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Bcl-2, survivin and variant CD44 v7–v10 are downregulated and p53 is upregulated in breast cancer cells by progesterone: Inhibition of cell growth and induction of apoptosis

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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, bcl-2 and survivin 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% inhibition of cell proliferation with T47-D breast cancer cells after exposure to 10 μM progesterone for 72 h. Control progesterone receptor negative MDA-231 cancer cells were unresponsive to 10 μM progesterone. The earliest sign of apoptosis is translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane and can be monitored by the calcium-dependent binding of annexin V in conjunction with flow cytometry. After 24 h of exposure to 10 μM progesterone, cytofluorometric analysis of T47-D breast cancer cells indicated 43% were annexin V-positive and had undergone apoptosis and no cells showed signs of cellular necrosis (propidium iodide negative). After 72 h of exposure to 10 μM progesterone, 48% of the cells had undergone apoptosis and 40% were annexin V positive/propidium iodide positive indicating signs of necrosis. Control 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 h of exposure of T47-D cells to either 1 or 10 μM progesterone, we observed a marked down-regulation of protooncogene bcl-2 protein and mRNA levels. mRNA levels of survivin and the metastatic variant CD44 v7–v10 were also downregulated. Progesterone increased p53 mRNA levels.

These results demonstrate that progesterone at relative high physiological concentrations, but comparable to those seen in plasma during the third trimester of human pregnancy, exhibited a strong antiproliferative effect on breast cancer cells and induced apoptosis. (Mol Cell Biochem **202**: 53–61, 1999)

Key words: breast cancer cells, anti-apoptotic genes, apoptosis, progesterone

Introduction

Tumor regression occurs when the rate of cell death is greater than the rate of cell proliferation. 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 chromatin condensation and DNA fragmentation [1]. However, the recent observation that apoptosis can be induced in anucleate cells [2] implies

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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 both 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]. Survivin is a new inhibitor of apoptosis protein (IAP) expressed during development and in human cancer *in vivo* [7]. Expression of survivin with other anti-apoptosis genes like bcl-2 translate in drastically reduced apoptosis of cancer cells *in vivo* [8].

CD44, the prominent vertebrate cell surface receptor for hyaluronan, exists in a variety of isoforms resulting from alternative splicing of a single gene. Since the spliced variant of CD44 expressing exons v7–v10 in tandem correlate with increased cell motility, and with poor clinical prognosis in several kinds of carcinomas [9], we investigated the effect of progesterone on transcripts levels of this isoform.

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 [10, 11]. Thus the physiological role of bcl-2 expression and control of homeostasis in the normal breast and endometrium should involve an upregulation 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 [12], and in a recent work Kandouz *et al*. [13] found that estradiol stimulated and a progesterone inhibited bcl-2 protein expression in cells of the breast cancer cell line T47-D.

Cells from the breast cancer cell line MCF-7 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 [14]. 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 [15]. Additional support for this observation are the experiments of Miyashita *et al*. [16, 17] showing the existence of a negative response element in the bcl-2 gene through which p53 either directly or indirectly transcriptionally downregulate expression of this gene. Tumor suppressor p53 may also negatively regulate survivin gene expression in a mechanism potentially similar to its role in transcriptional repression of bcl-2 [18].

Our objective in this study was therefore to investigate the level of p53 as well as levels of bcl-2, survivin and variant CD44 v7–v10 transcripts primary in T47-D breast cancer cells after treatment by progesterone. The results of our work confirm the fact that the levels of bcl-2, survivin and variant CD44 are downregulated and p53 upregulated by progesterone concomitantly with inhibition of growth and induced apoptosis.

Materials and methods

Materials

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

Cell culture and hormone treatment

Cell cultures were done in a 5%-CO_2 enriched atmosphere at 37°C in RPMI without phenol-red but containing 2 mM glutamine and antibiotics plus 5%-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 [19]. 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 (~ 6×10^4/ml) were treated with two different concentrations of progesterone for durations of 24 or 72 h.

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 h. During the last 4 h of a culture period 1 μCi of ^{3}H-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 3 independent experiments are presented as percentages ± S.D. of controls.

Cytofluorometric analysis of PS exposure on cells undergoing apoptosis

After being treated with 10 μM progesterone for either 24 or 72 h, 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 $CaCl_2$). Five μl of Annexin V-FITC and 10 μl propidium iodide (50 μg/ml) were added, and the cells were incubated for 15 min at room temperature in the dark. Cells were then analyzed for fluorescence using FACScan software (Becton Dickinson).

Cytofluorometric analysis of bcl-2 protein

For the detection of bcl-2 protein, the cells were fixed with 0.25% formaldehyde for 30 min at room temperature, washed once, and suspended in a PBS medium containing 10% fetal bovine serum (FBS). Permeabilization of the cells was achieved by adding 0.025% saponin (Sigma, St. Louis, MO, USA) for 10 min before adding the antibody. The permeabilized cells were then incubated at 4°C with FITC-conjugated hamster anti-human bcl-2 antibody (clone 6C8, Ig2a isotype from Pharmigen, San Diego, CA, USA) for 30 min. 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 [20].

Semiquantitative reverse transcriptase (RT)-PCR analysis

A sensitive RT-PCR technique previously described by us (21) was used to access the relative levels of mRNA (± 10–25%) for specific gene transcripts. Briefly, poly-adenylated RNA (poly A RNA) was extracted from T47-D cells cultured 24 h with 0, 1 or 10 μM of progesterone using the mRNA isolation kit from Invitrogen (San Diego, CA, USA). The mRNA (~ 1 μg) was used directly as template for first strand synthesis of cDNA in a reaction mixture containing 1000 U 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 min, after which they were diluted 1:2 with DEPC-treated water. For hot start PCR 3 μl cDNA was 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 overlayed with light mineral oil and heated to 99°C. After 5 min, the reactions are cooled to 94°C and a 10 μl vol. 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 60 sec, annealing at 58°C for 30 sec, 72°C for 30 sec, with a 15 min final enzymatic primer extension at 72°C. Reactions are cycled 28–32 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 never were 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 intron spanning amplimers (supplied commercially by Operon Technologies, Alameda, CA, USA) were used; human bcl-2 (GenBank accession #M14745) forward primer was 5′-GGTGCCACCTGTGGTCCA-3′, and the reverse primer was 5′-ACTTGTGGCCCAGATAGG-3′, amplifying a product of 451 bp. Human p53 forward primer was 5′-GAGCCC-CCTCTGAGTCAG-3′, and the reverse primer was 5′-GCA-AAACATCTTGTTGAG-3′, amplifying a product of 375 bp. Human survivin (GenBank accession #U75285) forward primer was 5′-CCCTGCCTGGCAGCCCTTTC-3′ and the reverse primer was 5′-CTGGCTCCCAGCCTTCCA-3′, amplifying a product of 188 bp. Variant CD44 v7–v10 forward primer (exon 10) was 5′-GATGGCATGAGGGATATCGCC-3′ and reverse primer (exon 14) was 5′-TTC-CTTCGTGTG-GTGGTAATG-3′ amplifying a product of 501 bp. Human

β-actin forward primer was 5′-GCTCTCTTCCA-GCCTTC-CTTC-3′, and the reverse primer was 5′-AGAGCCACC-AACCCACA-CAGAG, 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% in T47-D breast cancer cells

As shown in Fig. 1, proliferation of T47-D breast tumor cells was inhibited by progesterone. After 24 h, 1 μM progesterone inhibited ^{3}H-thymidine uptake by 46 ± 12% (n = 3, $p < 0.001$) and 10 μM by 63 ± 4% (n = 3, $p < 0.001$), respectively. After 72 h, 1 μM progesterone inhibited the uptake 56 ± 14% (n = 3, $p < 0.001$) and 10 μM 90 ± 10% (n = 3, $p < 0.001$), respectively. When MCF-7 breast tumor cells were cultured 24 h with 10 μM progesterone an inhibition of 35 ± 7% (n = 3, $p < 0.001$), was found (this is not shown in Fig. 1). After 72 h of culture, neither 10 nor 1 μM progesterone significantly inhibited proliferation of the control MDA-231 cells.

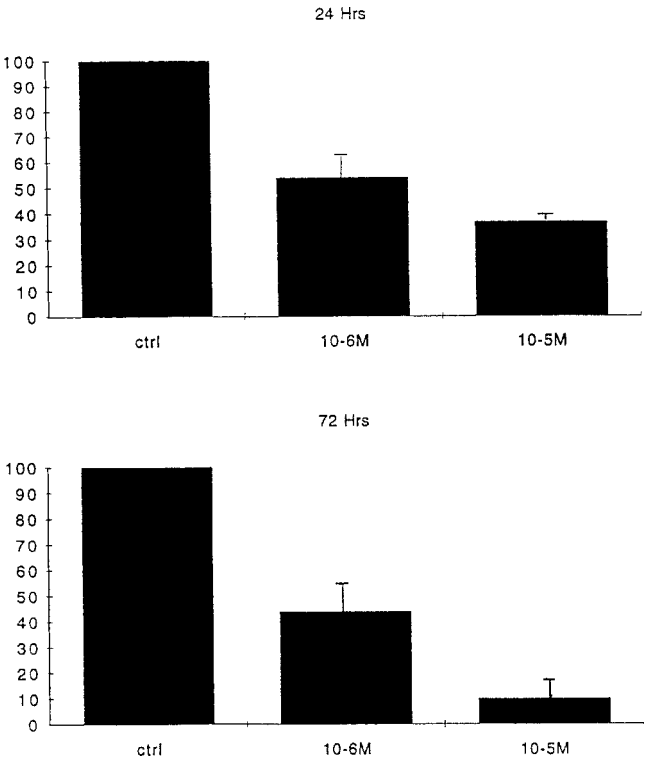

Fig. 1. The effect of progesterone on ^{3}H-thymidine uptake into DNA of T47-D human breast cancer cells. The cells were exposed to two concentrations of progesterone as indicated during a culture period of 24 and 72 h, respectively. The percentages represent the mean of 3 individual experiments. See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

Analysis of progesterone-induced apoptosis

T47-D breast tumor cells undergo apoptosis in response to progesterone. After 24 or 72 h 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 Fig. 2.

As shown in Fig. 2B, 24 h progesterone treatment strongly induced PS externalization and 43% of the cells displayed a uniform high binding to annexin-FITC. After 72 h of exposure to progesterone, 48% of the cells displayed binding to annexin V-FITC (Fig. 2C). No binding to annexin V-FITC was observed in the control population of cells (Fig. 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 have plasma membrane damage.

As documented in Fig. 2B, a negligible portion of the cells after 24 h of exposure to progesterone were annexin V-FITC^{+}/propidium iodide^{+} (fluorescence 1 and 2, respectively in Fig. 2), hence confirming annexin V was indeed binding to the external PM leaflet of the cells undergoing apoptosis. However, after 72 h of exposure to progesterone, 40% of the cells now stained positive for propidium iodide indicating signs of necrosis (Fig. 2C). After 24 h exposure to 1 μM progesterone, 23% of the cells were annexin V-FITC positive (data not shown). Apoptosis introduced after 72 h 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 h (Fig. 3, right lane).

Semiquantitative RT-PCR analysis of bcl-2, p-53 survivin and variant CD44 v7–v10 transcripts in progesterone treated T47-D 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 h. As shown in Fig. 4A, the mRNA level of bcl-2 was markedly decreased after treatment with progesterone. Inversely, the mRNA level of p53 was markedly increased. Expression of survivin and variant CD44 v7–v10 was also tested after cells were treated with 0, 1 and 10 μM progesterone

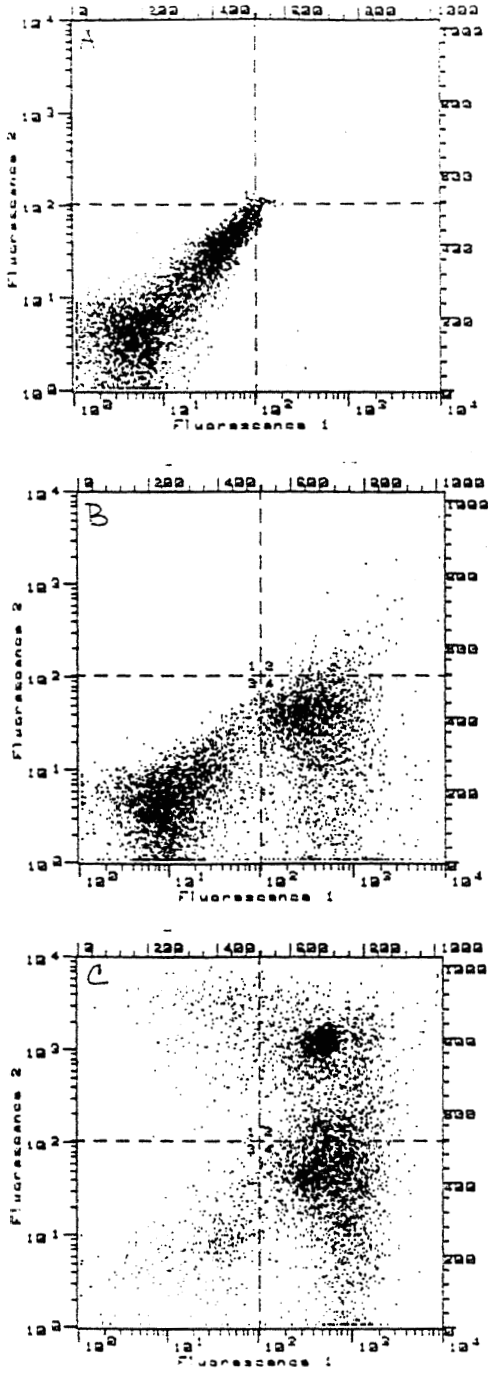

Fig. 2. Annexin V binding and propidium iodide uptake in T47-D breast tumor cells undergoing apoptosis vs. necrosis. T47-D cells were induced to undergo apoptosis by exposure to none (A) or 10 μM progesterone for 24 h (B) or 72 h (C). Cells were stained with annexin V-FITC and propidium iodide. Cells (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% (single cells undergoing apoptosis). The percentage of annexin V^+/propidium$^-$ in the lower right quadrant in C is 48%. The percentage of annexin V^+/propidium$^+$ in upper right quadrant in B is < 2% and in C is 40% (cellular necrosis). Results are respresentative of 4 individual experiments.

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

for 24 h. As shown in Fig. 4B, the mRNA levels of survivin were markedly decreased. Interestingly, transcripts of variant CD44 v7–v10 were completely eliminated after treatment with progesterone (Fig. 4B). Ratios of the relative amounts of the target gene (i.e. bcl-2, p53, or survivin) 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) vs. 10 μM progesterone bcl-2: 0.16 ± 0.03 (n = 3, p = 0.022), for control p53: 0.54 ± 0.17 (n = 3) vs. 10 μM progesterone p53: 1.06 ± 0.08 (n = 3, p = 0.017) and for control survivin: 0.61 ± 0.04 (n = 3) vs. 10 μM progesterone survivin: 0.20 ± 0.05 (n = 3, $p < 0.001$).

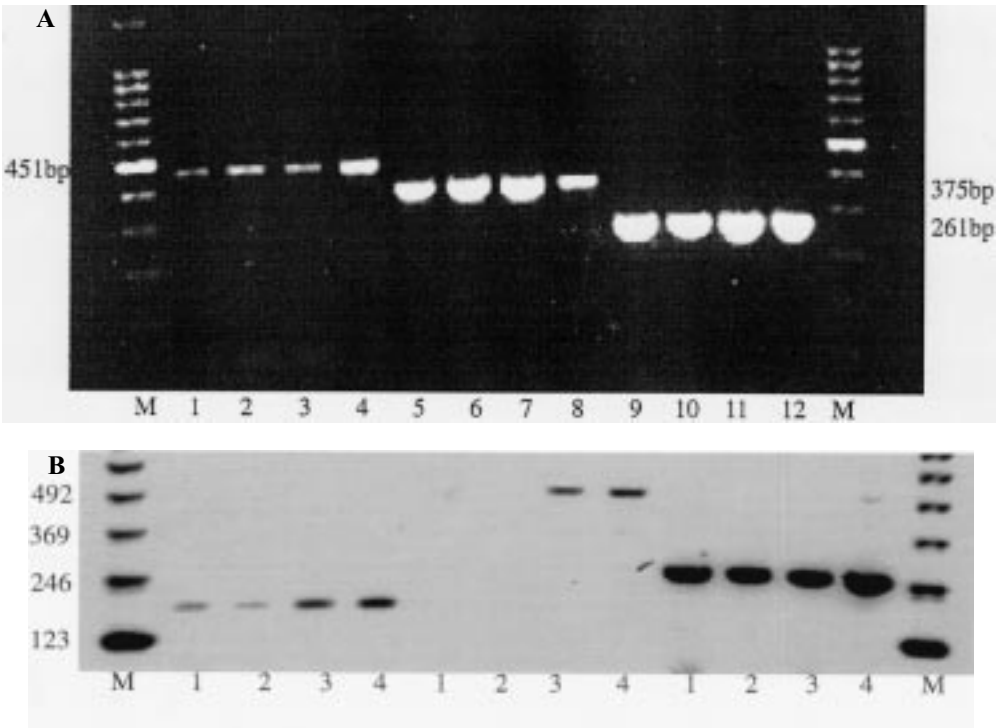

Fig. 4. Representative data of 3 individual experiments showing effect of progesterone on steady state levels of bcl-2, survivin, variant CD44 v7–v10, p53 and β-actin mRNAs in T47-D cells as analysed by subplateau RT-PCR. The products of PCR amplification were separated by agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV light. Gels were photographed (Polaroid 667 film), and scanned using commercially available software (see also Methods). A: cDNA were amplified with bcl-2 primers to produce a product of 451 bp (lanes 1–4), with p53 primers to produce a product of 375 bp (lanes 5–8), with β-actin primers (internal control) to produce a product of 261 bp (lanes 9–12). T47-D breast cancer cells were exposed 24 h to 1 μM progesterone in lane 1, 5 and 9 or 10 μM progesterone in lane 2, 3,6,7,10 and 11. Untreated controls are shown in lane 4,8 and 12. Note progesterone downregulates bcl-2 mRNA and upregulates p53 mRNA. M is a 100-bp DNA ladder for size comparison; B: cDNA were amplified with survivin primers to produce a product of 188 bp (first 4 lanes), with variant CD44 v7–v10 primers to produce a product of 501 bp (middle 4 lanes), with β-actin primers to produce a product of 261 bp (last 4 lanes). T47-D breast cancer cells were exposed 24 h to 1 μM progesterone (all lanes 1) or 10 μM progesterone (all lane 2). Untreated controls are shown in lanes 3 and 4. Note progesterone downregulates survivin and eliminates variant CD44 v7–v10 levels of mRNA.

Bcl-2 protein expression in progesterone treated T47-D cells

To examine whether bcl-2 expression was correlated with the sensitivity to progesterone induced apoptosis, intracytoplasmatic bcl-2 was analyzed after cells were treated with 10 μM progesterone for 0, 24 and 72 h. As shown in Fig. 5A, T47-D cells expressed bcl-2, but a significant decrease in bcl-2 expression was observed after exposure to progesterone. Thus, the bcl-2 positivity dropped from 78 to 65% after 24 h of exposure to progesterone (Figs 5A and 5B). After 72 h the bcl-2 positivity dropped to 3% (Fig. 5C). Downregulation of bcl-2 gene transcripts was detected by RT-PCR (Fig. 4); a relative faint band (Fig. 4A, lanes 2 and 3) was observed after 24 h exposure to 10 μM progesterone, despite measurable amounts of protein (65% positivity in Fig. 5B). This may actually suggest that the bcl-2 protein in T47-D tumor cells has a relative long half-life.

Discussion

In 1973 Sherman *et al*. [22] 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 al*. [23] categorized into two groups 1083 women as to the cause of their infertility; those with endogenous progesterone deficiency 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 a 5.4 times the risk of premenopausal breast cancer and 10-fold increase in deaths from all malignant neoplasms compared to women in the nonhormonal group.

To further determine 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

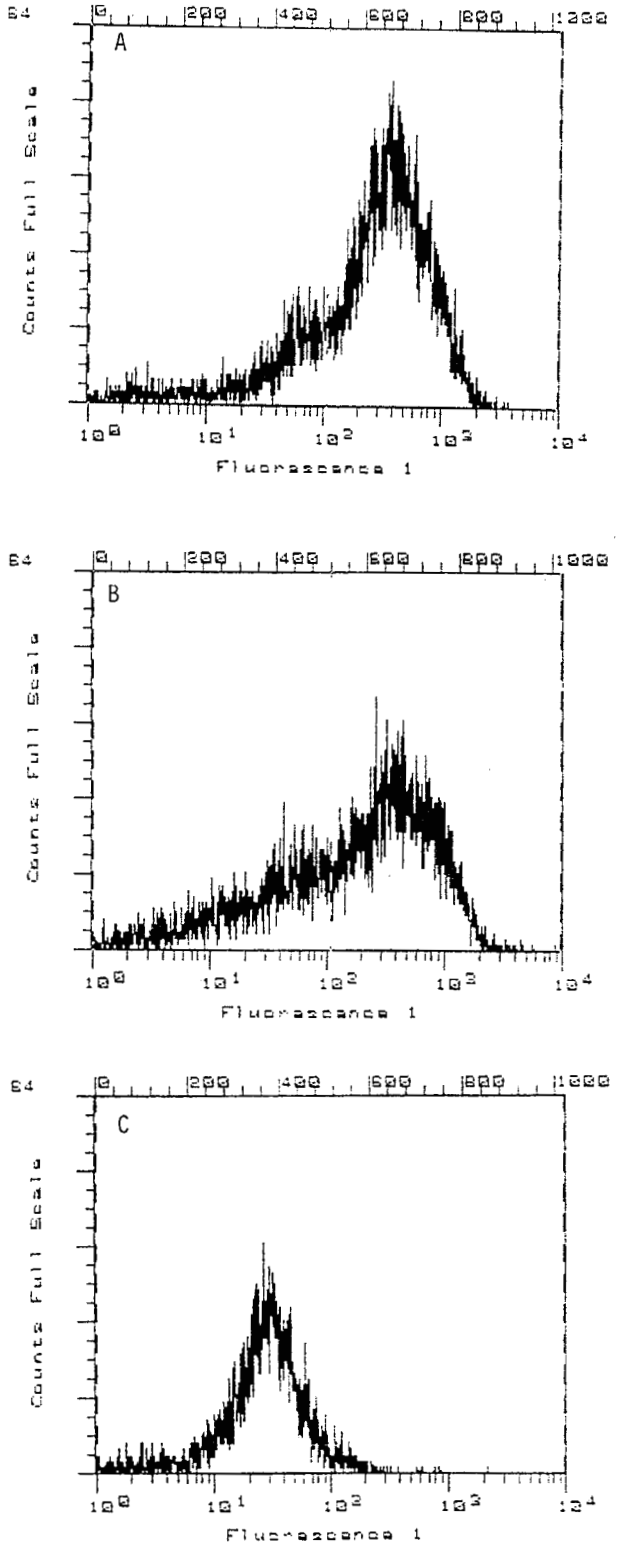

Fig. 5. Cytofluorometric analysis of cytoplasmatic bcl-2 protein in T47-D tumor cells exposed to 10 μM progesterone for 24 h (B), or 72 h (C). (A) is control. Within 24 h bcl-2 positivity dropped from 78 to 65% (A and B). Within 72 h bcl-2 dropped from 78 to 3% (A and C).Representative data of 3 individual experiments.

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 cells lines, T47-D and MCF-7. Progesterone inhibition was dose dependent with a maximal 90% inhibition observed after 72 h of culture in the presence of 10 μM 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 [24, 25], especially the strong progesterone receptor positive T47-D cells [19]. It is important to note, however; that normal breast epithelial cells respond in the same way to progesterone. Thus, it was found [12] that the progesterone R5020 at a concentration of 1 μM inhibited > 90% the growth after 72 h in culture. Estradiol at a concentration of 0.01 μM stimulated proliferation of normal breast epithelial cells, which was blocked > 90% by 0.1 μM progesterone.

Also, *in vivo* studies show that progesterone decreases the mitotic activity of normal human breast epithelial cells. Thus, Chang *et al*. [26] in a double-blind randomized study topically applied progesterone, estradiol, or a combination of estradiol and progesterone, or placebo daily for period of a 10–13 days, exactly the same duration as a normal luteal phase *in vivo*. In the surgically biopsied breast epithelium, it was found that increased concentration of estradiol increased the number of cycling epithelial cells; whereas, increasing concentrations of progesterone significantly decreased the number of cycling epithelial cells. Progesterone reduced estradiol-induced proliferation. This is an important observation that recently was confirmed by Foidart *et al*. in a double-blind randomized study [27]. It indicates that natural progesterone replacement (NPR) can prevent normal breast epithelium from transforming into estradiol-induced hyperplasia. Similar, Barrat *et al*. [28] found that sustained levels of progesterone in breast tissue 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, e.g. 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 progesterone receptor. This explains their growth-stimulatory potential [29, 30]. In a broader context, an evaluation of the 'total estrogenicity' of combinations of synthetic progestins may provide clues likely to be

detrimental to women, which is documented in a recent study [31], where 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 [32]. 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 [32].

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 [32].

Several studies have concluded that progesterone can induce apoptosis in normal breast and endometrial tissue [11, 12]. Of great importance, recently, Bu *et al*. [33] reported progesterone at a concentration of 10 μM induced apoptosis in two *ovarian* carcinoma cell lines. We found, that 43% of T47-D breast tumor cells were annexin V positive after exposure to 10 μM progesterone for 24 h, indicating apoptotic pathways were activated in these tumor cells. After 72 h of culture in the presence of 10 μM progesterone, 48% of the T47-D cancer cells were annexin V-FITC positive, but 40% 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 not 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; and bcl-2 inhibits apoptosis [4, 5]. It is of interest to note, that Lee *et al*. [34] analysed 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% of cases, and suggested bcl-2 could be an estrogen-related protein.

Bu *et al*. [33] found by Northern blots that exposure of ovarian tumor cells to 10 μM progesterone for 72 h 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 μM 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*. [15], who found 8 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, is that others [13] have reported progesterone R5020 down-regulates expression of bcl-2 protein in T47-D breast cancer cells. Apoptosis in this study was not investigated.

We examined the bcl-2 protein expression in T47-D cells after 24 and 72 h exposure to 10 μM progesterone, because the protein of proto-oncogene bcl-2 inhibits apoptosis [6]. As shown in Figs 4 and 5, we observed a significant correlation between progesterone induced downregulation of bcl-2 gene transcripts and bcl-2 protein, suggesting this protein might regulate progesterone-mediated apoptosis in T47-D tumor cells. Expression of p53 and survivin proteins were not examined in this study.

CD44 functions in cell motility, lymphocyte homing, and in a variety of cell-matrix interactions. Alternatively spliced variants of CD44 are associated with enhanced invasiveness of cancer cells. Particular the isoform expressing exons v7–v10 in tandem show increased metastatic potential [35]. Elimination of an entire viable region of CD44 (exons v7–v10) was observed following 24 h incubation with 10 μM progesterone. Similar observation has not been reported before and the molecular implications of this finding, remain to be determined.

Bcl-2 and p53 are established modulators of tumor cell progression and viability. We found that the expression of anti-apoptotic gene survivin correlated with the expression of bcl-2. Both genes were downregulated by progesterone which translated in drastically enhanced apoptosis of T47-D tumor cells. The significant coassociation of survivin and bcl-2 in T47-D breast cancer cells imply a strong mechanism of apoptosis inhibition, which potentially contribute to tumor progression and multidrug resistance. The intriguing finding of this study is the novel role of progesterone in transcriptional repression, potentially involving apoptosis inhibitors like bcl-2 and survivin, which may provide important insights into a potential broad mechanism [36, 37] eliminating cancer cell viability *in vivo*.

Acknowledgements

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