Requirement for the von Hippel-Lindau Tumor Suppressor Gene for Functional Epidermal Growth Factor Receptor Blockade by Monoclonal Antibody C225 in Renal Cell Carcinoma

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ABSTRACT

Renal cell carcinoma (RCC) is a cytologically and histologically diverse disease in which a spectrum of distinct molecular alterations occurs, including the inactivation of the von Hippel-Lindau (VHL) tumor suppressor gene, which is specific for the clear cell variant of RCC. The prognosis for RCC is poor, and, to date, no effective systemic treatment is available for this cancer. In the present study, we assessed the extent to which the transforming growth factor α-epidermal growth factor receptor (EGFR) autocrine loop could be used as a potential therapeutic target for RCC. Northern blot analysis of transforming growth factor α and EGFR revealed variable but consistent expression of these transcripts in cell lines derived from both clear cell and non-clear cell RCC variants, indicating the potential for this autocrine loop in both tumor types. The therapeutic utility of interruption of this feedback loop was determined by examining growth inhibition after the exposure of these cell lines to a humanized anti-EGFR monoclonal antibody, C225. In vitro treatment of clear cell RCC-derived cell lines lacking VHL resulted in only a modest decrease in growth rate. In contrast, non-clear cell RCC-derived cell lines that retained VHL responded significantly to C225 treatment. Transfection of VHL into VHL-negative RCC cell lines restored responsiveness to C225, indicating that this tumor suppressor gene is required for effective EGFR blockade. Growth inhibition by C225 in VHL-positive cells was linked to a requirement for VHL to up-regulate p27 in response to C225. These data provide compelling evidence that treatment modalities for RCC are likely to be strongly influenced by the molecular etiology of this phenotypically diverse cancer.

INTRODUCTION

RCC is a phenotypically heterogeneous neoplasm whose early detection is hindered by the absence of early clinical manifestations. Subsequent therapy of RCC is impaired by both a high incidence of metastasis at the time of presentation (1) and resistance to conventional cytotoxic treatment regimens (2, 3). Increased presentation of this cancer (a 38% increase was observed between 1974 and 1990) is thought to be linked to an increase in smoking, obesity, high-protein diet, and hypertension and, to a lesser degree, to an earlier diagnosis (4). Diverse histological and cytological variants are a hallmark of RCC, and distinct molecular etiologies are thought to underlie the different phenotypes observed in this disease. The most common type of RCC, solid RCC of the clear cell type, is thought to arise as a result of loss of function of the VHL tumor suppressor gene (5–7). Other RCC variants, which can be collectively classified as non-clear cell RCC, have various genetic alterations (8–10) but no VHL defects (6, 7). In light of these differences, it is not unreasonable to propose that the outcome of treatment modalities for this disease may be strongly influenced by the molecular etiology of a particular RCC variant. The recent demonstration in a retrospective study that effectiveness of RCC treatment with cytokines correlated with tumor histology (11) provides support for this hypothesis.

In RCC, as in many other cancers, the TGF-α-EGFR autocrine loop is regulated aberrantly as compared to epithelial cells of the proximal tubule of the renal cortex, from which RCC originates (12–14). TGF-α is a potent mitogen, and its interaction with the EGFR is thought to play a significant role in neoplastic transformation and the subsequent deregulated cell growth observed during tumorigenesis. Interruption of the TGF-α-EGFR loop may therefore provide a potential therapeutic for the treatment of RCC. In the present study, we explored this possibility by using a humanized monoclonal antibody against the EGFR, C225, on cell lines derived from both clear and non-clear cell RCC. This antibody has been shown to be effective in suppressing the growth and, in some cases, inducing apoptosis (15) in cancer cell lines of epithelioid origin (16–18) and in RCC xenografts in nude mice (16, 19). In the present study, we report the anti-proliferative effect of C225 on RCC-derived cell lines and provide evidence that the molecular etiology of the RCC variant may have a strong influence on the efficacy of this treatment. Specifically, RCC variants retaining VHL exhibited a significantly greater growth inhibition upon C225 treatment as compared with RCC cells that harbor VHL.

The abbreviations used are: RCC, renal cell carcinoma; EGFR, epidermal growth factor receptor; TGF-α, transforming growth factor α; VHL, von Hippel-Lindau; FBS, fetal bovine serum.
deleted. Cell line 101wt expresses the full-length VHL protein. Cell lines 112 and 124 were derived from papillary tumors and have been previously shown to contain wild-type VHL (22). Cell lines ACHN, CAKI-1, and A498 were purchased from the American Type Culture Collection (Manassas, VA). Nontransfected cells were cultured in DMEM or MEM containing 10% FBS. Cell lines transfected with either wild-type or mutant VHL were grown in DMEM with 10% FBS and 800 µg/ml G418. To confirm VHL status in the transfected cell lines used in the study, total RNA was obtained by conventional CsCl centrifugation, and reverse transcription-PCR was performed with a forward primer located in exon 1 (22–5’TCTTTCTTGCAATCGCAGTCCGCGCTGTT-3’) and a reverse primer located in the vector (5’-ACTTTCCACACCCTAACTGACACATCTTCCAGTCCGCGCTGTT-3’). As expected, PCR using primers located in exon 1 and the vector did not result in VHL cDNA amplification in cell line 101. In contrast, cell lines bearing VHL constructs had reverse transcription-PCR products of the expected size of 501 bp for the wild-type constructs and 333 bp for the mutant VHL constructs (data not shown).

RNA Isolation and Northern Analysis. Total RNA was isolated from cells in log phase by conventional centrifugation in CsCl, and poly(A)+ RNA was obtained by affinity chromatography. Five µg of poly(A)+ RNA from each cell line were used for Northern blot analysis using the standard protocol. Blots were hybridized with 32P-labeled probes for TGF-α (24) and EGFR (25).

Proliferation Assays. Four RCC cell lines with mutant VHL (VHL−) and four RCC cell lines with wild-type VHL (VHL+) were plated in triplicate into 24-well dishes. Twenty-four h before exposure to C225, cells were switched to low-serum growth conditions (0.5% FBS in growth medium). Cells were treated with 80 or 160 nM C225 or vehicle, and the number of cells in each well at different time points was determined using a Coulter counter. For some cell lines, the experiment was repeated three times. Statistical analysis was performed using StatView (Abacus Concepts, Berkeley, CA). Percentage of inhibition at each time point was determined as a ratio of a mean number of cells treated with C225:vehicle-treated cells.

Analysis of Cell Cycle and p27 Expression in Response to C225. Isogenic cell lines 786-0/157Δ and 786-0/g7f were grown in media containing 10% FBS. When cells were at 20–40% confluence, they were switched to the low-serum conditions (0.5% FBS in growth media) for 24 h. Cells were then exposed to 160 nM C225 and harvested at 24 and 48 h for either protein or DNA content analysis. For protein analysis, total cell lysates were obtained by resuspending cells in PBS containing 0.1% SDS, 0.01% NP40, 4 mg/ml aprotinin, 0.1 mM phenylmethylsulfonyl fluoride, 25 mg/ml soybean trypsin inhibitor, and 50 mM leupeptin. Cell lysates with an equal amount of total protein were electrophoresed on 4–20% Tris-glycine gradient gels (Novex, San Diego, CA) and transferred to polyvinylidene difluoride membranes (Pierce, Rockford, IL). Blots were probed with a p27 monoclonal antibody, and a protein detector Western blot kit, LumiGLO System (Kirkgaard and Perry Laboratories, Gaithersburg, MD), was used to visualize the bands. For DNA content analysis, cells were trypsinized, washed twice in ice-cold PBS, and resuspended in 200 µl of PBS. Cells were fixed by slowly adding the cell suspension drop-wise to 4 ml of

**Materials and Methods**

**Reagents.** The humanized anti-EGFR monoclonal antibody C225 has been described previously (20, 21) and was kindly provided by Imclone Systems (New York, NY). The p27 monoclonal antibody used for Western blot analysis was obtained from Transduction Laboratories (Lexington, KY).

**Tumor Cell Lines and Culture Conditions.** A panel of cell lines derived from clear cell and non-clear cell RCCs that have previously been characterized for VHL mutations (22) was used. Cell lines 112, 124, 786-0, and 101 and their derivatives stably transfected with either wild-type or mutant VHL constructs (23) were a gift from Dr. J. Gnarra (Louisiana State University Medical Center, New Orleans, LA). Cell line 786-0/g7f expresses a FLAG-tagged full-length VHL protein (tagged at the COOH terminus), and cell line 786-0/157Δ expresses VHL amino acids 1–157, with amino acids 158–213 being

**Fig. 1** Northern blot analysis of EGFR and TGF-α RNA expression in RCC cell lines. A major 10.3-kb EGFR transcript was detected in both clear cell (VHL−) and non-clear cell (VHL+) RCC cell lines. The slight difference in the band size is an artifact resulting from differences in electrophoretic mobility in different experiments. Normalization to glyceraldehyde-3-phosphate dehydrogenase revealed variable expression of both EGFR and TGF-α in different RCC histological variants.
ice-cold 70% ethanol with gentle agitation and incubated on ice for 30 min. The suspension was then centrifuged, and cells were resuspended in 1 ml of PBS containing 40 μg/ml propidium iodide and 100 μg/ml RNase A and incubated at 37°C for 30 min. DNA content analysis was performed on a Coulter EPICS Elite flow cytometer, and data were analyzed using MultiCycle DNA cell cycle analysis software (Phoenix Flow Systems, San Diego, CA). Aggregate discrimination mode was used to analyze single cells only.

RESULTS

Expression of TGF-α and EGFR mRNA. A panel of RCC cell lines derived from clear cell (mutant VHL) and non-clear cell (wild-type VHL) tumors was examined for expression of mRNA transcripts for TGF-α and EGFR to establish the potential for blockade of this autocrine loop in different RCC variants. The three expected RNA transcripts of EGFR were detected in all cell lines, with the 10.3-kb transcript being the most abundant (Fig. 1). Although expression was variable, TGF-α RNA transcripts were also detected in all of the cell lines examined, indicating that the TGF-α-EGFR autocrine loop was present in both VHL+ and VHL− RCC cell lines and was not a hallmark of any specific histological variant.

Effect of C225 on Cell Proliferation. The panel of RCC-derived cell lines, including VHL− and VHL+ isogenic cell lines, was examined for growth inhibition by C225. During log phase, maximum growth inhibition of all cell lines was observed at day 4 after C225 treatment. A summary of growth inhibition at this time point for all cell lines is shown in Table 1. Statistical analysis of inhibition data revealed that the anti-proliferative effect of C225 on VHL− cell lines was, in most instances, only marginal, with an average maximal growth suppression of 14.58 ± 6.22%. In contrast, cell lines with wild-type VHL had, on average, a 42.25 ± 1.88% inhibition of growth, which was significantly greater by unpaired Student’s t-test (P < 0.02) compared with cell lines with mutant VHL.

Fig. 2 VHL impact on growth kinetics of RCC cell lines treated with C225. VHL− cell lines 101 and 786-0/157Δ and isogenic matched VHL+ cell lines 101wt and 786-0/g7f were treated with 160 nM C225, and growth kinetics were obtained. ○ and ●, mean number of untreated and treated cells, respectively, determined for three wells at each time point.
The specific contribution of VHL in mediating the effect of the C225 antibody on cell growth was demonstrated in two clear cell RCC-derived cell lines, 786-0 and 101, which have mutant VHL. In both cell lines, the introduction of wild-type VHL dramatically changed the profile of growth inhibition, conferring responsiveness to C225 on VHL+ cells (Fig. 2). Interestingly, a statistically significant growth inhibition effect of C225 became evident much earlier in VHL+ cells (days 3 and 2 for 101wt and 786-0/g7f, respectively) than in VHL− cells (day 5 for both 101 and 786-0/157Δ).

**Effect of C225 on Cell Cycle and p27 Expression.** To determine whether inhibition of growth of VHL+ cells by C225 affects the progression of the cell cycle, DNA content was determined in 786-0/g7f cells at various time points after the treatment. After 24 h of exposure to this antibody, DNA content analysis demonstrated that the percentage of treated cells in S phase had decreased from 29.5% to 13.8%, with a commensurate increase in the G1 fraction from 68% to 85%, suggesting that C225 treatment had induced a G1 arrest in these cells (Fig. 3). The same distribution of cells within the cell cycle phases was observed at 48 and 96 h after the treatment. Interestingly, the sub-2n fraction of cells also increased in the C225-treated cells, suggesting a possible induction of apoptosis in RCC by C225 (data not shown).

The VHL gene product has been previously shown to regulate response to growth factor deprivation, and this activity has been attributed to the ability of VHL to regulate p27 levels (26). Up-regulation of p27 in tumors other than RCC in response to C225 has been previously demonstrated (17, 18); therefore, we examined the involvement of p27 in growth inhibition by C225 in RCC. Western blot analysis of p27 revealed that exposure of the VHL− cell line 786-0/157Δ to C225 did not change the level of expression of this protein (Fig. 4). In contrast, treatment of the isogenic matched cell line 786-0/g7f (transfected with the wild-type VHL) with C225 increased the p27 level after 24 h of exposure (Fig. 4).

**DISCUSSION**

The relative ineffectiveness of traditional systemic chemotherapy modalities for RCC clearly warrants the exploration of novel approaches to the treatment of this disease. In the present study, we report the effectiveness of the humanized anti-EGFR monoclonal antibody C225 in suppressing the growth of a panel RCC cell lines derived from clear cell and non-clear cell tumors. Although the growth-inhibitory action of C225 on RCC cell lines has been reported previously (15, 16, 27), possible differences in response between RCC tumor variants to this potential therapeutic agent have not been explored. Consistent with the previously reported TGF-α-EGFR autocrine loop in RCC (13, 27, 28), our Northern blot analysis detected the expression of both EGFR and TGF-α RNA transcripts in cell lines derived from different RCC histological variants, namely, clear and non-clear cell RCCs. We then investigated whether the response to C225 was affected by the molecular etiology of the RCC cell lines and hence the tumor type. The exposure of a panel of cell lines to C225 revealed a distinct dichotomy in response to this agent. Non-clear cell RCC cell lines with the wild-type VHL responded robustly to C225, whereas the cell lines that originated from clear cell variants of RCC and had mutations in the VHL gene showed a generally weaker response to this antibody. How the level of expression of either TGF-α or EGFR influences the response of RCC cells to C225 is not clear at present and requires further investigation. However, the TGF-α-EGFR autocrine loop appears to be in place in both histolog-
RCC variants studied, suggesting that, in addition to the autocrine loop, other molecular events impact the C225 inhibitory effect on RCC.

RCC of the clear cell variant is generally thought to result from VHL inactivation (5–7), whereas this gene is unaltered in non-clear cell variants (6, 7). The different response observed in VHL- and VHL+ RCC variants in the present study suggested that the VHL gene in RCC could mediate the inhibitory effects of C225. Direct evidence that the presence of wild-type VHL accounted for this difference was obtained for clear cell RCC cell lines 101 and 786-0, which had been shown to be poor responders to C225 (Table 1). These cell lines, after transfection with wild-type VHL, have shown a dramatic transition from a relatively weak response to a robust response to C225 treatment (Table 1; Fig. 2). Thus our data indicate that VHL function may be required for the therapeutic action of C225 in the treatment of RCC.

How VHL mediates the downstream events of EGFR blockade by C225 is not clear at present. The function or probable functions of VHL are currently being elucidated (29). To date, the protein product of this tumor suppressor gene is thought to be involved in a number of pathways including interaction with the elongin BC complex (30, 31), protein degradation (32), and negative regulation of hypoxia-inducible genes such as VEGF via interaction with Sp1 (33) or via posttranscriptional mechanisms (23). In the context of the present experiments, the role of VHL interaction with elongin BC in protein degradation is of special interest. Pause et al. (26) demonstrated that VHL function was necessary for RCC cell line 786-0 to up-regulate p27 and exit the cell cycle in response to serum starvation. This, as suggested by the authors, may result from the inability of mutant VHL to inhibit Hs-Cul-2-mediated p27 degradation. Consistent with this hypothesis, we found that the extent of C225 inhibition of cell growth and p27 up-regulation in RCC is significantly dependent on VHL function. The relative lack of response of the clear cell-derived RCC cell lines to C225 appears to be due to their inability to increase p27 levels. Our findings that VHL– RCC cell lines were unable to augment p27 in response to C225 and that the reintroduction of wild-type VHL can overcome this defect clearly point to a requirement for VHL function in p27 up-regulation and growth inhibition in response to C225. Interestingly, it has been shown for other tumors, which are not associated with alterations at the VHL locus, that the inhibition of cell growth by C225 is also mediated by the up-regulation of p27 (17, 34).

In summary, the present experiments show that blockade of the EGFR by the monoclonal antibody C225 may provide a potential therapeutic avenue for the treatment of RCC. However, the dramatic difference in response between different RCC variants clearly indicates that the molecular etiology of these tumors may have a critical impact on the utility of this and possibly other potential therapeutic agents in the treatment of this disease.

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