The expression profile of apoptosis-related genes in the chicken as a human epithelial ovarian cancer model

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Abstract. The purpose of our study was to examine the expression pattern of apoptosis-related genes in normal and cancerous ovaries of the hen. Localization of apoptosis-related gene mRNA was investigated in cancerous ovaries using in situ hybridization. The expression patterns of apoptosis-related genes were confirmed with RT-PCR in normal and cancerous ovaries. Differences of expression level between normal ovaries and ovarian cancers were analyzed using quantitative RT-PCR. In both normal and cancerous chicken ovaries, the expression of CASP1, CASP2, CASP3, CASP6, CASP8 and CASP9 were detected through RT-PCR analysis. The expression of BCL2, BCL2L1 and BID were confirmed in normal and cancerous ovaries of the hen. Quantitative RT-PCR showed that CASP1 expression was significantly increased in cancerous ovaries compared with normal ovaries, whereas BID expression was decreased. Our results showed a resistance to removal of abnormal cells via apoptosis in cancerous ovaries of the hen. Collectively, this phenomenon is closely associated with the dysregulation of CASP1 and BID expression in chicken ovarian cancer.

Introduction

Until recently, epithelial ovarian cancer presented a major risk among gynecologic cancers due to its high lethality (1). The survival rate of patients with epithelial ovarian cancer can sharply increase if it is diagnosed at an early stage. For diagnosis of ovarian cancer in the early stages, a moderate biomarker is insufficient; the specificity and sensitivity of diagnostic biomarkers is associated with its BH domains included in proteins is associated with its BH1, BH2, BH3 or BH4, and the number of domains (i.e., BH1, BH2, BH3 or BH4), and the number of BH domains contained in proteins is associated with its BH domains in mice (5). However, ovarian cancers of these transgenic mice do not closely resemble human ovarian cancer (6-8).

To discover appropriate chemopreventive or chemotherapeutic agents to conquer human ovarian cancer, the development of a suitable animal model is essential. Ovarian cancers are rarely spontaneously developed from the ovarian epithelial surface in rodents models, except rodent models, several genetic modifications have been applied to advance ovarian cancer research in mice (5). However, ovarian cancers of these transgenic mice do not closely resemble human ovarian cancer (6-8).

On the other hand, chicken epithelial ovarian cancers spontaneously occur at a high rate. Like human ovarian cancer, the incidence of chicken ovarian adenocarcinoma spontaneously increases in aged hens that no longer produce or produce fewer eggs after 2 years of age (9). It has been recently noted that chicken and human ovarian cancers share the same immunohistochemical biomarkers, such as CA125 and the erbB2 antibody (10,11).

Programmed cell death or apoptosis has a vital role in various biological events. The elimination of unwanted or damaged cells by apoptosis is indispensable for embryogenesis, maintaining cellular homeostasis, and normal development of the immune system (12). Because impaired apoptosis induces abnormal cell proliferation, the dysregulation of cell death mechanisms is a major hallmark of cancer development (13). Programmed cell death is activated by intracellular stresses and developmental cues. Well-known representative intrinsic regulators, the extended BCL2 family proteins play crucial roles in cell death regulation and are able to regulate several cell death mechanisms, including apoptosis, necrosis and autophagy (14-16). BCL2 family proteins contain at least one of four BCL2 homology (BH) domains (i.e., BH1, BH2, BH3 or BH4), and the number of BH domains included in proteins is associated with its apoptotic functions (17). Antiapoptotic BCL2 family members (e.g., BCL2, BCL2L1, MCL1 and BCL2A1) have all four BH domains. Proapoptotic BCL2 family members are categorized into two groups: the multiple-BH domain, which includes members BAX, BAK1 and BOK, and a single BH-3 group that includes members BID, BIM, BAD and NOXA.
The antiapoptotic BCL2 family prevents endoplasmic reticulum (ER)-mediated cell death by reducing basal Ca\(^{2+}\) concentrations in the ER for regulation of cell survival (18-20), whereas proapoptotic family members like BAX and BAK induce ER-mediated apoptosis by inhibiting the effect of antiapoptotic members or by opposing effects on ER Ca\(^{2+}\) concentrations for regulation of cell death (17). Proapoptotic BCL2 family proteins, such as BAX and BID, stimulate mitochondrial outer membrane permeabilization (MOMP) that induces the release of regulators or activators (such as caspase activator) and other cell-death mediators. In contrast, antiapoptotic family members, such as BCL2 and BCL2L1, guard the outer membrane and conserve its integrity by the action of BAX and BAK (17). There is a close association between tumor development, initiation and resistance to chemotherapy and the dysfunction of BCL2 family proteins (12).

Cysteine aspartate-specific proteases (CASPs or caspases) serve as intrinsic initiators of apoptosis by cleaving substrates at aspartate residues (21). The caspase protein family currently consists of 13 and 11 isoenzymes in mammals and humans, respectively (22). The function of caspases is closely connected to initiation and execution of apoptosis, with caspases categorized into either initiator or effector caspases. CASP2, CASP8, CASP9 and CASP10 are initiator caspases, and CASP3, CASP6 and CASP7 are effector caspases (23). Known as interleukin 1B converting enzyme, CASP1 plays an important role in both apoptosis and inflammation (24). Apoptosis is regulated by stepwise activation of caspases for the processing or cleaving of other caspases (25). Therefore, caspases are proapoptotic proteins that are likely to serve as tumor suppressors, and their dysregulation is involved in the initiation and development of tumors.

Chicken ovarian cancer is an excellent animal disease model for the development, treatment, or prevention of human epithelial ovarian cancer. Thus, we have investigated the apoptotic status of chicken ovarian adenocarcinoma and then analyzed the expression pattern of antiapoptotic or pro-apoptotic genes. The expression level of \(\text{BID}\) was decreased, whereas the expression level of \(\text{CASP1}\) was increased in chicken ovarian cancer compared with levels in normal ovaries. Although the expression level of \(\text{CASP1}\) was increased to initiate apoptosis of cancer cells, it was impaired by the down-regulation of the proapoptotic gene \(\text{BID}\). This dysregulation of the apoptosis pathway is believed to be closely linked to the occurrence of chicken ovarian cancer. Our study has provided insight into the study of apoptotic status using a chicken ovarian cancer model and can be applied for the discovery of therapeutic agents or the prevention of human ovarian cancer.

**Materials and methods**

**General information.** The care and experimental use of White Leghorns were approved by the Institute of Laboratory Animal Resources at Seoul National University (SNU-070823-5). White Leghorns were maintained in a standard management program at the University Animal Farm of Seoul National University in Korea. The procedures used for animal management, reproduction and embryo manipulation followed the standard operating protocols of our laboratory.

**TUNEL assay.** Normal and cancerous overy were cryosectioned and fixed in a 4% paraformaldehyde solution for 20 min, and tissues were incubated in a permeabilization solution (0.1% Triton X-100 and 0.1% sodium citrate in PBS). Apoptotic cells were detected using the In Situ Cell Death Detection Kit with the fluorescein-labeled cell marker TMR red (Roche Applied Science, Penzberg, Germany). Then, the tissues were counterstained with DAPI, mounted and analyzed under a confocal laser microscope (Carl Zeiss, Oberkochen, Germany).

**RNA isolation and RT-PCR.** cDNA was synthesized from total RNA (3 μg) using the Superscript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA). The cDNA was serially diluted 10-fold and was quantitatively equalized for PCR amplification. PCR reactions had a total volume of 20 μl and contained 100 ng of genomic DNA, 2 μl 10X PCR buffer (CoreBio, Seoul, Korea), 1.6 μl of dNTPs (10 mM each), 2 pmol of each gene-specific primer (Table I), and 0.25 U of Taq polymerase (CoreBio). PCR conditions were 94˚C for 3 min, followed by 35 cycles at 94˚C for 30 sec, 60˚C for 40 sec and 72˚C for 1 min. PCR products were analyzed using a 1% agarose gel with ethidium bromide.

**Quantitative real-time RT-PCR.** To confirm mRNA expression levels of apoptosis-related genes in normal ovaries and ovarian cancers of chickens, quantitative RT-PCR was performed. Based on RT-PCR analysis of apoptosis-related genes, we selected genes and designed primers using Primer 3 software (26) and sequences from the GenBank database (Table II). Quantitative RT-PCR was performed using the Real-time PCR Detection System (Applied Biosystems, Foster City, CA) with SYBR® Green (Sigma, St. Louis, MO). The glyceraldehyde-3-phosphate dehydrogenase (\(\text{GAPDH}\)) gene was simultaneously run as a control and used for normalization. Each test sample was run in triplicate. Following the standard curve method, expression quantities of target genes were determined using standard curves and \(\text{Ct}\) values and were normalized using \(\text{GAPDH}\) expression quantities. PCR conditions were 94˚C for 3 min, followed by 40 cycles at 94˚C for 20 sec, 60˚C for 40 sec and 72˚C for 1 min using a melting curve program (increased temperature from 55 to 95˚C with a heating rate of 0.5˚C per 10 sec). Continuous fluorescence measurement and ROX dye (Invitrogen) was used as a negative control of fluorescence measurement, and sequence-specific products were identified by generating a melting curve. With the \(\text{Ct}\) value representing the cycle number at which a fluorescent signal rises statistically above background, relative quantification of gene expression was analyzed by the 2\(^{-\Delta\Delta\text{Ct}}\) method (27) and was normalized to the \(\text{Ct}\) of the normal ovary control group.

**Hybridization probes.** To determine the exact localization of apoptosis-related genes, expression of target genes was examined using in situ hybridization as previously described (28). The cDNA from chicken ovarian cancer tissues was amplified with the appropriate forward and reverse primers. The amplified fragment of apoptosis-related genes was loaded onto a 1% agarose gel and separated by electrophoresis at 5 V/cm for ~30 min. The DNA from each cancer-related gene...
was recovered with a Wizard SV Gel and PCR Clean-up System (Promega, WI, USA) and cloned into a pGEM-T plasmid vector (Promega). After verification of sequences, the recombinant plasmids for each sequence were amplified with T7- and SP6-specific primers (T7, 5’-TGTAATACGAC TCACTATAGGG-3’; SP6, 5’-CTATTTAGGTGACACTAT AGAAT-3’) to prepare the template for labeling cRNA probes. Digoxigenin (DIG)-labeled sense and antisense cRNA probes (promoted by T7 or SP6 RNA polymerase according to the cloning directions) were transcribed in vitro with a DIG RNA labeling kit (Roche).

In situ hybridization. Chicken ovarian cancer tissue was cut into 8- to 10-mm pieces and frozen in liquid nitrogen. Frozen sections (10 μm) were mounted on 3-aminopropyltriethoxysilane-pretreated slides (Sigma), dried on a 50°C slide warmer and fixed in freshly prepared 4% paraformaldehyde in 1X phosphate-buffered saline (PBS; 1.4 M NaCl, 27 mM KCl, 100 mM NaH2PO4 and 18 mM KH2PO4, pH 7.4) for 1 h at room temperature. The sections were washed twice in 1X PBS, treated in 1% Triton X-100 for 20 min, and washed three times in 1X PBS. The sections were incubated with a prehybridization mixture containing 50% formamide and 5X standard saline citrate (SSC; 150 mM NaCl and 15 mM sodium citrate, pH 7.0) for 15 min at room temperature. After prehybridization, the sections were incubated with a hybridization mixture containing 50% formamide, 5X SSC, 10% dextran sulfate sodium salt, 0.02% bovine serum albumin,
250 μg/ml yeast tRNA and denatured DIG-labeled sense or antisense cRNA probes for target gene for 18 h in a 55˚C incubation chamber. The sections were washed for stringency in a series of solutions containing formamide and SSC. After non-specific binding was blocked with a 1% blocking reagent (Roche) in buffer I (1 M Tris-HCl at pH 7.5 and 2.5 M NaCl), the sections were incubated overnight with sheep anti-DIG antibody conjugated to alkaline phosphatase (Roche). The signal was visualized with a visualization solution containing 0.4 mM 5-bromo-4-chloro-3-indolyl phosphate, 0.4 mM nitroblue tetrazolium and 2 mM levamisole (Sigma) in buffer III (1 M Tris-HCl at pH 9.5, 2.5 M NaCl and 0.5 M MgCl2). All sections were counterstained with 1% (w/v) methyl green (Sigma), and photographs were taken with a Zeiss Axiophot light microscope equipped with an Axiocam HR-camera (Carl Zeiss).

Statistical analysis. Difference in variance between normal and cancerous ovaries was analyzed using the F-test, and the difference of means was analyzed using the Student’s t-test. A p-value of <0.05 was considered statistically significant. Microsoft Office Excel was used for statistical analysis.

Results

Apoptotic status of chicken ovarian cancer. For pathological analysis of chicken ovarian cancer in hens >2 years of age, paraffin-embedded normal ovaries and cancerous ovaries were stained with hematoxylin and eosin (H&E). The general stromal region was observed in normal ovaries; small follicles were detected, and abnormal proliferated cells were not developed (Fig. 1A, B). The abnormal neoplasia region was observed in cancerous ovaries; many tubular structures were infiltrated into the stromal region (Fig. 1C, D). To investigate the apoptotic status of chicken ovarian cancer, a TUNEL assay for detecting DNA fragmentation via labeling the terminal end of nucleic acids was conducted. Stained cells near the epithelial region were present in normal ovaries, whereas many unstained tubular structures were present in cancerous ovaries (Fig. 2).

The expression pattern of apoptosis-related genes in normal and cancerous ovaries. To elucidate the difference in apoptotic status between normal and cancerous ovaries, the expression pattern of apoptosis-related genes was examined by RT-PCR (Fig. 3). We first investigated the expression of the BCL-2 family (BCL2, BCL2L1, MCL1, BCL2A1, BID and NOXA). In both normal and cancerous ovaries, we observed expression of BCL2, BCL2L1 and BID, significant weak expression of MCL1, and no expression of BCL2A1 and NOXA. The expression pattern of the caspase gene family was confirmed, with CASP1, CASP3, CASP6, CASP8 and CASP9 expressed in normal and cancerous ovaries. Both tissues showed weak expression of CASP7 and no expression of CASP2, CASP10 and CASP18.

Quantification of expression level of apoptosis-related genes. Using quantitative RT-PCR, the expression levels of apoptosis-related genes in normal and cancerous ovaries were investigated (Fig. 4). No significant difference was found between the expression levels of BCL2 (p=0.11) and BCL2L1 (p=0.16) in normal and cancerous ovaries. A dramatically decreased expression of BID was observed in cancerous ovaries compared with that in normal ovaries (p=0.0003). No significant
A difference in the expression levels of CASP3 (p=0.38), CASP6 (p=0.10) and CASP8 (p=0.16) was found between normal and cancerous ovaries, whereas increased expression of CASP1 (p=0.007) was observed in cancerous ovaries.

Localization of CASP mRNA in normal ovary and ovarian cancer. To determine where CASP1 is expressed, in situ hybridization using a CASP1-specific RNA probe was performed. CASP1 gene mRNA was not detected in normal
Apoptosis serves as a safety process by removing abnormally-controlled cells, such as DNA-damaged, abnormal-proliferated or dedifferentiated cells (13). Studying the apoptotic status dysregulation of cancer cells will provide meaningful findings for human cancer therapy. In this study, we investigated dysregulation of apoptosis in a chicken ovarian cancer model to enhance the value of the animal model for human epithelial ovarian cancer. Our results showed that few apoptotic cells were detected in chicken ovarian cancer in contrast to the apoptotic status in normal ovaries. To better understand apoptotic dysregulation in cancer, the expression pattern of antiapoptotic and proapoptotic genes were examined. Among the BCL2 gene family, BCL2, BCL2L1 and BID were expressed, and the expression of MCL-1 was weakly detected in both normal and cancerous ovaries. Among the CASP gene family, CASP1, CASP3, CASP6, CASP8 and CASP9 were expressed, and the expression of CASP7 was faintly observed in both normal and cancerous ovaries. Furthermore, the expression level of BID was decreased in chicken ovarian cancer compared with that in normal ovaries, and the expression level of CASP1 was greatly increased. CASP1 mRNA was also detected in chicken ovarian cancer cells with tubular structure.

Resistance to apoptosis is a hallmark of several human cancers, including ovarian cancer, and is closely related to chemotherapy resistance (12). Although CASP1 is well known as one of the cytokine-processing caspases for producing IL-18 and IL-18, it also plays a major role in apoptosis and is linked to certain pathologic conditions (29,30). The down-regulation of CASP1 is particularly associated with the occurrence of human ovarian cancer, with overexpression of CASP1 triggering apoptosis (24). However, our results revealed overexpression of CASP1 in cancerous ovaries of chickens compared with normal ovaries and detected CASP1 mRNA in cancerous ovaries. This may be due to an association between CASP1 overexpression and endogenous stimuli for obstruction of tumor progression. INF-γ prohibits growth of human pancreatic carcinoma cells through the up-regulation and activation of CASP1 (31), and treatment of tumor necrosis factor-α (TNF-α) in A549 cells increases mRNA levels of CASP1, resulting in the stimulation of apoptosis (32). It is assumed that the overexpression of CASP1 by external signals is an endogenous response to block cancer progression.

Another possibility is that the up-regulation of CASP1 does not induce apoptosis in cancerous ovarian cells, with this status closely connected to modified BID expression levels. Our results indicated decreased expression of BID in cancerous chicken ovaries compared with normal ovaries. Generally, the activation of the RIP2/CASP1 pathway is converged to the cleavage of BID, an intrinsic apoptotic pathway, which subsequently triggers activation of CASP9 and CASP3 to induce apoptosis (29). Our results support the hypothesis that despite CASP1 overexpression to induce apoptosis of transformed cells, a successive intrinsic apoptotic pathway cannot progress due to down-regulation of BID. Considered to be the origin of epithelial ovarian carcinomas, ovarian surface epithelial cells (OSE) greatly express CASP1, resulting in the production of proinflammatory cytokines such as IL-1 and IL-18 (33,34). In light of induction of apoptosis by CASP1, CASP1 may also contribute to the removal of damaged cells generated by continuous ovulation. Likewise, CASP1 is highly expressed in CaOV3 human ovarian cancer cells, and CASP1-dependent apoptosis is dysregulated (24). It is hypothesized that the dysregulation of the intrinsic apoptosis pathway, which includes BID cleavage stimulated by CASP1, is subsequently able to survive damaged cells and could increase the risk of cancer incidence.

In conclusion, we have shown that CASP1 is overexpressed and CASP1 mRNA is detected in cancerous ovarian tissue of chickens in vivo. Also, BID acts as an intrinsic apoptosis
A regulator that is down-regulated in chicken ovarian cancer. Through this study, we have provided a new application of the apoptotic status of chicken ovarian cancer as an excellent model of human epithelial ovarian cancer. Through the combination of our transgenic techniques in chickens (35), our findings will be useful in addressing the incidence of cancer due to dysregulated apoptosis. In conclusion, our study provides new insight into the development of therapeutic agents based on the apoptotic pathway in human ovarian cancer.

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