999). The increase in Fas was the first step in the initiation of extrinsic pathway followed by Fas/FasL system activation and thereby apoptotic cascade proceeded forward (Roughton et al 1999). Based on the assumption that TNFα and IFNγ produced by immune cells during luteal regression contributed to the increased levels of Fas (Sugino and Okuda 2007), it is possible that the decline in cFLIP levels during luteal regression observed in the present study coincides with high levels of Fas. In contrast, the overexpression of cFLIP protein during the early, developing, mid and late luteal stages in this study may be directly or indirectly compromised by low levels of Fas during these stages (Taniguchi et al 2002). Therefore, the down regulation of cFLIP during luteal regression can facilitate the extrinsic apoptotic cascade to proceed without disturbance at the DISC site. Moreover, in the present study, TNFa/IFNg-induced apoptosis in cultured luteal cells resulted in a reduction in cFLIP

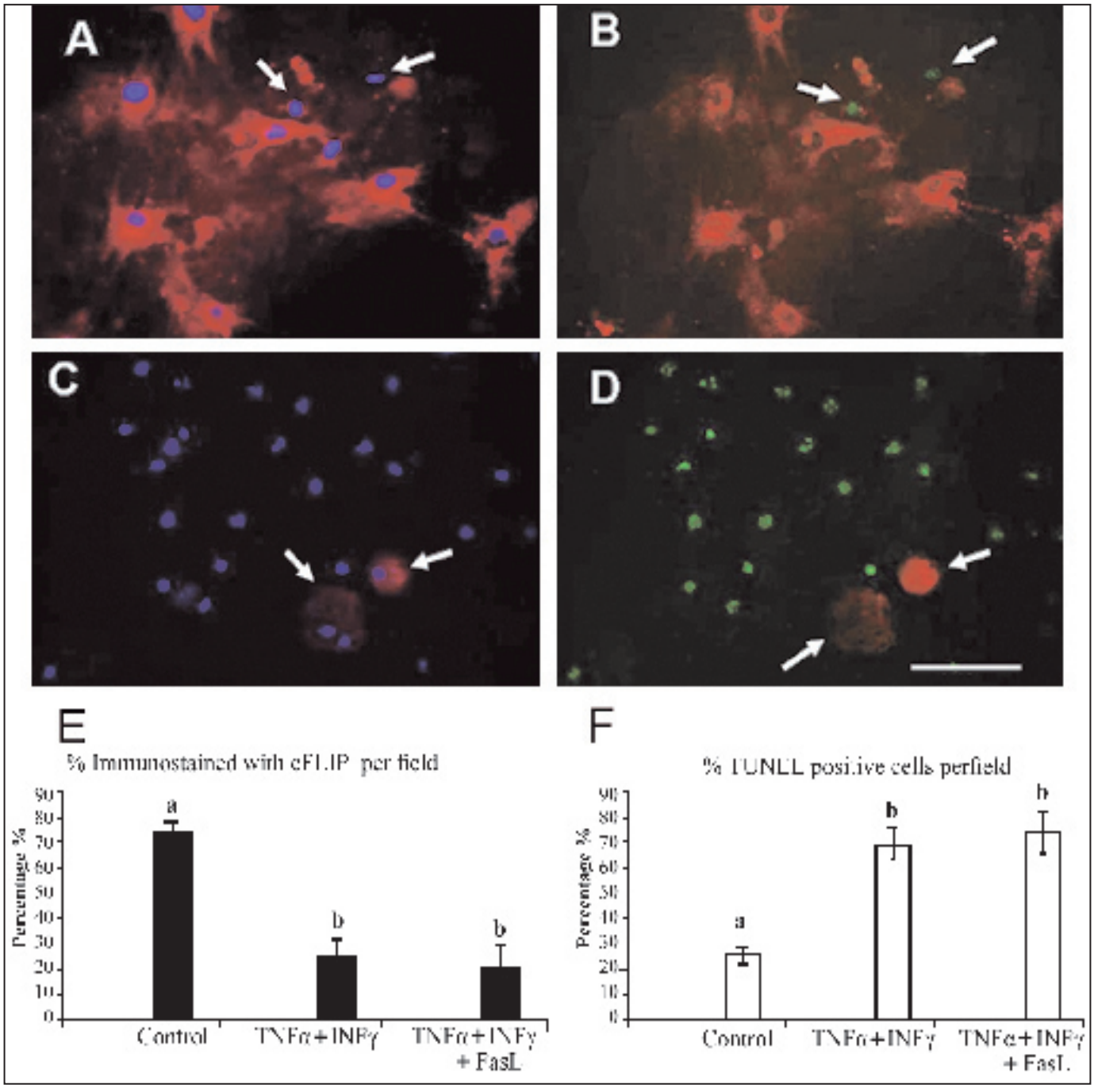

Fig. 3. Immunofluorescence staining of cFLIP and TUNEL in cultured luteal cells. A) untreated luteal cells showed positive immunofluorescence for cFLIP. B) the same macroscopic field of A, few numbers of untreated cultured cells was positive for TUNEL (white arrows). C) few numbers of luteal cells treated with IFN and TNFα showed immunofluorescence for cFLIP (white arrows), D) same macroscopic field of C, the majority of the cells were positive for TUNEL and few cells stained with cFLIP (white arrows). E) Percentage of positive cells for cFLIP were significantly higher ($P<0.01$) in the control than in treated luteal cells. F) the percentage of positive cells for TUNEL were significantly lower ($P<0.01$) in the control compared with treated cells with TNFα/IFNγ.. Three separate experiments were carried out and all data are shown as means ± SEM, different letters indicate significant difference ($P<0.01$). Scale bar = 50 µm.

positive cells and an increase in TUNEL positive cells, confirming our present data in luteal tissue. However, apoptosis induced by death receptors is tightly regulated by genes that are activated by the transcription factor nuclear factor- Kappa B (NF-κB) (Wang *et al.,*1998). There is evidence that cFLIP is one of several anti-apoptotic genes that are under the control of the NF-κB (Micheau *et al.,*2001). Moreover, protein kinase C delta (PKCδ) (Wang *et al.,*2006), phosphatidylinositol 3-kinase, mitogen-activated protein kinase (MAPK) (Panka *et al.,*2001) and p53 (Fukazawa et al 2001) have been reported to play a critical role in regulating cFLIP expression in various cell types. Further studies are needed to elucidate the regulatory mechanism of cFLIP expression in the bovine CL.

Both short and long isoforms of cFLIP have been shown to prevent apoptosis (Irmler et al., 1997). In this study, polyclonal antibody cFLIP (short and long) was used. A previous report (Mastsuda-minehata et al., 2005) demonstrated that $cFLIP_L$ is the most abundant isoform to be expressed in the porcine granulosa cells at protein levels. Moreover, porcine granulosa cell line JC-410 was shown to express $cFLIP_S$ at 28 kDa but only traces of $cFLIP_S$ was found in granulosa tumor cell line KGN and granulosa cells from healthy follicles (Mastsuda-minehata et al., 2005, 2007). Another study demonstrated that over-expression of $cFLIP_S$ but not $cFLIP_L$ prevented apoptosis induced by TNFα in the rat's granulosa cells (Xiao et al 2002). The discrepancy of cFLIP isoforms expression may highlight inter-species differences and/or differences in experimental approaches used in the above studies.

cFLIP is expressed in virtually all cells irrespective of their sensitivity to death-receptor-mediated apoptosis (Peter, 2004). It is well known that luteal and endothelial cells in the CL preserve their physiological function and structural integrity during the early, developing and mid luteal stages in many species including sheep (Jablonka-Shariff et al 1993), horse (Al-zi'abi et al 2002) and cow (Zheng et al 1994). In the present study, the overexpression of cFLIP in both luteal and endothelial cells during the early, developing and mid luteal stages prevents these cells from undergoing apoptosis. In contrast, when apoptosis took place during structural regression at the regressed stage, cFLIP was under-expressed in luteal and endothelial cells. The down-regulation of cFLIP appears to be a cell death feature in both luteal and endothelial cells. The same scenario was also observed in cultured luteal cells from the mid luteal stage where cFLIP was overexpressed in the control group and down-regulated when apoptosis was induced by IFNγ, TNFα. These findings indicate that overexpression of cFLIP is a promoter of cell survival in the bovine CL. However, duringe luteal regression, luteal and endothelial cells were not immunostained, but macrophage like cells were positively stained to cFLIP. This phenomenon suggests that cFLIP plays a role as survival factor for these cells. However, immune cells including macrophages tended to increase in numbers during luteolysis in bovine corpus luteum (Penny et al 1999) and showed immunostaining for Ki67 proliferation marker during luteal regression in bovine (Bauer et al 2001) and equine (Al-zi'abi et al 2003). The relevance of cFLIP overexpression in macrophages like cells during luteolysis may highlight their significant role in clearing up cellular debris and apoptotic cells (Al-zi'abi et al 2002) as well as

cytokine production such as IFNγ/TNFα which are assumed to magnify the Fas-mediated apoptosis system in the bovine CL (Taniguchi et al 2002).

In conclusion, the present study showed a downregulated cFLIP during structural luteal regression, suggesting that cFLIP plays a crucial role in the bovine CL.

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