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Advances in Brief

Anti-Epidermal Growth Factor Receptor Monoclonal Antibody 225 Up-Regulates $p27^{KIP1}$ and Induces G_1 Arrest in Prostatic Cancer Cell Line DU145[1]

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Abstract

Autocrine production of transforming growth factor α and overexpression of the epidermal growth factor receptor (EGFR) may contribute to androgen-independent prostatic cancer growth at both primary and metastatic sites. Previously, we showed that human EGFR-blocking monoclonal antibody mAb225 inhibited the growth of DU145 human prostatic cancer cells. Here we explore the hypothesis that mAb225 may act by interfering with cell cycle traversal in these cells. Treatment with mAb225 induced G_1 arrest, which was accompanied by a marked decrease in CDK2-, cyclin A-, and cyclin E-associated histone H1 kinase activities, and a sustained increase in cell cycle inhibitor $p27^{KIP1}$. The increased $p27^{KIP1}$ levels were attributable to elevation of both transcription and translation. CDK2 associated with $p27^{KIP1}$ was increased in mAb225-treated DU145 cells. The retinoblastoma-related protein p130 remained hypophosphorylated in these retinoblastoma-negative cells. These studies demonstrate that the antiproliferative effect of EGFR blockade in DU145 cells may be mediated by up-regulation of $p27^{KIP1}$ at both the mRNA and protein levels.

Introduction

Prostatic cancer, the most prevalent malignancy in North American males, is the second leading cause of cancer death in men (1, 2). Androgen ablation can provide effective palliation. However, in the majority of cases the response is short lived, and proliferation resumes despite castrated levels of androgen. Although the mechanisms are multifactorial, functional autocrine and paracrine growth factor/growth factor receptor interactions are believed to be contributory.

Signaling mediated by receptor tyrosine kinases plays an essential role in the control of cell proliferation. In previous studies, EGF and TGF-α[4] and their receptor (EGFR) have been implicated in the regulation of prostatic cell mitogenesis (3). Human prostatic cancer cell lines derived from hormone-independent cancer express high levels of EGFRs (4, 5). Moreover, coexpression of EGFR and TGF-α has been demonstrated in advanced and metastatic prostatic cancer specimens examined by immunohistochemistry (6). This suggests that a functional autocrine loop may contribute to hormone-independent cancer growth and successful proliferation of prostate cancer at metastatic sites.

We have produced mAbs 225 and 528, which bind to EGFRs with high affinity, block the binding of EGF/TGF-α, and prevent activation of receptor tyrosine kinase (7–10). mAb225 significantly reduced phosphorylation of the EGFR in cultured nontransformed prostatic epithelial cells as well as human prostate carcinoma cell lines PC-3 and DU145, and it inhibited growth of these cells (4, 5).

The capacity of growth factors to regulate cell cycle progression has been characterized as an important aspect of their function. The cell cycle is controlled by the periodic activation of a family of CDKs, which are in turn controlled by interactions with other proteins, including the cyclins and the CDKIs (11). In this study, we define the mechanisms of inhibition of DU145 cell proliferation by EGFR blockade with mAb225. We provide evidence that mAb225 induces G_1 arrest, which is associated with the inhibition of CDK2 activity and induction of the CDKI $p27^{KIP1}$.

Materials and Methods

Cells, Cell Culture, and Cell Proliferation Assays. The DU145 human prostate adenocarcinoma cell line was purchased from the American Type Culture Collection (Bethesda, MD). Cells were grown as monolayers in DMEM with 10% fetal bovine serum. The cell proliferation assay was performed in six-well culture plates in the presence or absence of mAb225. For time points of longer than 3 days, medium was changed every 2 days with re-addition of the antibody. Cells were harvested by trypsinization and counted with a Coulter counter. For flow cytometric analysis of DNA content, cells were cultured with or without mAb225 for various periods and processed for fluorescence-activated cell-sorting analysis as reported previously (12).

CDK Kinase Assay. DU145 cells were treated with mAb225 for indicated time intervals, harvested and washed with PBS by centrifugation, and sonicated. CDK2-, cyclin A-, and cyclin E-associated kinase activities were measured by histone H1 kinase assays as described (12, 13). Briefly, sonicated cell lysates were immunoprecipitated with antibodies against CDK2, CDK4, CDK6, or cyclin A, E, or D (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Washed protein A-Sepharose beads (Repligen Corp., Cambridge, MA) containing immunoprecipitates were resuspended in kinase buffer and [γ-^{32}P]ATP (New England Nuclear) and histone H1 were added. The reactions were stopped after 30 min incubation and analyzed by SDS-PAGE followed by autoradiography. Rb kinase assays were performed on similar preparations as described previously (13), except that a glutathione *S*-transferase-Rb fusion protein (Santa Cruz Biotechnology, Inc.) was used as substrate in the current study.

Western and Northern Blot Analyses. Equal amounts of lysates were used for Western immunoblotting and immmunoprecipitation analyses with indicated antibodies as described (12, 13). Briefly, cells were lysed in NP40 lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% NP40, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and 25 μg/ml of leupetin and aprotinin) and sonicated at 4°C. Proteins from total cell extracts or from immunoprecipitates were separated by SDS-PAGE and blotted with specific antibodies (Santa Cruz Biotechnology, Inc.). For Northern blots, total cellular RNA was extracted by ultracentrifugation of cell lysates in guanidine thiocyanate over cesium chloride cushions. Hybridizations were performed as described (12, 13). The $p27^{KIP1}$ probe was a reverse transcription-PCR-generated human cDNA fragment using a primer pair flanking the whole $p27^{KIP1}$ coding region (primer 1, 5′- ATGTCAAACGTGCGAGTGTC-3′; primer 2, 5′-TTACGTTTGACGTCTTCTGAG-3′).

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[4] The abbreviations used are: EGF, epidermal growth factor; EGFR, EGF receptor; TGF-α, transforming growth factor α; mAb, monoclonal antibody; CDK, cyclin-dependent kinase; CDKI, CDK inhibitor; Rb, retinoblastoma.

Results and Discussion

Effects of mAb225 on DU145 Proliferation and Cell Cycle Traversal. Initial experiments defined the reduction in DU145 cell proliferation and change in cell cycle phase distribution induced by EGFR blockade with mAb225. As shown in Fig. 1*A*, DU145 proliferation was inhibited by mAb225 treatment. Flow cytometric (fluorescence-activated cell-sorting) analysis indicated that a G_1 arrest was induced by mAb225 in cultured DU145 cells after 24 h treatment (Fig. 1*B*). The increase in the G_1 population was accompanied by a decrease in cells in S phase, whereas the G_2-M population was essentially unchanged.

The Rb protein is one of the key regulators of the G_1-S phase transition (14). Rb and its related proteins, p107 and p130, can bind to various members of the E2F-transcription factor family, preventing their activity. Hyperphosphorylation of Rb, p107, and p130 by CDKs dissociates these proteins from E2F (15). The released E2F may mediate cell cycle progression by activating the genes required for the S phase. Interestingly, DU145 has been demonstrated to be an Rb-deficient cell line (16). Therefore, we investigated the other Rb-related proteins, p107 and p130 (17). As shown in Fig. 1*C*, Western blot analysis indicated that mAb225-treated cells accumulated increased amounts of the hypophosphorylated form of p130, whereas p107 did not show a significant change (data not shown). This suggests that in these Rb-deficient cells, Rb-related protein p130 may be used to regulate cell cycle traversal. Indeed, recent data suggested that phosphorylation of p130 is controlled by the cell cycle machinery and that it may be another key G_1-S phase regulator (18).

Inhibition of CDK2 Activity Is Associated with mAb225-induced Cell Cycle Arrest. Cell cycle traversal is controlled by an interacting set of proteins, including cyclins, CDKs, and CDKIs (11). The transition from G_1 to S phase is thought to be regulated by CDK4 or CDK6 complexed with cyclin D and by CDK2 complexed with cyclins E or A (15). We analyzed the expression and functional status of these molecules in DU145 cells culture with mAb225. CDK2 kinase activity, as measured in CDK2 immunoprecipitates using histone H1 as substrate, was decreased as result of mAb225 treatment of DU145 cells (Fig. 2*A*). The cyclin A- and cyclin E-associated kinase activities also showed a parallel decrease (Fig. 2*A*). These altered activities remained low for at least 48 h (data not shown). In contrast, CDK4- and CDK6-associated kinase and cyclin D1 kinase activities measured using Rb as substrate were not altered significantly after mAb225 treatment (Fig. 2*B*). Except for a slight decrease in D1 protein, mAb225 treatment did not significantly change the amounts of any of these proteins (Fig. 2*C*).

Inhibition of CDK2 Is Associated with Up Regulation of CDKI, $p27^{KIP1}$. The decrease in CDK2-associated kinase activity could not be explained by a change in the levels of CDK2 protein or its cyclin partners. This suggested that mAb225 action on DU145 may activate an additional factor that is responsible for inhibition of CDK2 kinase activity. We therefore investigated whether mAb225 might regulate the levels of expression of the specific inhibitors of CDK2 activity. There are two classes of CDKI that can inhibit CDK activity: the KIP/CIP family, consisting of $p21^{CIP/WAF1}$, $p27^{KIP1}$, and $p57^{KIP2}$, and the INK family consisting of $p15^{INK4B}$, $p16^{INK}$, $p18^{INK4C}$, and $p19^{INK4D}$ (13). Western blot analysis of CDKIs showed that $p27^{KIP1}$ increased after mAb225 treatment, whereas $p21^{CIP/WAF1}$, $p15^{INK4B}$, and $p19^{INK4D}$ proteins did not change significantly (Fig. 3*A* and data not shown). Next, we analyzed expression of $p27^{KIP1}$ and $p21^{CIP/WAF1}$ at the mRNA level. Northern blot analysis showed that $p27^{KIP1}$ mRNA displayed a moderate increase after addition of mAb225, which could be detected as early as 2 h after treatment (Fig. 3*B*). The $p21^{CIP/WAF1}$ mRNA levels did not change after mAb225 treatment (data not shown). Recent data suggest that translational control is primarily responsible for the regulation of $p27^{KIP1}$ protein levels under various conditions (19). It also is reported that $p27^{KIP1}$ can be regulated through ubiquitin-dependent degradation in serum-starved fibroblasts (20). Our results demonstrate that $p27^{KIP1}$ mRNA can be up regulated by the blockade of EGF/EGFR signal transduction pathways. The fact that the increase of $p27^{KIP1}$ mRNA preceded the increase in $p27^{KIP1}$ protein implies that the up-regulation of mRNA can be at least partially responsible for the increase in $p27^{KIP1}$ protein. Thus, the expression of $p27^{KIP1}$ protein in DU145 cells may be

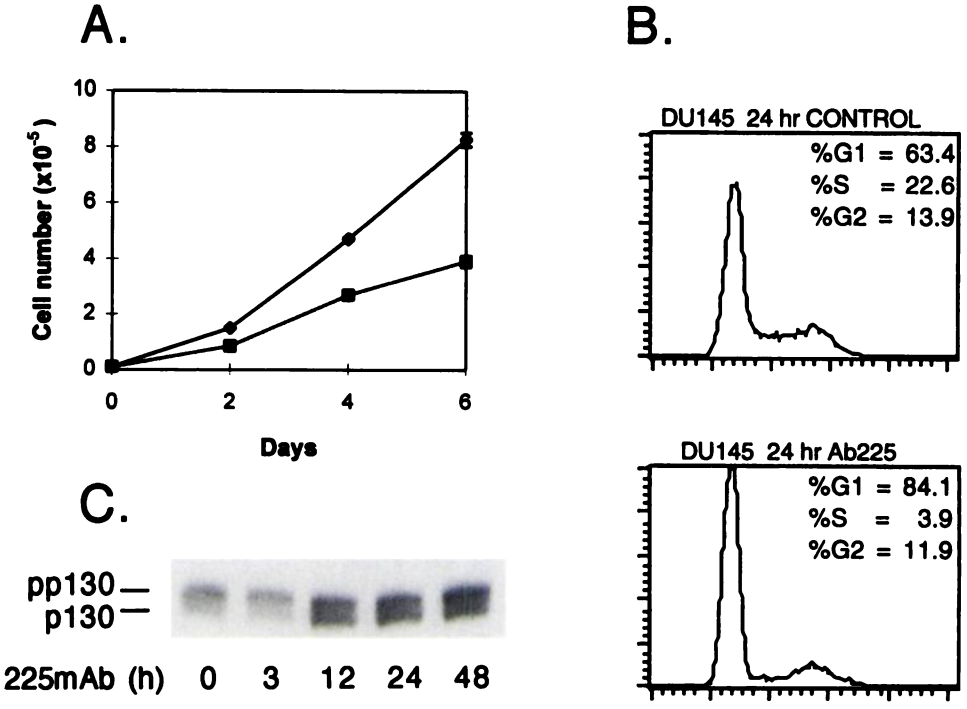

Fig. 1. Cell proliferation, cell cycle distribution and p130 phosphorylation of DU145 cells treated with mAb225. *A*, DU145 cells (1×10^4 cells per well) were grown in six-well culture plates in the presence and absence of 40 nM mAb225. The medium and mAb225 were replaced every two days. Cell numbers were counted with a Coulter Counter at indicated time intervals after treatment. ◆, controls; ■, mAb225 treated. *B*, exponentially growing DU145 cells were treated with mAb225 for 24 hours, and harvested cells were stained with propidium iodide and analyzed for DNA content by flow cytometric analysis. *C*, effect of mAb225 on phosphorylation of the p130 protein. Whole cell lysates from DU145 cells and cells treated with mAb225 for varying time intervals were subjected to electrophoresis for Western blot analysis with an anti-p130 antibody.

regulated at the more usual translational/posttranslational level, as well as the mRNA level (Fig. 3, *A* and *B*).

To further investigate whether the up-regulation of p27^{KIP1} contributes to the observed inhibition of CDK2 but not CDK4 and CDK6 activities, we analyzed the physical association of p27^{KIP1} with CDK complexes. Cell lysates of control and mAb225-treated cells were immunoprecipitated with a p27^{KIP1} antibody, and Western blot assays were performed with antibodies against p27^{KIP1}, CDK2, and CDK4. As shown in Fig. 3*C*, there was a significant increase of p27^{KIP1}-associated CDK2, whereas CDK4 associated with p27^{KIP1} did not change after mAb225 treatment, nor did p27^{KIP1}-associated CDK6 (data not shown).

In summary, we have demonstrated that EGFR-blocking antibody mAb225 inhibits proliferation of androgen-independent DU145 prostatic cancer cells by arresting cell cycle progression in G_1. This is mediated by inhibition of CDK2 activity, which is attributable to p27^{KIP1} up-regulation. These changes can explain the marked increase in the amount of hypophosphorylated p130 that we observed in these Rb-negative cells. It is worth noting that in the DiFi human colon adenocarcinoma cell line, which also expresses high levels of EGFR, mAb225 treatment also induced p27^{KIP1} up-regulation and Rb hypophosphorylation with G_1 arrest, followed by apoptosis (13). The DU145 cell line differs from DiFi cells in that it is Rb deficient and the G_1 arrest induced by mAb225 is not followed by apoptosis. Furthermore, the up regulation of p27^{KIP1} mRNA in DU145 cells by treatment with mAb225 is unique. These differences demonstrate the complexity involved in the regulation of cell cycle progression. Further study will attempt to ascertain whether p27^{KIP1} is necessary and sufficient for the G_1 arrest induced by blockade of EGFR-mediated signal transduction pathways with mAb225.

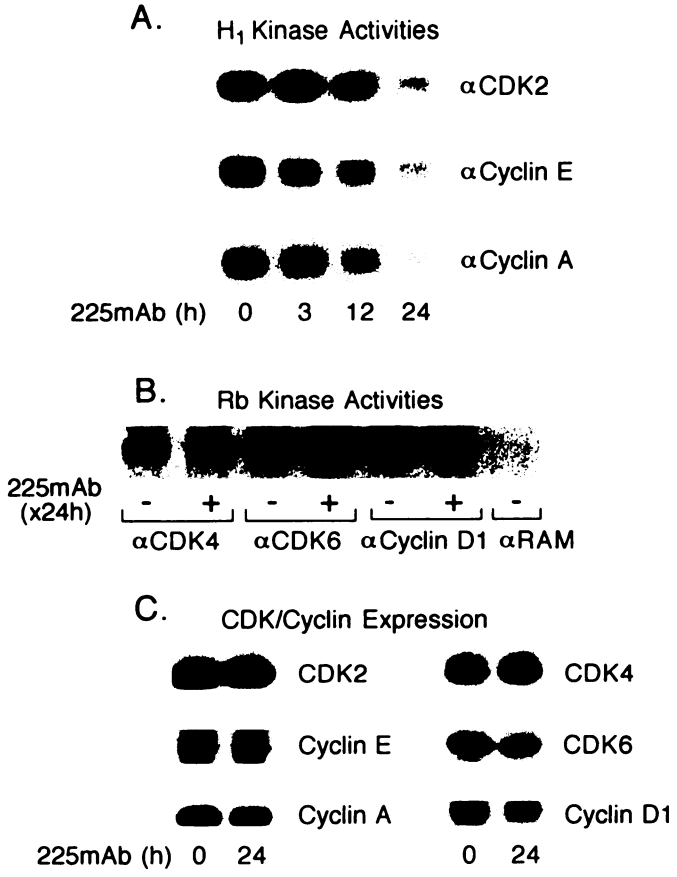

Fig. 2. Effects of mAb225 on G_1 cell cycle CDK kinases. *A*, inhibition of CDK2-, cyclin A-, and cyclin E-associated histone H1 kinase activities with mAb225 treatment. Immunoprecipitates using antibodies against CDK2, cyclin A, or cyclin E were prepared from whole-cell lysates of control and mAb225-treated DU145 cells. Histone H1 kinase assays were carried out with these immunoprecipitates, and histone H1 protein was separated by SDS-PAGE. *B*, effect of mAb225 on *in vitro* CDK4-, CDK6-, and cyclin D1-associated Rb kinase activities. Assays were carried out as in *A*, except that different antibodies were used to immunoprecipitate and a recombinant glutathione *S*-transferase-Rb protein was used as substrate. *C*, effects of mAb225 treatment on the expression of cyclins A, E, and D1 and CDK2, CDK4, and CDK6 proteins. Whole-cell lysates of control and mAb225-treated DU145 cells were separated by SDS-PAGE, and Western blots were probed with antibodies to indicated proteins.

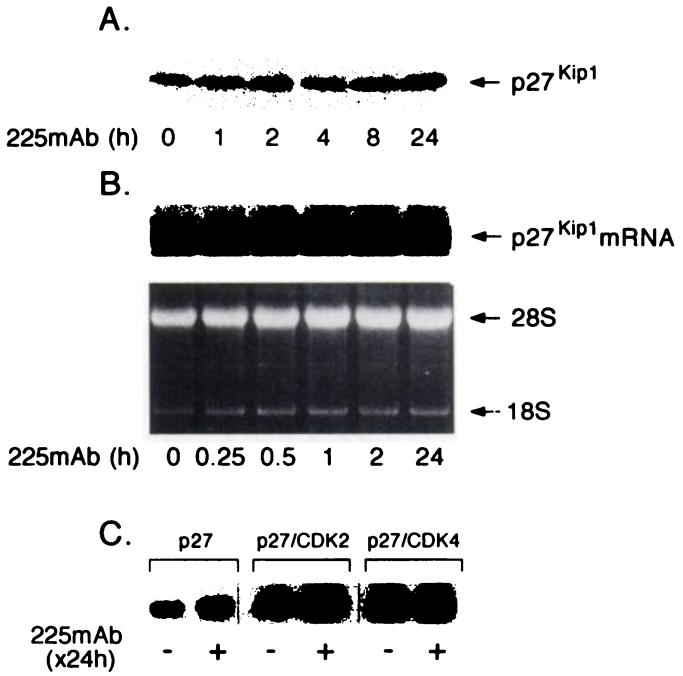

Fig. 3. Induction of p27^{KIP1} protein and mRNA expression by treatment with mAb225. *A*, equal amounts of protein from lysates of control cells and mAb225-treated cells were separated by SDS-PAGE and transferred to filter membranes. Specific antibody was used to detect p27^{KIP1}. *B*, samples containing 10 μg total RNA from control and mAb225-treated cells were separated on formaldehyde-agarose gels. RNA was transferred to nitrocellulose filter membranes and probed with a p27^{KIP1} probe (see "Materials and Methods"). *C*, effects of mAb225 on association of p27^{KIP1} with CDK2 and CDK4. Equal amounts of lysates from control and mAb225-treated cells were immunoprecipitated with an anti-p27^{KIP1} antibody. These were then separated by SDS-PAGE and immunoblotted with antibodies against p27^{KIP1}, CDK2, or CDK4.

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