

Progesterone Inhibits Growth and Induces Apoptosis in Breast Cancer Cells: Inverse Effects on Bcl-2 and p53*

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ABSTRACT

Progesterone inhibits the proliferation of normal breast epithelial cells *in vivo*, as well as breast cancer cells *in vitro*. But the biologic mechanism of this inhibition remains to be determined. We explored the possibility that an antiproliferative activity of progesterone in breast cancer cell lines is due to its ability to induce apoptosis. Since p53 and bcl-2 genetically control the apoptotic process, we investigated whether or not these genes could be involved in the progesterone-induced apoptosis.

We found a maximal 90 percent inhibition of cell proliferation with T47-D breast cancer cells after exposure to 10 μ M progesterone for 72 hours. Control progesterone receptor negative MDA-231 cancer cells were unresponsive to these two concentrations of progesterone. After 24 hours of exposure to 10 μ M progesterone, cytofluorometric analysis of T47-D breast cancer cells demonstrated 43 percent had undergone apoptosis without signs of necrosis. After 72 hours of exposure to 10 μ M progesterone, 48 percent of the cells had undergone apoptosis and 40 percent demonstrated "leaky" membranes. Untreated cancer cells did not undergo apoptosis.

Evidence proving apoptosis was also demonstrated by fragmentation of nuclear DNA into multiples of oligonucleosomal fragments. After 24 hours of exposure to either 1 μ M or 10 μ M progesterone, the expression by T47-D cancer cells of bcl-2 was down-regulated, and that of p53 was up-regulated as detected by semiquantitative RT-PCR analysis.

These results demonstrate that progesterone at a concentration similar to that seen during the third trimester of pregnancy exhibited a strong antiproliferative effect on at least two breast cancer cell lines. Apoptosis was induced in the progesterone receptor expressing T47-D breast cancer cells.

Introduction

Tumor regression occurs when the rate of cell death is greater than the rate of cell pro-

liferation. Apoptosis is an active, energy-dependent process in which a distinct series of biochemical and molecular events leads to the death of cells by specific signals which are mediated by two different pathways: (A) a ligand to a plasma membrane receptor, or (B) negative induction by loss of a suppressor signal.¹ Apoptosis is mostly characterized by

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chromatin condensation and DNA fragmentation.¹ However, the recent observation that apoptosis can be induced in anucleate cells² implies that cytoplasmatic structures must control the apoptotic process by at least two connected mechanisms: (i) apoptotic cells exhibit intact membranes until an advanced stage of the death process; and (ii) apoptotic cells exhibit changes of their plasma membrane that allow for their recognition and elimination by adjacent cells. This recognition event is mediated by phosphatidylserine (PS) residues,³ which usually are only present on the inner leaflet of the plasma membrane, but in apoptotic cells PS residues are aberrantly expressed on the outer leaflet, hence allowing cytofluorometric analysis of apoptotic cells with high affinity binding proteins for PS.

Both *bcl-2* and *p53* genes and their products have been linked to the second apoptotic pathway.^{4,5} The function of *bcl-2* is to counteract the occurrence of apoptosis and to prolong cell survival. *Bcl-2* proteins are found as dimers facing the cytosol predominantly in the outer mitochondrial membrane with less in the endoplasmatic reticular and nuclear membranes.⁶ It has been shown that the expression of *bcl-2* in normal human breast epithelial cells and the glandular endometrium is hormonally regulated with maximal expression being at the end of the follicular phase and a decrease seen during the luteal phase.^{7,8} Thus the physiological role of *bcl-2* expression and control of homeostasis in the normal breast and endometrium should involve an up-regulation by estradiol and down-regulation by progesterone.

Indeed, a proliferative effect of estrogens on normal breast cells as well as breast cancer cell lines has been reported,⁹ and in a recent work Kandouz et al¹⁰ found that estradiol stimulated and a progesterone inhibited *bcl-2* protein expression in T47-D breast cancer cells. MCF-7 cells normally express high levels of *bcl-2* and low levels of *p53* protein. In addition, *p53* in those cells is nonfunctional because it is segregated in the cytoplasm.¹¹

But more importantly, when MCF-7 cells were transfected with mutant *p53*, the level of *bcl-2* was significantly reduced in comparison

with the parental MCF-7 cells, thus disclosing a possible mechanism for an inverse correlation between the function of the two proteins. In essence, an up-regulation of *p53* induces apoptosis by down-regulating *bcl-2*.¹²

In order to verify our assumption, we have studied the level of mutant *p53* (codon 194) and *bcl-2* transcripts in T47-D breast cancer cells after treatment by progesterone. The results of our work confirm the fact that the levels of *bcl-2* and *p53* expression are inversely regulated by progesterone concomitantly with induced apoptosis and inhibition of growth.

Materials and Methods

MATERIALS

Progesterone-water soluble, propidium iodide and reagents for cell culture were from Sigma (St. Louis, MO). The Ca^{2+} -dependent phosphatidylserine (PS) binding protein annexin V-FITC was from Pharmingen (San Diego, CA). ³H-thymidine was from New England Nuclear (Boston, MA). T47-D, MCF-7 and MDA-231 cell lines were purchased from ATCC (Rockville, MD).

CELL CULTURE AND HORMONE TREATMENT

Cell cultures were done in a 5 percent- CO_2 enriched atmosphere at 37°C in RPMI without phenol-red but containing 2 mM glutamine and antibiotics plus 5 percent-steroid stripped FCS (using dextran-coated charcoal). The effect of progesterone on cancer was studied mainly in T47-D cells, which highly express progesterone receptors.¹³ We used progesterone receptor negative MDA-231 tumor cells as controls to ensure the effect of progesterone. Up to a relative high physiological dose of 10 μM did not cause cell death in a non-specific manner. Exponentially growing cells ($\sim 6 \times 10^4$ /ml) were treated with two different concentrations of progesterone for durations of 24 or 72 hours.

PROLIFERATION ASSAYS

Breast cancer cells treated with two concentrations (1 μM or 10 μM) of progesterone were plated in triplicate wells of 96-well microtiter plates and cultured 24 or 72 hours. During the last four hours of a culture period, 1 μCi of ^3H -thymidine was added to each well. The cultures were harvested onto glass fiber disks by a cell harvester. The disks were assayed for radioactivity in 3 ml Aquasol-2 in a β -counter. The data from three independent experiments are presented as percentages \pm SD of controls.

CYTOFLUOROMETRIC ANALYSIS OF PS EXPOSURE ON CELLS UNDERGOING APOPTOSIS

After being treated with 10 μM progesterone for either 24 hours or 72 hours, the cells were centrifuged, and the pellets washed twice with cold PBS and then resuspended in binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl and 2.5 mM CaCl_2). 5 μl of Annexin V-FITC and 10 μl propidium iodide (50 $\mu\text{g}/\text{ml}$) were added, and the cells were incubated for 15 minutes at room temperature in the dark. Cells were then analyzed for fluorescence using FACScan software (Becton Dickinson).

ASSESSMENT OF DNA FRAGMENTATION

DNA fragmentation was determined by electrophoresis according to a standard method previously described.¹⁴

SEMIQUANTITATIVE REVERSE TRANSCRIPTASE (RT)-PCR ANALYSIS

A sensitive RT-PCR technique previously described by us¹⁵ was used to access the relative levels of mRNA (± 10 –25 percent) for specific gene transcripts. Briefly, poly-adenylated RNA (poly A RNA) was extracted from T47-D cells cultured 24 hours with 0, 1 or 10 μM of progesterone using the mRNA isolation kit from Invitrogen (San Diego, CA). The mRNA ($\sim 1 \mu\text{g}$) was used directly as template for first-strand synthesis of cDNA in a reaction mixture containing 1000U Moloney murine leukemia

virus reverse transcriptase, 50 μM random hexamer or oligo(dT)_{12–14}, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 10 mM DTT, 3 mM MgCl_2 , 0.5 mM each of dGTP, dATP, dTTP and dCTP, all in 60 μl DEPC-treated water. A sample not treated with RT was used as a control for eventual contaminating DNA.

Reactions proceeded at 37°C for 60 minutes, after which they were diluted 1:2 with DEPC-treated water. For hot start PCR, 3 μl cDNA is added to 30 μl of a master mix containing 0.8 μM of each primer, 10 mM Tris-HCl (pH 8.3) and 50 mM KCl. Water is added to the cDNA reactions to a final volume of 40 μl . All reactions are overlaid with light mineral oil and heated to 99°C. After 5 minutes, the reactions are cooled to 94°C and a 10 μl volume containing 7.0 mM MgCl_2 , 1 mM each of dATP, dTTP, dGTP and dCTP, and 1.2 U taq polymerase is added directly through the oil. Cycle parameters are: 95°C for 60s, annealing at 58°C for 30s, 72°C for 30s, with a 15-minute final enzymatic primer extension at 72°C. Reactions are cycled 28 to 32 times (*vide infra*) using an automated thermocycler (Perkin Elmer Cetus). PCR products are separated on agarose gels, stained in ethidium bromide, visualized in UV light, photographed (Polaroid 667 film) and scanned at 600 dpi in 8-bit (256 level) gray scale. A gray scale value (GSV) was defined as the mean gray scale level (0–255) multiplied by the total number of pixels in the scanned PCR band, and used to indicate DNA content. These measurements were performed using commercially available software.

Upon PCR amplification of the serial dilutions of sample cDNA, a concomitant decrease in PCR product was observed. For semiquantitative comparisons, template dilutions were chosen to fall within the range in which input template concentration correlated with the intensity of amplification product. Similarly, a varying the number of PCR cycles did not change the relative differences between samples. These basic studies indicated that our PCR conditions were never within the plateau phase of amplification. Plateau occurs at higher cycles during PCR when band intensity no longer increases or even

decreases due to build-up of higher molecular weight products. Therefore, PCR reactions, in the linear phase of amplification, are used for comparisons of gene regulation following treatment with progesterone. The following amplimers (supplied commercially by Operon Technologies, Alameda, CA) were used: Human bcl-2 (GenBank accession #M14745) forward primer was 5'-GGTGCCACCTGTG-GTCCA-3', and the reverse primer was 5'-ACTTGTGGCCCAGATAGG-3', amplifying a product of 451 bp. Human p53 forward primer was 5'-GAGCCCCCTCTGAGTCAG-3', and the reverse primer was 5'-GCAAAACA-TCTTGTTGAG-3, amplifying a product of 375 bp. Human β -actin forward primer was 5'-GCTCTCTTCCA-GCCITCCTTC-3', and

the reverse primer was 5'-AGAGCCACCAAC-CCACACAGAG, amplifying a product of 261 bp. β -actin was an internal control to confirm equal amounts of RNA were reversed transcribed and loaded on the gel.

Results

PROGESTERONE INHIBITS PROLIFERATION OF BREAST CANCER CELLS UP TO 90 PERCENT IN T47-D BREAST CANCER CELLS

As shown in figure 1, proliferation of T47-D breast tumor cells was inhibited by progesterone. After 24 hours, 1 μ M progesterone inhibited 3 H-thymidine uptake by 46 ± 12 percent ($n = 3$, $p < 0.001$) and 10 μ M by 63 ± 4 percent ($n = 3$, $p < 0.001$), respectively. After 72 hours,

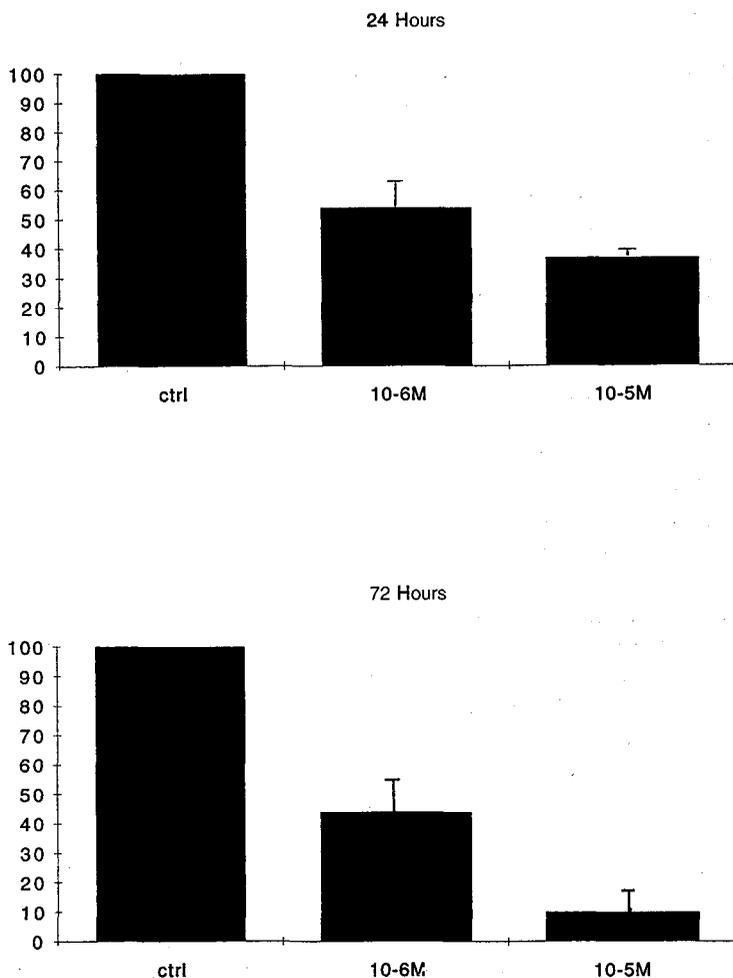


FIGURE 1. The effect of progesterone on 3 H-thymidine uptake into DNA of T47-D human breast cancer cells. The cells were exposed to two concentrations of progesterone as indicated during a culture period of 24 and 72 hours, respectively. The percentages represent the mean of three individual experiments. See text for statistics.

1 μM progesterone inhibited the uptake 56 ± 14 percent ($n = 3, p < 0.001$) and 10 μM 90 ± 10 percent ($n = 3, p < 0.001$), respectively. When MCF-7 breast tumor cells were cultured 24 hours with 10 μM progesterone, an inhibition of 35 ± 7 percent ($n = 3, p < 0.001$) was found (this is not shown in figure 1). After 72 hours of culture, neither 10 μM nor 1 μM progesterone significantly inhibited proliferation of the control MDA-231 cells.

ANALYSIS OF PROGESTERONE-INDUCED APOPTOSIS

T47-D breast tumor cells undergo apoptosis in response to progesterone. After 24 hours or 72 hours of culture in the presence of 10 μM progesterone, the aberrant surface exposure of phosphatidylserine (PS) was monitored by means of an FITC-annexin V conjugate. Loss of membrane integrity was measured by the ability of cells to exclude the DNA-binding fluorescent dye propidium iodide. Representative data are shown in figure 2.

As shown in figure 2B, 24 hours progesterone treatment strongly induced PS externalization and 43 percent of the cells displayed a uniform high binding to annexin-FITC. After 72 hours of exposure to progesterone, 48 percent of the cells displayed binding to annexin V-FITC (figure 2C). No binding to annexin V-FITC was observed in the control population of cells (figure 2A). Since it cannot be excluded that the appearance of annexin V-FITC binding cells coincided with the appearance of cells with "leaky" membranes in these cultures, thereby allowing annexin V to gain access to PS inside the cell, control and progesterone-treated cells were stained with annexin V-FITC, along with propidium iodide, to reveal cells that bound annexin V to have plasma membrane damage.

As documented in figure 2B, a negligible portion of the cells after 24 hours of exposure to progesterone were annexin V-FITC⁺/propidium iodide⁺ (fluorescence 1 and 2, respectively, in figure 2), hence confirming annexin V was indeed binding to the external PM leaflet of the cells undergoing apoptosis. However, after 72 hours of exposure to pro-

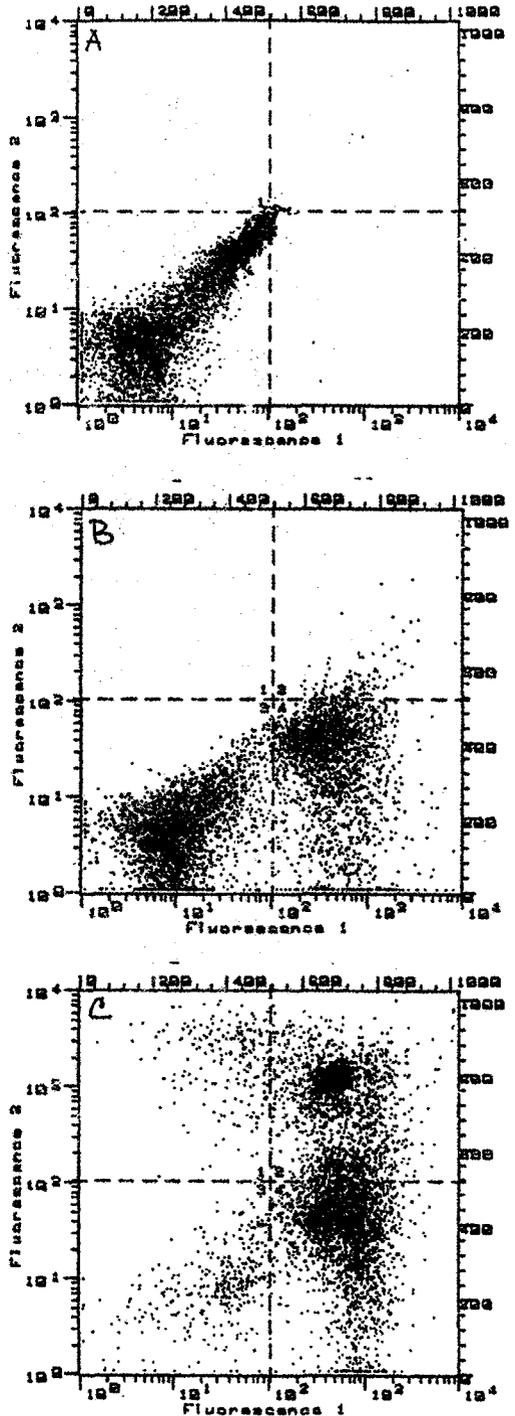


FIGURE 2. Annexin V binding and propidium iodide uptake in T47-D breast tumor cells undergoing apoptosis versus necrosis. T47-D cells were induced to undergo apoptosis by exposure to none (A) or 10 μM progesterone for 24 hours (B) or 72 hours (C). Cells were stained with annexin V-FITC and propidium iodide. Cells (10,000 per assay) were then analyzed by flow cytometry. The percentage of annexin V⁺/propidium⁻ in the lower right quadrant in B is 43 percent (single cells undergoing apoptosis). The percentage of annexin V⁺/propidium⁻ in the lower right quadrant in C is 48 percent. The percentage of annexin V⁺/propidium⁺ in upper right quadrant in B is <2 percent and in C is 40 percent (cellular necrosis). Results are representative of four individual experiments.

gestosterone, 40 percent of the cells now stained positive for propidium iodide, indicating signs of necrosis (figure 2C). After 24 hours exposure to 1 μM progesterone, 23 percent of the cells were annexin V-FITC positive (data not shown). Apoptosis introduced after 72 hours exposure to 10 μM progesterone was confirmed by DNA electrophoresis. DNA laddering and nuclear fragmentation, characteristic of apoptosis, was observed only in cells treated with progesterone for 72 hours (right lane in figure 3)

SEMIQUANTITATIVE RT-PCR ANALYSIS OF bcl-2 AND p-53 TRANSCRIPTS IN PROGESTERONE TREATED CELLS

Expression of the apoptosis-related genes bcl-2 and p53 was tested after cells were treated with 0, 1 and 10 μM progesterone for 24 hours. As shown in figure 4, the mRNA level of bcl-2 was markedly decreased after treatment with progesterone. Inversely, the mRNA level of p53 was markedly increased. Ratios of the relative amounts of the target gene (*ie* bcl-2 or p53) to constitutively expressed β -actin gene were calculated after scanning of PCR products from the serial dilutions (1/2, 1/4, 1/8) of template cDNA during the linear phase of amplification (gels not shown). The relative densitometric values (target gene/ β -actin gene) were: for control bcl-2: 0.66 ± 0.06 ($n = 3$) versus 10 μM progesterone bcl-2: 0.16 ± 0.03 ($n = 3$, $p = 0.022$), and for control p53: 0.54 ± 0.17 ($n = 3$) versus 10 μM progesterone p53: 1.06 ± 0.08 ($n = 3$, $p = 0.017$).

Discussion

In 1973 Sherman et al¹⁶ hypothesized that breast cancer risk factors are the "manifestation of inadequate corpus luteum secretory function." In that light the absence of sufficient cyclic progesterone secretion does provide a situation favorable to estrogen mediated development of mammary carcinoma. Cowan et al¹⁷ categorized into two groups 1083 women as to the cause of their infertility: those with endogeneous progesterone deficiency



FIGURE 3. DNA fragmentation in T47-D cells induced by 10 μM progesterone after 72 hours of culture (right lane), and untreated control T47-D cells (left lane). Data are representative of three experiments.

due to unovulatory cycles, and those with "nonhormonal causes." During a 33 year follow-up period, it was found that women in the progesterone deficient group had 5.4 times the risk of premenopausal breast cancer and a 10-fold increase in death from all malignant neoplasms compared to women in the nonhormonal group.

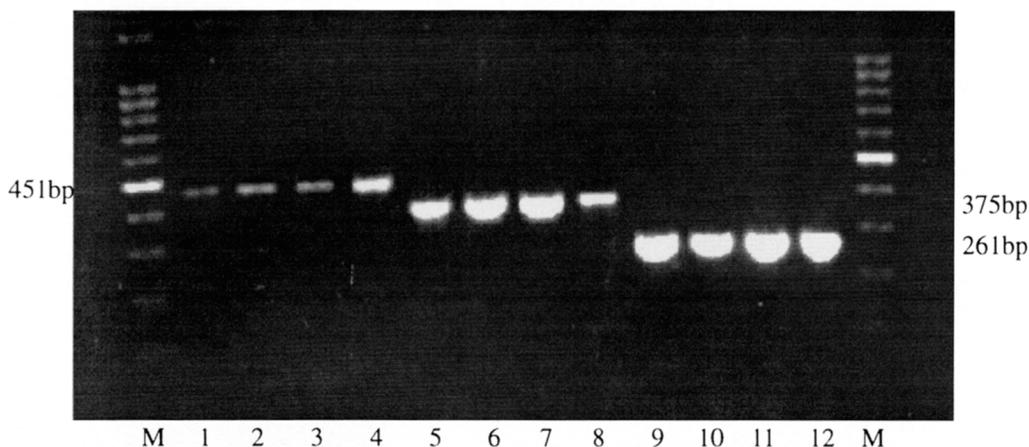


FIGURE 4. Representative data of three individual experiments showing effect of progesterone on steady state levels of *bcl-2*, *p53* and β -actin mRNAs in T47-D cells as analysed by subplateau RT-PCR. The products of PCR amplification were separated by agarose gel electrophoresis, stained with ethidium bromide and visualized under UV light. Gels were photographed (Polaroid 667 film) and scanned, using commercially available software (see also Methods). cDNA were amplified with *bcl-2* primers to produce a product of 451 bp (lane 1 to 4), with *p53* primers to produce a product of 375 bp (lane 5 to 8), with β -actin primers (internal control) to produce a product of 261 bp (lane 9 to 12). T47-D breast cancer cells were exposed 24 hours to 1 μ M progesterone in lane 1,5 and 9 or 10 μ M progesterone in lane 2,3,6,7,10 and 11. Untreated controls are shown in lane 4,8 and 12. Note progesterone down-regulates *bcl-2* mRNA and upregulates *p53* mRNA. M is a 100-bp DNA ladder for size comparison.

To determine further a possible association of progesterone deficiency and breast cancer risk, we investigated the effect of progesterone on the growth of breast tumor cells and determined whether progesterone induces apoptosis in breast cancer cells and which apoptosis-related genes might be involved. Here, we report that progesterone *in vitro* inhibits proliferation of at least two breast tumor cell lines, T47-D and MCF-7. Progesterone inhibition was dose dependent with a maximal 90 percent inhibition observed after 72 hours of culture in the presence of 10 μ M progesterone. Growth of progesterone receptor negative control MDA-231 breast cancer cells was not affected by progesterone at any of the concentrations used, hence excluding any progesterone-induced non-specific cell death.

Our results confirm data reported by other investigators on breast cancer cell lines in culture,^{18,19} especially the strong progesterone receptor positive T47-D cells.¹³ It is important to note, however, that normal breast epithelial cells respond in the same way to progesterone. Thus, it was found⁹ that the progesterone R5020 at a concentration of 1 μ M inhibited

>90 percent the growth after 72 hours in culture. Estradiol at a concentration of 0.01 μ M stimulated proliferation of normal breast epithelial cells, which was blocked >90 percent by 0.1 μ M progesterone.

Also, *in vivo* studies show that progesterone decreases the mitotic activity of normal human breast epithelial cells. Thus, Chang et al²⁰ in a double-blind randomized study topically applied progesterone, estradiol, or a combination of estradiol and progesterone, or placebo daily for period of a 10 to 13 days, exactly the same duration as a normal luteal phase *in vivo*. In the surgically biopsied breast epithelium, it was found that the number of cycling epithelial cells increased with the concentration of estradiol; whereas, increasing concentrations of progesterone significantly decreased the number of cycling epithelial cells. Progesterone reduced estradiol-induced proliferation. This is an important observation. It indicates that natural progesterone replacement (NPR) can prevent normal breast epithelium from transforming into estradiol-induced hyperplasia. Similarly, Barrat et al²¹ found that sustained levels of progesterone in breast tis-

sue maintained for >10 days decreased the mitotic activity in the normal breast epithelial cells.

Collectively, these data strongly support the concept, as is also the case in endometrium, that during a normal 14-day luteal phase, progesterone controls the human breast epithelial cell cycle. Such control could be mediated by apoptosis. Inversely to these observations, some synthetic progestins in oral contraceptives, *eg*, 19-nortestosterone, have been reported to stimulate proliferation of breast cancer cells, but it should be emphasized that many progestins have estrogenic properties with the ability to activate the estrogen receptor, but not the progesterone receptor. This explains their growth-stimulatory potential.^{22,23} In a broader context, an evaluation, recently documented, of the "total estrogenicity" of combinations of synthetic progestins may provide clues likely to be detrimental to women: medroxyprogesterone (progestin), contrary to natural progesterone, stimulated the development of coronary vasospasm in ovariectomized rhesus monkeys.

A critical event during programmed cell death appears to be the acquisition of plasma membrane changes. In the apoptotic cells with still intact cell membranes, there is observed a loss of phosphatidylserine bilayer asymmetry.²⁵ This effect is also obtained in anucleate cells, indicating that the nucleus does not intervene in the sequence of initial events coupled to phosphatidylserine exposure. Thus, phosphatidylserine residues, which usually are only present on the inner leaflet of the plasma membrane, are expressed on the outer leaflet of apoptotic cells in a sequence followed by full-blown DNA fragmentation.³ The aberrant exposure on the outer plasma membrane leaflet of phosphatidylserine can be quantified cytofluorometrically at the single-cell level as an FITC-annexin V conjugate. Such observations have been reported in Jurkat cultures where apoptosis was induced by Fas ligation.²⁵ Phosphatidylserine exposure on apoptotic Jurkat cells preceded the increase in membrane permeability and nuclear condensation. Of interest was the observation that the effector

element(s) responsible for activating phosphatidylserine translocase activity was likely to be downstream of bcl-2, because the morphological features of apoptosis were prevented when bcl-2 was overexpressed in transfected cell lines.²⁵ Several studies have concluded that progesterone can induce apoptosis in normal breast and endometrial tissue.^{8,9} Of great importance, recently, Bu et al²⁶ reported progesterone at a concentration of 10 μ M induced apoptosis in two ovarian carcinoma cell lines. We found that 43 percent of T47-D breast tumor cells were annexin V positive after exposure to 10 μ M progesterone for 24 hours, indicating apoptotic pathways were activated in these tumor cells. After 72 hours of culture in the presence of 10 μ M progesterone, 48 percent of the T47-D cancer cells were annexin V-FITC positive, but 40 percent of the cells now being propidium iodide positive is a significant sign of "leaky" membranes (necrosis). Similar studies using breast cancer cells, to the best of our knowledge, have never been reported heretofore.

Apoptosis is an active process which depends on the expression of specific sets of genes. Among these genes, p53 can induce apoptosis; bcl-2 inhibits apoptosis.^{4,5} It is of interest to note that Lee et al²⁷ analyzed 101 invasive ductal carcinomas of the breast for the expression of bcl-2 and p53. These investigators found reciprocal expression of bcl-2 and p53 present in 71.3 percent of cases, and suggested bcl-2 could be an estrogen-related protein. Bu et al²⁶ found by Northern blots that exposure of ovarian tumor cells to 10 μ M progesterone for 72 hours also strongly increased the levels of p53 mRNA. Combined with our data on breast cancer, we speculate that progesterone occurs naturally as a homeostatic control of normal proliferation in healthy young women. Cancer then, together with other factors, could be a natural body-wide phenomenon when anovulation becomes prevalent.

Using a sensitive, semiquantitative RT-PCR technique, we found 1 and 10 μ M progesterone strongly down-regulated expression of bcl-2 and up-regulated expression of p53 at the

transcriptional level. A similar inverse relationship of bcl-2 and p53 at the translational level has been reported by Haldar et al,¹² who found eight of 12 breast cancer cell lines had an inverse and reciprocal level of expression of those two genes, suggesting that p53 determines bcl-2 down-regulation. This was confirmed in MCF-7 cells transfected to express high levels of p53 protein, which significantly reduced the level of bcl-2 protein. Of note, others¹⁰ have reported progesterone R5020 down-regulates expression of bcl-2 protein in T47-D breast cancer cells. Apoptosis in this study was not investigated.

Although the role of p53 in bcl-2 regulation is not clear, the correlation between the down-regulation of bcl-2 and apoptosis as reported in the present study is a possibility that must be further investigated, along with p21WAF-1. p21 is a critical component in the control and initiation of apoptosis, and recently it was reported that progesterone up-regulates the expression of p21 in PR-positive T47-D breast cancer cells.²⁸

Finally, it should be emphasized that *in vitro* studies do not adequately reproduce interactions between epithelium and stroma *in vivo*, but the present data do support the premise that progesterone in substitutive doses could have a therapeutic value in the prevention and treatment of patients with breast cancer.

Acknowledgments

The work was supported by the University of California Breast Cancer Research Program (1RB-0008) and by a gift from Mrs. L. Domz.

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