Histol Histopathol (2012) 27: 241-248
DOI: 10.14670/HH-27.241

http://www.hh.um.es

Histology and Histopathology
Cellular and Molecular Biology

Analysis of pituitary gonadotropin concentration in blood serum and immunolocalization and immunoexpression of follicle stimulating hormone and luteinising hormone receptors in ovaries of postmenopausal women

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Summary. The participation of gonadotropins in ovarian carcinogenesis is well known and is supported by studies with inhibition of pituitary gonadotropin secretion, which results in a diminished risk of cancer. However, there are few data on localization and expression of Follicle Stimulating Hormone and Luteinising Hormone Receptors (FSHR and LHR) in ovaries of healthy postmenopausal women, and their correlation with FSH and LH concentration in blood serum is unknown.

The aim of our study was to analyze gonadotropin concentration in blood serum and the expression of FSHR and LHR in ovaries of 207 postmenopausal women. Patients included in the study were divided into three groups depending on the number of years since menopause. We analyzed the concentration of FSH and LH in blood serum and the expression of FSHR and LHR in ovaries.

Ovaries of postmenopausal women showed numerous morphological changes in the cortex and medulla when compared to the structure of ovaries of women at reproductive age. In all groups of patients clefts in the surface epithelium and epithelial inclusion cysts were found.

The concentration of FSH and LH in the blood serum of women studied increased significantly with time from menopause. Significant differences between analyzed menopausal groups were found. The highest FSH and LH concentration in blood serum were found in women with the longest period of time from menopause. Quantitatively similar expression of FSHR and LHR was found in ovarian surface epithelial cells, in epithelial inclusion cysts and in the connective tissue cells of ovarian stroma. The intensity of the immunohistochemical reaction decreased with time from menopause and with age.

Key words: Postmenopausal ovary, Immunohistochemistry, FSH and LH receptors, Epithelial inclusion cysts, Pathology

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Introduction

The results of our previous studies have shown expression of steroid hormone receptors in postmenopausal ovaries (Brodowska et al., 2007b, 2010; Starczewski et al., 2008).

Current published data indicates that ovarian cancer occurs most commonly in the postmenopausal period and the involvement of gonadotropins in ovarian carcinogenesis is well known. The results of many studies have shown that by inhibiting the secretion of pituitary gonadotropins eg. by using hormonal contraception, long lasting breast feeding or with frequent pregnancies, the risk of ovarian cancer is diminished. Additionally, numerous studies of women with ovarian cancer have shown increased expression of FSH and LH receptors (Parrot et al., 2001; Chu et al., 2002; Choi et al., 2002, 2007; Chudecka-Głaz et al., 2004). Interestingly, however, there are few data on the localization and expression of FSHR and LHR in ovaries of healthy postmenopausal women in relation to

hormonal therapy, indications for this treatment, dosage of hormones or period of therapy. It was previously unknown whether the localization and expression of the above-mentioned receptors in cells of healthy gonads after menopause correlates with FSH or LH concentration in blood serum.

The first study on FSHR and LHR in ovaries of postmenopausal women was conducted in 1976 and showed the localization of these receptors in stromal and hilar cells of the ovary (Peluso et al., 1976). Several years later Nakano showed that FSHR and LHR are localized mainly in stromal cells and surface epithelial cells of postmenopausal ovaries (Nakano et al., 1989), while Vihko et al. did not detect the presence of FSHR and LHR in postmenopausal ovaries (Vihko, 1996).

Previous analysis of FSH and LH concentrations in the blood serum of postmenopausal women has shown a 10 to 20-fold increase in FSH concentration and an approximately 3-fold increase in LH concentration compared to women of reproductive age (Buffet and Bouchard, 2001; Skałba, 2005; Speroff and Fritz, 2007). After the menopause the concentration of pituitary gonadotropins in blood serum remains constant without changes in amplitude or frequency of secretion. Maximal concentrations of FSH and LH in blood serum were found from one to three years after menopause with a subsequent lower secretion (Buffet and Bouchard, 2001; Skałba, 2005; Speroff and Fritz, 2007). Some published data have shown that the concentrations of FSH and LH in blood serum do not correlate with the risk of ovarian cancer in the general population (Kramer et al., 1998; Arslan et al., 2003), while other studies have found a negative correlation (Helzlsouer et al., 1995; Blaakaer, 1997). Elevated blood serum concentration of LH only has been found in BRCA-1 positive postmenopausal women (Kramer et al., 1998; Vartiainen et al., 2001). It follows that diminished activity of ovarian steroidogenesis probably increases the risk of ovarian cancer via the interaction of FSH and its receptors on ovarian surface epithelium and peritoneal cells (Wang et al., 2003; Vanderhyden, 2005).

Studies performed on women with diagnosed ovarian cancer have shown significantly higher concentration of free beta Human Chorionic Gonadotropin (ß-HCG) in blood serum (normal concentration is to 5 mIU/ml) (Cole et al., 1996; Vartiainen et al., 2008) whilst the concentration of FSH and LH was significantly higher in ovarian cyst fluid and/or in fluid from the peritoneal cavity but not in blood serum (Kramer et al., 1998; Halperin et al., 2003; Chudecka-Głaz et al., 2004). It was shown that, for both FSH and LH, the values of the ratios of concentrations in blood serum / tumor fluid was the lowest in malignant neoplasm (Rzepka-Górska et al., 2004). Some studies on women of reproductive age have shown that administration of gonadotropins may induce ovarian tumors (eg. Kuroda et al., 1998), but not all published results support this observation (Venn et al., 1995; Konishi et al., 1999).

Patients treated with gonadotropins to stimulate ovulation in the case of infertility have shown significantly increased risk of granulosa cell tumor (Willemsen et al., 1993; Rossing et al., 1994; Anderson and Dimitrievich, 1996); and Kuroda et al. (1998) found that 6 out of 131 patients (4.6%) were diagnosed with ovarian cancer during the stimulation of ovulation.

The study on different histological types of ovarian cancers by Chu et al. showed the highest expression of FSH receptors in granulosa cell tumor cells, much higher than in the normal ovary or in serous or mucinous cancers. On the other hand the expression of LH receptors was the highest in ovarian cancer cells, lower in the normal ovary and the lowest in granulosa cell tumor cells (Chu et al., 2002, 2004). The analysis of immunoexpression of FSH and LH receptors at different stages of ovarian cancer showed a positive correlation between the expression of both receptors and a favorable outcome. The higher the FSH expression the lower the stage of the disease, while increased expression of LH receptors indicated better prognosis, higher effectiveness of chemotherapy and a lower risk of metastases compared to double-negative tumors (Lu et al., 2000; Wang et al., 2003; Weiss et al., 2004).

The aim of our study was to look for correlations between gonadotropin concentrations in blood serum and immunolocalization and immunoexpression of FSHR and LHR in ovaries of postmenopausal women.

Materials and methods

Patients

The study was performed on 207 postmenopausal women operated on in the Department of Reproduction and Gynecology at the Pomeranian Medical University in Szczecin between 2003 and 2008, due to uterine leiomyomas, endometriosis and/or prolapsed uterus. All patients fulfilled inclusion criteria: a minimum of one year had elapsed after the menopause, non-appliance of hormonal postmenopausal therapy, normal prophylactics tests (PAP smear, mammography); and exclusion criteria: regular periods, usage of hormonal therapy, pathologic PAP smear and/or mammography. The study was approved by the Bioethics Commission of Pomeranian Medical University and it was supported by grant No. 2 PO5E-10527 from the Polish State Committee for Scientific Research.

The concentrations of FSH and LH were measured in blood serum of the women included in the study a day before their operation. Patients were divided into three groups: A, B and C depending on the time from menopause: Group A included women with menopause from one to five years previously (79 women), group B-women with menopause from five to ten years previously (66 women) and group C-women with menopause more than ten years before their operation (62 women). During the laparotomy, hysterectomy or removal of uterine corpus with adnexa was performed.

Immunolocalization and immunoexpression of membrane FSH and LH receptors were analyzed in 104 selected ovary samples: Group A (42 women); group B (40 women); group C (22 women).

Hormone measurements

Total concentrations of LH and FSH in blood serum was measured by electochemiluminesence assay (ECLISA) with monoclonal antibodies, using a COBAS E analyzer (Roche Diagnostics GmbH, Poland). The sensitivity limit was 0.10 mIU/ml for both LH and FSH.

Immunohistochemistry

FSHR and LHR were detected immunohistocemically in 4% buffered-formalin-fixed, paraffin-embedded tissue samples. Thick sections (5 μm) were cut and mounted on microscope slides. Slides were heated in pH 9.0 buffer for 30 minutes at 99°C and incubated with primary polyclonal rabbit anti-human antibodies against either FSHR or LHR in a humidified chamber for 30 minute, at room temperature (FSHR-F3929, LHR-L6792, Sigma, USA). Immunohistochemical reactions were visualized using an ABC Staining System with diaminobenzidine (DAB; Santa Cruz Biotechnology, USA). Slides were counterstained with Mayer's hematoxylin and washed in phosphate buffered saline (PBS). Negative controls were without primary antibodies.

Quantitative image analysis of immunohistochemistry

Analysis of digital images was performed in order to measure objectively the immunohistochemically-detected expression of LHR and FSHR. Slides of examined ovary tissue sections, stained for LHR and FSHR, were studied using light microscopy (Olympus BX41) "using 40x objective lens type" with built-in digital camera (Olympus SP350) and acquisition software (QuickPHOTO CAMERA 2.3 PROMICRA, Czech Republic). For each slide five areas of interest (AOI) were carefully selected from each of three tissue types, the ovarian surface epithelium (OSE), epithelial inclusion cysts and stromal cells. Selected AOI were photographed using the digital camera with fixed exposition parameters. For analysis ImageJ 1.44p software (National Institutes of Health, Bethesda, MD, USA) was used. Regions of interest (ROI) were manually selected from each digital picture of FSHR and LHR stained tissues - separately for OSE, epithelial inclusion cysts and stromal cells. Mean optical density (MOD) was calculated for each ROI using the image analysis software. Mean optical density for negative controls was calculated from immunohistochemistry slides stained without primary antibody, using ROI selection. Maximal optical density (MxOD) was taken as the MOD of the ROI with the highest expression pattern (hot spot). The mean MOD values for each postmenopausal group and tissue type, negative controls and mean MxOD values are presented in table (3A and 4A). Ratios of mean MxOD to mean MOD are also given.

Statistical analysis

All statistical analyses were done using Statistica 6.0 for Windows (StatSoft, Poland). Quantitative variables assessed were: minimal and maximal values, arithmetic mean and standard deviation. The Shapiro-Wilk test was used to assess the distribution of measured variates. The significance of the difference between two samples was assessed using the Student's t test, the Mann–Whitney test and the Kruskal-Wallis test. Correlations were assessed using the Pearson correlation coefficient. Parameters of mean optical density (MOD) were analyzed by the Mann-Whitney U test using Graph Pad Prism software (Ver. 5.0). A P value less than $p<0.05$ (or $P>0.05$ for the Shapiro-Wilk test) was considered significant.

Results

General morphology

Ovaries of postmenopausal women showed numerous morphological changes to the cortex and medulla when compared to the structure of ovaries of reproductive women.

There were numerous corpora albicantia, well developed fibrous connective tissue with cells tightly attached to each other forming a dense stroma in ovaries of women in group A, five years from menopause. Corpora albicantia consisted of connective tissue cells: fibroblasts, macrophages and collagen fibers. Moreover numerous arterial and venous vessels, lymphatic vessels and nerves were seen in ovarian stroma. The lumen of arterial and venous vessels was wide, much wider than the thickness of their walls. In ovaries of women five to ten years from menopause (group B) corpora albicantia were less frequent and they were fragmented. The connective tissue was more fibrotic but less tight, yielding thinner stroma compared to ovaries of women from group A. Blood vessels were partially hyalinated, their walls were thickened and their lumen was narrowed compared to ovaries of women from group A. In ovaries of women of more than ten years from menopause single corpora albicantia were found and their fragments compared to group A. The connective tissue was highly reduced and it formed thin stroma, with rare blood vessels of very thin walls and narrow lumen and very rare lymphatic vessels and nerves. In all analyzed groups of patients clefts of surface epithelium and epithelial inclusion cysts were found. There were no differences in number and size of epithelial clefts and inclusion cysts between analyzed groups (Brodowska et al., 2007a;

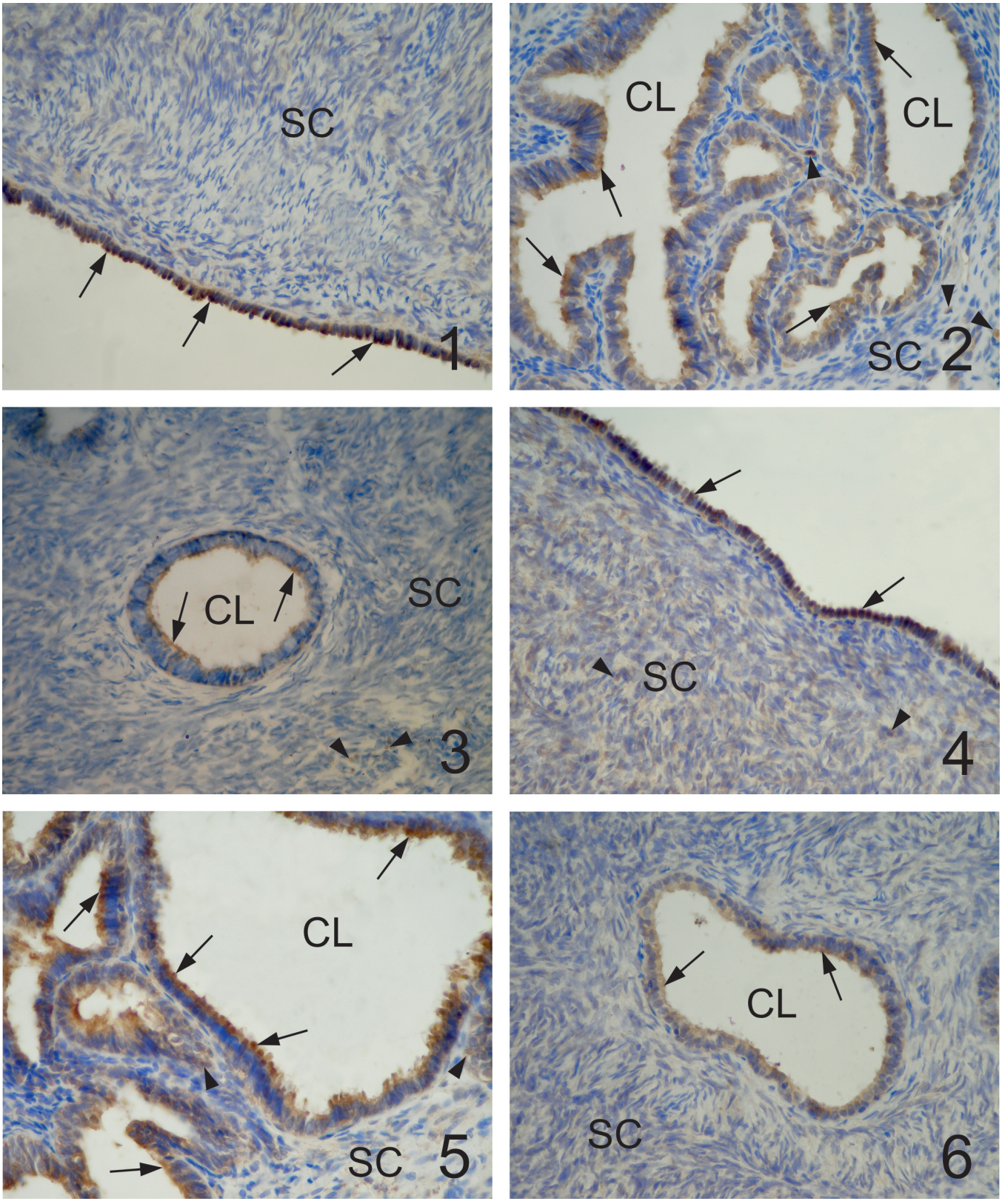
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Table 1. Description of serum FSH levels in the study groups.

Description of distribution	FSH [mIU/ml]		
	Group A (n=79)	Group B (n=66)	Group C (n=62)
min-max	2.7-146.2	4.0-184.9	18.9-146.1
Q_1-Q_3	40.1-81.6	40.5-85.2	59.5-92.8
Me	61.3	59.3	84.1
x±SD	62±33.4	62±32.3	78.4±24.1
N	>0.05 (+)	<0.03 (-)	>0.71 (+)

n: the number of people in the group; min-max: range of values; Q1-Q3: interquartile range; Me: median; x ± SD: arithmetic mean and standard deviation; N: normality of distribution P value from the Shapiro-Wilk test, yes (+), no (-).

Table 2. Description of serum LH levels in the study groups.

Description of distribution	LH [mIU/ml]		
	Group A (n=79)	Group B (n=66)	Group C (n=62)
min-max	2.1-123.8	3.1-115.8	12.2-142.8
Q_1-Q_3	28.1-66.1	29.1-62.2	41.2-76.2
Me	44.2	40.9	58.3
x ± SD	48.6±30	44.7±24.7	59.8±24.6
N	<0.02 (-)	<0.03 (-)	>0.15 (+)

n: the number of people in the group; min-max: range of values; Q1-Q3: interquartile range; Me: median; x ± SD: arithmetic mean and standard deviation; N: normality of distribution P value from the Shapiro-Wilk test, yes (+), no (-).

Table 3A. Mean optical density (MOD) of FSHR immunostaining in ovaries of postmenopausal women with time from menopause (groups A to C). Results in bracket are the ratio of maximal optical density (MxOD) to mean optical density fraction (MOD).

Study group	Ovarian surface epithelium (MxOD/MOD)	Epithelial inclusion cysts (MxOD/MOD)	Stromal cells MxOD/MOD)
Group A	85 (0.84)	88 (0.81)	124 (0.57)
Group B	111 (0.64)	112 (0.63)	131 (0.54)
Group C	137 (0.52)	139 (0.51)	149 (0.48)

Mean optical density for negative control: 164 (0.43); Maximal optical density: 71 (1)

Table 3B. Immunolocalization and immunoexpression of FSHR in the ovaries of postmenopausal women with time from menopause (Groups A to C).

Study group	Ovarian surface epithelium	Epithelial inclusion cysts	Stromal cells
Group A	++	++	+
Group B	+	+	+
Group C	+/-	+/-	+/-

Legend: strong reaction (++), weak reaction (+), lack of reaction (-)

Table 4A. Mean optical density (MOD) of LHR immunostaining in ovaries of postmenopausal women depending on the time from menopause (Groups A to C). Results in brackets are the ratios of maximal optical density (MxOD) to mean optical density fraction.

Study group	Ovarian surface epithelium (MxOD/MOD)	Epithelial inclusion cysts (MxOD/MOD)	Stromal cells (MxOD/MOD)
Group A	82 (0.87)	77 (0.92)	129 (0.55)
Group B	115 (0.62)	89 (0.80)	134 (0.53)
Group C	140 (0.51)	116 (0.61)	142 (0.50)

Mean optical density for negative control: 164 (0.43); Maximal optical density: 71 (1)

Table 4B. Immunolocalization and immunoexpression of LHR in the ovaries of postmenopausal women depending on the time from menopause (Groups A to C).

Study group	Ovarian surface epithelium	Epithelial inclusion cysts	Stromal cells
Group A	++	+++	+
Group B	+	++	+
Group C	+/-	+	+/-

Legend: very strong reaction (+++), strong reaction (++), weak reaction (+), lack of reaction (-)

Fig. 1. Ovary of group A postmenopausal woman (examined no more than 5 years after menopause). Immunohistochemical cytoplasmic localization of FSH receptor (FSHR) in the ovarian surface epithelium; strong expression of FSHR (arrows). Stromal cells (SC). x 400

Fig. 2. Ovary of group A postmenopausal woman (examined no more than 5 years after menopause). Immunohistochemical cytoplasmic localization of FSHR in an epithelial inclusion cyst; strong expression of FSHR in the apical part of columnar cells (arrows). Expression of FSHR in some stromal cells (arrowheads). Stromal cells (SC), cyst lumen (CL). x 400

Fig. 3. Ovary of group C postmenopausal woman (examined more than 10 years after menopause). Immunohistochemical cytoplasmic localization of FSHR in an epithelial inclusion cyst; weak expression of FSHR in apical part of columnar cells (arrows). Expression of FSHR in some stromal cells (arrowheads). Stromal cells (SC), cyst lumen (CL). x 400

Fig. 4. Ovary of group A postmenopausal woman (examined no more than 5 years after menopause). Immunohistochemical, cytoplasmic localization of LH receptor (LHR) in the ovarian surface epithelium; strong expression of LHR (arrows). Expression of LHR in some stromal cells (arrowheads). Stromal cells (SC). x 400

Fig. 5. Ovary of group A postmenopausal woman (examined no more than 5 years after menopause). Immunohistochemical cytoplasmic localization of LHR in epithelial inclusion cysts; very strong expression of LHR in apical part of columnar cells (arrows). Expression of LHR in some stromal cells (arrowheads). Stromal cells (SC), cyst lumen (CL). x 400

Fig. 6. Ovary of group C postmenopausal woman (examined more than 10 years after menopause). Immunohistochemical cytoplasmic localization of LHR in an epithelial inclusion cyst, weak expression of LHR in apical part of columnar cells (arrows). Stromal cells (SC), cyst lumen (CL). x 400

Laszczyńska et al., 2008).

Hormone measurements (FSH and LH) in blood serum of postmenopausal women

The concentration of FSH and LH in the blood serum of women studied increased significantly with time from menopause (Tables 1 and 2). Significant differences between analyzed groups were found. The highest FSH concentration, 78.4 mIU/ml, was found in group C and it was significantly higher than in group A and in group B (Table 1). The highest mean concentration of LH, 59,8 mIU/ml, was also found in group C (Table 2). This was lower than the mean maximal concentration of FSH (Table 1) and was significantly higher than the mean LH concentration in group A and in group B.

Immunolocalization and immunoexpression of membrane FSH and LH receptors in ovaries of postmenopausal women

Immunolocalization and immunoexpression of FSHR and LHR was found in three tissue types: surface epithelial cells (Figs 1 and 4), in epithelial inclusion cysts (Figs. 2, 3, 5 and 6), and in connective tissue cells of ovarian stroma (Figs. 2-5). The intensity of immunohistochemical reaction decreased with time from menopause and with age. Immunoexpression of FSHR and LHR was similar in these three tissue types. Only expression of LHR was higher in epithelial inclusion cysts in all groups studied (Table 4A and 4B) compared to the expression of FSHR (Table 3A and 3B). In group A the immunoexpression of LHR varied from very strong or strong to weak depending on the localization of the receptor (Figs.4-6), while the expression of FSHR varied from strong to weak (Figs.1-3). In group C the expression of LHR and FSHR was weak or sometimes undetectable (Tables 3B and 4B).

Discussion

Immunolocalization and immunoexpression of FSH and LH receptors in normal postmenopausal ovaries was found in the cytoplasm of surface epithelial cells, in epithelial inclusion cysts, and in connective tissue cells of ovarian stroma. The expression of FSHR and LHR decreased with time from menopause; however it was also detected in patients more than 10 years from menopause.

Some published data indicate that 85% to 90% of ovarian cancers develop from surface epithelial clefts or epithelial inclusion cysts (Scully, 1995; Mittal et al., 1995; Aoki et al., 2000; Auersperg et al., 2001; Heller et al., 2003; Scott and McCluggage., 2006), but the surgical removal of detected cysts based on ultrasound imaging did not reduce mortality from ovarian cancer (Crayford et al., 2000). The surface epithelial clefts and epithelial inclusion cysts found in postmenopausal ovaries in our study may provide one explanation as to why there is a higher incidence of ovarian cancer in postmenopausal women.

The results of our own studies correspond partially to the results of other authors. Previously, FSHR and LHR were found in stromal and hilar cells (Peluso et al., 1976) and in the surface epithelial cells (Nakano et al., 1989) of postmenopausal ovaries. In contrast to our own results these authors did not find the presence of FSHR and LHR in epithelial inclusion cysts (Peluso et al., 1976; Nakano et al., 1989). Vihko did not find the expression of FSHR and LHR in postmenopausal ovaries, while he found the expression of FSHR in 27% and LHR in 68% in ovaries of perimenopausal, menstruating women, depending on the phase of the menstruation cycle (Vihko, 1996).

The localization and expression of FSHR and LHR in cells typical for ovarian steroidogenesis, mainly in stromal cells as presented in the current paper, provides evidence that pituitary gonadotropins may influence steroidogenesis in postmenopausal ovaries. Similar data have been published by many authors (Adashi, 1994; Bufet and Bouchard, 2001; Burger, 2002; Elder et al., 2008; Broekmans et al., 2009).

The negative correlation between receptor (FSHR and LHR) expression in ovarian cells and mean blood serum concentration of gonadotropins may indicate one of the reasons why there is a limited ovarian reaction to gonadotropin stimulation in postmenopausal women. The reduced ovarian response to FSH and LH observed in older women may be related to the lower expression of FSHR and LHR in ovarian cells, but also to the progressive dispersion of ovarian stromal cells, disturbances in paracrine regulation of FSHR and LHR and to lower blood supply. Finally, based on our results, it may be supposed that the activity of ovarian steroidogenesis in postmenopausal women depends more on the ovarian expression of FSHR and LHR than on the stimulation of pituitary gonadotropins. However, the immunoexpression and immunolocalization of FSHR and LHR in normal ovarian cells, in surface epithelial cells and in inclusion cysts, typical in the most frequent neoplastic transformations (Sherman et al., 1999; Wang et al., 2003) indicate the possible participation of gonadotropins and their receptors in ovarian carcinogenesis in postmenopausal women. The higher blood serum concentration of FSH and LH found in our studies in postmenopausal women may indicate the possible role of gonadotropins in ovarian carcinogenesis, as there is a continuous increase in age-adjusted annual incidence rate of ovarian carcinoma (Goodman and Shvestsov, 2009). However, a recent study by McSorley et al. showed a decreased risk of ovarian cancer with increase in FSH concentration in serum, which raises the question of a protective role of gonadotropins in ovarian carcinogenesis (McSorley et al., 2009).

The interpretation of our own results is difficult due to a very limited number of publications on the presence of FSH and LH receptors in normal postmenopausal

ovaries, and differing results of studies of localization of those receptors in ovaries. In particular, older studies in which methods used have now been invalidated (eg. with radionuclide-labelled gonadotropins) and which used different interpretation methods, gave different results. Rajanemi et al. found the presence of LHR only in 27% of epithelial malignant neoplasms of the ovary (Rajaniemi et al., 1981) while Stouffer (Stouffer et al., 1984) did not find gonadotropin receptors at all in ovarian cancer cells. Only contemporary studies based on molecular biology techniques confirm the presence of FSHR and LHR in ovarian cancer cells. Mandai et al. (1997) found the expression of LHR in 40% of women with ovarian cancer, in 71% of borderline tumors and in 80% of cystadenomas. Other studies have shown a different distribution of gonadotropin receptors in ovarian cancer cells in relation to age, histological type of cancer and stage (Rajaniemi et al., 1981; Mandai et al., 1997; Minegishi et al., 2000; Lu et al., 2000; Parrott et al., 2001; Choi et al., 2007; Kobayashi et al., 2008).

We conclude that immunohistochemical studies on the expression and localization of FSHR and LHR in normal postmenopausal ovaries and their correlation with FSH and LH concentration in blood serum should be continued to create an assessment of the risk of ovarian cancer related to these hormones.

Acknowledgements. This paper was supported by the National Committee of Scientific Research of Poland, Grant. no. PG-2-PO5E-10527

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Accepted September 28, 2011