ROLE OF RECEPTOR-DEPENDENT APOPTOSIS SYSTEM ON REGULATION OF FOLLICULAR ATRESIA IN PIG OVARY

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Abstract: Several hundred thousand primordial follicles are present in the mammalian ovary, however, only a limited number develop to the preovulatory stage, and then finally ovulate. The others, more than 99%, will be eliminated via a degenerative process called "atresia". The endocrinological regulatory mechanisms involved in follicular development and atresia have been characterized to a large extent, but the precise temporal and molecular mechanisms involved in the regulation of these events have remained unknown. From many recent studies, it is suggested that the apoptosis in ovarian granulosa cells plays a crucial role in follicular atresia. Notably, death ligand and receptor interaction and subsequent intracellular signaling have been demonstrated to be the key mechanisms regulating granulosa cell apoptosis. Here, we provide an overview of granulosa cell apoptosis regulated by death ligand-receptor signaling in pig ovary. The roles of death ligands and receptors [Fas ligand (FasL)-Fas, tumor necrosis factor (TNF)α -TNF receptor (TNFR), and TNFα-related apoptosis-inducing ligand (TRAIL)-TRAIL receptor (TRAILR)] and intracellular death-signal mediators [Fas-associated death domain protein (FADD), TNF receptor 1-associated death domain protein (TRADD), caspases, apoptotic protease-activating factor 1 (Apaf1), TNFR-associated factor 2 (TRAF2), and cellular FLICE-like inhibitory protein (cFLIP) etc.] in granulosa cells of each follicle will be discussed.

Keywords: Apoptosis, Apoptosis inhibitingfactor, Cell death ligand and receptor, Follicular atresia, Pig

Introduction

In cells of both vertebrate and invertebrate animal species, apoptosis plays a significant role in almost all physiological functions. Apoptosis is a form of cell death essential for the elimination of cells that are damaged, senescent, potentially harmful, or no longer useful. Apoptosis is characterized by internucleosomal DNA fragmentation, cell shrinkage, plasma membrane blebbing, and the formation of apoptotic bodies (Schwartzman and Cidlowski 1993). Stimulation by death ligands or deprivation of key survival-promoting growth factors is the main contributor to apoptosis, while stress inducers, including drugs, toxicants, oxidative stress, and radiation, are also known to cause apoptosis (Hengartner 2000). Recent studies have revealed that apoptosis also plays a crucial role in maintaining ovarian homeostasis in mammals (Hughes and Gorospe 1991; Tilly et al. 1991; Kaipia and Hsueh 1997; Manabe et al. 1996). During follicular growth and development, more than 99% of follicles disappear primarily due to apoptosis of granulosa cells as the biochemical and morphological characteristics of apoptosis have been observed in the granulosa cells of atretic follicles growth (Grant et al. 1989; Guthrie et al. 1995). Apoptotic stimuli and intracellular signal transduction pathways involved in the apoptosis of granulosa cells remain to be determined, and investigators are studying potential triggers of apoptosis and how intracellular apoptotic signals are transmitted in granulosa cells (Manabe et al. 2003, 2004). Many apoptosis-related factors are implicated in follicular atresia, including death ligands and receptors, intracellular pro- and anti-apoptotic molecules, cytokines, and growth factors (Matsuda et al. 2006). In particular, cell death ligand-receptor signaling has been revealed to be the major regulatory system for apoptosis in granulosa cells (Nakayama et al. 2003; Matsuda et al. 2008).

Follicular Growth, Development and Atresia in Pig Ovary

After puberty, a number of primordial follicles start to grow during each estrus cycle in adult females.

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Initiation of follicular growth involves endocrinological factors, mainly follicle stimulating hormone (FSH), and local modulating factors from granulosa cells, theca cells, stromal-interstitial cells, and oocytes (Hirshfield 1991; Guthrie et al. 1995). Primary follicles (follicles with a monolayer of follicular epithelial/granulosa cells) develop into secondary follicles (follicles with stratified granulosa cells but without an antrum) and subsequently into tertiary follicles (follicles with a follicular antrum) (Grant et al. 1989). Due to a large increase in the proliferation of granulosa cells and an increase in the size of the antrum, tertiary follicles show an exponential rate of growth. In the oocyte, meiosis then restarts and the first polar body divides. Finally, selected follicles burst, and the oocytes ovulate. With increasing serum FSH concentrations at the start of the estrous cycle, follicles produce increasing amounts of estrogen and inhibin, produced by granulosa cells. As a feedback mechanism by inhibin, FSH secretion falls, and the remaining small follicles undergo atresia. Although atresia can occur at any time during follicular development, the majority of follicles become atretic during the early antral stage of development. The transition from preantral to antral follicular development occurs after exposure of the granulosa cells to gonadotropin. Then, the differentiation of granulosa cells is initiated, which renders them susceptible to apoptosis. The endonuclease, DNase-I was demonstrated to exist in granulosa cells from antral, but not preantral, follicles (Manabe et al. 1996). However, the presence of the endonuclease is not sufficient to cause apoptosis; a signal to activate DNase-I and induce cell death is required. Death ligand-receptor interaction is thought to be a major trigger of apoptosis in granulosa cells (Manabe et al. 2003, 2004).

Death Ligand and Receptor System Regulates Follicular Atresia in Pig Ovary

Death ligands, which are grouped the tumor necrosis factor (TNF) family, are synthesized as type-II membrane proteins (Ashkenazi and Dixit 1998). Most death ligands can be cleaved from the cell membrane to become a soluble form and act as trimers. Death receptors, which are classified as type-I membrane proteins, constitute a subfamily within the TNF receptor superfamily (TNFRsf) that has a cytoplasmic death domain (DD) necessary for the activation of apoptosis (Hengartner 2000). These receptors are trimerized and then bind to death ligands, which are a trigger for apoptosis. Cell death ligand-receptor systems known in mammals include the Fas ligand (FasL) and Fas (CD95, APO-1 or TNFRsf6), TNFα and TNFα receptors (TNFRs), and TNFα-related apoptosis-inducing ligand (TRAIL, Apo2L) and TRAIL receptors (Nagata 1997; Ashkenazi and Dixit 1998; Wallach et al. 1999). In most cases, the cell death receptor-mediated apoptotic signaling pathway is as follows. (1) Cell death ligands, binding with cell membranes or existing as a soluble form, bind to the extracellular domain of trimerized cell death receptors, each of which contains an intracellular DD. (2) The DD of the death receptor binds with the DD of the adaptor proteins (TNF receptor 1-associated death domain protein: TRADD and Fas-associated death domain protein: FADD) through homophilic interaction (Inoue et al. 2007). (3) An initiator caspase (procaspase-8) binds to FADD through homophilic interaction with the death effector domain (DED; the resulting complex is called the "death-inducing signaling complex": DISC) (Medema et al. 1997). (4) Dimerization of procaspase-8 induces auto-proteolytic cleavage and activation (Boldin et al. 1995). (5) The activated caspase-8 subsequently activates downstream caspases either directly ("type I") or via mitochondrial perturbation ("type II; mitochondrion-dependent") (Matsui et al. 2003). (6-a) In type I apoptotic cells, caspase-8 directly activates the effector enzyme, caspase-3. (7-a) Trancated caspase-3, active form, activates endogenous endonucleases that results in apoptosis. (6-b) In type II apoptotic cells, caspase-8's activation leads to the release of cytochrome c from mitochondrion, which results in the interaction of procaspase-9 with apoptotic protease-activating factor 1 (Apaf1) [formation of cytochrome c-Apaf1-caspase-9 complex (apoptosome)] . (7-b) Activated caspase-9 cleaves procaspase-3. (8-b) Active caspase-3 activates the endonucleases that results in apoptosis (Nagata 1997; Ashkenazi and Dixit 1998; Matsui et al. 2003). FasL-Fas and other cell death ligand-receptor systems in granulosa cell: The FasL-Fas system is one of the most studied paradigms of instructive apoptosis, with strong apoptosis-inducing activity (Wallach et al. 1999). The apoptosis signal triggered and mediated by FasL and Fas is as follows. (1) FasL binds to the extracellular domain of Fas. (2) The intracellular DD of Fas interacts with the adaptor protein (FADD) via DD. (3) FADD interacts with procaspase-8 via their DED and the active caspase-8 is

formed. (4) A caspase cascade is activated and eventually apoptosis is induced. The FasL-Fas system is the most characterized apoptosis signaling machinery in granulosa cells (Inoue et al. 2006). In many species, both FasL and Fas are expressed in granulosa cells. In murine ovaries, FasL and Fas mRNA and protein are expressed in granulosa cells of healthy and atretic follicles (Sakamaki et al. 1997). Moreover, treatment of female mice with Fas-activating antibody promoted granulosa cell apoptosis and follicular atresia, suggesting a pro-apoptotic function of FasL-Fas signaling in vivo. In rat ovaries, FasL and Fas protein occur in granulosa cells, which tend to be more abundant in atretic follicles than healthy follicles (Hakuno et al. 1996). In human females, the granulosa cells of antral follicles express Fas during the early stages of atresia, the levels of Fas expression increase as atresia progresses, and granulosa FasL protein also increases with atresia in antral follicles (Cataldo et al. 2000). In bovine ovaries, FasL mRNA levels are higher in granulosa cells from atretic follicles than those from healthy follicles, and Fas mRNA expression is stronger in granulosa cells of atretic than healthy follicles (Porter et al. 2000, 2001). Moreover, CD45, a pan-leucocyte marker, was not detected in bovine granulosa cells, indicating that immune cells were not a source of FasL or Fas in the granulosa cell layer (Hu et al. 2001). Trace levels of FasL and Fas mRNA and protein were detected in granulosa cells of healthy follicles but the levels increased during atresia in porcine ovaries (Inoue et al. 2006). These expression patterns of FasL and Fas strongly indicate the important role of FasL-Fas signaling in follicular atresia. Both FasL and Fas exist on the granulosa cell membrane and their interaction may induce apoptosis. The pro-apoptotic effect of the FasL-Fas system in granulosa cells was also demonstrated by experiments in vitro. Stimulation of Fas-signaling can induce apoptosis in primary cultured granulosa cells of many species including pigs (Matsuda et al. 2006). However, pre- or co-treatment with interferon-γ or cycloheximide (CHX) is necessary for inducing the apoptosis in vitro, indicating the existence of other essential factor(s) in the FasL-Fas signaling pathway of granulosa cell apoptosis. Moreover, we found not only FasL-Fas syatem, but also TRAIL- TRAILRs (Wada et al. 2002; Inoue et al. 2003), TNFα-TNFRs (Nakayama et al. 2003), and unknown lihgand-PFG receptors (Manabe et al. 2000) systems in porcine granulisa cells. However, we have no confirmed information which system plays dominant role in regulation of granulosa cell apoptosis (Matsuda et al. 2008).

Intracellular regulator (apoptosis-inhibitory factor) in porcine granulosa cell: Death ligand-receptor interaction is indeed essential for triggering apoptotic signaling, however, it does not necessarily result in apoptotic cell death, indicating the importance of intracellular inhibitors of the apoptotic signaling pathway. Recently we found that cellular FLICE-like inhibitory protein (cFLIP: also called CASH, Casper, CLARP, FLAME, I-FLICE, MRIT, or usurpin) (Goltsev et al. 1997; Han et al. 1997; Hu et al. 1997; Krueger et al. 2001), which is a homologue of procaspase-8 (also called FLICE) (Muzio et al. 1996) and one of the intracellular proteins that interferes with the apoptotic effects of death ligands, plays crucial role in regulation of granulosa cell apoptosis in porcine ovaries (Goto et al. 2004). FLIP was firstly identified in several viruses as viral FLIP (vFLIP), which contains two DEDs that interact with FADD to avoid the host's apoptotic response (Thome et al. 1997). In mammalian cell, homologue of vFLIP was found and named cFLIP (Irmler et al. 1997). There are two splicing variants of cFLIP, short and long forms (cFLIPS and cFLIPL, respectively). cFLIPS is very similar in structure to vFLIP, containing two DEDs, while cFLIPL contains an additional pseudo-enzymatic domain that is similar to the enzymatic domain of procaspase-8 but lacks enzymatic activity. Recently, we found that cFLIPS and cFLIPL are expressed in porcine granulosa cells and both of them to be important regulators of apoptosis that block death ligand-inducible apoptosis, by competing with procaspase-8 and inhibiting the activation of caspase-8 (Thome and Tschopp 2001). The homology of porcine cFLIP with human and murine cFLIP is very high (more than 75% for both the mRNA and amino acid levels) (Goto et al. 2004), and we have proposed that cFLIP also has cell survival-promoting effects in the pig. As described above, the FasL-Fas system is well-characterized as a pro-apoptotic signal in granulosa cells in sows, and the expression of FasL and Fas in granulosa cells increases during atresia, however, both proteins are also expressed in granulosa cells of healthy pre-antral and antral follicles, which rapidly grow and have many proliferating granulosa cells. Moreover, Fas cannot induce apoptosis

in primarily cultured porcine granulosa cells without either IFN- or CHX. It has been suggested that the factor(s) that blocks FasL- Fas-mediated apoptotic signaling is essential for maintaining granulosa cells and keeping follicles healthy. By reverse-transcription- polymerase chain reaction (RT-PCR) and Western blottingting, the mRNA and protein of cFLIPL were found to be highly expressed in the granulosa cells of healthy follicles and decreased during atresia (Goto et al. 2004). The mRNA levels of cFLIPS in granulosa cells are low and showed no changes among the stages of follicular development (Matsuda et al. 2005, 2007). Furthermore, the protein level of cFLIPS is extremely low. By in situ hybridization, cFLIPL was found to be abundant in the granulosa cells of healthy follicles in comparison with those of atretic follicles. Immunohistochemical analyses showed that cFLIP protein was found to be highly expressed in the granulosa cells of healthy follicles but weakly expressed in those of atretic follicles. We presume that cFLIP, especially cFLIPL, plays an anti-apoptotic role in the granulosa cells of healthy follicles from pig ovaries. Since the anti-apoptotic activity of porcine cFLIP (pcFLIP) had not been confirmed in granulosa cells, we examined the effect of pcFLIP on survival using granulosa-derived cells (Matsuda et al. 2007). Human ovarian granulosa tumor cell derived KGN cells (Nishi et al. 2001) transfected with pcFLIPS or pcFLIPL vectors survive the induction of ant-Fas antibody-Fas-mediated apoptosis, while almost all cells transfected with empty vector die, indicating the anti-apoptotic activity of pcFLIP in granulosa cells. When both cFLIPS and cFLIPL, or cFLIPL only, were suppressed by small interfering RNA (siRNA), the viability of KGN and J porcine granulosa-derived JC-410 cells (Chedrese et al. 1998) decreased significantly. Thus, we conclude that porcine cFLIP functions as an anti-apoptotic factor in granulosa-derived cells. These our findings strongly suggest that cFLIP acts as a survival-promoting factor in granulosa cells and determines whether porcine ovarian follicles survive or undergo atresia. To date, investigations have revealed that cFLIP can also inhibit TNFα - and TRAIL-signaling (Cheng et al. 2007), not only Fas-signaling (Manabe et al. 2003). Further investigations are needed to determine wether cFLIP affects intracellular signaling transduction in other cell death ligand and receptor systems (ex. TNFα -TNFRs, TRAIL-TRAILRs etc.) in porcine granulosa cells or not. More researches are also needed how to regulate the expression of cFLIP in granulosa cells during follicular growth, development, and atresia. Our previous findings have shown that interleukin-6 (IL-6) up-regulates TNFα expression in porcine granulosa cells (Maeda et al. 2007a, 2007b), and that TNFα up-regulates cFLIPL expression and acts a survival factor (Nakayama et al. 2003). However, there is no confirmed information what is the initial trigger to control cFLIP expression.

To date, inducers of apoptosis including FasL-Fas, TNFα -TNFRs, TRAIL-TRAILRs have been the main targets in the studies of regulation mechanism of granulosa cell apoptosis, and their contributions to follicular atresia have been clarified. However, it has become apparent that an intracellular anti-apoptotic/inhibitory factor(s), like cFLIP, is critical for inhibiting granulosa cell apoptosis from our recent research. Granulosa cell apoptosis is likely to be regulated by a sophisticated balance of pro-apoptotic and anti-apoptotic factors. The mechanisms of death ligand-receptor signaling should be determined to entirely define granulosa cell apoptosis. Solving this problem will help to establish methods (1) of selecting healthy oocytes or to improve damaged oocytes that result in an increased rate of gestation for in vitro fertilization in domestic animals and humans, and (2) of rescue oocytes in the ovaries of wild animals and livestock sacrificed at the slaughterhouse.

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