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Growth Inhibition of Androgen-Insensitive Human Prostate Carcinoma Cells by a 19-Norsteroid Derivative Agent, Mifepristone

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ABSTRACT: Mifepristone, also known as RU 486, is a 19-norsteroid derivative. Currently, mifepristone is being tested in clinical trials on meningioma and breast cancer. In this study we analyzed whether mifepristone could inhibit the growth of human prostate cancer cells including androgen-insensitive (PC-3 and DU145) and androgen-sensitive (LNCaP) cell lines. At 1-nM concentration, mifepristone exhibited a marginal stimulatory action on LNCaP and PC-3 cells. Nevertheless, a dose-dependent growth inhibition on those same cell lines was observed at concentrations of 1 μM and 10 μM. Twenty-day exposure to the clinically achievable concentration of 1 μM mifepristone resulted in consistent inhibition of all three cell lines studied. Furthermore, this in vitro growth inhibition was reflected in an in vivo nude mouse system. Mifepristone at the dosage of 4 mg/100 g body weight completely suppressed the growth of PC-3 tumors for 21 days, although this was followed by a growth rate similar to that of the control tumor. To understand the possible mechanism of mifepristone inhibition, PC-3 cells were exposed to mifepristone in comparison with dexamethasone (Dex), progesterone, and 5 alpha-dihydrotestosterone (DHT), each at 1-μM concentration. The results demonstrated that while both DHT and Dex alone had essentially no effect on cell growth, progesterone alone resulted in a 20% growth inhibition, while mifepristone had more than 60% inhibition with a 16-day exposure. At an equal concentration, the degree of growth inhibition of PC-3 cells by mifepristone or progesterone was partially diminished by simultaneous exposure to Dex. In conclusion, our results demonstrated that the growth of androgen-insensitive prostate cancer cells can be directly inhibited by mifepristone in cultures. This in vitro inhibition is reflected in xenografted tumors.

KEY WORDS: **human prostate cancer, chemotherapy, mifepristone (RU 38486)**

INTRODUCTION

Cancer of the prostate is now the most commonly diagnosed male malignancy in the United States. In this country over 200,000 new cases of prostate cancer are expected to be identified in 1994 [1]. In addition, its significance as a cause of cancer-related mortality will be realized when, as our third leading cause of male cancer deaths, over 35,000 men will succumb to the effects of this malignancy in that same year [1].

Treatment options for prostate cancer are tailored

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to the stage of the disease at the time treatment is initiated [2,3]. Localized prostate cancer including extracapsular, locally invasive disease can be managed by radical prostatectomy or radiation therapy (external beam or interstitial implant). Metastatic, advanced prostate cancer is generally managed by hormonal manipulation. Orchiectomy, estrogen therapy, luteinizing hormone releasing hormone (LHRH) agonist therapy, and antiandrogen therapy are now being utilized. The common mode of action for these treatments is essentially the androgen deprivation of the prostate cancer cell [2,4–6]. Hormone therapy, however, is not curative in advanced cancer, and disease relapse will inevitably occur [2,6]. Despite the variety of methods, they all share an objective/subjective response rate of between 60–80% [2,5,6]. The duration of response to hormonal therapy generally only lasts for between 18–24 months, at which point patients become resistant to further hormonal manipulation. Once disease progression occurs on hormone therapy, survival is usually less than one year [2,6].

Systemic therapy for metastatic prostate cancer could be an optimal strength. However, chemotherapeutic treatment for this group of patients has shown limited activity (for review, see [7]). The response rate for single or combination chemotherapy has been less than 30% overall. Thus, along with continued efforts to identify effective chemotherapeutic agents, efforts must be made to explore other facets of prostate cancer tumor biology [8,9].

Mifepristone, a 19-norsteroid derivative also known as RU 486, possesses many intriguing biochemical functions with a high affinity for the progesterone receptor [10,11]. Clinically this antiprogestational property has been utilized for its efficacy as an abortifacient and as a contraceptive agent [10]. Additionally, mifepristone has been found to bind to the glucocorticoid receptor, and as such can block the effects of dexamethasone (Dex) [10,12,13]. Based on this antiglucocorticoid property, mifepristone has been used to ameliorate the symptoms of Cushing's syndrome [14,15]. Additionally, its antiglucocorticoid properties have been used in the local treatment of skin wounds, burns, abrasions, and in those forms of glaucoma proposed to be caused by increased levels of cortisone [16].

Potential applications in the field of oncology are currently under investigation. Mifepristone has demonstrated antitumor activity in vitro against meningiomas [17,18], which have been shown to express high levels of steroid-binding proteins including progesterone-binding protein/receptor [19]. Grunberg et al. [20,21] have now noted improvement in 8 of 28 patients with unresectable meningioma receiving long-term mifepristone. Breast cancer trials with mifepristone have also suggested therapeutic activity. In two clinical trials studying advanced breast cancer patients, partial response has been observed in 4 of 33 patients [22,23] who were refractory to other available forms of treatment.

In view of the hormone dependence of prostate carcinoma, investigation of this unique form of hormonal modulation would be of interest. Observation of transient increase in bone pain (resembling a tamoxifen flare) in one patient with meningioma and metastatic prostate cancer receiving mifepristone (S. Grunberg, unpublished observation) further encouraged us to pursue this line of research. We have now examined whether mifepristone can directly inhibit the growth of human prostate carcinoma cells in vitro. We have also examined whether the in vitro growth inhibition could be reflected in an in vivo animal model, and we have investigated the possible mechanisms by which this may occur.

MATERIALS AND METHODS

Materials

Fetal bovine serum (FBS) and RPMI-1640 culture medium were purchased from Gibco/BRL, (Grand Island, NY). FBS (Gibco Control No. 43N8314) was a certified grade serum and was accompanied by a manufacture certificate indicating that the concentration of steroid was as follows: estradiol, 4.7 pg/ml; progesterone, undetectable; testosterone, undetectable (the sensitivity of detectable limit for testosterone is 0.02 ng/ml). FBS (100 ml) was further dialyzed in a membrane tubing (m. wt. cut-off 3,500) against 20 mM N-[2-Hydroxyethyl]piperazine-N-[2-ethanesulfonic acid] (Hepes)-0.9% NaCl, pH 7.0 (1 l), at 4°C with 3 changes of buffer over a 24-hr period, filtered by a 0.2-μ membrane, and then heat-inactivated at 56°C for 30 min. The dialysis membrane tubing (m. wt. cut-off 3,500) was purchased from Spectrum (Los Angeles, CA). The disposable filterware with 0.2-μ membrane was from Nalge (Rochester, NY). Dex, progesterone, [D-Trp]^6-luteinizing hormone releasing hormone (LHRH), 5α-dihydrotestosterone (DHT), trypan blue solution, normal mouse immunoglobulin G (IgG), Hepes, and NaCl were all from Sigma (St. Louis, MO). Tissue culture plasticwares were purchased from Becton Dickinson Company (Franklin Lakes, NJ). Mifepristone (also known as RU 38486, 11-[4-(Dimethylamino)phenyl]-17-hydroxy-17-[1-propynyl]-[11β, 17β]-estra-4,9-dien-3-one) was a gift from Roussel-UCLAF Research Center (Romainville, France). Affinity isolated goat antibody (Ab) (anti-mouse IgG) fluorescein conjugate was from Tago, Inc. (Burlingame, CA). Mouse monoclonal anti-progesterone receptor Ab (IgG1) was purchased from Affinity

BioReagents (Neshanic Station, NJ). Monoclonal antiglucocorticoid receptor GR49 Ab was produced against purified rat glucocorticoid receptor provided by Dr. H. Westphal [24].

Cell Lines

Three distinctive prostate carcinoma cell lines, LNCaP-FGC (LNCaP), DU 145, and PC-3 cells [25–27], were obtained from the American Type Culture Collection (ATCC) and maintained in RPMI-1640 medium supplemented with 1% glutamine, 0.5% gentamicin, and 10% FBS for LNCaP cells, and 7% FBS for DU 145 and PC-3 cells as described previously [28,29]. LNCaP cells have been known to be androgen-sensitive [25], while DU 145 cells [26] and PC-3 cells [27] are androgen-insensitive. T-47D breast cancer cells that express progesterone receptors [30] were also obtained from ATCC and maintained in RPMI-1640 medium containing 10% FBS and 0.2 I.U. bovine insulin per ml.

Mifepristone Treatment

For experiments, LNCaP and PC-3 as well as DU 145 cells were plated onto 6-well culture plates in RPMI-1640 medium containing 10% or 7% FBS, respectively, at a density of 1.0×10^5 cells/well. LNCaP cells were left undisturbed for 2 days in a 37°C incubator (7% CO_2) to facilitate the attachment of cells to plates, while PC-3 and DU 145 cells could attach to plates efficiently with overnight incubation. Attached cells were fed with steroid-reduced medium (RPMI-1640 containing 2% heat-inactivated dialyzed FBS) and maintained at 37°C for an additional one or two days [29]. One set of attached cells was harvested and counted as day 0. The remaining cells were then grown in 3 ml of fresh steroid-reduced medium containing various concentrations of mifepristone as indicated in the figure (Fig. 1). Mifepristone was dissolved in 100% ethanol and then diluted 1/1,000 on a volume basis in the same culture medium to achieve desired concentrations. Experimental cells received mifepristone solution daily 5 times per week. Control cells received ethanol in medium daily. The fresh steroid-reduced medium (3 ml) was added every 3–4 days. After a period of time, as described in the figure legend (Fig. 1), all cells including suspended cells and attached cells were harvested and counted with a hemocytometer.

Competitive Inhibition Assay

PC-3 cells were plated in 6-well culture plates as described in the previous section. Steroids including mifepristone were each dissolved in 100% ethanol and then diluted 1/2,000 on a volume basis in the same culture medium to achieve desired concentrations as specified in the figure legend (Fig. 7). For competition experiments, experimental cells simultaneously received mifepristone and the competing steroid daily. Two other sets of cells received mifepristone and the competing steroid, respectively. The control cells received ethanol daily. The final concentration of ethanol in medium in all cells was 0.1% (v/v). After a period of time, cells were harvested and counted as described in the previous section.

Immunofluorescent Staining of Steroid Hormone Receptor

Cells were plated in 6-well culture plates containing a coverglass on the bottom in RPMI-1640 medium containing 7% FBS as described previously [31]. After being fixed with 3.7% formaldehyde in 20 mM Hepes, pH 7.2, containing 0.9% NaCl, cells were permeabilized by 0.2% Triton X-100 in Hepes-saline buffer and then rinsed with medium containing 5% FBS. Permeabilized cells were incubated with monoclonal antiglucocorticoid receptor Ab, antiprogesterone receptor Ab, or normal mouse IgG at 37°C for 1 hr with gentle shaking. Then they were rinsed to remove excess Ab, incubated with fluorescein isothiocyanate conjugated goat anti-mouse IgG Ab in medium containing 5% FBS for an additional 1 hr, and rinsed with the same medium-FBS to remove excess fluorescein-conjugated Ab, and with Hepes-saline to remove FBS. Cells were mounted in 50% glycerol in 0.2 M Hepes, pH 8.0, on a microslide, visualized, and photographed with an epifluorescent microscope.

Mifepristone Effect on Tumor Growth in Athymic Nude Mice

Homozygous mutant Balb/c nu/nu male athymic mice (6–8 weeks old) were purchased from the Simonson Lab (Gilroy, CA). The animals were maintained in the USC vivaria facility for approximately 1 week before experimentation. For tumor induction, the exponentially growing PC-3 cells were trypsinized, neutralized with the RPMI-1640 medium containing 10% FBS, washed once in the same medium-FBS and twice in the medium alone, and then resuspended in the same medium [25]. Animals were weighed before the injection of cancer cells. Viable cells (3.5×10^6 cells), as determined by trypan blue exclusion in 0.15 ml medium, were injected subcutaneously (s.c.) between the shoulder blades in the right side of the flank.

After palpable tumors were established, animals were weighed and tumor sizes were obtained by measurement of two perpendicular diameters, including the longest diameter with calipers. The tumor

volume was calculated using the formula: $W^2 \times L/2$ cu mm (W, shorter diameter; L, longer diameter) [25]. Tumor-bearing animals were distributed into two groups with the same averaged tumor volume.

Experimental animals were treated with mifepristone at a dose of 4 mg/100 g body weight in 0.1 ml oil containing 10% ethanol. Control animals received 0.1 ml oil containing 10% ethanol. All injections were given intradermally (i.d.) daily with a total of 5 times per week in the left side of the flank. Tumor growth and animal weight were measured weekly. Animals were fed an autoclaved, commercially available sterilizable chow (rodent diet #8656, Harland-Teklad Co., Madison, WI) and autoclaved water ad libitum. All animal experimentations followed the guidelines set by the National Institutes of Health, and the specific protocols used were approved by the University of Southern California Institutional Animal Care and Use Committee.

Statistical Analysis

The data for inhibitory activity on cell growth by steroids were statistically analyzed. The significance between two groups of data was analyzed by two-tailed Student's t test (P), as described previously [29]. $P < 0.05$ is considered significant.

RESULTS

Mifepristone Effect on the Growth of Prostate Carcinoma Cells

Since mifepristone had been reported to inhibit the growth of several cancer cells [20–23,30], we examined whether mifepristone can inhibit the growth of androgen-sensitive LNCaP and androgen-insensitive PC-3 and DU 145 human prostate carcinoma cells in cultures. With a 12-day exposure, a biphasic effect of mifepristone on the growth of LNCaP and PC-3 cells was observed. As shown in Figure 1, at low concentrations such as 1 nM, mifepristone stimulated the growth of LNCaP and PC-3 cells by approximately 20% over controls; while at high concentrations (100 nM or more), the growth of cells was inhibited in a dose-dependent fashion. There was greater than 80% inhibition on cell growth by mifepristone at 10 μM. High concentrations of mifepristone (100 nM or more) also inhibited the growth of DU 145 cells with a period of 20-day exposure, although no stimulation was observed at the 1-nM concentration (Fig. 1). Thus, mifepristone at concentrations of 100 nM or more directly inhibits the growth of both androgen-sensitive and androgen-insensitive human prostate carcinoma cells.

The time-dependence of mifepristone inhibition

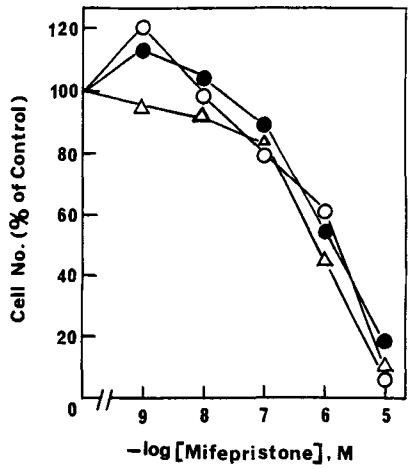

Fig. 1. Mifepristone effect on the growth of prostate carcinoma cells. LNCaP (○), PC-3 (●), and DU 145 (△) cells were plated onto 6-well culture plates and maintained in growth medium with dialyzed FBS at a density of 1.0×10^5 cells/well as described in Materials and Methods. Experimental cells received various concentrations of mifepristone in ethanol, as indicated. Control cells received ethanol in medium daily. After 12 days, LNCaP and PC-3 cells were trypsinized and counted with a hemacytometer. DU 145 cells were treated for 20 days. The cell number of LNCaP, PC-3, and DU 145 cells in control groups was 1.3×10^6, 1.7×10^6, and 2.5×10^6 cells, respectively, and referred to as 100% each. The data shown is the average of duplicates in one set of three independent experiments in which similar results were obtained.

on the cell growth was investigated. In human clinical trials, 1 μM appears to be an achievable and sustainable serum concentration for mifepristone [11]. All three cell lines were therefore exposed to 1 μM of mifepristone for a period of up to 20 days. As shown in Figure 2, over time we see up to 90% inhibition on cell growth of LNCaP, 83% on PC-3 cells, and 60% on DU 145 cells. Therefore, mifepristone inhibits the growth of three human prostate carcinoma cells in a time-dependent fashion at a clinically achievable concentration.

Further investigation was performed to determine whether the mechanism of mifepristone inhibition was by a reversible cytostatic or a cytotoxic effect, since a cytotoxic effect on breast cancer cells has been proposed [30]. In the presence of mifepristone (Fig. 3), the cell number increased by approximately 70% during the first 2-day period, and subsequently by a diminished growth rate with only an additional increase of approximately 50% from day 2 to day 10. In contrast, in the absence of mifepristone, the cell number increased approximately 2-fold in the first 2 days and more than 3-fold from day 2 to day 10 (Fig. 3). At day 10, as indicated by an arrow in the figure, the removal of mifepristone from the second set of experimental cells resulted in releasing cells from growth

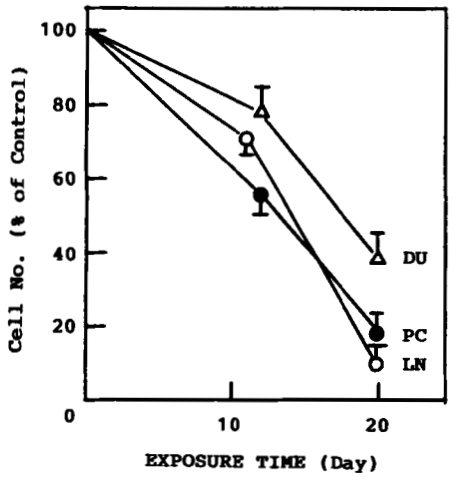

Fig. 2. Time-dependence of mifepristone effect on prostate carcinoma cell growth. LNCaP, DU 145, and PC-3 cells were plated as described in Materials and Methods. Experimental cells received 1 μM of mifepristone daily for a period of up to 20 days, as indicated. Control cells received the solvent alone. Cells in triplicate were harvested and counted. The cell number of LNCaP, DU 145, and PC-3 cells in each control group (referred to as 100%) was 1.6 × 10^6, 2.0 × 10^6, and 2.5 × 10^6 cells, respectively. Bar, the range of triplicates in one set of experiments.

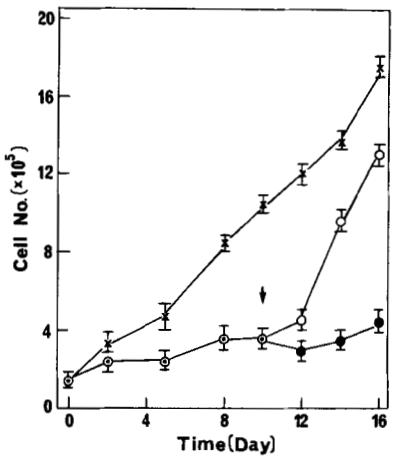

Fig. 3. Effect of mifepristone on cell growth. Three sets of PC-3 cells were plated. The first (●) and second (X) sets of cells daily received 1 μM of mifepristone/ethanol and ethanol, respectively, through the entire experimental period. The third set (○) of cells received mifepristone/ethanol for 10 days, and then medium containing ethanol alone for an additional 6 days. Cells from each set were harvested and counted at a 2- to 3-day interval. Arrow, the 10-day time point. Bar, the range of triplicate cells.

inhibition. After a latent period of approximately 2 days, cells grew exponentially. Therefore, with a 10-day exposure to 1 μM of mifepristone, prostate cancer cell growth inhibition was apparently achieved by a cytostatic effect.

In Vivo Response of PC-3-Induced Tumors to Mifepristone

Since growth inhibition on androgen-insensitive prostate cancer cells could be clinically useful, we investigated whether the in vitro mifepristone inhibition could be reflected in an in vivo system by utilizing PC-3 cells as the model system, since PC-3 cells have been used widely in cell growth regulation by cDNA transfection [29] and in drug trials, including suramin [8]. Athymic nude mice were injected with PC-3 cells. Eighteen-day post tumor cell injection, all animals developed a palpable tumor. Body weight and tumor size were measured and the treatment was initiated.

The administration of mifepristone induced a growth retardation of PC-3 tumors at a dose of 4 mg/100 g body weight/day (Fig. 4). At this dose, the PC-3 tumor growth was totally suppressed for a period of 3 weeks ($P<0.001$). By a 3-week period of treatment, the average tumor volume was approximately one-third the size of those in the untreated animals. After 3 weeks of growth suppression, the PC-3 tumors exhibited a similar growth rate to that of control tumors in spite of continued administration of mifepristone. Thus, 4 mg/100 g body weight/day was apparently sufficient to suppress temporarily tumor growth in this animal model study. Further, mifepristone treatment did not produce a decrease in body weight or food intake, and did not produce any obvious outward signs of toxicity. Thus, mifepristone alone was able to produce a total growth retardation on androgen-insensitive PC-3 human prostate carcinoma cells in vivo for a period of 21 days in this study.

Other Steroid Modulation on the Growth of Prostate Carcinoma Cells

We investigated the possible mechanism by which steroid hormone receptors mediate this inhibition of PC-3 cells. Cell growth modulation by mifepristone was compared with that of various steroids including progesterone, Dex, and DHT, and peptide hormone LHRH. LHRH was used as a control to demonstrate the steroid hormone receptor-mediating event. As shown in Figure 5, progesterone at 1 μM concentrations exhibited an unexpected inhibitory activity on the growth of PC-3 cells by approximately 20% ($P<0.05$, n = 3), while mifepristone exhibited a 60% inhibition ($P<0.001$, n = 3). Dex exhibited no significant effect, with an average of approximately 5% inhibition on the growth of PC-3 cells. Neither DHT nor LHRH at 1 μM had any significant effect on the growth of androgen-insensitive PC-3 cells (Fig. 5).

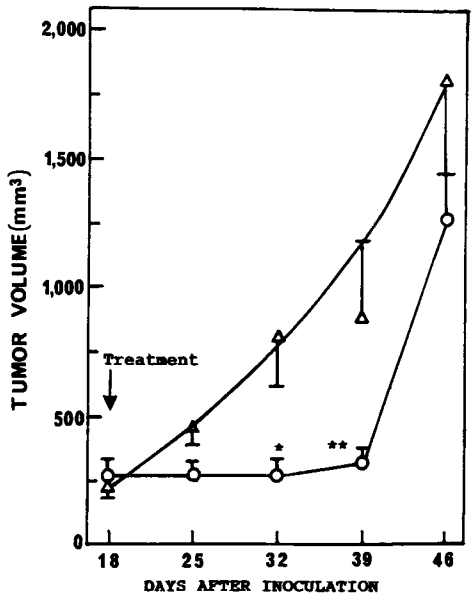

Fig. 4. Mifepristone effect on the growth of xenografted PC-3 tumors. Viable PC-3 cells (3.5×10^6 cells) were injected s.c. in the flank of male nude mice. After tumors were established, four animals (tumor volume, 261 ± 59 mm^3, mean ± SE) were treated with mifepristone in 4 mg/100 g body weight (○), and six animals (tumor volume, 240 ± 60 mm^3) received solvent alone (△) with 5 times per week. The data shown was the mean of tumor volume ± SE. For statistical analysis, two tumors in the control group were not included, since these were not within the normal distribution range. The average size of these two tumors was 1,223 mm^3, while that of all six tumors was 880 mm^3 at day 39. *, $P < 0.01$ (n = 4); **, $P < 0.001$ (n = 4), compared with the corresponding tumors in animals that received the solvent alone.

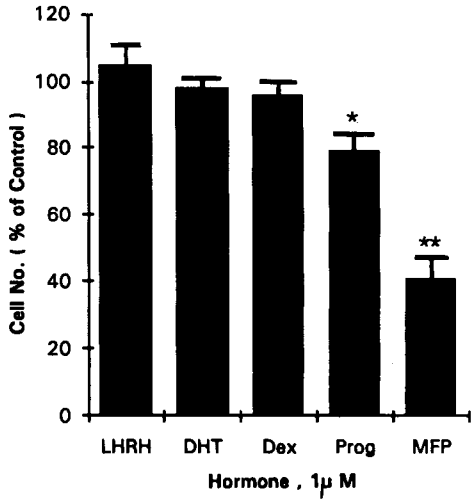

Fig. 5. Growth modulations by steroids on PC-3 cells. PC-3 cells were seeded at a density of 1×10^5 cells/well in 6-well culture plates as described above. After one day in the steroid-reduced medium, cells were fed with the same fresh steroid-reduced medium and various hormones (1 μM each) daily, as indicated. Control cells received solvent ethanol in medium daily. Fresh medium was added every 3–4 days. After 16 days, experimental and control cells in triplicates were harvested and cell numbers were counted. The control cells (2.0×10^6 cells/well) were referred to as 100% (data not shown). Dex, dexamethasone; Prog, progesterone; MFP, mifepristone. Bar, the range of triplicates in one set of experiments. *$P < 0.05$ (n = 3) vs. control; **$P < 0.001$ (n = 3) vs. control.

Progesterone Modulation of Prostate Cancer Cell Growth

The inhibitory effect of progesterone on PC-3 cells was further examined in these three cells. Progesterone modulated the growth of both PC-3 and LNCaP cells with a pattern that is very similar to that of mifepristone effects (Fig. 6 vs. Fig. 1). At low concentrations such as 1 nM, progesterone exhibited a low, but reproducible, stimulatory activity on the growth of both PC-3 and LNCaP cells, while at the concentration of 10 μM, the growth of both cell lines was suppressed by up to 80%. An inhibition of DU 145 cells was also obtained although to a lesser extent (data not shown). Thus, progesterone and mifepristone appeared to modulate the growth of human prostate carcinoma cells in a somewhat similar fashion.

Effect of Dexamethasone on Mifepristone or Progesterone Inhibition

Since Dex could function as an antagonist to mifepristone, we examined whether Dex could modify

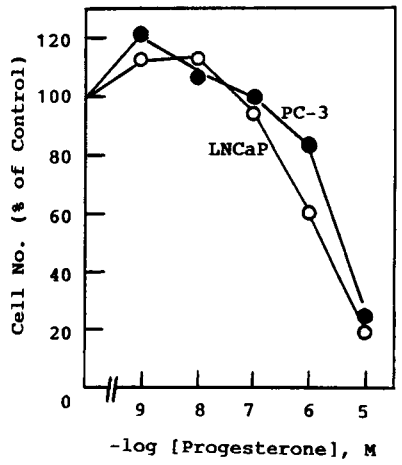

Fig. 6. Progesterone effects on the growth of prostate carcinoma cells. LNCaP and PC-3 cells were plated as described in Figure 1. Experimental cells received various concentrations of progesterone daily for 16 days, as indicated. Control cells received the solvent alone. Cells were harvested and counted as described above. The data shown is the average of duplicates in one set of experiments.

growth inhibition by mifepristone. As shown in Figure 7A, in the presence of 1 nM or 10 nM Dex, mifepristone (1 μM) continued to exhibit an inhibitory activity with a minor synergistic effect, as observed in our early phase of the investigation (data not shown). However, in the presence of 100 nM Dex, the synergistic inhibition disappeared. At the concentration of 1 μM Dex, the inhibitory activity of mifepristone (1 μM) was partially reversed ($P<0.05$, n = 4). This competition, however, was a transient response. After an exposure period of over 15 days, the competition by Dex disappeared (data not shown). Thus, at an equal concentration, Dex could partially and significantly rescue cells from mifepristone inhibition with an exposure period of 10 days.

The possible effect of Dex on progesterone cell growth inhibition was also investigated. Two-μM concentrations of progesterone were chosen to obtain an increased inhibition, allowing us to observe any significant competition. In the presence of 20 nM and 200 nM of Dex, progesterone inhibited the growth of PC-3 cells with a minor, but not significant, synergistic activity, as observed in mifepristone inhibition (Fig. 7A vs. 7B). In the presence of 2 μM of Dex, the majority of the inhibitory activity of progesterone was competed ($P<0.001$, n = 4). Therefore, at the same concentration, Dex could significantly rescue cells from progesterone inhibition.

Immunofluorescent Staining of Steroid Receptors

We examined the expression of steroid receptors in PC-3 cells by immunofluorescent staining with antiprogesterone receptor Ab or antiglucocorticoid receptor Ab to understand the possible mechanism for this inhibition. In PC-3 cells, a specific staining of glucocorticoid receptor at the peripheral area of nucleus was clearly observed (Fig. 8). The expression of progesterone receptor was not detected, in comparison with control cells that were reacted with normal IgG. The sensitivity of antiprogesterone receptor Ab was further reexamined by reacting with T-47 breast carcinoma cells which express progesterone receptors [30]. A positive staining was observed (data not shown). Therefore, PC-3 cells express detectable levels of glucocorticoid receptors.

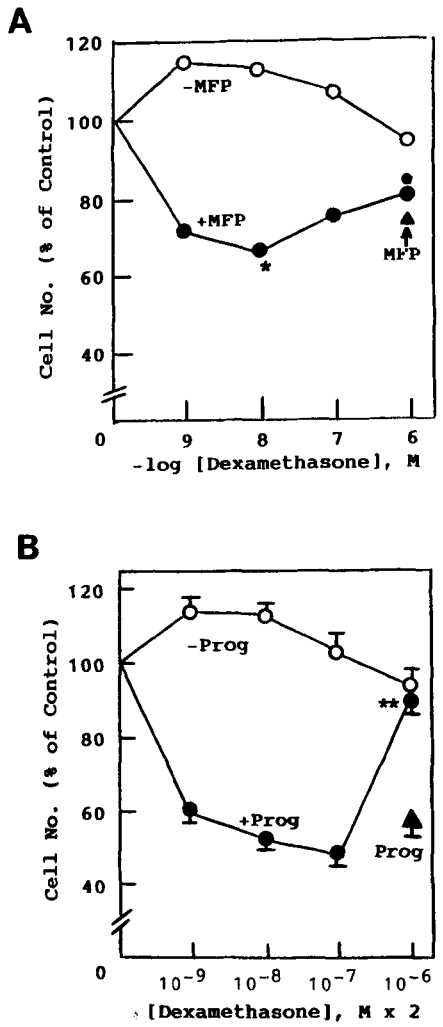

Fig. 7. Dexamethasone modulations on mifepristone and progesterone growth inhibition. PC-3 cells were plated in three sets with duplicates as described above. **A:** Competition of Dex with mifepristone. One set of cells received various concentrations of Dex as indicated, plus mifepristone (1 μM) (●). The second set of cells received Dex alone (○). The last set of cells received mifepristone (1 μM) alone (▲). **B:** Competition of Dex with progesterone. As described in **A**, one set of cells received various concentrations of Dex plus 2 μM progesterone (●). The second set of cells received Dex alone (○). The last set of cells received 2 μM progesterone alone (▲). After 10 days of exposure to steroids, cells in duplicates were harvested and counted. *$P < 0.05$ (n = 4), compared with the cells which received MFP alone; **$P < 0.001$ (n = 4), compared with the cells which received progesterone alone. Bar, the range of duplicates in two sets of experiments.

DISCUSSION

Our results clearly demonstrate the in vitro effect of mifepristone on human prostate carcinoma cell lines. Several human prostate carcinoma cell lines were studied, including androgen-sensitive and androgen-insensitive varieties. Without exception, mifepristone exposure resulted in growth inhibition in both androgen-sensitive (LNCaP) and androgen-insensitive (PC-3 and DU 145) cell cultures. This growth inhibition followed a dose dependent fashion at concentrations of 1 μM and 10 μM. Furthermore, this in vitro growth inhibition of androgen-insensitive prostate cancer cells was reflected in in vivo xenografted tumors in a nude mouse system.

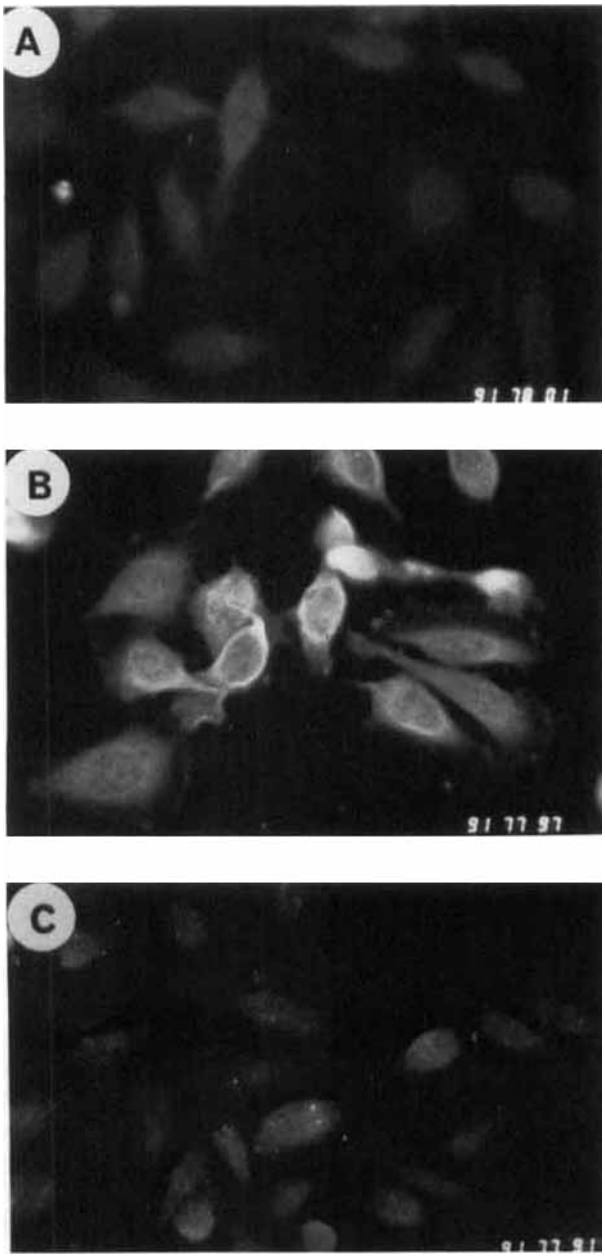

Fig. 8. Immunofluorescent staining of steroid receptors. PC-3 cells were plated on a coverglass in 6-well culture plates. Expression of steroid receptors was visualized by staining with (**A**) normal mouse IgG, (**B**) mouse monoclonal antiglucocorticoid receptor Ab, and (**C**) mouse monoclonal antiprogesterone receptor Ab, followed by fluorescein isothiocyanate-conjugated goat anti-mouse IgG Ab.

The role of steroid receptors in the inhibitory effect of mifepristone is well established in some cancer cells, including human breast carcinoma cells [30] and rat prostate carcinomas [32]. The mechanism by which mifepristone inhibition of androgen-insensitive human prostate cancer cell growth occurs is as yet still under investigation. Nevertheless, the data presented here may imply a possible mechanism of this growth inhibition. Since mifepristone binds with great affinity to the glucocorticoid receptor [10,33,34], and since active glucocorticoid-specific receptors are apparently presented in rat [35,36] as well as human (Fig. 8 and [37]) prostate carcinoma cells, we suggest that this receptor may be the functional receptor.

Dex per se does not cause significant growth inhibition of PC-3 cells (Figs. 5, 7). For example, 10 μM of Dex only depressed 20% of PC-3 cell growth (data not shown). However, at the same 10 μM, progesterone inhibited those same cells with 75% growth suppression (Fig. 6). Specific Ab staining demonstrated the presence of cytoplasmic glucocorticoid receptors, and more importantly failed to demonstrate the presence of progesterone receptors (Fig. 8 and [38]). These results imply that progesterone was acting as an antagonist at a nonprogesterone receptor. Furthermore, competitive studies (Fig. 7B) indicate that Dex and progesterone are competing for the same receptor. This hypothesis is compatible with the observation that progesterone exhibits weak antagonist activity at the glucocorticoid receptor [39].

Exposure of PC-3 cells to mifepristone demonstrates inhibitory activity quite similar to that of progesterone. However, mifepristone induces a much greater degree of cell growth inhibition at equal molar concentrations (Fig. 5, and Fig. 1 vs. Fig. 6). The relative efficacies of growth inhibition by progesterone and mifepristone are clearly compatible with the relative affinities of the two ligands for the glucocorticoid receptor. Mifepristone exhibits a 2- to 4-fold higher binding affinity for the glucocorticoid receptor than that of Dex [10,33,34], while progesterone exhibits a Kd value approximately 10% that of Dex [39]. The high binding affinity of mifepristone for Dex receptors may also provide an explanation for the observation on the temporary competition by Dex (data not shown). Furthermore, the observation that there was no significant competition between progesterone and mifepristone, nor a significant added effect of the simultaneous exposure to the two steroids, indicates that these two agents are acting by similar mechanisms (M. Kawachi and M.F. Lin, unpublished observations). In addition, Dex can competitively reduce growth inhibition by mifepristone and progesterone. The data therefore indicate that the effect of both mifepristone and progesterone on PC-3 cells is possibly mediated through the glucocorticoid receptor.

The mechanism of mifepristone inhibition of LNCaP cells is unknown. LNCaP cells express a mutant form of androgen receptor with one single-point mutation in the ligand binding domain [40,41]. The mutant receptor can bind various steroids [40,41]. There-

fore, steroid competition experiments cannot be performed in LNCaP cells.

Mobbs and Johnson in 1991 [32] investigated the effects of mifepristone on androgen-insensitive R3327 HI rat prostate carcinoma in tumor-bearing rats. Mifepristone alone had essentially no inhibitory activity on the growth of R3327 HI cells in rats. However, when diethylstilbestrol (DES) exposure was used to induce significant progesterone receptor concentrations, mifepristone exposure resulted in significant inhibition of tumor growth. Furthermore, the essential progesterone receptor population was localized to stromal cells, and was actually absent from the prostate carcinoma glandular epithelial cells [38,42]. Thus, it was postulated that mifepristone-induced growth inhibition of rat prostate carcinoma epithelium in tumor-bearing rats is a secondary effect mediated by progesterone receptor-positive stromal cells [32].

One possible explanation for the different conclusions about inhibitory mechanisms proposed by Mobbs and Johnson [32] and by ourselves may be due to cell type specificity. We used human prostate carcinoma cells, while Mobbs and Johnson used rat prostate carcinoma cells [32]. It was recently demonstrated that glucocorticoids at 10-nM concentrations inhibit the growth of rat prostate androgen-sensitive R3327H-G8-A1 tumor cells [35], and hamster androgen-responsive as well as androgen-nonresponsive leiomyosarcoma DDT1 MF-2 cells [43]. However, in our experiments, there was no significant inhibition of PC-3 cells by up to 1-μM concentrations of Dex (Figs. 5, 7). Further experiments are required to resolve this discrepancy.

There are still some puzzling findings. For example, it is not clear why high concentrations of mifepristone were required to achieve significant inhibition by its antagonist activity. Mifepristone exhibits a more than 2-fold higher binding affinity for glucocorticoid receptors than the corresponding hormone Dex [10,33,34]. This may indicate that there was some glucocorticoid agonist activity (e.g., the remaining glucocorticoid hormone in the dialyzed FBS) to compete with the added mifepristone. Alternatively, growth inhibition by high concentrations of mifepristone may be a nonreceptor-mediated, toxic effect. Nevertheless, by using charcoal-treated serum, a similar μM concentration of mifepristone is required to obtain a receptor-mediated growth inhibition of breast cancer cells [30]. A quantitative analysis on steroid binding protein in FBS may provide us with an answer [44]. Furthermore, it is not known why low concentrations (e.g., 1 nM) of mifepristone exhibited a minor stimulatory activity. Mifepristone has been proposed to be a pure antagonist to steroid receptors [10]. A model that was proposed for mechanisms of action of progesterone and mifepristone [45] may be able to rationalize this observation. Further experiments are needed to answer these questions and to delineate the mechanism by which mifepristone inhibits prostate cell growth.

We observed that mifepristone causes a growth inhibition of androgen-insensitive human prostate carcinoma cells both in vitro and in vivo. Mifepristone therapy may therefore be a potential therapeutic approach to supplement antiandrogen therapy or androgen deprivation of human prostate cancer cells. This possibility is supported by the observations that recurring metastatic lesions of human prostate tumors contain high levels of glucocorticoid receptors [37], and that mifepristone inhibition of androgen-insensitive human prostate carcinoma cells may act via glucocorticoid receptors. Furthermore, minimum side effects in long-term human clinical trials of mifepristone have been well documented [21,45,46]. In conclusion, we have demonstrated that exposure to mifepristone alone directly results in the growth inhibition of androgen-insensitive human prostate carcinoma cells in vitro. Most significantly, this in vitro growth inhibition was reflected in an in vivo animal model. This observation could have potential clinical importance in human prostate cancer therapy.

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