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—Original—

Roles of Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand Signaling Pathway in Granulosa Cell Apoptosis During Atresia in Pig Ovaries

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Abstract. To reveal the molecular mechanism of selective follicular atresia in porcine ovaries, we investigated the changes in the expression of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and its receptor (DR4) proteins and TRAIL mRNA in granulosa cells during follicular atresia. Immunohistochemical, Western immunoblotting and reverse transcription-polymerase chain reaction analyses (RT-PCR) revealed that significant increases in TRAIL protein and mRNA levels but not DR4 protein were changed during atresia. The RT-PCR product was confirmed to be porcine TRAIL by the cDNA sequence determination. An *in vitro* apoptosis inducing assay using cultured granulosa cells prepared from healthy follicles showed that TRAIL could activate caspase-3 and induce apoptotic cell death in the cells. The present findings confirm that TRAIL induces apoptosis in granulosa cells during atresia in porcine ovaries.

Key words: Apoptosis, Follicular atresia, Granulosa cell, Porcine ovary, Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)

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In mammalian ovaries, more than 99% of follicles undergo a degenerative process known as "atresia", and only a few follicles ovulate during ovarian follicular development [1–7]. We have investigated the molecular mechanism of selective follicular atresia in porcine ovaries, and have reported that follicular selection dominantly depends on granulosa cell apoptosis [8–10]. Unfortunately, we have little knowledge of the molecular mechanisms that control apoptotic cell death in granulosa cells during follicle selection.

To date, at least four cell death ligand-receptor systems, tumor necrosis factor-α (TNFα), Fas-ligand (also called APO-1/CD95-ligand), TNF-related apoptosis-inducing ligand (TRAIL; also called APO-2 ligand) and APO-3 ligand, have been found [11]. TRAIL was initially identified as the homologic gene of TNFα. The biological roles of TRAIL are not yet fully understood. TRAIL has been reported to induce apoptosis in various tumor cells but not in normal cells [12–14]. However, a recent study revealed that TRAIL induces apoptosis in normal hepatocytes of human, but not in those of rat, mouse or rhesus monkey, indicating that there are species-specific differences in the mode of action of TRAIL and the receptor systems [15]. Moreover, our preliminary experiments showed that TRAIL and its receptor proteins, death receptor (DR)-4 (also called TRAIL-R1), DR-5 (TRAIL-R2, TRICK2 or KILLER), which specifically binds with TRAIL and induces apoptosis, and

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decoy receptor-1 (DcR1; TRAIL-R3, TRID or LIT), which has a higher affinity for TRAIL than death receptors (DRs) and competes with DRs [16, 17], were immunohistochemically demonstrated in granulosa cells of porcine ovaries [18]. DcR1 protein disappeared in granulosa cells of atretic follicles. However, no experiments on whether their mRNAs are expressed in granulosa cells or not have been performed. Our preliminary *in vitro* study showed that when DcR1 was removed from the cell membrane of cultured granulosa cells, TRAIL could induce apoptotic cell death, indicating that DcR1 may act as an inhibitor against TRAIL-induced apoptosis in granulosa cells [19]. However, we had no evidence of whether TRAIL can induce apoptotis cell death in granulosa cells without treatment or not, or whether intracellular transducing factor, caspase-3, in granulosa cells is activated or not. Thus, there was insufficient information about the physiological roles of TRAIL in granulosa cells during follicular atresia.

In the present study, to confirm the physiological roles of TRAIL and its receptor system, we determined detailed changes in the expression of TRAIL and its receptor proteins in granulosa cells by immunohistochemical staining and Western immunoblotting, and of TRAIL mRNA in granulosa cells by reverse transcription-polymerase chain reaction (RT-PCR) analyses during follicular atresia. Moreover, we assessed the apoptosis-inducing ability of TRAIL in cultured granulosa cells prepared from healthy follicles by the caspase-3 activation assay.

Materials and Methods

Immunohistochemistry

Ovaries obtained from mature sows weighing more than 120 kg at a local slaughterhouse were fixed in 10% phosphate buffered formalin (pH 7.4), dehydrated through a graded ethanol series and embedded in Histosec-paraffin (Merck, Darmstadt, Germany). Sections 3 μm thick were mounted on glass slides, deparaffinized, rehydrated, and then washed well with 20 mM Tris-HCl (pH 7.6) containing 137 mM NaCl and 0.1% (v/v) Tween-20 (TBST; Sigma Aldrich Chemicals, St. Louis, MO, USA). Immunohistochemical stainings for TRAIL and DR4, major TRAIL-death receptors in granulosa cells [18], and for single strand DNA (ssDNA), which is a marker of DNA fragmentation caused by a single break in nuclei during apoptotic cell death, were performed using a CSA immunostaining system (Dako, Glostrup, Denmark) according to the manufacturer's protocols. Briefly, the slides were pretreated with 0.1% (v/v) H_2O_2 in methanol for 5 min, washed with TBST, and then incubated with each primary antibody, rabbit anti-TRAIL and rabbit anti-DR4 polyclonal antibodies (1:100 diluted with 20 mM Tris-HCl, pH 7.6, and 137 mM NaCl: TBS; Sigma), and rabbit anti-ssDNA polyclonal antibody (1:300 diluted with TBS; Dako), at 4 C for 18 h. After washing well with TBST, the slides were incubated with biotinylated anti-rabbit antibody (Dako) for 15 min at room temperature (23–25 C), washed well with TBST and post-treated with streptavidin-peroxidase complex (Dako) for 15 min at room temperature. Next, they were washed with TBST, incubated with amplification reagent (Dako), washed with TBST again, and then treated with substrate-chromogen solution (Dako) for 1 min. The slides were rinsed with distilled water, counter-stained with methyl green, dehydrated, mounted with Entelan (Merck), and then examined by light microscopy (BX-51, Olympus, Tokyo, Japan). In each experimental run, adjacent sections incubated without each primary antibody or without any antibodies were prepared as negative controls.

Preparation of granulosa cells

Each antral follicle, approximately 3 mm in diameter, was dissected from the ovaries under a surgical dissecting microscope (SZ40, Olympus) and classified as morphologically healthy or atretic, and further subdivided into early and progressed atretic. Follicular fluid from each follicle was collected using a 1-ml syringe, separated by centrifugation at 3,000 g for 10 min at 4 C, frozen and kept at –80 C. After biochemical analyses and cell culture experiments were performed, estradiol-17β and progesterone levels were retrospectively measured using [^{125}I]-RIA kits (Bio-Mérieux, Marcy-l'Etolle, France) to confirm the classification of the follicles. Follicles with a progesterone/estradiol-17β ratio of less than 15 were classified as healthy according to previous findings [8, 9, 20, 21]. Each follicle was opened using fine #5-watchmaker forceps, and granulosa layers and oocyte-cumulus complexes were removed. The granulosa

cells were isolated with Pasteur's pipettes, collected and washed 3 times in Medium 199 (Gibco BRL Life Technologies, Rockville, MD, USA) containing 50 μg/ml gentamicin sulfate (Sigma) and 5% (v/v) fetal calf serum (FCS; Gibco) by centrifugation at 600 g for 5 min at room temperature. Cell number was counted using a hemocytometer plate, and cell viability was determined by the trypan blue exclusion method. The cells with a viability of more than 95% were used for biochemical analyses (Western immunoblotting and RT-PCR) and cell culture experiment (TRAIL-induced apoptosis assay).

Western immunobloting analysis

As previously reported [22], for Western immunoblotting analysis, the protein fraction (50 μg/lane) prepared from each cell lysate sample of granulosa cells, which were prepared from healthy or atretic follicles, was separated by 10–20% gradient SDS-PAGE (Wako), and then transferred onto nitrocellulose membranes (Hybond-C, Amersham Pharmacia). The membranes were stained with 0.2% (w/v) Ponceau-S solution (Serva Electrophoresis, Heidelberg, Germany), and then immersed in blocking solution, TBST containing 5% (w/v) skim milk (Sigma), for 30 min, followed by incubation with each primary antibody, rabbit anti-TRAIL and rabbit anti-DR4 polyclonal antibodies (1:200 diluted with blocking solution), at 4 C for 18 h. After washing, they were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1:5,000 diluted with blocking solution; Amersham Pharmacia Biotech, Piscataway, NJ, USA) for 1 h at 25 C, and then chemiluminescence was visualized using an ECL system (Amersham Pharmacia Biotech) according to the manufacturer's protocol. The chemiluminescence was recorded with a digital fluorescence recorder (LAS 1000; Fuji Film, Tokyo, Japan). Each protein expression level, the chemiluminescence intensity of each protein band, was quantified using Image-Gauge program (Fuji Film) on a Macintosh computer, and then the percentage of increase against each vehicle control was calculated.

RT-PCR analysis

As described previously [22], for RT-PCR analysis for TRAIL mRNA, mRNA was extracted from granulosa cell samples using a Quick-Prep micro mRNA purification kit (Amersham Pharmacia Biotech), and then reverse-transcribed using You-Primed first-strand beads (Amersham Pharmacia Biotech) to synthesize cDNA. Primers for the amplification of partial cDNA sequences of TRAIL and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; GenBank accession number: AF017079, used as an intrinsic control) were as follows: TRAIL forward, 5′-AGTGG CTCAC ACCTG TAATC CC-3′ and reverse 5′-GGTTC AAACG ATTCT CCTGC C-3′; and GAPDH forward, 5′-GATGG TGAAG GTCGG AGTG-3′ and reverse, 5′-CGAAG TTGTC ATGGA TGACC-3′. The expected PCR product sizes of TRAIL and GAPDH were 194 and 500 bp, respectively. PCR amplification was performed as follows: Platinum *Taq* DNA polymerase (10,000 U/ml; Gibco) was added to cDNA mixture (cDNA was mixed with the PCR reaction mix containing 1 × PCR buffer, 0.1 mM dNTP mixture, 1.5 mM $MgCl_2$ and 0.5 μM each primer pair) and denatured. The mixture was subjected to PCR in a thermal cycler (GeneAmp PCR Systems 2400; PE Applied Biosystems, Foster City, CA, USA). The hot-start PCR cycles for TRAIL and GAPDH were as follows: 96 C for 4 min, and then 30 cycles of 94 C for 1 min, 58 C for 1 min, 72 C for 1 min, followed by a final extension period at 72 C for 10 min. PCR products were electrophoresed in 2% (w/v) agarose gels (Sigma) and stained with ethidium bromide (Wako). Ready-load 100 bp DNA ladder (Gibco) was used as a molecular weight marker for electrophoresis. After electrophoresis, the stained gels were recorded with a digital fluorescence-recorder, and each mRNA expression level, fluorescence intensity of each band of PCR product, was quantified using ImageGauge on a Macintosh computer. The relative abundance of specific mRNA was normalized to the relative abundance of GAPDH mRNA, and then the percentage of increase against vehicle control was calculated. To confirm the expression of porcine TRAIL mRNAs, the DNA sequence of the PCR product was determined using an automatic DNA sequencer (ABI Prism 310; PE Applied Biosystems). All the above procedures were performed according to the manufacturer's instructions.

TRAIL-induced apoptosis assay

The granulosa cells were seeded in Medium 199 containing 50 μg/ml gentamicin sulfate and 5% (v/

v) FCS, and cultured in culture flasks (Falcon 3009; Becton Dickinson, Lincoln Park, NJ, USA) in a 5% CO_2-air incubator (TE-HER; Hirasawa Co., Tokyo, Japan) for 1 h at 37 C. Non-adherent cells, the granulosa cells, were collected by centrifugation, 500 g for 10 min, and then the cells (1×10^6 cells/ml) were seeded and preincubated on type I collagen coated cell culture slide glasses with four chambers (Iwaki Glass, Tokyo, Japan) for 24 h at 37 C. After washing with culture medium, TRAIL (0, 50, 100, 200 or 400 ng/ml dissolved with culture medium; Upstate Biotechnology, New York, NY, USA) was added to each chamber, and then incubated for 0, 4, 6, 8, 12 or 16 h at 37 C. After the incubation with TRAIL, the activation of caspase-3, which is a central player in mediating apoptosis and is used as a marker of apoptosis, was cytochemically assayed. Briefly, the cells were washed with PBS, fixed with 10% (w/v) formaldehyde (Wako) in phosphate buffered saline (PBS; pH 7.4) for 25 min at room temperature, and washed with PBS containing 0.1% (v/v) Tween 20 (PBST). The cells were incubated with blocking buffer, PBST containing 5% (v/v) normal goat serum (Dako), for 1 h at room temperature in a humidified chamber, washed with PBST, and then incubated with affinity-purified rabbit anti-activated caspase-3 polyclonal antibody (a peptide from the p18 fragment of caspase-3 was used as antigen; 1:250 diluted with PBST; Promega, Madison, WI, USA) for 1 h at room temperature. After washing with PBST, the cells were incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG antibody (1:200 diluted with PBS; Molecular Probe) for 90 min, and then nuclear-stained with 20 μg/ml propidium iodide solution (PI: Sigma) for 90 min. The slides were washed with PBS, mounted with glycerol, and then examined with a confocal laser-scanning microscope (Fluoview 300, Olympus). The number of activated caspase-3-positive cells (apoptotic cell number)/PI-positive nuclei (total cell number) was calculated, and then the percentage of increase against each vehicle control was determined. In each experimental run, adjacent chambers incubated without primary and/or secondary antibodies were prepared as negative controls.

Statistical analysis

All experiments involving follicle isolation were repeated with separate groups (nine sows/group) for independent observation. ANOVA with Fisher's least significant differences test comparison for biochemical data and Wilcoxon's signed-rank test for histological estimation were carried out using StatView-4.5 program (Abacus Concepts, Berkely, CA) on a Macintosh computer. Differences at $P < 0.05$ were considered significant.

Results

Immunohistochemistry

Positive immunostaining for TRAIL (Fig. 1A, B and C) was observed in apoptotic granulosa cells located in the nearby basement membrane (outer region of granulosa layers). Positive staining for DR4 was seen in scattered granulosa cells of granulosa layers (Fig. 1D, E and F). The intensity of TRAIL-staining was increased during follicular atresia, but notable changes in the intensity of DR4-staining were not observed.

Changes in TRAIL and DR4 protein levels in granulosa cells

Weak expression of TRAIL protein (37 kDa) was seen in granulosa cells of healthy follicles (Fig. 2A). The chemiluminescence intensity quantified using an automatic image-analyzer showed that TRAIL protein expression levels increased during follicular atresia ($P<0.01$; Fig. 2B). However, no significant differences in the expression levels of DR4 protein (70 kDa) among healthy, early atretic and progressed atretic follicles were shown (Fig. 2C and D).

Expression of TRAIL mRNA in granulosa cells

The RT-PCR products detected in the present study were confirmed to be porcine TRAIL by the cDNA sequence determination. The cDNA sequence of the corresponding domain of TRAIL was 5'-AGTGG CTCAC ACCTG TAATC CCAGC ATTTG GGAAG CCGTG GCGGG GGGGT TCATG AGGTC GGGAG TTCGA GACCA GTGTG GTCAA CACGG AGAGA CCGTG TCTAT CCTAA AAACA CAAAC TTCAC CGGGG TGGGG GGGCG CGGGC GCGTA ATCCG ATCTT CTGGG GGGGC CCGGG TCTGC GAACC CGGTA CCCC-3' (194 bp). The degree of homology between porcine and human TRAIL (Gen Bank accession number: U37518) was 80%. The high degree of homology shows that the PCR product detected in the present study was porcine

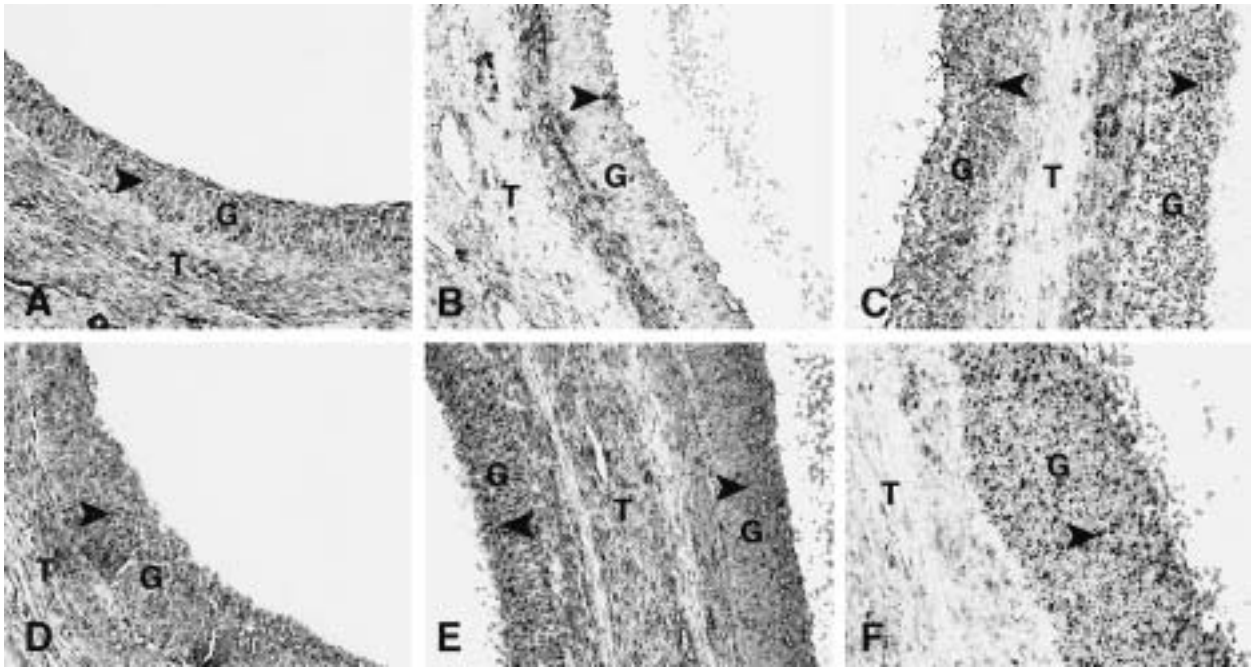

Fig. 1. Ovarian sections from healthy (A and D), early atretic (B and E) and progressed atretic (C and F) follicles. They were immunohistochemically stained for tumor necrosis factor-related apoptosis-inducing ligand (A-C) and for death receptor-4 (D-F). × 200.

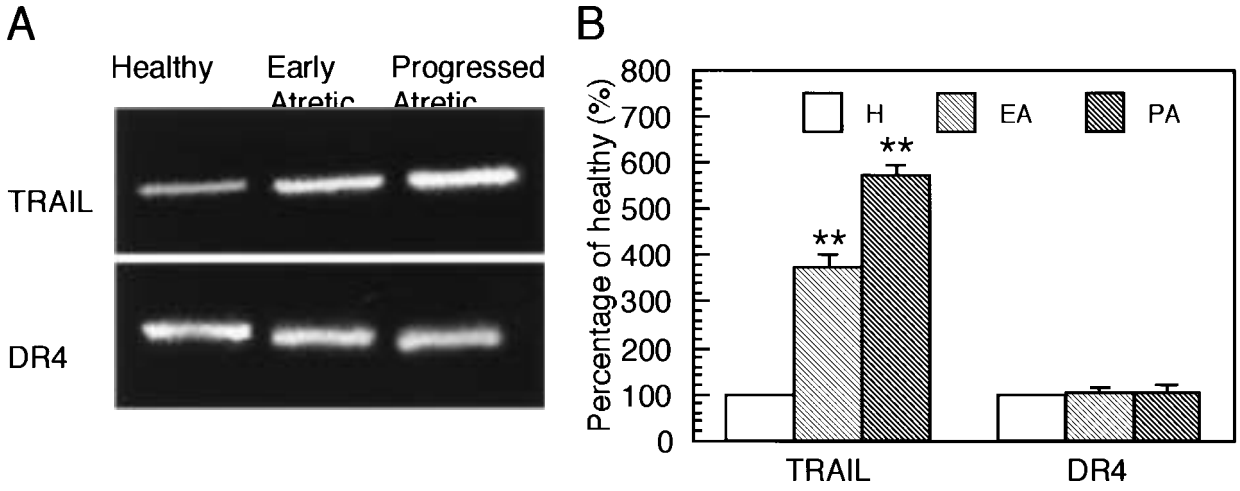

Fig. 2. Representative photographs of chemiluminescence-Western blotting for tumor necrosis factor-related apoptosis-inducing ligand and death receptor-4 proteins in granulosa cells from healthy, early and progressed atretic follicles are shown in A. These proteins in granulosa cells from healthy, early atretic and progressed atretic follicles were quantified by Western blotting image analysis and shown as chemiluminescence levels, then the percentage of increase against healthy follicles was calculated (B). All data in B are shown as mean ± SEM. **: $P<0.01$ vs each healthy sample.

TRAIL.

TRAIL and GAPDH mRNAs were detected in isolated granulosa cells of healthy, early atretic and progressed atretic follicles by RT-PCR (Fig. 3A). The fluorescence intensity quantified using an automatic image-analyzer showed that TRAIL mRNA expression increased during follicular atresia ($P<0.05$; Fig. 3B). Higher levels of TRAIL mRNA were seen in granulosa cells of early atretic and progressed atretic follicles (TRAIL/GAPDH

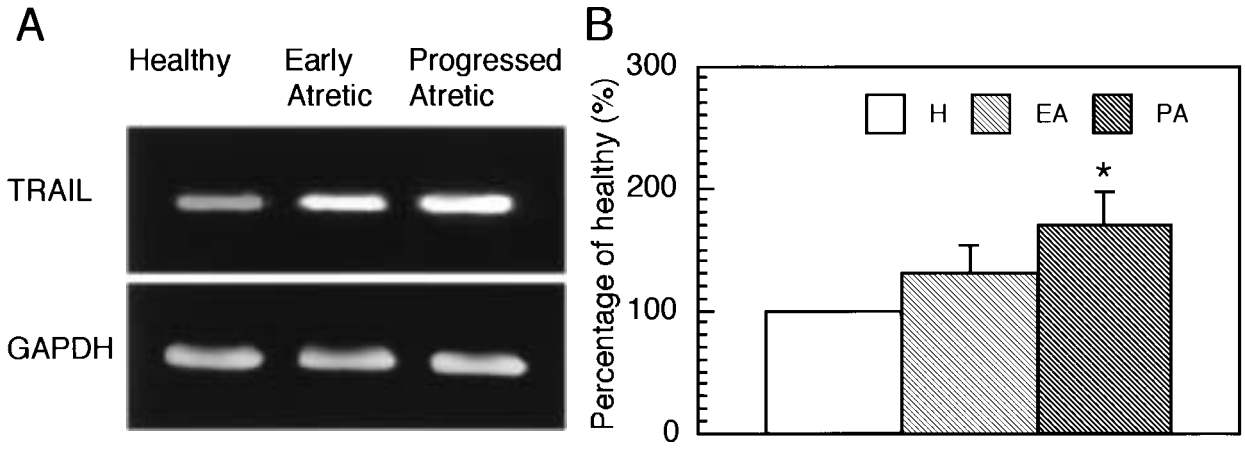

Fig. 3. Representative photographs of RT-PCR products for tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs are shown in A. TRAIL mRNA expression levels (TRAIL mRNA/GAPDH mRNA ratio) in granulosa cells of healthy (H), early atretic (EA) and progressed atretic (PA) follicles were determined, and then the percentage of increase against healthy follicles was calculated (B). All data in B are shown as mean ± SEM. *: P<0.05 vs each healthy sample.

ratio: 0.58 ± 0.06 and 0.98 ± 0.06; 29.9 and 71.0% increase, respectively) than in granulosa cells of healthy follicles (0.45 ± 0.06). Changes in TRAIL mRNA level during follicular atresia corresponded well to changes in TRAIL protein level.

Caspase-3 activation induced by TRAIL

To confirm the apoptosis inducing ability of TRAIL, we measured the caspase-3 activation rate (activated caspase-3-positive cells/PI-positive nuclei; apoptosis induction rate) in cultured granulosa cells with TRAIL treatment by an immunocytochemical technique. The caspase-3 activation indicates the intracellular activation of the apoptosis signal transduction system. When cultured granulosa cells were incubated without TRAIL (0 ng/ml, vehicle control) for 0, 4, 6, 8, 12 and 16 h, caspase-3 activation rates were 0.11, 0.08, 0.08, 0.14, 0.16 and 0.14, respectively. As shown in Fig. 4, when they were incubated with more than 200 ng/ml TRAIL from 0 to 12 h, TRAIL induced apoptotic cell death in dose- and time-dependent manners. After 6 h incubation, the caspase-3 activation rate reached a plateau level.

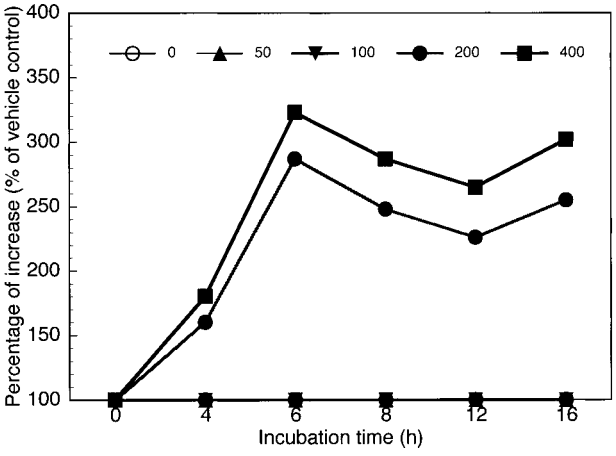

Fig. 4. Apoptosis inducing ability of TRAIL. Apoptosis induction rate (activated caspase-3-positive cells/PI-positive nuclei) in cultured granulosa cells was determined by an immunocytochemical technique, and then the percentage of increase against each vehicle control was calculated. All data are shown as mean ± SEM. *: P<0.05 vs each healthy sample.

Discussion

In mammalian ovaries, more than 99% of follicles undergo atresia during follicular growth and development, and apoptosis signal induction in granulosa cells plays a key role in the regulation of follicular selection [6, 23–26]. To date, many investigators have examined the molecular mechanism of this regulation of follicular selection, but the detailed molecular mechanism in follicular selection is still unknown. Recently, cell death ligand and receptor systems have been shown to play crucial roles in the control of apoptosis induction in granulosa cells [8, 27, 28]. TRAIL belongs to the TNF ligand family and induces

apoptotic cell death, and its mRNA is biochemically detected in a wide range of tissues [29], but the role of TRAIL and receptor systems in ovarian tissues has not been revealed. We performed the present study to confirm the role of TRAIL and its receptor systems in the regulation of granulosa cell apoptosis.

Until now, at least four TRAIL receptors (DR4, DR5, DcR1 and DcR2) have been identified [30–36], and the present findings and/or preliminary studies [18, 37] confirmed by immunohistochemical and biochemical analyses that TRAIL and its three receptors proteins (DR4, DR5 and DcR1) are expressed in porcine granulosa cells. Positive immunostainings for TRAIL, DR4, DR5 and DcR1 proteins were demonstrated in granulosa cells of healthy follicles. However, positive stainings for TRAIL, DR4 and DR5 proteins, but not DcR1 protein, were seen in granulosa cells of atretic follicles. The intensity of immunostaining for DR5 was at trace or weak levels [18]. TRAIL induces apoptotic cell death in normal healthy human hepatocytes [38]. Such TRAIL-induced apoptosis is massive and rapid, and occurred in more than 60% of hepatocytes exposed to TRAIL within 10 h. However, in the present study, the dose of TRAIL inducing apoptosis (more than 200 ng/ml) was extremely high compared with the cases of tumor cells. When cultured granulosa cells prepared from porcine healthy follicles were incubated with TRAIL, more than 200 ng/ml TRAIL could induce apoptotic cell death in a dose- and time-dependent manner, but less than 100 ng/ml of TRAIL could not induce apoptosis. It is believed that the sensitivity to TRAIL in granulosa cells of porcine healthy follicles is the same as normal healthy human hepatocytes. When DcR1 was removed from the cell membrane of granulosa cells, a lower dose of TRAIL could induce apoptosis [19]. We conclude that TRAIL can induce granulosa cell apoptosis, and that DcR1 blocks TRAIL-induced apoptosis in granulosa cells of healthy follicles. Based on our findings, we hypothesize that the mode of action of TRAIL and receptor systems in the regulation of granulosa cell apoptosis is as follows. (1) In healthy follicles, a sufficient amount of DcR1, whose ligand binding affinity is higher than those of DR4 and DR5 [31, 36], is expressed on the cell membrane of granulosa cells. TRAIL and DR4, and trace levels of DR5 also exist. DcR1 strongly binds with TRAIL, and inhibits TRAIL-DRs binding. (2) In early atretic follicles, DcR1 on the cell membrane of granulosa cells decreases. Finally, it disappears in progressed atretic follicles. Consequently, TRAIL can bind with DRs. (3) Ligand-receptor binding is the initial signal for granulosa cell apoptosis. The TRAIL-dependent apoptosis signal is transduced into cytoplasmic adaptor proteins (TNF receptor-associated death domain protein and Fas-associated death domain protein). Initiator caspase (procaspase-8) is activated, and thereby the caspase cascade (caspase-3) is activated for intracellular transduction of the apoptotic signal. However, we have no knowledge of what factor(s) control the expression of DcR1 and how it is regulated in the extremely early stage of follicular atresia. Further studies are needed to determine the regulation mechanism of DcR1 expression.

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