

Overexpression of wild-type p53 gene renders MCF-7 breast cancer cells more sensitive to the antiproliferative effect of progesterone

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Abstract

We have recently shown that growth inhibition of breast cancer cells by progesterone is due to the induction of cell differentiation, but not apoptosis. Because the tumor suppressor protein p53 plays a central role in normal cell growth and in tumor suppression, we have examined the effect of progesterone on the levels of this protein in MCF-7 cells. We show here that the antiproliferative effect of progesterone is accompanied with down-regulation of endogenous p53 protein. To study the effect of progesterone on cell growth in the presence of normal levels of p53 protein, we used transient transfection to overexpress p53 protein. MCF-7 cells were transfected

with a p53 expressing vector that contains p53 human cDNA under the control of a cytomegalovirus promoter. Cell growth, cell viability, and apoptosis were analyzed in the transfected cells after six days of exposure to 100 nM progesterone. We show here that progesterone significantly enhances growth inhibition and apoptosis in MCF-7 cells overexpressing p53, but not in cells transfected with the control vector. These data suggest that re-establishing p53 function in MCF-7 breast cancer cells renders them more sensitive to the growth inhibitory effect of progesterone.

Journal of Endocrinology (2003) **179**, 55–62

Introduction

The steroid hormone progesterone is an important growth regulator of normal and malignant breast epithelial cells, and is used to treat hormone-dependent breast tumors (Clarke & Sutherland 1990, Santen *et al.* 1990, Pasqualini *et al.* 1998, Sutherland *et al.* 1998). However, the exact molecular mechanism of the antiproliferative effect of progesterone is poorly understood and apart from the requirement of its receptor (Horwitz 1993, Osborne 1998), little is known about the mechanism by which it mediates growth inhibitory effects on hormone responsive cells. Several studies have shown that growth inhibition of breast cancer cell lines by progesterone is associated with altered expression of genes that have a role in growth arrest and differentiation (Alkhalaf & Murphy 1992, Musgrove *et al.* 1996, Kester *et al.* 1997, Swarbrick *et al.* 2000). The p53 tumor suppressor protein, which is a transcription factor capable of inducing either growth arrest or apoptosis (Lane 1994, Levine 1997), is frequently inactivated by mutation or other mechanisms in human breast cancer (Olivier & Hainaut 2001). There are conflicting reports about the regulation of the p53 protein level by progesterone in breast cancer cells. For instance, Moudgil *et al.* (2001) reported that the treatment of T47D breast cancer

cells with the synthetic progestin, R5020, lowered the p53 levels to 30% of the control level. On the other hand, Formby & Wiley (1998) showed that the antiproliferative effect of progesterone was due to the induction of apoptosis and that p53 mRNA levels were upregulated as detected by semi-quantitative RT-PCR analysis. The T47D human breast cancer cells used by both authors contained a mutated p53 gene (Schafer *et al.* 2000). Moore *et al.* (2000) suggested that the effects of progesterone on p53 and on other apoptotic proteins might contribute to the survival of breast cancer cells and to the development of resistance to progestin therapy. In this report we used the MCF-7 human breast cancer cell line which harbors a wild-type p53 gene. By using these cells, we have previously shown that growth inhibition of breast cancer cells by progesterone is due to the induction of cell differentiation and not to apoptosis (Alkhalaf *et al.* 2002). Our data show that progesterone inhibits the growth of MCF-7 cells and this growth inhibition is associated with down-regulation of p53 levels. We report here that progesterone may protect breast cancer cells from apoptosis by altering p53 protein levels. We present evidence that re-establishing p53 expression in these cells enhances the growth inhibitory effect of progesterone by activating apoptosis.

Materials and Methods

The MCF-7, T47D and ZR75-1 breast cancer cell lines were kindly provided by Bohdan Wasyluk (IGBMC Core Facility, Strasbourg, France). All three cell lines are classified as progesterone receptor positive and estrogen receptor positive. The MCF-7 cells contain functional p53 protein localized at the nucleus, whereas T47D cells have a mutated type of p53 which is localized at the cytoplasm (Schafer *et al.* 2000). ZR75-1 contains normal levels of p53, but it is not known whether the protein is functional or not.

The cells were grown in RPMI 1640 medium (Gibco BRL) supplemented with 5% fetal bovine serum (FBS), glutamine and gentamicin. Cells were plated at 5×10^5 cells/35 mm diameter multi-wells (for cell proliferation, apoptosis and morphology analysis) or at 3×10^6 cells/25 cm² (for Western blotting assay) and maintained in a 5% CO₂ humidified atmosphere at 37 °C for 48 h.

Western blot analysis

Cells were washed twice with PBS buffer and then the preheated (95 °C) lysis buffer (20 mM Tris-HCl pH 7.4, 20 mM dithiothreitol (DTT), 2 mM EDTA (sodium salt), 1% Triton X-100, 1% NP40, 1% sodium deoxycholate, 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate (prepared in Tris buffer) and 1 mM phenylmethylsulfonyl-fluoride) was added directly to the cell monolayer. The cells were scraped and mixed with a rubber policeman, transferred to Eppendorf tubes and centrifuged at 13 000 g for 5 min. The resulting supernatant was saved and the protein was determined by the Bradford method. Extracts were boiled for 3 min in 2 × SDS buffer. Equal amounts of protein were loaded on 10% SDS-PAGE according to the method of Laemmli, then electrotransferred to nitrocellulose membranes. The blots were incubated with p53 (DO1) monoclonal antibodies (Oncogene Research, Cambridge, MA, USA) at a 1/500 dilution for 1 h, then incubated with peroxidase-conjugated anti-mouse IgG (Jackson Laboratory, West Grove, PA, USA) at a 1/2000 dilution. Immunoreactive bands were visualized by incubation with luminol (according to the manufacturer's instructions; ECL Western blotting detection system from Amersham). TATA-binding protein (TBP) monoclonal antibody was used as a loading control (kindly provided by IGBMC Core Facility, Illkirch, France).

Transient transfection of MCF-7 with p53 cDNA expression vector

MCF-7 cells were maintained in RPMI 1640 medium + 10% FBS + antibiotics at 37 °C with 5% CO₂. They were transfected either with 3 µg cytomegalovirus (CMV) expression vector (pcDNA3, obtained from Invitrogen), in 36 mm plates (6-well cluster, Nunc, Naperville, IL, USA) or with 3 µg CMV vector containing human p53 cDNA

(kindly provided by the IGBMC Core Facility, Illkirch, France) by using lipofectamine (Gibco-BRL). Twenty-four hours later the cells were fixed with methanol and analyzed by immunocytochemistry with antibodies against p53 followed by FITC-conjugated goat anti-mouse and propidium iodide to detect nuclei. The cells were visualized with a fluorescence microscope equipped with dual filters and photographed. To study the effect of progesterone on MCF-7 cells that overexpress p53, the p53-transfected as well as the control cells (transfected with the empty vector) were treated with vehicle alone or increasing concentrations of progesterone (1, 10, 100, 1000 and 5000 nM). The medium with or without progesterone was changed every second day. The cells were left for 6 days then harvested and cell numbers were counted on a Coulter counter. In each experiment, triplicate dishes were used for each data point.

4,6-Diamidino-2-phenylindol (DAPI) and Trypan Blue staining for the detection and quantification of apoptosis

The cells (5×10^4) were seeded into 35 mm diameter multi-wells containing 5% FBS for 48 h. The cells were then treated with vehicle (control) or with 100 nM progesterone in the presence of 5% FBS. Before the processing of cells for DAPI staining, the cell morphology was examined and photomicrographs were taken for each treatment by using phase contrast microscopy. To identify cells undergoing apoptosis we used the DAPI staining method. Cells were washed with PBS, fixed with 100% methanol and then stained with DAPI (1 µg/ml). The stained nuclei were visualized under a Zeiss Axioplan microscope equipped with a DAPI filter using a 40 × oil objective. Photographs were taken with Kodak TMax 400 film. Condensed or fragmented nuclei were scored as apoptotic cells. To quantify apoptosis, three random fields were counted, with each field consisting of ~100 cells.

In another set of experiments, the rate of cell death with each type of treatment was estimated by the Trypan Blue exclusion assay. After three days of treatment with progesterone, the medium containing floating cells was collected and the adhering cells were detached by trypsinization and pooled with the floating ones. Cell viability was estimated after adding an equivalent volume of a 0.125% Trypan Blue solution to an aliquot of the whole suspended cells followed by counting, under the microscope, the proportion of unstained versus total cells deposited in a hemacytometer. At least five counts over different fields of about 100 cells were made to evaluate the average cell viability in each group of treated cells. The viability in control cells was considered to be 100%.

Statistical analysis

Data were analyzed by Student's *t*-test, and $P < 0.05$ was considered significant.

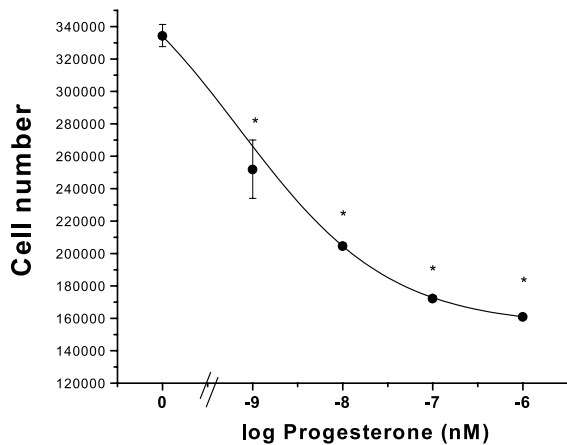


Figure 1 Dose-dependent effect of progesterone on the proliferation of MCF-7 cells. Cells were incubated with increasing concentrations of progesterone in 5% serum for 4 days. Results are presented as means \pm S.D. from two experiments using triplicate sets of dishes. * $P < 0.05$, treated samples were significantly different from the control.

Results

Effect of progesterone on cell growth of non-transfected MCF-7 cells

For the dose-response experiment, MCF-7 cells were incubated with vehicle (ethanol) or with increasing concentrations of progesterone (0, 1, 10, 100, 1000 nM) in the presence of 5% FBS for 4 days. Cell growth was determined by counting the cell number in triplicate sets of dishes. At all concentrations, progesterone caused significant growth inhibition ($P < 0.05$) (Fig. 1). For the time-course experiment, MCF-7 cells were incubated with 100 nM progesterone or with vehicle in the presence of 5% FBS and cell number was determined after 1, 2, 3 or 4 days of treatment. Note the decrease in cell number with increasing length of progesterone treatment. The cell number in progesterone-treated MCF-7 cells corresponded to approximately 50% of the control after 4 days of treatment (Fig. 2).

Growth inhibition by progesterone is associated with p53 down-regulation

The MCF-7 cell line, which contains the wild-type p53 gene, was used to study the regulation of p53 by progesterone. Our aim was to evaluate the involvement of p53 in progesterone induction of growth inhibition. We used Western blotting analysis with p53 antibody to assess the level of p53 protein in MCF-7 cells treated with progesterone for 0, 2, 4, 6, 12, 24 and 30 h. Figure 3 shows that 100 nM progesterone caused down-regulation of the endogenous p53 protein. The decrease in p53 levels starts as early as 12 h after treatment and maximal inhibition was

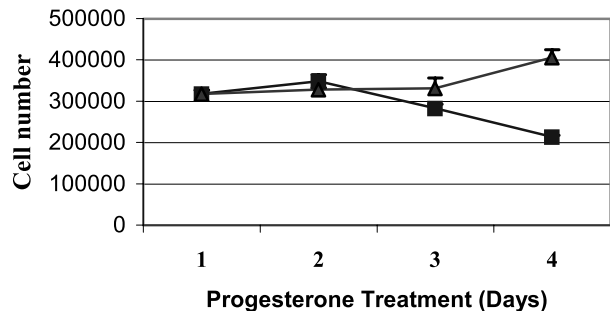


Figure 2 Time-course effect of progesterone on the proliferation of MCF-7 cells. MCF-7 cells were incubated with 100 nM progesterone (■) or with vehicle (▲) in the presence of 5% FBS. Cell number was determined after 1, 2, 3 or 4 days of treatment. The control MCF-7 cells are growing over after 4 days in the presence of 5% FBS. Note the decrease in cell number with increasing length of progesterone treatment (50% as of control in day 4).

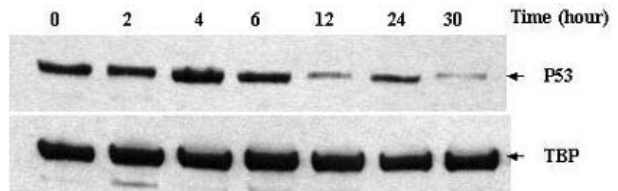


Figure 3 Time-course effect of progesterone on p53 protein levels in MCF-7 cells. Cells were treated with 100 nM progesterone and harvested after different periods of time. Twenty micrograms of extract were subjected to Western blotting analysis to determine the relative amounts of p53 and TBP (used as a loading control) proteins.

observed after 30 h of treatment. To show that down-regulation of p53 is specific to progesterone treatment, the same blotting membrane was re-probed with the TBP antibody. Figure 3 shows that TBP was not altered by progesterone treatment in MCF-7 cells. Therefore this antibody was used as a loading control. This inhibitory effect on p53 expression in MCF-7 cells was specific for progesterone treatment (Fig. 4, lane 6) and was not observed with the use of another type of growth inhibitory drug, the antiestrogen 4-hydroxytamoxifen (Fig. 4, lane 7) or with the growth stimulating estrogen, estradiol-17 β (Fig. 4, lane 8). The inhibitory effect of progesterone in MCF-7 cells was not detected in another breast cancer cell line, ZR75-1 (Fig. 4, lane 2). In the same culture conditions, progesterone inhibited the growth of another breast cancer cell line, T47D, in a dose- and time-dependent fashion (data not shown). T47D cells contain a mutated form of p53 protein and treatment with progesterone has no effect on p53 levels (data not shown). These results show that growth inhibition induced by progesterone is independent of p53 regulation, at least in the T47D and ZR75-1 cell lines.

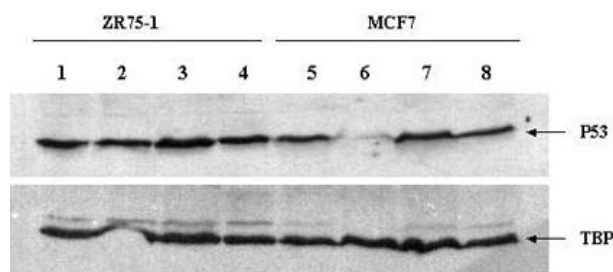


Figure 4 Effect of medroxyprogesterone acetate (MPA), 4-hydroxytamoxifen (OH-TAM) and estradiol on p53 protein levels in breast cancer cell lines. The results of Western blotting analysis of cells treated for 24 h with vehicle (lanes 1, 5), 100 nM MPA (lanes 2, 6), 100 nM OH-TAM (lanes 3, 7), or 100 mM estradiol (lanes 4, 8) are shown. Immunoblotting was performed with monoclonal antibodies specific for p53 and TBP (loading control).

p53 overexpression enhances the progesterone effect on cell growth and apoptosis

The observation that endogenous p53 protein levels were down-regulated by progesterone in MCF-7 cells prompted us to check the consequences of re-establishing the p53 expression in the cells by exogenous expression using transient transfection experiments. In the beginning, we examined the efficiency of transfection of MCF-7 cells with the CMV expression vector. Cells transfected with 3 µg p53 expression vector showed high levels of nuclei with p53 protein (Fig. 5, lower panel), as compared with cells transfected with the empty vector (Fig. 5, upper panel) which showed few positive nuclei representing endogenous p53. The total number of cells expressing p53 was scored in both CMV and CMV-p53 transfected cultures. The percentage of cells with nuclear staining in CMV and CMV-p53 transfected cells was 5% and 11% respectively. We used Western blot analysis to estimate the amount of p53 levels achieved by transient transfection. MCF-7 cells were transfected with expression vector (3 µg/well) for CMV vector and CMV vector containing human p53 cDNA. The cells were treated with vehicle or with 100 nM progesterone for 24 h. p53 levels were more than twofold higher in MCF-7 cells transfected with CMV-p53 vector than in CMV transfected cells as revealed by densitometry (Fig. 6).

To study the effect of exogenous p53 on progesterone-induced growth inhibition, the transfected cells were treated with vehicle alone or increasing concentrations of progesterone (1, 10, 100, 1000 and 5000 nM). Figure 7 shows that MCF-7 cells overexpressing p53 are more sensitive to progesterone-induced growth inhibition. Cell number in CMV-p53 transfected cells was reduced to 71.6, 70.5 and 65% in cells treated with 0.1, 1 or 5 µM progesterone respectively, as compared with cells transfected with the empty vector ($P < 0.05$). This significant sensitivity to progesterone in cells overexpressing exogenous p53 was due to decreasing cell viability, tested by

using the Trypan Blue exclusion test. Figure 8 shows a decrease in cell viability in CMV-p53 transfected cells in response to progesterone compared with cells transfected with the CMV empty vector. Cell viability in CMV-p53 transfected cells was reduced to 78.6, 61.9 and 44.8% in cells treated with 0.1, 1 or 5 µM progesterone respectively, as compared with cells transfected with the CMV vector ($P < 0.05$). To further ascertain the cell viability findings in the transfected cells, we examined the effect of progesterone on DNA fragmentation by using the DAPI staining method. Figure 9A shows a representative staining pattern observed in MCF-7 cells transfected with CMV vector. DAPI staining did not reveal any nuclear changes characteristic of apoptosis, whereas a marked alteration is observed with cells transfected with the CMV-p53 vector after treatment with 100 nM progesterone (Fig. 9B). Quantification of apoptotic cells by scoring morphologically aberrant nuclei (condensed and fragmented nuclei) showed that the percentages of progesterone-induced apoptosis with control (CMV vector) and p53 overexpressing cells (CMV-p53) were 8.5 ± 3 and 54.5 ± 5.5 respectively. However, the DNA ladder test performed on MCF-7 cells treated under the same conditions did not show significant DNA fragmentation which may be attributed to the high serum concentration present in the culture medium. When the cells were transfected with CMV-p53 cDNA or with CMV vector and kept in culture for six days without the addition of progesterone, CMV-p53 had no significant effect on cell growth or viability (Figs 7 and 8). In other words, the overexpression of p53 *per se* has no effect on cell growth or viability, implying that the progesterone machinery is crucial for the induction of apoptosis.

Discussion

Depending on the experimental model system, progesterone can elicit either proliferative or antiproliferative effects on breast epithelial growth (Lange *et al.* 1999, Thuneke *et al.* 2000). The antiproliferative effect may be due to the induction of cell differentiation (Kester *et al.* 1997) or to the stimulation of apoptosis (Formby & Wiley 1999, Horita *et al.* 2001). We and others showed that progesterone can protect MCF-7 cells from dying by apoptosis (Ory *et al.* 2001, Alkhalaf *et al.* 2002). We showed evidence that progesterone may protect breast cancer cells from apoptosis but promotes their differentiation by activating the Akt/P13-kinase pathway. Moore *et al.* (2000) suggested that progestins in T47D cells (known to have a mutated p53 gene) serve as survival factors and also stimulate proliferation, implying a possible similar role in breast cancer patients. They showed that the effects of progestin on breast cancer cell survival were accompanied by upregulation of the antiapoptotic protein bcl-xL, consistent with a defective p53 function in these cells. We have extended

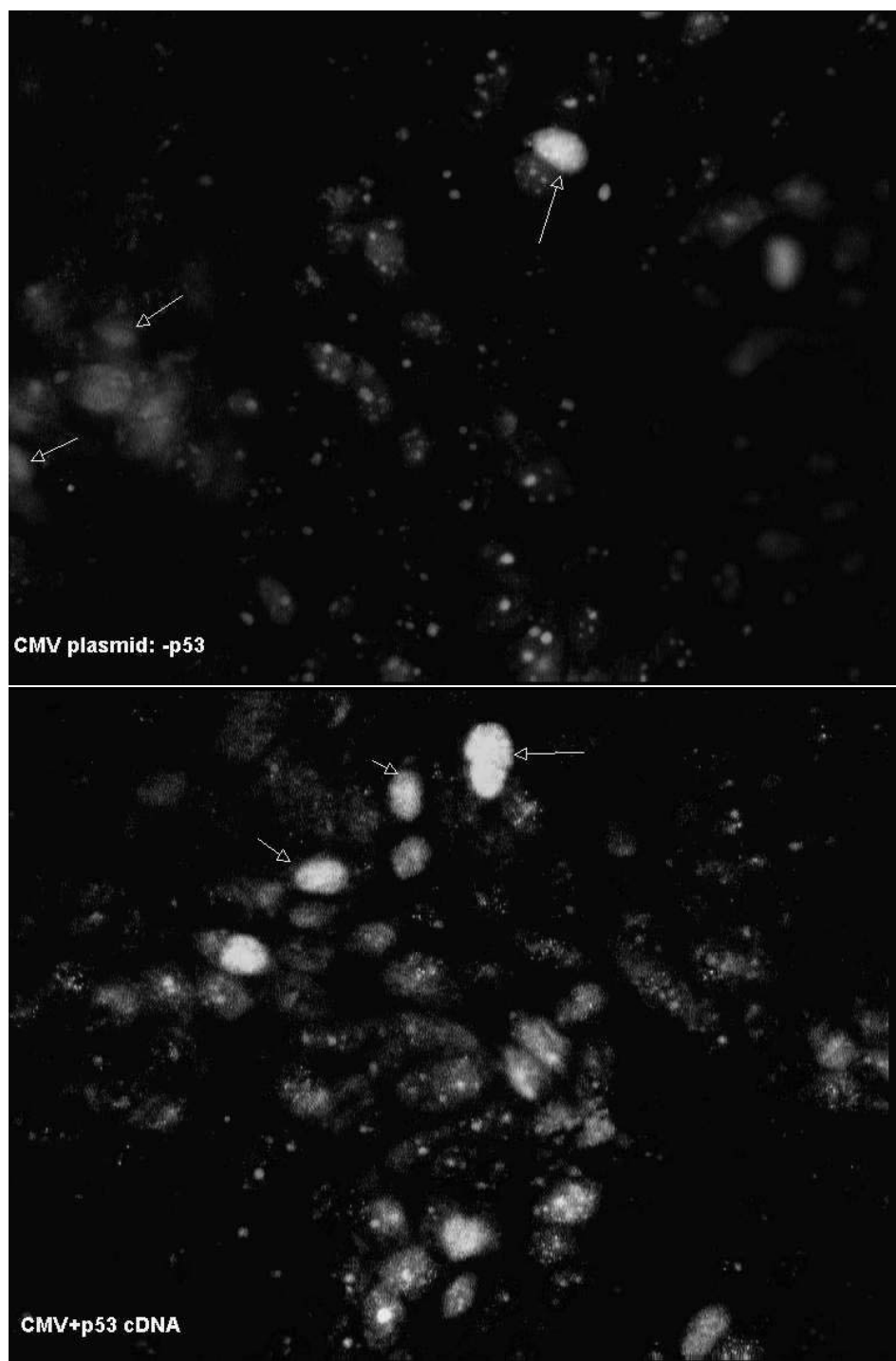


Figure 5 Transient transfection of MCF-7 human breast cancer cells with human p53 cDNA. MCF-7 cells were transfected with expression vector (3 μ g/well) for CMV vector (control) (upper panel) and CMV vector containing human p53 cDNA (lower panel) by using lipofectamine (Gibco-BRL). Twenty-four hours later the cells were fixed with methanol and analyzed by immunocytochemistry with antibodies against p53 followed by FITC-conjugated goat anti-mouse and propidium iodide to detect nuclei. The cells were visualized with a fluorescence microscope equipped with dual filters, and photographed. Arrows indicate nuclei with p53 protein.

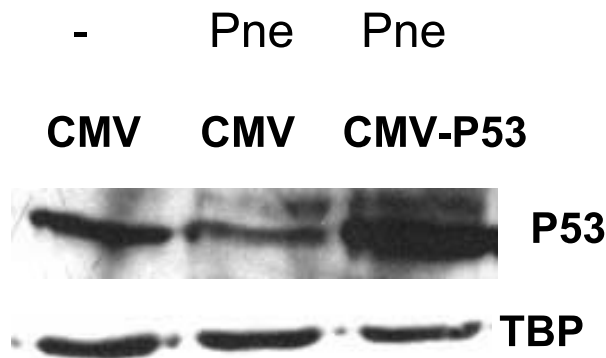


Figure 6 Western blotting of transiently transfected MCF-7 human breast cancer cells with human p53 cDNA. MCF-7 cells were transfected with expression vector (3 µg/well) for CMV vector and CMV vector containing human p53 cDNA. The cells were treated with vehicle or with 100 nM progesterone for 24 h. Twenty micrograms of extract were subjected to Western blotting analysis to determine the relative amounts of p53 and TBP (used as a loading control) proteins. MCF-7 cells transfected with CMV-p53 vector contained twofold more p53 than CMV transfected cells. Pne, 100 nm progesterone. -, vehicle.

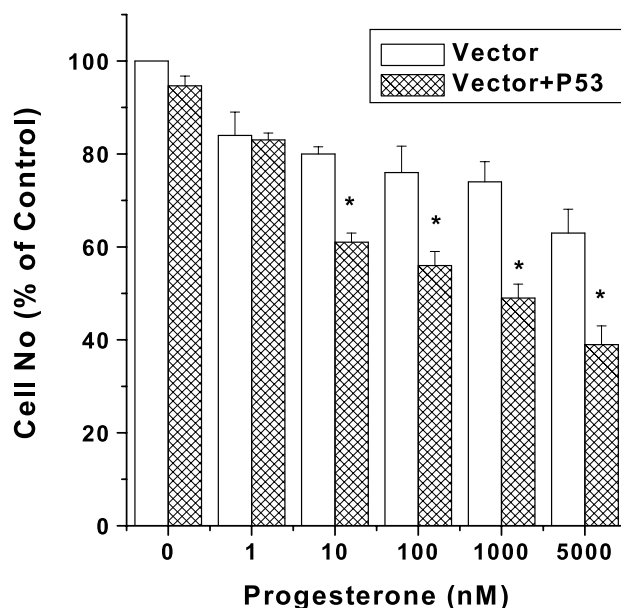


Figure 7 Effect of altered p53 expression on the growth response of MCF-7 cells to progesterone. Cells were either transiently transfected with CMV-p53 vector or with the empty CMV vector as described in Materials and Methods. The cells were then treated with vehicle alone or increasing concentrations of progesterone as indicated. The medium with or without progesterone was changed every second day. The cells were left for 6 days then harvested and cell numbers were counted on a Coulter counter. In each experiment, triplicate dishes were used for each data point. Note that MCF-7 cells transfected with p53 cDNA are more sensitive to progesterone compared with cells transfected with the control vector. * $P < 0.05$ significantly different compared with vector alone.

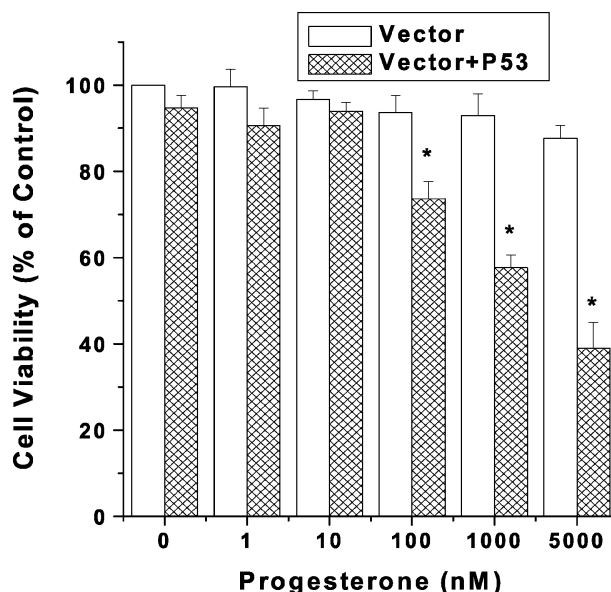


Figure 8 Effect of altered p53 expression on the cell viability of MCF-7 cells to progesterone. Cells were either transiently transfected with CMV-p53 vector or with the empty CMV vector. The cells were then treated with vehicle alone or increasing concentrations of progesterone, as indicated, for 4 days. After this incubation, the medium containing floating cells was collected and the adhering cells were detached by trypsinization and pooled with the floating ones. Cell viability was estimated after adding an equivalent volume of a 0.125% Trypan Blue solution to an aliquot of the whole suspended cells and counting under the microscope the proportion of unstained versus total cells deposited in a hemacytometer. At least five counts over different fields of about 100 cells were made to evaluate the average cell viability in each group of treated cells. The viability in control cells was considered as 100%. Note the decrease in cell viability in CMV-p53 transfected cells in response to progesterone compared with cells transfected with the CMV empty vector. * $P < 0.05$ significantly different compared with vector alone.

these observations by showing that the absence of apoptosis in MCF-7 cells treated with progesterone is associated with down-regulation of the endogenous p53 protein. In other words, it seems that progesterone, by stimulating the expression of antiapoptotic protein and/or by down-regulating apoptotic protein, enables the cells to survive and to escape from dying by apoptosis. Whether progesterone directly regulates the transcription of the p53 gene or alternatively acts by an indirect or postranscriptional mechanism remains to be determined. However, Castoria *et al.* (1999) using MCF-7 cells presented evidence that progestins act on cell cycle progression by a non-transcriptional action.

Several reports showed that the accumulation of p53 mutations in human breast cancer is associated with a high proliferation rate in the tumor, an association that is expected in view of the role of p53 as a negative regulator of cell proliferation (Isola *et al.* 1992, Hartmann *et al.* 1997, van Slooten *et al.* 1999). Therefore, the observed decrease

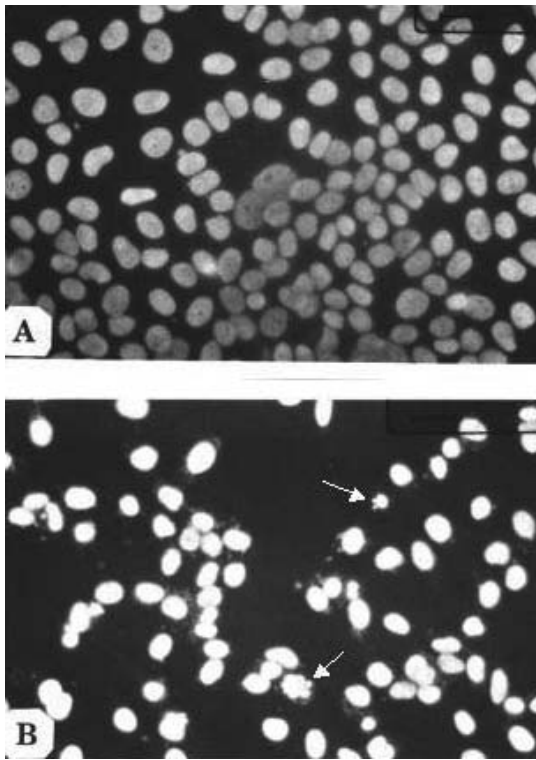


Figure 9 Effect of progesterone on apoptosis in transfected MCF-7 cells. Cells were either transiently transfected with CMV-p53 vector or with the empty CMV vector. The cells were then treated with vehicle alone (A) or with 100 nM progesterone for 4 days (B). The 4,6-diamidino-2-phenylindol (DAPI, 1 µg/ml) staining method was used to identify cells undergoing apoptosis. The stained nuclei were visualized under a Zeiss Axioplan microscope using a UV filter in the range of 395–450 nm. Condensed or fragmented nuclei were scored as apoptotic cells. Arrows indicate fragmented nuclei.

in p53 levels in MCF-7 breast cancer cells treated with progesterone may be involved in the protection of MCF-7 cells from dying by apoptosis. In order to check whether the sensitivity of MCF-7 cells can be altered by the level of cellular p53 protein, we overexpressed p53 by using a p53 cDNA expression vector. We show in this report that re-establishing p53 protein levels in the cells by exogenous expression renders the cells more sensitive to progesterone and that the observed increase in growth inhibition rate was due to the induction of cell death by apoptosis. In pre-clinical model systems it was demonstrated that many types of tumors with wild-type p53 status responded better to certain oncological therapeutic modalities than tumors with an altered p53 status (Clarke *et al.* 1993, Lowe *et al.* 1993, 1994, O'Connor *et al.* 1993, Fan *et al.* 1994). However, none of these therapeutic modalities included progesterone. In the absence of the tumor suppressor gene p53 in the mammary epithelial cell model, progesterone facilitates chromosomal instability (aneuploidy) (Goepfert *et al.* 2000). This supports the hypothesis that progesterone

markedly enhances mammary tumorigenesis in the absence of p53 protein. At the present time, little is known regarding the efficacy of progesterone treatment in relation to endogenous p53 levels in the tumor (O'Connor *et al.* 1998). To our best knowledge, our data is the first to correlate the effect of progesterone on human breast cancer cell proliferation with the status of p53 in the cells. In conclusion, our data show that the effect of progesterone on growth inhibition and apoptosis is enhanced in cultured MCF-7 cells that overexpress p53 protein and suggest that re-establishing p53 function may render breast cancer cells more sensitive to the growth inhibitory effect of progesterone. In other words, p53 levels appear to dictate the mechanism(s) whereby progesterone exerts antiproliferative effects on MCF-7 cells. Down-regulation of p53 favors cellular differentiation, whereas its upregulation forces the cells additionally to undergo apoptosis. Therefore, the rationale of the use of progesterone in hormone replacement therapy for breast cancer should be evaluated in relation to the p53 status of the tumors.

Acknowledgements

We thank Professor Christopher HJ Ford for his critical reading of the manuscript and Maya Bakir and Lilly Verghese for technical assistance.

Funding

M A was supported by Kuwait University Research Administration grant MB 028.

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Received 16 June 2003

Accepted 25 June 2003

Made available online as an

Accepted Preprint 27 June 2003