# Medroxyprogesterone Acetate Inhibits Human Pancreatic Carcinoma Cell Growth by Inducing Apoptosis in Association with Bcl-2 Phosphorylation

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**BACKGROUND.** A previous study found that medroxyprogesterone acetate (MPA) delayed the in vivo growth of three (AsPC-1, Capan-2, and MiaPaCa-2) of nine human pancreatic carcinoma cell lines transplanted into nude mice (*Cancer* 1995; 75:1263–72). The current study was undertaken to evaluate the basis for this inhibitor.

**METHODS.** The estrogen receptor (ER) and progesterone receptor (PgR) status in nine human pancreatic carcinoma cell lines, AsPC-1, BxPC-3, Capan-1, Capan-2, Hs-700T, Hs-766T, MiaPaCa-2, PANC-1, and SUIT-2, was assessed using an enzyme immunoassay (EIA). The authors tested the growth inhibitory activity of MPA and the morphologic changes in these nine pancreatic carcinoma cell lines. Cell cycle progression and DNA fragmentation also were evaluated in these cell lines. Immunoblot analysis was used to determine *bcl-2* expression and phosphorylation.

**RESULTS.** In the EIA assay, ER was detected in three cell lines (BxPC-3, Capan-2, and MiaPaCa-2), and PgR was also detected in three (AsPC-1, Capan-2, and MiaPaCa-2). Medroxyprogesterone acetate inhibited the growth of three cell lines (AsPC-1, Capan-2, and MiaPaCa-2) with $IC_{50}$ values ranging from $2.3 \times 10^{-7}$ to $6.1 \times 10^{-7}$ M. In these three responsive cell lines, MPA caused cell detachment and decreased cell density. The nuclei of the MPA-treated cells were condensed and often fragmented. Cell cycle analysis of these three cell lines showed that MPA induced the appearance of a sub-$G_1$ peak, which is characteristic of early apoptotic cells. DNA degradation assay after MPA treatment showed a typical DNA ladder pattern consistent with apoptosis. Immunoblot analysis of MPA-treated cells that overexpressed *bcl-2* revealed a pattern consistent with *bcl-2* phosphorylation.

**CONCLUSIONS.** Clinically attainable concentrations of MPA can inhibit the growth of some human pancreatic carcinoma cells in vitro by inducing apoptosis, probably through their PgR, in association with the phosphorylation of *bcl-2*. This agent may be useful for treating patients with pancreatic carcinoma. ***Cancer* 2000;88: 2000–9.** *© 2000 American Cancer Society.*

**KEYWORDS: pancreatic carcinoma, medroxyprogesterone acetate (MPA), flow cytometry, DNA fragmentation, apoptosis, *bcl-2*.**

Carcinoma of the pancreas accounts for approximately 14,000 deaths per year in Japan. Despite considerable progress in diagnostic techniques, early detection of pancreatic carcinoma is still rare, and the overall 5-year survival rate is less than 3%.[1] Even after curative surgical resections, the 5-year survival rate in Japan is only 15%.[1] Irradiation and cytotoxic chemotherapy alone or in combination have only a minor influence on survival.[2–4] No other treatment regimen has been shown to significantly increase patient survival, and there is

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a need for an effective therapy in this aggressive and often rapidly fatal disease.

A possible new approach to the endocrine treatment of pancreatic carcinoma was suggested in the 1980s when the presence of the estrogen receptor (ER) and a specific estrogen binding protein were demonstrated in pancreatic carcinomas.5–7 Since then, although there have been a number of trials using tamoxifen, an antiestrogenic agent, in patients with pancreatic carcinoma, the results have been inconclusive; several investigators have reported that tamoxifen therapy may prolong survival in patients with pancreatic carcinoma,[8,9] whereas others have not.[10,11] In this regard, we previously have investigated the relation between the estrogen dependency of the production of tissue-type plasminogen activator, which is an estrogen-inducible enzyme and therefore reflects an intact ER system in breast carcinoma cells,[12–14] in human pancreatic carcinoma cells and the in vivo effects of tamoxifen and other various endocrine agents on the growth of pancreatic carcinoma cells transplanted into nude mice. During this investigation, we found that medroxyprogesterone acetate (MPA) treatment exerts a significant antitumor effect on the growth of three (Capan-2, AsPC-1, and MiaPaCa-2) of the nine human pancreatic carcinoma cell lines (the other six cell lines studied were BxPC-3, Capan-1, Hs-700T, Hs-766T, PANC-1, and SUIT-2).[15]

Medroxyprogesterone acetate is a synthetic steroid derived from progesterone and exhibits marked progestogenic activity in animals and humans. Although the mechanism for the antitumor activity of MPA is still controversial, it appears to be complex.[16] A decrease in the concentration of the ER and the estrogen binding capacity of the ER positive cells after MPA treatment would be expected to make these cells less responsive to estrogen.[17,18] Medroxyprogesterone acetate also has a systemic effect resulting from hormonal interference, producing suppression of the hypothalamo-pituitary-adrenal and hypothalamo-pituitary-gonadal axes and leading to a drop in estrogen levels in women.[19,20] Furthermore, MPA has been known to act directly on the growth of breast carcinoma and/or endometrial carcinoma through its inhibitory effect on DNA and/or RNA synthesis via the progesterone receptor (PgR).[21]

Based on these observations, we initially investigated whether the above nine human pancreatic carcinoma cell lines express ER and/or PgR. Second, we determined whether MPA has a direct antiproliferative effect on the growth of the nine human pancreatic carcinoma cells in vitro. Last, we investigated whether the growth-inhibitory effect of MPA on human pancreatic carcinoma cells is due to the induction of apoptosis. We demonstrate here that clinically attainable concentrations of MPA can inhibit the growth of some pancreatic carcinoma cells in vitro by inducing apoptosis, probably through their PgR, in association with the phosphorylation of *bcl-2*.

## MATERIALS AND METHODS

### Cell Culture

Nine human pancreatic carcinoma cell lines (AsPC-1, BxPC-3, Capan-1, Capan-2, Hs-700T, Hs-766T, MiaPaCa-2, PANC-1, and SUIT-2) were purchased from the American Type Culture Collection (Rockville, MD). Of these nine cell lines, seven (Capan-1, Capan-2, Hs-700T, Hs-766T, MiaPaCa-2, PANC-1, and SUIT-2) are derived from male pancreatic carcinoma, and two (AsPC-1 and BxPC-3) are derived from female pancreatic carcinoma. Experimentally, Capan-2, Hs-700T, Hs-766T, and MiaPaCa-2 are known to be responsive to testosterone. In contrast, AsPC-1, PANC-1, and SUIT-2 are known to be unresponsive to testosterone. Testosterone responsiveness of BxPC-3 and Capan-1 is not known. All these nine cell lines were maintained in 25-$cm^2$ plastic tissue culture flasks containing RPMI-1640 medium (GIBCO Laboratories, Detroit, MI) with 10% fetal bovine serum (FBS), penicillin (100 units/mL), and streptomycin (100 mg/mL) at 37 °C in a 95% room air–5% $CO_2$ humidified incubator.

### Enzyme Immunoassay for ER and PgR

The nine human pancreatic carcinoma cells were harvested at subconfluence by using 0.125% trypsin/0.01% ethylenediamine tetraacetic acid (EDTA) in phosphate-buffered saline (PBS). The assays were set up in a steroid-depleted basic medium (BM) composed of RPMI-1640 (phenol red free) supplemented with 2 mM glutamine plus 10% FBS treated with dextran-coated charcoal to remove endogenous steroids. Cytosols were prepared from cells harvested from cultures approaching confluence. Preservation of the receptors, which are heat labile, was ensured by performing all further manipulations at 4 °C by using prechilled solutions and equipment. Pellets of 2–5 $\times$ $10^7$ cells were washed in PBS and sonicated for 10 seconds on ice in 1 mL of sonication buffer (10 mM Tris-HCl, pH 7.4, 1.5 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol). The supernatant was retained and pooled after pelleting and washing of the macroscopic cell debris in the same buffer. This then was ultracentrifuged at 100,000$\times g$ for 1 hour. The cytosols were analyzed immediately for ER and PgR by using an enzyme immunoassay (EIA) kit (Abbott Diagnostics, Maidenhead, Berks, UK) as previously reported for breast carcinoma.[22] Cytosols with protein concentrations of 1–4 mg/mL (at the higher end of the range

recommended in the kit protocols) were used routinely for the receptor analysis. This was based on the premise from previously reported work[23] that only low levels of these receptors were present and thus might be detected more readily in more concentrated cytosols.

### Cell Proliferation Assay

Medroxyprogesterone acetate was kindly provided by Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan). Cells were treated with either various concentrations of MPA ($10^{-8}$ to $10^{-4}$ M) in a 0.1% ethanol solution or the vehicle alone for 72 hours.

The MTT assay was performed according to the method described by Tada et al.[24] Briefly, human pancreatic carcinoma cells were plated into 96-well flat-bottom plates (Falcon; Becton Dickinson and Company, Lincoln Park, NJ) in 200 $\mu$L of medium containing a suitable number of cells (3–10 $\times$ $10^3$ per well). The MTT solution was freshly prepared at a concentration of 5 $\mu$g/mL in PBS and filtered through a 0.2-$\mu$m pore filter. A 20-$\mu$L aliquot of the MTT stock solution was added to each well, and the plate was incubated for 3 hours at 37 °C in a humidified 5% $CO_2$ incubator. One hundred microliters of dimethyl sulfoxide was added to each well to solubilize the formazan. Fifteen minutes later, the plates were read on a microplate reader (EAR 340AT; Slt-Lab Instruments, Vienna, Austria), by using a test wavelength of 570 nm and a reference wavelength of 620 nm. The MTT assay results were linear over the range from 3000 to 400,000 cells per well, corresponding to a range of 0.004 to 0.33 in the optical density values. Therefore, all experiments were designed to remain within this linear range.

All experiments were performed five times, and the values reported as the mean $\pm$ standard deviation (SD) of the individual experiments. The 50% inhibitory concentration ($IC_{50}$) was determined from the dose–response data from the five experiments with CalcuSyn software (Biosoft, Cambridge, UK).

### Microscopic Studies

Adherent growing pancreatic carcinoma cells were incubated with $10^{-6}$ M MPA, and the morphology of the cells was assessed microscopically. Photographs of the adherent cells were taken under a phase-contrast microscope after the medium containing the floating cells was removed.

The human pancreatic carcinoma cells were fixed with 70% ethanol at −20 °C and then stained with 1 $\mu$g/mL propidium iodide containing 100 $\mu$g/mL DNase-free RNase A for 30 minutes at 37 °C to visualize the nuclei. The cells were examined with a fluorescence microscope.

### Cell Cycle Analysis

Flow cytometric analysis was performed by a modification of the method of Evan et al..[25] Briefly, the human pancreatic carcinoma cells were detached from the plastic by trypsinization and then washed in PBS. The cells ($1 \times 10^6$ per sample) were fixed in 70% ethanol and stored at 4 °C until the analysis. These cells were rehydrated in PBS and then treated with RNase (500 units/mL) for 30 minutes. Next, the cells were stained with propidium iodide. Cell cycle analysis was performed using a Becton Dickinson (San Jose, CA) FACScan flow cytometer, and the data were analyzed using cell lysis software (Cell Quest, Becton Dickinson & Company, Lincoln Park, NJ).

### DNA Fragmentation Assay

The extent of DNA fragmentation in the cells was determined by a modification of the method of Sellins and Cohen.[26] Briefly, a fraction of the human pancreatic carcinoma cells was harvested by centrifugation at 200$\times g$ for 10 minutes. The pellet was lysed with 0.1 mL hypotonic lysing buffer (10 mM Tris, 1 mM EDTA, pH 7.5) containing 0.2 % Triton X-100, and the lysates were centrifuged at 13,000$\times g$ for 10 minutes to separate intact from fragmented chromatin. The supernatant, containing the fragmented DNA, was placed in a separate microfuge tube.

RNase A (0.4 mg/mL; Sigma Chemical Co., St Louis, MO) was added to the supernatants, followed by incubation at 37 °C for 1 hour. Subsequently, proteinase K (0.4 mg/mL; Wako Junyaku Kogyo Co., Osaka, Japan) was added to the supernatants followed by incubation at 37 °C for 1 hour. The lysate was precipitated overnight at −20 °C in 50% isopropanol and 0.5 M NaCl. The precipitates were pelleted by centrifugation at 13,000$\times g$ for 15 minutes, air-dried, and resuspended in 10 mM Tris, 1 mM EDTA, pH 7.4. Loading buffer, containing 15 mM EDTA, 2% sodium dodecyl sulfate, 50% glycerol, and 0.5% bromphenol blue, was added to the samples at a 1:5 (v/v) ratio. The DNA was electrophoresed on a 2% agarose gel at 50 volts. The DNA was visualized with ethidium bromide.

### Immunoblot Analysis

Immunoblot analysis was used to determine *bcl-2* expression and phosphorylation according to the method of Ali et al.[27] Cells were treated with either the indicated concentrations of MPA or the vehicle before the extraction of total cellular protein. Briefly, the cells were pelleted, washed twice with cold PBS, and resuspended in lysis buffer (PBS, 20 $\mu$g/mL aprotinin, 20

**TABLE 1**
**ER, PgR, and $IC_{50}$ Value in Nine Human Pancreatic Carcinoma Cell Lines**

| | Receptor concentration (fmol/mg protein)[a] | | |
|---|---|---|---|
| | ER | PgR | |
| Cell line | Mean ± SD (n) | Mean ± SD (n) | $IC_{50}$ ($\times 10^{-7}$ M)[b] |
| AsPC-1 | < 1.5 (10) | 9.4 ± 2.5 (8) | 4.5 |
| BxPC-3 | 9.1 ± 1.5 (6) | < 2.0 (6) | — |
| Capan-1 | < 1.5 (6) | < 2.0 (6) | — |
| Capan-2 | 5.1 ± 1.3 (8) | 8.8 ± 3.0 (8) | 6.1 |
| Hs-700T | < 1.5 (6) | < 2.0 (6) | — |
| Hs-766T | < 1.5 (6) | < 2.0 (6) | — |
| MiaPaCa-2 | 6.9 ± 2.2 (8) | 10.4 ± 2.2 (8) | 2.3 |
| PANC-1 | < 1.5 (6) | < 2.0 (6) | — |
| SUIT-2 | < 1.5 (6) | < 2.0 (6) | — |

ER: estrogen receptor; PgR: progesterone receptor; IC: inhibitory concentration; SD: standard deviation; MPA: medroxyprogesterone acetate.

[a] n denotes the number of determinations for each pancreatic carcinoma cell line. The lower detection limit of the assay for ER and PgR is 1.5 and 2.0 fmol/mg protein, respectively.

[b] Exponentially growing cells were incubated in the presence or absence of MPA for 72 hours, harvested, and counted. $IC_{50}$s were determined from plots of the percentage of control cell number versus the logarithm of the MPA concentration (Fig. 1). Values reported are the averages from five experiments.

$\mu$g/mL leupeptin, 1.0 mM phenylmethylsulfonyl fluoride) before sonication and high-speed centrifugation. Soluble protein then was quantitated by the Bradford method. Equivalent amounts of protein from each sample were loaded onto 10% acrylamide gels and separated via sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by electroblot transfer to supported nitrocellulose membranes (Hybond C-Super; Amersham, Arlington Heights, IL). For immunodetection, membranes were blocked overnight in 10% nonfat dried milk at 4 °C and incubated with mouse monoclonal anti–*bcl-2* or anti–B-actin primary antibody (Calbiochem, Cambridge, MA) at a 1:100 dilution for 1 hour, followed by incubation with sheep anti-mouse immunoglobulin horseradish peroxidase (HRP)-conjugated secondary antibody (Amersham) at a 1:1000 dilution for 1 hour. Chemiluminescent detection was performed using enhanced chemiluminescence (ECL) reagents (Amersham).

## RESULTS

### Estrogen receptor and PgR in Human Pancreatic Carcinoma Cell Lines

Table 1 shows the mean ER and PgR concentrations (fmol/mg cytosolic protein) ±SD in nine human pancreatic carcinoma cell lines by using an EIA kit. The range of receptor levels in the cell lines for both ER and PgR were similar whether the cytosols were prepared from cells grown in the standard growth medium or in the steroid-depleted basic medium. Therefore, the data sets were combined (n = 6–10 depending on the cell line). In the EIA assay, the ER was detected in the three of the nine pancreatic carcinoma cell lines (BxPC-3, Capan-2, and MiaPaCa-2) with mean ER levels between 5.1 and 9.1 fmol/mg protein. The PgR was detected in the three of the nine pancreatic carcinoma cell lines (AsPC-1, Capan-2, and MiaPaCa-2) with mean PgR levels between 8.8 and 10.4 fmol/mg protein.

### Effect of MPA on the In Vitro Growth of Pancreatic Carcinoma Cells

The nine human pancreatic carcinoma cell lines were exposed to a range of concentrations of MPA for 72 hours before the determination of cell viability with a standardized MTT assay. Cells exposed to the vehicle served as controls and all experiments were repeated five times. Three of the nine pancreatic carcinoma cell lines (AsPC-1, Capan-2, and MiaPaCa-2) exhibited concentration-dependent growth inhibition from $10^{-8}$ to $10^{-4}$ M MPA. The $IC_{50}$ values for MPA ranged from $2.3 \times 10^{-7}$ to $6.1 \times 10^{-7}$ M (Fig. 1 and Table 1).

### Morphologic Change

Treatment of 80% confluent cultures of human pancreatic carcinoma cell lines with $10^{-6}$ M MPA resulted in cell detachment and decreased cell density in three (AsPC-1, Capan-2, and MiaPaCa-2) of the nine pancreatic carcinoma cell lines. Figure 2 shows representative examples of the Capan-2 cells.

When untreated human pancreatic carcinoma cells were stained with propidium iodide and observed under a fluorescence microscope, the nuclei appeared as homogeneously fluorescent oval discs. In contrast, the nuclei of cells treated with $10^{-6}$ M MPA appeared as condensed, often fragmented, intensely fluorescent discs, indicating that the cells may have become apoptotic.[28] Figure 3 shows representative examples with AsPC-1 cells treated with $10^{-6}$ M MPA. Similar results were also observed in the two other PgR positive pancreatic cell lines (Capan-2 and MiaPaCa-2) but not in the six PgR negative cell lines (data not shown).

### Effect of MPA on Cell Cycle Distribution

To examine further the effect of MPA on the cell cycle progression of human pancreatic carcinoma cells, we studied DNA histograms of MPA-treated cells in the nine cell lines by flow cytometric analysis. Figure 4 shows representative examples of the cell cycle effects of MPA in the MiaPaCa-2, BxPC-3, and Capan-1 cells.

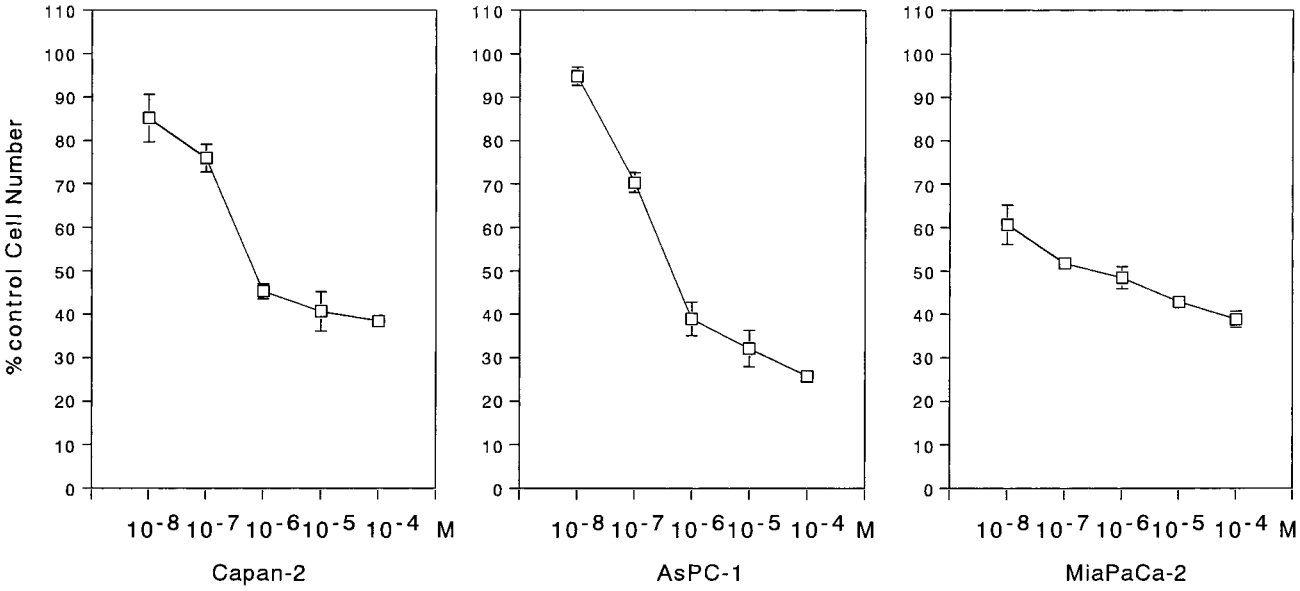


**FIGURE 1.** Concentration-dependent growth inhibition of human pancreatic carcinoma cells by MPA is shown. Exponentially growing cells were incubated in the presence or absence of MPA for 72 hours as described in "Materials and Methods." Three of the nine pancreatic carcinoma cell lines (AsPC-1, Capan-2, and MiaPaCa-2) exhibited concentration-dependent growth inhibition from $10^{-8}$ to $10^{-4}$ M MPA. Values reported are the mean ± SD of five individual experiments.

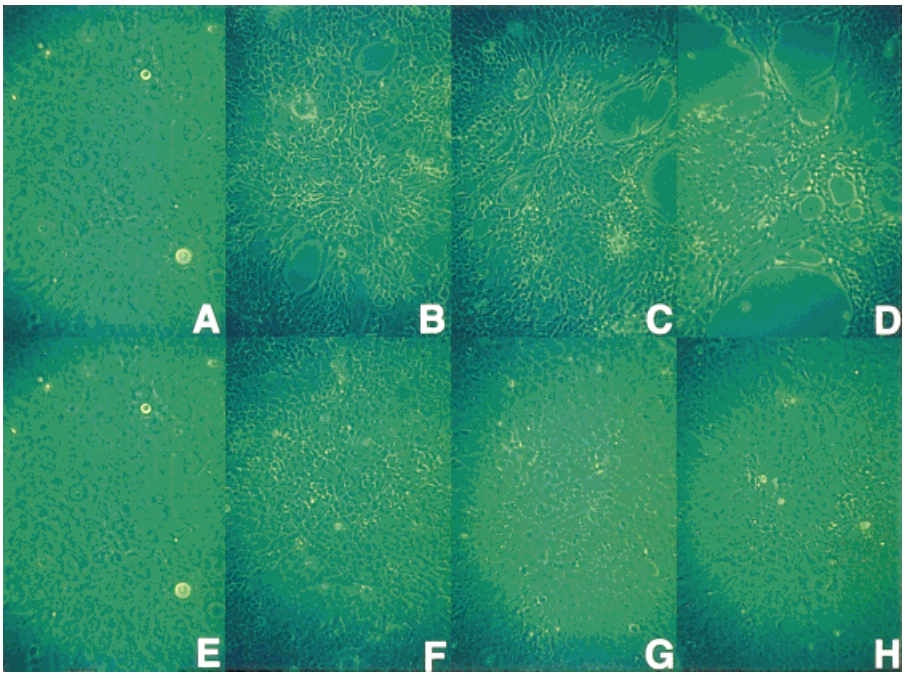


**FIGURE 2.** Effect of MPA on the morphology of the Capan-2 cell line is shown. Adherent Capan-2 cells growing in medium were incubated in the presence (A–D) or absence (E–H) of $10^{-6}$ M MPA, and the cells were inspected microscopically. At various times, photographs of the adherent cells were taken with a phase-contrast microscope after the media containing the floating cells were removed. A and E: 0 hour; B and F: 24 hours; C and G: 48 hours; D and H: 72 hours.

The appearance of cells with a DNA content less than $G_1$, a characteristic of early apoptotic cells (sub-$G_1$ peak), was observed 72 hours after the addition of $10^{-6}$ M MPA in the MiaPaCa-2 cells but not in BxPC-3 and Capan-1 cells. Continuous exposure of the MiaPaCa-2 cells to $10^{-6}$ M MPA for 120 hours resulted in a further increase in the proportion of apoptotic cells with decreased DNA content. Quite similar results were observed in the other two PgR positive cell lines including AsPC-1 and Capan-2 but not in Hs-700T, Hs766T, PANC-1, and SUIT-2 (data not shown).

### Effect of MPA on DNA Degradation

Cleavage of DNA by endonucleases is a typical feature of apoptosis. The nucleosome pattern can be visual-

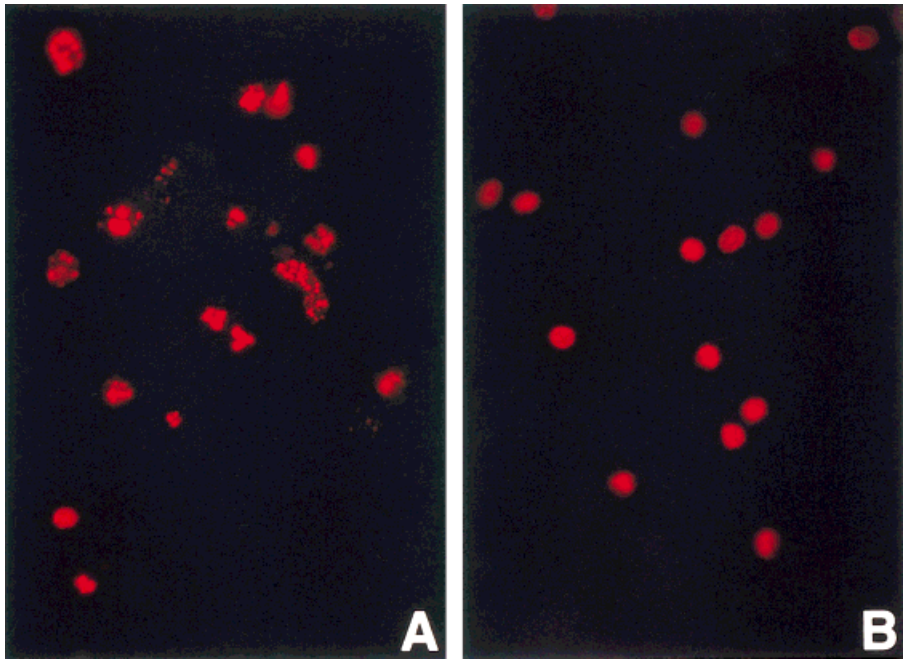


**FIGURE 3.** Fluorescent micrographs of AsPC-1 cells incubated in the presence or absence of MPA are shown. AsPC-1 cells were incubated for 72 hours in the presence (A) or absence (B) of $10^{-6}$ M MPA and were fixed with 70% ethanol and then stained with 1 $\mu$g/mL of propidium iodide. In the absence of MPA, the nuclei appear as homogeneously fluorescent oval discs with propidium iodide staining (B). After treatment with MPA, the nuclei of the cells appear fragmented, condensed, and brightly fluorescent with propidium iodide staining (A). These nuclei are easily distinguished from intact normal nuclei (B).

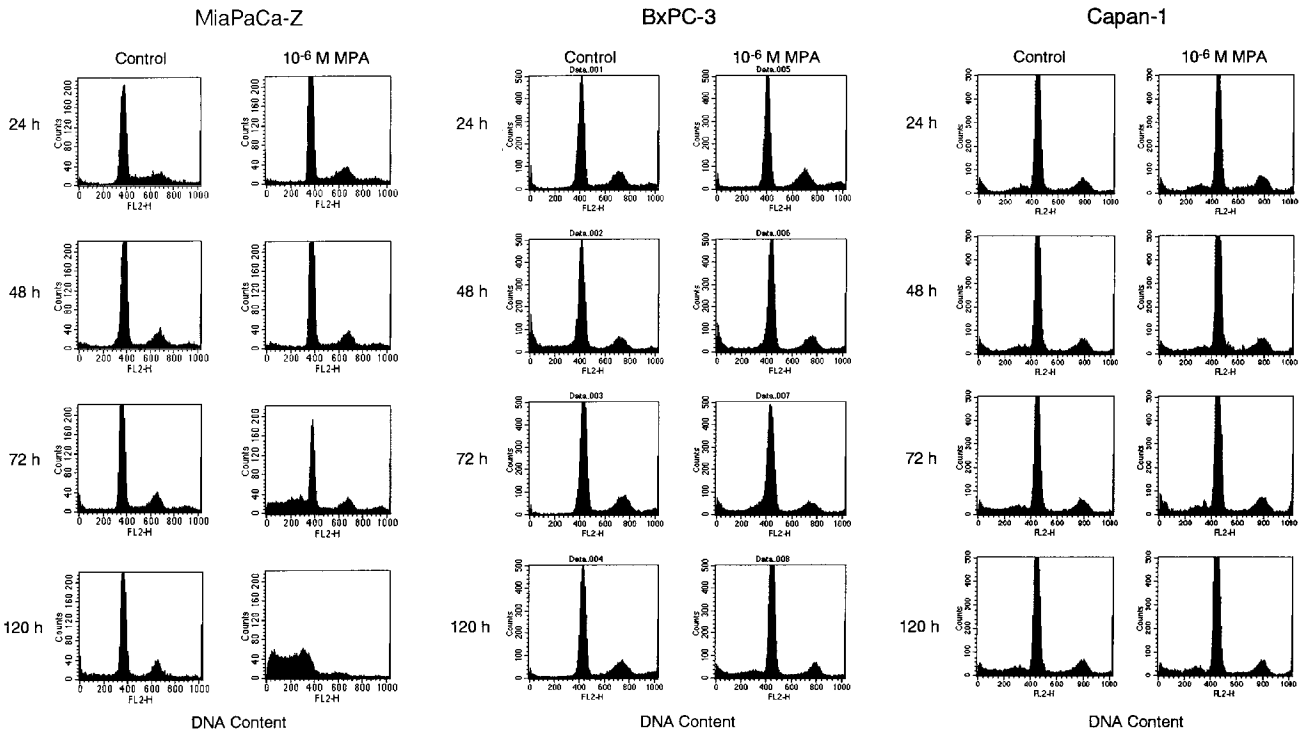


**FIGURE 4.** Effect of MPA on the cell cycle distribution of the human pancreatic carcinoma cell line MiaPaCa-2, BxPC-3, and Capan-1 is shown. Cells with a DNA content less than $G_1$ (sub-$G_1$ peak) are observed 72 and 120 hours after $10^{-6}$ M MPA treatment in MiaPaCa-2 cell line.

ized on agarose gels after the induction of apoptosis and electrophoresis of the DNA. To investigate whether the mechanism of MPA-mediated cytotoxicity on human pancreatic carcinoma cells involves apoptosis, the three PgR positive cell lines (AsPC-1, Capan-2, and MiaPaCa-2) and BxPC-3 and Capan-1 cells as controls were incubated for 72 hours with $10^{-5}$ and $10^{-6}$ M MPA, and their DNA was isolated and run on an agarose gel. Medroxyprogesterone acetate treatment of the three PgR positive cell lines, but not

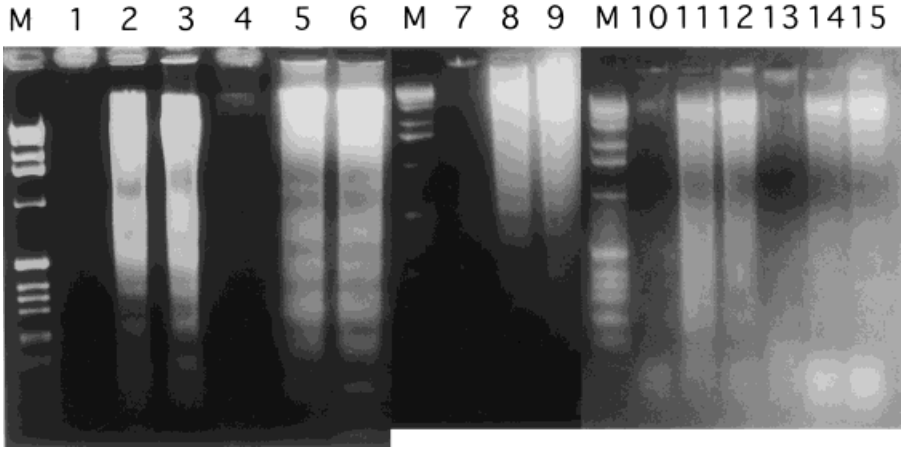


**FIGURE 5.** DNA degradation assay after MPA treatment of the human pancreatic carcinoma cell lines AsPC-1, Capan-2, MiaPaCa-2, BxPC-3, and Capan-1. Lanes M: DNA ladder marker; lane 1: untreated Capan-2 cells; lane 2: Capan-2 cells treated with $10^{-6}$ M MPA; lane 3: Capan-2 cells treated with $10^{-5}$ M MPA; lane 4: untreated AsPC-1 cells; lane 5: AsPC-1 cells treated with $10^{-6}$ M MPA; lane 6: AsPC-1 cells treated with $10^{-5}$ M MPA; lane 7: untreated MiaPaCa-2 cells; lane 8: MiaPaCa-2 cells treated with $10^{-6}$ M MPA; lane 9: MiaPaCa-2 cells treated with $10^{-5}$ M MPA; lane 10: untreated BxPC-3 cells; lane 11: BxPC-3 cells treated with $10^{-6}$ M MPA; lane 12: BxPC-3 cells treated with $10^{-5}$ M MPA; lane 13: untreated Capan-1 cells; lane 14: Capan-1 cells treated with $10^{-6}$ M MPA; and lane 15: Capan-1 cells treated with $10^{-5}$ M MPA.

BxPC-3 and Capan-1, was associated with a DNA laddering pattern that is characteristic of apoptosis (Fig. 5). It is unclear why the two control cells, BxPC-3 and Capan-1, appear to show DNA degradation, albeit not with a laddering pattern. A possible explanation is that a portion of culture cells might die during the cell culture maintenance including medium replacement, and the DNA degradation of the control cells seen in Figure 5 may reflect such cell death during the cell handling.

### Bcl-2 Phosphorylation in Pancreatic Carcinoma Cells after MPA Treatment

The nine human pancreatic carcinoma cells express relatively high but variable basal levels of *bcl-2*. The three PgR positive cell lines (AsPC-1, Capan-2, and MiaPaCa-2) and BxPC-3 and Capan-1 cells as controls were exposed to $10^{-8}$ to $10^{-4}$ M of MPA or the vehicle for 48 hours before the extraction of total cellular protein. After exposure to MPA, immunoblot analysis revealed a dose-dependent alteration in the *bcl-2* electrophoretic pattern in the three PgR positive cell lines but not in BxPC-3 and Capan-1 cells (Fig. 6). This altered pattern consisted of the appearance of two bands with slightly slower mobility, similar to those reported after serine phosphorylation of *bcl-2*.[29]

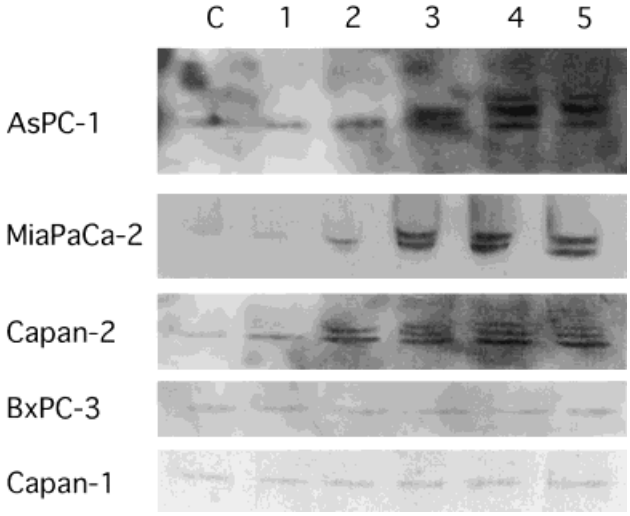


**FIGURE 6.** Immunoblot analysis with total protein from AsPC-1, Capan-2, MiaPaCa-2, BxPC-3, and Capan-1 cells exposed to either vehicle alone (lane C) or $10^{-8}$ to $10^{-4}$ M (lanes 1–5) of MPA for 48 hours is shown. The membrane was sequentially probed with a monoclonal *bcl-2* antibody followed by chemiluminescent immunodetection.

## DISCUSSION

Medroxyprogesterone acetate is a well established therapeutic agent for patients with advanced or recurrent breast carcinoma. This agent usually is administered as a second-line hormonal therapy after tamoxifen for these patients. Although the mechanism for the antitumor activity of MPA is still controversial, there appears to be at least two components: 1) a reduction in the number of ER and a decrease in the estrogen binding capacity of the ER positive cells after MPA treatment would be expected to make them less responsive to estrogen,[17,18] and 2) MPA exerts suppressive effects on the hypothalamo-pituitary-adrenal and hypothalamo-pituitary-gonadal axes in women, and the antitumor activity of MPA has correlated with the extent of endogenous cortisol suppression and subsequent estrogen deprivation in patients.[19,20] Furthermore, MPA has been known to act directly on the

growth of breast carcinoma and/or endometrial carcinoma through its inhibitory effect on DNA and/or RNA synthesis via the PgR[21].

In the current study, we demonstrated that MPA acts directly on the growth of human pancreatic carcinoma cells. Medroxyprogesterone acetate had a dose-dependent cytotoxic effect on three (AsPC-1, Capan-2, and MiaPaCa-2) of the nine human pancreatic carcinoma cells tested. Medroxyprogesterone acetate treatment resulted in cell detachment and decreased cell density in these cells. The nuclei of the cells appeared to be condensed, often fragmented, and intensely fluorescent after propidium iodide staining. Using flow cytometry, we found that MPA arrested the responsive pancreatic carcinoma cells mainly at the $G_0/G_1$ phase, and an accumulation of cells with less DNA content than $G_0/G_1$ cells (sub-$G_1$ peak) suggested that the MPA-induced cytotoxicity was the result of apoptosis. The observation of DNA fragmentation into multimers of approximately 180 base pairs, called a DNA ladder, confirmed our observation that the MPA-induced cytotoxicity was the result of cell apoptosis.

We also demonstrated that three of the nine pancreatic carcinoma cell lines (AsPC-1, Capan-2, and MiaPaCa-2) contained measurable levels of PgR by EIA, although the PgR concentration in the three pancreatic carcinoma cell lines was near or lower than the cutoff value usually used in human breast carcinoma to categorize between PgR positive and PgR negative tumors (5–10 fmol/mg protein). Nevertheless, low-level PgR expression in the three pancreatic cell lines was consistently observed in our series of experiments, and these results were quite compatible with the in vitro results, as well as the finding that MPA exerted a significant antitumor effect on the in vivo growth of these three pancreatic cell lines in a prior study (AsPC-1, Capan-2, and MiaPaCa-2) but failed to exert any antitumor effect on the other six pancreatic cell lines (BxPC-3, Capan-1, Hs-700T, Hs-766T, PANC-1, and SUIT-2) transplanted into nude mice. This suggests that the presence of PgR appears to correlate with MPA sensitivity in human pancreatic carcinoma cells, and hence MPA is likely to exert its antitumor effect via the PgR pathway.

The induction of apoptosis is a major mechanism of action of many common cytotoxic agents, and the dysregulation of apoptotic pathways can play a significant role in the growth and therapeutic responsiveness of cancer cells. The product of the *bcl-2* oncogene is an inhibitor of apoptotic cell death, and overexpression of *bcl-2* correlates with relative resistance to many cytotoxic agents.[30–32] Therefore, the use of pharmacologic agents that decrease the apoptotic threshold by suppressing *bcl-2* expression and/or activity represents a rational strategy for novel anticancer therapy. Drug-induced *bcl-2* phosphorylation recently has been associated with decreased *bcl-2* function and the induction of apoptosis in a number of malignant cell types.[33,34]

A dose–response relation has been demonstrated with MPA, and a minimum daily dosage, a "high dose," is necessary for optimal antitumor effects in metastatic breast carcinoma or endometrial carcinoma. 35–37 An oral dose of approximately 1000 mg/day usually yields a high enough MPA plasma concentration for antitumor activity, i.e., 100–200 ng/mL.[38] This plasma concentration of MPA is comparable to a final concentration of $10^{-6}$ to $10^{-7}$ M MPA in culture, which was sufficient to inhibit the growth of the three PgR positive pancreatic carcinoma cell lines.

Severe wasting (cachexia) has been associated with advanced cancer and is a significant problem in the clinical management of patients. Recently, we have demonstrated that oral MPA treatment reduced the serum levels of interleukin-6, a cytokine postulated to be associated with cancer cachexia,[39,40] in patients with metastatic breast carcinoma, regardless of whether their tumors responded to the treatment.[41] In addition, previous studies have shown that megestrol acetate has a potential role in producing subjective improvement, a sense of well-being, and an increase in appetite and weight in patients with various cancers.[42–44] Thus, it is possible that oral MPA treatment also may contribute to an improvement in the quality of life in patients with pancreatic carcinoma.

In conclusion, this is the first study to our knowledge demonstrating that clinically attainable concentrations of MPA are effective in inhibiting the growth of some human pancreatic carcinoma cells in vitro by inducing apoptosis. Our results indicate that it may be useful for the treatment of human pancreatic carcinoma. We propose that high dose oral MPA treatment should be evaluated in well controlled clinical trials of patients with this aggressive and often rapidly fatal cancer.

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