Effects of Anti-EGFR Antibody Cetuximab on Androgen-independent Prostate Cancer Cells

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Abstract. *Aim: Epidermal growth factor receptor (EGFR) is a novel molecular target for anticancer therapy. This study examined the effects of anti-EGFR antibody cetuximab on two human androgen-independent prostate carcinoma cell lines, Du145 and PC-3. Materials and Methods: Cell proliferation was monitored with a trypan blue viability assay. Cell apoptosis and cell cycle profile was evaluated by flow cytometry. The expression of various signaling molecules was examined by Western immunoblotting. Results: Cetuximab (100 μg/ml) caused a significant growth inhibition by inducing cell apoptosis in Du145 cells, but not in PC-3 cells. It caused EGFR down-regulation and inhibited EGFR Tyr-845 autophosphorylation in both Du145 and PC-3 cells. However, EGFR phosphorylation at Tyr-1173 and MAPK 44/42 phosphorylation were inhibited in Du145 cells, but not in PC-3 cells. Cetuximab was not able to inhibit Akt phosphorylation in either prostate cancer cell line. Conclusion: Du145 cells only showed a very moderate response to cetuximab whereas PC-3 cells showed resistance. Persistent activation of EGFR downstream signaling likely contributes to cell resistance to cetuximab.*

Prostate cancer is the most frequently diagnosed form of malignancy in men and responsible for the second leading cause of cancer related death (1). Prostate cancer initiation and progression involves a transformation from normal epithelium to androgen-sensitive tumors and finally to the aggressive androgen-independent cancer (2). Chemotherapy is often used for patients with androgen-independent prostate cancer. However, the median patient survival time with current standard docetaxel-based combination chemotherapy is only about 18 months (3), which necessitates new therapeutic approaches for this disease.

Activation of receptor tyrosine kinase (RTK)-mediated downstream pathways is known to partially contribute to prostate cancer androgen independence by stimulating tumor cell proliferation at low androgen levels (2). Epidermal growth factor receptor (EGFR), the first and most studied RTK, is overexpressed in about one third of epithelial cancers including head and neck, colorectal, breast, ovarian, prostate, bladder and lung cancer (4). Aberrant and persistent EGFR signaling stimulates tumor cell proliferation, tumor angiogenesis, invasion and metastasis through the activation of multiple downstream signaling pathways such as MAPK, PI3K/Akt, NF-κB and PLC-γ pathways (2). Studies have shown that EGFR expression correlates with disease progression from being androgen-dependent to androgen-independent (5, 6). Thus, EGFR may serve as a potential molecular target for androgen-independent prostate cancer.

Antibody therapy is one promising approach for targeting EGFR. Anti-EGFR antibodies block the ligand binding to EGFR, thus preventing EGFR-mediated tyrosine kinase activation (7). Cetuximab (C225), a chimeric anti-EGFR monoclonal antibody, has been approved by the U.S. Food and Drug Administration for the treatment of head and neck cancer and metastatic colorectal cancer (8). In this study, the effect of cetuximab on human androgen-independent prostate cancer cell lines was investigated. Two cell lines, PC-3 and Du145, with different levels of EGFR expression were studied in comparison to a human skin cancer cell line A431 with high expression of EGFR.

Materials and Methods

Cell culture and reagents. Two human androgen-independent prostatic cancer cell lines PC-3 and Du145, and one human epidermoid carcinoma cell line A431 were purchased from the

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American Type Culture Collection (Manassas, VA, USA). All cell lines were cultured in RPMI-1640 medium with glutamine (Mediatech, Manassas, VA, USA) supplemented with 10% fetal bovine serum and 100 units/ml penicillin-streptomycin solution in an environment maintained at 37˚C and 5% CO_2. The chimeric anti-EGFR monoclonal antibody cetuximab (2 mg/ml) manufactured by ImClone Systems Inc. (New York, NY, USA) was purchased and stored at 4˚C until use.

Trypan blue viability assay. The effect of cetuximab on tumor cell survival was determined by trypan blue exclusion assay. Briefly, Du145, PC-3 and A431 cells suspended in complete RPMI were plated in 35 mm dishes. After 24 h incubation, the medium was replaced with either fresh RPMI medium containing cetuximab (100 μg/ml) for treated dishes or fresh RPMI without drug for control dishes. Tumor cells were treated with cetuximab for 24, 48 or 72 h. The number of viable cells at different times after treatment was counted using trypan blue exclusion and plotted as a three-day growth curve. The data shown were based on 4 repeated experiments.

Cell cycle distribution and apoptotic analysis by flow cytometry. Du145, PC-3 and A431 cells were plated in 35 mm culture dishes. After 24 h incubation, medium was replaced with either fresh RPMI medium containing 100 μg/ml cetuximab for treatment or RPMI medium without drug for 72 h. Cells were harvested at the end of treatment and cell pellets were obtained by centrifugation. The pellets were resuspended in pH 7.6 citrate buffer including 250 nM sucrose, 40 mM trisodium citrate-$2H_2O$ and 5% dimethylsulfoxide (DMSO). After treatment with solution containing 0.01% ribonuclease A, cells were incubated with 0.04% propidium iodide solution for 1 h for DNA staining. Flow cytometric analysis of cell cycle distribution was performed using precalibrated FACS Calibur flow cytometer and 10,000 events were recorded for each sample. Analysis was performed using CellQuest software (BD Biosciences, San Jose, CA, USA). The percentage of apoptotic cells in each sample was determined by quantifying the percentage of cells in sub-G_1 (M_1) phase.

Western blot analysis. To determine the effects of cetuximab treatment on the expression of EGFR and its downstream signaling pathways, western blots were carried out on whole cell lysates. Briefly, cells were treated with 100 μg/ml cetuximab for 72 h and lysed with ice-cold 4x Nupage LDS (lithium dodecyl sulfate) buffer (422 mM Tris HCl, 563 mM Tris Base, 8% LDS, 2.04 mM EDTA, 40% Glycerol) containing 200 mM DTT. Cell lysates were briefly sonicated and subsequently denatured using Laemmeli buffer at 90˚C for 5 min. Sample protein concentration was determined by the Amido black staining method. An equal amount of protein (40 μg) from each sample was resolved by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. After overnight blocking at 4°C, membranes were probed with primary antibodies followed by incubation with horseradish peroxidase-conjugated secondary antibodies (Jackson Immuno Research Laboratories, West Grove, PA, USA). Immunoreactive bands were detected with enhanced chemiluminescence and quantified using NIH Image J software (V1.39; National Institute of Health, Bethesda, MD, USA). The primary antibodies used in this study were monoclonal total EGFR (clone H9B4) antibody (Invitrogen Inc., Camarillo, CA, USA), polyclonal phospho-EGFR (Tyr 845) antibody (Invitrogen), monoclonal phospho-EGFR (Tyr 1173) antibody (Upstate Cell Signaling Solutions, Lake Placid, NY, USA), polyclonal total Akt and phospho-Akt (Ser 308) antibodies (Cell Signaling Technology, Inc. Danvers, MA, USA), and polyclonal total p44/42 MAPK and phosphor-p44/42 MAPK (Ser 202/Tyr 204) antibodies (Cell Signaling Technology). Protein β-actin probed with monoclonal β-actin antibody (Sigma-Aldrich, St. Louis, MO, USA) was used as a loading control in all blots.

Statistical analysis. Student's *t*-test was used to determine the statistical significance between different groups and statistical significance was accepted at $p<0.05$.

Results

Effect of cetuximab on tumor cell growth. The effect of cetuximab treatment on tumor cell growth were determined by trypan blue assay. Figure 1 shows the percentage increase in cell number as a function of time after incubation with 100 μg/ml concentration of cetuximab in Du145, PC-3 and A431 cell lines. Cetuximab did not inhibit tumor cell growth after 24 h of incubation in all three cell lines. After 48 h of incubation, cetuximab caused a significant decrease of Du145 cells, but not of PC-3 or A431 cells. After 72 h of incubation, cetuximab significantly inhibited tumor cell proliferation in both Du145 and A431 cells ($p<0.01$). The cell number was decreased to 67.2% and 58.2% of the control value for Du145 and A431 cell lines, respectively. However, cetuximab did not significantly inhibit PC-3 cell proliferation.

Effects of cetuximab on tumor cell cycle. The effect of cetuximab treatment on tumor cell cycle was examined by fluorescence-activated cell-sorting analysis, where cells were gated into sub G_1 (M1), G_0/G_1 (M2), S (M3) and G_2-M (M4) phases based on DNA fluorescence staining intensity. Cell population in sub G_1 (M_1) phase was used to estimate apoptotic cells because apoptosis causes DNA fragmentation, leading to decreased DNA staining. Figure 2 shows a representative experimental dataset involving all three tumor cell lines. The proportion of cells in the apoptotic sub G_1 phase in Du145 cells increased by about 4.5% after cetuximab treatment. There was a corresponding decrease in the percentage of cells in S and G2-M phases, suggesting inhibition of cell proliferation. However, cetuximab treatment did not induce any significant change in cell cycle in PC-3 cells compared to control cells. In A431 cells, cetuximab caused a significant increase (about 8%) in the proportion of cells in sub-G_1 phase and a corresponding decrease in cell populations in S and G_2-M phases.

Effects of cetuximab on EGFR signaling. Western blot analysis was performed to determine the effects of cetuximab on EGFR signaling. Figure 3 shows that there is a significant variation in total EGFR expression among the three tumor cell lines. A431

cell line exhibited the highest EGFR expression while PC-3 had the lowest level of expression. Treatment with cetuximab (100 μg/ml) for 72 h caused down-regulation of total EGFR in all three cell lines. EGFR phosphorylation was also inhibited as a result of treatment. EGFR phosphorylation at tyrosine 845 site was decreased in all three tumor cell lines after treatment. However, phosphorylation at tyrosine 1173 site was only inhibited in Du145 and A431 cells, but not in PC-3 cells. Two downstream signaling pathways of EGFR signaling, the MAPK and PI3K/Akt pathways, were also evaluated. Cetuximab caused a slight inhibition (4-7%) of 44/42 MAPK phosphorylation as well as total 44/42 MAPK down-regulation in Du145 cells. Similar inhibition of 44/42 phosphorylation MAPK and total 44/42 MAPK downregulation was also observed in A431 cells. However, no significant change in 44/42 MAPK was found in PC-3 cells. Cetuximab did not induce any significant change in Akt phosphorylation in either the Du145 or PC-3 cell lines. In contrast, significant inhibition of Akt phosphorylation was observed in A431 cells.

Discussion

EGFR-targeted therapy represents a potential novel therapeutic strategy for the treatment of solid tumors. Both monoclonal antibodies and small molecules targeting EGFR and its signaling pathway are being actively pursued for cancer treatment (9). Cetuximab is the most clinically advanced anti-EGFR antibody and has been approved for the treatment of colorectal and head and neck cancer (8). In this study, its effect on prostate cancer cell lines was evaluated.

Results from this study demonstrated that cetuximab was able to inhibit prostate cancer cell proliferation and induce cell apoptosis. However, this effect was time and cell dependent. Relatively long incubation with cetuximab was necessary to obtain a significant effect of cell growth inhibition (Figure 1). In fact, incubation of cetuximab with cancer cells for less than 48 h failed to achieve a significant effect in all three cancer cell lines tested in this study. These data also demonstrated that Du145 and A431 cells, with a relatively higher EGFR expression, were responsive to cetuximab whereas PC-3 cells with the lowest EGFR expression were resistant to cetuximab. However, previous studies in colon, gastric and lung tumor models have shown that EGFR tumor expression was not correlated with tumor responses to EGFR therapy (10). Moreover, a recent study in prostate cancer also indicated that tumor response to the small molecular EGFR tyrosine kinase inhibitor erlotinib had an inverse correlation with the ratio of EGFR/Her2 rather than EGFR expression (11). These results suggest that EGFR expression might not be a reliable predicator of tumor response to EGFR targeted therapy.

To better understand the difference in tumor response to cetuximab, the effect of cetuximab treatment on EGFR

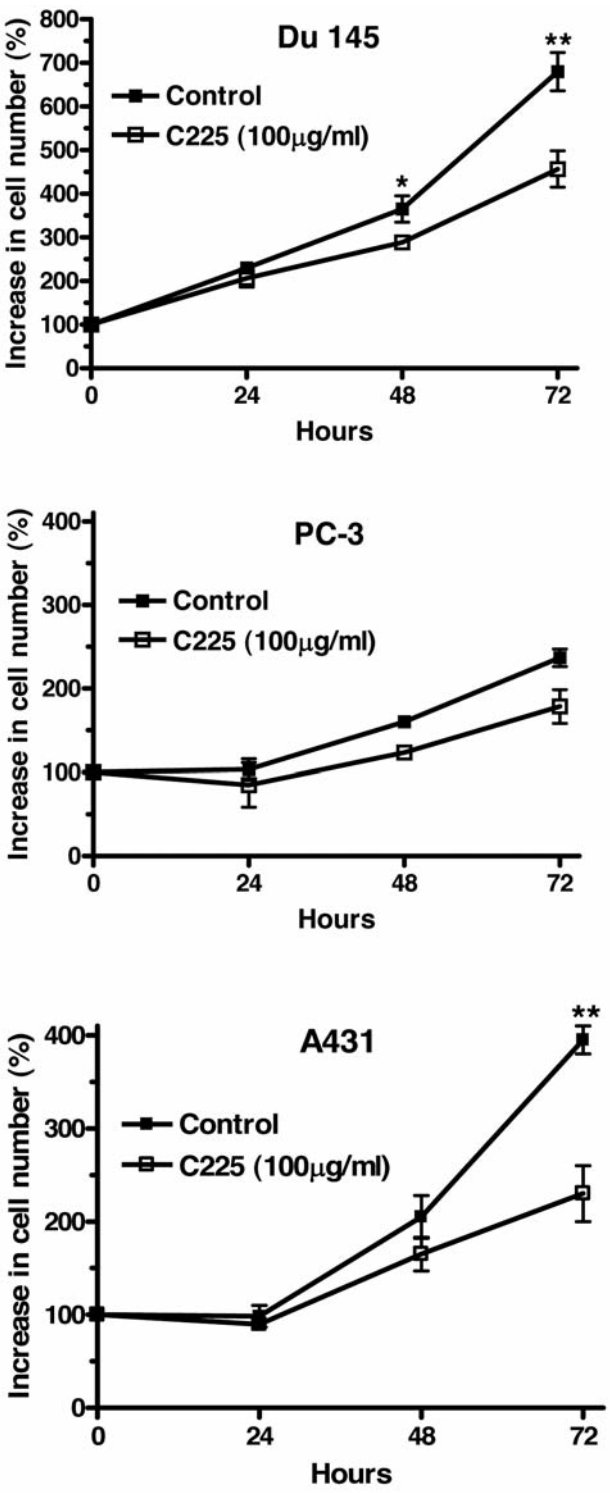

Figure 1. *Effects of cetuximab (C225) on tumor cell growth. Du145, PC-3 and A431 cells were incubated with 100 μg/ml cetuximab for up to 72h. Control cells received no treatment. Cell number was counted using trypan blue viability assay and normalized to the pretreatment value. The data shown are based on four independent experiments. Bars, SE. *p<0.05, **p<0.01, Compared to the control at the corresponding time points.*

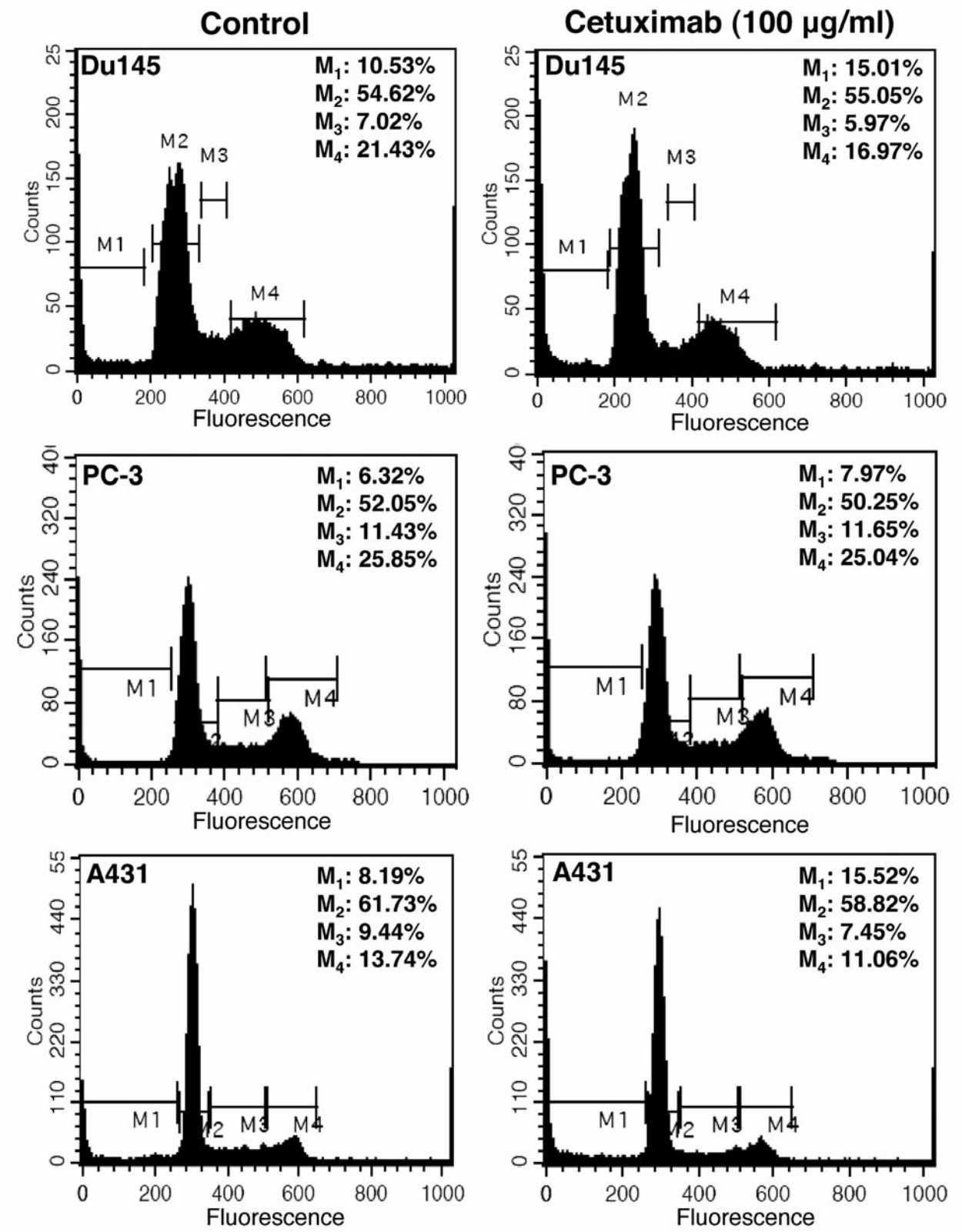

Figure 2. *Representative flow cytometry histograms showing the population of cells in M1 (Sub-G1), M2 (G_0/G_1), M3 (S) and M4 (G_2-M) phases. Du145, PC-3 and A431 cells were incubated with 100 µg/ml cetuximab (C225) for 72 h. Control cells received no treatment. At the end of treatment, cells were collected and prepared as described in the Materials and Methods. Cellular DNA content was analyzed with flow cytometry after staining.*

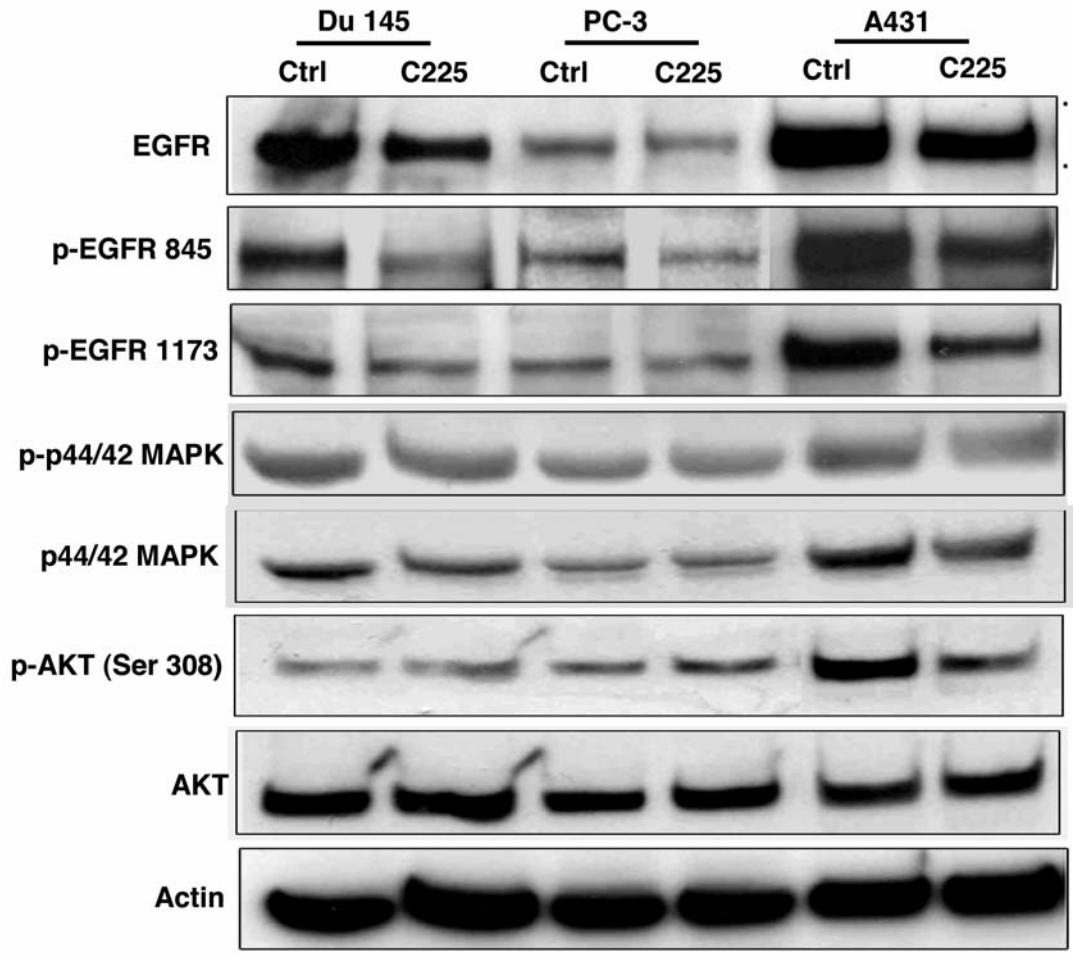

Figure 3. *Western blots showing the effects of certuximab (C225) on EGFR signaling. Du145, PC-3 and A431 cells were incubated with 100 μg/ml cetuximab (C225) for 72 h. Control cells received no treatment. At the end of treatment, cells were lysed and prepared as described in the Materials and Methods. Cell samples were resolved by SDS-PAGE.*

expression and phosphorylation was examined. It was found that cetuximab treatment induced down-regulation of total EGFR in all three cell lines (Figure 3), suggesting that cetuximab is able to bind to the EGFR and triggers receptor internalization. Since EGFR phosphorylation on specific tyrosine residues is essential for EGFR signal transduction and receptor regulation (12), EGFR phosphorylation at two different tyrosine sites Tyr-845 (Src-dependent phosphorylation site) and Tyr-1173 (Shc adaptor protein/phospholipase C binding site) was then examined. These data indicated that cetuximab inhibited the Src-mediated EGFR Tyr-845 phosphorylation in all three tumor cell lines and, in agreement with cell growth inhibition data, inhibition of EGFR Tyr-845 phosphorylation in Du145 cells was stronger than in PC-3 cells (Figure 3). Cetuximab also inhibited EGFR phosphorylation at Tyr-1173 site in Du145 and A431 cells. However, it failed to inhibit EGFR Tyr-1173 phosphorylation in PC-3 cells. These results strongly indicate that, although cetuximab can bind effectively to EGFR receptor on tumor cells, it may not be able to inhibit receptor phosphorylation effectively and block EGFR down stream signal transduction.

The effects of cetuximab on MAPK and PI3K pathways, two major downstream signaling pathways leading to cell survival following EGFR activation were then examined. Consistent with previous studies (13, 14), PC-3 cells were found to express a higher level of phosphorylated Akt than Du145 cells (Figure 3). This is in accordance with the observation that PC-3 cells are PTEN negative, which leads to a constitutive activation of PI3K pathway, whereas Du145 cells are PTEN positive (13). Results from this study clearly indicate that cetuximab treatment had little effect on MAPK 44/42 and Akt phosphorylation in PC-3 cells. A slight inhibition of MAPK 44/42, but not Akt phosphorylation, was observed in Du145 cells. However, inhibition of both MAPK 44/42 and Akt phosphorylation was observed in A431 cells.

These results confirmed that inhibition of EGFR phosphorylation (*e.g.* at Tyr-845) by cetuximab was not associated with downstream signaling inhibition in prostate cancer cells, which suggests that continued activation of downstream MAPK and PI3K pathways likely contributed to the resistance to cetuximab in hormone-dependent prostate cancer cells. Resistance to cetuximab and other EGFR inhibitors due to constitutive activation of Ras-mediated MAPK and PI3K pathways have been reported in different types of cancer including pancreatic, colorectal, lung and breast cancer cells (15). EGFR-independent PI3K activation might occur as a result of activating mutations of kinase itself, loss-of-function mutation of PTEN and Akt overexpression. Functional inactivation of PTEN due to genetic mutation exists in about 50% of prostate carcinomas, resulting in abnormal Akt activation and uncoupling of Akt activity from EGFR activation (as shown in the present study). Thus, it is not surprising to see that PTEN-deficient PC-3 cells are resistant to cetuximab (16) and other EGFR-targeted agents (14). Indeed, it has been shown recently that reintroduction of PTEN into PC-3 cells significantly increases cell response to cetuximab (17).

In summary, this study indicates that cetuximab exhibits a limited anticancer activity in prostate cancer cells with EGFR expression. Therapeutic resistance to this EGFR antibody is partially due to the continued activation of EGFR downstream signaling pathways in spite of EGFR receptor downregulation and inhibition of receptor autophosphorylation. Selective inhibition of these pro-survival signaling pathways will likely enhance the therapeutic response of cetuximab.

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