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Localization of apoptotic and proliferating cells and mRNA expression of caspases and Bcl-2 in gonads of chicken embryos

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ABSTRACT

The aim of the present study was to analyze participation of apoptosis and proliferation in gonadal development in the chicken embryo by: (1) localization of apoptotic (TUNEL) and proliferating (PCNA immunoassay) cells in male and female gonads and (2) examination of mRNA expression (RT-PCR) of caspase-3, caspase-6 and Bcl-2 in the ovary and testis during the second half of embryogenesis and in newly hatched chickens. Apoptotic cells were found in gonads of both sexes. At E18 the percentage of apoptotic cells (the apoptotic index, AI) in the ovarian medulla and the testis was lower ($p < 0.05$) than in the ovarian cortex. In the ovarian medulla, the AI at E18 was lower ($p < 0.05$) than on E12. In the testis, the AI was significantly lower ($p < 0.05$) at E18 than at E15 and 1D. The percentage of proliferating cells (the proliferation index: PI) within the ovary significantly increased from E15 to 1D in the cortex, while proliferating cells in the medulla were detected only at E15. In the testis, the PI gradually increased from E12 to 1D. The mRNA expression of caspase-3 and -6 as well as Bcl-2 was detected in male and female gonads at days 12 (E12), 15 (E15) and 18 (E18) of embryogenesis and the day after hatching (1D). The expression of all analyzed genes on E12 was significantly higher ($p < 0.05$) in female than in male gonads. This difference was also observed at E15 and E18, but only for the caspase-6. The results obtained showed tissue- and sex-dependent differences in the number of apoptotic and proliferating cells as well as mRNA expression of caspase-3, -6 and Bcl-2 genes in the gonads of chicken embryos. Significant increase in the number of proliferating cells in the ovarian cortex and lack of these cells in the ovarian medulla (stages E12, E18, 1D) simultaneous with decrease in the intensity of apoptosis only in the medulla indicates that proliferation is the dominant process involved in the cortical development, which constitutes the majority of the functional structure of the fully developed ovary. No pronounced changes in the expression of apoptosis-related genes found during embryogenesis suggest that they cannot be considered as important indicators of gonad development. The molecular mechanisms of the regulation of balance between apoptosis and proliferation in developing avian gonads need to be further investigated.

Introduction

The gonadal sex in chicken embryos is morphologically indistinguishable and bipotential up to day 6 of incubation (E6) (Romanoff, 1960). By embryonic days 8–10 (E8–E10), the gonads differentiate enough to be morphologically recognized. Gonads of ZW heterozygotes develop as asymmetric ovaries, while those of ZZ homozygotes develop as symmetric testes. The embryogenic growth and development makes the left ovary 2.4 times longer than the right ovary in newly hatched chicks. Starting at E7, the ovarian cords of the left ovary develop to form a cortex, whereas the medulla is composed of epithelium derived from medullary cords (primary cords). The right ovary ceases further developed from day 7 onwards and finally the right ovary is composed only of a medulla (Ukeshima and Fujimoto, 1991; Smith and Sinclair, 2004).

In the female, the hormonally regulated loss of germ cells at various stages of pre- and postnatal development is a normal physiological process ensuring the greatest chance for ovulation of viable, fertilizable oocytes (Johnson and Woods, 2007). In male vertebrates, 75% of testicular cells are discarded *via* apoptosis during embryogenesis as well as in adults (Hikim et al., 2003).

Caspases, key regulators of apoptosis, are a family of intracellular cysteine proteases linked both to the initial and final stages of apoptosis. Six caspase orthologs have been characterized in birds (chicken caspase-1, -2, -3, -6, -8 and -9), and all are expressed in the ovary (Johnson et al., 1997, 1998; Johnson and Bridgham, 2000; Hrabia and Jankoś, 2011). Two general pathways involving initiator caspases have been identified. One upstream pathway, promoted

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by the activation of several members of the tumor necrosis factor receptor (TNF-R) family, results in the processing of the initiator caspase-8 and/or caspase-10. Fas-induced cell death is mediated by the recruitment and activation of caspase-8 *via* Fas-associated death domain (FADD) (Strasser and Newton, 1999). The alternative pathway involving an initiator caspase occurs after a perturbation of the mitochondria, which promotes the release of mitochondrial cytochrome C (Ekert et al., 2001). Cytosolic cytochrome C mediates the formation of the apoptosome complex, a high molecular weight complex consisting of Apaf-1 and caspase-9. Autoactivation of either caspase-8 or caspase-9 eventually initiates the processing of an effector caspase (-3, -6 or -7), and the full potentiation of the caspase cascade. The caspase activation is regulated by a variety of cellular pro- and antiapoptotic proteins, including members of the Bcl-2-related family. Anti-apoptotic members of the Bcl-2 family, such as Bcl-2, form ion channels within the mitochondrial membrane, and help maintain mitochondrial integrity by allowing the export of H^+ ions from the inner mitochondrial space. Bcl-2 family members are involved in the regulation of oocyte and early embryo survival (Boumela et al., 2011) and in spermatogenesis (Vilagrasa et al., 1997).

Both from the perspective of improving gamete production in infertile individuals and treatment of testicular disorders, study of ovary and testis development is of great relevance for the improvement of reproductive health. Building a knowledge base on the molecular components of the apoptotic programme in ovarian and spermatogenic cells is an essential step towards the development of novel therapeutic regimens to treatment of germ cell tumors and infertility. Numerous studies on cell death in embryonic chicks have been mostly focused on individual organ systems including limb buds (Hurle et al., 1995; Lee et al., 1999), heart (Pexieder, 1975) sclerotome (Sanders, 1997), tail bud (Schoenwolf, 1981; Miller and Briglin, 1996) or neural crest (Jeffs et al., 1992; Homma et al., 1994; Lawson et al., 1999). Moreover, TUNEL-positive cells have been detected in chickens as early as 3 h after the onset of incubation (Hirata and Hall, 2000). Surprisingly, despite numerous studies concerning the differentiation and development of gonads in the chicken, participation of apoptosis and proliferation in these processes has not yet been elucidated. Therefore, in this study the pattern of apoptosis and proliferation was examined in the chicken gonads during the second half of embryogenesis and after hatching. Since many specific genes are involved in these events, the mRNA expressions of selected pro- and anti-apoptotic molecules were additionally determined.

Materials and methods

The experiment was carried out in accordance with the principles and procedures of the Local Animal Ethics Committee in Krakow. Fertilized eggs ($n = 180$) of the Hy-Line Brown strain were set in an incubator (Masalles 65 Digital Incubator) and were incubated under standard conditions [1–18 days of incubation: $t = 37.8\,^{\circ}C$, relative humidity (RH) = 55%; 19–21 days of incubation: $t = 37.2\,^{\circ}C$, RH = 70%]. They were candled on day 5 of the incubation to eliminate unfertilized eggs and dead embryos. The embryos were decapitated at E12, E15, E18 and just after hatching (1D). After sexing, the left ovary and testes were collected for immunohistochemical analyses: TdT-mediated dUTP nick-end labeling (TUNEL) and proliferating cell nuclear antigen (PCNA) immunoassay ($n = 6$ ovary or testis in each age group), and for RNA isolation followed by reverse transcription and polymerase chain reaction (RT-PCR) ($n = 6$ ovaries or testis in each age group). In the histochemical methods, the ovarian cortex and ovarian medulla were analyzed separately.

TUNEL assay

For evaluation of apoptosis, chicken gonads were fixed in 4% formaldehyde in phosphate-buffered saline (pH 7.4) and embedded in paraffin wax by routine procedure. Deparaffinized and rehydrated 6 μm-thick sections of gonadal tissues were incubated with proteinase K (20 μg ml^{-1}) in 10 mM Tris–HCl, pH 7.4 at 37 °C for 20 min. Apoptotic cells were detected by TUNEL (Gavrieli et al., 1992) using an *in situ* cell death detection kit, POD according to manufacturer's instructions (Roche Diagnostics GmbH, Mannheim, Germany). Negative controls were prepared by incubation without terminal deoxynucleotidyl transferase. To visualize the immunoreaction products, sections were incubated with a DAB and H_2O_2 mixture for approximately 2 min. Slides were examined under a light microscope (Jena Zeiss, Jena, Germany). Apoptotic cells (TUNEL-positive) were counted with a computerized image analysis system (MultiScanBase v. 14.02, Computer Scanning System, Warsaw, Poland) on 10–15 random areas of each left ovary (medulla or cortex) or testis and averaged for each embryo. The resulting value (the apoptotic index: AI) was shown as the number of TUNEL positive cells per 100 cells counted. The mean value was calculated from six embryos.

Immunostaining for PCNA

Microtome sections (6 μm thick) of gonads prepared as described above were deparaffinized in xylene, rehydrated by passing through graded alcohols, rinsed in water and treated with 0.5% H_2O_2 in methanol to block endogenous peroxidase activity. After washing in water, slides were heated in a citric buffer (pH 6.0, 75 °C, 20 min) and non-specific binding of the secondary antibody was blocked by incubation with 5% (v/v) normal goat serum in TBST buffer (Tris buffer saline + 0.1% Tween 20, RT, 10 min). Sections were then incubated (60 min) with specific mouse monoclonal antibody against PCNA (a marker of S phase; dilution 1:150) followed by washing with TBS and incubation with biotinylated goat anti-mouse antibody (35 min, dilution 1:300) and the avidin–biotin–peroxidase complex StreptABComplex/HPR (Dako-Cytomation, Glostrup, Denmark; 30 min). The color reaction was developed by incubation with diaminobenzidine (DAB) and H_2O_2 solution. Control slides were prepared by replacement of the primary antibody with normal mouse serum or TBST. Slides were examined and proliferating cells (PCNA-positive) were counted in the same way as apoptotic cells. The resulting value (the proliferation index: PI) was shown as the number of PCNA-positive cells per 100 cells counted. The mean value was calculated from six embryos.

RT-PCR

The collected tissues were snap frozen and kept in liquid nitrogen until RNA extraction. The total RNA was isolated (Chomczynski and Sacchi, 1987) by using TRI-Reagent (Molecular Research Center, Cincinnati, OH, USA). The tissues were homogenized with UltraTurrax T25 (IKA-Labortechnik, Staufen, Germany), and phase separation was made with bromochloropropane. RNA was precipitated from the aqueous phase by mixing with isopropanol, washing with 75% ethanol and dissolving in pure RNAase-free water (Promega, Madison, WI, USA). The concentration of RNA was measured spectrophotometrically using a Biophotometer (Eppendorf, Hamburg, Germany). Each sample was tested for RNA degradation on agarose gel and spectrophotometry. The 260/280 nm ratio of RNA was 1.65–1.85. To exclude genomic DNA contamination, RNA samples were treated with DNAse (RQ1 RNase-Free DNase, Promega). RT was performed in 20 μl volume. The reaction mixture contained 5 μg of total RNA, 200 U M-MuLV reverse transcriptase