Activation of Mitogen-activated Protein Kinase Pathway by the Antiandrogen Hydroxyflutamide in Androgen Receptor-negative Prostate Cancer Cells

Yi-Fen Lee, Wen-Jye Lin, Jiaoti Huang, Edward M. Messing, Franky L. Chan, George Wilding, and Chawshang Chang

INTRODUCTION

Prostate cancer is the most common noncutaneous cancer in men and the second leading cause of cancer-related death (1). The lack of effective therapies for advanced prostate cancer reflects, in part, the lack of knowledge about the molecular mechanism involved in the development and progression of this disease (2, 3). In particular, little is known about the mechanisms that trigger the conversion of an initially androgen-dependent cancer to androgen independence. When prostate cancers first occur, they are dependent on androgens for growth and can be treated successfully with androgen ablation therapy. However, after prolonged antiandrogen therapy, eventually the cancer acquires the ability to proliferate (4, 5). Defining the mechanism other than AR mutations that contribute to tumor progression and incomplete nature of the response to antiandrogen withdrawal, as well as its failure to occur in many patients, implies that there are mechanisms other than AR mutations that contribute to tumor progression.

We demonstrated that, at a clinically relevant concentration, 1 μM HF rapidly activates the Ras-MAP kinase signal pathway that consequently leads to cell proliferation in an AR-independent manner. This finding not only helps explain the flutamide withdrawal syndrome, in which patients who experience an increase in PSA while taking flutamide have a PSA decrease after cessation of flutamide treatment (20–22). However, the transient and incomplete nature of the response to antiandrogen withdrawal, as well as its failure to occur in many patients, implies that there are mechanisms other than AR mutations that contribute to tumor progression.

MATERIALS AND METHODS

Immunocytochemistry. Immunocytochemical stainings were performed on formalin-fixed, paraffin-embedded tissue sections using antibodies to ERK1 and ERK2 (SC-94 and SC-154, respectively; 1/400 dilution), and phospho-ERK1/2 (SC-7383; 1/50 dilution) all from Santa Cruz Biotechnology (Santa Cruz, CA). Sections were cut at 4–5 μm and deparaffinized according to.
established procedures and quenched with 3% hydrogen peroxide for 6 min. Antigen unmasking with heat retrieval in citrate buffer (pH 6.0) was accomplished by placing slides in a microwave (1500 W) pressure cooker for 30 min. Slides were rinsed and stained for 45 min with primary antibody, and then incubated for 20 min with secondary antibody and streptavidin-horseradish peroxidase. Slides were developed with 3-amin-9-ethylcarbazole and counterstained with Mayer Hematoxylin Blue in 0.3% ammonia water.

**Transient Transfection Assay.** DU145 cells were obtained from American Type Culture Collection and cultured in DMEM supplemented with 10% fetal bovine serum (FBS). Cells (1 x 10⁶) were seeded in 35-mm plates and transfected with the SuperFect transfection reagent (Qiagen, Chatworth, CA). After 4 h, the medium was changed to serum-free DMEM for 24 h. Thereafter, the cells were treated with HF, EGF, and vehicle for 18 h and then lysed for luciferase assay. Luciferase activity was normalized for transfection efficiency using pRL-TK as an internal control. Luciferase assays were performed using dual-luciferase reporter system (Promega, Madison, WI).

**Immunoblot of Phosphorylated p44/42 MAP Kinase.** This assay uses a polyclonal antibody specific against the activated p44/42 MAP kinases (Cell Signaling Technology, Beverly, MA), which detects p44 and p42 MAP kinase (ERK1 and 2) only when catalytically activated by dual phosphorylation at Thr202 and Tyr204. DU145 cells were seeded at 1 x 10⁵ cells/100-mm plate and were allowed to attach overnight; then the medium was replaced with serum-free DMEM for 24 h. The cells were pretreated with MAP kinase inhibitors, EGFR antibodies, or cycloheximide for 1 h, followed by HF, EGF, or vehicle treatment. Cells were washed twice with PBS, harvested at indicated times (Fig. 2), and lysed with RIPA buffer for 30 min on ice. The protein concentrations were determined by Bradford assay, and equal amounts of protein were resolved by SDS-PAGE, and immunoblotted with p44/42 MAP kinase antibody (1/1000), and then incubated with secondary antibody. Proteins were visualized by the enhanced chemiluminescence system (NEN Life Science Products, Boston, MA). p44/42 MAP kinase (total ERK1/2) was blotted as a control.

**Cell Proliferation Assay.** DU145 cells were seeded at 2 x 10⁴/ml on 35-mm plates and allowed to attach overnight. Cells were kept in serum-free DMEM for 24 h, and then medium was replaced with either DMEM-10% FBS or DMEM-0.5% FBS and treated as indicated in Fig. 5. Cells were trypsinized and counted with a hemacytometer at different times after treatments. For the antisense oligonucleotide experiments, cells were incubated with a fixed ratio of oligonucleotide versus SuperFect (2.5 µl of SuperFect;100 nM oligo) for 4 h, and then the oligo-containing medium was replaced with DMEM-0.5% FBS. Cells were trypsinized and counted by hemacytometer at indicated times (Fig. 5).

**Immunoprecipitation.** Serum-starved and treated DU145 cells were lysed with RIPA buffer for 30 min on ice. Lysates were then centrifuged at 12,000 x g for 10 min at 4°C, and protein concentrations were determined by the Bradford assay. Cell lysates (500 µg) were incubated for 4 h with either anti-Ras or anti-EGFR antibody (1/100; Santa Cruz Biotechnology) to the protein-antibody mixture and then added in 20 µl of protein A/G plus-agarose (Santa Cruz Biotechnology) for another 4-h incubation with constant rotation. The immunoprecipitates were washed four times with cold PBS, resolved by SDS-PAGE, and immunoblotted by anti-Raf-1 antibody, or anti-phosphotyrosine monoclonal antibody (Oncogene Research; Calbiochem, La Jolla, CA). The results were visualized by chemiluminescence.

**RESULTS**

**Increase of Phospho-ERK1/2 Level in the Patients’ Tumors after Patients Developed Flutamide Withdrawal Syndrome.** To evaluate the role that the activation of MAP kinase plays in HF withdrawal in prostate cancer, four prostate cancer patients undergoing androgen ablation therapy with flutamide were examined. We compared their phospho-ERK1/2 levels in prostate cancer biopsies before and after the development of the flutamide withdrawal syndrome. We have examined each slide and scored the number of the cells stained positive for phospho-ERK1/2 under the ×400 field. We found that phosphorylated or activated MAP kinase was undetectable before androgen ablation therapy with flutamide treatment (Fig. 1C). In contrast, 30, 41, 52, and 45% of the cells were stained positive for phospho-ERK1/2 in the recurrent tumors of all four patients whose disease was progressing while receiving flutamide and who developed flutamide withdrawal syndrome when the medication was discontinued immediately after the second biopsy (Fig. 1D). Mayer hematoxylin blue staining for pathological morphological examination is shown in Fig. 1, A and B. It is possible that

Fig. 1. Elevated levels of active MAP kinase in the prostate cancer specimens from a patient whose tumors progressed on androgen ablation therapy. Prostate tumor tissue sections from the same patient with either the pre-androgen ablation (A and C) or post-androgen ablation plus flutamide therapy (B and D) were immunohistochemically stained for phospho-ERK1/2. Sections were counterstained with Mayer hematoxylin blue (A and B) ×400.
plasmid was cotransfected with or without pSG5AR into DU145 cells. After 18 h, cells were harvested, and cell lysates were collected and assayed for the luciferase activity.

Activation of MAP Kinase Pathway by HF in DU145 Cells. To examine whether HF has any effect on the MAP kinase signal transduction pathway, we performed Western blotting using an anti-ERK1/2 antibodies. In [Fig. 2A], 100-mm dishes and serum starved for 24 h. The cells were treated with 1 mM HF, EGF, or both, for another 18 h, and then harvested. Western blots were performed with antibodies against phospho-ERK1/2 and -ERK1/2. The density of phospho-ERK1/2 shown as absorbance (OD) was determined by the Versa Doc Imaging System (Bio-Rad) and quantified by Quantity One software. kDa, kDa, in thousands.

Factors other than HF during complete androgen ablation therapy may contribute to the activation of MAP kinase. The effects of HF-mediated activation of MAP kinase in the prostate cancer cells was examined as follows.

Activation of MAP Kinase Pathway by HF in DU145 Cells. To investigate whether HF has any effect on the MAP kinase signal transduction pathway, we performed Western blotting using an antibody that specifically recognizes dually phosphorylated MAP kinase (phospho-ERK1/2) in AR-negative DU145 human prostate cancer cells. Our results showed that HF has no influence on total MAP kinase (ERK1/2) protein expression. HF at 1 μM can activate MAP kinase within 15 min and reach maximum activation in 30 min (Fig. 2A, Lanes 2 and 4). DU145 cells (passage number 61–65, ATCC HTB-81) are documented as an AR-negative cell line. This is also demonstrated in our Fig. 2B, which shows no visible AR band using AR antibody NH27. Our data in Fig. 1, A and B, demonstrate that HF can activate MAP kinase via a non-AR-mediated mechanism.

Next, we found that U0126, a specific MEK inhibitor, but not U0124, a structurally similar compound without inhibiting effects, can block HF-mediated MAP kinase activation (Fig. 2C, Lanes 7 versus Lanes 8 and 9).

For comparison, we also found that HF-mediated MAP kinase activation could be detected in other selected prostate cancer cells with endogenous AR expression. For example, HF activates MAP kinase in the CWR22 and in the PC3-AR2; Fig. 2C, D, and E), but not in LNCap (data not shown). It is unclear whether endogenous AR in these cells plays any role in the activation of the MAP kinase pathway. Because our data demonstrate that HF can activate the MAP kinase pathway in AR-negative cells, such as DU145 cells, we concluded that AR may not be a determining factor to mediate the activation of MAP kinase by HF. We noted that the activation of MAP kinase by HF is, however, relative weak as compared with EGF activation of MAP kinase. Nevertheless, because the concentration of HF needed to activate the MAP kinase is much lower than the concentration commonly available in the treatment of prostate cancer, our data implies that HF at pharmacological concentrations (10⁻⁶ to 10⁻⁵ M) may become a potent activator to stimulate MAP kinase pathway. To ensure that the HF that we used indeed exerts an antiantiandrogenic effect, the AR/dihydrotestosterone (DHT)-mediated PSA transcriptional activity was examined by adding HF. As shown in Fig. 2F, 1 μM of HF represses DHT-induced PSA-Luciferase (Luc) activity. Together, data from Fig. 2 suggest that HF can activate the MAP kinase pathway within 15 min without the involvement of the AR-mediated mechanism.

HF-mediated MAP Kinase Activation Is via Ras and Raf Pathway. To assess whether Ras and Raf are upstream regulators of HF-mediated MAP kinase activation, we used a Ras-Raf immunoprecipitation assay. As shown in Fig. 3A, Lane 2 versus Lane 3 and Lane 4 versus Lane 3, Raf was detected in the Ras immunocomplex
The treatment of DU145 cells with monoclonal antibody EGFRmAb-528 or polyclonal antibody EGFRpAb-1005 for 1 h also abrogated activation of phospho-ERK1/2 induced by either EGF or HF (Fig. 4B) in a dose-dependent manner (Lanes 3–5). Because these antibodies have been demonstrated to bind to a cell surface epitope of the EGFR and to antagonize ligand-stimulated EGFR tyrosine kinase activity, our data suggest that the activation by HF of the MAP kinase pathway could be exerted through a membrane receptor tyrosine kinase-mediated pathway without the involvement of new protein synthesis in an AR-deficient environment. Whether the EGFR itself, or an EGFR isoform that could be recognized by EGFR antibodies, served as the membrane mediator for the activation by HF of the Ras-Raf-MAPK pathway remains unclear.

The M<sub>1</sub> 170,000 EGFR exercises its biological effects in response to the binding of specific polypeptide ligands, including EGF and transforming growth factor α. This leads to the activation of EGFR catalytic tyrosine kinase domain, autophosphorylation of specific residues in its COOH terminus, and recruitment and phosphorylation of signaling proteins. As shown in Fig. 4C, HF causes the autophosphorylation of EGFR as detected by the immunoprecipitation with an antibody to the EGFR and immunoblotting with anti-phosphotyrosine. However, AG1478 can inhibit both EGF- and HF-mediated EGFR autophosphorylation, without affecting total EGFR concentration.

HF Promoted the Cell Proliferation and Cyclin D1 Expression.

Because the activation of MAP kinase might result in cell proliferation, we determined whether HF treatment affected cell proliferation. We found that HF, like EGF, can promote cell proliferation in a low-serum (0.5% FBS) environment after serum starvation (Fig. 5A). In contrast, if we maintain the cells in 10% FBS medium, HF or EGF causes no significant stimulation of cell proliferation (data not shown). To further link cell proliferation with the HF-mediated activation of Ras/MAP kinase pathway, we transfected the DU145 cells with antisense of Ras, Raf, and HIV as a control antisense. As expected, cells transfected with antisense of Ras and Raf oligomers that block the activation of MAP kinase did not show any significant increase of cell number after 24-h HF or EGF treatment. However, the cells doubled in number when we transfected control antisense oligomers and treated with HF or EGF (Fig. 5B). In conclusion, we demonstrated that HF activated the MAP kinase pathway and promoted cell proliferation. The blocking of Ras and Raf can reverse the proliferation mediated by HF and EGF. These data provided evidence that HF, like EGF, promotes cell proliferation through the Ras-Raf-MAPK pathway.

To further dissect the mechanism of how HF, like EGF, promotes cell proliferation, we tested several potential G<sub>1</sub> or S-phase targets that might be influenced by HF or EGF. We found that cyclin D1 is induced after HF or EGF treatment (Fig. 6A, Lane 1 versus Lane 2; Lane 1 versus Lane 3). In contrast, there is no significant change in other cell cycle-related gene products including p27, p21, Ki67, and proliferating cell nuclear antigen (data not shown). The HF- or EGF-mediated cyclin D1 gene expression was confirmed by a cyclin D1 promoter study (23). As shown in Fig. 5B, 10<sup>–6</sup> M HF or 30 ng/ml EGF induced cyclin D1 promoter (~174SD1-Luc) activity about 2-fold (Lane 1 versus Lane 2). Importantly, cotransfection of the dominant negative of Ras and Raf attenuated this HF- or EGF-mediated induction. An increase in cyclin D1 concentration may trigger transition from G1 to S phase and may eventually result in increased cell proliferation. That the expression of cyclin D1 could be induced by HF further clarifies how HF promotes DU145 cell growth.

**DISCUSSION**

Flutamide was the first androgen-receptor blocker to achieve widespread use. It is metabolized into HF, the biologically active form of...
the drug. In 1993, Kelly and Scher described four patients with progressive metastatic prostate cancer combined-androgen-blockade (simultaneous administration of castration and anti-androgen therapy) therapy. After selective discontinuation of flutamide treatments, the patients showed a biochemical and objective improvement (20). This phenomenon has also been reported for cyproterone acetate (24), nilutamide (25), and bicalutamide (26), as well as for progestational agents (27). It is apparent that prolonged therapy with flutamide may select for tumor cells that are stimulated by HF and, thus, contribute to prostate cancer progression. Discontinuation of treatment with stimulatory antiandrogens results in a related withdrawal syndrome. The pathophysiology of anti-androgen-withdrawal syndrome is not completely understood, although AR gene mutations seem to be a part of the explanation. However, the transient and incomplete response of the tumor to anti-androgen withdrawal, as well as the failure of the withdrawal syndrome to occur in many patients, implies that there are...
mechanisms other than AR mutations that contribute to tumor progression. The progression of prostate cancer to androgen-independent disease is also associated with the elevation and autocrine production of multiple polypeptide growth factors (6, 7). It is widely suspected that the paracrine and autocrine loops that exist play an important role in the loss of hormone dependence, as well as in the dependence of metastatic potential. The growth factors and receptors associated with prostate cancer progression regulate cell growth, at least partly, through regulation of the activity of Ras family members. Ras, a proto-oncogene, is dependent on protein-protein interactions to cause the clinical arena (28).

In this report, we provide another possible mechanism to explain HF withdrawal syndrome. We demonstrated that HF may have dual roles in the modulation of prostate tumor growth; these data are summarized in Fig. 7. First, when prostate cancer patients have relatively high concentrations of androgens, HF may function as an effective antiandrogen, competing with androgens for binding to the AR. The effect is the inhibition of androgen-mediated prostate cancer growth. At later stages, when prostate tumors become androgen independent, the continuation of HF treatment triggers MAP kinase pathway activation, with subsequent stimulation of prostate tumor growth. This phenomenon may contribute to the flutamide withdrawal syndrome and may explain why, in the case metastatic carcinoma, little prolongation of survival can be demonstrated for combined-androgen blockade (simultaneous administration of castration and anti-androgen therapy) compared with androgen-deprivation monotherapy.

Moreover, this observation offers potential therapeutic targets that may prolong the anti-tumor effect of flutamide. Finally, our studies demonstrate that advanced prostate cancer cells have variable responses to therapeutic strategies; hence, the characterization and treatment of hormone-refractory prostate cancer remains a difficult, yet intriguing, challenge for continuing research in this field.

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