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—Original Article—

Effect of RNA Interference of BID and BAX mRNAs on Apoptosis in Granulosa Cell-derived KGN Cells

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Abstract. In mitochondrion-dependent type II apoptosis, BH3-interacting domain death agonist (BID) and BCL-2-associated X protein (BAX) promote death ligand and receptor-mediated cell death. In porcine ovaries, the levels of BID and BAX increase in follicular granulosa cells during atresia. In the present study, to confirm the pro-apoptotic activity of BID and BAX in granulosa cells, we examined the effect of RNA interference of BID or BAX on apoptosis using a human ovarian granulosa tumor cell line, KGN. By reverse transcription polymerase chain reaction (RT-PCR) and Western blotting, expression of BID and BAX was detected in KGN cells. Then, we suppressed *BID* and *BAX* mRNA expression in KGN cells using small interfering RNA (siRNA). When BID or BAX was suppressed, a significant decrease in the apoptotic cell rate was noted. In granulosa-derived cells, BID and BAX showed pro-apoptotic activity. These results suggest that BID and BAX act as signal-transducing factors in mitochondrion-dependent type II apoptosis.
Key words: BCL-2-associated X protein (BAX), BH3-interacting domain death agonist (BID), Mitochondrion-dependent type II apoptosis, RNA interference (RNAi)

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In mammalian ovaries, more than 99% of follicles degenerate at various stages of follicular growth and development [1]. The degeneration is explained, at least in part, by the apoptosis of granulosa cells [2–6]. In porcine ovarian follicles in the early stages of atresia, characteristics typical of apoptosis are observed in scattered granulosa cells, but not in cumulus cells, oocytes or the cells of internal or external thecal layers [7]. As previously reported [8], it is considered that selective granulosa cell apoptosis is induced by death ligand and receptor systems as follows: When trimerized death ligands bind with trimerized death receptors located on the cell membrane, the receptors are activated. An adaptor protein (Fas-associated death domain protein) binds with activated receptors to construct death-inducing signal complex (DISC), which binds procaspase-8. Then, procaspase-8 is truncated and activated in DISC. Two types of cell death ligand and receptor-dependent signaling pathways, type I and II, have been found. Granulosa cells undergo mitochondrion-dependent type II apoptosis. Activated caspase-8 shortens BH3-interacting domain death agonist (BID) protein [9, 10], and the truncated-BID (tBID) stimulates BCL-2-associated X protein (BAX). Then, BAX is transferred to the outer membrane of the mitochondrion and releases cytochrome c [11, 12]. Cytochrome c binds with apoptotic protease-activating factor 1 and procaspase-9, and this complex is called an apoptosome [13]. The procaspase-9 is activated in the apoptosome and shortens procaspase-3, and the activated caspase-3 truncates caspase-3-activated DNase (CAD) [14, 15]. The shortened CAD moves into the nucleus and cuts the genomic DNA leading to apoptosis.

A large amount of caspase-8 is activated in type I apoptosis, but a small amount of procaspase-8 is activated in type II apoptosis [16]. Because the intracellular apoptotic signal has to be amplified in type II cells, activated caspase-8 stimulates BCL-2 family members, BID and BAX, to transduce the apoptotic signal into mitochondria. The mitochondrion provides a key amplification step in the type II apoptotic pathway [17, 18]. However, details of the intracellular signal transducing pathway in the follicular granulosa cell, which is a mitochondrion-dependent type II apoptotic cell, have not been revealed. In the present study, to reveal the roles of BID and BAX in mitochondrion-dependent apoptotic signaling in granulosa cells, we examined the effect of suppressing *BID* or *BAX* mRNA expression in human granulosa tumor-derived (KGN) cells using small interfering RNA (siRNA).

Materials and Methods

Cell culture

KGN cells (provided by Drs, Y Nishi and H Nawata, Kyushu University, Fukuoka, Japan) were precultured in DMEM/F-12 (1:1) medium (Invitrogen, Carlsbad, CA, USA), containing 10% (v/v) fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA), 100 units/ml of penicillin (Sigma) and 100 μg/ml of streptomycin (Sigma) in an incubator (MCO-18AIC; Sanyo, Osaka, Japan; 5% of CO_2-air) for 24 h at 37 C.

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Reverse transcription-polymerase chain reaction (RT-PCR) assay for BID and BAX mRNA

As previously reported [19, 20], total RNA was extracted from cells using a QIAshredder and RNeasy Mini Kit (Qiagen, Chatsworth, CA, USA) according to the manufacturer's directions. First strand cDNA was synthesized from the total RNA using a Ready-To-Go T-Primed First-Strand Kit (GE Healthcare, Little Chalfont, UK). The primers used for PCR were as follows: 5′-ATG GAC TGT GAG GTC AAC AA-3′ and 5′- TCA GTC CAT CCC ATT TCT GG-3′ for *BID* (GenBank Accession No. NM197966), 5′- AAG AAG CTG AGC GAG TGT -3′ and 5′-GGA GGA AGT CCA ATG TC-3′ for *BAX* (NM004324) and 5′-TCC TCT GAC TTC AAC AGC GAC ACC-3′ and 5′-TCT CTC TTC CTC TTG TGC TCT TGG-3′ for *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*; AF017079, used as an intrinsic control). PCR amplification was performed as follows: Platinum Quantative PCR Supermix-UDG (Invitrogen) and 0.2 μM of each primer pair were added to the cDNA mixture and denatured. The mixture was subjected to PCR in a thermal cycler (GeneAmp PCR System 9700; Invitrogen). The PCR profile was 94 C for 5 min, and then 30 cycles of 94 C for 30 sec, 53 C for 30 sec and 72 C for 1 min, followed by a final extension at 72 C for 7 min. The product sizes of *BID*, *BAX* and *GAPDH* were 588, 265 and 201 bp, respectively. Each PCR product was electrophoresed in 2% (w/v) agarose gels (Cambrex Bio Science, Rockland, ME, USA) and stained with ethidium bromide (Wako Pure Chemical Industries, Osaka, Japan). The stained gels were scanned with a digital fluorescence recorder (LAS-1000; Fujifilm, Tokyo, Japan), and the intensity of each mRNA band was quantified using the Image Gauge software (Fujifilm) on a Macintosh computer. The expression levels of each mRNA was normalized by the level of *GAPDH* mRNA. To confirm the characteristic of each PCR product, a representative PCR product was sequenced using an automatic DNA sequencer (ABI PRISM 310; Invitrogen) according to the manufacturer's instructions.

Western blot analysis for BID and BAX proteins

As previously reported [21–23], each protein fraction (10 μg/lane) prepared from a cell sample was separated by 10–20% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Atto, Tokyo, Japan) and then transferred onto nitrocellulose membranes (Hybond-C; GE Healthcare). The membranes were stained with 0.2% (w/v) Ponceau S solution (Serva Electrophoresis, Heidelberg, Germany) and immersed in a blocking solution [0.1 M Tris HCl, (pH 7.6), 5% (w/v) skim milk, 0.05 M NaCl and 0.1% (v/v) Tween 20; Sigma] for 1 h at 25 C. Each membrane was incubated with the appropriate primary antibody for 1 h at room temperature (22–25 C): for BID protein, a rabbit polyclonal anti-human BID antibody (diluted 1:1,000 with the blocking solution; Santa Cruz Biotechnology, Santa Cruz, CA, USA); for BAX protein, a rabbit polyclonal anti-human BAX antibody (diluted 1:500 with the blocking solution; Santa Cruz Biotechnology); and for GAPDH protein, a goat polyclonal anti-human GAPDH antibody (diluted 1:200 with the blocking solution; Santa Cruz Biotechnology). After being washed with the blocking solution, each membrane was incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at 25 C: for BID and BAX, an HRP-conjugated goat anti-rabbit IgG antibody (diluted 1:2,000; Dako, Copenhagen, Denmark), and for GAPDH, an HRP-conjugated rabbit anti-goat IgG antibody (diluted 1:2,000; Dako). After incubation, chemiluminescence was visualized using an ECL system (GE Healthcare) according to the manufacturer's protocol. The chemiluminescence was recorded with a digital fluorescence recorder, and the intensity of each protein band was quantified using the Image Gauge software on a Macintosh computer. The relative abundance of each protein was normalized to the relative abundance of GAPDH protein.

Small interfering RNA (siRNA) transfection and cell viability

As previously reported [24], the RNAi-Ready pSIREN-RetroQ-ZsGreen vector (BD Bioscience, Palo Alto, CA, USA) was used for intracellular expression of siRNA. Briefly, the 19-mer siRNA target sequence for BID was 5′-GAA GAC ATC ATC CGG AAT A-3′ (700^{th}-717^{th}; GenBank accession number: NM197966). The target sequence for BAX was 5′-GCG CAT CGG GGA CGA ACT G-3′ (261^{st}-279^{th}; NM004324). These sequences were designed to make hairpin siRNA and inserted into pSIREN vector (Clontech Laboratories, Madison, WI, USA) according to the manufacturer's directions. First, 40,000 KGN cells/ml of medium were cultured without penicillin or streptomycin in 96-well culture plates (4,000 cells/100 μl in each well; Falcon, San Jose, CA, USA) for 24 h at 37 C, and then 0.2 μg/100 μl of the plasmid was transfected using Lipofectamine 2000 (Invitrogen). All procedures were performed according to the manufacturer's protocols. Empty vector (mock control), or the siRNA vector of *BID* or *BAX* was introduced into the KGN cells. As previously reported [25], to induce apoptosis, 100 ng/ml of anti-human Fas monoclonal antibody (CH-11; MLB, Nagoya, Japan) and 5 μg/ml of cycloheximide (CHX; Sigma) were added to the culture medium 48 h after the transfection, and the cells were incubated for 10 h. At 10 h after the transfection and 10 h after the induction of apoptosis, we examined the green fluorescence protein (GFP) fluorescence of KGN cells to assess the transfection under a fluorescence microscope (BX-51; Olympus, Tokyo, Japan). At 10 h after the induction of apoptosis, cell viability was assessed using an MTS Assay Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, 20 μl of the Cell Titer 96-Aqueous One Solution was added to each well and incubated for 1 h. The absorbance at 490 nm was measured using a 96-well plate reader (Model 680; Bio-Rad, Hercules, CA, USA).

Inhibition of cytochrome c release and cell viability

KGN cells (40,000 cells/ml of medium) were cultured without penicillin or streptomycin in 96-well culture plates (4,000 cells/100 μl in each well) for 24 h at 37 C, and then a cytochrome c release inhibitor (500 nM of Cell-Permeable; Merck, Darmstadt, Germany) was added. After another 24 h, 100 ng/ml of CH-11 antibody and 5 μg/ml of CHX were added to the culture medium to induce apoptosis, and then the cells were incubated for 10 h. At 10 h after the induction of apoptosis, cell viability was assessed using an MTS Assay Kit.

Statistical analysis

An analysis of variance (ANOVA) with Fisher's least significant differences test was carried out using the Stat View 4.5 program

(Abacus Concepts, Berkeley, CA, USA) on a Macintosh computer. Differences at $P<0.05$ were considered significant.

Results

Expression of mRNA and protein of BID and BAX and confirmation of the suppression of BID and BAX mRNA by the siRNA in KGN cells

As shown in Fig. 1, mRNA and protein of both BID and BAX were detected in KGN cells. The RT-PCR analysis confirmed that *BID* as well as *BAX* mRNA expression was completely suppressed by the siRNA (Fig. 2). At 10 h after the transfection, Western blot analysis showed no BID or BAX protein expression in KGN cells with siRNA treatment (data not shown).

Effect of suppressing BID and BAX mRNA on apoptosis

When empty vectors were transfected (mock control), most of the cells died at 10 h after apoptosis induction; however, when BID or BAX was suppressed, many cells with fluorescence survived (Fig. 3A). Cell viability rates in BID- and BAX-suppressed cells were approximately 171 and 137% of the mock control, respectively, (Fig. 3B).

Effect of inhibiting the release of cytochrome c on apoptosis

When KGN cells were coincubated with the inhibitor of cytochrome c release, the cell viability was approximately 140% of the negative control (without treatment) at more than 10 h after apoptosis induction.

Discussion

Granulosa cell apoptosis is the first event in follicular atresia and is mediated by cell death ligand and receptor systems [7, 26–28]. However, there is insufficient information on the role of intracellular signaling molecules in granulosa cells, especially downstream of the activated caspase-8. Previously, we revealed high levels of BID and BAX mRNA and protein in the granulosa cells of early atretic follicles but low levels in those of healthy follicles, indicating that BID and BAX are involved in granulosa cell apoptosis during atresia in porcine ovaries [8]. In the present study, to confirm the roles of BID and BAX in the apoptosis, we examined the effects of suppressing *BID* or *BAX* mRNA expression with the RNAi technique on apoptosis in human granulosa cell-derived KGN cells. When the expression of BID and BAX was suppressed by RNAi and the release of cytochrome c was inhibited by a specific inhibitor, the rate of apoptosis decreased in KGN cells. We conclude that BID and BAX are essential for apoptotic cell death in the mitochondrion-dependent type II apoptosis cell such as the granulosa cell. Though ovarian phenotypes have not been reported, a deficiency of Bid caused resistance to apoptosis mediated by cell death ligand and receptor in mice hepatocytes [29]. Moreover, a deficiency of Bax disrupted granulosa cell apoptosis (aberrant atresia) and affected rates of atresia among primordial and primary follicles in mice [30, 31]. Considering these reports, BID and BAX are necessary for apoptotic signal transduction in granulosa cells of ovarian follicles. However, the precise mechanism underlying the mitochondrion-dependent type II pathway and its control by regulating factors, the BCL-2 family, in ovarian tissues remains unclear. In the mitochondrial signalling pathway, BCL-2 family proteins have dual roles, as pro- or anti-apoptotic factors

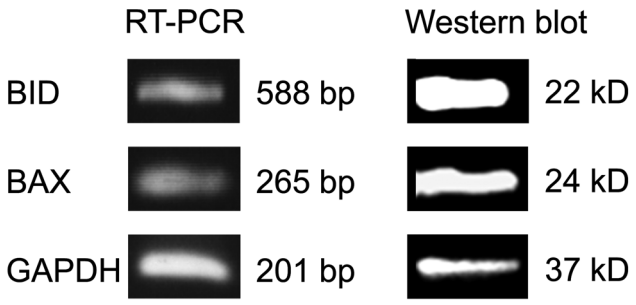

Fig. 1. Expression of *BID* and *BAX* mRNA in KGN cells examined by RT-PCR (left lane). Bands of *BID* and *BAX* mRNA were detected at 588 and 265 bp, respectively. Internal BID and BAX protein expression in KGN cells was examined by Western blotting (right lane). Bands of BID and BAX were detected at 22 and 24 kDa, respectively.

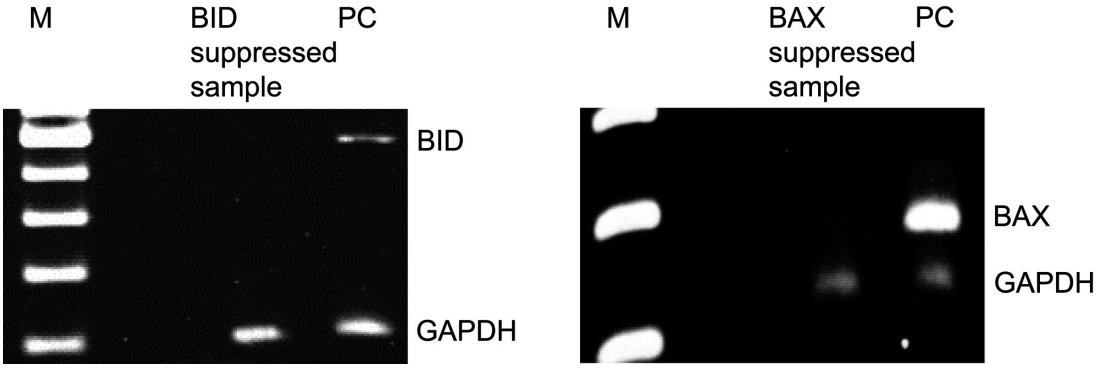

Fig. 2. BID and BAX expression was suppressed by transfection of the p*Silencer* siRNA vectors in KGN cells, and down-regulation of *BID* and *BAX* mRNA by siRNA was confirmed by RT-PCR. M: Molecular size marker. PC: Positive control.

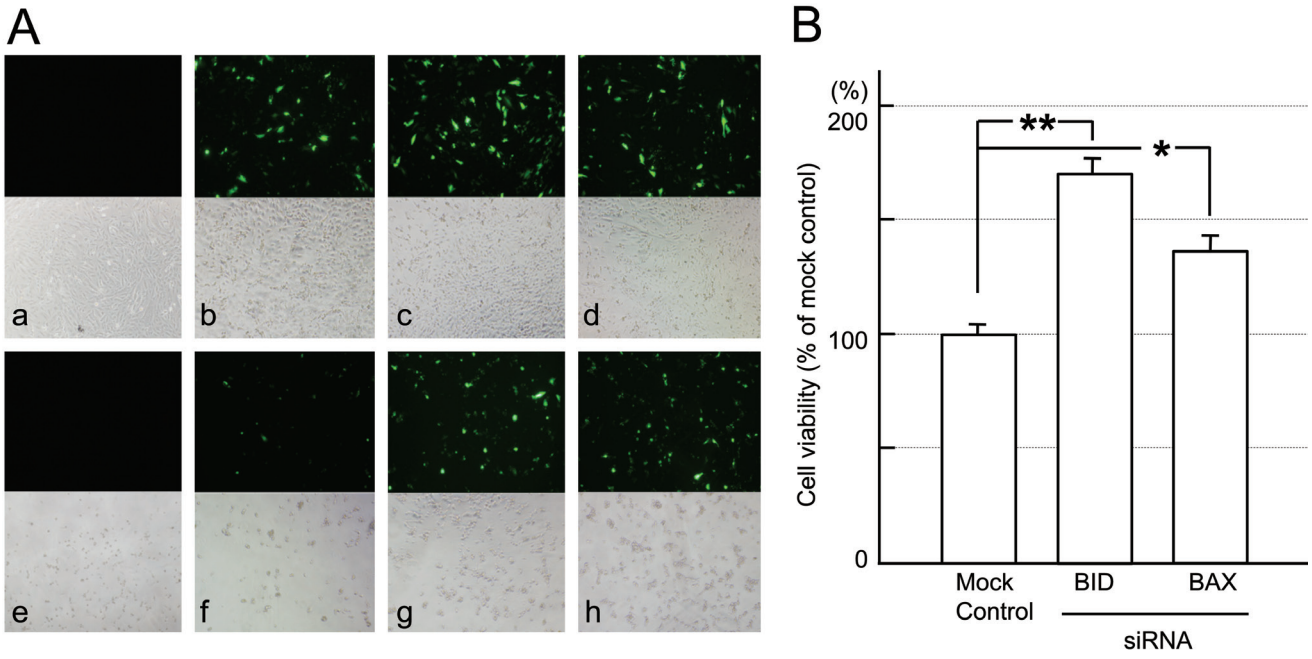

Fig. 3. Representative photographs of GFP fluorescence (upper) and phase contrast (lower) micrographs of KGN cells treated with siRNA for BID (A-c and g, respectively) or BAX (A-d and h), the mock control (A-b and f) and cells without any treatment (A-a and e: no positive fluorescent was shown) before (A-a, b, c and d) and 10 h after (A-e, f, g and h) apoptosis was induced. Many cells survived when BID or BAX was suppressed (A-g and h) than in the mock control (A-f). Moreover, cell viability assessed by an MTS assay increased to 171 and 137% that of the mock control (B). Values represent the means ± SEM for six independent experiments. * and **: $P < 0.05$ and 0.001 relative to the corresponding mock control.

[17, 18]. Transcription of *BID* and *BAX* was upregulated by activated p53 [32], but BAX was inhibited by anti-apoptotic proteins, BCL-2, BCL-xL, 14-3-3, Ku70 and so on [17]. Moreover, most of these previous reports were performed using the cells derived from tumors, which have aberrations in cell death. More studies are necessary to reveal the mechanisms regulating the expression of BID and BAX in granulosa cells in connection with physiological condition, follicular growth, development and atresia.

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