Progestins Initiate a Luminal to Myoepithelial Switch in Estrogen-Dependent Human Breast Tumors without Altering Growth

Carol A. Sartorius,1 Djuana M.E. Harvell,1 Tianjie Shen,1 and Kathryn B. Horwitz1,2

1Division of Endocrinology, Department of Medicine and 2Department of Pathology, University of Colorado Health Sciences Center, Aurora, Colorado

Abstract

Although long-term clinical use of progestins is associated with an increased incidence of breast cancers, their role in established cancers is unclear. Estrogens are considered to be the main mitogens in the majority of breast cancers. Whether progesterone affects proliferation and/or differentiation is under debate. To assess the role of progesterone in established breast cancers, we used T47D human breast cancer cells that are estrogen receptor (ER) positive and either progesterone receptor (PR) negative or positive for PRA, PRB, or both. These cells were grown as strictly estrogen-dependent solid tumors in ovariectomized female nude mice. Progesterone or medroxyprogesterone acetate (MPA) alone did not support tumor growth, nor did progesterone or MPA given simultaneously with estrogen significantly alter estrogen-dependent tumor growth. However, treatment of mice bearing ER+PR− but not ER+PR+ tumors with either progesterone or MPA increased expression of the myoepithelial cytokeratins (CK) 5 and 6 in a subpopulation of tumor cells. These CK5+/CK6+ cells had decreased expression of luminal epithelial CK8, CK18, and CK19. We conclude that progestins exert differentiative effects on tumors characterized by transition of a cell subpopulation from luminal to myoepithelial. This may not be beneficial, however, because such a phenotype is associated with poor prognosis. (Cancer Res 2005; 65(21): 9779-88)

Introduction

The ovarian steroid hormones estrogen and progesterone are usually present together physiologically, and their cognate estrogen receptor (ER) and progesterone receptor (PR) are coexpressed in the same tissues and/or cells (1). Each of the receptors exists as two forms—ERα and ERβ for estrogens and PRA and PRB for progesterone—that subserve different, at times, opposing functions in the murine mammary gland (13). In summary, progesterone can be stimulatory, inhibitory, or differentiative depending on the context of the experiment. The presence of other growth factors during these phases may help explain the various effects of progesterone in the normal breast.

Other studies address the role of progesterone in tumorigenesis. The seminal studies of Huggins et al. (14) underscore the complex actions of progesterone in carcinogen-induced mammary tumor development. Exogenous progesterone given before a carcinogen or the physiologically high progesterone levels of pregnancy are both protective (reviewed in ref. 15), whereas progesterone given after carcinogen exposure exacerbates tumor formation. Thus, progesterone has either inhibitory or stimulatory effects on breast cancer formation depending on dose and timing. The possible deleterious effects of progesterone recently resurfaced on demonstration in the Women’s Health Initiative studies of hormone replacement therapy at menopause (16) that increased risk of breast cancer is associated with an estrogen plus progesterone regimen but not estrogen alone.

Also contentious is the effect of progesterone on proliferation of established breast cancers. The majority of primary human tumors are “hormone dependent” by virtue of the fact that they are ER+ and/or PR+ (17). It is assumed that such tumors proliferate in response to estrogen; indeed, antiestrogen or estrogen suppression therapies are highly successful (18). However, the proliferative effects of progesterone, if any, in established breast cancers remain unclear. Long-term in vivo studies of progesterone effects in tumor models are sparse. Progesterone treatment inhibits proliferation of established rat mammary tumors (19, 20) and PR-transfected MDA-231 (ER−PR−) human breast cancer cells grown into tumors in severe combined immunodeficient mice (21). On the other hand, Michna et al. (22) report inhibitory effects of antiprogestins, in which case progesterone would be growth stimulatory.

We have developed in vivo human breast cancer models in which to test the effects of progesterone either alone or in the presence of estrogen. This is done in ER+ T47D cells that vary in their PR content (PR−, PRA+, PRB+, or PRA+PRB+; ref. 23). This is of interest because the equimolar PRA/PRB ratios of the normal mammary gland change during malignant progression so that >70% of invasive breast cancers express excessive levels of one or the other PR isoform (24, 25). This in turn influences hormone responsiveness (26). We show that regardless of whether the
tumors are PR− or PR+ for either form their growth is unaffected by progesterone or medroxyprogesterone acetate (MPA) alone or together with estrogen. However, long-term treatment with progesterone or MPA leads to the appearance of cell subpopulations expressing the myoepithelial/basal markers cytokeratins (CK) 5 and 6. T47D cells originated from the pleural effusion of an ER+/PR+ luminal epithelial ductal cancer (27). Such tumors are marked by expression of glandular or secretory (luminal) intermediate filament CK8, CK18, and CK19 (28). Expression of these luminal CKs was decreased in progestin-treated tumors. Therefore, steroid hormones can influence the differentiation state of proliferating tumors as measured by their CK expression profile. In the case of myoepithelial CK5 and CK6, tumors expressing these markers are characterized by poor prognosis (29-31).

Materials and Methods

Cell lines. We described previously the selection of a PR− subpopulation of T47D human breast cancer cells (T47D-Y or T47Dwet) from the original wild-type cells, which express high levels of both PR forms, PRA and PRB (T47Dwt, ref. 23). T47Dwet cells were used to create stable cell lines that exclusively express PRA (T47DPrA) or PRB (T47DPRB) by reintroducing cDNA expression plasmids (23). Cells were maintained in DMEM (Life Technologies, Carlsbad, CA) supplemented with 5% fetal bovine serum (Life Technologies, 30%).

Experimental animals and xenograft tumor growth. All animal procedures were done under a protocol approved by the University of Colorado Institutional Animal Care and Use Committee. Ovariectomized female athymic nu/nu mice were obtained from Harlan Sprague-Dawley (Indianapolis, IN) at 5 to 6 weeks of age. Inoculation of T47D breast cancer cells into animals is essentially as described (32). Briefly, anesthetized animals were injected with 5 × 10^6 cells suspended in 50% Matrigel (Becton Dickinson, Bedford, MA) in MEM (Life Technologies). For estrogen plus progesterone or estrogen plus MPA experiments, progesterone or MPA were added to estrogen either at the start of the experiment or after 4 weeks of estrogen alone. Two tumors per animal were grown on the left and right flanks. All animals were implanted with silastic pellets containing hormone mixtures (described in ref. 32). These included placebo (10 mg cellulose), estrogen (2 mg 17β-estradiol + 8 mg cellulose), progesterone (10 mg progesterone), or MPA (10 mg 6α-methyl-17α-hydroxyprogesterone acetate). Growth of tumors was monitored weekly for 8 weeks by measuring the length (l) and width (w) with a digital caliper, with l being the smaller measurement. Tumor volume was estimated by the formula l*w/2. At termination of the experiment, mice were euthanized by CO₂ asphyxiation, and tumors were excised and weighed. Tumors that were overly necrotic were omitted from the study. In some cases, uteri (wet weight) and mammary glands (whole mount stains) were removed.

Serum levels of progesterone. Whole blood was collected from animals by aortic puncture at time of euthanasia and fractionated by centrifugation at 9,000 × g for 5 minutes at 4°C. Serum supernatants were removed and stored at −20°C. Serum from two animals were combined for each assay. Progesterone concentrations (ng/mL) in sera were determined by chemiluminescence microparticle immunoassay (Abbott Laboratories, North Chicago, IL) at the University of Colorado Hospital.

Mammary gland whole mounts. Whole mammary glands (no. 4) were removed from select animals at euthanasia and spread onto glass slides. Tissue was dried for 5 minutes before fixation in Carnoy’s fixative (60% ethanol, 30% chloroform, 10% acetic acid) for 24 hours. After gradual dehydration for 1 hour each in 70% ethanol, 50% ethanol, and distilled water, slides were stained with carmine aluminum (0.2% carmine and 0.5% aluminum potassium sulfate) overnight. Slides were washed in 70%, 95%, and 100% ethanol for 1 hour each then cleared with xylene for 1 hour before mounting with Permount (Fisher, Fair Lawn, NJ). Photographs were taken at ×4 magnification.

3-Bromo-2-deoxyuridine incorporation. Animals were injected i.p. with 5-bromo-2-deoxyuridine (Brdu; Sigma, St. Louis, MO) at a concentration of 50 mg/kg in sterile PBS 2 hours before euthanasia. Tumors were removed and fixed in formalin, processed, and paraffin embedded. Sections (5 μm) taken from the middle portion of each tumor were stained with an anti-BrdUrd antibody (BD Biosciences, San Jose, CA) and counterstained with hematoxylin. Five random fields at high magnification (~40) from each tumor were counted for BrdUrd-positive cells in a blinded fashion. Small intestines were stained as a positive control with epithelial cells positive for BrdUrd.

Gene expression analysis. T47DPRA and T47DPRB tumors grown bilaterally on the same experimental animals were removed for gene expression analysis. Triplicate samples were obtained from three animals each treated with estrogen plus placebo, estrogen plus progesterone, or estrogen plus MPA. Total RNA was prepared from tumors using TRizol reagent according to the manufacturer’s instructions (Invitrogen, San Diego, CA). Polyadenylated RNA was prepared from total RNA and processed for hybridization to HG-U95Av2 arrays according to a detailed protocol by Affymetrix (Santa Clara, CA). Expression profiling was done by the University of Colorado Cancer Center Microarray Core Laboratory. Data were analyzed using Microarray Suite (Affymetrix) and GeneSpring software (Silicon Genetics, Redwood City, CA). Data were normalized for each chip, and statistical significance among expression levels in estrogen, progesterone plus progesterone, and estrogen plus MPA tumors was determined by one-way ANOVA followed by a Tukey post-test using a cutoff value of P < 0.05 (described in detail in ref. 33). Fold changes were determined by dividing the normalized intensities for the estrogen plus progesterone and estrogen plus MPA sets by the intensities in the estrogen set.

Immunohistochemistry and immunofluorescence. Whole tumors were removed from animals and fixed in 10% buffered formalin. Tissue was processed, paraffin embedded, and cut into 5-μm sections. After high-temperature antigen retrieval in citrate buffer, sections were stained with monoclonal antibodies specific for CK5 or CK8/CK18 (both from Novocastra, United Kingdom). Sections were counterstained with hematoxylin and mounted. Representative photographs were taken under a light microscope at ×40 magnification. For dual immunofluorescence, samples were processed as above and then stained simultaneously with a mouse monoclonal antibody specific for CK5 (Novocastra) and rabbit polyclonal antibodies to either CK18 (Calbiochem, La Jolla, CA) or human PR (DAKO, Carpinteria, CA). Sections were stained with secondary antibodies that fluoresce green (goat anti-mouse Alexa 488) and red (goat anti-rabbit Alexa 555) both from Molecular Probes, Eugene, OR. Nuclei were stained with 1 μg/mL 4,6-diamidino-2-phenylindole (DAPI) in methanol for 15 minutes at room temperature. Sections were mounted with Gelmount (Biomeda, Foster City, CA) and photographs were taken under ×100 magnification for the same field using the UV, FITC, and TRITC filters. Images were merged using ImagePro software (Media Cybernetics, Silver Springs, MD).

Statistical analyses. Statistics were done using GraphPad software (San Diego, CA) and statistical significance was determined by comparison of three or more groups using one-way ANOVA followed by a Tukey post-test. P < 0.05 was considered significant.

Results

In vivo model of T47D tumor growth during estrogen plus progestin treatment. We established previously an in vivo model in which four T47D cell lines (T47Dwet, T47DPRA, T47DPRB) are grown into tumors in ovariectomized, estrogen-supplemented female nude mice (32). We showed that the rate of ER+, estrogen-dependent tumor growth can be modified by the type of PR coexpressed with ER: tumors were generally smaller if PRA was present compared with no PR or PRB. This was discussed in relation with the reported inhibitory effects of PRA on ER action in vitro and in vivo (34, 35). Importantly, these studies were done in the absence of PR ligand. In the present study, we tested the effects of the progestins, progesterone or MPA, either alone or in the
presence of estrogen, on tumor growth in vivo. Mice were implanted bilaterally with two of the four cell lines as follows: T47DPRB with T47DPRA and T47DPR with T47Dwt. To study the effects of estrogen or progestins alone, mice received implants of estrogen (2 mg), progesterone, or MPA (10 mg each) for 8 weeks, and tumor size was measured weekly. For estrogen plus progestin experiments, mice were implanted with estrogen pellets (2 mg) and tumors were allowed to establish for 4 weeks, at which time they received a second implant of either placebo, progesterone, or MPA (10 mg each), and tumor size was measured for an additional 4 weeks.

Effects of hormones on the physiology of experimental animals. To confirm that progesterone and MPA were being delivered efficiently, the mice were monitored for several physiologic side effects of estrogen and progestin exposure. Figure 1A shows the circulating levels of progesterone in blood obtained by aortic puncture at the time of euthanasia. Serum fractions from two mice were combined per measurement. Control mice (cycling) had an average progesterone level of 4.7 ng/mL. Ovariectomy reduced this to less than half or 2.0 ng/mL. Addition of progesterone pellets for 8 weeks led to an ~4-fold increase in progesterone levels over controls to 20.6 ng/mL. Mice treated with estrogen for 8 weeks (plus addition of placebo for the last 4 weeks) had low levels of progesterone (3.1 ng/mL). In the estrogen plus progesterone group, in which animals received progesterone for the last 4 weeks, circulating progesterone levels were 22.1 ng/mL. These levels represent a high physiologic dose of the hormone.

MPA levels in sera are not reported due to lack of a commercially available assay. However, the structural resemblance of MPA to progesterone, plus the ensuing physiologic data (see below), indicate that MPA is present at least at levels similar to progesterone. Circulating levels of estrogen (17β-estradiol) were similar to those reported previously (32) with blood levels after 8 weeks of estrogen pellet implantation of 145 ± 47 pg/mL.
compared with 35 ± 9 pg/mL for ovariectomized controls (n = 4 each). Animals in the estrogen plus progesterone and estrogen plus MPA groups had average estrogen levels of 126 ± 37 and 133 ± 33 ng/mL, respectively (n = 4 each).

The effect of seven different hormonal states on body mass are shown in Fig. 1B before (filled symbols) and after (open symbols) 8 weeks of treatment. The average starting mass of intact female mice at ~6 weeks of age ranged from 20 to 22 g. Ovariectomized mice 7 to 10 days postsurgery were slightly larger than intact age-matched controls, but these differences were not significant. After 8 weeks, intact female nude mice weighed 23.7 g on average compared with 30.0 g for ovariectomized mice. Progestosterone and MPA alone maintained the higher body mass of ovariectomized mice. However, estrogen treatment suppressed growth of ovariectomized mice. The lower mass of estrogen-only mice (23.6 g) was partially reversed by estrogen plus progesterone or estrogen plus MPA after only 4 weeks of the progestins to 28.5 and 28.3 g, respectively.

Hormone treatment also affected uterine wet weight (Fig. 1C). At the time of dissection, uteri from intact animals (including ovaries) weighed 74.0 mg, which was reduced to 23.2 mg by ovariectomy. Progestins alone increased uterine mass to 29.5 mg (progesterone) and 40.1 mg (MPA), which was only statistically significant for MPA (P < 0.01). Estrogen treatment led to extensive hypertrophy (126.0 mg), which was partially reduced by estrogen plus progesterone (109.3 mg) or estrogen plus MPA (95.2 mg). Whole mammary gland ductal branching and lobuloalveolar development at the time of euthanasia is shown in Fig. 1D. Ovariectomized animals have underdeveloped mammary glands. Estrogen alone minimally increases ductal branching, whereas progesterone or MPA alone had no noticeable effect. However, estrogen plus progesterone or estrogen plus MPA led to extensive branching and lobuloalveolar development, indicating that the levels of circulating progesterone and MPA achieved in mice by the implants were physiologic.

**Progestins alone do not promote tumor growth.** Ovariectomized mice bearing bilateral T47DPRB and T47Dwt or T47DPRB and T47DPRB tumors were implanted with pellets containing placebo (10 mg cellulose), progesterone (10 mg), or estrogen (2 mg) and tumor volumes were measured weekly for 8 weeks (Fig. 2A). Cells inoculated into ovariectomized mice implanted with placebo pellets remain viable but do not grow, with final average volumes ranging from 80 to 130 mm³. Importantly, neither progesterone nor MPA alone promoted tumor growth regardless of the PR content of the cells. In contrast, estrogen alone led to rapid growth with maximum average tumor volumes at 8 weeks ranging from 250 to 600 mm³ depending on tumor type. Note the smaller average size of PRB+ tumors (T47DPRB and T47Dwt) discussed in ref. 32.

Final tumor masses are shown in Fig. 2B. The average mass of estrogen-treated tumors compared with placebo and progesterone was 282 versus 70 and 58 mg for T47DPRB, 172 versus 30 or 28 mg for T47Dwt, 161 versus 36 and 35 mg for T47DPRB, and 321 versus 67 and 80 mg for T47DPRB. For each cell line, the estrogen-treated tumors were significantly larger than either placebo or progesterone alone (P < 0.001). Therefore, in the absence of estrogen, progesterone alone cannot promote tumor growth of ER+ T47D cells in the absence or presence of PR. MPA alone also did not promote tumor growth in vivo (data not shown).

**Progestins do not alter estrogen-dependent tumor growth.** We next tested whether progesterone or MPA modify estrogen-dependent tumor growth in vivo (Fig. 3). T47DPRB and T47Dwt or T47DPRB and T47Dwt cells were implanted on opposite flanks of the same mice and tumors were established for 4 weeks under estrogen stimulation. A second pellet containing placebo, progesterone, or MPA (10 mg each) was then implanted (arrows), while estrogen was continued, and tumors were grown for an additional 4 weeks. Changes in tumor volume per week are shown in Fig. 3A. T47DPRB tumors grew to an average final volume of ~400 mm³ and were unaffected by 4 weeks of progesterone or MPA treatment. T47DPRB tumors grew to an average final volume of 667 mm³, which was increased ~20% by progesterone or MPA. Estrogen-treated T47DPRB tumors grew to 217 mm³, which decreased by 10% to 20% when progesterone or MPA were added. Although these subtle differences in growth were reproducible in separate experiments, none reached statistical significance. Estrogen-dependent growth of T47Dwt (193 mm³) was not significantly altered by estrogen plus progesterone or estrogen plus MPA.

Final tumor masses are shown in Fig. 3B. There was no significant difference in the mean mass of T47DPRB tumors treated with estrogen (338 mg), estrogen plus progesterone (307 mg), or estrogen plus MPA (324 mg). Estrogen-treated T47DPRB tumors were 601 mg, whereas estrogen plus progesterone- and estrogen plus MPA–treated tumors were 618 and 801 mg, respectively. T47DPRB tumors treated with estrogen had an average mass of 131 mg, which decreased to 120 and 104 mg with estrogen plus progesterone and estrogen plus MPA. Size differences comparing hormone treatments of T47DPRB (estrogen, estrogen + progesterone, and estrogen + MPA) and T47Dwt (estrogen, estrogen + progesterone, and estrogen + MPA) tumors were not significant (P > 0.05). There was no difference in the final average masses of T47Dwt tumors (113 mg for estrogen, 101 mg for estrogen + progesterone, and 91 mg for estrogen + MPA). Therefore, progestins have only subtle effects on the growth of ER+/PR+, estrogen-dependent tumors depending on the PR isoform present. Experiments in which progesterone or MPA were added at the start of the experiment simultaneously with estrogen also showed no significant differences in tumor growth after 8 weeks (data not shown).

To assess proliferation rates, several mice bearing T47D tumors treated with estrogen, estrogen plus progesterone, or estrogen plus MPA described above were injected with BrdUrd 2 hours before sacrifice. Sections were stained with an anti-BrdUrd antibody, and five fields from separate areas of each tumor were photographed and scored for the number of BrdUrd-positive cells. Figure 3C shows representative sections from estrogen and estrogen plus MPA–treated tumors (T47DPRB and T47Dwt) used for BrdUrd analysis. Mean positive cells per field ranged from 61 to 90 as indicated, with no significant differences noted between tumors from estrogen and estrogen plus MPA–treated animals. T47DPRB and T47Dwt tumors treated with estrogen plus progesterone had 89 ± 13 and 81 ± 11 BrdUrd-positive cells per field, respectively, which also do not differ from estrogen alone (data not shown).

**Progestins increase expression of myoepithelial cytokeratins in a subset of progesterone receptor–positive tumor cells.** To investigate other potential physiologic changes in progesterin-treated tumors, we determined gene expression profiles in three pairs of T47DPRB and T47Dwt tumors treated with estrogen, estrogen plus progesterone, or estrogen plus MPA using Affymetrix HuFL-U95Av2 arrays. Data were normalized, comparisons among hormone treatments were made, and genes were identified that were significantly changed at least 2-fold (up or down) by the
progestins compared with the estrogen control. Genes with the highest fold regulation by progestins in both T47DPRA and T47DPRB tumors included CK5 and CK6—markers of myoepithelial cells. We then assessed gene expression levels of other CK family members on the array.

Figure 4A shows normalized expression levels of transcripts for the myoepithelial CK5 and CK6 in T47DPRA or T47DPRB tumors treated with estrogen, estrogen plus progesterone, and estrogen plus MPA. Whereas expression of CK5 and CK6 was nearly absent in estrogen-treated tumors, their levels were increased substantially by both progesterone and MPA (8.5- and 16.5-fold for CK5 and 13.7- and 30.7-fold for CK6 in T47DPRA; 4.5- and 5.5-fold for CK5 and 9.9- and 18.1-fold for CK6 in T47DPRB, respectively). * P < 0.001, after 8 weeks of growth, estrogen-treated tumors are significantly larger than placebo or progesterone-treated tumors for each tumor type.

Figure 4B shows expression levels of the luminal epithelial CK8, CK18, and CK19. Transcripts for these CKs were highly expressed in estrogen-treated T47DPRA and T47DPRB tumors. Expression was decreased by progestins 1.3- to 2-fold (significant only for estrogen plus MPA at 1.5-fold cutoff; P < 0.05). Of the ~20 known epithelial CKs, transcripts for CK10 and CK17 were the only others called "present" in the estrogen-treated tumors, and their levels (~10% that of CK18) were not significantly altered by progesterone or MPA. CK7 (a luminal cell marker) and CK14 (a myoepithelial cell marker) were not represented on the array.

We next determined if the CK transcript changes are paralleled by changes in their protein levels in tumors. Sections of T47DPRneg, T47DPRA, and T47DPRB tumors treated with estrogen, estrogen plus progesterone, and estrogen plus MPA. Whereas expression of CK5 and CK6 was nearly absent in estrogen-treated tumors, their levels were increased substantially by both progesterone and MPA (8.5- and 16.5-fold for CK5 and 13.7- and 30.7-fold for CK6 in T47DPRA; 4.5- and 5.5-fold for CK5 and 9.9- and 18.1-fold for CK6 in T47DPRB). Figure 4B shows expression levels of the luminal epithelial CK8, CK18, and CK19. Transcripts for these CKs were highly expressed in estrogen-treated T47DPRA and T47DPRB tumors. Expression was decreased by progestins 1.3- to 2-fold (significant only for estrogen plus MPA at 1.5-fold cutoff; P < 0.05). Of the ~20 known epithelial CKs, transcripts for CK10 and CK17 were the only others called "present" in the estrogen-treated tumors, and their levels (~10% that of CK18) were not significantly altered by progesterone or MPA. CK7 (a luminal cell marker) and CK14 (a myoepithelial cell marker) were not represented on the array.

We next determined if the CK transcript changes are paralleled by changes in their protein levels in tumors. Sections of T47DPRneg, T47DPRA, and T47DPRB tumors treated with estrogen or estrogen plus MPA were immunohistochemically stained with antibodies that recognize CK5 or CK8/CK18. Except for a rare positive cell (Fig. 4C, arrows), estrogen-treated tumors lack CK5. Addition of MPA to estrogen had no effect in the PRneg T47DPRneg tumors but...
greatly increased the number of CK5+ cells in PR+ T47DPR and T47DPRB tumors. Interestingly, this occurs in ~5% to 10% of tumor cells. CK6 showed a similar pattern of expression (data not shown). Virtually all tumor cells stain positive for the luminal epithelial CK8/CK18 regardless of tumor type or hormone treatment (Fig. 4D).

Tumors cells expressing cytokeratin 5 contain less cytokeratin 18. To specifically determine if individual CK5+ cells have altered expression of luminal CKs (not detectable by peroxidase staining), dual immunofluorescence studies were done. Sections from estrogen plus MPA–treated T47DPR tumors were dually stained with antibodies to CK5 (monoclonal) and CK18 (polyclonal).

Figure 3. Estrogen-dependent growth of T47D tumors is not significantly altered by progestins. T47D cells were implanted in female ovariectomized mice as described, with T47DPR and T47Dwt or T47DPR and T47Dwt grown on opposing flanks of the same animals. A, tumors were grown under estrogen stimulation (pellet) for 4 weeks, at which time animals were implanted with a second hormone pellet (arrows) containing placebo (○), progesterone (△), or MPA (▲). Points, mean tumor volume (mm³) for each cell line; bars, SE. Sample numbers (n) for the estrogen + placebo, estrogen + progesterone, and estrogen + MPA groups are 10, 10, and 10 for T47DPR; 20, 17, and 19 for T47DPRB; 21, 17, and 21 for T47DPR; and 10, 10, and 10 for T47Dwt, respectively. B, individual tumor masses for T47DPR and T47Dwt tumors treated with estrogen + placebo, estrogen + progesterone, or estrogen + MPA. Bar, mean. Note that scales are different depending on tumor size. No significant difference in tumor volumes (A) or masses (B) were noted between estrogen-, estrogen + progesterone–, or estrogen + MPA–treated groups for any tumor type (P > 0.05). C, mice were given i.p. BrdUrd injections (50 mg/kg) 2 hours before harvesting tumors. Sections from each tumor were stained with an anti-BrdUrd antibody. The number of BrdUrd-positive cells per field was counted in five fields per tumor; total tumors (n = 3). Representative fields used for quantitation for estrogen- and estrogen + MPA–treated T47DPR, T47Dwt, T47DPR, and T47Dwt tumors. Magnification, ×40. Bar, 100 μm. Mean ± SD BrdUrd-positive cells per field are indicated.
Sections were then stained with specific green and red fluorescing secondary antibodies, and after antibody incubations, nuclei were stained with DAPI. Figure 5A shows a representative field of a T47DPRA tumor at high magnification containing a cluster of CK5+ cells (green). CK18+ cells in the same field are shown in red. Most cells positive for CK5 have lost expression of CK18 compared with neighboring CK5+/CK18+ cells. Images were merged with or without the nuclear stain DAPI. As indicated in the merged image (arrow), there were always a few CK5+ cells in each cluster that retained some expression of CK18. This same pattern of CK5/CK18 expression occurs in T47DPRB tumors. Dual immunofluorescence was also done with antibodies to CK5 and PR. A representative image of an estrogen plus MPA–treated T47DPRB tumor is shown in Fig. 5B. Individual CK5+ cells are shown in green and PR in red. Merged images (DAPI) show nuclear PR staining in the same cells with cytoplasmic CK5 staining. Results were identical in T47DPRA tumors.

Discussion

Progesterone and progesterone receptor as modulators of estrogen and estrogen receptor proliferative activity. The aim of these studies was to assess the effects of progesterone and...
Indeed, in the absence of chemotherapy, high-dose MPA increases counteract the proliferative effects of estrogen in breast cancers. This fosters the assumption that progesterone would similarly progestins therapeutically (6, 38). The success of these regimens with the nuclear stain DAPI (merged + DAPI CK5 (polyclonal; green) and PR (green) was done simultaneously with antibodies to CK5 (monoclonal; green) was linked to poor clinical outcome (30). In a study of >1,900 cases, >600 tumors and found that expression of CK5/CK6 and/or CK17 was linked to poorly differentiated grade 3 cancers and shorter disease-free interval and ER negativity (31). Conversely, expression of CK5/CK6 was linked to shorter (1%). Malzahn et al. found that expression of myoepithelial CKs was linked to poorly differentiated grade 3 cancers and shorter disease-free interval and ER negativity (31). Conversely, expression of CK5/CK6 was linked to poorly differentiated grade 3 cancers and shorter disease-free interval and ER negativity (31). Conversely, expression of CK5/CK6 was linked to poorly differentiated grade 3 cancers and shorter disease-free interval and ER negativity (31). Conversely, expression of CK5/CK6 was linked to poorly differentiated grade 3 cancers and shorter disease-free interval and ER negativity (31). Conversely, expression of CK5/CK6 was linked to poorly differentiated grade 3 cancers and shorter disease-free interval and ER negativity (31). Conversely, expression of CK5/CK6 was linked to poorly differentiated grade 3 cancers and shorter disease-free interval and ER negativity (31). Conversely, expression of CK5/CK6 was linked to poorly differentiated grade 3 cancers and shorter disease-free interval and ER negativity (31). Conversely, expression of CK5/CK6 was linked to poorly differentiated grade 3 cancers and shorter disease-free interval and ER negativity (31). Conversely, expression of CK5/CK6 was linked to poorly differentiated grade 3 cancers and shorter disease-free interval and ER negativity (31). Conversely, expression of CK5/CK6 was linked to poorly differentiated grade 3 cancers and shorter disease-free interval and ER negativity (31). Conversely, expression of CK5/CK6 was linked to poorly differentiated grade 3 cancers and shorter disease-free interval and ER negativity (31). Conversely, expression of CK5/CK6 was linked to poorly differentiated grade 3 cancers and shorter disease-free interval and ER negativity (31). Conversely, expression of CK5/CK6 was linked to poorly differentiated grade 3 cancers and shorter disease-free interval and ER negativity (31). Conversely, expression of CK5/CK6 was linked to poorly differentiated grade 3 cancers and shorter disease-free interval and ER negativity (31). Conversely, expression of CK5/CK6 was linked to poorly differentiated grade 3 cancers and shorter disease-free interval and ER negativity (31). Conversely, expression of CK5/CK6 was linked to poorly differentiated grade 3 cancers and shorter disease-free interval and ER negativity (31). Conversely, expression of CK5/CK6 was linked to poorly differentiated grade 3 cancers and shorter disease-free interval and ER negativity (31). Conversely, expression of CK5/CK6 was linked to poorly differentiated grade 3 cancers and shorter disease-free interval and ER negativity (31). Conversely, expression of CK5/CK6 was linked to poorly differentiated grade 3 cancers and shorter disease-free interval and ER negativity (31). Conversely, expression of CK5/CK6 was linked to poorly differentiated grade 3 cancers and shorter disease-free interval and ER negativity (31). Conversely, expression of CK5/CK6 was linked to poorly differentiated grade 3 cancers and shorter disease-free interval and ER negativity (31). Conversely, expression of CK5/CK6 was linked to poorly differentiated grade 3 cancers and shorter disease-free interval and ER negativity (31). Conversely, expression of CK5/CK6 was linked to poorly differentiated grade 3 cancers and shorter disease-free interval and ER negativity (31). Conversely, expression of CK5/CK6 was linked to poorly differentiated grade 3 cancers and shorter disease-free interval and ER negativity (31). Conversely, expression of CK5/CK6 was linked to poorly differentiated grade 3 cancers and shorter disease-free interval and ER negativity (31). Conversely, expression of CK5/CK6 was linked to poorly differentiated grade 3 cancers and shorter disease-free interval and ER negativity (31). Conversely, expression of CK5/CK6 was linked to poorly differentiated grade 3 cancers and shorter disease-free interval and ER negativity (31). Conversely, expression of CK5/CK6 was linked to poorly differentiated grade 3 cancers and shorter disease-free interval and ER negativity (31). Conversely, expression of CK5/CK6 was linked to poorly differentiated grade 3 cancers and shorter disease-free interval and ER negativity (31). Conversely, expression of CK5/CK6 was linked to poorly differentiated grade 3 cancers and shorter disease-free interval and ER negativity (31). Conversely, expression of CK5/CK6 was linked to poorly differentiated grade 3 cancers and shorter disease-free interval and ER negativity (31). Conversely, expression of CK5/CK6 was linked to poorly differentiated grade 3 cancers and shorter disease-free interval and ER negativity (31). Conversely, expression of CK5/CK6 was linked to poorly differentiated grade 3 cancers and shorter disease-free interval and ER negativity (31). Conversely, expression of CK5/CK6 was linked to poorly differentiated grade 3 cancers and shorter disease-free interval and ER negativity (31). Conversely, expression of CK5/CK6 was linked to poorly differentiated grade 3 cancers and shorter disease-free interval and ER negativity (31). Conversely, expression of CK5/CK6 was linked to poorly differentiated grade 3 cancers and shorter disease-free interval and ER negativity (31). Conversely, expression of CK5/CK6 was linked to poorly differentiated grade 3 cancers and shorter disease-free interval and ER negativity (31). Conversely, expression of CK5/CK6 was linked to poorly differentiated grade 3 cancers and shorter disease-free interval and ER negativity (31). Conversely, expression of CK5/CK6 was linked to poorly differentiated grade 3 cancers and shorter disease-free interval and ER negativity (31). Conversely, expression of CK5/CK6 was linked to poorly differentiated grade 3 cancers and shorter disease-free interval and ER negativ...
individual tumor. Microarray and dual-immunofluorescence studies confirmed that many of these CK5+/cells are negative for or express reduced levels of the luminal CK8, CK18, and CK19. Interestingly, we could not detect smooth muscle actin (SMA) expression, a marker of terminally differentiated myoepithelial cells, in CK5+ tumor cells (data not shown). Bocker et al. have described such CK5+/CK6+ cells that are negative for luminal CKs and SMA (46). They propose that these are progenitor cells that may later become fully differentiated luminal (CK18+) or myoepithelial (SMA+) cells. Cells containing dual expression of CK5 and CK18 have also been described in both mammary and prostate epithelia and are postulated to be in an "intermediate" state of differentiation (47, 48). Thus, CK5+ cells in our tumors may be myoepithelial precursor cells, which have lost expression of CK18 but which lack expression of the terminal marker SMA. Because phenotypic transitions in tumors are postulated to be long-term processes (40), longer progesterone treatment times or additional exogenous factors may be required to induce complete transition to the myoepithelial state.

Implications. We conclude that progesterone induces a subpopulation of cells within the tumor to gradually switch to a myoepithelial differentiation state. This occurs without any significant changes in overall tumor growth and in a PR-dependent manner. It remains to be determined whether progesterone targets unique cells within the tumor directly, which then expand clonally by cell division, or whether random cells within the tumor exhibit heterogeneous sensitivity to progesterone. In this regard, it is interesting that a very minor cell fraction (0.1%) constitutively expresses CK5. The expression of significant amounts of CK5/CK6 has certainly been correlated in primary human breast cancers with higher grade and worse prognosis (29–31). On the other hand, a few investigators hypothesize that some luminal cells in the normal breast express CK5/CK6, which is then lost during carcinogenesis (49, 50). In this regard, progesterone could be conferring a more "normal" morphology on tumor cells. It remains to be seen whether the differentiation state of tumor cells can be altered by exogenous factors in women; our data show, however, that such a phenotypic switch can be hormonally driven.

Acknowledgments

Received 2/14/2005; revised 6/24/2005; accepted 8/7/2005.

Grants and Funding:Supported by Susan G. Komen Breast Cancer Foundation grant BCTR0402682 and Department of Defense grant 17-01-1-0508 (C.A. Sartorius) and NIH grant CA186899, National Foundation for Cancer Research, and Avon Foundation grants (K.B. Horwitz).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Fatima Nawaz for technical assistance.

References

2. Matthews J, Gustafsson JA. Estrogen signaling: a Graham JD, Clarke CL. Physiological action of
3. Conneely OM, Mulac-Jericevic B, Lydon JP, De Mayo FJ. Reproductive functions of the progesterone
4. Cho H, Aronica SM, Katzenellenbogen BS. Regulation
5. Hewitt SC, Korach KS. Oestrogen receptor knockout
6. Brown PH, Lippman SM. Chemoprevention of breast
7. McGuire WL, Clark GM, Dressler LG, Owens MA. Growth inhibition of 7,12-dimethylbenz(a)anthracene-
8. Brown PH, Lippman SM. Chemoprevention of breast
9. Li S, Lepage M, Merand Y, Belanger A, Labrie F. Growth inhibition of 7,12-dimethylbenz(a)anthracene-
10. Robinson SP, Jordan VC. Reversal of the antitumor effects of tamoxifen by progesterone in the 7,12-
11. Laidlaw IJ, Clarke RB, Howell A, Owen AW, Potten CS, Southcott BM. Carcinoma of the endometrium. Drugs
12. Southcott BM. Carcinoma of the endometrium. Drugs
18. Li S, Lepage M, Merand Y, Belanger A, Labrie F. Growth inhibition of 7,12-dimethylbenz(a)anthracene-
19. Robinson SP, Jordan VC. Reversal of the antitumor effects of tamoxifen by progesterone in the 7,12-
20. Robinson SP, Jordan VC. Reversal of the antitumor effects of tamoxifen by progesterone in the 7,12-
Progestins Initiate a Luminal to Myoepithelial Switch in Estrogen-Dependent Human Breast Tumors without Altering Growth

Carol A. Sartorius, Djuana M.E. Harvell, Tianjie Shen, et al.