Anti-Epidermal Growth Factor Receptor Monoclonal Antibody 225 Up-Regulates p27KIP1 and Induces G1 Arrest in Prostatic Cancer Cell Line DU145

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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 G1 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 p27KIP1. The increased p27KIP1 levels were attributable to elevation of both transcription and translation. CDK2 associated with p27KIP1 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 p27KIP1 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, autonomous 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, EGFR and TGF-α2 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 G1 arrest, which is associated with the inhibition of CDK2 activity and induction of the CDKI p27KIP1.

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-32P]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 immunoprecipitation 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 leupeptin 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 p27KIP1 probe was a reverse transcription-PCR-generated human CDNA fragment using a primer pair flanking the whole p27KIP1 coding region (primer 1, 5'-ATGTCAAACGTGCGAGTGTC-3'; primer 2, 5'-TTACGTTTGACGTCTTCTGAG-3').

Received 5/23/96; accepted 7/15/96.

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This study was supported in part by grants from CaPUCURE and the Pepsi Co. Foundation. J. M. was supported by NIH Grants CA42060 and CA37641. H. S. was supported by NIH Grant CA05826.

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* The abbreviations used are: EGFR, epidermal growth factor; EGFR, EGFR 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. 1A, DU145 proliferation was inhibited by mAb225 treatment. Flow cytometric fluorescence-activated cell-sorting analysis indicated that a G₁ arrest was induced by mAb225 in cultured DU145 cells after 24 h treatment (Fig. 1B). The increase in the G₁ population was accompanied by a decrease in cells in S phase, whereas the G₂-M population was essentially unchanged.

The Rb protein is one of the key regulators of the G₁-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. 1C, 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 G₁-S phase regulator (18).

Inhibition of CDK2 Activity Is Associated with Up Regulation of CDKI, p27KIP₁. 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 p21CIP/WAF1, p27KIP₁, and p57KIP₂, and the INK family consisting of p15INK4B, p16INK4, p18INK4C, and p19INK4D (13). Western blot analysis of CDKs showed that p27KIP₁ increased after mAb225 treatment, whereas p21CIP/WAF1, p15INK4B, and p19INK4D proteins did not change significantly (Fig. 3A and data not shown). Next, we analyzed expression of p27KIP₁ and p21CIP/WAF1 at the mRNA level. Northern blot analysis showed that p27KIP₁ mRNA displayed a moderate increase after addition of mAb225, which could be detected as early as 2 h after treatment (Fig. 3B). The p21CIP/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 p27KIP₁ protein levels under various conditions (19). It also is reported that p27KIP₁ can be regulated through ubiquitin-dependent degradation in serum-starved fibroblasts (20). Our results demonstrate that p27KIP₁ mRNA can be up regulated by the blockade of EGFR signal transduction pathways. The fact that the increase of p27KIP₁ mRNA preceded the increase in p27KIP₁ protein implies that the up-regulation of mRNA can be at least partially responsible for the increase in p27KIP₁ protein. Thus, the expression of p27KIP₁ protein in DU145 cells may be...
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 p27KIP1 contributes to the observed inhibition of CDK2 but not CDK4 and CDK6 activities, we analyzed the physical association of p27KIP1 with CDK complexes. Cell lysates of control and mAb225-treated cells were immunoprecipitated with a p27KIP1 antibody, and Western blot assays were performed with antibodies against p27KIP1, CDK2, and CDK4. As shown in Fig. 3C, there was a significant increase of p27KIP1-associated CDK2, whereas CDK4-associated p27KIP1 did not change after mAb225 treatment, nor did p27KIP1-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 G1. This is mediated by inhibition of CDK2 activity, which is attributable to p27KIP1 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 p27KIP1 up-regulation and Rb hypophosphorylation with G1 arrest, followed by apoptosis (13). The

DU145 cell line differs from DiFi cells in that it is Rb deficient and the G1 arrest induced by mAb225 is not followed by apoptosis. Furthermore, the up-regulation of p27KIP1 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 p27KIP1 is necessary and sufficient for the G1 arrest induced by blockade of EGFR-mediated signal transduction pathways with mAb225.

References
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