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ARTICLES

Suppression of the Transformed Phenotype and Induction of Differentiation-Like Characteristics in Cultured Ovarian Tumor Cells by Chronic Treatment With Progesterone

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Epidemiological evidence suggests that elevated levels of the pregnancy hormone progesterone might play a role in the reduced risk of women to develop ovarian cancer. In vitro studies have supported this hypothesis by demonstrating negative effects of this hormone on the growth and proliferation of cultured ovarian carcinoma cells. However, little is known about the underlying molecular processes and how progesterone might decrease the risk for ovarian tumors. Therefore, we investigated the effects of chronic hormone treatment on the cell-cycle and transformed phenotype of ovarian carcinoma cell lines in vitro. We found that long-term treatment of these cells with progesterone caused a concomitant reduction of cyclin-dependent kinase (CDK) activity. In parallel, these cells lost their transformed phenotype as indicated by the acquisition of contact inhibition and the loss of anchorage-independence, as well as the reduced expression of tumor markers such as heat shock protein (HSP) 72 and carcinoma antigen (CA) 125. In addition, progesterone-treated cells exhibited characteristics that resembled a more differentiated phenotype. Taken together, our data indicated that progesterone was able to suppress the transformed phenotype of ovarian tumor cells. This observation could serve to explain progesterone's alleged protective effect in ovarian carcinogenesis.

Key words: ovarian carcinoma; cell-cycle; differentiation; progesterone

INTRODUCTION

Ovarian carcinoma is the fourth most common cause of cancer death among women in the United States, and is the most lethal gynecological malignancy. Estimates by the American Cancer Society for the year 2002 predict 23 300 new cases and 13 900 deaths from this disease in the US [1]. Ovarian cancer is difficult to treat because patients frequently present late in the course of the disease, which may be asymptomatic until advanced stages. There is strong epidemiological evidence for an association between ovulatory activity and risk of ovarian tumorigenesis; i.e., pregnancy and the use of oral contraceptives, both of which suppress ovulation, are associated with decreased risk [2–7]. This latter observation prompted the hypothesis of ''incessant ovulation,'' which holds that factors that suppress ovulation may reduce the risk of developing cancer of the ovary [8,9]. Similarly, it was noted that reduced gonadotropin stimulation may occur during pregnancy and oral contraceptive use and that this factor could possibly reduce the risk of ovarian cancer as well [10]. This view is supportive of the ''gonadotropin stimulation'' hypothesis which holds that increased gonadotropin release may stimulate ovarian tumorigenesis [see detailed refs. in 7].

Several epidemiological observations cannot be readily explained by the incessant ovulation theory or by the gonadotropin stimulation hypothesis [e.g., see refs. 7,11,12]. Risch [7] suggested that elevated levels of the pregnancy hormone progesterone might play a role in the reduced risk of developing ovarian cancer. Progesterone as a protective factor would be completely compatible with the observed

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Abbreviations: CDK, cyclin-dependent kinase; HSP, heat shock protein; CA, carcinoma antigen; P4, progesterone.

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risk reduction associated with pregnancy, breast feeding, oral contraceptives, physical exercise, and twin pregnancies [7].

Recent studies have analyzed the effects of progesterone on ovarian cell growth in vitro. In this regard, it has been consistently observed that progesterone acts as a negative regulator of the growth of normal, precancerous, and cancerous ovarian cells [13–18]. Moreover, at higher concentrations, progesterone is able to induce apoptotic cell death in various ovarian cancer cell lines [13,15], an observation that has been considered by Syed et al. [17] as a mechanistic explanation of the ''pregnancy clearance effect'' [11], which postulates that pregnancy rids the ovaries of cells that have undergone malignant transformation.

While these epidemiological and experimental data strongly suggest that elevated levels of progesterone might reduce the incidence of ovarian carcinomas, little is known about the molecular mechanism that may underlie such a process. Therefore, we have set out to investigate the effects of progesterone on transformation-specific characteristics of ovarian carcinoma cells during long-term treatment in culture. In the present report, we have shown that chronic treatment with progesterone caused ovarian carcinoma cells to lose typical tumor cell characteristics; i.e., the cells assumed growth in a contact-inhibited manner, lost their ability to grow anchorage-independently, and downregulated their expression of two tumor-associated markers. Moreover, the cells acquired characteristics that resembled a more differentiated phenotype. These observations supported the idea that elevated levels of progesterone may directly downregulate the malignant potential of ovarian epithelial tumors.

MATERIALS AND METHODS

Source of Reagents

Progesterone (4-pregnene-3,20-dione) was purchased from Sigma (St. Louis, MO) and dissolved in absolute ethanol at 100 mM. Before addition to cell cultures to establish the various final concentrations, the stock solution was appropriately diluted so that each culture would receive the same amount of the vehicle ethanol.

Cell Lines and Culture Conditions

HOC-7 human ovarian carcinoma cells were originally obtained from R. Buick (University of Toronto, Canada) [19]. OVCAR-3 cells were purchased from the American Tissue Culture Collection (ATCC, Manassas, VA: #HTB161). Both cell lines are positive for progesterone receptor expression [18]. The cells were maintained in RPMI 1640 (Gibco BRL, Grand Island, NY) supplemented with 2% fetal bovine serum (qualified, Australia sourced from Gibco BRL). In addition, the culture medium contained 100 U/mL penicillin and 0.1 mg/mL streptomycin. The cells were kept in a humidified incubator at 37°C and a 5% CO_2 atmosphere.

Western Blotting

Total cell lysates were prepared by lysis of cells with RIPA buffer [20]. Thirty micrograms of each sample was processed by Western blot analysis as described [21]. Antibodies against cell-cycle–regulatory proteins were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against heat shock protein (HSP) 72 were kindly provided by Roger Duncan (University of Southern California, Los Angeles, CA). The secondary antibodies were coupled to horseradish peroxidase, and were detected by chemiluminescence with the SuperSignal™ substrate from Pierce (Rockford, IL).

In Vitro Kinase Assays

Cells were lysed in RIPA buffer [20] and protein concentrations were determined with the bicinchoninic acid (BCA) protein assay reagent (Pierce). One hundred micrograms of each lysate was used for immunoprecipitation with 1 μg of either anti-cdk2 or anti–cyclin B antibodies. The antigen-antibody complexes were collected with proteinA-sepharose, washed twice with RIPA and three times with kinase buffer (50 mM Tris pH 7.4, 10 mM $MgCl_2$, 1 mM DTT). Twenty-five microliters of kinase buffer containing 2 μg histone H1 protein, 50 μM ATP, and 5 μCi [γ-^{32}P]ATP (3000 Ci mmol^{-1}) was added, and the mixture was incubated on a hard rocking platform at room temperature for 20 min. The kinase reaction was stopped by the addition of 40 μl 2× Laemmli sample buffer [20] and boiling for 8 min. The reaction products were separated on a 12% acrylamide gel and exposed to Kodak X-AR film. After exposure, the gel was stained with Coomassie blue and dried. All reactions were repeated and quantitated with the AMBIS Radioanalytic Imaging System.

Soft Agar Assays

The bottom of 6-well plates was covered with 2 mL 0.8% agar in RPMI 1640 supplemented with 10% fetal bovine serum and the antibiotics penicillin and streptomycin. On top of this layer, 10^4 cells in 3 mL 0.35% agar, mixed with the same medium and supplements as well as different concentrations of progesterone, was added. These soft agar–embedded cells were kept in a humidified incubator at 37°C and a 5% CO_2 atmosphere. After 5 d, 1 mL of medium containing the appropriate concentrations of progesterone was added on top of the agar and replaced with fresh medium (±progesterone) every 5 d for up to a month.

Determination of Carcinoma Antigen (CA) 125 Level

The levels of CA125 in conditioned media from cultured cells were determined by microparticle

enzyme immunoassay (MEIA) on the AxSYM immunoassay analyzer (Abbott Laboratories, Abbott Park, IL). The specific protocol provided by the manufacturer was followed (Protocol CA125, pp 1-8, October 1999, Abbott Laboratories), which is similar to the one published by others [22]. The whole procedure was performed by an analytical laboratory here at USC that routinely executes this same assay with patient samples and as a rule includes appropriate controls.

Measurement of Transepithelial Resistance (TER)

HOC-7 cells were plated on tissue culture-treated polycarbonate (Nucleopore) filter cups (0.4 μm pore size; 1.1 cm^2; Transwell [Corning-Costar, Cambridge, MA]) and cultured in regular medium with and without progesterone for several weeks. In parallel, the cells were also cultured on clear filters (0.4 μm pore size; 1.1 cm^2; Transwell-clear [Corning-Costar]) to allow for microscopic inspection of confluency and cell shape. TER was measured aseptically with a rapid screening device (Millicell-ERS [Millipore, Bedford, MA]) and normalized for the area of the filter after background subtraction [23]. All measurements were repeated.

RESULTS

Treatment of HOC-7 or OVCAR-3 ovarian carcinoma cells with increasing concentrations of progesterone (P4) in vitro resulted in concentration-dependent growth inhibition (Figure 1). While 3 μM P4 had a negligible effect, higher concentrations of 10 and 30 μM significantly reduced the proliferation rate of these cells. These results are in agreement with earlier observations that P4 is able to reduce the rate of DNA synthesis in various ovarian tumor cell lines [13–18].

Because we had shown earlier that P4 is able to affect cell-cycle–regulatory mechanisms of nontumorigenic ML5 ovarian cystadenoma cells [18], we next determined whether this would also be the case in malignant carcinoma cells. For this purpose, HOC-7 cells were treated with different concentrations of P4, and the activity of one of the cyclin-dependent kinase (CDK) complexes, cyclinB/cdk1, was analyzed. As shown in Figure 2A, there was a concentration-dependent decrease in CDK activity. At 10 μM, P4 led to a 50% decrease in kinase activity within 24 h, which was further reduced slightly to 42% over the course of the following 2 d. At 30 μM, P4 caused a similar reduction initially; however, the decline continued and after 3 d CDK activity was barely detectable. Thus, the observed concentration-dependent growth-inhibitory effects of P4 (Figure 1) correlated with a decrease in CDK activity (Figure 2A). Similar inhibitory effects on CDK activity were observed with other malignant ovarian carcinoma cell lines as well, such as OVCAR-3 and Hey cells (not shown).

In order to determine the underlying molecular events that led to the observed decrease in CDK enzymatic activity, we analyzed the expression level of various cell-cycle-regulatory proteins that are known to impinge on this activity. Cyclin B and, to a lesser extent, cyclin A are the regulatory subunits of CDK complexes containing cdk1. We found that the expression of these two subunits was differentially affected by treatment of cells with P4 (Figure 2B). Cyclin A was only marginally affected by 10 μM and weakly reduced at 30 μM P4. In contrast, cyclin B was strongly reduced by 10 μM and was undetectable at 30 μM P4. In addition, we analyzed the expression of two CDK inhibitors, $p21^{Cip1}$ and $p27^{Kip1}$, which are known to bind to various CDK complexes and inhibit their activity. In this case, we found significantly increased levels of both of these proteins (Figure 2B), similar to what we had observed earlier with the use of ML5 ovarian cystadenoma cells [18].

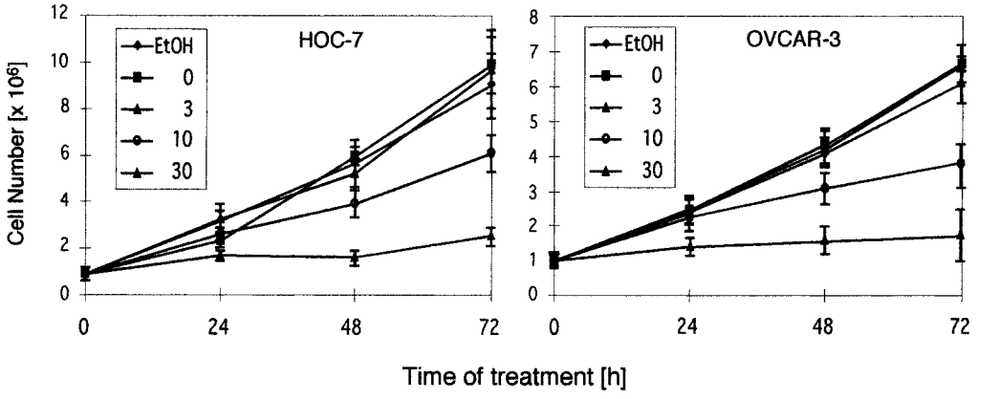

Figure 1. Cell growth in the presence of progesterone. HOC-7 and OVCAR-3 cells were seeded into cell culture dishes 1 d before the experiment. At time 0 h, different concentrations of progesterone were added to the cell culture medium as indicated. As a control, the same amount of vehicle only (ethanol [EtOH]) was added to parallel cultures. Every 24 h thereafter, the cells were counted for up to 72 h. Shown is the mean (±SD) of three measurements for each point. This experiment was repeated with essentially the same results.

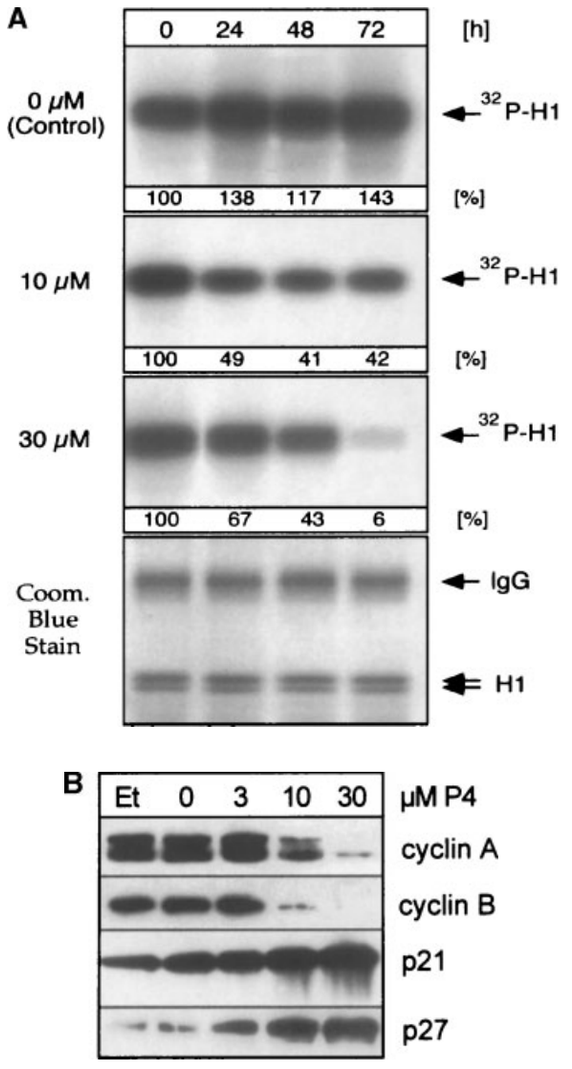

Figure 2. Cell-cycle–regulatory components during short-term culture. HOC-7 cells were cultured in the absence or presence of different concentrations of progesterone as indicated. Part A presents autoradiographs of in vitro kinase reactions where antibodies to cyclin B had been used to immunoprecipitate the cyclin-dependent kinase (CDK) complex cyclinB/cdk1. The bottom panel shows one of the gels stained with Coomassie blue. This staining was performed with every gel as a control to verify that the same amount of antibody (IgG) was recovered from each immunoprecipitation, and also to confirm that the same amount of the substrate histone H1 (H1) was added to each in vitro kinase reaction. This experiment was repeated twice with very similar outcomes; a typical result is shown here. Part B represents Western blots that demonstrate the expression levels of the cell-cycle-regulatory proteins cyclin A and cyclin B, as well as the two CDK inhibitors $p21^{Cip1}$ and $p27^{Kip1}$ after 60 h of progesterone treatment. Repetition of this experiment essentially yielded the same results.

As the proliferation of cells critically depends on CDK activity, the above experiments indicate that the growth inhibitory effects of P4 are likely mediated by a combination of two effects, namely the strongly increased expression of CDK inhibitors together with a pronounced decrease in the overall amount of cyclin B.

To investigate whether the reduction of CDK activity would be maintained for extended periods of time if P4 was continuously present in the culture medium, HOC-7 cells were cultured for up to a month in the presence or absence of P4. As shown in Figure 3A, after several weeks, the CDK kinase activity pattern was still comparable to what was observed within the first 3 d of treatment (compare Figure 2A); i.e., 10 μM led to a pronounced reduction in CDK activity, which was even further reduced at 30 μM P4. At the protein level, both cyclin A and cyclin B were strongly reduced at 30 μM P4, whereas only cyclin B was reduced at 10 μM. Conversely, the CDK inhibitor $p21^{Cip1}$ was elevated at 10 μM P4 and

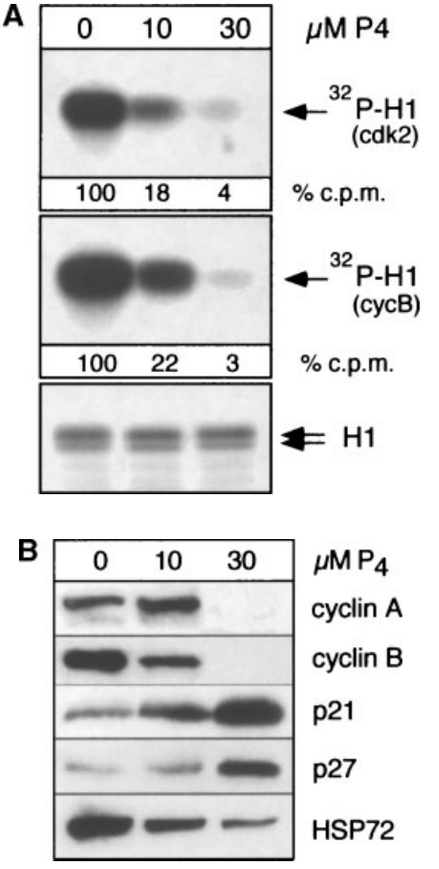

Figure 3. Cell-cycle–regulatory components during long-term culture. HOC-7 cells were cultured in the absence or presence of different concentrations of progesterone for 26 d. Part A presents autoradiographs of in vitro kinase reactions where antibodies to either cdk2 or cyclin B (cycB) were used to immunoprecipitate the respective CDK complexes. After exposure to X-ray film, all gels were stained with Coomassie blue to verify that the same amount of the substrate H1 was added to each in vitro kinase reaction. Only one of the stained gels is shown exemplarily in the bottom panel. The same experiment was repeated with either 22 or 31 d of incubation with progesterone and essentially yielded the same results. Part B represents Western blots that demonstrate the expression levels of the cell-cycle–regulatory proteins cyclin A and cyclin B, as well as the two CDK inhibitors $p21^{Cip1}$ and $p27^{Kip1}$. In addition, the same lysates were also analyzed for the amount of HSP72 present.

even more so at 30 μM, whereas $p27^{Kip1}$ showed no increase at 10 μM P4 but a prominent induction at 30 μM (Figure 3B).

Cells cultured in the presence of 10 or 30 μM P4 continued to proliferate (although at a reduced rate; see Figure 1) and eventually formed a confluent monolayer. While control cells completely covered the cell culture dish after approximately 4–5 d, it took them 6–7 d at 10 μM, and approximately 10 d at 30 μM P4. However, when confluent cell layers were inspected microscopically, an obvious alteration in the morphology of P4-treated cells became apparent. As displayed in Figure 4, untreated control HOC-7 cells formed a disorganized monolayer structure with numerous three-dimensional foci. Thus, these cells exhibited the typical appearance of transformed cells, including the loss of contact inhibition. In stark contrast, cells treated with 10 μM P4 appeared more cubic, and the resulting monolayer exhibited a rather uniform cobblestone pattern. Not a single focus was observed, indicating that these cells might have regained contact inhibition (Figure 4). Cells chronically treated with 30 μM P4 displayed yet another morphology. They were considerably enlarged and presented elongated, round extensions. Similar to the cells treated with 10 μM, these cells also did not grow three-dimensionally and did not form foci, even after a month of reaching confluency.

The new phenotype of contact inhibition of P4-treated cells indicated that these cells had lost a characteristic feature of tumor cells in response to hormone treatment. To investigate whether this would also hold true for other ovarian tumor cell lines, we cultured two more cell lines, OVCAR-3 and Hey cells, in the presence of P4 for extended time periods. However, we noticed that Hey cells were not useful for this type of study because even the untreated control cells did not form foci under our culture conditions (not shown); thus, the acquisition of contact inhibition could not be studied with this cell type. OVCAR-3 cells, however, did form large three-dimensional foci and therefore were used for this analysis. As can be seen in Figure 4, similar to the case of HOC-7 cells, OVCAR-3 cells treated with 10 μM P4 grew only two-dimensionally and did not form three-dimensional structures. Similarly, OVCAR-3 cells treated with 30 μM P4 were considerably enlarged and did not form foci either (Figure 4).

Because the above results indicated that treatment with P4 might have influenced the manifestation of the transformed phenotype, another typical feature of transformed cells, anchorage-independent growth, was analyzed. For this purpose, cells were seeded into soft agar to determine whether P4 would affect their ability to grow in suspension, i.e., anchorage-independently. As shown in Table 1, this

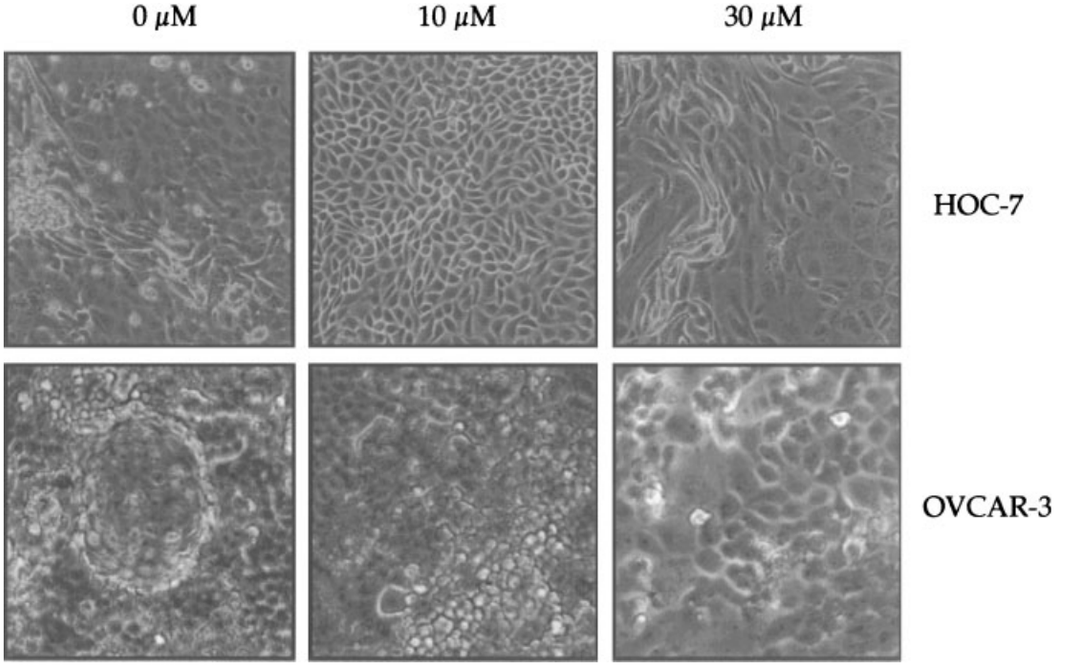

Figure 4. Morphology of cells during long-term culture. HOC-7 cells (top panel) or OVCAR-3 cells (bottom panel) were cultured in the absence or presence of different concentrations of progesterone. After reaching confluency, the cell cultures were not trypsinized, but rather kept on the same dish. The medium (±progesterone) was replaced every 2–3 d and photos were taken after 22 d. Note that in the absence of progesterone, both cell lines have developed three-dimensional foci, whereas in hormone-treated cells no foci are present. This experiment was repeated several times with HOC-7 cells, and the absence of foci was consistently observed when progesterone was present. All photos were taken at the same magnification (light microscope at 40×).

Table 1. Growth in Soft Agar

Concentration of P4 (μM)	Number of colonies			
	HOC-7		OVCAR-3	
	Expt. no. 1	Expt. no. 2	Expt. no. 1	Expt. no. 2
0	>100	>100	67	88
10	0	0	7	3
30	0	0	0	0

HOC-7 or OVCAR-3 (10^4) cells were suspended in 3 mL of soft agar in the presence or absence of either 10 or 30 μM progesterone. Control cells received the same volume of vehicle (ethanol). In the absence of progesterone, colonies of cells started to appear at around day 10 and were counted on day 16. Colonies were defined as clusters of cells that clearly contained more than approximately 10 cells (most colonies contained substantially more than 10 cells). In the presence of progesterone, very small aggregates of cells (<10 cells) became apparent, but did not increase in size, even after an additional 10 d of incubation.

was indeed the case. While untreated cells were able to form numerous colonies in soft agar, cells treated with 10 or 30 μM P4 were unable to do this. Even when incubated for extended time periods of up to 4 wk, few or no colonies became apparent in P4-treated cultures. Thus, it appeared that hormone treatment was able to abrogate the ability of these cells to grow anchorage independently.

The above results suggested that chronic treatment with P4 might cause a suppression of the transformed phenotype. To obtain further evidence of such a process, we investigated two common tumor markers, HSP72 and CA125. The amount of HSP72 is frequently found elevated in many types of tumor cells, whereas CA125 is a secreted antigen that is often found elevated in the serum of patients with ovarian carcinoma.

The amount of HSP72 was determined by Western blotting of lysates from cells that were chronically treated with P4. As shown in Figure 3B (bottom panel), the expression level of this tumor marker was diminished in cells treated with 10 μM P4, and was even further reduced in cells treated with 30 μM hormone. Similarly, the amount of secreted CA125, which was determined by an ELISA-based assay, was strongly reduced by increasing concentrations of long-term P4 treatment (Figure 5). Thus, in combination with the above observations that P4-treated cells lose specific characteristics of tumor cells, such as anchorage independence and the ability to form foci, these results indicated that long-term treatment with P4 might suppress the transformed phenotype of these HOC-7 cells.

As shown above (Figure 4), HOC-7 cell cultures treated with 10 μM P4 appear more organized and form a cobblestone pattern characteristic of normal epithelial cells in general [24,25]. This led us to speculate that P4-treated cells might have reverted to a more differentiated state. A characteristic feature of epithelial tissues is the formation of tight junctions at cell-cell borders that block the paracellular transport of ions; this leads to increased electrical resistance when voltage and current are measured on opposite sides of the epithelial layer [26,27]. When we determined this TER in hormone-treated cells, we found that 10 μM P4 caused a fivefold increase in TER (Figure 6). At 30 μM P4, however, no increase was observed. Thus, the development of the cobblestone pattern at 10 μM P4 correlated with substantially increased TER. Untreated control cells or cells treated with 30 μM P4, neither of which demonstrate the cobblestone pattern, do not display high levels of TER. Because the measurement of

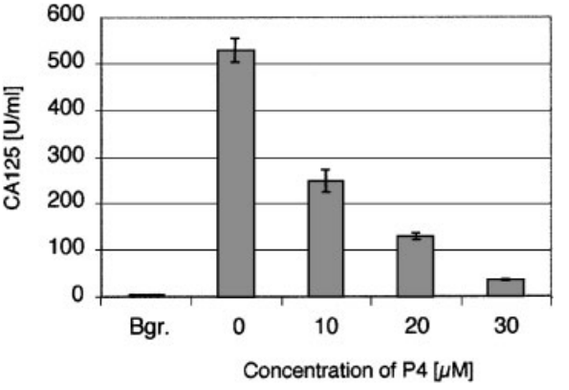

Figure 5. Secretion levels of CA125. HOC-7 cells were cultured in the presence or absence of progesterone and allowed to form a confluent monolayer. After 15 d, 2-d–old culture medium was collected, centrifuged briefly to eliminate any cells, and analyzed for the levels of CA125 by ELISA assay. Shown is the mean (±SD) from three separate measurements. Control background (Bgr.) indicates medium only (not supernatant); in this case, no CA125 was detected (the detection limit of this assay is <10 U/mL). See further details in Materials and Methods. In parallel, cell counts were performed to determine the number of cells constituting the confluent monolayers. Monolayers established in the presence of 10 or 20 μM progesterone contained essentially the same number of cells as untreated control cultures, whereas cultures grown in the presence of 30 μM progesterone had only a third of the cells as control cultures. Thus, when secreted CA125 levels are adjusted to the number of cells per culture (not shown), the significant reduction in CA125 levels in the presence of progesterone remains apparent.

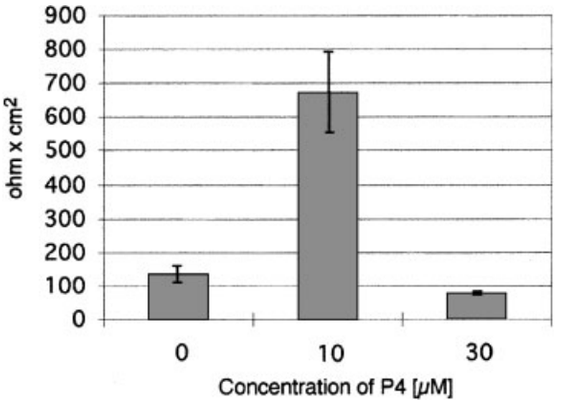

Figure 6. Transepithelial resistance (TER) measurements. HOC-7 cells were grown on polycarbonate filters in the presence or absence of progesterone at the concentrations indicated. After 28 d, TER was determined. The values shown are the mean (±SD) of three measurement after subtraction of background resistance. The whole experiment was repeated with very similar results.

increased TER reveals the formation of efficient tight junctions, these results indicate that P4 at 10 μM might induce a functional epithelialization of HOC-7 carcinoma cells.

DISCUSSION

It has been suggested that elevated levels of the pregnancy hormone progesterone might reduce the risk of developing ovarian cancer [7]. The observed risk reduction associated with pregnancy, breast feeding, oral contraceptives, physical exercise, and twin pregnancies would be compatible with this hypothesis, as all these conditions involve elevated levels of P4 [7]. In further support, in vitro studies have shown that the proliferation of cultured normal and tumorigenic ovarian cells is reduced in the presence of this hormone [13–18]. While some details of the cell-cycle–regulatory effects of progesterone have been studied in breast cancer cell lines [28–31], little is known about these mechanisms in malignant ovarian carcinoma cells. In order to gain insight into the processes by which P4 affects ovarian carcinoma cell growth, we used HOC-7 and OVCAR-3 ovarian carcinoma cells and cultured them in the continuous presence of elevated levels of P4. Unlike the above cited studies that investigated P4's effect on cellular proliferation, we did not terminate our observations after a few days, but continued some of the experiments for more than 4 wk.

Our analysis of the cell-cycle machinery clearly demonstrated that the initial downregulation of CDK activity in response to P4 treatment was maintained in these cells for up to a month, and probably permanently for as long as elevated levels of P4 remained present. The observed decline in CDK activity was likely due to a twofold mechanism: the increased expression of the two CDK inhibitors, $p21^{Cip1}$ and $p27^{Kip1}$, in combination with a reduction of cyclin B expression (see Figs. 2B and 3B). Elevated levels of $p21^{Cip1}$ are known to affect CDK activity at two different levels: the first is posttranslationally, where $p21^{Cip1}$ is able to bind to CDK complexes and thereby inhibit their enzymatic activity [32]; the second is at the transcriptional level, where $p21^{Cip1}$ has been shown to inhibit cyclin B expression through the downregulation of cyclin B promoter activity [33,34]. Although it needs to be formally established, it is reasonable to assume that a similar process might take place in HOC-7 cells.

A prominent feature of HOC-7 carcinoma cells that have been treated with 10 μM P4 is their striking morphological change (Fig. 4). Whereas monolayers of untreated control cells show the typical disorganized growth pattern of tumor cells, including the strong propensity to focus formation, cells treated with P4 develop an obvious cobblestone pattern that is remarkably similar to the one reported for normal ovarian epithelial cells [24,25]. This observation prompted us to investigate additional features that in general distinguish tumor cells from normal cells. In this regard, we found that P4-treated cells did not develop three-dimensional foci, indicating that they had acquired contact inhibition. Furthermore, these cells were not able to form colonies in soft agar, which revealed that they had lost their anchorage-independence. Thus, hormone-treated cells had lost several of the typical phenotypic hallmarks of cancer cells and had assumed characteristics associated with normal cells.

Our supposition that P4 had reverted the ovarian tumor cells towards a more normal phenotype was further asserted at the molecular level by demonstrating the significantly reduced expression of two tumor markers, HSP72 and CA125. HSP72 is an oncogene [35] and its elevated expression has been documented for a great variety of tumors and tumor cell lines, including ovarian carcinomas [36,37]. In nontransformed cells under normal conditions, it is expressed at very low levels. Similarly, expression of the CA125 antigen is associated with epithelial ovarian carcinomas, but has not been detected in normal adult ovarian cells [38–40]. It is the only marker to have an established role in the diagnosis, monitoring of treatment, and detection of relapse of epithelial ovarian cancer [41]. In the present study, we demonstrated that both tumor markers, HSP72 and CA125, were downregulated by P4 treatment. Thus, these observations are supportive of our conclusion that long-term treatment with P4 suppresses the transformed phenotype of HOC-7 carcinoma cells. This effect does not appear to be restricted to HOC-7 cells, as at least some of the consequences were also observed with another tumor cell line; i.e., OVCAR-3 ovarian carcinoma cells did not form foci nor grew anchorage-independently in the presence of P4 (Figure 4, Table 1). Considered together with recent findings by others

who have used SKOV-3 ovarian carcinoma cells and shown that progesterone inhibits the invasive activity and tumor-forming potential of these cells [42,43], our results indicate the possibility that the suppression of the transformed phenotype by progesterone might occur in some other ovarian carcinoma cells as well.

In addition to losing the transformed phenotype, cells treated with 10 μM P4 appear to acquire functional characteristics that resemble the phenotype of normal differentiated epithelial cells. In polarized epithelial cells in general, establishment of functional tight junctions is a property that correlates with the differentiated phenotype. Conversely, loss of intercellular junctional complexes and normal cell-cell contacts is characteristic of transformed cells with a less differentiated phenotype [44,45]. In this regard, the measurement of TER is a commonly applied physiologic indicator of functional tight junctions [26,27]. For example, in mammary epithelial Con8 tumor cells, dexamethasone treatment coordinately suppresses cell proliferation and stimulates tight junction formation; in parallel, increases in TER correlate with a decrease in paracellular permeability to [^{3}H]inulin, indicating the formation of a tight monolayer [46]. Similarly, treatment of HT-29 human colon carcinoma cell lines with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) prevents the accumulation of differentiation markers and induces the loss of functional integrity of tight junctions in monolayers, which is reflected in a decrease in TER [47]. In this present study, when we measured TER, we found that in response to long-term treatment with 10 μm P4, HOC-7 cells had developed measurable TER, indicating the formation of functional tight junctions. Thus, the distinct cobblestone appearance of these P4-treated cells, which looked strikingly similar to monolayers of primary ovarian epithelial cells [24,25] correlated closely with strongly increased TER and suggested the functional epithelialization of these cells.

An interesting observation in the above effects is the fact that the acquisition of properties that resemble normal, differentiated cells, i.e., the cobblestone pattern of cellular growth and the formation of tight junctions, only became apparent at 10 μM P4, but not at 30 μM. Thus, it appeared that the induction of this epitheloid appearance by P4 was strongly concentration-dependent and went through an apparent optimum at around 10 μM. In contrast, the effects of P4 on cell growth and various indicators of transformation were continuously augmented by increasing concentrations of this hormone; i.e., 30 μM P4 exhibited stronger inhibitory effects on cell growth, the activity of CDK, the expression of cell-cycle-regulatory proteins, and the downregulation of tumor markers, than 10 μM. Thus, it seems that P4 exerted quantitatively different effects, depending on the concentration used. In this regard, we also observed that increasing the concentration of P4 even higher, i.e., to 50 μM and above, stimulated apoptotic processes in these cells (not shown). This latter observation was not entirely surprising, as others have reported the induction of apoptosis in SNU-840 ovarian cancer cells by 100 μM P4 [13].

Alternatively, the known steroid receptor–independent intercalation of progesterone into the plasma membrane [48,49] might serve to explain the observed differential effects of P4. It has been shown that supraphysiological concentrations of progesterone are able to disrupt the normal fluid dynamics of plasma membranes of SKOV-3 ovarian carcinoma cells [42,43]. We observed that when cells were cultured in the presence of 30 μM P4, a drastic change in morphology occured that neither resembled the typical appearance of untreated tumor cells nor the cobblestone look of cells exposed to 10 μM P4 (Fig. 4). As we deem it unlikely that the nongenomic disruption of normal fluid dynamics would generate an organized cell monolayer inclusive of functional tight junctions (as observed at 10 μM P4), we speculate that alterations of membrane fluidity might have taken place only at 30 μM, but not yet at 10 μM P4. This scenario could furthermore explain the unusual morphology of cells treated with 30 μM or higher.

In general, all of the above described effects of P4 occurred at concentrations above the ones reported for serum levels in vivo [50]. However, because the ovary is a producer of P4 [16,51,52], cells within and even surrounding this organ are undoubtedly exposed to concentrations of this hormone that are orders of magnitude higher than circulating levels. Indeed, measurements of P4 levels in the ovarian vein of fertile women showed concentrations in the range of what was used in our study [53]. Therefore, it is conceivable that our in vitro observations might represent mechanisms that contribute to the protective effect of P4 against the development of ovarian epithelial tumors.

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