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)

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 CO2-air) for 24 h at 37 C.
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 QiAshedder 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 TTC TTG TGC TCT TGG-3′ for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; AF017079, used as an intrinsic control). PCR amplification was performed as follows: Platinum Quantitative 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, 55 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 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′-GCA GAC ATC ATC CGG AAT A-3′ (700th-717th; GenBank accession number: NM197966). The target sequence for BAX was 5′-GCC CAT CGG GGA CGA ACT G-3′ (261st-279th; 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.
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 pSilencer 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.
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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) 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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