Cytotoxic Effects Induced by Docetaxel, Gefitinib, and Cyclopamine on Side Population and Nonside Population Cell Fractions from Human Invasive Prostate Cancer Cells

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Abstract

The present study has been undertaken to establish the therapeutic benefit of cotargeting epidermal growth factor receptor (EGFR) and sonic hedgehog pathways by using gefitinib and cyclopamine, respectively, for improving the efficacy of the current chemotherapeutic drug docetaxel to counteract the prostate cancer progression from locally invasive to metastatic and recurrent disease stages. The data from immunofluorescence analyses revealed that EGFR/Tyr1173-pEGFR, sonic hedgehog ligand, smoothened coreceptor, and GLI-1 were colocalized with the CD133+ stem cell–like marker in a small subpopulation of prostate cancer cells. These signaling molecules were also present in the bulk tumor mass of CD133+ prostate cancer cells with a luminal phenotype detected in patient’s adenocarcinoma tissues. Importantly, the results revealed that the CD133+/CD44high/AR−/low side population (SP) cell fraction endowed with a high self-renewal potential isolated from tumorigenic and invasive WPE1-NB26 cells by the Hoechst dye technique was insensitive to the current chemotherapeutic drug, docetaxel. In contrast, the docetaxel treatment induced significant antiproliferative and apoptotic effects on the CD133−/CD44low/AR+ non-SP cell fraction isolated from the WPE1-NB26 cell line. Of therapeutic interest, the results have also indicated that combined docetaxel, gefitinib, and cyclopamine induced greater antiproliferative and apoptotic effects on SP and non-SP cell fractions isolated from WPE1-NB26 cells than individual drugs or two-drug combinations. Altogether, these observations suggest that EGFR and sonic hedgehog cascades may represent the potential therapeutic targets of great clinical interest to eradicate the total prostate cancer cell mass and improve the current docetaxel-based therapies against locally advanced and invasive prostate cancers, and thereby prevent metastases and disease relapse.

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Introduction

Major progress in prostate cancer research has led to an earlier diagnosis and effective treatment of patients diagnosed with low-grade and organ-confined prostate cancers by surgical tumor resection, antihormonal therapies, and/or radiation therapy (1–3). Unfortunately, the disease progression to highly invasive and metastatic and hormone-refractory prostate cancers (HRPC), which are generally refractory to current conventional androgen ablation and chemotherapy therapies, generally culminate in the death of patients after about 12 to 19 months (1, 4–7). This inefficacy of current therapies against locally invasive and metastatic HRPCs underlines the urgent need to develop novel therapeutic strategies for countering disease progression and overcoming treatment resistance and disease relapse.

Recent lines of experimental evidence indicated that the accumulation of genetic and/or epigenetic alterations in multipotent CD133+/CD44+/low androgen receptor (AR+/low) adult prostatic stem/progenitor cells resident in the basal cell compartment and/or their early progenies, transit-amplifying/intermediate cells, may culminate in their malignant transformation into highly tumorigenic prostate cancer stem/progenitor cells and tumor formation (1, 8–17). In support with the critical functions of prostate cancer stem/progenitor cells in prostate cancer initiation and progression, the small subpopulations of immature prostate cancer cells have been identified by immunohistochemical analyses as well as isolated from primary prostatic adenocarcinoma and metastatic tissue specimens of patients and well-established human prostate cancer cell lines (8–23). These prostate cancer stem/progenitor cells typically expressed different stem cell–like markers such as telomerase, CD133, CD44+/high, α5β1-integrinhigh, cytokeratin (CK5/14), CK18, CXC chemokine receptor 4, and/or ATP-binding cassette (ABC) multidrug transporters. The
highly tumorigenic prostate cancer stem/progenitor cells isolated from patient’s malignant tissues and prostate cancer cell lines were able to give rise to the total prostate cancer cell mass, including differentiated prostate cancer cell lines with a secretory luminal phenotype in vitro and in animal models in vivo that recapitulated the architectural phenotype of patient’s original tumors (8, 9, 12–14, 19–21, 23).

In addition, the prostate cancer progression to invasive and metastatic stages is typically characterized by a downregulation of diverse tumor suppressor gene products combined with an upregulation of the expression and/or activity of numerous oncogenic signaling elements in prostate cancer stem/progenitor cells and their progenies (1, 10, 11, 15, 24). In general, the interplay of a complex network of distinct oncogenic pathways initiated by hormones, growth factors, cytokines, and chemokines through their cognate receptors is involved in sustained growth, survival, invasion, and metastasis of prostate cancer cells as well as the development of an androgen-independent phenotype by tumor cells and treatment resistance (1, 10, 15).

Importantly, it has been reported that the persistent activation of epidermal growth factor receptor (EGFR) and sonic hedgehog cascades frequently occurs in prostate cancer cells, including prostate cancer stem/progenitor cells, during prostate cancer initiation and progression to androgen-independent and metastatic stages (1, 10, 24–34). These tumorigenic cascades may cooperate in the acquisition of a more malignant behavior and resistance of prostate cancer cells to current clinical therapies, metastases at distant tissues, and disease relapse (1, 10, 24–35). Consequently, the combination therapies targeting different oncogenic products, including EGFR and hedgehog pathways, in highly tumorigenic prostate cancer stem/progenitor cells and their differentiated progenies with a luminal phenotype, may represent more promising approaches than monotherapy to counteract disease progression and relapse (1, 10, 15, 16, 24, 32). In this regard, our recent works combined with several prior studies revealed that the blockade of the EGFR and hedgehog tumorigenic cascades resulted in a growth arrest and a massive rate of apoptotic death of metastatic prostate cancer cell lines (31–34). Importantly, we have shown that the cotargeting of EGFR and sonic hedgehog pathways by using gefitinib and cyclopamine with the chemotherapeutic drugs docetaxel or mitoxantrone resulted in supra-additive antiproliferative, antinvasive, and apoptotic effects on diverse metastatic parental prostate cancer cell lines compared with individual agents and two-drug combinations (32, 33). Additional studies are required to ascertain the efficacy of these cytotoxic drugs to eradicate the prostate cancer–initiating cells and their differentiated progenies at earlier stages of prostate carcinogenesis, and thereby prevent the transition from localized prostate cancers to invasive and metastatic HRPCs, disease recurrence, and the death of patients.

The present investigation was undertaken to establish the therapeutic benefit of cotargeting EGFR and hedgehog cascades by using gefitinib and cyclopamine for eradicating the total prostate cancer cell mass, including prostate cancer–initiating cells and their progenies, and improving the current docetaxel-based chemotherapeutic treatments against locally advanced and invasive prostate cancers. Therefore, the antiproliferative and apoptotic effects of docetaxel, gefitinib, and cyclopamine, alone or in combination, were estimated on side population (SP) cells and the non-SP cell fraction isolated from parental highly tumorigenic and invasive WPE1-NB26 cell line by the Hoechst dye exclusion method.

Materials and Methods

Materials
Human nonmalignant immortalized RWPE-1 prostatic epithelial cell line and its prostate cancer cell line derivatives comprised of RWPE-2, WPE1-NA22, WPE1-NB14, and WPE1-NB26 as well as the metastatic and androgen-independent PC3 cell lines were originally purchased from the American Type Culture Collection. All prostate cancer cells were maintained routinely in keratinocyte serum-free medium supplemented with 1% l-glutamine, antibiotics (100 IU/mL penicillin and 100 μg/mL streptomycin), bovine pituitary extract, and EGF according to the instructions of the American Type Culture Collection in a 37°C incubator supplied with 5% CO2. Keratinocyte serum-free medium and all other culture materials were from Life Technologies. Cyclopamine was obtained from Toronto Research Chemicals, Inc. Docetaxel, 3′,3′-dihexyloxacarbocyanine iodide [DiOC6(3)], MTT, and EGF were purchased from Sigma, and broad caspase inhibitor, N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-FMK) were from Calbiochem Corp. The gefitinib was synthesized according to a modification of a described procedure (36). The mouse monoclonal anti-CK5 antibody (RCK103), rabbit polyclonal anti-Ck18 antibody (H-80), rabbit polyclonal anti-EGFR antibody (1005), goat polyclonal anti-Tyr1173-phospho-EGFR antibody (1173) recognizing EGFR form phosphorylated at tyrosine 1173, rabbit polyclonal anti-sonic hedgehog ligand (SHH) antibody (H-160), and goat polyclonal anti-GLI-1 antibody (H300) were purchased from Santa Cruz Biotechnology, Inc. The rabbit polyclonal anti-smoothened coreceptor (SMO) antibody was provided by Abcam, Inc. The phycocerythrin-conjugated monoclonal anti-CD133/2 antibody (293C3) was purchased from Miltenyi Biotec, Inc. and was used according to the manufacturer’s instructions. The Vectastain avidin-biotin complex method peroxidase kit and 3,3′-diaminobenzidine dihydrochloride (DAB) were purchased from Vector Laboratories. The amounts of proteins were estimated by using a detergent-compatible protein assay kit from Bio-Rad Laboratories, Inc.

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Molecular Cancer Therapeutics
Immunohistochemical and Double Immunohistofluorescence Analyses

Immunohistochemical studies on the localization of the activated Tyr1173-pEGFR–phosphorylated form and the hedgehog signaling effector, GLI-1 transcription factor, in nonmalignant and malignant patient’s prostatic tissues were done as previously described using the Vector avidin-biotin complex method kit as indicated in the manufacturer’s instructions (33, 34). Briefly, the immunostaining was carried out on 32 pairs of AccuMax array tissue sections (Petagen, Inc.) from patients with primary prostatic adenocarcinoma (Gleason scores 4–10) with their corresponding normal adjacent tissues from the same patients. A reddish brown color precipitate observed on tissue sections indicates a positive immunoreactivity with the tested primary antibody. For each tissue section, the intensity of immunoreactivity for each tested signaling element was semiquantitatively graded by a urological pathologist (S.L.J.) on a 0 to +3 scale (0, no staining; 1+, week staining; 2+, moderately strong; and 3+, strong staining). The staining intensity of Tyr1173-pEGFR or GLI-1 in prostatic adenocarcinoma samples was scored and compared with the corresponding nonneoplastic prostatic tissues, and the value was considered enhanced if the staining intensity was higher by one or more points.

In addition, the double immunohistofluorescence analyses of the colocalization of the stem cell–like marker CD133 antigen (prominin-1) with unphosphorylated EGFR or its activated Tyr1173-pEGFR–phosphorylated form and hedgehog signaling elements (SHH ligand, SMO coreceptor, or GLI-1 transcription factor) were carried out on deparaffinized and rehydrated nonmalignant and malignant human prostatic tissue specimens from the patients obtained from the University of Nebraska Medical Center’s tissue bank. The tissue slides were blocked in the presence of 10% goat serum for 30 min followed by incubation with the phycoerythrin-conjugated pEGFR, anti-SHH, anti-SMO, or anti-GLI-1 antibody for 2 h. The slides were washed twice with PBS and processed for immunofluorescent detection as described below for the confocal microscopic analyses of fixed cells.

Reverse Transcriptase-PCR Analyses

All prostate cancer cells were maintained in serum-free keratinocyte medium for 48 h. The expression levels of stem cell–like (CD133 and CD44), ABCG2 multidrug transporter, basal (CK5), and luminal (CK18 and AR) markers as well as EGF, EGFR, SHH, patched receptor 1 (PTCH-1), SMO, and GLI-1 were estimated in total prostate cancer cell samples by reverse transcriptase-PCR (RT-PCR). After incubation, the cells were collected by centrifugation, and the total cellular mRNA was extracted from cultured cell pellets using the RNeasy kit (Qiagen) according to the manufacturer’s instructions. RT-PCR was done with the SuperScript II reverse transcriptase and Taq DNA Polymerase (Invitrogen) on 2 μg of total RNA. The reaction medium contained 2 μL of each primer. Equivalent amounts of primers (5 nmol/μL) were added to a 40.5 μL master mix of PCR reagents. After the denaturation of the aliquots at 95°C for 10 min, RT-PCR was done as previously described (31, 34). The samples were analyzed by electrophoresis on a 1.5% agarose gel staining with ethidium bromide. The primer sequences used to estimate the mRNA expression levels of human signaling products are presented in Table 1 (19, 29, 31, 32, 37–40).

Confocal Microscopy Analyses

All the cells were grown at a low density on sterilized coverslips for 24 h, washed with PBS, and fixed in ice-cold methanol at −20°C for 2 min (33, 34). The cells were blocked in 10% goat serum for 30 min and incubated with rabbit polyclonal anti-EGFR antibody (1105), goat polyclonal anti–Tyr1173-pEGFR antibody (1173), rabbit polyclonal anti-SHH antibody (H160), goat polyclonal anti-SMO antibody (H161), anti-pEGFR, anti-SHH, anti-pSMO, or anti-GLI-1 antibody for 2 h. The slides were washed twice with PBS and processed for immunofluorescent detection as described below for the confocal microscopic analyses of fixed cells.

Table 1. Sequences of primers used for RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5'-3')</th>
<th>Reverse primer (5'-3')</th>
<th>Reference</th>
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<tr>
<td>CD133</td>
<td>5′-CACCTACGGGCACTTCTACCT-3′</td>
<td>5′-TGCACGATGCCACCTTTCAG-3′</td>
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<td>CD44</td>
<td>5′-TCCATCAAGGGATGAGGCG-3′</td>
<td>5′-AACCTGGCCCTGCTGGAGTCA-3′</td>
<td>(19)</td>
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<td>5′-GGTGTGCTGGCTCGAC-3′</td>
<td>5′-GCCGAGCTTTGGACCAAGGT-3′</td>
<td>(37)</td>
</tr>
<tr>
<td>CK5</td>
<td>5′-AGTGAACCACCTCCTGGCTAC-3′</td>
<td>5′-GGAATGCTGCTGCTGCTGAGC-3′</td>
<td>(32)</td>
</tr>
<tr>
<td>CK18</td>
<td>5′-CGCCGCCCGCCGCCGCTTGC-3′</td>
<td>5′-CTCTGGATCTGGATCTTG-3′</td>
<td>(38)</td>
</tr>
<tr>
<td>AR</td>
<td>5′-CTGTTCGACACGGACAGATCT-3′</td>
<td>5′-CAACTTGCCGACAGATCTTGGC-3′</td>
<td>(32)</td>
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<tr>
<td>EGF</td>
<td>5′-ACAGGCTGTTAGGATGGAG-3′</td>
<td>5′-GGTGTGCTGGCTGGAGTCA-3′</td>
<td>(39)</td>
</tr>
<tr>
<td>EGFR</td>
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<td>5′-TCCGCTATGGCTGGATG-3′</td>
<td>(31)</td>
</tr>
<tr>
<td>SHH</td>
<td>5′-GATGGGCCACCATAGGAG-3′</td>
<td>5′-CGTCTGCGGACTAGAAGAG-3′</td>
<td>(31)</td>
</tr>
<tr>
<td>PTCH-1</td>
<td>5′-TTTTCGAGCTGTCGGAGAC-3′</td>
<td>5′-CGTCTGCGGACTAGAAGAG-3′</td>
<td>(31)</td>
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<tr>
<td>SMO</td>
<td>5′-ATTTCCGAGGGAAGAAGAC-3′</td>
<td>5′-AAATGGGACCCTGGAGTCA-3′</td>
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<tr>
<td>GLI-1</td>
<td>5′-TACTTACGGCTGCAAAC-3′</td>
<td>5′-GTCTGTTTCTCCTCCTGATG-3′</td>
<td>(29)</td>
</tr>
<tr>
<td>β-Actin</td>
<td>5′-GTTGTATCCAGGCTGTCG-3′</td>
<td>5′-GATCCGCTGGGGAATC-3′</td>
<td>(31)</td>
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anti-SMO antibody, or goat polyclonal anti-GLI-1 antibody (N-16) diluted in PBS for 1 h at room temperature. After three washes with PBS, the cells were then incubated with FITC-conjugated goat anti-mouse, FITC-conjugated donkey anti-goat, and/or Texas red-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, Inc.) for 1 h. Cells were washed again with PBS, nuclei counterstained with 4',6-diamidino-2-phenylindole, and mounted on glass slides in antifade VestaShield mounting medium (Vector Laboratories). Immunofluorescence staining was observed under a confocal laser scanning microscope (LSM 410, Zeiss).

**Isolation of the SP and Non-SP Cell Fractions from the Human Tumorigenic and Invasive WPE1-NB26 Cell Line by Flow Cytometry and Colony-Forming Assays**

The parental WPE1-NB26 cells (1 × 10^6 cells/mL) were stained with Hoechst buffer containing a final concentration of 2 μg/mL fluorescent Hoechst dye at 37°C for 2 h in the absence or presence of a broad ABC transporter inhibitor and 50 μmol/L verapamil. The small subpopulations of SP and non-SP cells were isolated by fluorescence-activated cell sorting (FACS) as previously described (22). The analyses and sorting of the viable SP and non-SP cell fractions were done using a FACS Aria flow cytometer with a DIVA software (Becton Dickinson Biosciences). The SP and non-SP cell fractions were collected after FACS and the expression level of the CD133 marker without apparent further phenotypic changes in these two cultured cell subpopulations was obtained by maintaining the cells in serum-free keratinocyte culture medium containing exogenous EGF (10 ng/mL) plus fibroblast growth factor at 8 ng/mL before their use.

The monolayer clonogenic assays were then done to estimate the self-renewal capacity of SP versus non-SP cell fractions isolated from the tumorigenic and invasive WPE1-NB26 cell line by FACS. For each assay, 500 viable SP or non-SP cells obtained after cell sorting were suspended in serum-free keratinocyte medium onto a 120-mm dish. All samples were plated in triplicate. After 14 d, the cultures were fixed and directly stained with a crystal violet solution and colonies were counted.

**Cell Culture and Growth Assays**

The SP and non-SP cell fractions isolated from the total WPE1-NB26 cell mass were maintained in serum-free keratinocyte culture medium. For growth assays, the cells were seeded on 96-well plates at a density of 3 × 10^4 cells per well in a total volume of 200 μL culture medium as previously mentioned (31, 33, 34, 41). After 3 d, the cell growth assays were done in the serum-free medium. Different concentrations of 2 nmol/L docetaxel, 0.5 μmol/L gefitinib, and 1 μmol/L cyclopamine, alone or in combination, were also added to the culture medium. After incubation for 48 h, the rate of cell growth was estimated by a MTT colorimetric test (42).

**Flow Cytofluorometric Analyses**

The SP and non-SP cells were grown at a density of 5 × 10^5 cells on 25-cm² dishes as previously described (31, 33, 34, 41). The cells were treated with different concentrations of docetaxel, gefitinib, and cyclopamine, alone or in combination, in the absence or presence of broad caspase inhibitor, Z-VAD-FMK. In all experiments, the cells were kept at a subconfluent level to avoid contact inhibition. More specifically, to determine the influence of drugs on the cellular cycle progression of SP and non-SP cell fractions, the cytometric analyses by FACS were done 48 h after the addition of different concentrations of tested drugs, alone or in combination. Moreover, the apoptotic effect induced by the tested drugs, alone or in combination, on the SP and non-SP cell fractions were estimated by FACS analyses after 4 d of drug treatment initiation. The DNA content estimation of each sample was done after staining with propidium iodide by FACS analyses essentially as previously described (31, 33, 34, 41).

**Estimation of Mitochondrial Membrane Potential and Cytosolic Cytochrome c Release**

To determine whether the apoptotic effect induced by docetaxel, gefitinib, and/or cyclopamine in the SP and non-SP fractions is mediated through a mitochondrial pathway, the mitochondrial membrane potential (MMP) and the amount of cytosolic cytochrome c were estimated as previously described (31, 33, 34, 41). Briefly, the SP and non-SP cells were nontreated (control) or treated with 2 nmol/L docetaxel, 1 μmol/L gefitinib, and 2 μmol/L cyclopamine, either alone or in combination, for 4 d. The adherent and floating cells were collected and washed in PBS. The pellets corresponding to ~1 × 10^6 prostate cancer cells were resuspended in 1 mL PBS containing the cationic, lipophilic, and fluorescent dye, 40 nmol/L DiOC6(3), which specifically accumulates within the mitochondrial compartment in a MMP-dependent manner (43). After incubation at 37°C for 20 min, the accumulation of DiOC6(3) within the mitochondria of SP and non-SP cell fractions was measured by FACS analyses. Moreover, after 4 d of cell growth on 25 cm² dishes in the absence or presence of different tested agents, which was done under the same conditions as described above, the floating and adherent cells were collected by centrifugation, rinsed twice with PBS, and centrifuged. Then, the amounts of cytochrome c present in the cytosolic extracts of each sample were estimated following the method described in the ELISA kit from Zymed Laboratories with a human anti-cytochrome c antibody.

**Statistical Analyses**

Statistical analyses were done using the Student's t test to compare the results, with P values of <0.05 indicating statistically significant differences.
Results

Immunohistochemical Analyses of Tyr1173-pEGFR and GLI-1 Expression Levels in Nonmalignant and Malignant Prostatic Tissues

To establish the potential implication of the activation of EGFR and hedgehog cascades in prostate cancer cells during prostate carcinogenesis, the expression levels of the activated Tyr1173-pEGFR–phosphorylated form of EGFR and hedgehog signaling effector, GLI-1 transcription factor, were examined by immunohistochemical stains on nonmalignant and malignant human prostatic tissue sections (Fig. 1A). The results from immunohistochemical analyses indicated a very weak cytoplasmic and membrane immunostaining for the activated Tyr1173-pEGFR–phosphorylated form of EGFR in certain prostatic epithelial cells in nonmalignant prostatic tissues (Fig. 1A). In contrast, Tyr1173-pEGFR expression levels varied from weak to strong within the cytoplasm and at the membrane, respectively, in the malignant epithelial cells localized in the intermediate and luminal compartments in a subset of primary prostatic adenocarcinomas as shown in Fig. 1A. The staining intensity associated with the Tyr1173-pEGFR protein expression was enhanced in 34% of tested primary prostatic adenocarcinomas (32 samples, 11 positive, and 21 negative cases; Gleason scores 4–10) compared with the corresponding nonneoplastic tissues. In addition, a positive immunoreactivity for the hedgehog signaling effector, GLI-1 transcription factor, was also observed in the cytoplasm and nuclei of prostate cancer cells detected in primary prostatic adenocarcinoma tissues, whereas this protein was not expressed at significant levels in the cytoplasm and nuclei of epithelial cells detected in nonmalignant prostatic tissues (Fig. 1A). The data from immunohistochemical analyses revealed that the expression of the hedgehog effector GLI-1 transcription factor was enhanced in 38% of the primary prostatic adenocarcinomas analyzed (32 samples, 12 positive, and 20 negative cases; Gleason scores 4–10), relative to the corresponding nonmalignant prostatic tissues from the same patients.

Immunohistochemical fluorescence Confocal Microscopy Analyses of the Expression Level of CD133 Stem Cell–Like Marker and Its Colocalization with EGFR and Hedgehog Signaling Elements in Nonmalignant and Malignant Prostatic Tissues

To obtain further experimental evidence of the implication of EGFR and sonic hedgehog cascades in the malignant transformation of CD133+ adult prostatic stem/progenitor cells into CD133+ prostate cancer stem/progenitor cells, we have characterized the colocalization of the CD133 stem cell–like marker with the signaling effectors of these tumorigenic cascades in nonmalignant and malignant prostatic tissues. The immunoconfocal coanalyses of the expression of the CD133 cell surface antigen with the basal CK5 and luminal CK18 markers revealed that this stem cell–like marker is detectable only in a very rare subpopulation of CK5/18-expressing prostatic epithelial cells in the basal compartment in nonmalignant prostatic tissue specimens (Fig. 1B). In contrast, the CD133 protein was principally detected in a small subset of intermediate prostate cancer cells (CK5/18) dispersed through the intermediate compartment in high-grade prostate intraepithelial neoplasias (PIN) and prostatic adenocarcinoma tissues from patients (Fig. 1B).

As shown in Fig. 1B, the results from immunohistochemical analyses have also revealed that the expression levels of EGFR and hedgehog signaling elements were significantly enhanced in a small subset of CD133+ prostate cancer cells and the bulk tumor mass of CD133+ prostate cancer cells in high-grade PINs and prostate cancers relative to nonmalignant prostatic tissue specimens from patients. Particularly, a positive immunoreactivity was observed for EGFR and its Tyr1173-pEGFR–activated form as well as the SHH ligand and the SMO coreceptor in the cytoplasm and at the cell surface in intermediate and luminal CD133+ tumor cells detected in high-grade PINs and prostatic adenocarcinoma specimens (Fig. 1B). Furthermore, a positive cytoplasmic and nuclear staining was detected for GLI-1, which acts as a transcriptional signaling effector of the activated hedgehog pathway, in intermediate and luminal CD133+ tumor cells (Fig. 1B). Importantly, a double-positive immunostaining was also seen for the CD133 stem cell–like marker with EGFR, Tyr1173-pEGFR, SHH, SMO, or GLI-1 signaling element in a similar small subset of prostate cancer cells dispersed through the intermediate compartment in malignant prostatic tissues (Fig. 1B).

RT-PCR and Confocal Microscopy Analyses of the Expression Levels of Stem Cell–Like Markers and EGFR and Sonic Hedgehog Signaling Elements in Different Nonmalignant and Malignant Prostatic Cell Lines and the SP and Non-SP cell Fractions Isolated from the Parental WPE1-NB26 Cell Line

To further assess whether an enhanced expression and activation of EGFR and sonic hedgehog cascades occur in prostate cancer cells during disease progression, a characterization of the expression levels of distinct signaling components of these tumorigenic pathways was done on well-established prostate cancer cell lines miming the multiple stages of prostate carcinogenesis and metastases at bone marrow. The data from RT-PCR and immunofluorescence analyses have indicated that the expression levels of EGF/EGFR and hedgehog signaling elements (SHH/SMO/GLI-1) were enhanced in tumorigenic and invasive WPE1-NB14 and WPE1-NB26 cell lines and metastatic PC3 cells compared with weakly tumorigenic WPE1-NA22 and RWPE-2 and nonmalignant RWPE-1 prostasitic cell lines (Fig. 2). Moreover, all of the tested prostatic cell lines expressed significant levels of stem cell–like markers, including CD133, CD44, and ABCG2 transporter as well as CK5 (basal) and CK18 (luminal) markers, suggesting that these cell lines possess an intermediate phenotype (CK5/18; Fig. 2).
Figure 1. Immunohistochemical and immunofluorescence analyses of expression levels of EGFR and hedgehog signaling elements in nonmalignant and malignant prostatic tissues. A, immunohistochemical analyses of expression levels of activated Tyr1173-pEGFR–phosphorylated form and sonic hedgehog effector GLI-1 transcription factor in nonmalignant and malignant prostatic tissues. AccuMax array sections of nonmalignant prostatic tissues and adenocarcinomas from the same prostate cancer patients were probed with antibody directed against the activated Tyr1173-pEGFR–phosphorylated form or GLI-1 transcription factor after blocking with serum as described in Materials and Methods. All tissue sections were examined under a microscope, and the Tyr1173-pEGFR or GLI-1 immunoreactivity was judged by dark brown staining. Representative pictures of stained tissue samples of normal prostate and adenocarcinoma are shown at original magnifications of ×100 and ×400. B, immunofluorescence analyses of the colocalization of the expression of the CD133 stem cell–like marker with CK5/18, EGFR, and its activated Tyr1173-pEGFR–phosphorylated form, and SHH hedgehog ligand, SMO coreceptor or GLI-1 transcriptional effector, in nonmalignant and malignant prostatic tissue specimens from patients. Double immunofluorescence staining was simultaneously done with the phycoerythrin-labeled anti-CD133 antibody (red) plus Alexa 340–labeled anti-CK5 antibody (blue) or fluorescein-labeled anti-CK18, EGFR, Tyr1173-pEGFR, Tyr1173-SHH, Tyr1173-SMO, or Tyr1173-GLI-1 antibody (green) after blocking with goat serum as described in Materials and Methods. Arrow, double staining (yellow/purple) detected by confocal analyses, which is indicative of the colocalization of these markers. Representative pictures are shown at original magnification of ×630.
In addition, the results from RT-PCR and immunoconfocal microscopy have also revealed that the small SP cell fraction isolated from WPE1-NB26 cells by FACS was characterized by higher expression levels of different stem cell–like markers, including CD133, CD44, and ABCG2, but lower to undetectable level of AR relative to the non-SP cell subpopulation (Fig. 3C and D). Importantly, the SP and non-SP cell fractions isolated from the WPE1-NB26 cell line also expressed significant levels of EGF/EGFR and hedgehog signaling elements (Fig. 3C and D). Moreover, the results from the clonogenicity assays have also revealed that the SP cell subpopulation isolated from the WPE1-NB26 cell line displayed a higher self-renewal ability, which was retained upon serial passage, than the non-SP cell fraction in serum free-keratinocyte medium (Fig. 3B).

Antiproliferative Effects Induced by Docetaxel, Gefitinib, and Cyclopamine on the SP and Non-SP Cell Fractions Isolated from the Parental Tumorigenic and Invasive WPE1-NB26 Cell Line

To establish whether the antiproliferative effect induced by docetaxel on the prostate cancer cell proliferation may be improved by the combined use of gefitinib and cyclopamine, the growth-inhibitory effects either of a single agent, two-agent, or triple drug combinations were evaluated on SP and non-SP cell fractions from parental tumorigenic and invasive WPE1-NB26 cells (Fig. 4). The low concentrations of tested drugs, which induced about 15% to 25% inhibition, were used in experiments in the present combination study with other drugs. As shown in Fig. 4A, the results from MTT assays revealed that 2 nmol/L docetaxel induced a significant antiproliferative effect on...
non-SP cells whereas the SP cell fraction was insensitive to a treatment with 2 nmol/L docetaxel. Interestingly, 1 μmol/L gefitinib or 2 μmol/L cyclopamine was however effective to induce the antiproliferative effect on both SP and non-SP cell fractions from WPE1-NB26 cells (Fig. 4A). Moreover, a bicombination of 2 nmol/L docetaxel plus 0.5 μmol/L gefitinib or 1 μmol/L cyclopamine induced a greater growth-inhibitory effect on SP and non-SP cell fractions relative to individual drugs. Of therapeutic interest, the SP and non-SP cell growth was more markedly inhibited by a triple drug combination of docetaxel, gefitinib, and cyclopamine compared with two-drug combinations (Fig. 4A). As shown in Fig. 4B, the results from FACS analyses have also indicated that the combined use of 2 nmol/L docetaxel and 0.5 μmol/L gefitinib plus 1 μmol/L cyclopamine caused a marked reduction of the growth of the SP and non-SP cell fractions isolated from the WPE1-NB26 cell line through a blockade in the G1 and G2-M phases of the cellular cycle.

**Apoptotic Effect Induced by Docetaxel, Gefitinib, and Cyclopamine on the SP and Non-SP Cell Fractions Isolated from the Parental Tumorigenic and Invasive WPE1-NB26 Cell Line**

To determine the benefit of combining gefitinib and cyclopamine to improve the efficacy of the current chemotherapeutic drug docetaxel, the percentages of apoptotic cell death induced by docetaxel, either alone or in drug combinations, were estimated by the flow cytometric analyses and the apoptotic cell number in the sub-G1 phase was quantified. The lowest effective concentrations for each tested drug that can trigger apoptotic death in prostate cancer cells were used. As shown in Fig. 5A, the results of FACS analyses revealed that SP cell fraction was insensitive to a treatment with 2 nmol/L docetaxel. In contrast, 2 nmol/L docetaxel alone caused a significant increase in apoptotic population of non-SP cell fraction compared with nontreated non-SP cells (control) after 4 days of treatment. Moreover, the bicombination of 2 nmol/L docetaxel plus 1 μmol/L gefitinib or 2 μmol/L
cyclopamine resulted in a higher rate of apoptotic death of SP and non-SP cells compared with individual drugs (Fig. 5A). Importantly, the triple combination of docetaxel, gefitinib, and cyclopamine was also more effective than two-drug combinations and caused the death of the majority of SP and non-SP cells (Fig. 5A).

Estimation of the Role of the Caspase Pathway in the Apoptotic Effect Induced by Docetaxel, Gefitinib, and Cyclopamine on the SP and Non-SP Cell Fractions Isolated from the WPE1-NB26 Cell Line

To assess whether the cytotoxic effects induced by the tested drugs on the SP and non-SP cell fractions from the WPE1-NB26 cell line is mediated through a mitochondrial pathway-dependent caspase activation, an estimation of the effects of drug treatment on MMP and cytosolic cytochrome c was done by FACS analyses and ELISA assays. As shown in Fig. 5B, the continuous treatment of the non-SP cells for 4 days with 2 nmol/L docetaxel induced a small decrease of MMP whereas SP cell fraction was nonresponsive to docetaxel treatment. Furthermore, 1 μmol/L gefitinib or 2 μmol/L cyclopamine alone caused only a slight decrease of MMP, as indicated by the weak shoulder of peak compared with the stained cells that were nontreated (control). The treatment of the SP and non-SP cell fractions with 2 nmol/L docetaxel plus 1 μmol/L gefitinib or 2 μmol/L cyclopamine, however, was accompanied with a marked decrease of MMP. The triple drug combination of 2 nmol/L docetaxel plus 1 μmol/L gefitinib and 2 μmol/L cyclopamine also induced a higher mitochondrial membrane depolarizing effect and cytochrome c amount released in the cytosol in the SP and non-SP cells compared with the treatment of...
Figure 5. Assessment of the stimulatory effect induced by docetaxel, gefitinib, and cyclopamine on the apoptotic death, mitochondrial membrane depolarizing, and cytosolic cytochrome c releasing in the SP and non-SP cell fractions isolated from parental tumorigenic and invasive WPE1-NB26 cells. The prostate cancer cells were nontreated (control) or treated with the indicated concentrations of docetaxel (Doc.), gefitinib (Gef.), and cyclopamine (Cycl.), alone or in combination, for 4 d. A, the cell nuclei were stained with propidium iodide and the number of apoptotic prostate cancer cells detected in the sub-G1 phase was analyzed by flow cytometry. Plots showing the percentages of apoptotic prostate cancer cell death induced by tested drugs, alone or in combination, obtained from three separate experiments. *, P < 0.05, a significant difference between the apoptotic effects induced by docetaxel, gefitinib, and cyclopamine on the SP and non-SP cell fractions. After the treatments, the cells were prepared by staining with 40 nmol/L DIOC6(3) for analyses of MMP by flow cytometry. Moreover, the amounts of cytochrome c released into cytosol were estimated by ELISA as described in Materials and Methods. B, representative profiles of effects induced by tested drugs, alone or in combination, on MMP in SP and non-SP cell fractions isolated from WPE1-NB26 cells are shown. C, plots showing the percentages of the stimulatory effects induced by tested drugs, alone or in combination on cytochrome c release in SP and non-SP cell fractions. *, P < 0.05, a significant difference between the stimulatory effect induced by docetaxel, gefitinib, and cyclopamine, alone or in combination, on cytochrome c release in the SP and non-SP cell fractions.
cells with the two-drug combinations (Fig. 5B and C). Consistent with the drug-induced caspase activation, we have also observed that the broad spectrum caspase inhibitor, Z-VAD-FMK, at 5 μmol/L abrogated the rate of apoptotic death induced by these cytotoxic agents on the SP and non-SP cell fractions (Fig. 5A).

Discussion

Recent accumulating lines of experimental evidence have revealed that the highly tumorigenic CD133+/CD44+/high/AR−/low prostate cancer stem/progenitor cells and/or their early progenies endowed with the stem cell-like properties can provide a critical role for prostate cancer initiation and progression (1, 8–17, 21, 23, 44). Moreover, the prostate cancer transition to invasive and metastatic disease stages is typically associated with the deregulation of diverse signaling cascades, such as hedgehog and EGFR pathways, in prostate cancer stem/progenitor cells and their progenies that may confer them with more malignant phenotypes and survival advantages contributing to treatment resistance, metastases, and disease relapse (1, 10, 11, 15, 44, 45). In this regard, the data from the present investigation indicated that EGFR and its activated Tyr1173-pEGFR coreceptor and ABCG2 multidrug transporter (Fig. 3). These data agree with numerous prior studies that have revealed that higher expression levels of EGFR, SHH, PTCH-1, GLI-1, and/or GLI-2 signaling components were detected in high-grade PIN and localized advanced prostate cancer, androgen-independent, and metastatic tumor specimens as well as prostate cancer cell lines relative to nonmalignant prostatic tissues and prostatic epithelial cell lines (10, 24–26, 28, 29, 31–35, 49–56). Especially, an increase in the number of prostate cancer cells expressing EGFR and basal luminal cell-specific markers CK5/18, respectively, has been observed in a subset of patients with HRPCs (26, 55). This observation suggests that the proportion of EGFR- and CK5/18-positive prostate cancer cells with an intermediate phenotype may increase during androgen deprivation, supporting the benefit of targeting EGFR for eliminating this small neoplastic cell subpopulation. Moreover, higher expression levels of SMO hedgehog coreceptor and β-catenin were also detected in CD44+/DU145 progenitor cells displaying a higher tumorigenic potential compared with the corresponding CD44−/low DU145 cell subpopulation (19). It is worth mentioning here that a small subpopulation of CD133+/CD44+/α2β1-integrinhigh DU145 stem/progenitor cells purified from the CD44+DU145 total population has also been shown to possess a higher tumorigenic potential in vivo than the CD133+/CD44+/α2β1-integrinhigh cell fraction (21). On the other hand, it has also been reported that the putative prostate stem/progenitor cells and their malignant counterpart, prostate cancer-initiating cells in nonmalignant and malignant primary prostatic tissues from patients may be detected within the SP cells endowed with stem cell-like properties (13, 18, 57). Collectively, these observations suggest that an enhanced expression and sustained activation of EGFR and sonic hedgehog oncogenic signaling elements in
CD133+ /CD44+/high /AR−/low prostate cancer stem/progenitor cells and their differentiated CD133−/CD44+/low /AR+ prostate cancer progenies may contribute to prostate cancer initiation and disease progression to locally invasive, androgen-independent, metastatic and recurrent disease stages.

Although a high curative rate is associated with the treatment of patients diagnosed with prostate cancers confined to prostate gland by prostatectomy, antiandrogen deprivation therapy, and/or radiation therapy, the locally advanced, invasive, and metastatic prostate cancers remain incurable with the current therapeutic regimens (1, 4, 6, 7). Moreover, the first-line systemic docetaxel-based chemotherapies used as care for patients with high-risk or metastatic HRPCs are only palliative and typically culminate in the development of diverse mechanisms of resistance by prostate cancer cells, disease relapse, and the death of prostate cancer patients after a short time period of about 15 to 19 months after treatment initiation (1, 4, 5, 7). Of particular interest, the results from the present study have revealed that the cotargeting of EGFR and hedgehog cascades could constitute a potential therapeutic strategy for reversing docetaxel resistance and improving its efficacy to eradicating the total tumor cell mass in organ-confined prostate cancers, and thereby prevent prostate cancer progression to metastatic, recurrent, and lethal disease stages. Indeed, despite the fact that SP cells isolated from WPE1-NB26 cell line was insensitive to 2 nmol/L docetaxel treatment compared with the non-SP cell fraction, a combination of 2 nmol/L docetaxel with selective inhibitor of EGFR or SMO hedgehog coreceptor, orally active gefitinib, and cyclopamine resulted in significant antiproliferative and apoptotic effects on this immature prostate cancer cell subset. Moreover, the combined use of low concentrations of gefitinib and cyclopamine cooperatively improved the antiproliferative and apoptotic effects induced by docetaxel on the SP and non-SP cell fractions compared with two drug combinations (Figs. 4 and 5).

More specifically, the antiproliferative effect induced by the combined drugs, docetaxel plus gefitinib and cyclopamine, was mediated through a blockade of SP and non-SP cells in the G1 and G2-M phases of the cell cycle (Fig. 4). Moreover, the results have also revealed that this triple drug combination mediated a massive rate of apoptotic cell death, at least in part, through a depolarization of the mitochondrial membrane, cytochrome c release into cytosol, and activation of caspase pathway (Fig. 5). Hence, together, these data revealed a beneficial effect of combining EGFR and hedgehog signaling inhibitors, gefitinib and cyclopamine, with current clinical chemotherapeutic drug, docetaxel, to improve the antiproliferative and apoptotic responses induced by low drug concentrations on the total prostate cancer cell mass.

These data support several studies conducted by us and other research groups that indicated that the treatment of metastatic prostate cancer cells with docetaxel and/or specific inhibitors of EGFR and SMO hedgehog signaling cascades was associated with an inhibition of growth, invasion, and metastatic spread of prostate cancer cells in vitro and animal model in vivo (1, 10, 28, 29, 31, 33, 34, 49, 50, 58–62). More specifically, gefitinib has been observed to sensitize the PC3 cells to ionizing radiations and diverse chemotherapeutic agents such as docetaxel, paclitaxel, and platinum compounds cisplatin and carboplatinum (1, 33, 34, 63). The continuous cyclopamine treatment of PC3 cell–derived tumor xenografts in nude mice in vivo also led to the apoptotic death of PC3 cells and tumoral endothelial cells whose molecular events were accompanied by a tumor growth regression and without major toxicity on normal cells (29). Additionally, our prior studies have also revealed that the simultaneous blockade of EGFR and hedgehog cascades significantly improved the cytotoxic effects induced by docetaxel or mitoxantrone on metastatic prostate cancer cells including enriched CD44+/high PC3 and DU145 cell fractions (32, 33).

Taken together, the results from the present investigation revealed that an enhanced expression and activation of EGFR and sonic hedgehog signaling elements may occur in CD133+ prostate cancer cells and their differentiated CD133− progenies during disease progression to locally invasive prostate cancers. Moreover, the cotargeting of EGFR and hedgehog cascades was effective for improving the efficacy of current chemotherapeutic drug docetaxel to eradicate the total prostate cancer cell mass, including SP cells with stem cell–like properties and the non-SP cell fraction from tumorigenic and invasive WPE1-NB26 cells. Further in vivo studies should allow us to corroborate these in vitro data supporting the benefit of cotargeting EGFR and hedgehog pathways for improving the efficacy of current clinical docetaxel-based chemotherapeutic regimens against locally invasive prostate cancers and/or metastatic HRPCs and prevent disease recurrence.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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