TRAIL-Decoy Receptor-1 Disappears in Granulosa Cells of Atretic Follicles in Porcine Ovaries

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Abstract. To reveal the specific regulatory molecules that control granulosa cell apoptosis during follicular atresia, we immunohistochemically examined the localization of tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) and its receptors in porcine ovaries. A marked reduction in the expression of decoy receptor-1 (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 or other TRAIL-receptors (death receptor-4 or death receptor-5) in granulosa cells between healthy and atretic follicles. No positive staining for DcR2 was seen. We presume that TRAIL and its receptors are involved in induction of apoptosis in granulosa cells during atresia, and that DcR1 plays an inhibitory role in granulosa cell apoptosis.

Key words: Apoptosis, TRAIL-decoy receptor, Follicular atresia, Granulosa cell, Pig

In mammalian ovaries, more than 99% of the follicles undergo a degenerative change known as atresia at varying stages of follicle development [1, 2]. A number of studies of follicular atresia have revealed the morphological and biochemical characteristics of atretic follicles [3, 4]. Recent findings have suggested that apoptosis, originally described by Kerr et al. [5], is the mechanism underlying ovarian follicular atresia [6, 7]. The degeneration of atretic follicles in porcine ovaries can be explained, at least in part, by apoptosis of granulosa cells [8–11]. We previously confirmed that granulosa cells undergo apoptosis but that no apoptosis occurs in cumulus cells during follicular atresia in porcine ovaries [12–14]. In rodents, Fas ligand (FasL) and its receptor Fas/APO-1/CD95 (Fas) may regulate granulosa cell apoptosis in ovarian follicle atresia and luteal cell degeneration [15–19]. However, it has not been determined whether the FasL-Fas system mediates apoptosis in pig ovaries. Previously, we revealed species-specific differences in the apoptotic process in granulosa cells and indicated that local mechanisms of regulation of granulosa cell apoptosis may be different among mammalian species [20].

As apoptotic stimuli and intracellular signal transduction pathways involved in granulosa cell apoptosis remain to be determined. We have been studying which trigger molecules induce granulosa cell apoptosis, and how intercellular apoptotic signals are transmitted in granulosa cells. A novel cell death ligand, TNF-related apoptosis-inducing ligand (TRAIL; also known as Apo-2 ligand), a cytotoxic cytokine homologous to FasL, was identified in 1995 [21–23]. Apoptosis mediated by TRAIL is regulated by the expression of two death receptors, death receptor-4 (DR4; also known as TRAIL-R1) and death receptor-5 (DR-5; also known].
as TRAIL-R2 or TRICK 2), and three unique decoy receptors, decoy receptor-1 (DcR1; also known as TRAIL-R3, LIT or TRID), decoy receptor-2 (DcR-2; also known as TRAIL-R4 or TRUNDD) and osteoprotegerin (OPG), that inhibit apoptosis [24–36]. DcR1 and DcR2 have two extracellular cysteine-rich domains which show close homology to those of DR4 and DR5. TRAIL-induced apoptosis was inhibited by cellular transfection with DcR1 or DcR2 [32]. Although the biological functions of the TRAIL receptor system are largely unknown, this system has been suggested as contributing to the selective abolishment of unnecessary cells under physiological conditions [37].

In the present study, to determine the physiological roles of TRAIL and its receptors on granulosa cell apoptosis in porcine ovarian follicles, we immunohistochemically examined the changes in localization of TRAIL and its receptors in porcine follicles during follicular atresia.

Materials and Methods

Immunohistochemistry for TRAIL and its receptors

Frozen sections of follicular sections of pig ovaries were prepared as described previously [38–42]. Briefly, individual preovulatory antral follicles, 3–5 mm in diameter, were dissected from ovaries obtained from mature sows at a slaughterhouse. Under a surgical dissecting microscope (SZ40, Olympus, Tokyo, Japan), the follicles were classified as morphologically healthy or atretic, and further subdivided into early and progressed atretic follicles [40]. A part of the follicular fluid of each follicle was collected by a 1-ml syringe. Fluid from each follicle was separated by centrifugation at 3,000 g for 10 min, and then estradiol-17β and progesterone levels in the follicular fluid were quantified using [125I]-RIA kits (Bio-Mérieux, Marcy-l’Etoile, France). When the progesterone/estradiol-17β ratio was less than 15, the follicle was classified as healthy according to our previous findings [9–14]. Then, each follicle was put on filter paper, mounted in OTC compound (Miles Lab., Elkhart, IN, USA), and rapidly frozen in dry ice-isopentane (Wako Pure Chemical, Osaka, Japan) mixture. Serial sections 5 µm thick were cut on a cryostat (Jung CM1500; Leica, Heidelberg, Germany), and mounted on glass slides precoated with 3-aminopropytriethoxysilane (Sigma Aldrich Chemicals, St. Louis, MO, USA). The sections were fixed with precooled acetone for 5 min at – 80 C. After washing with PBS (pH 7.4; Wako), the sections were preincubated with diluted normal rabbit serum (1/200 dilution with PBS; Wako) for 10 min at RT. Goat polyclonal antibodies against human TRAIL, DR4, DR5, DcR1 and DcR2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were diluted at 1/150 with PBS containing 40 mg/ml BSA (PBS-BSA; Sigma), and then applied to the sections. The sections were incubated with each first antibody for 18 h at 4 C, washed with PBS containing 0.05% Tween 20 (PBS-Tw; Sigma), and then incubated with 25 µg/ml of FITC-conjugated rabbit anti-goat IgG (American Qualex, La Mirada, CA, USA) diluted with PBS-BSA containing 20 µg/ml of propidium iodide (PI; Sigma) for 90 min at RT for nuclear staining. They were washed with PBS-Tw, mounted with glycerol, and then examined with a confocal laser scanning microscope (FV3000, Olympus).

Statistical analysis

All experiments involving follicle isolation were repeated with separate groups (six sows/group) for independent observation. Wilcoxon’s signed-rank test for histological estimation was carried out using StatView IV on a Macintosh computer. Differences at P<0.05 were considered significant.

Results

TRAIL and its receptors, DR4, DR5, DcR1 and DcR2, in frozen sections of healthy and atretic follicles of porcine ovaries were visualized by the immunofluorescence technique, and nuclear morphology was visualized by PI staining. Positive signals for TRAIL (Fig. 1A and C), DR4 (Fig. 1E and G) and DR5 (Fig. 2A and C) were observed in granulosa cells and the cells of theca internal and external layers of both healthy (Fig. 1A, E and 2A) and atretic follicles (Fig. 1C, G and 2C). Intense staining for TRAIL was seen in granulosa cells lining the antral cavity of healthy antral follicles (Fig. 1A) and those separating the antral cavity from the basement membrane of atretic follicles (Fig. 1C). Extremely strong immunoreactivity for DR4 was detected in granulosa cells of both healthy and atretic follicles (Fig. 1E and G) and theca
TRAIL RECEPTORS IN PIG GRANULOSA CELLS

169

Intense staining for DR5 was detected in granulosa cells lining the antral cavity of healthy follicles (Fig. 2A) and those suspended in the cavity of atretic follicles (Fig. 2C), but weak signals were seen throughout the theca interna and externa layers in both follicles. Positive staining for DcR1 was demonstrated in granulosa cells and theca internal and external layers of healthy follicles (Fig. 2E). Intense positive immunostaining was seen in granulosa cells located on the inner surface of the follicular wall. In atretic follicles, however, no positive signal for DcR1 was observed in granulosa cells, but scattered weak signals were seen throughout the theca internal and external layers (Fig. 2G). No positive staining for DcR2 was shown (data not shown). Nuclear morphology was examined by PI staining in the frozen sections (Fig. 1B, D, F and H). Large oval nuclei were seen in granulosa cells of healthy follicles (Fig. 1B and F, and 2B and F), but many small condensed nuclei, a morphological hallmark of apoptotic cells, were seen in granulosa cells of atretic follicles (Fig. 1D and H, and 2D and H).

Discussion

Over the last decade, several cell death ligands (TNF-α, FasL, TRAIL etc.) and their receptors (TNFR1, Fas, DR4, DR5, DcR1, DcR2 etc.) have been found [24–26, 43–45]. Recent studies have increased our understanding of selective apoptotic cell death under both physiological and pathological conditions through the cell death ligand-receptor interaction. Most follicles selectively undergo
atresia and disappear during follicular development in mammalian ovaries [1, 2]. Such atretic degeneration may be explained by selective apoptosis of granulosa cells [6, 7]. Many researchers believe that the cell death ligand-receptor system plays critical roles in selective apoptosis of granulosa cells during follicular atresia.

Recently, a novel cell death ligand, TRAIL, and its receptors were found [24–26, 43–45]. TRAIL and its receptors are widely expressed on normal tissues and are considered to act as regulators of selective cell death under normal conditions. Although recombinant FasL and TNFα trigger apoptosis in many tumor cells, the severe toxicity of these ligands toward normal tissues impedes their therapeutic application [46]. As DR4 and DR5 are expressed in normal tissues and many types of tumor cells, whereas DcR1 and DcR2 are expressed frequently in normal tissues but not in tumor cells [24–26], TRAIL is considered to be a safer agent. Although the biological functions of the TRAIL-receptor system are largely unknown, this system may dominantly contribute to the selective abolished of unnecessary cells under physiological conditions [37]. The apoptotic stimuli and intracellular signal transduction pathways involved in granulosa cell apoptosis, which occur under normal physiological conditions, remain to be determined. Therefore, we histochemically examined the changes in expression and localization of the TRAIL receptor system during follicular atresia in porcine ovaries.

Fig. 2. Localization of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-death receptor-5 (DR5) and TRAIL-decoy receptor-1 (DcR1) in sections of healthy (A, B, E and F) and atretic (C, D, G and H) follicles of porcine ovaries. The frozen sections were double-stained with anti-DR5 (A and C) and -DcR1 (E and G) antibodies and with propidium iodide (B, D, F and H). G, granulosa layer; BM, basement membrane; TI, theca interna layer. × 200.
atretic follicles, but no marked differences were seen in the expression of TRAIL or other receptors (DR4 or DR5) in granulosa cells between healthy and atretic follicles. Moreover, no positive reaction for DcR2 was seen. Remarkably intense TRAIL, DR5 and DcR1 immunostaining were seen in granulosa cells lining the antral cavity of healthy follicles. Our previous studies in nulliparous and multiparous sows showed that apoptosis primarily occurs in granulosa cells located on the inner surface of the follicular wall at the early stage of atresia [12, 14, 20]. We presume that DcR1 competes with TRAIL receptors, DR4 and/or DR5, for binding to TRAIL and blocks granulosa cells from apoptotic cell death, with the result that healthy follicles do not undergo atresia. In atretic follicles, however, DcR1 disappears in granulosa cells, TRAIL binds with DR4 and/or DR5, and then a TRAIL-dependent apoptosis signal is transmitted into the cytoplasm of the cells. We considere that TRAIL and its receptors play critical roles in induction and regulation of apoptosis in granulosa cells during follicular atresia of porcine ovaries, and that DcR1 may have a protective effect against the induction of apoptosis by TRAIL.

Previous studies by other authors [15–19] as well as the findings of both the present and our previous studies [38–40] indicate that cell death ligands and receptors play critical roles in selective apoptosis of folicular cells during atresia. Our previous experiments using production of monoclonal IgM and IgG antibodies against unique cell-membrane proteins that exist only in granulosa cells of porcine ovaries indicated that other unknown receptor(s) may be involved in granulosa cell apoptosis [38–40]. Briefly, the IgM antibody recognized two cell-membrane proteins (55 kD-pI 5.9 and 42 kD-pI 5.2), and induced apoptosis in cultured granulosa cells prepared from healthy porcine follicles. The IgG antibody recognized the 42 kD-pI 5.2 protein, and inhibited IgM antibody-induced apoptosis in vitro. We consider the 55 kD-pI 5.9 protein to be a novel cell death receptor, which is different from Fas, TNFR or TRAIL-death receptors. We also think that the 42 kD-pI 5.2 protein may act as a decoy receptor, which is different from the TRAIL-decoy receptors. Further studies are considered necessary to determine which ligand-receptor system dominantly causes or regulates granulosa cell apoptosis in the selection of follicles in mammalian ovaries and to characterize the detailed mechanism of apoptosis-signal transmission in granulosa cells during follicular atresia.

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