The Role of Interleukin-6 in the Regulation of Granulosa Cell Apoptosis During Follicular Atresia in Pig Ovaries

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Abstract. More than 99% of follicles in mammalian ovaries undergo a degenerative process known as atresia, and only a few follicles actually ovulate during follicular growth and development. Follicular selection mostly depends on granulosa cell apoptosis, but the molecular mechanism behind the regulation of this selective atresia is still largely unknown. In the present study, to examine whether or not interleukin-6 (IL-6), a multifunctional cytokine, is involved in apoptosis during atresia in pig ovaries, the expression of IL-6 mRNA in granulosa cells was quantified by real-time reverse transcription-polymerase chain reaction (RT-PCR). The level of mRNA decreased during atresia. Enzyme-linked immunosorbent assay (ELISA) showed that the level of IL-6 protein in follicular fluid also decreased during atresia. Moreover, recombinant human IL-6 upregulated the expression of an intracellular apoptosis inhibitor, cellular FLICE-like inhibitory protein long form (cFLIPL), in cultured cells derived from human granulosa cells. It is possible that IL-6 is produced in the granulosa cells of healthy follicles, that it increases the cFLIPL level, and cFLIPL then prevents apoptotic cell death.

Key words: Apoptosis, Follicular atresia, Granulosa cell, Interleukin-6 (IL-6), Pig ovary

In mammalian ovaries, more than 99% of the follicles undergo a degenerative process known as atresia, and only a few follicles ovulate during ovarian follicular development [1, 2]. We have investigated the molecular mechanism of selective follicular atresia in porcine ovaries and have reported that follicular selection predominantly depends on granulosa cell apoptosis [3, 4]. However, we have little knowledge of the molecular mechanisms that control apoptotic cell death in granulosa cells during follicular selection.

Interleukin-6 (IL-6), a multifunctional cytokine originally identified as B-cell differentiated factor [5, 6], is produced by fibroblasts, keratinocytes, and vascular endothelial cells as well as immune cells such as lymphocytes, monocytes, and macrophages [7]. IL-6 binds with a transmembrane receptor, IL-6 receptor (IL-6 receptor α: IL-6Rα), or a soluble receptor (IL-6 soluble receptor: IL-6sR). In most cytokine signal transduction systems, the soluble receptor binds with its ligands and inhibits signal transduction by competing with the transmembrane receptor, but IL-6sR can transduce IL-6 signals the same as IL-6Rα [8]. Briefly, IL-6 binds with IL-6sR, and the IL-6/IL-6sR complex binds with a signal transducer, gp130. This binding leads to homodimerization of gp130, which results in activation of the gp130-associated Janus kinases (JAKs) [9, 10]. Subsequently, the activated JAKs phosphorylate gp130 at several tyrosine residues,
and these phosphorylated molecules recruit various Src homology 2 (SH2) domain-containing proteins, such as signal transducer and activator of transcription 3 (STAT3) and Src homology 2-containing tyrosine phosphatase 2 (SHP-2). These intracellular events lead to activation of multiple signal-transduction pathways, including STAT3, Ras/mitogen-activated protein kinase (MAPK), and phosphoinositide 3-kinase (PI3K) [11–14]. Finally, the IL-6 signal is transduced into the nucleus and regulates the transcription of specific gene sets. The mechanisms regulating these transduction pathways are not fully understood. Recently, it has been reported that IL-6 plays important roles in cell survival because it inhibits apoptosis by upregulating expression of the antiapoptotic factor Mcl-1 through activation of the STAT3, MAPK, and PI3K pathways [12–14] and by inducing phosphorylation of the proapoptotic factor Bad through activation of the PI3K pathway [11]. The roles of IL-6 in the apoptosis of granulosa cells remain to be investigated.

In the present study, we examined the changes in the levels of IL-6 mRNA in granulosa cells and IL-6 protein in follicular fluid during atresia and examined the changes in localization of IL-6 mRNA to confirm the physiological roles of IL-6 in granulosa cell apoptosis. Moreover, we assessed whether IL-6 affects the expression of an antiapoptotic factor, cFLIP L, in cultured KGN cells, a human granulosa cell-derived cell line.

Materials and Methods

Preparation of granulosa cells

Ovaries were obtained from mature sows weighing more than 120 kg at a local slaughterhouse. Individual antral follicles, approximately 3 mm in diameter, were dissected from the ovaries under a surgical dissecting microscope (SZ40; Olympus, Tokyo, Japan). Each follicle was classified as morphologically healthy or atretic and was further subdivided into early and progressed atretic follicles. Follicular fluid from each follicle was collected using a 1-ml syringe, separated by centrifugation at 3,000 g for 10 min at 4 C, frozen, and kept at –80 C. After biochemical analyses were performed, 17β-estradiol (E2) and progesterone (P4) levels were retrospectively measured using [125I]-RIA kits (Bio-Mérieux, Marcy-l’Etoile, France) to confirm classification of the follicles. Follicles with a P4/E2 ratio of less than 15 were classified as healthy according to previous findings [15–18]. Each follicle was opened using fine watch maker forceps, and granulosa layers and oocyte-cumulus complexes were removed. The granulosa cells were isolated with Pasteur’s pipettes, collected, washed 3 times in phosphate-buffered saline (PBS; pH 7.2) by centrifugation at 600 g for 5 min at room temperature (20–25 C), and used for real-time RT-PCR analysis.

Quantitative real time RT-PCR analysis and DNA sequencing

To quantify the expression of porcine IL-6 mRNA in granulosa cells prepared from healthy, early atretic, and progressed atretic follicles, total RNA was isolated from the cells using a RNeasy mini kit (Qiagen, Chatsworth, CA, USA) and then reverse-transcribed using the T-primed first-strand kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) to synthesize cDNA. Porcine IL-6 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; used as an intrinsic control) mRNAs were quantified using a Light-Cycler system (Roche Diagnostics, Rotkreuz, Switzerland) according to the manufacturer’s instructions. Briefly, PCRs were performed in a 20-µl reaction mixture (Light-Cycler DNA master SYBR green I; Roche) containing 0.5 µM of each primer, nucleotides, Taq DNA polymerase, and 3 mM MgCl2. The primers for amplification of the partial cDNA sequences of IL-6 and GAPDH were as follows: 5’- GACCT GCTTG ATGAG AATCA CC -3’ (forward) and 5’- ATCCA CTCGT TCTGT GACTG C -3’ (reverse) for IL-6 and 5’- GGACT CATGA ACCAC GTCCA T -3’ (forward) and 5’- TCAGA TCCAC AACCG ACACG T -3’ (reverse) for GAPDH. The expected PCR products of IL-6 and GAPDH were 231 and 220 bp, respectively. The conditions of amplification were as follows: denaturation at 95 C for 10 min and 40 cycles of denaturation at 95 C for 15 sec, annealing at 64 C for 4 sec, and extension at 72 C for 9 sec for IL-6 and denaturation at 95 C for 10 min and 35 cycles of denaturation at 95 C for 15 sec, annealing at 64 C for 5 sec, and extension at 72 C for 8 sec for GAPDH. Quantification was performed using the Light-Cycler analysis software (Roche) on an IBM computer. To confirm the expression of IL-6 mRNA, the DNA sequence of the PCR product was determined using an automatic DNA
sequencer (ABI Prism 310; PE Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s directions.

**Enzyme-linked immunosorbent assay**

The expression level of porcine IL-6 in follicular fluid prepared from healthy, early atretic, and progressed atretic follicles was measured by ELISA using an IL-6 ELISA kit (Quantikine; R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions. After the immunochemical reaction, absorbance at 450 nm was measured with a microplate reader (Model 680; Bio-Rad Laboratories, Melville, NY, USA).

**Immunohistochemistry**

Ovaries obtained at a local slaughterhouse were fixed in 10% (v/v) phosphate-buffered formalin (pH 7.4; Wako, Osaka, Japan), dehydrated through a graded ethanol series, and embedded in Histosec paraffin (Merck, Darmstadt, Germany). To determine the localization of apoptotic cells, proliferating cells, and macrophages, serial paraffin-embedded sections 3 µm thick were cut, mounted on glass slides precoated with 3-aminopropyltri-methoxysilane (silane; Sigma Aldrich Chemicals, St. Louis, MO, USA), deparaffinized, rehydrated, and washed with PBS. As previously reported [19], to visualize the apoptotic cells in ovarian tissue, the sections were stained by the terminal deoxynucleotidyl transferase-mediated biotinylated deoxyuridine triphosphate nick end-labeling (TUNEL) method using an apoptosis in situ detection kit (ApopTag; Intergen, Manhattanville, NY, USA) according to the manufacturer’s directions. They were counterstained with methyl green, dehydrated, mounted with Entellan (Merck), and examined by light microscopy. As negative controls, sections were incubated without either terminal deoxynucleotidyl transferase or anti-digoxigenin (DIG) antibody. As positive controls, sections of rat intestine were stained. Proliferating cells in ovarian tissues were detected as follows [20]. Serial paraffin sections were treated with 1% (v/v) HCl for 10 min and digested with proteinase K (10 µg/ml; Roche) for 23 min. They were postfixed with 4% (w/v) paraformaldehyde (Wako) in PBS, rinsed well with PBS, and prehybridized with 50% (v/v) deionized formamide in hybridization solution (Ambrosco, Solon, OH, USA) for 1 h at room temperature. They were hybridized with the DIG-labeled antisense cRNA probe for IL-6 mRNA (1 µg/ml diluted with hybridization solution) for 18 h at 45 °C. The DIG-labeled antisense and sense cRNA probes were synthesized using a Lig’n scribe kit (Ambion, Austin, TX, USA) and DIG RNA labeling kit (Roche) according to the manufacturer’s directions [19, 21]. The sections were washed with 2 × standard saline citrate (SSC; Invitrogen, Carlsbad, CA, USA) for 1 h, 2 × SSC containing 0.075% Brij35 (Sigma) for 1 h, 0.5 × SSC containing 0.075% Brij35 for 1 h, and then 0.2 × SSC containing 0.075% Brij35 substrate-chromogen solution (Dako) for 1 min. After being washed with distilled water, they were counterstained with methyl green, dehydrated, mounted with Entellan, and examined by light microscopy. In each experimental run, adjacent sections incubated without antibody were prepared as a negative control. Sections of rat testis were stained as a positive control. To determine the localization of macrophages in ovarian tissue, the slides were treated with 3% H2O2 in methanol for 5 min to inactivate endogenous peroxidase, washed with PBS, and incubated with mouse anti-macrophage scavenger receptor (diluted 1:100 with PBS; Transgenic, Kumamoto, Japan) for 18 h at 4 C. After a thorough washing with PBS, they were incubated with biotinylated anti-mouse antibody (Vector Laboratories, Burlingame, CA, USA) for 30 min at room temperature, washed well with PBS, and incubated with Vectastain ABC solution (Vector Laboratories) for 1 h. Then they were washed with PBS and incubated with substrate-chromogen solution (Dako) for 1 min. After being washed with distilled water, they were counterstained with methyl green, dehydrated, mounted with Entellan, and examined by light microscopy (BX-51; Olympus).
for 30 min at 45°C. They were rinsed with 100 mM Tris-HCl (pH 7.5) containing 150 mM NaCl (THS), immersed in blocking solution (Roche) for 1 h at room temperature, incubated with alkaline phosphatase (AP)-conjugated sheep anti-DIG antibody (diluted 1:500 with the blocking solution; Roche) for 2 h, and washed with THS. They were then equilibrated with 100 mM Tris-HCl (pH 9.5) containing 100 mM NaCl and 100 mM MgCl₂ (THSM), incubated with 0.4 mM nitroblue tetrazolium chloride (Roche), 0.4 mM 5-bromo-4-chloro-3-indolyl-phosphate 4-toluidine salt (Roche), and 1 mM levamisole (Dako) in THSM for 15 h at 4°C, and washed well with PBS. The sections were mounted with Histofine (Nichirei, Tokyo, Japan) and examined with a light microscope. As negative controls, serial sections were hybridized with the DIG-labeled sense cRNA probe or without a probe, or incubated without anti-DIG antibody.

KGN cell culture and Western immunoblotting for cFLIPLₐ

Human granulosa tumor cell-derived KGN cells were cultured in DMEM-F12 (Invitrogen) containing 10% (v/v) fetal calf serum (FCS; Invitrogen), 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen) in an incubator (Sanyo, Osaka, Japan; 5% CO₂ and air) at 37°C. The cells were seeded in 6-well culture plates (Becton Dickinson Labware, Franklin Lakes, NJ, USA) at a density of 5.0 × 10⁴ cells/ml and precultured in DMEM-F12 containing 10% fetal calf serum for 24 h, DMEM-F12 containing 0.5% fetal calf serum for 24 h, and then DMEM-F12 for 2 h. After preculture, they were incubated with 100 ng/ml recombinant human IL-6 (rhIL-6; R&D Systems) in DEME-F12 for 0, 6, 12, and 24 h. After incubation, they were washed with PBS, fixed in 10% (w/v) trichloroacetic acid (Wako) for 30 min at 4°C, and collected by centrifugation at 15,000 g for 5 min at 4°C. The cells were then treated with extraction medium [9 M urea (Wako), 2% (v/v) Triton X-100 (Sigma) and 1% (w/v) dithiothreitol (DTT; Wako)]. The protein fraction (30 µg per lane) prepared from each extraction sample was separated by 10–20% gradient SDS-PAGE (Atto, Tokyo, Japan) and then transferred onto PVDF membranes (Immobilon Transfer Membrane; Millipore, Marlborough, MA, USA). The membranes were stained with 0.2% (w/v) Ponceau S solution (Serva Electrophoresis, Heidelberg, Germany) and then immersed in blocking solution [TBST containing 2.5% (w/v) BSA (Sigma)] for 30 min. This was followed by incubation with mouse monoclonal anti-cFLIP_p antibody (diluted 1:100 with blocking solution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C for 18 h. After washing, they were incubated with HRP-conjugated anti-mouse IgG antibody (diluted 1:2,000 with blocking solution; Dako) for 1 h at room temperature and then chemiluminescence was visualized using an ECL system (Amersham Pharmacia) according to the manufacturer’s instructions. The chemiluminescence was recorded with a digital fluorescence recorder (LAS-1000; Fuji Film, Tokyo, Japan). Using the same membrane, the GAPDH protein level was also examined as an internal control. Goat monoclonal anti-GAPDH antibody (diluted 1:2,000 with blocking solution; Santa Cruz Biotechnology) and HRP-conjugated anti-goat IgG antibody (1:2,000 diluted with blocking solution; Dako) were used as the primary and secondary antibodies, respectively. Protein expression (cFLIPₐ/GAPDH) was quantified using the Image-Gauge program (Fuji Film) on a Macintosh computer.

Statistical analysis

All experiments including isolation of follicles and preparation of granulosa cells were repeated three times with separate groups (nine sows/group) for independent observation. Before ANOVA processing, the homogeneity of variance was assessed using the StatView-4.5 program (Abacus Concepts, Berkely, CA, USA) on a Macintosh computer. The StatView-4.5 program was used to conduct ANOVA with Fisher’s least significant differences test for biochemical data and Wilcoxon’s signed-rank tests for histological estimation. Differences of P<0.05 were considered significant.

Results

Changes in IL-6 mRNA levels in porcine granulosa cells during atresia

Detection of porcine IL-6 mRNA was confirmed based on the DNA sequence of the RT-PCR product. The DNA sequence of the corresponding domain of IL-6 was 5'-GACCT GCTTG ATGAG AATCA CCACC GGTCT TGTGG AGTTT CAGAT AATCA CCACC GGTCT TGTGG AGTTT CAGAT ATACC TGGAC TACCT CCAGA AAGAG
TATGA GAGCA ATAAG GGAAA TGTCG AGGCT GTGCA GATTA GTACC AAAGC ACTGA TCCAG ACCCT GAGGC AAAAG GGAAA GAATC CAGAC AAAGC CACCA CCCCT AACCC CACCA CAAAT GCCGG CCTGC TGGAT AAGCT GCAGT CACAG AACGA GTGGA T -3' (231 bp). The sequence of this PCR product corresponded with the known sequence of porcine IL-6 (GenBank accession number: M80258). The degree of homology between porcine and human IL-6 (GenBank accession number: M14584) was 79%.

IL-6 and GAPDH mRNAs were detected in isolated granulosa cells from healthy, early atretic, and progressed atretic follicles by quantitative real-time RT-PCR. Quantification using Light-Cycler showed that IL-6 mRNA expression (IL-6 mRNA/GAPDH mRNA ratio) decreased during follicular atresia (P<0.05; Fig. 1). Higher levels of IL-6 mRNA were seen in granulosa cells prepared from healthy follicles than in those from early atretic and progressed atretic follicles.

Changes in IL-6 protein levels in porcine follicular fluid during atresia

IL-6 protein was quantified in follicular fluid from healthy, early atretic, and progressed atretic follicles by ELISA. As shown in Fig. 2, the IL-6 protein level in the follicular fluid decreased during follicular atresia.

Localization of TUNEL-positive cells, PCNA-positive cells, macrophages, and IL-6 mRNA in porcine ovaries

No apoptotic (TUNEL-positive) cells were found in healthy follicles, but many proliferating (PCNA-positive) cells were present in the granulosa layer (Fig. 3C and D). Strong staining for IL-6 mRNA was demonstrated in the granulosa layer and negative/weak expression was shown in theca internal and external layers (Fig. 3A). In early atretic follicles, a few apoptotic cells were located on the inner surface of the granulosa layer and many proliferating granulosa cells were detected (Fig. 3G and H). As in our previous studies [17, 20, 22, 23], apoptosis occurred in granulosa cells located on the inner surface of the follicular wall in the follicles at the early stages of atresia (Fig. 3H). Weaker expression of IL-6 was observed in granulosa cells than in healthy follicles, and negative/trace expression was observed in theca internal and external layers (Fig. 3E). In the follicles at the progressed stage of atresia, many apoptotic cells, but no proliferating cells, were detected in the granulosa layer (Fig. 3K and L). Nuclear morphological changes such as condensation of nuclei and apoptotic bodies were observed. No expression of IL-6 mRNA was observed in the granulosa or theca layers (Fig. 3I). No positive reactions were seen in sense probe stainings used as negative controls (Fig. 3B, F and J). As shown in Fig. 4, immunohistochemical reactions for

![Fig. 1](image1.png)

![Fig. 2](image2.png)
macrophage-specific antigen were observed in the theca layers of healthy follicles, in which no apoptotic cells were detected (Fig. 4A and B), and progressed atretic follicles, in which many apoptotic cells were observed (Fig. 4C and D). Immunohistochemical reactions for macrophage-specific antigen were demonstrated in the follicular antral cavities of progressed atretic follicles, but not in healthy follicles (Fig. 4C). No local IL-6 mRNA expression was detected for macrophages that had invaded the follicular antrum of progressed atretic follicles (Fig. 5A, B and C).

**Effect of IL-6 stimulation on cFLIP L expression in KGN cells**

The protein levels of cFLIP L in KGN cells were assessed by Western blotting (Fig. 6A). When the KGN cells were treated with 100 ng/ml rhIL-6, the expression levels of cFLIP L protein in the cells increased (P<0.05; Fig. 6B).

**Discussion**

The balance between pro-survival and pro-death
factors is crucial for the regulation of cell growth, proliferation, differentiation, and death in granulosa cells, which are unique in that they die when a follicle begins atresia but differentiate into luteal cells after ovulation [24]. Although it has been revealed that many cytokines are involved in granulosa cell apoptosis during follicular atresia [25–27], we have no clear knowledge of the role of IL-6 in the regulation of granulosa cell apoptosis. In the present study, we investigated whether or not IL-6 is involved in granulosa cell apoptosis during atresia. It has been reported that IL-6 is produced by granulosa cells in human, rat, and bovine ovaries and that it affects the folliculogenesis and steroidogenesis of granulosa cells [28–31], but in porcine ovaries, IL-6 is produced by immune cells such as macrophages and T cells, not granulosa cells [32]. In the present study, however, we confirmed that IL-6 mRNA was expressed in granulosa cells of healthy, early atretic, and progressed atretic follicles in porcine ovaries. Interestingly, the level of IL-6 mRNA in the granulosa cells decreased during follicular atresia. Moreover, the level of IL-6 protein in the follicular fluid decreased in the progressed atretic follicles. Thus, the IL-6 signal transduces into the cells, especially in healthy follicles. In other organs such as spleen, mostly macrophages produce IL-6. However, histochemistry in the present study showed that IL-6 was not produced by macrophages in the ovaries. Cytokines produced by immune cells such as macrophages are involved in various physiological events, such as follicular development, atresia, ovulation, and luteolysis in ovaries [33, 34]. Macrophages are present in the ovaries of many animal species, but it has not been clear whether they are located in the theca layer, granulosa layer, or follicular antrum [35–37]. In the

Fig. 4. Ovarian sections from (A and B) healthy and (C and D) progressed atretic follicles. Macrophages (A and C) and apoptotic cells (B and D) were immunohistochemically detected using anti-macrophage scavenger receptor antibody and the TUNEL method, respectively (an arrow in C indicates macrophages). A, B, C and D were serial sections. g: granulosa cell layer. ti: theca internal layer.

Fig. 5. Ovarian sections from progressed atretic follicles. Macrophages were immunohistochemically detected (arrows in A indicate macrophages). Representative sections were subjected to in situ hybridization with an anti-sense cRNA probe for IL-6 mRNA (B) and a sense probe (C) as a negative control. A, B and C were serial sections. g: granulosa cell layer. ti: theca interna layer.
present study, we clearly demonstrated that macrophages are located only in theca external and internal layers of healthy follicles, and that they are not in the granulosa layer or follicular antrum. In progressed atretic follicles, they invaded the follicular antrum. No IL-6 mRNA was expressed by macrophages that invaded the follicular antrum in the progressed atretic follicles. Thus, in porcine ovaries, IL-6 is predominantly produced in granulosa cells, not macrophages. To date, no report concerning the inhibiting effect of IL-6 on apoptosis in granulosa cells has been published, and the present findings constitute the first report on IL-6-dependent regulation of apoptosis in granulosa cells.

To investigate the molecular mechanism regulating IL-6-dependent apoptosis in granulosa cells, we investigated the expression and changes in apoptosis-related factors in cultured granulosa cells treated with IL-6. IL-6 elevated the expression of cFLIP${}_L$, which is an inhibitor for death receptor-mediated apoptosis signal transduction. The inhibiting mechanism of cFLIP${}_L$ is as follows: cFLIP${}_L$ has two death effector domains (DEDs). One DED of cFLIP${}_L$ binds with the DED of Fas-associated death domain (FADD) and/or procaspase-8. This binding prevents FADD from interacting with procaspase-8 and inhibits apoptosis [38–40]. Recently, we identified a porcine cFLIP gene [41] and revealed cFLIP${}_L$ to be dominantly involved in the regulation of apoptosis in granulosa cells [21]. Based on these findings, we conclude that the mode of action of IL-6 in the regulation of granulosa cell apoptosis is as follows. (1) In healthy follicles, granulosa cells express higher levels of IL-6 and secrete higher levels of IL-6 into follicular fluid. (2) The IL-6 signal is transduced in an autocrine fashion, promoting FLIP${}_L$ production, which results in inhibition of apoptosis. (3) In atretic follicles, the expression level of IL-6 decreases and IL-6 signal transduction is arrested. Thus, cell death is induced and follicular atresia proceeds. This is the first report showing that IL-6 upregulates the expression of cFLIP${}_L$. Previous studies have reported upregulation of cFLIP${}_L$ expression by STAT3, which is activated by IL-6 [42], and decreased expression of cFLIP${}_L$ in IL-6 knockout mice [43]. The molecular mechanisms behind the upregulation of cFLIP${}_L$ expression by IL-6, especially which pathway is activated and how the transcription of genes is regulated, are currently being studied in our laboratory.

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