IN VITRO EFFECTS OF INHIBIN ON APOPTOSIS AND APOPTOSIS RELATED PROTEINS IN HUMAN OVARIAN GRANULOSA CELLS

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Objective. To clarify the in vitro effects of inhibin A (I) on apoptotic cell death and its mechanisms in ovarian granulosa cells the immunoexpression patterns of the apoptosis markers caspase-3 and pro- and anti-apoptotic proteins (Bcl-2, Bcl-xl, Bak) were evaluated in ovarian granulosa cells collected from women with different hormonal status.

Materials and Methods. Granulosa cells were isolated from follicles of women participating in an in vitro fertilization (IVF) program, normally cyclic (NC) and premenopausal women (PrM). The obtained cells were cultured for 72 h with inhibin A (Sigma, USA) – 10 ng/ml. The concentration of estradiol in the culture medium was determined by radioimmunoassay using the Coat-A-Count kit (Nippon, Japan), whose intra- and interassay coefficients of variations were 6,8 % and 6,2 % respectively. The expression of caspase-3, Bak, Bcl-2, Bcl-xl was investigated immunohistochemically. The percentages of immunopositive cells were calculated and Student's t-test was used for statistical analysis.

Results. Addition of inhibin A (10 ng/ml) to granulosa cells cultures resulted in increased estradiol production. Maximal stimulation was observed in granulosa cells collected from women participating in IVF whereas minimal effect of inhibin treatment on estradiol production was demonstrated in premenopausal women. Inhibin A exposition enhanced the immunoexpression of pro-oncogenes (Bcl-2, Bcl-xl) and reduced the expression of caspase-3 and pro-apoptotic protein Bak in ovarian granulosa cells from the three experimental groups.

Conclusions. Our findings suggest that inhibin A in vitro stimulates the estradiol secretion by granulosa cells dependently of the woman hormonal status, while it inhibits apoptotic process in granulosa cells independently of the hormonal status.

Key words: Human ovarian granulosa cells – Inhibin – Apoptosis – Apoptosis-related proteins H

During ovarian follicle growth and development the follicular atresia is a negatively selective degenerative process which involves granulosa cell (GC) death by apoptosis (Tilly et al.. 1991; Huges et al. 1991; Vaskivuo et al. 2002). Apoptosis (programmed cell death) is a distinct physiological form of cell death of characteristic morphology and biochemistry. When the cells proceed to apoptosis, the death cascade leads to activation of caspases, such as caspase-3, which break down the cells (Otala et al. 2002). The balance between proliferation and apoptosis of granulosa cells is crucial for the growth, development and differentiation of ovarian follicles both before birth and during the reproductive life. Diverse stimuli are known to compromise this balance leading to either degenerative diseases or via uncontrolled cell proliferation to cancerogenesis (Tal et

al. 1995; Coates et al. 1996; Otala et al. 2002; Catz and Johnson 2002). The cyclic proliferation, differentiation and programmed cell death of ovarian granulosa cells are under the complicated and still obscure control of ovarian (steroid) and pituitary hormones, paracrine and/or autocrine regulators and growth factors . In order to clarify whether inhibin (a member of transforming growth factors-ß super family) is involved in the control of programmed cell death, in vitro studies were undertaken to characterize the effects of this growth factor on the apoptosis and the immunoexpression patterns of apoptosis-related proteins in human ovarian granulosa cells from women of different age and hormonal status.

Materials and Methods

Granulosa cells were isolated by laparoscopy via the non-enzymatic needle puncture method from ovarian antral follicles of: 1. women participating in an in vitro fertilization program (age: 27-31 years, n=18); 2. young normally cycling women (age:21-26 years, n= 6) during sterilization via salpingo-oophorectomy or hysteroctomy for benign indications (myoma uteri, bleeding disorders), 3. premenopausal women (without any hormonal therapy for at least one year; (age: 45-51 years, n= 12) at the Institute of Sterility and Assisted Reproduction Technologies in Sofia. All patients have given their informed consent to the study.

Granulosa cells were cultured in Dulbecco minimal essential medium (DMEM, Sigma, USA) in the presence of 10 % fetal calf serum (FCS, Sigma) either with or without Inhibin A (Sigma, 10 ng/ml) for 72 hours. The cells grown as monolayer on microscopic slides were then washed in 0.1M PBS, fixed in 4% formaldehyde, freshly prepared from paraformaldehyde, in 0.1M phosphate buffer with 7.5 sucrose added for 30 min, washed again in 0.1M PBS and preserved until use at – 20 °C in a preserving medium. The cell monolayers were conditioned for 30 min in a blocking solution, consisting of 1 % immunoglobulin-free bovine serum albumin (BSA Fraction V, Sigma, USA) in 0.1% PBS and incubated overnight at 4 °C in a humidified chamber with one of the primary monoclonal antibodies: Caspase-3 (CPP32) (diluted 1:500, Serotec), Bcl-2 (diluted 1:5, BioGenx, CA, USA), Bcl-xl (diluted 1:500, Serotec), Bak (diluted 1:400, Dako, Denmark). To calculate the percentage of immunopositive cells by light microscopy at 400 X magnification, positive cells were counted out of 450 randomly selected granulosa cells from three slides per patient (3 patients per group).

Statistical evaluation. Results reported as mean ± SD throughout the study were statistically analyzed by Student's t-test.

Results

The results shown in Fig.1 illustrated the effect of inhibin on estradiol production by granulosa cells collected from women with different hormonal status, while progesterone secretion remained unaffected. The amount of estradiol, secreted by granulosa cells was highest in women taking part in IVF, whereas the amount of estradiol, secreted by granulosa cells of PrM women was lowest as compared to controls (C) – granulosa cells cultured in the absence of inhibin. Apoptotic granulosa cells in the cultures were identified by their condensed chromatin and darkly stained fragmented nuclei using light microscopically evaluation. Taken into consideration the activity of caspase-3 (Fig.2) the smallest amount of apoptotic cells was noted in IVF women (7 %), larger amount in normally cyclic women (16 %) and the largest in premenopausal patients (27 %). The obtained values of the percentages of caspase-3 positive cells in granulosa cells cultures after inhibin exposure pointed out decrease of the number of enzyme positive cells as compared to controls (IVF-5 %; NC-11 %; PrM-25 %) (Fig.3). The number of Bak positive cultured granulosa cells (Fig.4) varied from 8 % for women undergoing IVF to 16 % for premenopausal women. The applied inhibin provoked depletion of the values of Bak positive cells in all experimental groups (3 %; 4 %; 12 %) for women after IVF, normally cyclic and premenopausal women, respectively (Fig.5). The number of cultured granulosa cells with pro-oncogene Bcl-2 expression was almost similar in both groups of normally cycling patients (23 %) and patients participating in an IVF (24 %) and was lower in premenopausal women (16 %). After inhibin application some changes in the percentages of positive cells were observed (IVF-27 %; NC-26 %; PrM-15 %) (Fig.6). The reaction for Bcl-xl in granulosa cells cytoplasm showed numerous positive cells in women after IVF (26 %) and in normally cycling patients (25 %), but almost 2 fold less in granulosa cells cultures of premenopausal women (14 %). The applied inhibin changed slightly the proportions of Bcl-xl stained granulosa cells (IVF-28 %; NC-27 %; PrM-15 %) (Fig.7).

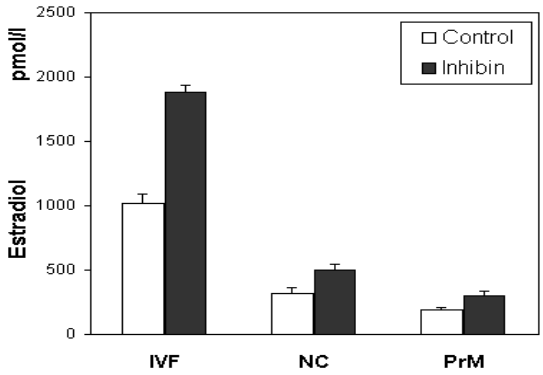

Fig.1 In vitro inhibin A action on estradiol secretion by cultured human ovarian granulosa cells. Values are means ±SD of 9 cultures from 3 experiments, p<0.001 when compared to control. IVF = in vitro fertlisation group, NC = normally cycling group, PrM =premenopausal group

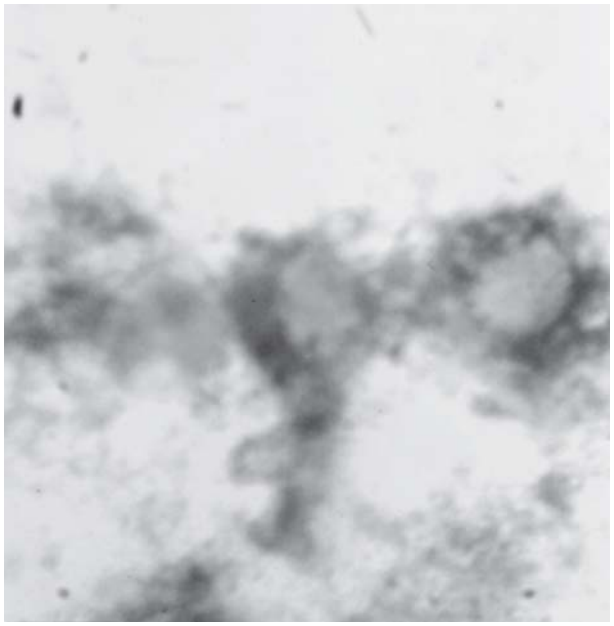

Fig.2 Caspase-3 immunoexpression in cultured ovarian granulosa cells. X400

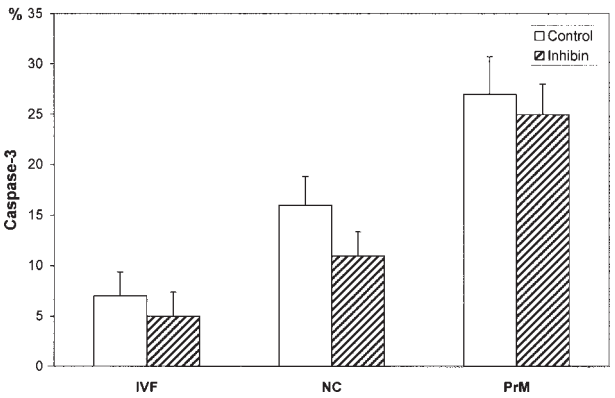

Fig.3 Percentage of caspase-3 immunopositive ovarian granulosa cells from women with different hormonal status (IVF, NC and PrM) after inhibin treatment. Values are mean ± SD of 12 cultures from 3 experiments, IVF, PrM (p<0.01 vs.control); NC (p<0.05 vs. control). IVF = in vitro fertlisation group, NC = normally cycling group, PrM =premenopausal group

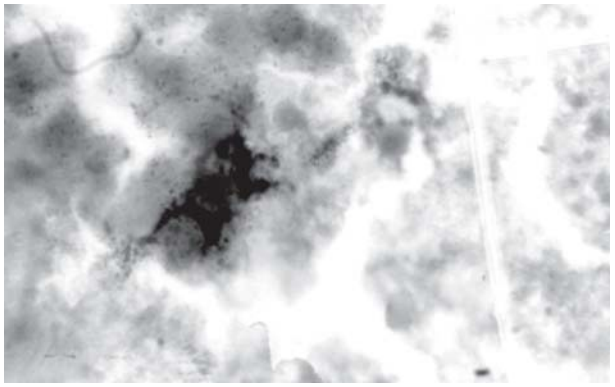

Fig.4 Bak immunoexpression in cultured ovarian granulosa cells. X100

Discussion

With regards to the inhibin A action we established that this growth factor reduced apoptosis in granulosa cells isolated from women with different hormonal status, suggesting its apoptotic effect. Granulosa cell death in vitro through apoptosis was shown by the detection of caspase-3 enzyme activity (one of the specific cell human death enzyme after GALLAHAR et al. 2001) and by the immunoexpression of pro- and anti-apoptotic proteins respectively. The enhancement of granulosa cells apoptosis in our study was paralleled by up-regulated expression of proapoptotic protein Bak and down regulated expression of anti-apoptotic Bcl-2 and Bcl-xl proteins. Immunohistochemical and biochemical analyses of BOONE and TSANG 1998; KUGH et al. 1998; SAKAMAKI 2003; GABRIEL et al. 2003; ELISEEV et al. 2003 have also shown apoptosis in animal and human granulosa cells to be regulated by caspases and Bcl-2 gene family members. The role of apoptosis in human reproduction has been discussed repeatedly (BENIFLA et al. 2002; HSUEH 2002; TAKAGIMORISHITA et al. 2003). In our study inhibin has been shown to enhance estradiol secretion by cultured granulosa cells. Based on the

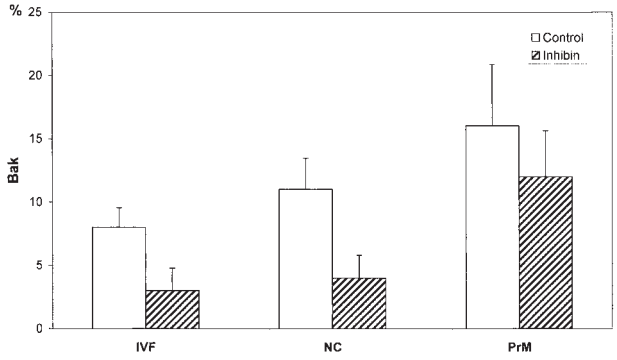

Fig.5 Percentage of Bak immunopositive granulosa cells from IVF, NC and PrM women after inhibin treatment. Values are mean ± SD of 12 cultures from 3 experiments, p<0.001 when compared to control. IVF = in vitro fertlisation group, NC = normally cycling group, PrM =premenopausal group

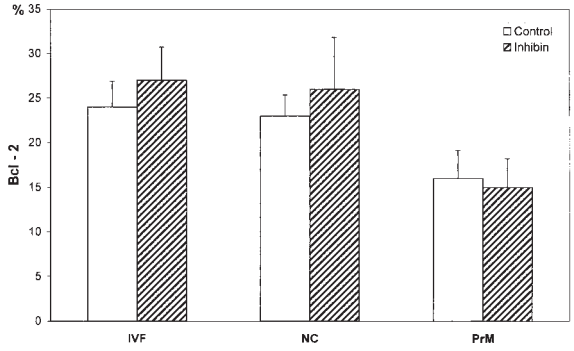

Fig.6 Percentage of Bcl-2 immunopositive granulosa cells from IVF, NC and PrM women after inhibin treatment. Value are mean ± SD of 12 cultures from 3 experiments, p<0.01 when compared to control.
IVF = in vitro fertlisation group, NC = normally cycling group, PrM =premenopausal group

obtained radioimmunoassay of estradiol levels in granulosa cells culture we found that the enhanced production of estradiol by cultured granulosa cells depend on the woman hormonal status (the most intensive stimulation of the basal estradiol production was observed in IVF patients and the less one in premenopausal patients). The enhanced estradiol secretion correlate positively with the apoptosis suppression. The number of granulosa cells immunopositive for caspase-3 revealed highest number in cultures from premenopausal women, contrary to the small number of the enzyme positive granulosa cells in women undergoing IVF, when the estrogen in the follicular fluid is the highest. The obtained data revealed parallel alterations of the increased estradiol production after inhibin treatment, reduced apoptotic process and weakly expressed pro-apoptotic proteins. This is in agreement with the data of Billig et al. 1993; Campbell and Baird 2001; Song and Santen 2003) who considered estrogen as an inhibitor of ovarian granulosa cells apoptosis and in contrast to the negative effect of inhibin on estradiol production established by Jimenez-Krassel et al. (2003). According to these authors it is possible that the variable results among the laboratories that examined the direct effect of inhibin treatments on granulosa cell function were attributed to the length of cultures and hormonal or growth additives during culture, causing a different basal inhibin production. It is interesting that the inhibitory effect of inhibin on cultured granulosa cell apoptosis was uniform intense regardless to the hormonal status of the investigated women. Our findings provide some evidence that inhibin A has a key local autocrine role on stimulating estradiol secretion by granulosa cells and inhibitory effects on granulosa cells apoptotic process in addition to its endocrine role.

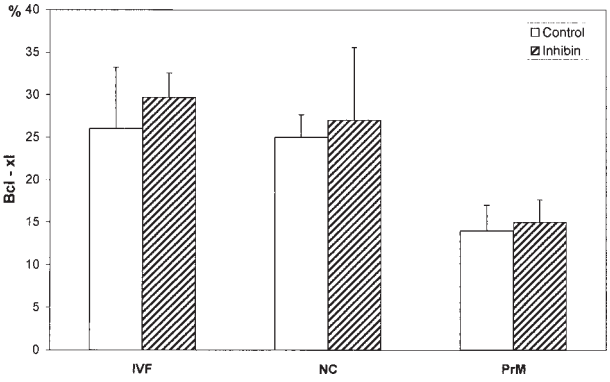

Fig.7 Percentage of Bcl-xl immunopositive granulosa cells of IVF, Y and PrM women after inhibin treatment. Values are mean ± SD of 12 cultures from 3 experiments, p< 0.05 when compared to control.
IVF = in vitro fertlisation group, NC = normally cycling group, PrM =premenopausal group

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