The developing human ovary: immunohistochemical analysis of germ-cell-specific VASA protein, BCL-2/BAX expression balance and apoptosis

Mirta S. Albamonte, Miguel A. Willis, María I. Albamonte, Federico Jensen, María B. Espinosa and Alfredo D. Vitullo

Departamento de Estudios Biomédicos y Biotecnológicos, Centro de Estudios Biomédicos, Biotecnológicos, Ambientales y Diagnóstico, CEBBAD, Universidad Maimonides, Hidalgo 775, C1405BCK Buenos Aires, Argentina

1Correspondence address. E-mail: vitullo.alfredo@maimonides.edu

BACKGROUND: Germ cell number during ovarian organogenesis is regulated through programmed cell death. We investigated the expression of germ-cell-specific VASA protein, apoptosis-related proteins BAX and BCL-2 and DNA fragmentation in developing human ovaries from gestation week 12 to term. METHODS: Human fetal ovaries from 13 women undergoing spontaneous abortion were fixed, paraffin-embedded and processed for immunohistochemistry to analyse temporal and cellular localization of VASA, BCL-2 and BAX, and to detect apoptosis by TUNEL assay. RESULTS: VASA showed a differential pattern of expression throughout the differentiation and proliferative phase and prophase I to finally associate with Balbiani’s body in primordial and primary follicles. BCL-2 was detected from week 12 to 17 and became undetectable thereafter. Strong BAX signal was detected in oogonia and oocytes from week 12 to term. Low levels (<10%) of TUNEL positive germ cells were detectable throughout gestation with a higher incidence (around 20%) at 18–20 weeks. CONCLUSIONS: VASA was specifically expressed in germ cells and displayed a stage-specific intracellular localization enabling one to follow oogenesis throughout gestation. Apoptosis-inhibiting BCL-2 was associated with the germ cell proliferative phase and prophase I, whereas BAX remained positive throughout gestation. The highest incidence of apoptotic germ cells was coincident with the lack of detectable BCL-2 protein, and when primordial follicle formation became widespread.

Keywords: human fetal ovary; female germ cells; apoptosis; VASA; BCL-2/BAX

Introduction

Primordial germ cells (PGC) migrate early during mammalian fetal life to colonize the genital ridges where they undergo an extensive mitotic proliferation giving rise to a large number of oogonia, and finally entering meiosis to produce by birth a pool of oocytes arrested at the diplotene stage of the first meiotic division (Hirschfield, 1991). As meiosis progresses, somatic cells surround the oocytes to form primordial follicles and thereafter folliculogenesis dominates the formation of germinal tissue (Hirschfield, 1991). Once PGCs differentiate to enter meiosis and then folliculogenesis, a degenerative process classically referred to as atresia will continuously clear them (Kaipia and Hsueh, 1997). In the rat, germ cell number decreases by two-thirds from the onset of meiosis to 48 h after birth (Hirschfield, 1991). In mice, 66% of the original oogonia are decimated by the end of gestation (Flaws et al., 2001). In humans, the process of oocyte loss is particularly intensive; from $7 \times 10^6$ potential oocytes in a 20-week-old fetus, around 85% are already lost by birth, and >99% will be lost by the beginning of puberty (Baker, 1963; Forabosco et al., 1991). This massive, constitutive germ cell death depends on the genetic machinery of apoptosis, programmed cell death (PCD) (Hsueh et al., 1996; Tilly, 1996; Tilly et al., 1997). The majority of studies of ovarian germ cell demise in mammals have shown that the expression of BCL-2, BCL-X and BAX genes plays an essential role (Tilly, 2001). The balance between apoptosis-inducing BAX and BCL-X, and apoptosis-inhibiting BCL-2 and BCL-Xl proteins determines death or survival of the germ cell (Tilly, 1996; Boise et al., 1993; Oltvai et al., 1993). As a general rule, it has been observed that expression of BAX is normally enhanced in the mammalian ovary, whereas BCL-2 protein is found at low levels or undetected at all (Kim and Tilly, 2004). This observation gives support to the high rate of PCD characterizing the mammalian ovary, and especially the human ovary.

The causes that determine massive constitutive death of mammalian female germ cells are poorly understood. This massive elimination may avoid the persistence in the ovary
of germ cells exhibiting nuclear or mitochondrial chromosomal/genetics defects (Tilly, 2001). Alternatively, death may relate to the exhaustion of germ cells acting as nurse cells to the surviving oocyte pool (Pepling and Spradling, 2001). Finally, it has been suggested that massive death may enable the appropriate association between germ cells and pregranulosa cells during ovigerous cords or ovarian cysts breakdown, just before primordial follicles begin to form (Guigon and Magre, 2006). In any case, the balance between germ cell death and survival seems to be critical to preclude ovarian dysgenesis or premature ovarian failure and to ensure reproductive success.

Most of our understanding of the processes acting during prenatal conformation of the mammalian female germinal tissue comes from studies in rat and mouse. In humans, a few contributions on ovarian apoptosis have been made (De Pol et al., 1997, 1998; Quenby et al., 1999; Vaskivuo et al., 2001; Abir et al., 2002; Hartley et al., 2002; Modi et al., 2003; Fulton et al., 2005) due to the scarcity of fetal germinal tissue. Most of the reports have concentrated on mitotic proliferation and prophase I periods (De Pol et al., 1997, 1998; Hartley et al., 2002; Modi et al., 2003; Fulton et al., 2005), and only one has spanned a wider gestation period including proliferative stage, prophase I and primordial follicle formation (Vaskivuo et al., 2001). Consequently, a large systematic analysis of the apoptosis-dependent germ cell degeneration from initial proliferation to early folliculogenesis throughout gestation is still lacking. Although the results so far published for human ovarian apoptosis yielded some discrepancies, several important features of fetal germ cell attrition in humans came from these studies. First, the apoptotic activity in the human fetal ovary is restricted to the germ cells proper, both oogonia and oocytes, rather than to pregranulosa/granulosa cells (Vaskivuo et al., 2001), a situation that will be reversed in the adult ovary in which apoptosis involves primarily the granulosa cells (Billig et al., 1996; Albamonte et al., 2005), especially during the final maturation of antral follicles (Hurst et al., 2006). Second, major apoptosis activity, as detected by TUNEL assay in different reports (Vaskivuo et al., 2001; Hartley et al., 2002) during ovarian genesis, is coincident with mitotic proliferation of oogonia and the entrance to meiosis occurring from 14 to 18–20 gestation weeks. Finally, the level of apoptosis increases notably in the ovaries of fetuses with chromosomal abnormalities. As shown by Modi et al. (2003) in their analysis of germ cell demise, sex-chromosome aneuploid fetal human ovaries had increased levels of apoptosis. This is an observation of particular interest since, in many cases, materials available for analysis come from abortions performed because of fetal malformations and/or chromosomal abnormalities (Abir et al., 2002).

In order to further investigate the dynamics of massive constitutive germ cell degeneration in the developing human ovary, we undertook an extensive study on the distribution of the specific germ cell marker VASA, on the evaluation of apoptotic germ cells by a DNA in situ labelling assay, and on the balance of expression of BCL-2 and BAX genes in normal fetal ovarian tissue spanning the period between 12 and 38 gestational weeks; this includes the transition from oogonial mitotic proliferation to the entrance to meiosis and primordial follicle formation.

Materials and Methods

Ovarian material

This study was reviewed and approved by the Research Ethics Committee of Universidad Maimonides. Human fetal ovaries were collected from spontaneous abortions at the Hospital Municipal de Merlo, Buenos Aires province, Argentina, after obtaining the patients consent. The age of fetuses was estimated from the date of the last menstrual period and the crown-heel length, and foot length at autopsy. All fetuses appeared morphologically normal. A total of 13 ovaries corresponding to 12 (n = 1), 16 (n = 1), 17 (n = 1), 18 (n = 3), 20 (n = 1), 22 (n = 1), 27 (n = 1), 28 (n = 1), 33 (n = 1), 34 (n = 1) and 38 (n = 1) gestation weeks were analysed. Fetuses in which death was suspected to have recently occurred before autopsy, with no sign of necrosis, were used. Ovarian tissues were recovered in the surgery room and immediately fixed in 10% buffered formalin. After 24 h fixation, samples were processed for routine paraffin embedding. Embedded samples were serially sectioned at 5 μm, mounted onto cleaned, coated slides and stored at room temperature until used. All samples were examined for tissue integrity and general histology by haematoxylin–eosine staining.

Immunofluorescence analysis of VASA expression

To assess VASA expression, samples were blocked in 10% bovine fetal serum (BFS)/15% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 h at room temperature, thoroughly rinsed in PBS and incubated with polyclonal rabbit anti-Mvh (mouse vasa homologue) primary antibodies kindly provided by Dr Noce (Mitsubishi Kagaku Institute of Life Sciences, Tokyo, Japan) overnight at 4°C. Although the primary antibody has been raised against mouse Vasa protein, cross-reaction with human VASA protein can be anticipated since the level of protein homology between species is >90% (Castrillon et al., 2000), especially for the epitopes against which antibodies were raised. After overnight incubation, slides were appropriately rinsed in PBS and incubated with goat anti-rabbit IgG conjugated with FITC (Santa Cruz Biotechnology, CA, USA) 45–60 min at room temperature. All slides were counterstained with 1 μg/ml propidium iodide. Negative controls were performed by omitting the primary antibody. Samples were examined in an Olympus BX40 microscope by conventional epifluorescence with ultraviolet illumination and images were captured with an Olympus Camedia C-5060 camera.

Immunohistochemical detection of BCL-2/BAX expression

Dewaxed and re-hydrated sections were incubated with rabbit polyclonal anti-BAX or anti-BCL-2 (Santa Cruz Biotechnology) primary antibodies overnight at 4°C. Double-stainings were performed by using EnVision® Doublestain System (Dako Cytomation, USA) according to the recommendations of the manufacturer. Immunoenzymatic reactions were performed with horse-radish peroxidase (HRP) or alkaline phosphatase labelled polymers, revealed with 3,3′-diaminobenzidine (DAB) and fast-red for BAX and BCL-2, respectively. All slides were counterstained with haematoxylin. Negative controls were performed by omitting the primary antibodies.

TUNEL assay

Detection of DNA fragmentation was performed in paraffin-embedded sections by terminal deoxynucleotidyl transferase (TdT)-mediated
deoxyuridine triphosphate (dUTP) nick end labelling technique, using the ‘In Situ Cell Death Detection Kit’ (Roche Diagnostics, Germany) with fluorescein tagged nucleotides. Procedure followed the suppliers’ recommendations. Treated sections were examined in an Olympus BX40 microscope by conventional epifluorescence with ultraviolet illumination. In order to confirm negative results, TUNEL-processed sections were incubated with 10 IU/ml DNase II (Sigma Chemical Co., USA) in 50 mM Tris–HCl pH 7.5, 10 mM MgCl2 and 1 mg/ml BSA for 10 min at room temperature. After incubation, slides were thoroughly rinsed and treated again according to the TUNEL protocol. Images were captured with an Olympus Camedia C-5060 camera.

Semi-quantification of expression levels
In order to assess trends in the expression of BCL-2, BAX and VASA, cells showing a positive signal were counted using an Olympus AX-0049, OC-M, 10/10 × 10 mm counting frame. Six frames were entirely counted per section in all samples (magnification ×200) in order to have comparable areas of homogeneous cortical tissue for each time-point. Counting was performed in the cortical region of the ovary by two independent observers, and the results were expressed as a mean value of absolute number for each time-point.

Results
VASA expression in the developing human ovary
VASA immunolabelling was detectable in the cytoplasm of germ cells, both oogonia and oocytes, at all gestational ages analysed. The immunolabelling pattern of VASA expression in the earliest sample (gestation week 12) was faint and homogeneously distributed within the germ cell cytoplasm (Fig. 1A). At weeks 16 and 18, the signal intensity increased progressively, became brighter and punctuated, and was localized mainly as a perinuclear ring (Fig. 1B and C). From week 20 to the end of gestation, VASA showed the strongest signal observed, with a clear para-nuclear localization, corresponding to the Balbiani’s vitelline space, in oocytes lying in primordial follicles (Fig. 1D). This was also the case for primary follicles found at week 38.

During the mitotic proliferative stage, week 12, most germ cells in any visual field were positive for VASA although the signal was weak (see above). As germ cells progressed to meiosis and finally to primordial follicle formation, absolute numbers of VASA positive cells decreased but remained easily detectable at any time-point.

In all gestational ages analysed, ovarian sections incubated in the absence of primary antibodies exhibited no detectable immunolabelling.

BCL-2/BAX expression balance
At gestational week 12, the majority of cells (around 95%), morphologically identified as oogonia, were labelled with anti-BCL-2 antibody (Figs. 2 and 3A). However, this number dropped to around 50% by week 16, <10% by week 17, and BCL-2 immunolabelling was no longer detectable afterwards (Figs 2 and 3B and C). It is interesting to note that the lack of detectable BCL-2 was coincident with the appearance of primordial follicles and was maintained from 20 to 38 week. In all cases, the BCL-2 signal localized to the germ cell cytoplasm; it is worthwhile, however, to mention that immunolabelling in somatic cells was also detected.

The pattern of BAX expression was quite different to that of BCL-2. Immunolabelling with anti-BAX antibodies remained positive throughout the entire gestation period, both in oogonia and oocytes, and during primordial follicle formation (Fig. 4). Positive immunostaining for BAX was stronger than that found for BCL-2 (compare Figs 3 and 4) although the treatments followed in both cases were the same. In order to confirm that differences in signal intensity were not related to the enzymatic reaction employed in each case, we reversed in some sections the use of DAB or fast-red for revealing immunoenzymatic reactions. Irrespective of this, signals were always stronger for BAX than BCL-2. BAX immunostaining was widespread and pregranulosa and granulosa cells were found to be positive for BAX.

Figure 1: Immunodetection of the specific germ cell marker VASA in fetal human ovaries.
(A) VASA is detected as a faint cytoplasmic staining in oogonia at gestation week 12, (B) the signal becomes stronger by gestation week 16 and (C) localizes mainly as a brightly and punctuated perinuclear ring by week 18. (D) With the development of primordial follicles, VASA localizes in a half-moon area with a para-nuclear position corresponding to the Balbiani’s vitelline space. Magnification: ×1000.

Figure 2: Absolute number of germ cells showing positive staining for BCL-2 and BAX proteins in the developing human ovary from gestation week 12 to term. BAX remains positive throughout prenatal oogenesis, whereas BCL-2 is specially associated with differentiation and proliferation of germ cells.
Plotting the results of semi-quantitative assessment of BCL-2 and BAX clearly showed that the expression of BCL-2 was specially associated with germ cell differentiation and proliferation, from gestation week 12 to 18, whereas BAX remained detectable throughout gestation including differentiation, proliferation, entrance into meiosis and follicle formation (Fig. 2). The majority (around 95%) of round celled oogonia, with a chromatin-homogeneous central nucleus, and early oocytes showing a heterogeneous chromatin distribution indicative of variable degrees of chromosome condensation as they progress through prophase I were found positive for BCL-2 protein at week 12, dropping to 50% by week 16 and 1% at week 18, a situation also reflected by absolute numbers (Fig. 2). Occasional primordial follicles, easily distinguishable by a layer of flattened somatic cells surrounding the oocyte, were also found positive for those time-points. BAX protein was expressed throughout gestation associated with the three stages of germ cell development described above, e.g. oogonia, early oocytes and primordial follicle-enclosed oocytes. Although the absolute number reflects a decrease from week 18 to term, the percent proportion of BAX positive cells remained constant, just fluctuating between 95% and 98% of the cells from week 12 to 38. It has to be taken into account that germ cells increase in size with maturity and the decrease in absolute number reflects this fact.

DNA fragmentation in germ cells

Samples from all of the gestation time-points analysed showed positive cells for TUNEL reaction both in oogonia and oocytes (Fig. 5). In all cases, no >10% of the cells showed a positive reaction except for samples between weeks 18 and 20, in which the extent of apoptosis attained a 20% detection level. TUNEL assayed sections were incubated with DNase in order to confirm DNA integrity in negative cells. After
enzymatic treatment all nuclei became positive (Fig. 5C). Negative controls performed by omitting TdT showed no positive reaction.

Discussion

During the period analysed in this study, germ cells in the developing human ovary progress in oogenesis through mitotic proliferation of PGC, enter into meiotic prophase I and finally associate with somatic cells to form primordial follicles. All those essential changes in ovarian organogenesis were reflected by the stage-specific immunodetection pattern displayed by VASA protein. In agreement with previous observations (Castrillon et al., 2000; Stoop et al., 2005), signal intensity was stronger as germ cells progressed from oogonia to oocytes, and cellular localization changed from widespread cytoplasmic distribution to a restricted aggregation in Balbiani’s vitelline space, in primordial-follicle enclosed oocytes. Balbiani’s body (Balbiani, 1864) is a cytoplasmic, para-nuclear complex composed of mitochondria, endoplasmic reticulum and lamellar aggregates (Hertig and Adams, 1967). The association of VASA with Balbiani’s body is intriguing; however, its presence has been considered as a distinct trait in fetal oocytes since it was not observed in adult ovaries (Castrillon et al., 2000). During the third trimester, we found Balbiani’s-associated VASA signal as a dominant feature of rodent L. maximaus in which over-expression of BCL-2 precludes germ cell attrition and oocyte degeneration is <10% (Jensen et al., 2005). It is tempting to speculate that the association to Balbiani’s body may relate to the final fate of the oocyte, especially considering that VASA is a RNA-binding protein belonging to the DEAD box family, involved in germ cell survival before primordial follicles appear in the developing ovary. Interestingly, Hartley et al. (2002) have reported that another member of the BCL-2 family, the anti-apoptotic Mcl-1 protein, is strongly expressed in germ cells from gestation week 18, and plays a central role in inhibiting cell death at the time of germ cell—somatic cell association during primordial follicle formation. Both BCL-2 and Mcl-1 are known to interact with proapoptotic BCL-2 members, such as BAX, to form heterodimers and determine cell survival (Hsu et al., 1998). Thus, it seems that different apoptosis-inhibiting BCL-2 members act in a concerted temporal way during prenatal oogenesis and folliculogenesis in the developing human ovary, to regulate the default pathway commitment germ cells to death.

Unlike BCL-2 protein which showed a time-restricted expression pattern, apoptosis-inducing BAX protein displayed conspicuous signals from week 12 to term associated with oogonia, primordial follicle-enclosed oocytes, and pregranulosa and granulosa cells. This result is comparable with previous reports showing widespread immunolocalization of BAX throughout proliferation, meiotic prophase and primordial follicle formation in the developing human ovary (Vaskivuo et al., 2001; Hartley et al., 2002). It is worthwhile to note that throughout the period studied, BAX signal was strong, in contrast with the slight staining found in ovaries expressing BCL-2. Repetition of staining on different days and using different enzymatic reactions for revealing BAX protein confirmed that the staining was reproducible. Thus, BAX is found throughout gestation displaying a strong and stable pattern of immunostaining in germ cells. It is clear that BAX is a central player in determining germ cell death in the human ovary. The widespread expression pattern found for apoptosis-inducing BAX in the developing human ovary is commensurate with the levels of massive germ cell degeneration occurring throughout ovarian genesis, affecting 85% of the original pool of oogonia by the end of gestation (Baker, 1963; Gondos et al., 1986; Forabosco et al., 1991). Nevertheless, detection of BAX by immunohistochemistry not necessarily indicates that apoptosis is active since BAX activity is dependent on intracellular localization (Hsu et al., 1997; Zamzami et al., 1998), and other anti-apoptotic Bcl-2 members, like Bcl-2 or Mcl-1 (Hsu et al., 1998), may be interacting with Bax to inhibiting its functional proapoptotic effect. Studies performed mainly in rat and mice have established that BCL-2 family members are essential players in activation or repression of apoptosis in the mammalian ovary (Kim and Tilly, 2004). In particular, BAX and BCL-2 genes function as a rheostat determining cell death or survival depending on the levels of each protein (Tilly, 2001; Tilly et al., 1997). In fact, proapoptotic BAX seems to be up-regulated by default in the mammalian ovary, being responsible for massive germ cell degeneration by attrition of fetal germ cells and apoptosis-dependent follicular atresia in the adult (Kim and Tilly, 2004). Experimental studies in the rat have shown that reducing the
level of BAX expression increases granulosa cell survival and diminishes follicular atresia, even in the absence of any change in BCL-2 level (Tilly et al., 1995). On the other hand, increased BAX expression is responsible for granulosa cell demise and follicular atresia in the rat (Tilly et al., 1995), human (Kugu et al., 1997), monkey (Uma et al., 2003) and quail (Van Nassauw et al., 1999) ovary. Recently, it has been shown in a caviomorph rodent that natural over-expression of BCL-2 in the ovary, accompanied by a down-regulation of BAX, promotes continuous folliculogenesis with suppressed apoptosis-dependent follicular atresia and reduced degeneration of germ cell in fetal life (Jensen et al., 2005, 2006). The results presented here and previous reports (Quenby et al., 1999; Vaskivuo et al., 2001; Abir et al., 2002; Hartley et al., 2002; Fulton et al., 2005) indicate that Bcl-2 family members are involved in oocyte degeneration in the human developing ovary acting in a concerted way during mitotic proliferation, entrance to meiosis and primordial follicle formation, with Bax being a central player.

The levels of apoptosis revealed by TUNEL did not correlate with the high incidence of germ cell attrition reported in classical studies (Baker, 1963; Gondos et al., 1986; Forabosco et al., 1991). The prevalence of TUNEL positive cells from week 12 to term was low, <10%, except for samples belonging to gestation weeks 18–20 in which we recorded around 20% apoptotic nuclei. The increase in apoptosis level at 18–20 weeks was coincident with the lack of detectable anti-apoptotic BCL-2. This may well reflect degeneration of oocytes in which apoptosis started earlier in oogenesis since it is not possible to relate the positive stain to the actual time it takes to disappear a dying cell from the tissue, and DNA degradation is a late event in the sequence of cell death (Collins et al., 1997). Similarly, an increased incidence of apoptosis reaching 14–17% has been shown by others to take place between weeks 14 and 20, and <10% apoptotic cells are found at week 13 and >22 gestation weeks (Vaskivuo et al., 2001). In other reports, the levels of apoptosis revealed by TUNEL were <5% for gestation periods spanning 19–33 weeks (Abir et al., 2002) or 13–18 weeks (Hartley et al., 2002). In agreement with our results, an analysis of cleaved caspase-3 immunodetection has shown that an increase in apoptosis takes place preceding primordial follicle formation (Fulton et al., 2005). Thus, it seems that a critical increase in apoptosis occurs before the developing ovary reaches the primordial follicle formation stage. Classical studies have shown that progression from mitosis to meiotic prophase I spans from weeks 11 to 20 as overlapped processes in the human ovary (Kurilo, 1981), and it is believed that cell death increases from these periods to reach a final 85% oocyte degeneration at the time of birth (Baker, 1963; Gondos et al., 1986). Nevertheless, the apoptosis incidence reported in this study and by others is far too low to explain this intensive level of cell death reported in classical studies. An incidence of 10–20% TUNEL positive cells is close to the level of oocyte attrition at week 20 recorded in classical studies (Baker, 1963; Baker and Franchi, 1967), but from this time-point to term no increase in apoptosis was detected. It is worthwhile to consider that other mechanisms may be at work in the developing human ovary. For instance, it has been demonstrated that germ cell exfoliation from the ovarian surface contributes to eliminate PGC as well as oogonia and oocytes (Motta et al., 1997).

In conclusion, these data demonstrate that Bcl-2 family plays an essential role in regulating germ cell number in the human developing ovary through the concerted temporal expression of both pro- and anti-apoptotic members at the different cell compartments during oogenesis. It is worthwhile to note that although some data have accumulated on apoptosis in the human developing ovary, we are still far from having a detailed knowledge on the regulation of germ cell number that is drastically reduced from $7 \times 10^6$ cells at the fifth month post-conception to around 750 000 oocytes at birth (Baker, 1963; Forabosco et al., 1991). Future analysis spanning the entire ovarian organogenesis and including more members of the Bcl-2 family and other apoptosis-related proteins will help to conduct a meta-analysis on fetal germ cell degeneration which is still pending.

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