

Androgen and Epidermal Growth Factor Down-Regulate Cyclin-dependent Kinase Inhibitor p27^{Kip1} and Costimulate Proliferation of MDA PCa 2a and MDA PCa 2b Prostate Cancer Cells¹

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ABSTRACT

Low levels of p27^{Kip1} in primary prostate cancer specimens have been shown to be associated with higher rates of disease recurrence and poor rates of disease-free survival in patients with localized disease. In this study, we provide the first direct evidence showing that dihydrotestosterone (DHT), a major proliferation regulator of prostate cancer, can down-regulate p27^{Kip1} and stimulate cyclin-dependent kinase-2 (CDK2) activity in established prostate cancer cell lines. We investigated the cooperative effects of DHT and epidermal growth factor (EGF) on the proliferation of androgen-responsive MDA PCa 2a and MDA PCa 2b prostate cancer cells. DHT and EGF each stimulated proliferation of these cells, but exposure of the cells to DHT and EGF together stimulated greater proliferation. Stimulation of cell proliferation by DHT and/or EGF was associated with increased CDK2 activity and a decreased level of p27^{Kip1}. There seems to be a positive feedback stimulation loop between androgen-induced gene transcription and EGF-stimulated signal transduction, as one could stimulate the synthesis of the receptors for the other. Dual blockade of androgen receptor function with the antiandrogen hydroxyflutamide and EGF receptor superfamily-mediated signal transduction with the anti-EGF receptor monoclonal antibody C225 and the anti-HER2 receptor monoclonal antibody Herceptin significantly enhanced growth inhibition of the MDA PCa 2a cells. Our results demonstrate the importance of counteracting both androgen receptors and EGF receptors in the development of novel therapies for prostate cancer.

INTRODUCTION

Despite increased public alertness, significant progress in early diagnosis, and improved treatment modalities in recent years, adenocarcinoma of the prostate remains a leading cause of cancer-related deaths in North American men (1). Prostate cancer has traditionally been believed to be regulated by androgens because the prostate gland is an androgen-sensitive organ. Presently, patients with local or distant metastatic prostate cancer are treated primarily either by castration or with drugs that eliminate the activity of endogenous androgen (2, 3). Although most patients with prostate cancer initially respond to this therapy, the majority will subsequently become unresponsive to androgen ablation; the cancer cells then often resume uncontrolled proliferation in an androgen-independent manner and eventually kill the patients (2, 3). This renewed proliferation is probably due to the emergence of a hormone-independent cell subpopulation or to the transition of prostate cancer cells from hormone dependence to hormone independence. Thus, the hormone-independent state that develops in prostate cancer is a major obstacle to the cure of patients with advanced disease (1–3).

Recent studies have demonstrated, however, that prostate cancer is also regulated by the mitogenic effects of many polypeptide growth factors, including IGF-I,³ the fibroblast growth factors, platelet-derived growth factor, EGF, and TGF- α (4–7). These pathways become much more essential to prostate cancer growth after androgen insensitivity has emerged. High levels of IGF-I have recently been shown to be associated with an increased risk for prostate cancer (8). Several autocrine and paracrine loops involving the EGF receptor and its ligands EGF and TGF- α are also postulated to stimulate the growth of prostate epithelial and stromal cells independent of androgen activity (4, 9). Large amounts of EGF are present in prostatic tissue (10). In fact, human prostatic secretions contain the highest EGF levels of all biological fluids (11). EGF levels are regulated by serum androgen concentrations. Castration in mice reduced prostatic EGF levels, which could be restored by administration of testosterone (12). EGF was also detected in the culture media from both androgen-responsive LNCaP cells and androgen-independent DU145 cells (13, 14).

In addition to directly activating androgen-responsive element gene transcription, androgens may also stimulate prostate cancer growth through an autocrine loop involving TGF- α and

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³ The abbreviations used are: IGF-I, insulin-like growth factor; EGF, epidermal growth factor; TGF, transforming growth factor; DHT, dihydrotestosterone; CDK, cyclin-dependent kinase; mAb, monoclonal antibody; MAP, mitogen-activated protein; FBS, fetal bovine serum; GST-Rb, glutathione S-transferase Rb fusion protein.

EGF receptor autocrine pathway (15). Exposure of the androgen-responsive human prostate carcinoma cell line ALVA101 to testosterone or to its active metabolite DHT up-regulated both TGF- α and EGF receptors at the messenger level. The anti-EGF receptor mAb 528 can block the cell proliferation induced by DHT (15). On the other hand, growth factors such EGF and IGF-I can stimulate androgen-mediated gene transcription in the absence of androgen, suggesting that the androgen-signaling pathway may be activated by an androgen-independent mechanism (9). A recent clinical study indicated that prostate cancer progression is characterized by a transition from a paracrine to an autocrine loop between the EGF receptor and TGF- α ; in primary tumors, the neoplastic cells expressed EGF receptor and the surrounding stromal cells expressed TGF- α , and in advanced disease, the neoplastic cells coexpressed both the EGF receptor and TGF- α (16). Another more recent clinical study examined the activation status of MAP kinase in primary and metastatic human prostate tumor specimens; the study showed that non-neoplastic prostate tissue had little or no MAP kinase activity. However, in prostate tumors, the level of activated MAP kinase was elevated with increasing Gleason score and tumor stage (17). In this latter study, two patients whose tumor samples originally showed no activation of MAP kinase before androgen ablation therapy exhibited high levels of activated MAP kinase in their recurrent tumors after androgen ablation treatment (17). Taken together, these results indicate that during prostate cancer progression after androgen ablation therapy, there may be a microenvironment in which peptide growth factors activate signal transduction pathways and help to drive prostate cancer cells to an androgen-independent state.

These studies suggest that the androgen-induced signaling and growth factor-mediated pathway may both regulate the proliferation of prostate cancer cells. In the present study, we investigated the cooperative effects of DHT and EGF on the stimulation of cell cycle traversal and of proliferation of the MDA PCa 2a and MDA PCa 2b cells and explored the cooperative effects of dual blockade of androgen receptor and the EGF receptor superfamily on the inhibition of the proliferation of these cells.

MATERIALS AND METHODS

Materials. Antibodies against human cyclin D1, cyclin D3, cyclin E, cyclin A, CDK2, CDK4, CDK6, and p27^{Kip1} were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-human androgen receptor antibody was purchased from Novocastra Laboratories, Ltd. (Newcastle, United Kingdom). Anti-EGF receptor antibody RK2 was provided by Dr. J. Schlessinger (New York University Medical Center, New York, NY). Recombinant EGF was obtained from Collaborative Research, Inc. (Bedford, MA), and DHT was purchased from Sigma Chemical Co. (St. Louis, MO). Protein A-Sepharose beads used for immunoprecipitation were purchased from Repligen Corp. (Cambridge, MA). [γ -³²P]ATP was obtained from New England Nuclear (Boston, MA). GST-Rb was purchased from Santa Cruz Biotechnology, Inc.

Cells. MDA PCa 2a and MDA PCa 2b human prostate adenocarcinoma cells were provided by Dr. Nora Navone at the University of Texas M. D. Anderson Cancer Center. The cells

were maintained in BRFF-HPC1 medium (Biological Research Faculty and Facility, Inc., Jamsville, MD) supplemented with 20% FBS in a 37°C humidified atmosphere containing 95% air and 5% CO₂ (18). The cells were used in our studies only within passages 60–70.

Cell Proliferation Assays. Cell proliferation assays were performed in six-well culture plates. Cells were seeded onto the plates in BRFF-HPC1 medium supplemented with 20% FBS and were allowed to attach the plates for 48 h prior to various treatments. To examine the stimulation of cell proliferation by EGF and/or DHT, the cells were removed from BRFF-HPC1 medium and cultured in an F12K medium (Life Technologies, Inc., Gaithersburg, MD) containing 1% charcoal-stripped FBS, 40 mM phosphoethanolamine, 10 μ g/ml hydrocortisone, 45 mM selenious acid, 5 μ g/ml insulin, and various concentrations of EGF with or without 5 nM DHT.

To examine the inhibitory effects of anti-EGF receptor mAb C225 (provided by Dr. Harlan Waksal, ImClone Systems, Inc., New York, NY), anti-HER2 mAb Herceptin (provided by Dr. Mark Sliwkowski, Genentech, Inc., San Francisco, CA) and hydroxyflutamide (provided by Dr. Rudolph Neri, Schering-Plough Corporation, Kenilworth, NJ), the cells were cultured in BRFF-HPC1 medium supplemented with 1% FBS. Cell numbers were obtained with a Coulter counter after harvesting the cells by trypsinization.

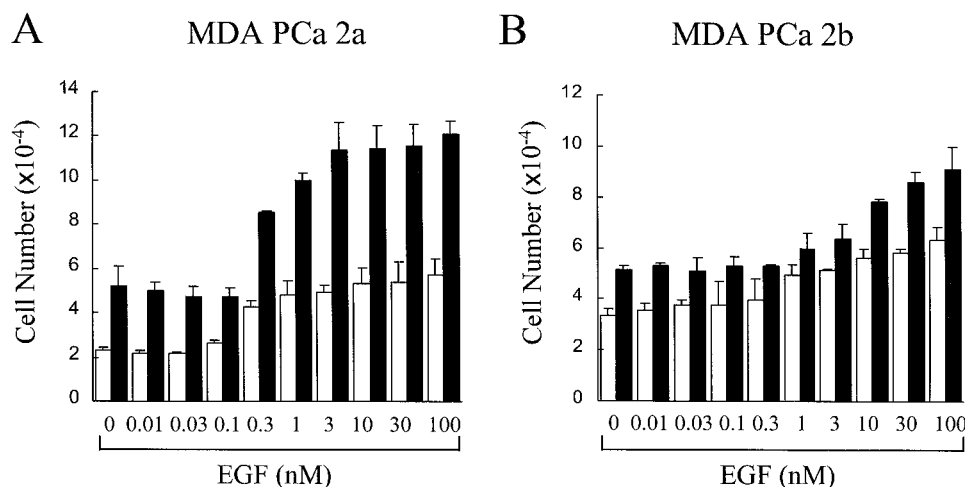
Western Blot Analysis. The procedures of Western blot analyses were described previously (19). Equal amounts of cell lysates were used for Western blot analyses with indicated antibodies described in "Materials." Cells were lysed in a buffer [containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% NP40, 50 mM NaF, 1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, 25 μ g/ml leupeptin, and 25 μ g/ml aprotinin] and were sonicated at 4°C.

CDK Kinase Assay. The kinase assay was performed as described previously (19). Briefly, CDK2, CDK4, or CDK6 was immunoprecipitated from sonicated lysates with corresponding antibodies and subjected to an *in vitro* kinase reaction in the presence of the CDK substrate GST-Rb and [γ -³²P]ATP.

RESULTS AND DISCUSSION

Enhanced Stimulation of Prostate Cancer Cell Proliferation by Androgen and EGF Combination. The human prostate carcinoma cell lines MDA PCa 2a and MDA PCa 2b were derived from a bone metastasis site from a patient with prostate cancer. These cells express androgen receptors and are stimulated by DHT (18). In the present study, we investigated the stimulatory effects on tumor cell proliferation of EGF alone and EGF combined with DHT. Exposure of the cells to 5 nM DHT stimulated cell proliferation (Fig. 1) as described previously (18). EGF stimulated proliferation of both MDA PCa 2a and MDA PCa 2b cells growing in F12K medium supplemented with 1% charcoal-stripped FBS in an EGF dose-dependent fashion (Fig. 1). Exposure of the cells to 5 nM DHT plus the increasing concentrations of EGF resulted in further stimulation of proliferation in both cell lines, particularly in the MDA PCa 2a cells (Fig. 1A). Our data demonstrating the responses of MDA PCa 2a and MDA PCa 2b cells to both DHT and EGF stimulation suggest that these cells are preferred models to

Fig. 1 Costimulation of MDA PCa 2a and MDA PCa 2b cells by DHT and EGF. MDA PCa 2a (A) and MDA PCa 2b (B) cells were seeded onto six-well plates in BRFF-HPC1 medium supplemented with 20% FBS. Forty-eight h later, the cells were switched to an F12K medium with additions as described in "Materials and Methods." The cells were exposed to the indicated concentrations of EGF with (■) or without (□) 5 nM DHT for 7 days. Cells were counted with a Coulter counter after harvesting by trypsinization. Bars, SD.



further investigate the regulatory mechanisms of prostate cancer cell proliferation by androgens and EGF.

Costimulation of CDK2 Activity with Androgen and EGF. Cell cycle is controlled by the sequential activation of a group of serine/threonine kinases known as CDK (20). The activities of CDKs are stimulated by their active regulatory subunits, called cyclins, and are opposed by a group of negative regulators called CDK inhibitors. We reported previously that treatment of androgen-independent DU145 prostate carcinoma cells with the anti-EGF receptor mAb 225 arrested the cell cycle at the G₁ phase and that this arrest was associated with inhibition of CDK2 activity and an increased level of a CDK inhibitor, p27^{Kip1} (21). Other researchers have reported that stimulation of androgen-dependent LNCaP cells with androgen up-regulated CDK2 and CDK4 gene expression, increased CDK2 kinase activity, and down-regulated the CDK inhibitor p16^{Ink4a} (22).

To investigate the effects of androgen and EGF on the regulation of cell cycle progression in the MDA PCa 2a and MDA PCa 2b prostate cancer cells, we examined the kinase activities as well as the protein levels of CDK2, CDK4, and CDK6 upon stimulation with DHT and/or EGF stimulation. In response to DHT and/or EGF stimulation, CDK2 activities markedly increased in both MDA PCa 2a and MDA PCa 2b cells. Costimulation of the cells with DHT and EGF resulted in a greater increase in CDK2 activities (Fig. 2). The activities of CDK4 and CDK6 in the two cell lines were not altered after exposure to EGF, DHT, or the combination of DHT and EGF. No changes in the protein levels of CDK2, CDK4, and CDK6 were observed within the 24-h period of exposure to DHT or EGF or both.

Accelerated Down-Regulation of CDK Inhibitor p27^{Kip1} by Androgen and EGF Combination. We next examined the protein levels of cyclins and CDK inhibitors upon exposure of the MDA PCa 2a and MDA PCa 2b cells to DHT or EGF or both. We found that, consistent with stimulation of CDK2 activity by DHT or EGF, both DHT and EGF down-regulated the levels of p27^{Kip1} in the MDA PCa 2a and MDA PCa 2b cells. The kinetics of p27^{Kip1} down-regulation by DHT or EGF was similar, with maximal down-regulation ~24 h after

EGF or DHT exposure (Fig. 3A). Combined stimulation with DHT and EGF resulted in accelerated down-regulation of p27^{Kip1}, which was observed as early as 8 h after coexposure to DHT and EGF (Fig. 3A). We also examined the levels of other cell cycle inhibitors such as p21^{Waf1}, p15^{Ink4b}, and p16^{Ink4a}. We found no change in the levels of these inhibitors after exposure to DHT or EGF or both (data not shown).

In contrast to a recent report that EGF induced expression of cyclin D1 in the androgen- and EGF-responsive LNCaP prostate cancer cell line (23), we found no change in the protein levels of cyclins D1, D3, and E upon EGF or DHT stimulation in the MDA PCa 2a and MDA PCa 2b cells (Fig. 2B). Levels of cyclin A increased upon EGF or DHT stimulation (Fig. 2B). The increased levels of cyclin A could be a consequence of EGF- and/or DHT-stimulated progression of cell cycle traversal as cyclin A synthesis peaks during S phase of the cell cycle (20).

In p27^{Kip1} knockout mice, the prostate was found to be significantly enlarged, indicating an important physiological role of p27^{Kip1} in regulating the normal development of the prostate gland (24–26). Low levels of p27^{Kip1} in primary prostate cancer specimens have been shown to be associated with higher recurrence rates and poor disease-free survival rates in patients with localized disease (27–35). Androgen is essential for the growth, differentiation, and function of the normal prostate as well as for the abnormal proliferation during the androgen-dependent stage of prostate cancer. To the best of our knowledge, our data are the first direct evidence showing that, in established cell lines, androgen can down-regulate p27^{Kip1} and stimulate CDK2 activity. Furthermore, costimulation of cell proliferation with DHT and EGF was accompanied by greater down-regulation of p27^{Kip1}, greater induction of CDK2 activity, and enhanced proliferation in the MDA PCa 2a and MDA PCa 2b cells.

Interactive Stimulation of EGF Receptor Synthesis and Androgen Receptor Synthesis by DHT and EGF. Previous studies have addressed the effect of androgen on EGF receptor regulation, which seems to be different in normal and carcinomatous prostates. Castration of mature rats resulted in a 3–6-fold increase in ¹²⁵I-EGF binding to the rat prostatic

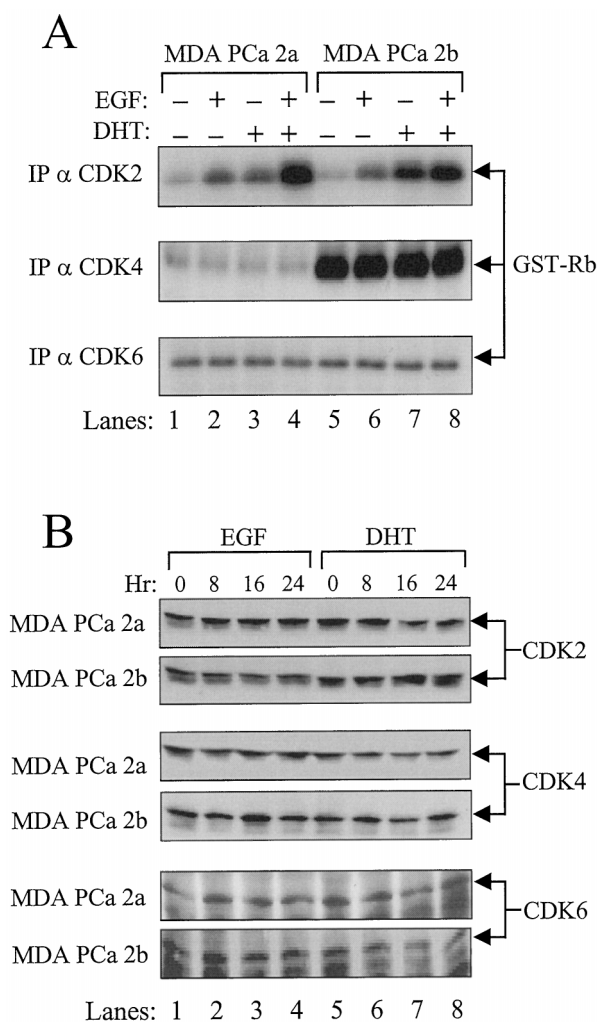


Fig. 2 Effects of DHT and EGF on the activities and expressions of CDKs. **A**, MDA PCa 2a and MDA PCa 2b cells were exposed to 5 nM DHT, 10 nM EGF, or 5 nM DHT plus 10 nM EGF in F12K medium supplemented with 1% charcoal-stripped FBS for 24 h. Immunoprecipitates were prepared from the treated cells using antibodies against CDK2, CDK4, and CDK6. A Rb kinase assay was performed as described in "Materials and Methods." **B**, MDA PCa 2a and MDA PCa 2b cells were exposed to either DHT or EGF for the indicated time intervals. Cell lysates were prepared as described in "Materials and Methods" and subjected to Western blot analysis with antibodies against CDK2, CDK4, and CDK6.

membranes, whereas treatment of the castrated rats with DHT decreased the number of ¹²⁵I-EGF binding sites (36). In contrast, an inverse pattern of EGF receptor level was observed in ALVA101 (15), LNCaP (37), and PC3 (38) prostate cancer cells, in which the levels of EGF receptor were increased by DHT or by the synthetic androgen methyltrienolone. In our study, exposure of the MDA PCa 2a and MDA PCa 2b cells to DHT also increased the levels of EGF receptors, which were maximally induced ~8 h after DHT treatment (Fig. 4A). Treatment with EGF down-regulated the levels of EGF receptors in these cells, as is regularly seen in many cell lines that show a mitogenic response to this growth

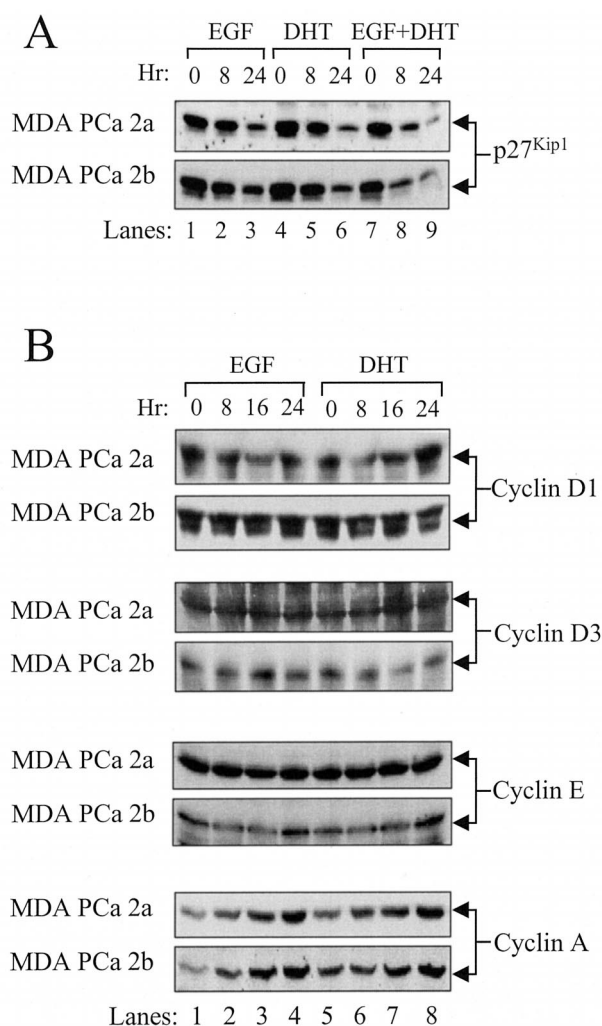


Fig. 3 Effects of DHT and EGF on the expressions of p27^{Kip1} and cyclins. **A**, MDA PCa 2a and MDA PCa 2b cells were exposed to 5 nM DHT, 10 nM EGF, or 5 nM DHT plus 10 nM EGF in F12K medium supplemented with 1% charcoal-stripped FBS for the indicated time intervals. Cell lysates were prepared as described in "Materials and Methods" and subjected to Western blot analysis with antibodies against p27^{Kip1}. **B**, MDA PCa 2a and MDA PCa 2b cells were exposed to either 5 nM DHT or 10 nM EGF for the indicated time intervals. Cell lysates were prepared as described in "Materials and Methods" and subjected to Western blot analysis with antibodies against cyclins D1, D3, E, and A.

factor. We further observed that the up-regulation of the EGF receptors in these cells by DHT could be prevented by coculturing the cells with cycloheximide, a protein synthesis inhibitor, suggesting that the increased levels of the EGF receptors after DHT stimulation were due to increased protein synthesis (Fig. 4B). Conversely, EGF could also up-regulate the levels of androgen receptors in these cells (Fig. 4C). The time course of androgen receptor up-regulation by EGF in both cell lines was similar to that for induction of androgen receptor by DHT, with a maximal induction ~8 h after exposure of the cells to DHT or EGF and then a gradual decrease (Fig. 4C). The levels of the androgen receptors after

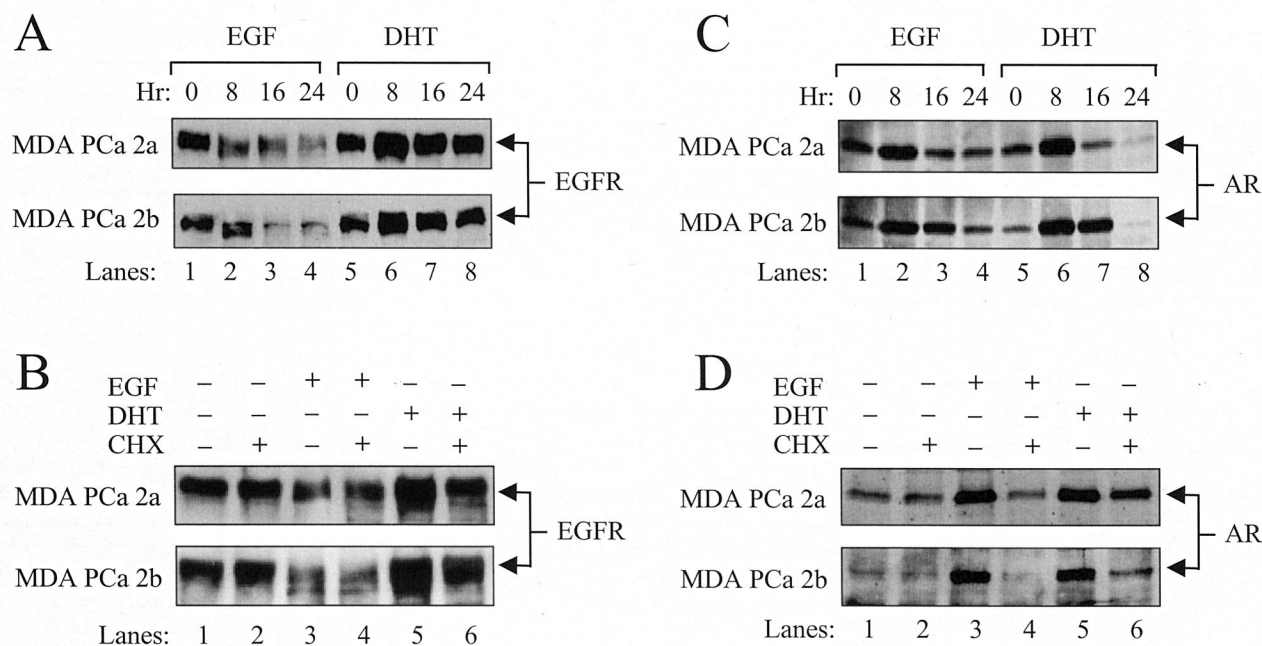


Fig. 4 Effects of DHT and EGF on the levels of androgen receptors and EGF receptors. **A** and **C**, MDA PCa 2a and MDA PCa 2b cells were exposed to 5 nM DHT or 10 nM EGF in F12K medium supplemented with 1% charcoal-stripped FBS for the indicated time intervals. Cell lysates were prepared as described in "Materials and Methods" and subjected to Western blot analysis with antibodies against EGF receptors (**A**) or androgen receptors (**C**). **B** and **D**, MDA PCa 2a and MDA PCa 2b cells were exposed to 5 nM DHT or 10 nM EGF for 8 h in the presence or absence of 50 μ g/ml cycloheximide (CHX). Cell lysates were prepared as described in "Materials and Methods" and subjected to Western blot analysis with antibodies against EGF receptors (**EGFR**; **B**) or androgen receptors (**AR**; **D**).

EGF or DHT exposure remained up-regulated longer in the MDA PCa 2b cells than in the MDA PCa 2a cells. Similar to the effect of cycloheximide on the up-regulation of the EGF receptors by DHT treatment, up-regulation of the androgen receptors by EGF or DHT could also be prevented by coculturing the cells with cycloheximide, suggesting that the increased levels of the androgen receptors after EGF stimulation were also due to increased protein synthesis (Fig. 4D).

Enhanced Inhibition of Prostate Cancer Proliferation by Dual Blockade of Androgen Receptor and EGF Receptor Superfamily. The costimulation of the MDA PCa 2a and 2b cell growth by DHT and EGF and the interactive stimulation of EGF receptor synthesis and androgen receptor synthesis by DHT and EGF in the prostate cancer cells indicated that DHT and EGF may play important complementary roles in regulating the proliferation of the prostate cancer cells. C225, a human-mouse chimeric anti-EGF receptor mAb, has been shown to block EGF binding to the receptor and inhibits EGF-induced receptor activation and signal transduction (39). To examine whether the dual inhibition of androgen receptor and EGF receptor can enhance the inhibition of prostate cancer cell proliferation, we evaluated the effect of hydroxyflutamide, a non-steroidal antiandrogen (40), and mAb C225, either alone or in combination, on the proliferation of MDA PCa 2a cells in culture. Because HER2 may heterodimerize with EGF receptor upon EGF stimulation and activate the ras/raf/MAP kinase signal cascade, we also evaluated the effects of a humanized anti-HER2 mAb, Herceptin (41), on the proliferation of MDA

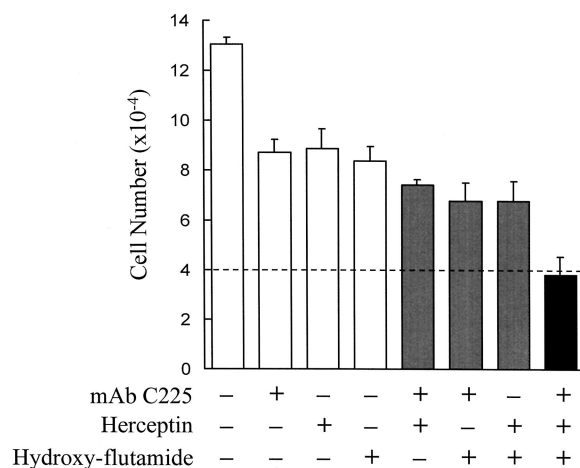


Fig. 5 Inhibition of MDA PCa 2a cell proliferation by dual inhibition of growth factor receptors and androgen receptors. MDA PCa 2a cells were seeded onto six-well plates in BRFF-HPC1 medium supplemented with 20% FBS. The cells were allowed to attach to the plate for 48 h. Dotted line, cell number before treatments were started. The cells were cultured for 7 days in BRFF-HPC1 medium containing 1% FCS and treated in triplicate with 20 nM anti-EGF receptor mAb C225, 20 nM anti-HER2 mAb Herceptin, 100 nM hydroxyflutamide, or in combination as indicated. Cells were counted with a Coulter counter after harvesting by trypsinization; bars, SD.

PCa 2a cells, using Herceptin alone or Herceptin combined with mAb C225 or hydroxyflutamide, or all three together agents together. Exposure of the MDA PCa 2a cells to any one agent (mAb 225 or Herceptin or hydroxyflutamide) inhibited growth by ~50% after a 7-day culture period compared with untreated cells (Fig. 5). The combined use of any two of these agents modestly enhanced this growth inhibition, but exposure to all three agents together markedly inhibited growth by ~100% compared with the initial cell numbers before the treatments were started.

In summary, we have demonstrated that both DHT and EGF can stimulate proliferation of the androgen-responsive MDA PCa 2a and MDA PCa 2b prostate cancer cells, and that this enhanced proliferation was mediated by stimulation of CDK2 activity in association with down-regulation of p27^{Kip1}. Coexposure of the cells to DHT and EGF resulted in greater stimulation of CDK2 and greater down-regulation of p27^{Kip1}, suggesting a convergent stimulatory effect of DHT and EGF on the traversal of the cell cycle in prostate cancer cells. Combination treatment with antiandrogen and inhibitors of the EGF receptor superfamily appears to be a promising new therapeutic strategy for prostate cancer.

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