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Production of Steroids by Human Ovarian Surface Epithelial Cells in Culture: Possible Role of Progesterone as Growth Inhibitor

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Objective. **The purpose was to investigate whether normal ovarian surface epithelial cells, harvested from premenopausal and postmenopausal women, are capable of steroid production, and to evaluate effects of estradiol and progesterone on growth regulation of such cells.**

Methods. **Ovarian surface epithelial cells were obtained by brushing of the ovarian surface of 9 premenopausal and 10 postmenopausal women undergoing surgery for benign gynecological diseases. The conditioned media after culture, with and without addition of FSH and LH, were analyzed for estradiol and progesterone. The proliferative effects of the steroids were analyzed using two different culture models, nonconfluent cells and confluent cells, and two different detection methods, [^{3}H]thymidine incorporation and a colorimetric method assaying cell number.**

Results. **The normal ovarian surface epithelial cells were found to secrete both estradiol and progesterone, a production that was not regulated by FSH or LH. Addition of steroids to the cultured cells did not induce any overall significant growth effects. However, progesterone significantly inhibited the growth of ovarian surface epithelial cells from three of the patients. Enhanced thymidine incorporation was observed in the presence of the progesterone receptor antagonist Org 31710 in the nonconfluent cultures of cells from postmenopausal women, but no effect of an estrogen receptor antagonist was observed.**

Conclusions. **The normal ovarian surface epithelium is capable of steroid production, which is also often observed in tissue from ovarian epithelial tumors. Progesterone appeared to be a negative regulator of ovarian surface epithelial growth, while estradiol had no effect.**

Key Words: **ovarian surface epithelium; progesterone; estradiol; proliferation.**

INTRODUCTION

The ovarian surface epithelium is the origin of about 90% of ovarian cancers [1]. The epithelium is of mesodermal origin and covers the ovary as a single layer of squamous or cuboidal cells. The function of the epithelium is closely connected to ovulation, since it is repeatedly degraded and reconstituted during every ovulatory cycle. These monthly intensive growth periods have been postulated to imply an increased risk of mutations, a suggestion that is the basis of one hypothesis for ovarian tumor development, the incessant ovulation theory [2]. Another hypothesis for ovarian cancer development is the gonadotropin theory [3], which is based on the coincidence of elevated levels of gonadotropins and the higher incidence of ovarian cancer after menopause. Steroids are associated with the major gonadotropin-regulated ovarian functions, follicle development and corpus luteum formation. However, their possible involvement in ovarian tumor development needs to be clarified. The role of steroids in ovarian surface epithelial growth regulation is especially important in view of the increasing use of hormonal replacement therapy (HRT) for climacteric symptoms. In a study on ovarian surface epithelial cells from four women, no significant effects of steroids on proliferation of nonconfluent cells were detected [4], despite the reported presence of both estrogen and progesterone receptors in ovarian surface epithelial cells [4–6]. In several studies on growth regulation of ovarian surface epithelial cells by factors other than steroids, epidermal growth factor, platelet-derived growth factor, interleukin-1β and tumor necrosis factor α were found to stimulate proliferation [7–9], while transforming growth factor β acted as a growth inhibitor [10]. In a recent study, FSH inhibited the growth of ovarian surface epithelial cells from postmenopausal women [11].

Numerous studies have indicated steroid involvement in ovarian tumor biology. For instance, progesterone receptors have been detected in ovarian tumors in several studies [12–14], and the effects of progesterone on ovarian cancer cell lines have been reported to be antiproliferative and apoptotic [15]. Estrogen receptors are also expressed in a subset of epithelial cancers [14, 16], and endogenous production of steroids by such tumors has been reported [17]. However, the effects of estradiol on ovarian tumor cell growth have been found to be

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variable, with reports of no effects [18], as well as both enhanced [18, 19] and inhibited [20, 21] proliferation of tumor cells. In the present study, we used normal ovarian surface epithelial cells from 19 women to study possible steroid production *in vitro*, using two different culture models reflecting different stages of epithelial function. In addition to the capacity for steroid production of these cells, we also investigated the possible proliferative influence of exogenous steroids.

MATERIAL AND METHODS

Harvest and Culture of Ovarian Surface Epithelial Cells

For the proliferation experiments, cells from 19 women (9 premenopausal, 10 postmenopausal) were used. Of these cell samples, 10 were obtained during laparoscopy, and the remaining during laparatomy. This study and the technique of cell sampling have been approved by the ethical committee of Göteborg University.

To obtain ovarian surface epithelial cells, cytobrushes (Cytobrush Plus, Medscand, Sweden), normally used for cervical cytology, were used. The brushes were rotated and at the same time moved back and forth over the ovarian surface with slight pressure. For every ovary, two brushes were used. The brushes were immediately placed in culture medium, MCDB 105/M199 (1:1) supplemented with 15% fetal bovine serum (FBS; Life Technologies Ltd., Paisley, Scotland) and penicillin–streptomycin (100 IU/ml, 100 μg/ml, Life Technologies Ltd.), and taken to the laboratory [22]. The cells were released from the brushes into the medium by rubbing the brushes against each other. The medium was then centrifuged for 5 min at 300*g*, then resolved in fresh culture medium and seeded into two culture dishes (30 mm in diameter, Falcon, Becton Dickinson, Meylan, France). The media were changed every second day until the cells reached confluence. In cases where a few cells were obtained, initially only half of the medium was changed.

To verify the epithelial origin of the cells, a portion of them were seeded in chamber slides, and immunohistochemistry was performed with antibodies against low-molecular-weight cytokeratin (AE1/AE3), vimentin, and blood factor VIII (Boehringer-Mannheim, Mannheim, Germany).

Proliferation Assays

Two different culture models were used to investigate the proliferation of ovarian surface epithelial cells: a nonconfluent model where cells were seeded at low density, and a confluent model where cells were seeded at a higher density and allowed to reach confluence before the proliferation experiments were initiated.

When the primary cultures reached confluence, the cells were trypsinized, counted in a Bürker chamber, and seeded at a density of 4000 cells per well (nonconfluent cultures) or 15,000 cells per well (confluent cultures) in 96-well microtiter plates (Falcon). The cells for nonconfluent culture were allowed to attach and resume growth (overnight or for 24 h), and the cells for the confluent cultures were allowed to reach confluence (approximately 4 days), before the medium was changed to the same medium containing only 1% FBS and the different agents added. The experiments were performed in six duplicate wells (nonconfluent cultures) or double or triple wells (confluent cultures) depending on the cell yield after trypsinization.

The cells were exposed to 17β-estradiol (10 ng/ml, Sigma Chemicals), progesterone (50 ng/ml, Sigma Chemicals), the estrogen receptor antagonist ICI 178,780 (10^{-7} M, Tocris Cookson Ltd, Bristol, UK) [23], and the progesterone receptor antagonist Org 31710 (10^{-7} M, Organon, Oss, The Netherlands) [24]. To control cultures, equal amounts of PBS with the equivalent percentages of ethanol and DMSO (dimethyl sulfoxide) were added instead of the agent studied.

The cultures were pulsed with [^{3}H]thymidine (2 μCi/ml) for 3 h before precipitation with 10% trichloroacetic acid (TCA) at 4, 24, and 48 h after the start of stimulation. The precipitates were washed twice with 5% TCA and dissolved in warm 0.1 M NaOH, and the radioactive content was determined in a liquid scintillation counter (Packard, Meriden, CT).

The change in cell number was also detected with the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI). This is an assay that measures bioreduction of the tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium: MTS) to a soluble formazan that absorbs light at 492 nm. This reaction is accomplished by dehydrogenase enzymes found in metabolically active cells, and thus the amount of formazan generated is proportional to the number of living cells. Twenty microliters of the MTS reducing agent was added to each well containing 0.1 ml medium, and incubated for 3 h, before being analyzed at 492 nm in a plate reader spectrophotometer (UVmax; Molecular Devices Corp., Sunnyvale, CA).

Detection of Estradiol and Progesterone

Media from FSH-exposed (0.5 IU/ml Puregon; Organon, Oss, The Netherlands) or LH-exposed (0.1 IU/ml LHADI; Serono Laboratories Inc., Rome, Italy) ovarian surface epithelial cells in both confluent and nonconfluent cultures, as well as from nonexposed control wells, were aspirated after 48 h of culture in 96-well plates, and assayed with Delfia Estradiol and Progesterone kits (Wallac Inc., Gaithersburg, MD).

Statistical Analyses

Student's paired *t* test was used to evaluate differences between control groups and treated groups. The original proliferation data were natural logarithm-transformed to enable comparison despite large differences in control levels between different experiments. Proliferation data are displayed as percentages of control in Figs. 1 and 2.

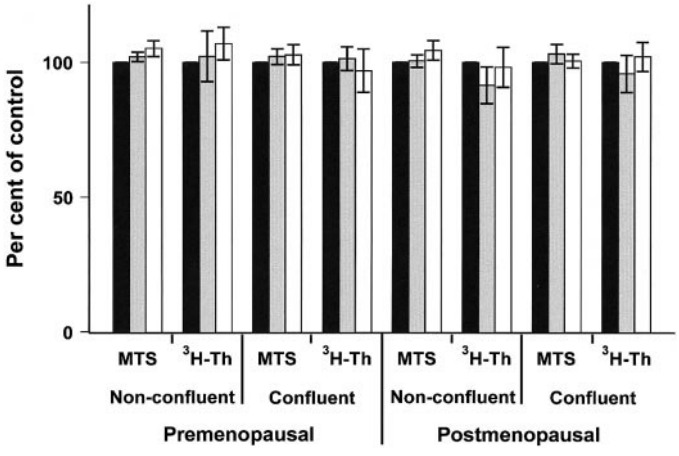

FIG. 1. Effects of progesterone on ovarian surface epithelial growth (mean ± SEM) evaluated with the MTS method and thymidine incorporation (^{3}H-Th). Effects after 24 h (gray bars) and 48 h (white bars) are compared with results for the untreated controls (black bars) expressed as 100%.

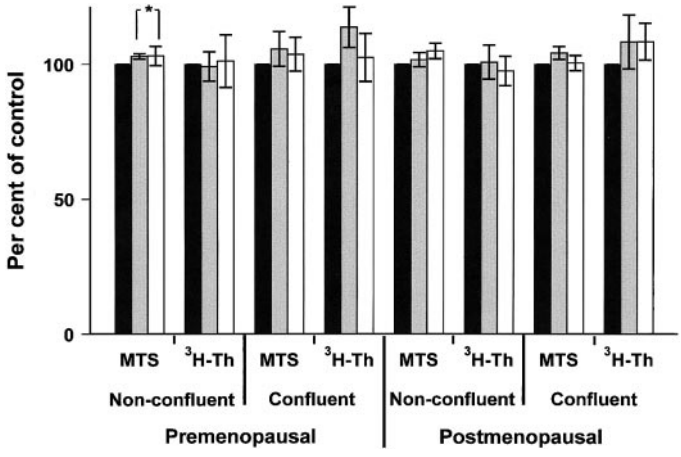

FIG. 2. Effects of estradiol on ovarian surface epithelial growth (mean ± SEM) evaluated with the MTS method and thymidine incorporation (^{3}H-Th). Effects after 24 h (gray bars) and 48 h (white bars) are compared with results for the untreated controls (black bars) expressed as 100%. *Significantly ($P < 0.05$) higher than control.

RESULTS

Effects of Progesterone on Growth of Ovarian Surface Epithelial Cells

Cells from 19 women (9 premenopausal, 10 postmenopausal) were used for studies on progesterone effects. No significant overall changes could be detected after stimulation with progesterone (Fig. 1). Even if no significant effect of progesterone was established when data from all cultures were analyzed together, one of the premenopausal and two of the postmenopausal cultures displayed significant ($0.05 > P > 0.001$) growth inhibition, approximately 30%, by progesterone (data not shown), as detected by thymidine incorporation.

Effects of Estradiol on Growth of Ovarian Surface Epithelial Cells

Cells from 19 women (9 premenopausal, 10 postmenopausal) were used for studies on estradiol effects. There were no significant effects on ovarian surface epithelial growth of estradiol, except in the nonconfluent cultures of premenopausal cells where a small increase in growth after 24 h was detected with the MTS method ($P < 0.05$) (Fig. 2).

Production of Steroids by Ovarian Surface Epithelial Cells in Culture

Media from 9 cultures (5 premenopausal, 4 postmenopausal), from both nonconfluent and confluent cultures, were assayed for steroids. The estradiol and progesterone levels in the unconditioned media containing 1% FBS were below the detection limit of the assays (<0.01 nmol/liter).

Progesterone was detected in 8 of the 9 cultures. In cultures of cells from premenopausal women the levels varied from nondetectable (<0.01 nmol/liter) to 0.6 nmol/liter, and the levels from postmenopausal cells varied between nondetectable and 6.6 nmol/liter. In the confluent model, cells from postmenopausal women secreted about 20 times more progesterone compared with cells from premenopausal women (Fig. 3). No such difference was observed in the nonconfluent model. No significant stimulatory or inhibitory effect of FSH or LH on progesterone production was established.

Estradiol was detected in all cultures analyzed, with levels varying between 2.3 and 423 nmol/liter (mean 192.3 nmol/liter). No difference between cultures from pre- and postmenopausal women was detected (Fig. 4). No significant effects of FSH or LH on estradiol levels were observed.

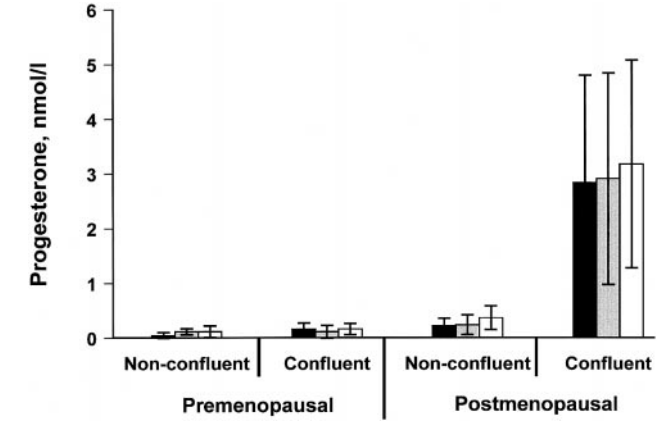

FIG. 3. Progesterone levels (mean ± SEM) in medium collected after 48 h of culture. FSH-stimulated (gray bars) and LH-stimulated (white bars) cultures are compared with untreated controls (black bars).

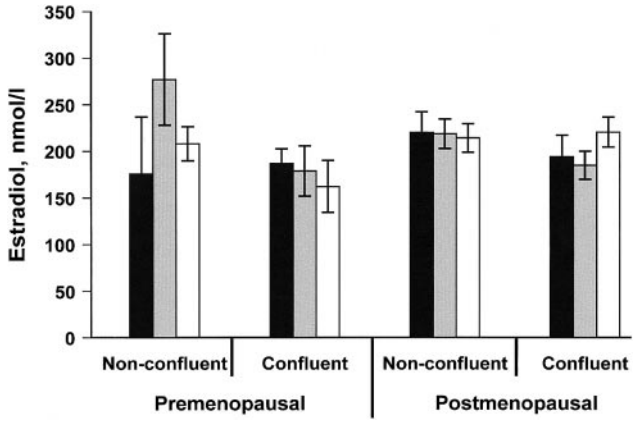

FIG. 4. Estradiol levels (mean ± SEM) in medium collected after 48 h of culture. FSH-stimulated (gray bars) and LH-stimulated (white bars) cultures are compared with untreated controls (black bars).

Effects of Steroid Receptor Antagonists on Growth of Ovarian Surface Epithelial Cells

To rule out possible growth effects of endogenously produced estradiol and progesterone, four additional experiments were performed in the presence of the progesterone receptor antagonist Org 31710 or the estrogen receptor antagonist ICI 182,780.

Org 31710 enhanced thymidine incorporation in all three analyzed nonconfluent cultures with cells from postmenopausal women (Fig. 5), while no effect was detected in the corresponding confluent cultures or in the culture with cells from a premenopausal woman (data not shown). No effects of ICI 182,780 were detected in these experiments (data not shown).

DISCUSSION

In this study we investigated the role of steroids in the biology of normal ovarian surface epithelial cells *in vitro.* We found that these cells are capable of both estradiol and progesterone production, and that progesterone may be an inhibitor of ovarian surface epithelial growth, since a progesterone receptor antagonist significantly increased the proliferation rate in cells from postmenopausal women.

The finding of endogenous production of steroids by ovarian surface epithelial cells from both pre- and postmenopausal women shows that the epithelium is contributing to the steroid production of the ovary. This finding also indicates that secretion of steroids by ovarian tumors [17] is not a tumor-specific property but rather a normal feature of ovarian surface epithelial cells that is preserved during malignant transformation. The ability for steroid production may be a reflection of the metaplastic potential of the normal ovarian surface epithelium [25], which is believed to be the embryological source of several steroid-producing cell types in the ovary [26, 27].

The levels of estradiol in conditioned media did not differ between cultures of cells from pre- and postmenopausal women. Progesterone secretion, on the other hand, was about 20 times higher from postmenopausal cells in the confluent model than from premenopausal cells in the same setting. This indicates that there is upregulation of steroidogenic pathways in these cells, an upregulation that could be acquired in the postmenopausal *in vivo* situation, possibly by long-time exposure to high gonadotropin levels. However, steroid production by ovarian surface epithelial cells *in vitro* was found not to be regulated by the added gonadotropins. This is in agreement with several studies on steroid production in ovarian cancer cells where gonadotropins appeared to have no effect [17, 28]; contrasting results also exist [29].

In the present study, exogenous progesterone did not induce any significant change in the growth rate of ovarian surface epithelial cells. However, individual effects were seen in cells from three patients, in which progesterone significantly inhibited ovarian surface epithelial growth. The lack of effects of exogenous progesterone in most of the cultures may be due to saturation of progesterone receptors by the endogenously produced steroid. The levels of endogenously produced progesterone were in the physiological range and about 50 times lower than the added dose. To evaluate any possible effects on proliferation of this endogenously produced progesterone, the progesterone receptor antagonist Org 31710 was added to the cultures. Org 31710 is a newly developed compound that exhibits binding affinity to the cytosolic progesterone receptor similar to that of RU 486, but displays only 1/30-th of the binding of RU 486 to the glucocorticoid receptor [24]. No agonistic growth effects of this compound have been reported. In our experiments, significant enhancement of thymidine incorporation was observed in all three nonconfluent cultures with cells from postmenopausal women. This may indicate that endogenous progesterone has a growth-inhibitory function that

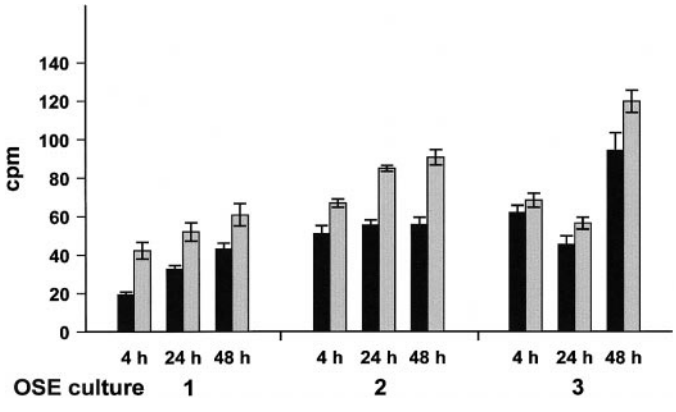

FIG. 5. Effects of Org 31710 on thymidine incorporation in nonconfluent cultures of cells from three postmenopausal women. Gray bars represent Org 31710-treated cultures and black bars represent untreated controls. Error bars indicate variations (SEM) between individual wells ($n = 6$) of each experiment.

can be blocked by Org 31710. These results are interesting with respect to ovarian cancer development, since the protective effect of oral contraceptives has recently been suggested to be exerted not only by counteracting incessant ovulation, but also through progesterone-induced activation of apoptotic pathways [7]. The suggestion of an antiproliferative function of progesterone seems even more relevant when the observation of decreasing levels of progesterone receptors in epithelial tumor cells compared with levels in normal ovarian surface epithelial cells is considered [6]. This hypothesis is also supported by the antiproliferative and apoptotic effects of progesterone that have been demonstrated in ovarian carcinoma cell lines [15]. The effects in that study correlated with upregulation of the tumor suppressor gene p53, indicating involvement of this protein in progesterone-induced apoptosis.

Increasing parity is associated with a reduction in the risk of ovarian cancer, and in a study by Adami and co-workers [30], the risk of cancer decreased by increment of age at first childbirth. Since those findings cannot be readily explained by theories involving incessant ovulation or high serum concentrations of gonadotropins, a pregnancy-dependent clearance, e.g., by the suggested progesterone-induced apoptotic mechanism, of cells that have undergone malignant transformation might explain the findings.

There are several reports of estrogen receptors in normal human ovarian surface epithelium, indicating that steroids are involved in the normal function of the surface epithelium of the ovary. Focal expression of estrogen receptors has been demonstrated in ovarian surface epithelium in sections of normal ovaries [31] and also in ovarian surface epithelial cells in primary culture and in early passages [4–6]. As in the case with progesterone, no significant general growth changes of ovarian surface epithelial cells were induced by exogenously added estradiol in the present study. Since the concentration of endogenously produced estradiol after 48 h of culture was found to be very high compared with physiological levels, and on average five times higher than the added dose, this was expected. The lack of effect of estradiol on ovarian surface epithelial proliferation in this study is in agreement with a previous report [4]. In the present study, we wanted to extend these investigations by using an estrogen receptor antagonist to evaluate possible effects of endogenous estradiol. However, not even addition of the estrogen receptor antagonist ICI 182,780 influenced proliferation in these cultures. Taken together, these results convincingly demonstrate that endogenous estradiol production by normal ovarian surface epithelial cells is not part of an autocrine loop influencing proliferation. Thus, the estradiol–estrogen receptor system in ovarian surface epithelium is probably regulating other functions, such as regulation of the secretion of matrix proteins or cytokines, properties that have been attributed to ovarian surface epithelial cells [32–34]. However, an alteration of the estradiol–estrogen receptor system during malignant transformation may lead to autocrine stimulation of proliferation in ovarian cancers. Such an autocrine mechanism would be supported by reports of estradiol production [17], presence of estrogen receptors [13, 35], and proliferative effects of estradiol [18, 19] observed in several ovarian tumors.

The use of hormone replacement therapy (HRT) has been accompanied by several studies on the possible risk of induction of ovarian tumors with this therapy. One meta-analysis indicates that there is an increased risk of ovarian cancer connected to long-term HRT [36]. The results of the present study, however, support several contradictory reports (for a review see [37]) and indicate that such a possible risk is not mediated through enhanced growth of the cells. In contrast to the reports of enhanced cancer risk connected to HRT, the gonadotropin theory proposes that the elevated levels of gonadotropins after menopause, due to the loss of steroid regulation, contribute to ovarian tumor development, and hence the effect of HRT would rather be protective.

In conclusion, the results of this study show that ovarian surface epithelial cells are capable of steroid production *in vitro.* We also show that a progesterone receptor antagonist induces increased growth of these cells, indicating that physiological levels of progesterone may have an inhibitory function on ovarian surface epithelial proliferation.

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