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—Review—

Regulation Mechanism of Selective Atresia in Porcine Follicles: Regulation of Granulosa Cell Apoptosis during Atresia

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Abstract. More than 99% of follicles undergo a degenerative process known as "atresia", in mammalian ovaries, and only a few follicles ovulate during ovarian follicular development. We have investigated the molecular mechanism of selective follicular atresia in mammalian ovaries, and have reported that follicular selection dominantly depends on granulosa cell apoptosis. However, we have little knowledge of the molecular mechanisms that control apoptotic cell death in granulosa cells during follicle selection. To date, at least five cell death ligand-receptor systems [tumor necrosis factor (TNF)α and receptors, Fas (also called APO-1/CD95) ligand and receptors, TNF-related apoptosis-inducing ligand (TRAIL; also called APO-2) and receptors, APO-3 ligand and receptors, and PFG-5 ligand and receptors] have been reported in granulosa cells of porcine ovaries. Some cell death ligand-receptor systems have "decoy" receptors, which act as inhibitors of cell death ligand-induced apoptosis in granulosa cells. Moreover, we showed that the porcine granulosa cell is a type II apoptotic cell, which has the mitochondrion-dependent apoptosis-signaling pathway. Briefly, the cell death receptor-mediated apoptosis signaling pathway in granulosa cells has been suggested to be as follows. (1) A cell death ligand binds to the extracellular domain of a cell death receptor, which contains an intracellular death domain (DD). (2) The intracellular DD of the cell death receptor interacts with the DD of the adaptor protein (Fas-associated death domain: FADD) through a homophilic DD interaction. (3) FADD activates an initiator caspase (procaspase-8; also called FLICE), which is a bipartite molecule, containing an N-terminal death effector domain (DED) and a C-terminal DD. (4) Procaspase-8 begins auto-proteolytic cleavage and activation. (5) The auto-activated caspase-8 cleaves Bid protein. (6) The truncated Bid releases cytochrome cfrom mitochondrion. (7) Cytochrome c and ATP-dependent oligimerization of apoptotic protease-activating factor-1 (Apaf-1) allows recruitment of procaspase-9 into the apoptosome complex. Activation of procaspase-9 is mediated by means of a conformational change. (8) The activated caspase-9 cleaves downstream effector caspases (caspase-3). (9) Finally, apoptosis is induced. Recently, we found two intracellular inhibitor proteins [cellular FLICE-like inhibitory protein short form ($cFLIP_S$) and long form ($cFLIP_L$)], which were strongly expressed in granulosa cells, and they may act as anti-apoptotic/survival factors. Further *in vivo* and i*n vitro* studies will elucidate the largely unknown molecular mechanisms, e. g.

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which cell death ligand-receptor system is the dominant factor controling the granulosa cell apoptosis of selective follicular atresia in mammalian ovaries. If we could elucidate the molecular mechanism of granulosa cell apoptosis (follicular selection), we could accurately diagnose the healthy ovulating follicles and precisely evaluate the oocyte quality. We hope that the mechanism will be clarified and lead to an integrated understanding of the regulation mechanism.
Key words: Apoptosis, Cell death ligand, Cell death receptor, Follicular atresia, Granulosa cell, Porcine ovary

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Uiviparity is a newly discovered characteristic of reproduction in mammals, and there are a wide variety of ovarian functions (follicular growth, development and selection, ovulation, luteinization and luteolysis), as well as fertilization, implantation and embryonic development. In female mammals, the ovaries release a constant small number of oocytes at appropriate intervals during the fertile years of the organism, and the duration of the ovulatory process is species-specific. During growth and development of the oocytes, which are enclosed and mothered by granulosa cells in follicles, less than 1% of oocytes are selected and the remainder are removed [1, 2]. The total number of ovulations, which is genetically regulated and called the "ovulation rate", is an important parameter of the reproductive efficiency of farm animals, especially of sows. Although the ovulation rate is a major limiting factor in determining the number of offspring born, numerous additional factors act upon the uterus and conceptuses throughout gestation and contribute to the number of healthy fetuses that develop to term [3]. Many investigators have studied which factors directly or indirectly regulate and modulate the ovulation rate, and how many follicles grow or die during atresia [4–14]. In mammalian ovaries, the process of follicular atresia is as follows [15–19]. (1) Scattered pyknotic nuclei (apoptotic bodies) are seen in the granulosa cells. (2) Detachment of the granulosa layer from the follicular basement membrane, whose structure is not destroyed, is observed. (3) Then, fragmentation of the basal membrane begins. (4) Cell debris and macrophages, which invade from the stromal area of the ovary, are seen in the antrum of the follicle. (5) Finally, the follicle disappears. In granulosa cells of atretic follicles, increase expression of some mRNAs is seen while reduced synthesis of DNA and protein is noted [20, 21]. At the early stage of atresia, endocrine cells of the theca interna layer undergo hypertrophy [22], the oocyte undergoes meiosis-like changes, followed by oocyte fragmentation, and disruption of the oocyte-cumulus connection [23]. The morphological changes in granulosa cells at the earliest stage of atresia are due to apoptosis [14], which is also confirmed by a biochemical hallmark of apoptosis, DNA fragmentation multiples of approximately 180 bp [14, 24, 25]. Moreover, there are species-specific differences in the detailed characteristics of granulosa cell apoptosis, for instance, in the localization of apoptotic cells in the granulosa and theca interna layers during atresia [26].

In the present review, we mainly focus on the follicle selection which is regulated by discriminating atresia of growing follicles, and describe the regulation factors that affect follicular atresia. A brief description of how some factors might regulate atresia or follicular development is given. The present review discusses some recent observations in our laboratory concerning the cell death receptor, decoy receptor and intracellular apoptosis-regulating protein roles in the regulation of follicle selection in porcine ovaries.

Follicular Development

During embryogenesis, primordial germ cells migrate from the yolk sac through the dorsal mesentery of the hindgut to the genital ridge. The germ cells undergo extensive proliferation, and somatic cells derived from the mesenchyme of the genital ridge also proliferate. Then the somatic cells, called follicular epithelial cells (granulosa cells), enclose the germ cells to form the primordial follicles. After mitosis (somatic cell division), the

first meiotic division begins in the germ cells (primary oocytes). The primary oocyte becomes arrested in the diplotene stage of meiosis, until the surrounding follicle leaves the primordial stage (primordial follicles), and starts to grow to reach ovulation. Approximately 5 million primordial follicles are present in both ovaries 10 days after birth in sows (1.2, 1.1, 4 and 1 million primordial follicles in cows, sheep, women and mice, respectively) [1–5, 27]. During fertile life in sows, at the most 1,600 oocytes (less than 0.14% of total primary oocytes) will ovulate, and all others will disappear. Such a low success rate is considered the result of an important selection process for quality of oocytes.

In adult sows, primordial follicles are not stimulated to grow and develop at the same time, and only a small number of primordial follicles begin their development, while the remainder remain quiescent. Initiation of follicular growth involves endocrinological actions, mainly follicular stimulating hormone (FSH), and local modulating factors from granulosa cells and endocrine cells of the theca interna layer and from the growing oocytes [5, 28]. The early growth stage of primary follicles (follicles with mono-layer follicular epithelial/granulosa cells) and/or secondary follicles (follicles with stratified granulosa cells but without antrum) is characterized by a dramatic increase in proliferation of granulosa cells (rapid increase in number and size). Subsequently, granulosa cells separate from each other resulting in the formation of the follicular antrum (tertiary follicle). In the tertiary follicles, cumulus cells are differentiated from granulosa cells and surround the primary oocyte, which has germinal vesicle (GV) [29]. Due to a large increase in proliferation of granulosa cells and an increase in the size of the antrum, growth rates of the tertiary follicles show an exponential curve [30]. In the oocyte (secondary oocyte), meiosis restarts (GV disappearance: called GV breakdown), and the first polar body divides. Finally, selected follicles burst, and the secondary oocytes ovulate.

Recent studies in FSH receptor (FSHR) knock out mice and experimental rodents with hypophysectomy showed that FSH is essential for antrum formation in secondary follicles and post-antral follicular development in tertiary follicles [31], and that FSH acts as an accelerator and a survival factor for the follicles [3]. In gilts, FSH administration induces follicular recruitment and increases the ovulation rate in a dose-dependent manner [32–34]. Active immunization against inhibin, which is produced by granulosa cells and inhibits FSH excretion, increased the number of ovulations [35, 36]. The number of recruited follicles might depend on blood FSH levels and/or on the number of follicles responsive to FSH present at the time of recruitment. Unfortunately, we have insufficient knowledge of the number of FSH-responsive follicles related to the endocrinological event occurring before recruitment, or what other factors might influence it, and how follicles are accurately selected during follicular growth and development.

Follicular Atresia

Preparatory to describing the cell death ligand, its receptor and intracellular signaling systems in porcine ovaries, here we briefly mention the outline profiles of growth, development and atresia of the follicles [14, 39–43]. Primary follicles require 84 days to grow to the tertiary stage, and an additional 19 days are necessary to grow up to the ovulatory size, approximately 10 mm in diameter [40]. The process of follicle selection during the tertiary stage growth and development probably takes place continuously from days 13 to 14 of the estrous cycle to ovulation (day 21 of the estrous cycle) [38]. During the final maturation stage, the growth rate of tertiary follicles from 3 to 10 mm in diameter is approximately 1.14 mm/day [37], and the endocrine cells of the theca layers and granulosa cells secrete significant amounts of steroid hormones, peptide hormones, prostaglandins and other physiologically active substances, which participate locally in follicular growth, development and atresia and act as coordinators of the hypothalamic-hypophyseal-ovarian axis. On day 16 of the estrous cycle, approximately 160 to 200 tertiary follicles/ovaries are present [39], and then 150 to 190 follicles degenerate and disappear from the ovaries through the process of atresia [42]. Atresia may occur at any time during growth and development of follicles, but most follicles disappear before reaching 6 mm in diameter in sows [30, 37–40]. On day 21 of the estrous cycle, secondary oocytes are ovulated from approximately 10 matured follicles, which are appropriately selected. Within each mammalian

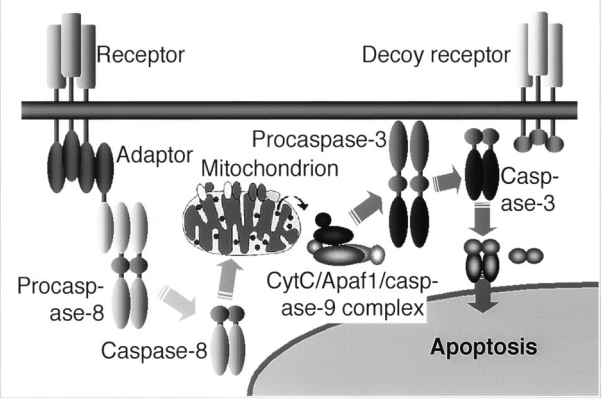

Fig. 1. Schematic illustration of our proposal for the cell death receptor-mediated apoptosis signaling pathway in granulosa cells of porcine ovarian follicles.

species, the ovulation rate is regulated within a relatively narrow range, and changes in rates of follicular atresia may alter the number of ovulated follicles. In pigs, the Meishan sow, a traditional Chinese breed, has a higher ovulation rate (maximum 30) and a larger number of piglets than conventional European breeds (Large White, Landrace, etc.) [44]. On day 16 of the estrous cycle, Meishan sows have more tertiary follicles than Large White sows, and the number of atretic follicles, which disappear from days 16 to 19 of the estrous cycle, is larger in Large White sows than in Meishan sows. Thus, the greater ovulation rate in the Meishan breed is related to differences in both follicular recruitment and atresia. Our preliminary study in Mangalica sows, which are native Hungarian pigs and have a lower ovulation rate and a smaller number of piglets (approximately 5) than conventional European breeds, showed that the lower ovulation rate in the Mangalica breed is also related to differences in both follicular recruitment and atresia.

Apoptosis in Granulosa Cells

During follicular growth and development in sows, more than 99% of follicles selectively disappear. Follicular atresia is primarily induced by granulosa cell apoptosis, which is characterized biochemically (internucleosomal DNA fragmentation) and morphologically (cell shrinkage, plasma membrane blebbing and formation of apoptic bodies) [6–14, 42–46]. Although considerable progress has been made in understanding the regulation mechanisms of apoptosis in granulosa cells during follicular atresia, the description of the apoptotic pathway in granulosa cells has not been completed [13]. Apoptotic stimuli and intracellular signal transduction pathways involved in granulosa cell apoptosis remain to be determined, and many investigators have been studying which trigger molecules induce granulosa cell apoptosis, and how intercellular apoptotic signals are transmitted in the granulosa cells [6–14, 42, 47, 48].

Recently, some cell-specific cell death ligands and cell death receptors were found by the researchers working in the fields of immunology and cancer therapy [49–50]. The major intracellular signaling pathway leading from the cell death ligand binding the cell death receptor to initiation of apoptosis is the caspase cascade system [57–60]. Each cell death receptor activates a specific caspase cascade within seconds of cell death ligand binding, causing apoptotic cell death within hours. To date, at least five cell death ligand-receptor systems [tumor necrosis factor (TNF)-α and receptors, Fas (also called APO-1/CD95) ligand and receptors, TNF-related apoptosis-inducing ligand (TRAIL; also called APO-2) and receptors, APO-3 ligand and receptors, and PFG-5 ligand and receptors] have been reported in granulosa cells of porcine ovaries [14]. Some cell death ligand-receptor systems have "decoy" receptors, which binds with ligand and act as extracellular inhibitors against cell death ligand-induced apoptosis in granulosa cells [14]. Moreover, we showed that the porcine granulosa cell is a type II apoptotic cell, which has the mitochondrion-dependent apoptosis-signaling pathway [14, 61–63]. The cell death receptor-mediated apoptosis signaling pathway in porcine granulosa cells has been suggested to be as follows (Fig. 1). (1) A cell death ligand binds to the extracellular domain of a cell death receptor, which contains an intracellular death domain (DD); (2) the intracellular DD of the cell death receptor interacts with the DD of the adaptor protein (Fas-associated death domain: FADD) through a homophilic DD interaction; (3) FADD activates an initiator caspase (procaspase-8; also called FLICE), which is a bipartite molecule, containing an N-terminal death effector domain

(DED) and a C-terminal DD; (4) procaspase-8 begins auto-proteolytic cleavage and activation; the activated caspase-8 cleaves Bid-protein, and the truncated Bid releases cytochrome c from mitochondrion. Cytochrome c and ATP-dependent oligimerization of the apoptotic protease-activating factor-1 (Apaf-1) allows recruitment of caspase-9 into the apoptsome complex. Activation of caspase-9 is mediated by means of conformational change. The activated caspase-9 cleaves downstream effector caspases (caspase-3), and apoptosis is induced. Recently, we found two intracellular inhibitor proteins (cellular FLICE-like inhibitory protein short form and long form: $cFLIP_S$ and $cFLIP_L$, respectively), which were strongly expressed in granulosa cells and may act as anti-apoptotic/survival factors [64]. Thus, porcine granulosa cells have some cell death ligand and receptor systems, however, as described below, their precise roles on the regulation of follicle selection have yet to be determined. Further *in vivo* and *in vitro* studies are necessary to elucidate which cell death ligand and receptor system is dominant in controling the granulosa cell apoptosis.

Interestingly, specific changes in glycoconjugates of cell surface membrane-glycoprotein, which act as regulators for phagocytosis in neighboring granulosa cells in atretic follicles, have been demonstrated in granulosa cells of atretic follicles [65, 66]. Expression of kinase cascades regulating entry into cell death and proliferation was also reported in them [67, 68]. Thus, the degeneration of atretic follicles in mammalian ovaries can be explained by apoptotic cell death of granulosa cells and endocrine cells of the theca interna layer [6–14, 21, 45–48], but the degenerating changes in cumulus cells during follicular atresia had not been investigated in detail. We examined them histologically, cytologically and biochemically and confirmed that apoptosis occurs in granulosa cells, but not cumulus cells or oocytes in tertiary follicles in the early stage of atresia, when no macrophages were detected [14, 19, 48, 69]. DNA fragmentation was visualized on ovarian sections using the terminal deoxynucleotidyl transferase-mediated biotinylated deoxyuridine triphosphate nick end-labeling (TUNEL) method, and cytological changes were determined by transmission electron microscopy (TEM). In healthy follicles, no apoptotic cells were observed among granulosa or cumulus cells, or the cells in internal or external theca layers, or oocytes. In the early stage of atresia, apoptosis was detected in scattered granulosa cells located on the inner surface of the follicular wall, but not in cumulus cells, oocytes or the cells of internal or external theca layers. Condensed nuclei, a typical apoptotic feature, were seen in scattered granulosa cells, but cumulus cells and oocytes with normal ultrastructure were seen in the same follicle. In the late stage of atresia, granulosa cells scattered on the inner surface of the follicular wall began to undergo apoptosis, but no TUNEL-positive cells were detected among the cumulus cells or oocytes. Finally, in the progressed stage of atresia, many apoptotic cells were located in the area abutting the basement membrane, which was partly broken, and macrophages had invaded the follicular antrum and ingurgitated the apoptotic granulosa cells and cell debris.

Our comparative studies of the progression of granulosa cell apoptosis during follicular atresia revealed that there are species-specific differences in the apoptotic process in granulosa cells [27, 48, 70]. In ovaries of rodents (rats and mice), randomly scattered apoptotic cells were observed in granulosa layers of follicles at the earliest stage. In bovine ovaries, granulosa cells located on the outer surface of the follicular wall appeared to undergo apoptosis at the earliest stage of atresia, followed by neighboring granulosa cells. In porcine ovaries, however, granulosa cells located on the inner surface of the granulosa layer appeared to undergo apoptosis in the earliest stage of atresia. In the ovaries of these mammalian species, detachment and degeneration of the granulosa layer, fragmentation of basement membrane, apoptotic endocrine cells in the theca interna layers were observed at the progressed stage. No apoptotic cells were observed in the theca externa layers during the early to middle stages of atresia. Thus, we concluded that apoptosis occurring in granulosa cells is an initial symptom of atresia in mammalian ovaries, but the initiation areas of granulosa cell apoptosis are different among the species, indicating local mechanisms of regulation of granulosa cell apoptosis, in particular the apoptotic stimuli induction mechanism may be different among mammalian species. To date, however, we have insufficient information on the induction mechanisms of granulosa cell apoptosis.

Many researchers have studied hormone control of follicular atresia through peptide hormones and

cytokines (FSH, luteinizing hormone: LH, gonadotropin-releasing hormone, insulin like growth factor-I, insulin-like growth factor binding protein, inhibin, activin, follistatin, interleukin-1β, etc.), as well as steroid hormones and their metabolites (estradiol-17β, progesterone, etc.) [71-93]. However, it is difficult to explain the locally regulated mechanism of granulosa cell apoptosis by a hormonally controlled system, which can well explain general regulation of follicular growth and development. Since the cell death ligand and receptor system is an appropriate system for understanding local cell to cell regulation mechanisms, we have focused on and examined the roles of the cell death ligand and receptor system in granulosa cell apoptosis during follicular atresia in porcine ovaries and describe here the physiological roles of them. To date, most investigators in the field of research of follicle selection have used the ovaries of neonatal and/or immature rodents, which have incomplete estrus cycles, because it is not possible to accurately isolate each component of the follicles and to evaluate the stages of follicular development in mature females. The ovaries of sows, which have complete estrous cycles, are suitable for the examination of the physiological roles of the cell death ligand and receptor system, since the stages of follicular development are easily evaluated by measurement of estradiol-17β and progesterone levels in follicular fluid [42, 81–86], and most importantly, it is easy to surgically manipulate and to accurately isolate each component of the follicle (oocyte, cumulus cells, granulosa cells and thecal layers) under a surgical microscope.

Apoptosis in Luteal Cells

The luteal cells of corpus lutea synthesize and secrete progesterone to provide uterine quiescence for the establishment and maintenance of pregnancy. In the absence of pregnancy, corpus lutea have a very limited life span. Functional and structural regressions of corpus lutea are necessary to maintain estrous-cyclicity and to avoid accumulation of non-functional luteal tissue within the ovary. Apoptotic cell death in luteal cells occurs during structural luteolysis. In luteolysis, most researchers suggest that the Fas-ligand and Fas system are the major mechanisms regulating luteal cell apoptosis [94–103]. Briefly, in murine luteal bodies, we demonstrated the expression of mRNA of the soluble form of Fas (soluble Fas; also called FasB: a decoy receptor), which binds to FasL and prevents apoptosis induction. By immunohistochemical staining and *in situ* hybridization, high expression levels of FasB protein and mRNA were demonstrated in normal luteal bodies, in which no apoptotic cells were detected, in constant to negative/trace expression in regressing luteal bodies, in which many apoptotic cells were observed. Immunohistochemical staining revealed that Fas and TNF-α were localized in both normal and regressing luteal bodies, but interferon (IFN)-γ was localized only in regressing luteal bodies. Apoptosis was induced in primary cultured luteal cells, when they were pretreated with TNF-α and IFN-γ and then incubated with TNF-α, IFN-γ and mouse recombinant FasL (rFasL). However, no apoptosis was detected in the cells, when they were treated with rFasL alone, TNF-α alone, IFN-γ alone, TNF-α and rFasL, IFN-γ and rFasL, or TNF-α and IFN-γ. Fas mRNA expression in cultured luteal cells was up-regulated by the treatment of TNF-α, IFN-γ or TNF-α and IFN-γ. The expression of FasB mRNA was down-regulated, when the cells were treated with TNF-α and IFN-γ, but its expression was not changed by treatment of TNF-α alone or IFN-γ alone. We concluded that FasB inhibits apoptosis induction in luteal cells of normal luteal bodies, and that decreased FasB production induced by TNF-α and IFN-γ made apoptosis induction possible in the luteal cells of regressing luteal bodies [102, 103].

Spontaneous oocyte apoptosis has been reported, but the detailed molecular processes of oocyte apoptosis have not been well explained [28, 93]. Hsueh *et al.* [13, 104] reported that targeted expression of Bcl-2, a major anti-apoptotic protein, in murine oocytes inhibits ovarian follicular atresia and prevents spontaneous and chemotherapy-induced oocyte apoptosis, indicating oocytes play important roles in the control of granulosa cell apoptosis in murine ovaries. In porcine ovaries, we could not detect apoptotic changes in oocytes by biochemical and morphological methods [14, 105-107].

Cell Death Ligand and Receptor Systems in Granulosa Cells

Fas ligand and Fas system

Apoptosis eliminates individual cells when they are no longer needed or have become seriously damaged by viral infection, cancerous transformation, etc. Mammals have evolved a signaling mechanism that actively directs cells to die by apoptosis. This process, which we refer to as instructive apoptosis, is critical particularly in the immune system [49–52]. The most studied paradigm for instructive apoptosis is that of the Fas ligand (FasL; also called Apo1L or CD95L) and Fas (Apo1 or CD95) [49–51]. FasL belongs to a family of proteins that have structural homology to TNF. FasL and TNFα define a subset of TNF-superfamily members that have apoptosis-inducing activity. FasL is predominantly expressed in activated T lymphocytes and natural killer cells and in several immune-privileged tissues and induces apoptosis in target cells through Fas. Fas, a transmembrane glycoprotein, belongs to the TNF/nerve growth factor (NGF) receptor superfamily and mediates apoptosis. Ligation of cell-surface Fas by FasL delivers an apoptotic signal that rapidly commits the cell to apoptotic cell death. *In vitro* studies show that FasL is critical for T-cell apoptosis. Furthermore, in mice or humans carrying spontaneous mutations in the genes encoding FasL or Fas, and in Fas gene knockout mice, accumulated lymphocytes resulting in a massive, lethal enlargement of lymph nodes have been observed. It is considered that the main biological role of the FasL and Fas system is to signal instructive apoptosis during peripheral deletion of lymphocytes. In addition, FasL and Fas-mediated apoptosis contributes to elimination of virus-infected cells and cancer cells by cytotoxic lymphocytes.

Recently, many researchers in the fields of immunology and oncology have been elucidating the intracellular signaling pathways that mediate instructive apoptosis. Their findings reveal that certain components of the instructive cell death pathway have roles not only in apoptosis but also in embryonic development, as well as in control of antigen-induced lymphocyte proliferation in adult mammals. Moreover, Fas mRNA expression was detected in the thymus, liver, heart, lung and ovary, but the physiological and pathological roles of the FasL and Fas system in the ovaries are not accurately understood. Immunohistochemical staining of rat ovaries revealed intense positive immunostaining for FasL and Fas in granulosa cells of small and medium antral follicles with atresia, and intense FasL staining was evident in the theca interna cells of healthy small-antral follicles [108]. We previously demonstrated that extreme accumulation of follicles and luteal bodies were seen in *lps* mice with hereditary abnormality of Fas [98]. Fas is expressed abundantly in surface epithelial cells, mediates apoptosis of the surface epithelial cells, and is involved with leuteolysis in luteal bodies of rodents, which contain luteal cells, stromal cells, endothelial cells, fibroblasts and surface epithelial cells [94–103]. In the ovaries of rodents, the FasL and Fas system may regulate granulosa cell apoptosis in ovarian follicle atresia and luteal cell degeneration [94–103, 108–112]. However, it has not been determined whether the FasL and Fas system mediates apoptosis in the ovaries of other species such as farm animals, including sows [14].

Our recent preliminary experiments indicated that constitutive expression of mRNAs of FasL and Fas estimated by conventional and quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) methods, and FasL and Fas proteins assessed by Western blotting analysis, were detected in granulosa cells of both healthy and atretic follicles of porcine ovaries [113]. Also, immunohistochemical staining for these proteins showed Fas localized in the cytoplasmic region but not in the cell-surface area in granulosa cells of both healthy and atretic follicles, and RT-PCR data showed constant mRNA expression of DcR3, a decoy receptor for FasL, in follicular cells of porcine ovaries. To date, we have not detected the expression of soluble decoy receptor (FasB), which is strongly expressed in functional luteal cells of murine ovaries and inhibits the induction of apoptosis [103], in follicular or luteal cells of porcine ovaries. Thus, we presume that the FasL and Fas system does not play a considerable role either in granulosa cell apoptosis during follicular atresia, or in luteal cells during luteolysis in porcine ovaries.

TNFα and TNF-receptor system

TNFα can induce both cell death and cell proliferation and exerts its effects by binding to

either TNF receptor (TNFR)-1 or TNFR2 [114, 115]. TNFα induces apoptosis in a variety of tumor cells, and the intracellular apoptosis signal pathway mediated by TNFR1 has been suggested to be as follows [116, 117]. (1) First, TNFα binds to the extracellular domain of TNFR1, which contains an intracellular death domain (DD). (2) The intracellular DD of the receptor interacts with the DD of the adaptor protein (TNF receptor-associated death domain protein: TRADD). (3) The DD of TRADD binds with the DD of another adaptor protein (Fas-associated death domain protein: FADD; also called MORT1). (4) FADD activates initiator caspase (procaspase-8). (5) Finally, the caspase cascade is activated for intracellular transduction of the apoptotic signal (named TNFR1-TRADD-FADD-caspase-8 signaling axis). In contrast, when TNFα acts as a survival/anti-apoptotic factor, the intracellular signal pathway mediated by TNFR1 has been suggested to be as follows [116–118]. (1) TNFα binds to the extracellular domain of TNFR1. (2) The intracellular DD of the receptor interacts with the DD of TRADD. (3) The DD of TRADD binds with the DD of receptor interacting protein (RIP). (4) RIP interacts with TNF receptor-associated factors (TRAF) 2. (5) Then, TRAF2 mediates the physical interaction of the TNFR1-signaling complex with the nuclear factor (NF)-κB-inducing inhibitor of κB kinase (IKK) and inhibitor of apoptosis proteins (cIAP) 1 (named TNFR1-TRADD-RIP-TRAF2 signaling axis). (6) Consequently expression of survival/anti-apoptotic genes is up-regulated. Thus, TRADD and RIP are key proteins at the point of divergence of cell death and cell proliferation in the TNFR1 signaling process, and TRAF2 is a good indicator of TNFα-dependent cell proliferation. When TNFα binds with TNFR2, which is a non-DD-containing TNF receptor. TNFα acts as a survival/anti-apoptotic and/or proliferating factor. The intracellular signal pathway mediated by TNFR2 has been suggested to be as follows [116–121]. (1) TNFα binds to the extracellular domain of TNFR2. (2) Although TRAF2 cannot bind directly to TNFR1 as described above, TRAF2 can interact directly with TNFR2. Thus, activated TNFR2 interacts with TRAF2. (3) NF-κB is activated. (4) Consequently expression of cell survival, growth and differentiation genes is up-regulated and apoptosis induced by TNFα is prevented. Thus, when TNFα bound TNFR2 interacts with TRAF2, expression of survival/anti-apoptotic/cell proliferation genes is up-regulated [121]. TRAF2 expression is considered to be a good indicator of TNFα-dependent cell proliferation in both TNFR1 and TNFR2 signaling cascades.

In primary cultured granulosa cells prepared from large antral follicles of porcine ovaries, TNFα could induce both cell proliferation and cell death [122]. However, there has been no *in vivo* confirmed evidence of whether TNFα acts as an apoptotic factor or as a survival/anti-apoptotic factor in porcine ovarian follicles. To determine the physiological roles of TNFα and its receptor system in granulosa cell apoptosis, we histochemically analyzed the *in vivo* changes in localization of TNFα signaling proteins (TNFα, TNF-receptors and TRAF2) in granulosa cells during follicular atresia in porcine ovaries, and examined the changes in localization and levels of expression of their mRNAs by *in situ* hybridization and by RT-PCR [121]. Moreover, the changes in expression level of TNFR2 in granulosa cells during follicular atresia were examined by Western blotting analysis. We demonstrated that in healthy follicles, intense signals for TNFα and TRAF2 and their mRNAs were demonstrated in the outer zone of the granulosa layer, where many proliferating cells but not apoptotic cells were seen. In the early atretic stage, decreased or trace staining for TRAF2 and its mRNA and decreased expression of TNFR2 were observed in the granulosa layer, where apoptotic cells were seen. Moreover, strong immunostaining for TNFR2, but not TNFR1, was demonstrated in granulosa cells. Our data indicate that TNFα acts as a survival factor in granulosa cells during follicular atresia in porcine ovaries. Although the biological roles of TNFα and its receptor system in ovarian tissues are largely unknown, TNFα and TNFR2 system may dominantly contribute to the selective survival of necessary cells under physiological conditions [114, 115]. Our findings suggest that TNFα and its receptor system, TNFR2, but not TNFR1, dominantly detected in ovarian tissue, play crucial roles in induction of survival/proliferating signals in granulosa cells during follicular growth in porcine ovaries. Further studies are necessary to determine which molecular system dominantly regulates the disappearance of TNFα receptor-associated proteins in granulosa cells in the early stage of atresia, and which intracellular signal transduction pathway

Table 1. The apoptosis-signaling pathways of cell death ligand and receptor system in mammalian cells

Incentive	Sensor	Adaptor	Initiator	Effector	Outcome
TRAIL	DR4, DR5 DcR1, DcR2 (Decoy)		Caspase-8	Caspase-3	Apoptosis
FasL	Fas DcR3 (Decoy)	FADD	Caspase-8	Caspase-3	Apoptosis
TNFα	TNFR1 TNFR2	TRADD/FADD	Caspase-8	Caspase-3	Apoptosis Proliferation
Apo3L	DR3	TRADD/FADD			
Unknown	DR6				
Unknown	PFG-5 PFG-6 (Decoy)		Caspase-8	Caspase-3	Apoptosis
Dexamethasone, ultraviolet etc.(Cytochrome c)(Apaf1) (mitochondrion-dependent)		(Caspase-9)	Caspase-3	Apoptosis	

Each pathway shares essential molecular components with those of other apoptotic stimuli, but the specific components of each pathway is different. For example, cell death receptors act as sensors for apoptotic stimulation by specific cell death ligands. Decoy receptors bind to specific cell death ligands and modulate apoptosis-induction by diverting specific cell death ligands from their cell death receptors. FADD, which acts as adaptor protein, couples Fas to initiator procapase-8, and also couples TNFR1 to procaspase-8. However, DR4 can transduce apoptotic signals without FADD. Caspase-3, which is one of the downstream components of caspase-8, acts as an effector. In type II apoptosis cells, activated caspase-8 transduces the apoptotic signal to mitochondrion.

dominantly causes granulosa cell survival.

TRAIL and TRAIL-receptor system

Through the screening of DNA databases for expressed sequence tags similar to TNFα, TNF-related apoptosis-inducing ligand (TRAIL; also called Apo2L), a novel cell death ligand, was identified in 1995 [123–125]. The cDNA sequence of TRAIL is similar to that of FasL, and *in vitro* functions of TRAIL are also like those of FasL and TNFα. TRAIL mRNA is expressed constitutively in many organs/tissues. TRAIL interacts with receptors that belong to the TNFR superfamily [53, 113, 126–137]. The receptors in the TNFR superfamily have several cysteine-rich domains in their extracellular N-terminal region. The cytoplasmic sequence divides the TNFR superfamily into two subgroups that either possess or lack a DD. The DD-containing receptors (called death receptors: DRs) include TNFR1, Fas, DR3 (also called Apo3, WSL-1, TRAMP or LARD), DR4 (TRAIL receptor 1: TRAILR1), DR5 (TRAILR2, TRICK2 or KILLER), and DR6 [53–56]. The DD couples each receptor to caspase cascades that induce apoptosis or to kinase cascades that turn on proliferating gene expression through NFκB and AP-1. Recently, an interesting subgroup of TNFR-homologues was found and named as "decoy" receptor (DcR). It has extracellular domains similar to active receptors, binds to ligands and acts as an inhibitor, rather than a transducer of apoptotic signaling. To date, the DcRs in the TNFR superfamily include DcR1 (TRAILR3, TRID or LIT) [131–135], DcR2 (TRAILR4 or TRUNDD) [134, 136], osteoprotegerin (OPG) [138], DcR3 [139] and PFG-6 [14, 105–107] (Table 1). DcR1, DcR2 and PFG-6 are cell-membrane proteins, and OPG and DcR3 are secreted/soluble proteins. Four of the novel cellular receptors (active receptors: DR4 and DR5, and decoy receptors: DcR1 and DcR2) bind to TRAIL and are structurally related [139]. Each has two extracellular cysteine-rich domains, and shows closer homology to the other TRAIL receptors than to the rest of the TNFR superfamily. TRAIL binds to all of these receptors with equivalent high affinities. DR4 and DR5 have cytoplasmic DDs. DcR1 lacks a cytoplasmic region, and appears to be attached to the cell surface through a glycosyl-phosphatidylinositol (GPI) anchor. DcR1-bearing cells pretreated with phosphatidylinositol specific phospholipase C (PI-PLC), which cleaves the GPI anchor and leads to removal of DcR1 from the cell

surface, showed a marked increase in apoptosis induced by TRAIL, indicating that DcR1 has an inhibitory effect on TRAIL-induced apoptosis [131–135]. DcR2 is also a cell-surface receptor and has a cytoplasmic DD, but it is two-thirds shorter than a typical DD of active receptors and does not signal apoptosis [134–136]. The extracellular domains of DcR1 or DcR2 compete with those of DR4 or DR5 for ligand binding. *In vitro* studies show that cell transfection with DcR1 or DcR2 inhibits apoptosis induction by TRAIL. Thus, DcR1 and DcR2 compete with DR4 and DR5 for ligand binding and can act as decoys for DR4 and DR5, preventing TRAIL from inducing apoptosis through DR4 and DR5. Hence, cells that express DcR1 and/or DcR2 at high levels relative to DR4 or DR5 may use the decoys as protection against the cytotoxic action of TRAIL [55, 56].

Unlike the limited localization of FasL, which is produced dominantly by immune cells, TRAIL is more abundantly expressed in many normal organs/tissues [52–56, 123, 140–144]. Moreover, DR4, DR5, DcR1 and DcR2 transcripts are also widely expressed in normal tissues. Although the biological functions of the TRAIL and TRAIL-receptor system are largely unknown, this apoptosis inducing system has been suggested to contribute to the selective abolishment of unnecessary cells under physiological (normal and healthy) conditions [145]. Recently, *in vitro* studies have shown that TRAIL can induce both apoptotic cell death and cell-proliferation [52–56, 146–148]. The cell death signal pathway mediated by TRAIL and TRAILRs has been suggested to be as follows. (1) TRAIL binds to the extracellular domain of active receptor (DR4 or DR5). (2) Intracellular DD of the receptor interacts with the DD of apoptosis-adaptor protein (TRADD). (3) The DD of TRADD binds with DD of an other adaptor protein (FADD). (4) FADD activates initiator caspase (procaspase-8). (5) Thereby, the caspase cascade is activated for intracellular transduction of the apoptotic signal. The cell proliferation signal pathway has been suggested to be as follows [146–148]. (1) TRAIL binds to the extracellular domain of active receptor (DR4 or DR5). (2) Intracellular DD of the receptor interacts with the DD of proliferation-adaptor protein (RIP). (3) The DD of RIP binds to the DD of the receptor. (4) NK-κB is activated. (5) Consequently expression of survival and cell proliferation genes is up-regulated. Thus, intracellular adaptor proteins (TRADD and RIP) are key proteins at the diverging point of cell death and cell proliferation, and TRADD expression is a good indicator of TRAIL-dependent apoptosis.

Our experiments [113, 149–151] were performed to determine the physiological roles of TRAIL and its receptor system on granulosa cell apoptosis during follicular atresia in porcine ovaries. Firstly, we histochemically examined the localization of TRAIL and its receptors in porcine ovaries [149]. A marked reduction in the expression of DcR1, which has high affinity for TRAIL, was demonstrated in granulosa cells of atretic follicles, but no marked differences were seen in expression of TRAIL, DR4 or DR5 in granulosa cells between healthy and atretic follicles. No biochemically positive reaction against DcR2 protein and mRNA was detected. Secondly, we examined the changes in expression and localization of mRNA of TRADD, which transmits the death signal from receptors to intracellular components, in granulosa cells during follicular atresia [159]. RT-PCR and *in situ* hybridization analyses revealed increased mRNA expression of TRADD in granulosa cells, demonstrated only in atretic follicles. Finally, to confirm the inhibitory activity of DcR1 in granulosa cells, primary cultured granulosa cells prepared from healthy follicles of porcine ovaries were treated with the enzyme, PI-PLC, to cleave GPI anchor of DcR1 and to remove DcR1 from the cell surface, and then incubated with TRAIL [151]. PI-PLC treatment increased the number of apoptotic cells induced by TRAIL. Our findings indicate that TRAIL and its receptors (DR4, DR5 and DcR1) are involved in induction of apoptosis in granulosa cells during atresia, and that DcR1 plays an inhibitory role in granulosa cell apoptosis, at least in porcine ovaries.

Novel cell death receptor and decoy receptor system

We prepared some monoclonal antibodies against granulosa cells prepared from porcine ovarian follicles, which recognized a novel cell death receptor and decoy receptor as follows [14, 105–107, 152–155]. Individual preovulatory tertiary follicles, approximately 3 mm in diameter, were dissected from porcine ovaries under a surgical dissecting microscope. Because the progesterone/estradiol-17β ratio of follicular fluid in each follicle provides a good index of follicular atresia in sows [42, 86], when the progesterone/estradiol-17β ratio

of follicular fluid quantified using (^{125}I)-RIA kits was less than 15, the follicle was classified as a healthy follicle. The granulosa layer was removed from each follicle and incubated in Ca^{2+}/Mg^{2+}-free Hanks' balanced salt solution containing 10 mM ethylenediaminetetraacetic acid disodium salt and 6.8 mM ethyleneglycol-*bis*-tetraacetic acid for 15 min at 22 C, and then granulosa cells were isolated by gentle pipetting. Female BALB/c mice were immunized intravenously with the isolated granulosa cells. The spleen cells from immunized mice, which produced anti-granulosa cell antibodies, were fused with Sp2/O-Ag14 mouse myeloma cells by the standard hybridization techniques. The hybridoma cells were selected by conventional hypoxanthine-aminopterin-thymidine and hypoxanthine-thymidine method [156]. Then, the hybridoma cells producing antibodies against the granulosa cells were screened by immunofluorescence staining using frozen sections of porcine ovaries, and antibody class was determined by an ELISA method. Three hybridoma cell lines (PFG-5, PFG-6 and PFG-7 clones) were selected and cloned by limiting dilution. PFG-5, PFG-6 and PFG-7 clones produced IgM, IgG and IgG antibodies to porcine granulosa cell surface components, respectively. To produce monoclonal antibody, 2,6,10,14-tetramethyl-pentadecane pretreated female BALB/c mice received an intraperitoneal injection of these hybridoma cells, and then ascites were obtained from the mice 2 weeks after the injection. Each antibody was purified using ultrafiltration equipment and preparative high-performance liquid chromatography. To determine the characteristics of each monoclonal antibody, frozen serial sections of porcine ovaries were cut on a cryostat, mounted on 3-aminopropyltri-ethoxysilane precoated glass slides, and fixed with precooled acetone for 5 min at –80 C, and then they were incubated with each purified monoclonal antibody after preincubation with normal goat serum. The sections were washed, incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgM or IgG antibody, and then examined with a confocal laser scanning microscope. PFG-5 and PFG-7 showed strong FITC-staining on granulosa cells of healthy and atretic follicles (Fig. 2). PFG-6 antibody reacted with granulosa cells of healthy follicles. These antibodies did not label the theca interna or externa cells, basement membrane, or ovarian stroma cells in either healthy or atretic follicles. Moreover, they showed neither specific binding to the luteal body, oviduct, uterus, testis, liver, kidney, adrenal gland, pancreas, stomach, small intestine, large intestine, spleen, thymus, brain, heart, lung or skeletal muscle. To determine the antigen profiles, we performed conventional Western blotting and two-dimensional (2D)-Western blotting analyses as follows. Homogenized ovarian tissues (granulosa cells, cumulus cells, oocytes and luteal bodies) and other organs were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Homogenized samples of cell membrane fractions prepared from isolated granulosa cells of healthy and atretic follicles were separated by 2D-PAGE. After electrophoresis, the proteins were transferred onto nitrocellulose membranes, and then the membranes were incubated with each monoclonal antibody. Immunological reaction products were visualized using a commercial staining kit. On conventional Western blotting, PFG-5, PFG-6 and PFG-7 antibodies reacted only with granulosa cells, but not with any other ovarian tissues or organs. On 2D-Western blotting analysis for cell membrane fractions, the two specific spots (molecular weight 42 kD and isoelectric point pI 5.2, and 55 kD, pI 5.9; named PFG-6 and PFG-5 antigens, respectively) of PFG-5 antibody were seen in the samples from healthy follicles, and only one specific spot of PFG-5 antigen was detected in those from atretic follicles (Fig. 3). Abundant PFG-6 antigen expression was noted in the granulosa cells of healthy follicles. PFG-6 antibody recognized only PFG-6 antigen, and PFG-7 antibody recognized PFG-5 antigen. The cell-killing activity of PFG-5 antibody, but not PFG-6 and PFG-7 antibodies, was found as follows. Isolated granulosa cells prepared from healthy follicles were cultured in tissue culture medium containing antibody (0.00001 to 1,000 μg/ml of PFG-5, PFG-6 or PFG-7) for 1 to 72 h at 37 C. After incubation, cultured granulosa cells were stained by the TUNEL method using a commercial kit to determine the apoptotic cells, and the nuclei of cultured cells were stained with Hoechst 33258 to observe their morphology. After staining, the cells were examined with a fluorescence microscope. Moreover, DNA samples prepared from cultured granulosa cells were electrophoresed to assess the DNA fragmentation. No TUNEL-positive

apoptotic cells were detected in the granulosa cells cultured with vehicle or with PFG-6 or PFG-7 antibodies, while many TUNEL-positive round nuclei and small condensed nuclear fractions (apoptotic bodies; a morphological hallmark of apoptotic cell death) were observed in the cells cultured with more than 0.01 μg/ml PFG-5 antibody for more than 6 h. The PFG-5 antibody-incubated DNA sample displayed a ladder pattern on electrophoresis (biochemical hallmark of apoptosis), while DNA samples of the granulosa cells cultured with vehicle or with PFG-6 or PFG-7 antibodies displayed no ladder pattern. Interestingly, when the cells were cultured in medium containing both PFG-5 and PFG-6 antibodies (PFG-6 antibody added first), an extremely low dose of PFG-5 antibody (0.0001 μg/ml) could induce apoptosis, indicating that PFG-6 antibody binds to PFG-6 antigen to block the binding activity of PFG-5 antibody to PFG-6 antigen. When the cells were cultured in medium containing both PFG-5 and PFG-7 antibodies (PFG-7 antibody added first), no apoptotic cell was seen, indicating the inhibitory effect of PFG-7 on PFG-5 antibody-inducible apoptosis. Based on our *in vivo* and *in vitro* preliminary experiments [14, 105–107, 152–155], we presume that interleukin-6 may control the expression of these novel receptors and intracellular inhibitors in granulosa cells of porcine ovarian follicles.

The molecular weights of well-known apoptosis-mediating receptors, Fas and TNFR1, are 45–46 and 65 kD, respectively [157–159], and the molecular weights of the granulosa cell surface antigens, PFG-5 and PFG-6 antigens, are 55 and 42 kD, respectively. Fas was immunohistochemically detected in the granulosa cells and luteal cells of both healthy and atretic follicles in rodent ovaries and in the thymus, liver, heart and lung [52, 98, 142], but TNFR1 was not detected in ovarian follicles or luteal bodies [121]. Both PFG-5 and PFG-6 antigens were only detected in the granulosa cells but not in luteal cells or other organs. Furthermore, PFG-5 antibody but not PFG-6 or PFG-7 antibodies could induce apoptotic cell death in primary cultured granulosa cells. Interestingly, the apoptosis inducing ability of PFG-5 antibody was heightened by pretreatment with PFG-6 antibody, which was added first into the culture medium. The cultured granulosa cells were not killed by PFG-5 antibody in the presence of PFG-7 antibody, which was added first into the culture medium. Abundant expression of PFG-6 antigen was noted in granulosa cells of healthy follicles, but no expression was demonstrated in granulosa cells of atretic follicles. Based on the biochemical and immunochemical findings, PFG-5 and PFG-6 antigens are different from the known apoptosis-mediating receptors, Fas or TNFR1. We hypothesize that PFG-5 antigen acts as a cell death receptor specifically expressed on the granulosa cells, and that PFG-6 antigen acts as a decoy receptor for PFG-5 antigen inhibiting apoptotic signals through PFG-5 antigen. A cell surface mechanism exists for the regulation of cellular responsiveness to pro-apoptotic stimuli [55, 56]. Cell death receptors transmit an apoptotic signal, and decoy receptors act as modulators of cell death receptors, and both are expressed on the same granulosa cells. It is considered that overexpression of the decoy receptor on the surface of granulosa cells inhibits apoptotic signal induced by the ligand for the apoptosis inducing receptor. Unfortunately, we have no data on the natural ligand for the novel cell death receptor, and the physiological properties of this receptor system are not well understood. These antibodies will require sensitive probes to investigate the unique cell death receptor on the granulosa cell membrane and its natural ligand, to elucidate cell surface mechanisms for the regulation of apoptosis, and to define the intercellular pathway of apoptotic signal transduction in granulosa cells of porcine ovaries.

As summarized in Fig. 1, we have demonstrated caspases and related proteins, and their mRNAs are expressed in porcine granulosa cells [63, 113, 121, 155]. Expression levels of Apaf-1, FAD were decreased during follicular atresia, but those of activated caspase-3, caspase-8 and caspase-9 were increased. No changes in expression levels of FasL or Fas during atresia were noted. We believe that these caspase cascade components, which locate downstream of the cell death ligand and receptor system, transduce the intracellular apoptotic signal. However, the detailed mechanisms of signal transduction have not been identified, and further studies are needed to understand the molecular mechanisms responsible for granulosa cell apoptosis.

Other cell death ligand and/or receptor systems

In vitro studies have demonstrated that other

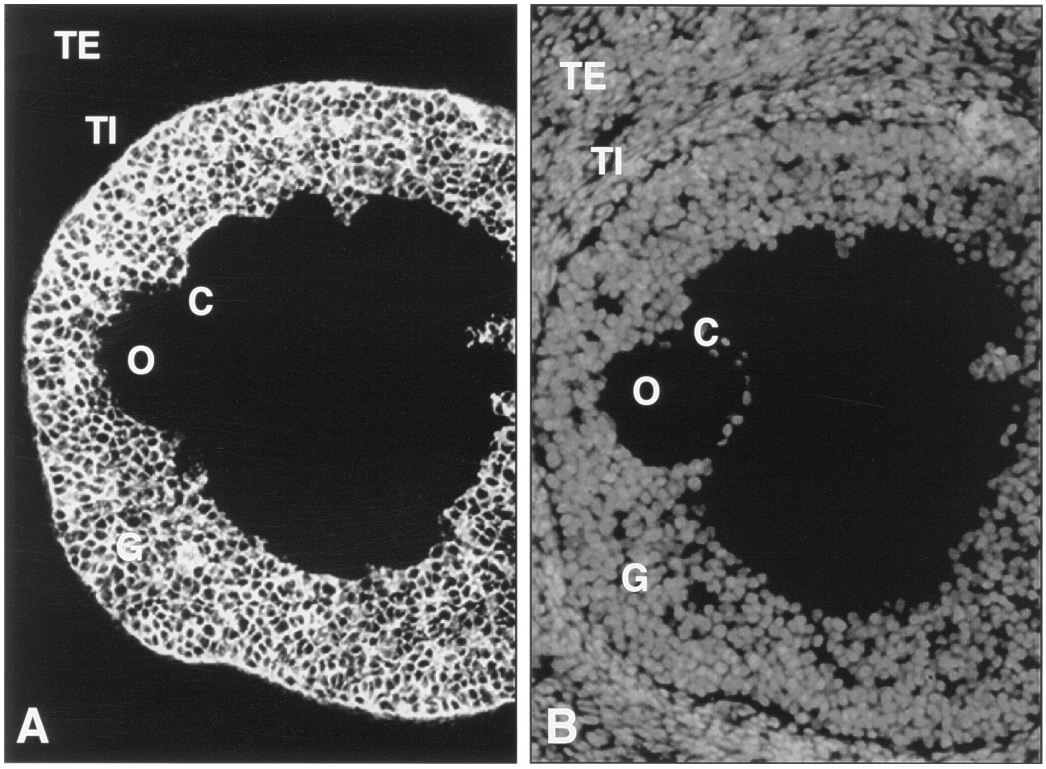

Fig. 2. Confocal images of follicular section of porcine ovary. Frozen sections were double-stained with PFG-5 antibody and with FITC-conjugated anti-mouse IgM, to show the distribution of granulosa cell membrane PFG-5 antigens (A), and then stained with Hoechst 33258 to visualize the cell nuclei (B). PFG-5 antibody showed strong fluorescent staining on granulosa cells (G). No immunostaining of cumulus cells (C), oocyte (O), theca interna (TI) or theca externa (TE) layers was observed (× 200).

receptors (angiotensin II type 2 receptor, gonadotropin-releasing hormone receptor etc.) may mediate follicular atresia [160, 161], but the *in vivo* physiological roles of these receptors in the selection of atretic folliculus have not been determined. Moreover, as in TRAIL findings, the screening of DNA databases for expressed sequence tags with similarity to TNFα revealed a novel cell death ligand (Apo3L or TWEAK) [162, 163]. The cDNA sequence of Apo3L is similar to that of TNFα, and Apo3L binds to DR3 and activates both the apoptosis signaling system and the proliferating system, NF-κB, in certain tumor cell lines [55, 56]. TNFα mRNA showed restricted expression in activated lymphoid and endothelial cells, but expression of Apo3L mRNA was found in many tissues. Our preliminary experiments showed no expression of Apo3L mRNA or DR3 mRNA in follicular cells of porcine ovaries.

The caspase cascade acts as an intracellular signal transducer of granulosa cell apoptosis as in lymphocytes and some tumor cells [61, 155]. Some members of the Bcl-2 family, which regulate apoptosis, interact with the cascade pathway and some of them have been shown to be functional in granulosa cells [13, 155, 164–166]. Bcl-2 family proteins, Bok [164], Bod [165], Boo [161], etc. are restrictively expressed in reproductive tissues. The expression of Boo, a novel anti-apoptotic member of the Bcl-2 family, was highly restricted to the ovary and epididymis in mice, implicating it controls ovarian atresia and sperm maturation. Boo homodimerizes and/or heterodimerizes with some cell death promoting and/or suppressing Bcl-2 family members, interacts with Apaf-1 to form a multimeric protein complex with Apaf-1 and caspase-9, and inhibit apoptosis. We showed that the porcine granulosa cell is a type II apoptotic cell,

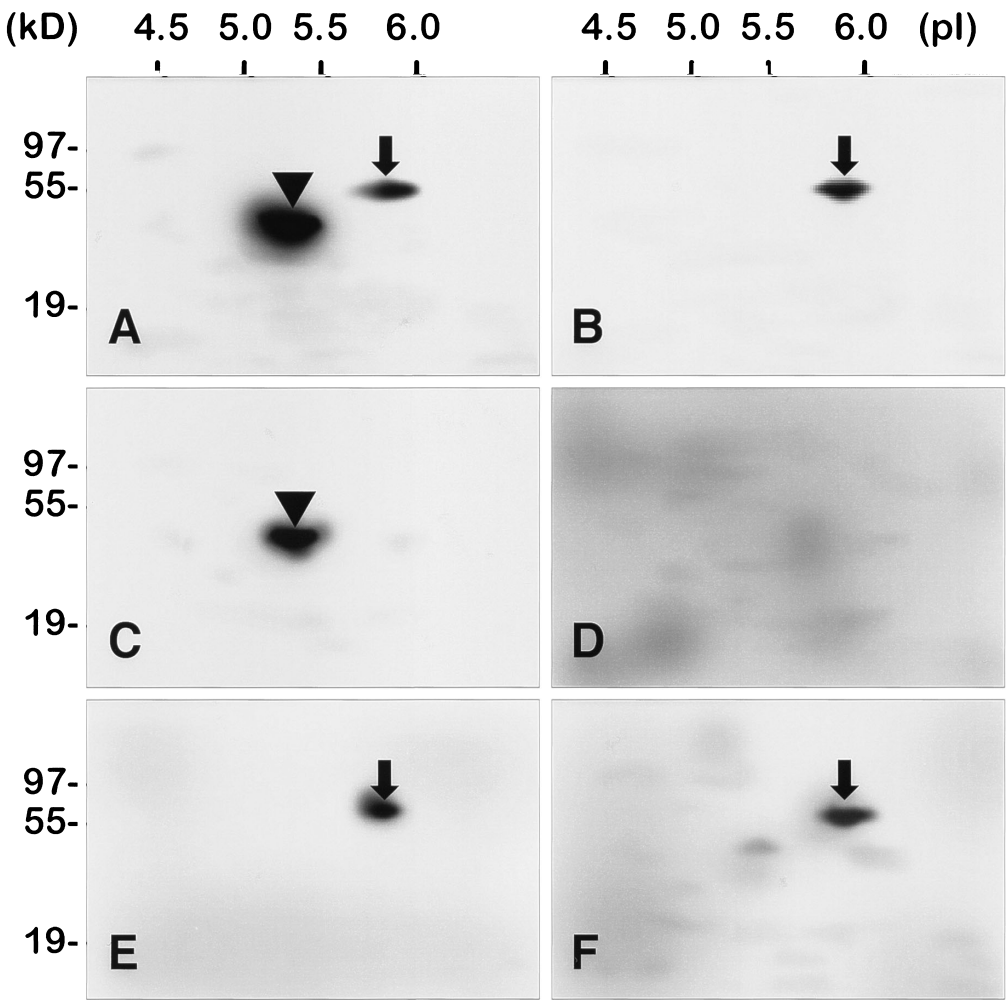

Fig. 3. Representative photos of two-dimensional (2D)-Western blot analysis of granulosa cell-membrane antigens recognized by PFG-5, PFG-6 and PFG-7. Granulosa cell membrane fractions were separated from granulosa cells of healthy (A, C and E) and early atretic (B, D and F) follicles, and analyzed by 2D-Western blot. Granulosa cell antigens were visualized by labeling with PFG-5 (A and D), PFG-6 (B and E) and PFG-7 (C and F). PFG-6 antigen (arrowheads: 42 kD, pI 5.2) and PFG-5 antigen (arrows: 55 kD, pI 5.9) were detected.

which has the mitochondrion-dependent apoptosis-signaling pathway [63], and we found that Boo-like proteins were expressed in the granulosa cells of healthy follicles in porcine ovaries, indicating that Bcl-2 members with restrictive expression in granulosa cells may contribute to the regulation of granulosa cell apoptosis. Further studies are in progress in our laboratory to reveal the intracellular regulation mechanism of granulosa cell apoptosis.

Intracellular Apoptosis Inhibitory Factors in Granulosa Cells

We recently isolated the porcine cDNA encoding cellular FLICE (procaspase-8)-like inhibitory protein (cFLIP), which inhibits death receptor-mediated apoptosis signal transduction (Fig. 4) [54]. In porcine organs, two alternative splicing isoforms of cFLIP, porcine cellular FLIP-short form (pcFLIP$_S$, 642 bp and 214-aa) and FLIP-long form (pcFLIP$_L$, 1446 bp and 482-aa), were identified in a

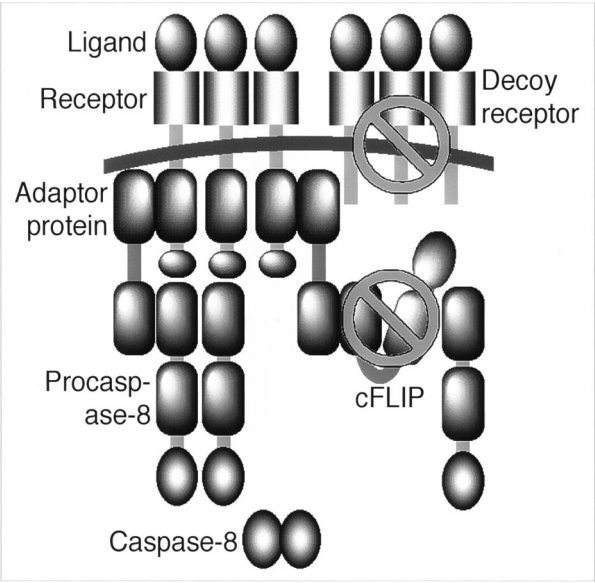

Fig. 4. Schematic illustration of the extracellular and intracellular inhibitory mechanisms [decoy receptor and cellular FLICE-inhibitory protein (cFLIP), respectively] of the cell death receptor-mediated apoptosis signaling pathway in granulosa cells of porcine ovarian follicles.

cDNA library prepared from granulosa cells of porcine ovarian follicles. pcFLIP$_S$ contained two tandem DEDs in the N-terminal region and a short C-terminal region. pcFLIP$_L$ contained two tandem DEDs in the N-terminal region and a caspase-like domain in the C-terminal region. High levels of pcFLIP$_S$ mRNA were detected in the colon, heart and lung, and high levels of pcFLIP$_L$ mRNA were detected in the heart and thymus. In the ovaries, both pcFLIP$_S$ and pcFLIP$_L$ mRNAs were highly expressed in granulosa cells of healthy follicles, suggesting that these cFLIPS play important roles in the regulation mechanism of apoptosis in ovarian follicular granulosa cells. We hypothesize that the cell death receptor-mediated apoptosis signaling pathway and intracellular inhibitory mechanism in granulosa cells are as follows [54]. (1) A cell death ligand binds to the extracellular domain of a cell death receptor, which contains an intracellular DD. (2) The intracellular DD of cell death receptor interacts with the DD of the adaptor protein (FADD) through a homophilic DD interaction. (3) FADD activates an initiator caspase (procaspase-8/FLICE), which is a bipartite molecule, containing an N-terminal DED and a C-terminal DD. (4) In healthy follicles, the DED of FADD interacts with the DED of cFLIP$_S$ and/or cFLIP$_L$ to inhibit the apoptosis signal transduction [167–173]. (5) In atretic follicles, procaspase-8 begins auto-proteolytic cleavage and activation. (6) The auto-activated caspase-8 cleaves Bid protein. (7) The truncated Bid releases cytochrome c from mitochondrion. (8) Cytochrome c and ATP-dependent oligimerization of Apaf-1 protein allows recruitment of procaspase-9 into the apoptsome complex. (9) Activation of procaspase-9 is mediated by means of conformational change. The activated caspase-9 cleaves downstream effector caspases (procaspase-3). (10) finally, apoptosis is induced. cFLIP$_S$ and cFLIP$_L$ may act as anti-apoptotic/survival factors in granulosa cells. Further *in vivo* and *in vitro* studies will elucidate the largely unknown molecular mechanisms (which cell death ligand-receptor system is the dominant factor for controling the granulosa cell apoptosis) of selective follicular atresia in mammalian ovaries.

Conclusions and Future Prospects

Biochemical and genetic studies have unraveled many of the signaling mechanisms that mediate induction of instructive apoptosis by the cell death ligand and cell death receptor system. Recently, genome sequencing has revealed some new cell death ligands, cell death receptors and their specific decoy receptors, which are categorized under the TNF-ligand and receptor superfamilies. It is likely that some novel cell death ligands and cell death receptors use similar intracellular signal inducing pathways to TNFα and TNFRs and the FasL and Fas system, although the molecular details of each pathway are still unknown. To date, many researchers in the field of immunology and oncology have revealed the unique biological roles of the cell death ligand-receptor systems and the molecular mechanisms that integrate their functions in the immune system (Table 1), but the biological roles and molecular mechanisms of these ligand-receptor systems in the reproductive system are largely unknown.

Apoptosis, an active form of cell suicide, plays a key role in the demise of the majority of mammalian gonadal cells (follicular granulosa and luteal cells in ovaries and germ cells in testes) during reproductive life. In mammalian ovaries, a

balance of cell proliferation and apoptosis in granulosa cells is maintained in healthy follicles and any imbalance of the two processes can lead to atretic change in follicles. Recent studies have indicated the crucial role of the cell death ligand and cell death receptor system as a survival factor and/or apoptotic factor in follicular granulosa cells, which act as inducers of follicular growth and/or atresia. Based on our findings in porcine ovaries, we presume that the TNFα and TNFR2 system, no TNFR1 is detectable, acts as a survival/growth factor in granulosa cells, and that the FasL and Fas system may control luteal cell death during luteolysis but not granulosa cell death during atresia. Also, TRAIL and its receptors (DR4, DR5 and DcR1, but not DcR2), and unknown ligand(s) and receptors (PFG-5 and PFG-6) contribute to selective cell death of granulosa cells during atresia. Furthermore, we believe that intracellular factors, which locate downstream of the cell-surface ligand and receptor complex, modulate cell death signal transduction from the receptors (for example $cFLIP_S$ and $cFLIP_L$) (Fig. 4), and that intragonadal survival factors in the ovary (estrogens, insulin-like growth factor-I, epidermal growth factor, basic fibroblast growth factor, interleukin-1β, nitrogen monoxide, etc.) and apoptotic factors (androgens, gonadotrophin releasing hormone-like peptide, interleukin-6, etc.) interact with the cell death ligand and cell death receptor systems. Unfortunately, we do not have enough detailed knowledge of the interaction between survival/growth and apoptotic factors and cell death ligand and cell death receptor systems, and such interaction may be important in regulating the induction of the death of ovarian follicles. Since our knowledge of the regulation mechanism of follicular selection is limited, further studies are essential to understand the cellular and molecular mechanisms responsible for follicular selection, in other words, granulosa cell apoptosis. If we could elucidate the molecular mechanism of follicular selection (the regulation mechanism of granulosa cell apoptosis), we could accurately predict/diagnose healthy follicles (ovulating follicles) and precisely evaluate the oocytes. We hope that the mechanism will be clarified and lead to an integrated understanding of the regulation mechanism.

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