IHC-P analysis of apoptotic process, in adult mouse ovary, by highlighting the Bcl-2 and Bax proteins

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Abstract

The occurrence of morphological changes and the triggering of biochemical events involved in the apoptotic process in ovaries, ultimately leading to the formation of apoptotic bodies and to their removal by phagocytes, are based on a genetic process mainly coordinated by the members of the Bcl-2 family. IHC-P images of sections taken from adult mouse ovaries point out the preferential localization of Bcl-2 and Bax proteins in the granulosa and internal theca cells. In the oocyte, the immune response of both proteins is weakly positive in the cytoplasm; in the primordial follicle stage, it increases in intensity in the primary and secondary follicle stages, becoming strongly positive and diffuse in preantral and antral follicle stages. In luteal cells, the reaction for Bcl-2 protein is positive and diffuse in the cytoplasm of luteal cells of the corpus hemorrhagicum and of the luteal body under formation; its intensity decreases in the active stage and becomes weakly positive or null in luteal regression. The immunoreaction of the Bax protein is weakly positive in the nucleus (especially in the nuclear membrane) and cytoplasm of luteal cells of corpus hemorrhagicum, in the corpus luteum under formation and in its active stage, becoming strongly positive in the corpus luteum in regression. The strong positive expression of the proapoptotic protein Bax during the follicular stage and the disappearance of corpus luteum, demonstrates a massive loss of ovarian cells during the reproductive life, involving this protein in the atresia of ovarian follicles, respectively in luteal regression.

Keywords: follicles, corpus luteum, Bcl-2 and Bax expression, oocyte, apoptosis

Introduction

The fertility of female domestic mammals declines in the course of time, a process explained by the depletion of follicular reserve as a consequence of cells action to hormonal titer but also of the involvement of several factors, such as oocytes quality and/or the pro- and antiapoptotic level of the Bcl-2 family proteins (McGee & Hsueh, [1], Vaskivuo & Tapainen, [2]).

The action of genes, namely that of Bcl-2 family proteins, to initiate or inhibit apoptosis in ovaries is a very complex one and occurs in some biological or biochemical subcellular structures, sometimes interfering with the normal development of several metabolic processes. In addition, the existence of very similar interaction domains in the...
structure of such proteins makes their homo-or heterodimerisation possible, an essential moment in the progress or blocking response to apoptotic stimulus (Antonsson & Martinou, [3], Svanberg, [4], Twomey & Mc Carthy, [5]).

By homodimerisation, the proapoptotic protein Bax causes the creation of a pore in the external mitochondrial membrane. This pore eases the leakage into the cytoplasm of the mitochondrial proteins, two of them with an important role in apoptosis, namely the cytochrome c and the AIF factor (Apoptosis Inducing Factor) (Bernardi & al., [6], Reed, [7], Sheau & Hsueh, [8], Hussein, [9], Boumela & al., [10]). The release of mitochondrial proteins, as a result of the transient pore opening, determines the blocking of the mitochondrial membrane potential and the activation of caspases, the proteases responsible for cell death (Svanberg, [4], Hussein, [9], Boumela & al., [10]). At the same time, the leakage of cytochrome c into the cells cytosol causes the release of a 50 kDa protein called Mitochondrial Factor which, together with endonucleases, facilitates chromatin condensation and internucleosomal DNA fragmentation (Kluck & al., [11]; Yang & al., [12]).

Bcl-2 antiapoptotic proteins have the property to prevent the hyperpolarization of mitochondrial membrane and hence the ballooning of such organelles, thus blocking homeostasis alteration and the transmission of apoptotic signals to subcellular structures (Reed, [7]).

Based on these issues, the object of this paper is an IHC-P study of the apoptotic process in ovaries, both in the follicular and luteal cells in adult mouse ovary. Using the IHC-P labeling index of apoptotic process by using anti Bcl-2 and anti-Bax monoclonal antibodies is a modern and objective method meant to decipher the cellular and molecular mechanisms that underlie this process, with a positive impact in the understanding of normal and pathological events leading to reproductive senescence and occurrence of several diseases, such as, for example, premature ovarian failure, polycystic ovarian syndrome, or even some forms of cancer.

Materials and methods

The animal method

The studies were performed on ovarian tissue sections of 15 adult female mice (90 days old) of the NMRI line, weighting 35-40 g, obtained from the Cantacuzino Institute, Baneasa Station, Bucharest. For a good reproductive performance, the room was lighted 12 hours from 24 by mounting an automatic lighting system. The females were fed ad libidum with combined granulated mice fodder, prepared in accordance with the product technical standards of the Cantacuzino Institute.

Tissue preparation

The females were sacrificed by cervical dislocation, then the gonads were removed and the samples were fixed in neutral formalin (10%) (Merck, Germany) for 18-24 hours. After the fixing operation, the ovaries were embedded in histological paraffin (Merck, Germany) and cut at the size of 2μ by means of a manual rotating microtome Leica RM2125RT.
Apoptosis detection by immunohistochemical study

The sections obtained were spread on slides specially treated with polylysine and then dewaxed in toluene (about 1 h, by thermostat). Afterwards, they were hydrated in three baths of ethyl alcohol in decreasing concentrations (100 %, 96 % and 80 %), about 5 min./bath. To expose the antigen, the sections were immersed one after another in a Target Retrieval solution (for Bax, pH 6, and for Bcl-2, pH = 9, Dako) for 20 minutes at room temperature. After their rinsing in distilled water, the sections were placed for 10 min. in a phosphate-buffered saline (PBS) and then incubated about 30 minutes with 3 % H₂O₂ to block endogenous peroxidase activity. Next, the sections were incubated with non-immune serum to block non-specific binding of IgG and afterwards they were incubated with primary polyclonal antibodies against Bcl-2 (Bcl-2 – RTU, 10-15 min., Dako) and against Bax (1:1500 dilution for 30 minutes, Dako), respectively. Subsequently, the sections were washed in buffer solution (PBS) and then incubated for 15-30 min. with the specific biotinylated secondary antibody, followed by the incubation with a 3,3'-diaminobenzidine (DAB) solution, to localize the antigen. The nucleic counterstaining was done with Mayer's hematoxylin

Immunohistochemical analysis

The positive expression of Bcl-2 and Bax proteins was assessed by highlighting the brown precipitate (in different shades of brown), localized diffusely in both cells’ cytoplasm and nuclei (especially in the nuclear membrane). Any detectable nuclear or cytoplasmic staining was considered as a positive immunoreactivity, regardless of the intensity of its expression. IHC-P images were taken and selected using a Cx41 Olympus optical microscope, equipped with an eyepiece with 10x magnification and optically corrected lens, with 40x and 100x magnification. The images obtained were printed with the digital camera Olympus and stored on our computer using Quick Photo Micro 2.2 program.

Immunostaining quantification: -/+ no marking/weak positive reaction, ++ positive reaction, +++ strong positive reaction, ++++ very strong positive reaction (many highly stained cells).

Results and discussions

The microscopic analysis of adult mouse ovary shows its organization in two completely defined areas, the cortex and medulla ones, the cortical area being populated by a large number of ovarian follicles and luteal bodies in different stages of evolution and involution. IHC-P images obtained by us from adult ovary suggest a weak positive staining in primordial follicles for both Bcl-2 and Bax proteins, both in the oocyte cytoplasm and the nucleus and cytoplasm of pregranulosa cells (Fig. 1, 2).
In primary follicles, immunoreactivity is positive for both proteins in oocyte cytoplasm and strongly positive and diffuse, especially for Bax protein, in the cytoplasm and nucleus of granulosa cells, an aspect correlated with an increased presence of this protein during the development in follicular stage (Fig. 1, 2).

Leng & al., [13], found that, in certain circumstances, the over-expression of Bcl-2 protein has the ability to protect ovarian follicular cells from apoptosis triggered by some factors. However, the inhibitory effect of this protein is influenced by its interaction with the Bax proapoptotic protein, when the homodimerisation of the Bax protein leads to the loss of the cytoprotective effect of Bcl-2 protein.

Starting with the secondary follicle stage, the intensity of reaction for Bcl-2 and Bax is positive in oocyte cytoplasm, strong positive and diffuse in the cytoplasm of granulosa cells and strong positive in the nucleus, especially in the nuclear membrane (Fig. 3, 4).

In preantral and antral follicles, IHC-P sections show an intense reaction for both proteins. Thus, in cytoplasm and nucleus of granulosa cell nucleus the reaction is very strong positive (particularly in granulosa cell delimiting follicular cavities), while in the oocyte and theca cells, the reaction is expressed mainly in cytoplasm, being strongly positive and diffuse (Fig. 5, 6 7).
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Fig 5. Strong positive and diffuse reaction for Bcl-2 protein in the cytoplasm and nucleus of granulosa cells, internal sheath cells and oocyte, in preantral follicle, in the adult stage; 1000x

Fig 6. Strong positive and diffuse reaction for Bax protein in the cytoplasm and nucleus of granulosa cells, internal sheath cells and oocyte in antral follicle, in the adult stage; 1000x

Fig 7. Strong positive and diffuse reaction for Bax protein in the cytoplasm and nucleus of granulosa cells and internal sheath cells, in preantral follicle, in the adult stage; 400x

Our results point out that both granulosa and theca cells are the main target of apoptosis in adult mouse ovary (Table 1, 2). These results are in accordance with data provided by Choi & al., [14], related to pubertal and adult rat ovary, where he found that the color for Bax protein is more intense in the granulosa cells of tertiary and preovulatory follicles than in the granulosa cells of the primary follicles. Kugu & al., [15], studying the expression of Bax protein in human and baboon ovary, found an overexpression of this protein in follicles being in atresia as compared to healthy follicles. Regarding the expression of the Bcl-2 and Bax proteins in the oocyte, the literature correlates this expression with the functional localization of the two proteins. The study of these proteins performed by Antczak & Van Blerkom, [16], with the confocal microscope, showed that they are preferentially expressed in oocyte subcortical region, with a tendency to accumulate at one pole of the cell. In addition, Van Blerkom, [17, 18] pointed out that this distribution is extremely important because the oocyte subcortical region is directly related to mitochondria, structures with an important role in regulating the Ca²⁺ ions flow until fecundation.

Table 1 Bcl-2 protein expression in ovarian follicle cells in adult mouse ovary

<table>
<thead>
<tr>
<th>Bcl-2 Protein</th>
<th>Primordial follicles</th>
<th>Primary follicles</th>
<th>Secondary follicles</th>
<th>Preantral follicles</th>
<th>Antral follicles</th>
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<tbody>
<tr>
<td>Adult stage</td>
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<tr>
<td>Oocyte</td>
<td>+</td>
<td>++</td>
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<td>+++</td>
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<tr>
<td>Granulosa cells</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
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<tr>
<td>Thecal cells</td>
<td>-</td>
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Table 2 Bax protein expression in ovarian follicle cells in adult mouse ovary

<table>
<thead>
<tr>
<th>Bax Protein</th>
<th>Primordial follicles</th>
<th>Primary follicles</th>
<th>Secondary follicles</th>
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Jurisikova & Acton, [19] and Wells & al., [20], studying by PCR techniques the expression of 15 genes of the bcl-2 family, in woman’s ovary, concluded that from each gene
there can be determined clear portions in each oocyte, thus resulting three profiles of the expression: oocytes susceptible to apoptosis, oocytes protected against apoptosis and oocytes with a balance between pro- and antiapoptotic genes. The result confirms a different level of apoptosis, the balance of the expression between the pro- and antiapoptotic members of bcl-2 family being a key element in the survival of oocytes and follicular reserve decline.

In corpus luteum, immunohistochemical images show a strong positive reaction for protein Bcl-2 in both the cytoplasm and the nucleus of luteal cells of the corpus hemorrhagicum and the corpus luteum in formation (Fig. 8, 9). The reaction diminishes in intensity in the active phase of the corpus luteum ((Fig. 10) and becomes weak positive or negative in the cells of the corpus luteum in regression (Fig. 11) (Table 3).

Regarding proapoptotic Bax protein expression, it is strongly positive in the cytoplasm and nucleus of luteal cells of corpus hemorrhagicum (Fig. 12), corpus luteum in development (Fig. 13) and corpus luteum in the active stage (Fig. 14) and strongly positive in the cells of corpus luteum in regression (Fig. 15) (Table 3).
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Fig. 12. Corpus hemorrhagicum - strong positive reaction for Bax protein in the nucleus and cytoplasm of luteal cells; 400x

Fig. 13. Corpus luteum in trening - positive reaction for Bax protein in the nucleus and cytoplasm of luteal cells; 400x

Fig. 14. Corpus luteum in the active phase - positive reaction for Bax protein in the cytoplasm of luteal cell; 400x

Fig. 15. Corpus luteum – first regression – strong positive reaction for Bax protein in the nucleus and cytoplasm of luteal cells. Destabilization of cellular cords. Involution of vascular network; 400x

**Table 3** Expression of Bcl-2 and Bax proteins in luteal cell

<table>
<thead>
<tr>
<th>Protein</th>
<th>Corpus hemorrhagicum</th>
<th>Corpus luteum in formation</th>
<th>Corpus luteum in active stage</th>
<th>Corpus luteum in regression</th>
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<tbody>
<tr>
<td>Bcl-2</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>+/-</td>
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<tr>
<td>Bax</td>
<td>+++</td>
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The issues raised by us are in correlation with the data provided by Sugino & al., [21], for human ovary and by Gürsoy & al., [22], for rat ovary, showing that when the level of Bax pro-apoptotic protein is high and the level of the anti-apoptotic protein Bcl-2 is low, luteal cells enter into apoptosis. Sugino & al., [21], Oltvai & al., [23] and Yang & al., [24], studied the action of Bcl-2 and Bax proteins and pointed out that responsible for the cells' death or survival is the ratio between the two proteins and not their concentration.
Conclusions

The immunohistochemical analysis of adult mouse ovary reveals the preferred localization of Bcl-2 and Bax proteins in the granulosa cells of ovarian follicles. At the same time, the low immunoreaction of Bcl-2 protein and the high immunoreaction of Bax protein in the active phase of corpus luteum and during luteal regression, demonstrates the involvement of Bax protein in luteal cell apoptosis in adult mouse ovary. With the intensification of the apoptotic process and the development of ovarian follicles, a natural selection of the cells involved in the gametogenesis process takes place. Thus, from a large number of precursory gamete cells there finally result a small number of highly viable gametes. The massive loss of cells in the ovary (99%) during the reproductive life is compensated by maintaining a balance between the two classes of Bcl-2 family proteins (pro- and antiapoptotic) and hence, implicitly, between the two well-known processes, namely cell proliferation and apoptosis. However, a random expression of the apoptotic process leads to premature ovarian failure and hence to the depletion of follicular reserve, with disastrous consequences upon the female reproductive life.

References

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