

## TRAIL-Decoy Receptor 1 Plays Inhibitory Role in Apoptosis of Granulosa Cells from Pig Ovarian Follicles

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**ABSTRACT.** Previously, we histochemically examined the localization of tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) and its receptors in porcine ovarian follicles, and demonstrated a marked reduction in the expression of TRAIL-decoy receptor-1 (DcR1) in granulosa cells of atretic follicles. In the present study, to confirm the inhibitory activity of DcR1 in granulosa cells, granulosa cells prepared from healthy follicles were treated with phosphatidylinositol-specific phospholipase C (PI-PLC) to cleave glycosphospholipid anchor of DcR1 and to remove DcR1 from the cell surface, and then incubated with TRAIL. PI-PLC treatment increased the number of apoptotic cells induced by TRAIL. The present finding indicated the possibility that TRAIL and its receptors were involved in induction of apoptosis in granulosa cells during atresia, and that DcR1 plays an inhibitory role in granulosa cell apoptosis. **KEY WORDS:** apoptosis, follicular atresia, granulosa cell, swine ovary, TRAIL-decoy receptor 1.

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Recent findings have suggested that granulosa cell apoptosis is associated with follicle selection in porcine ovaries [14–20], but the detailed regulatory mechanisms of this apoptosis are not yet understood. To reveal the specific regulatory molecules that control granulosa cell apoptosis during follicular atresia, we histochemically examined the localization of tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL; also known as Apo-2 ligand), that is a cytotoxic cytokine homologous to Fas ligand [21, 27, 35], and its receptors in porcine ovaries. A marked reduction in the expression of decoy receptor-1 (DcR1; also known as TRAIL-R3, LIT or TRID) [6, 7, 22, 25, 29] was demonstrated in granulosa cells of atretic follicles, but no marked differences were seen in expression of TRAIL or death receptors (death receptor-4, DR4/TRAIL-R1, or death receptor-5, DR5/TRAIL-R2/TRICK 2) [5, 8, 26], in granulosa cells between healthy and atretic follicles [32]. DR4 and DR5 are members of the TNF receptor family and have two extracellular cysteine-rich domains (CRDs), and their cytoplasmic regions contain death domains. DcR1 has two extracellular CRDs which show close homology to those of DR4 and DR5, has high affinity for TRAIL, and is a glycosylphosphatidylinositol (GPI)-anchored cell-surface receptor that lacks a cytoplasmic tail [29]. TRAIL induced apoptosis in normal cells may be inhibited by DcR1 [2]. However, TRAIL can induce both cell death and cell proliferation [1, 34]. Reverse transcription-polymerase chain reaction (RT-PCR) and *in situ* hybridization (ISH) analyses revealed increased mRNA expression of TNF receptor-associated death domain protein (TRADD), that transmits the death signal from DR4 and/or DR5 to intracellular apoptosis-signal transduction components [3, 13, 28], in granu-

losa cells was demonstrated only in atretic follicles, indicating that TRAIL-receptor system induces apoptosis in granulosa cells during atresia in porcine ovaries [33]. Moreover, our findings have indicated that DcR1 may play an inhibitory role in granulosa cell apoptosis, a key phenomenon in follicle selection. In the present study, primary cultured granulosa cells prepared from healthy follicles of porcine ovaries were pretreated with phosphatidylinositol-specific phospholipase C (PI-PLC), that cleaves the GPI anchor of DcR1 and leads to removal of DcR1 from the cell surface [29], and then the naked cells were incubated with TRAIL to confirm the inhibitory activity of DcR1 on granulosa cell apoptosis.

### MATERIALS AND METHODS

**PI-PLC treatment and TRAIL-induced apoptosis assay:** Granulosa cells from follicles of porcine ovaries were prepared as described previously [23]. Briefly, individual pre-ovulatory antral follicles, 3–4 mm in diameter, were dissected in Medium 199 (Gibco BRL, Grand Island, NY, U.S.A.) with 25 mM Hepes (Gibco) and 0.1% polyvinylalcohol (Wako Pure Chemical, Osaka, Japan) from the ovaries obtained from mature pigs at a slaughterhouse. Under a surgical dissecting microscope (SZ40, Olympus, Tokyo, Japan), follicles were classified as morphologically healthy or atretic follicles [4]. Because the progesterone/estradiol-17 $\beta$  (P/E2) ratio of follicular fluid in each follicle provides a good index of follicular atresia in sows [9], estradiol-17 $\beta$  and progesterone levels in follicular fluid collected from each follicle were quantified using [<sup>125</sup>I]-RIA kits (Bio-Mérieux, Marcy-l'Étoile, France) as described previously [12]. When P/E2 ratio was less than 15, the follicle was classified as healthy. The granulosa cell layers were removed from the follicles in 25 mM Hepes-buffered

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Medium 199 containing 80  $\mu\text{g/ml}$  of kanamycin sulfate (Sigma Aldrich Chemicals, St. Louis, MO, U.S.A.; Hepes-199), washed with Hepes-199 by centrifugation, incubated in  $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free Hanks' balanced salt solution (Gibco) containing 10 mM EDTA (Gibco) and 6.8 mM EGTA (Sigma) for 15 min at room temperature (RT; 22–25°C), and then granulosa cells were isolated by pipetting. The cells were washed twice in Hepes-199 containing 10% fetal calf serum (FCS; Gibco, Hepes-199-FCS) by centrifugation. Cell number was counted using a hemocytometer plate, and cell viability was determined by the trypan blue exclusion method. The cells with viability of more than 95% were used. Then, the isolated cells ( $1 \times 10^6$  cells/ml) were placed in 96-well cell culture plates (Falcon 3072, Becton Dickinson, Lincoln Park, NJ, U.S.A.) in Dulbecco's minimum essential medium (Gibco) containing 50  $\mu\text{g/ml}$  gentamicin sulfate (Sigma) and 10% FCS (DMEM-FCS) and precultured in a  $\text{CO}_2$  incubator (humidified atmosphere of 95% air and 5%  $\text{CO}_2$ ) at 37°C for 6 hr. After preculture, non-adherent cells were removed, and then the adherent granulosa cells were treated in DMEM-FCS with or without 1 U/ml PI-PLC (Molecular Probes, Eugene, OR, U.S.A.) for 1 hr at 37°C [29]. After wash with DMEM-FCS, the cells were incubated with DMEM-FCS containing TRAIL (Upstate Biotechnology, New York, NY, U.S.A.) at doses of 0 (vehicle control), 10, 50 and 100  $\text{ng/ml}$  for 10 hr at 37°C.

As described previously [19], cell viability was determined using a Cell survival and proliferation kit (Chemicon International, Temecula, CA, U.S.A.) according to the manufacturer's instructions. To determine the apoptotic cell rate, the cells were pre-fixed with 4% paraformaldehyde fixative buffered with sodium phosphate (4% PFA; pH 7.2) for 10 min at 4°C, washed with PBS containing 0.05% Tween 20 (PBS-Tw; Sigma), post-fixed in acid ethanol fixative (acetic acid/ethanol: 1/2), treated with 2% hydrogen peroxide to inhibit endogenous peroxidase activity, and washed well with PBS-Tw. Apoptotic cells were visualized by the terminal deoxynucleotidyl transferase (TdT)-mediated biotinylated deoxyuridine triphosphate nick end-labeling (TUNEL) method using an ApopTag peroxidase staining kit (Intergen, Purchase, NY, U.S.A.) according to the manufacturer's protocol. The cells were counterstained with methylgreen, dehydrated, mounted with Eukitt (Kindler, Freiburg, Germany), and examined with a microscope (BX51, Olympus). As a negative control, the cells were incubated without TdT. As a positive control, cell samples were treated with DNase I (1  $\mu\text{g/ml}$ ; Sigma), 140 mM sodium cacodylate, 4 mM  $\text{MgCl}_2$  and 0.1 mM dithiothreitol in 30 mM Tris-HCl (pH. 7.2) for 10 min at 20°C before exposure to TdT.

To assess the DNA fragmentation in cultured granulosa cells, DNA samples of cultured cells were electrophoresed as previously reported [19]. Briefly, DNA fractions were separated from the TRAIL-treated cells by centrifugation at 9,000 g for 20 min at 4°C, and then DNA contents were determined using a 4', 6-diamidino-2 phenylindole dihydrochloride (DAPI)-fluorescence assay kit (Wako) according to

the manufacturer's instructions. DNA samples were electrophoresed in 2% agarose gels, stained with ethidium bromide and recorded with a digital recorder (LAS 1000, Fuji Film Co. Ltd., Tokyo, Japan).

*Statistical analysis:* All experiments involving follicle isolation were repeated with separate groups (six sows/group) for independent observation. ANOVA with Fisher's least significant differences test comparison for cytochemical data were carried out using StatView IV on a Macintosh computer. Differences at  $p < 0.05$  were considered significant. All data are expressed as mean values  $\pm$  SD.

## RESULTS

The cultured granulosa cells were pretreated with or without PI-PLC, that cleaves the GPI anchor of DcR1 and leads to removal of DcR1 from the cell surface, and then incubated with TRAIL. Apoptotic cell death was confirmed by DNA fragmentation assessed by electrophoresis (Fig. 1A). Apoptotic cell rate was determined by TUNEL assay (Fig. 1B). The nuclei of TUNEL-positive cells (apoptotic cells) were stained brown, and those of negative cells were green.

As summarized in Fig. 2, TRAIL treatment (0, 10, 50 and 100  $\text{ng/ml}$ ) induced extremely low rates of apoptosis in granulosa cells without PI-PLC pretreatment ( $0.3 \pm 0.1$ ,  $0.9 \pm 0.5$ ,  $0.7 \pm 0.4$  and  $1.2 \pm 0.7\%$ , respectively). When the cells were pretreated with PI-PLC, however, apoptosis was induced by TRAIL treatment (0, 10, 50 and 100  $\text{ng/ml}$ ) in a dose-dependent manner ( $0.4 \pm 0.1$ ,  $5.5 \pm 1.3$ ,  $13.7 \pm 2.9$  and  $20.5 \pm 2.6\%$ , respectively).

## DISCUSSION

Over the last decade, several cell death ligands (TNF- $\alpha$ , FasL, TRAIL etc.) and their receptors (TNFR1, Fas, DR4, DR5, DcR1, DcR2 etc.) have been found [1, 5, 8, 24–26, 34]. 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 [10, 31]. Such atretic degeneration may be explained by selective apoptosis of granulosa cells [11, 30]. Many researchers believe that the cell death ligand-receptor system plays critical roles in selective apoptosis of granulosa cells during follicular atresia.

Previously, we examined the changes in expression and localization of the TRAIL, TRAIL-receptors and adaptor protein during follicular atresia in porcine ovaries to determine its involvement in regulation of granulosa cell apoptosis in follicular atresia, and to understand which trigger molecules induce granulosa cell apoptosis, or how intercellular apoptotic signals are transmitted in the granulosa cells [32, 33]. We demonstrated the expression of TRAIL and its receptors, DR4, DR5 and DcR1, in granulosa cells and theca internal and external layers of both healthy and atretic follicles [32]. Remarkably intense TRAIL, and DR5 and DcR1

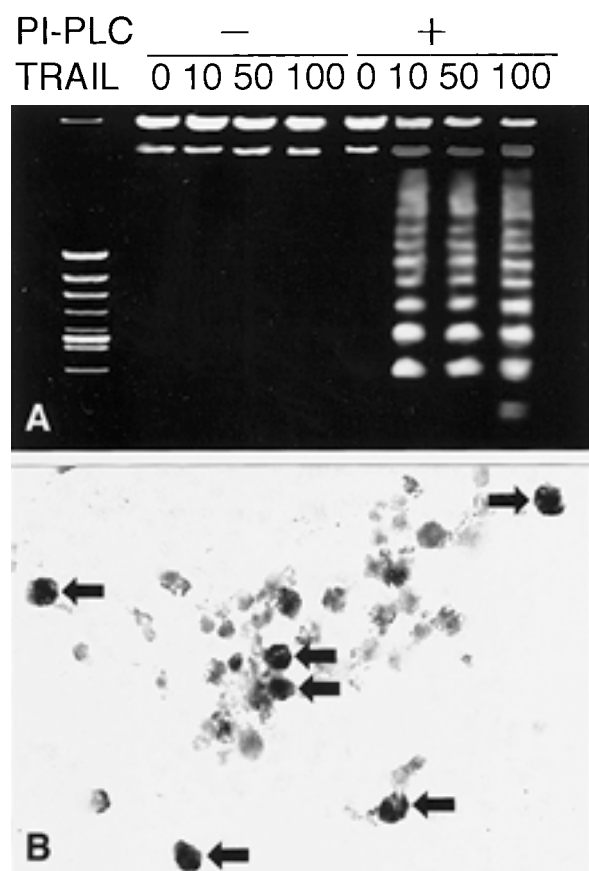


Fig. 1. Electrophoretic analysis of DNA fragmentation in DNA samples prepared from cultured granulosa cells (A). Representative photographs of the cultured granulosa cells stained by the TUNEL method to assess apoptosis (B). When the cells were pretreated with PI-PLC and incubated with TRAIL (100 ng/ml), many TUNEL-positive nuclei (stained brown; arrows in B) were observed.  $\times 200$ .

immunostaining were seen in granulosa cells lining the antral cavity of healthy follicles. Interestingly, no positive staining for DcR1 was seen in granulosa cells of atretic follicles. High levels of expression of TRADD mRNA were detected by RT-PCR in granulosa cells of early atretic follicles, and moderate expression was seen in those of progressed atretic follicles [33]. ISH staining indicated intense expression of TRADD mRNA in granulosa cells of atretic follicles, but no positive staining was seen in those of healthy follicles. These findings strongly suggested that TRAIL and its receptors play critical roles in induction and regulation of apoptotic cell death in granulosa cells during follicular atresia of porcine ovaries, and that DcR1 may have a protective effect against the induction of apoptosis by TRAIL and its receptors system.

In the present study, to confirm the protective effect of DcR1 on TRAIL-dependent apoptosis in granulosa cells, we examined the inhibitory effect of DcR1 during TRAIL-

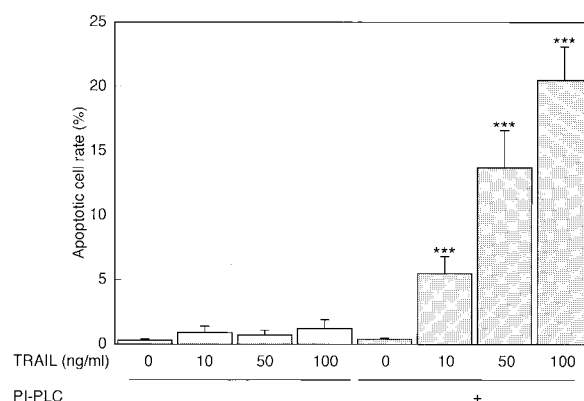


Fig. 2. Inhibitory effect of DcR1 on TRAIL-induced apoptosis. No apoptosis was induced by TRAIL treatment in porcine granulosa cells without PI-PLC pretreatment (—). In the cells with PI-PLC pretreatment (+), apoptosis was induced by TRAIL treatment in a dose-dependent manner. \*\*\*:  $p < 0.001$  vs vehicle control (0 ng/ml of TRAIL).

induced apoptosis in granulosa cells *in vitro*. Our preliminary experiments to estimate the removal of DcR1 by PI-PLC treatment showed that at least 0.5 U/ml of PI-PLC and more than 0.5 hr incubation were necessary to eliminate the positive immuno-staining for DcR1. In the present study, the granulosa cells were incubated in DMEM-FCS containing 1 U/ml of PI-PLC for 1 hr. It is considered that the PI-PLC treatment removes DcR1 from the cell surface of the granulosa cells. When primary cultured granulosa cells prepared from healthy follicles were pretreated with PI-PLC to remove endogenous GPI-linked DcR1 from the cell surface of the granulosa cells, apoptosis occurred in the cells incubated with TRAIL in a dose-dependent manner. Thus, it is considered in porcine ovarian follicles that DcR1 competes with TRAIL receptors, DR4 and/or DR5, for binding to TRAIL and blocks granulosa cells from apoptotic cell death, resulting follicles will not undergo atresia. In atretic follicles, however, DcR1 disappears in granulosa cells, TRAIL can bind with DR4 and/or DR5, and then TRAIL-dependent apoptosis signal transmits into cytoplasm of the cells. Further studies are considered necessary to determine which molecular system dominantly causes the disappearance of DcR1 in granulosa cells during follicular atresia in porcine ovaries and to characterize the detailed mechanism of apoptosis signal transduction in granulosa cells.

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