Epidermal Growth Factor Receptor-targeted Therapy with C225 and Cisplatin in Patients with Head and Neck Cancer

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ABSTRACT

C225, a human-mouse chimerized monoclonal antibody directed against the epidermal growth factor receptor (EGFr), has a synergistic effect with cisplatin in xenograft models. To determine the tumor EGFr saturation dose with C225 and the fate of infused C225, we conducted a Phase Ib study with C225 in combination with cisplatin in patients with recurrent squamous cell carcinoma of the head and neck. Using tumor samples, we assessed tumor EGFr saturation by antibody using immunohistochemistry studies, the EGFr tyrosine kinase assay, and detection of the EGFr/C225 complex formation by immunoblot. Potential candidates were screened for EGFr expression in their tumors, and 12 patients who had high levels of EGFr expression and tumors easily accessible for repeated biopsies (pretherapy, 24 h after first C225 infusion, 24 h before third C225 infusion) were entered at three different dose levels of C225 with a fixed dose of cisplatin. The median value of tumor EGFr saturation increased to 95% at the higher dose levels. EGFr tyrosine kinase activity was significantly reduced after C225 infusion, and EGFr/C225 complexes were also detected at higher doses of C225. The loading dose of C225 at 400 mg/m² with a maintenance dose at 250 mg/m² achieved a high percentage of saturation of EGFr in tumor tissue, and these doses were recommended for Phases II or III clinical trials. Six (67%) of nine evaluable patients achieved major responses, including two (22%) complete responses. Mild to moderate degrees of allergic reaction and folliculitis-like skin reactions were demonstrated. We conclude that infused C225 binds and significantly saturates tumor EGFr, which may render a high degree of antitumor activity, and provides a novel mechanism for targeting cancer therapy for patients who have EGFr expression in their tumors.

INTRODUCTION

EGFr⁴ is a 170,000-kDa transmembrane glycoprotein found primarily on cells of epithelial origin (1–3). EGFr represents one of the most important growth-regulatory signal-transduction molecules and exerts this function mainly through its intrinsic tyrosine kinase activity, which can be activated upon ligand binding (4–7). EGFr frequently is overexpressed in breast cancer, ovarian cancer, prostate cancer, bladder cancer, glioblastoma, non-small cell lung cancer, and head and neck cancer and has also been found to play a significant role in the progression of several human malignancies. EGFr positivity may also be an indicator of poor prognosis (8–12).

Earlier studies suggested that tumors with high EGFr expression appear to be more susceptible to chemotherapeutic agents or radiotherapy (13–15). When ovarian cancer cells bearing high levels of EGFr on the cell surface were pretreated with EGF, the sensitivity of these cells to cisplatin was substantially increased (13). Similarly, when squamous carcinoma cells were pretreated with EGF, their sensitivity to radiation was enhanced in relation to the number of EGFr on their surfaces (15). These observations indicate that EGFr expression and its signal transduction pathways may be crucial determinants of sensitivity to chemotherapeutic agents or irradiation, and alterations in receptor expression or function may influence response to these therapies.

Because of the relationship between overexpression of EGFr and aggressive behavior of tumor cells, monoclonal antibodies directed against this receptor might prove to be effective therapeutic agents. The anti-EGFr monoclonal antibody 225 was generated and has been shown to have antitumor activity in vitro and in xenograft models (16–18). This antibody has been chimerized with human IgG1 (C225) in its constant region to

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⁴The abbreviation used is: EGFr, epidermal growth factor receptor.
increase clinical utility by lowering the potential for generation of human antimusone antibodies in recipients (19). C225 was able to inhibit the growth of cultured EGFr-expressing tumor cell lines and to repress the in vivo growth of these tumors when grown as xenografts in nude mice (18–21). A therapeutic strategy combining C225 with chemotherapeutic agents such as doxorubicin or cisplatin was found to be markedly synergistic in well-established human xenograft models, and complete regression of tumor growth in these mice was noted (22, 23). Combination therapy of cisplatin and C225 may block activation of receptor tyrosine kinase and induce EGFr down-regulation (23).

We conducted a Phase Ib study of C225 in combination with cisplatin in patients with recurrent squamous cell carcinoma of the head and neck to determine the optimal biological dose of C225 (i.e., tumor EGFr-saturating dose) and to establish a safety profile of C225 in a different range of dose levels in combination with cisplatin.

MATERIALS AND METHODS

Study Design

A Phase Ib clinical and biological translational study using C225 and cisplatin was designed for patients with recurrent or metastatic squamous cell carcinoma of the head and neck. To be eligible for the study, patients had to have a histologically proven squamous cell carcinoma of the head and neck that was considered incurable with standard therapy, have a good performance status (Zubrod scale ≤ 2), have disease accessible to repeated biopsies, have measurable disease, have adequate major organ function, and sign a written informed consent. EGFr in tumor cells should have been overexpressed in a prescreening test of all eligible patients.

Monoclonal antibody C225, manufactured and supplied by ImClone System Incorporated (Somerville, NJ), was administered by i.v. infusion with a loading dose and maintenance dose weekly for 6 weeks, with a fixed dose of cisplatin (100 mg/m²) every 3 weeks. The cycle was repeated every 6 weeks. Because of potential allergic reaction to C225, all patients received premedication with 20 mg of dexamethasone i.v. and 50 mg of diphenhydramine i.v. 30 min before the C225 infusion. At the time of initial C225 infusion, emergency kits including i.v. epinephrine, solumedrol, and diphenhydramine were placed at each patient’s bedside to treat possible anaphylactic reaction. On the basis of a previous pharmacokinetic study (24), the starting dose of C225 was 100 mg/m² for the loading dose and 100 mg/m² weekly for the maintenance doses. Subsequent dose escalations in newly enrolled patients were 50 mg/m² (loading) plus 250 mg/m² weekly maintenance and 400 mg/m² (loading) plus 250 mg/m² weekly maintenance. Repeated doses of C225 were allowed every 6 weeks according to the tumor response status and the patient’s tolerance of the treatment.

Responses were assessed by computed tomography or magnetic resonance imaging scans of the head and neck before treatment began and after each 6-week course of treatment. Designation of complete or partial response, no change or stable disease, or progressive disease was based on standard World Health Organization criteria (25).

EGFr Saturation Studies

Tumor Specimens. Before therapy began, fresh tumor specimens were obtained by punch biopsy by a cytopathologist (H. J. S.); two specimens were obtained at each time point from each patient and distributed for immunohistochemistry, immunoprecipitation, and immunoblot analyses for tumor EGFr saturation and C225/EGFr complex studies. Posttherapy, fresh tumor specimens were also obtained by punch biopsy 24 h after the first infusion (posttherapy 1) and 24 h before the third infusion (posttherapy 2) of C225; two specimens again were obtained from each patient. All tumor specimens at each time point were stained with H&E to evaluate viability of tumor cells and processed accordingly for further studies.

Immunohistochemistry and Image Analysis. Tumor biopsy specimens obtained from patients at each time point were embedded in OCT (Miles Lab, Naperville, IL), snap-frozen in liquid nitrogen, and stored at −70°C until used. Four-μm sections were cut and mounted on saline-coated slides (HCS, Inc., Glen Head, NY). Briefly, the sections were fixed in acetone for 5 min and quickly transferred into PBS. Endogenous peroxidase activity was quenched by incubating the slides with 3% H₂O₂ in methanol for 30 min, followed by three rinses with PBS. The slides were then incubated for 30 min in 1% normal horse serum to reduce any nonspecific staining, allowed to react with 20 μg/ml M225 (murine monoclonal antibody; ImClone System) overnight at 4°C, and washed with PBS three times for 15 min each. Slides were then incubated with a secondary antibody (biotinylated horse antimumone IgG, diluted 1:200; Vector Laboratory, Burlingame, CA) for 1 h at room temperature, followed by incubation with avidin-biotin complex, and finally incubated with the peroxidase substrate, 0.1% diaminobenzidine in the presence of H₂O₂. Stained sections were mounted with Eukit mounting medium. The degree of EGFr staining was assessed quantitatively in each sample by image analysis (26); baseline samples (pretherapy) were compared with posttreatment samples (24 h after first infusion of C225 and/or 24 h before third infusion of C225).

Measurement of EGFr Tyrosine Kinase Activity. In early studies (Access No. 1–7), the EGFr level was determined after EGFr/C225 immune complexes were precleared with antihuman IgG and associated EGFr. Supernatants derived from antihuman IgG immunoprecipitates were normalized in volume, and equal aliquots were placed into two tubes. Anti-EGFr antibody (A108; 2.5 μg; Rhône-Poulenc Rorer) was added to one aliquot, and the other received BSA or an isotype-specific irrelevant antibody (ZME-018). Samples were incubated for 90 min, and 40 μl of Pansorbin were added to facilitate EGFr immunoprecipitation (as described below). The pellets were washed extensively in lysis buffer and resuspended in 20 μl of 20 mM HEPES buffer (pH 7.4) containing 0.4 mM Na₃VO₄. To initiate EGFr phosphorylation, 20 μl of HEPES buffer containing 20 μM MnCl₂ and 10 μCi of [32P]ATP were added to each tube, and samples were incubated at 30°C for 15 min. SDS sample buffer (5×) was added to each tube, samples were heated, and proteins were resolved by SDS-PAGE. The gel was fixed, dried, and exposed to X-ray film to detect 32P-labeled EGFr. Counts were quantitated by Phosphorimager, and samples receiving anti-EGFr were compared with those receiving...
irrelevant antibody to determine the level of specific versus nonspecific $^{32}$P incorporation. This technique was used previously to detect EGFr levels in clinical specimens and to estimate the level of EGFr saturation with an anti-EGFr monoclonal antibody (27).

**Detection of EGFr/C225 Complexes.** Tumor specimens were obtained prior to treatment and at indicated intervals after C225 infusion from patients whose disease was accessible to repeated biopsies. The frozen tumor tissues (\(~2.5\) mm$^3$ or larger) were stored at $-80^\circ$C until all specimens (pre- and posttherapy) from individual patients had been collected. Frozen sample weights were recorded (5–125 mg), and tissues were homogenized on ice (Polytron) in lysis buffer [20 mM sodium phosphate (pH 7.4), 5 mM EDTA, 150 mM NaCl, 50 mM NaF, 1 mM Na$_3$ VO$_4$, 2 mM phenylmethylsulfonyl fluoride, 10 $\mu$g/ml aprotinin, 10 $\mu$g/ml leupeptin, and 1% (v/v) Triton X-100] at a fixed tissue-to-volume ratio (20 mg of tissue:1 ml of lysis buffer). All procedures were carried out on ice unless otherwise specified. Homogenates were centrifuged (18,000 $\times$ g, 40 min, 4°C), and supernatants were retained. Equal volumes of supernatant from individual patient samples were incubated 60–90 min with 10 $\mu$g/ml affinity-purified goat antihuman IgG (Sigma Chemical Co.), and immune complexes were precipitated with 50 $\mu$l of Pansorbin (Calbiochem) for 30 min. Supernatants were transferred to a fresh test tube, and immunoprecipitation with goat antihuman IgG was repeated. The two resultant pellets were combined and washed extensively with lysis buffer by resuspension and brief centrifugation before heating in SDS sample buffer. Proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with anti-EGFr (Transduction Laboratory) followed by detection with secondary antibody (Bio-Rad) and ECL reagents (Amer sham). In some experiments, A431 cell lysate (1 $\mu$g) was loaded onto gels with immunoprecipitates as a positive control.

**RESULTS**

**Patient Characteristics.** Twenty-two potential patients were prescreened for tumor EGFr expression. In 20 (91%) of 22 patients, high ($\geq 2+$ in the scale from 0 (no expression) to 3+ (highest expression)) amounts of EGFr expression were seen. Among 20 candidates, 12 patients were enrolled on the protocol, and the remaining 8 patients were excluded because their tumors were not accessible for repeated biopsies. The characteristics of the 12 enrolled patients are summarized in Table 1. The median age was 55 years (range, 36–78 years). Seven of the 12 patients were men, and 5 were women. Six patients had dermal metastases, five had locoregional recurrence, and one had distant metastasis. Six patients had recurrent disease after surgery and postoperative radiation therapy, and six patients received systemic chemotherapy when disease recurred after surgery and/or radiation therapy (one patient received paclitaxel plus gemcitabine, one received 5-fluorouracil plus cisplatin, one received 5-fluorouracil plus cisplatin plus paclitaxel, one received 5-fluorouracil plus cisplatin and IFN-\(\alpha\) plus 13-cis-retinoic acid, one received p53 gene therapy, and one received bryostatin).

Five patients in the first cohort were treated with C225 at 100 mg/m$^2$ as a loading dose with maintenance doses at 100 mg/m$^2$ weekly and cisplatin at 100 mg/m$^2$ every 3 weeks. Four patients in the second cohort received C225 at 500 mg/m$^2$ as a loading dose with maintenance doses at 250 mg/m$^2$ weekly with the same dose of cisplatin every 3 weeks. Three patients in the third cohort received C225 at 400 mg/m$^2$ as a loading dose with maintenance doses at 250 mg/m$^2$ weekly with the same dose of cisplatin every 3 weeks.

**Immunohistochemistry Studies.** Immunohistochemistry studies with image analysis were performed in tumor specimens using pre- and posttherapy biopsied tissue (24 h after the first infusion of C225 and 24 h before the third infusion of C225) in eight cases (Acc 1, 2, 3, 4, 5, 7, 8, and 9) using frozen tissue sections as described in “Materials and Methods.” For the other four cases (Acc 6, 10, 11, and 12), we were not able to perform the assay because no tissue was available or tumor tissue became completely necrotic after therapy. The results are listed in Table 2. At a C225 loading dose of 100 mg/m$^2$ and maintenance doses of 100 mg/m$^2$, the median value of tumor EGFr saturation was 33% (range, 12–76%) 24 h after the first dose of C225, suggesting a modest degree of tumor EGFr saturation. At a C225 loading dose of 500 mg/m$^2$ with a maintenance dose of 250 mg/m$^2$, tumor EGFr saturation in two cases (Acc 7 and 8) after the first dose of C225 was 76 and 39%, respectively. EGFr saturation before the third dose of C225 in Acc 7 decreased to 36% from 76% possibly because of inter-

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**Table 1** Patient characteristics and C225 doses

<table>
<thead>
<tr>
<th>Acc no.</th>
<th>Loading/weekly</th>
<th>Age (yr)/Sex</th>
<th>Primary site</th>
<th>Recurrent site</th>
<th>Previous therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100/100</td>
<td>63/F</td>
<td>Base of tongue</td>
<td>Dermal</td>
<td>S$^*$ + RT</td>
</tr>
<tr>
<td>2</td>
<td>100/100</td>
<td>36/M</td>
<td>Oral tongue</td>
<td>Locoregional</td>
<td>S + RT + gene therapy</td>
</tr>
<tr>
<td>3</td>
<td>100/100</td>
<td>63/M</td>
<td>Alveolar ridge</td>
<td>Locoregional</td>
<td>S + RT</td>
</tr>
<tr>
<td>4</td>
<td>100/100</td>
<td>72/M</td>
<td>Tonsil</td>
<td>Locoregional</td>
<td>S + RT</td>
</tr>
<tr>
<td>5</td>
<td>100/100</td>
<td>46/F</td>
<td>Soft palate</td>
<td>Dermal</td>
<td>S + RT + 5-fluorouracil + cisplatin; IFN-(\alpha) + 13-cRA</td>
</tr>
<tr>
<td>6</td>
<td>500/250</td>
<td>74/M</td>
<td>Base of tongue</td>
<td>Dermal</td>
<td>S + RT; paclitaxel + gemcitabine</td>
</tr>
<tr>
<td>7</td>
<td>500/250</td>
<td>50/M</td>
<td>Base of tongue</td>
<td>Dermal</td>
<td>S + RT</td>
</tr>
<tr>
<td>8</td>
<td>500/250</td>
<td>55/F</td>
<td>Larynx</td>
<td>Dermal</td>
<td>S + RT + 5-fluorouracil + cisplatin</td>
</tr>
<tr>
<td>9</td>
<td>500/250</td>
<td>78/F</td>
<td>Mucosal lip</td>
<td>Locoregional</td>
<td>S + RT</td>
</tr>
<tr>
<td>11</td>
<td>400/250</td>
<td>55/M</td>
<td>Hypopharynx</td>
<td>Dermal</td>
<td>S + RT</td>
</tr>
<tr>
<td>12</td>
<td>400/250</td>
<td>68/M</td>
<td>Retromolar trigone</td>
<td>Distant</td>
<td>S + bryostatin</td>
</tr>
<tr>
<td>13</td>
<td>400/250</td>
<td>39/F</td>
<td>Oral tongue</td>
<td>Locoregional</td>
<td>S + RT + 5-fluorouracil + cisplatin + paclitaxel</td>
</tr>
</tbody>
</table>

$^*$ S, surgery; RT, radiation therapy; 13-cRA, 13-cis-retinoic acid.
nalization of the receptor or reduction in EGFr levels. However, EGFr saturation before the third dose of C225 in Acc 8 increased to 95%. In this case, complete tumor regression was noted, as shown in Fig. 1.

At a C225 loading dose of 400 mg/m² with a maintenance dose of 250 mg/m², tissue was available in one patient (Acc 9) for the EGFr assay; tumor EGFr saturation was 57% within 24 h after the first dose and 79% within 24 h before the third dose of C225.

Detection of EGFr/C225 Complexes by Immunoprecipitation. Additional evidence of an association between C225 and EGFr in tumor specimens was provided by independent biochemical analysis. Tumor samples taken before and after C225 infusion were collected and initially analyzed for residual EGFr tyrosine kinase activity after immunodepletion of C225/EGFr complexes with antihuman IgG. EGFr was collected in immunoprecipitates with A108 monoclonal antibody, which recognizes an epitope on EGFr distinct from that recognized by C225. As shown in Fig. 2, tumor specimens were available for analysis by this technique in 4 of 12 cases, and in each case, EGFr tyrosine kinase activity was detected in pretherapy tumor specimens. After the initial C225 infusion, EGFr tyrosine kinase activity was reduced by 50% or more in two cases (Acc 1 and 5), and 7), the decreased EGFr tyrosine kinase activity from antihuman IgG-immunodepleted supernatants (Fig. 2) correlated with the appearance of EGFr/C225 complexes in the antihuman IgG precipitate.

Clinical Responses and Toxic Effects. Although the primary objectives of this study were to assess tumor EGFr saturation and to determine the optimal biological dose and safety profile of C225 when combined with cisplatin, we also assessed the antitumor activity of C225 in patients who had recurrent squamous cell carcinoma of the head and neck. Among 12 patients entered in this protocol, 9 had disease response that could be evaluated, whereas 3 did not (2 at a C225 loading dose of 100 mg/m², 1 at a C225 loading dose of 500 mg/m²). One patient terminated therapy because of an allergic reaction during the loading dose of C225, one patient stopped treatment because of severe peripheral neuropathy, and one patient withdrew from the trial after three doses of C225. Among the nine patients, disease response was complete in two and partial in four. There was one case each of mixed response, stable disease, and progressive disease. The overall response rate was 67% (six of nine patients). Among the six responding cases, three had progressive disease during cisplatin-containing chemotherapy before enrollment in this protocol. Measurable diseases of the responders were located inside of the previous radiation port in four patients and outside of radiation port in the remaining two patients.

The toxic effects of C225 combined with cisplatin are listed in Table 3. No bone marrow suppression was observed in relation to C225 infusion. Four patients had grade 3 or grade 4 neutropenia, and one had neutropenic fever, which may have been related to cisplatin. Among nonhematological side effects, two patients had grade 3 fatigue, one had grade 3 peripheral neuropathy, and two had grade 3 orthostatic hypotension; all may have been related to cisplatin infusion. Allergic reaction developed in two patients: one grade 2 reaction, and one grade 3. Both patients experienced shortness of breath and chest tightness right after initiation of the loading dose of C225: one at 100 mg/m², and one at 500 mg/m². This was severe enough

<table>
<thead>
<tr>
<th>Acc no.</th>
<th>EGFr saturation by IHC</th>
<th>Tyrosine kinase activity unbound</th>
<th>EGFr/C225 complex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre tx</td>
<td>24 h post 1st tx</td>
<td>24 h pre 3rd tx</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>12</td>
<td>59ᵇ</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>76</td>
<td>32ᵇ</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>53</td>
<td>10ᵇ</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>33</td>
<td>45</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>26</td>
<td>25</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>76</td>
<td>36</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>39</td>
<td>95</td>
</tr>
<tr>
<td>9</td>
<td>0</td>
<td>57</td>
<td>79</td>
</tr>
<tr>
<td>12</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

ᵃ IHC, immunohistochemistry; tx, therapy; ND, not done or could not be evaluated.
ᵇ Biopsies obtained 48 h after third dose of C225.
ᶜ +, + +, + + +, weak, moderate, and strong band of EGFr/C225 complex, respectively; −, no EGFr/C225 complex formation.
ᵈ Tissue samples from Acc no. 8 may have undergone tumor necrosis and/or proteolysis.
Fig. 1 Tumor EGFR saturation with C225 detected by immunohistochemistry studies on tumor specimens from a patient with responding disease. Pretherapy photographic illustration of tumor showing multiple dermal metastases (A) in the anterior neck and upper chest were analyzed for EGFR expression (B) by immunohistochemistry (×200) and H&E staining (×200; C) of the adjacent section. After one course of C225 with cisplatin, multiple dermal metastases have completely regressed (D), and EGFR expression was marked down-regulated (E). F, H&E staining of the adjacent tissue section. Of note, 95% EGFR saturation with C225 in tumor tissue was observed by image analysis after therapy.
Fig. 1 D–F Continued.
for the second patient to receive epinephrine and steroids to recover from the allergic reaction, and the patient was taken off the study after recovering from the reaction. The allergic reaction was modest in the first patient, and the therapy was continued with additional premedication (20 mg of dexamethasone and 25 mg of diphenhydramine as i.v. infusion) and slower infusion of C225. Grade 3 folliculitis-like skin rashes developed in two patients (one had received a loading dose of 100 mg/m² C225 with a maintenance dose of 100 mg/m²; one had received a loading dose of 500 mg/m² C225 with a maintenance dose of 250 mg/m²). Both patients received more than six doses of C225, and the rashes appeared to gradually worsen with the cumulative amount of C225. However, topical and oral antibiotics helped to improve the folliculitis-like skin rashes in both patients.

DISCUSSION

Monoclonal antibody C225 against the human EGFr potentially blocks activation of receptor tyrosine kinase (16, 17, 28). Preclinical studies indicate that EGFr activation can be blocked by C225, which subsequently inhibits the growth of malignant tumor cells (18–20). Furthermore, C225-mediated cell growth inhibition involves down-regulation of EGFr expression (21, 29). Preclinical studies also suggest that blockade of EGFr allows augmentation of antitumor activities when combined with doxorubicin or cisplatin in A431 cell xenografts (23, 29, 30). In particular, C225 therapy in combination with the maximum tolerated dose of cisplatin (23, 29, 30) produced cures in tumor-bearing mice whose follow-up was observed for >6 months.

The current study describes the use of a dose escalation of C225 in combination with cisplatin in patients with head and neck cancer, a highly desirable tumor target for this combined therapy because a majority of squamous cell carcinomas have high EGFr expression (26, 31). One important aspect of this study was to understand the fate of the infused C225. Therefore, emphasis was placed on assessing the binding of infused C225 to tumor EGFr (i.e., EGFr/C225 complex formation). Three independent approaches were used to investigate this process. In the first approach, immunohistochemical methods were used to evaluate the tumor EGFr status before and after C225 infusion. Infused C225 may compete with receptor ligands (i.e., EGF or tumor growth factor-α) for EGFr on the cell surface (29). The tumor EGFr saturation status was assessed by measuring differential staining for EGFr expression by immunohistochemistry and quantitated by image analysis on tumor tissue obtained before and after C225 infusion. Tumor EGFr saturation with C225 was relatively low at lower C225 doses compared with higher amounts of C225. These findings are supported by our

![Image](https://cancerres.aacrjournals.org/content/61/22/8008/F1.large.jpg)
previous *in vitro* observation that inhibition of A431 cell proliferation by monoclonal antibody was more prominent in low-cell density cultures than in high-density cultures, possibly because of lower concentrations of antibody-competing autocrine factors. As had been predicted but not previously tested, higher amounts of C225 infusion in patients resulted in higher levels of tumor EGFr saturation, in agreement with preclinical studies (28).

To confirm the immunohistochemical results and to examine the association and saturation of tumor EGFr with C225 *in vivo*, two additional biochemical assays were used. The first assay estimated receptor saturation by gauging tyrosine kinase activity associated with murine monoclonal anti-EGFr antibody in patients with cancer (27). In the present study, tissue homogenates were immunoprecipitated with anti-EGFr (A108) *in vitro* after immunodepletion of EGFr/C225 complexes with human-specific anti-IgG. Signals were recorded by measuring the tyrosine kinase activity of the EGFr in the immune complex, and we were able to demonstrate EGFr autophosphorylation in the majority of the specimens obtained (some of the samples failed to provide a signal because of insufficient tissue or tissue necrosis). By comparing the autokinase activities of pre- and post-C225 specimens from the same patient (with pretherapy unbound kinase activity defined as 100% loss of EGFr kinase activity), we used measurements for C225-treated specimens as an estimate of tumor EGFr saturation. From this analysis, it appears that lower receptor expression in individual tumors correlates with more effective C225 saturation. However, other factors that are unrelated to C225 saturation may also contribute to this apparent loss of kinase activity in these fractions. The state of EGFr tyrosine phosphorylation, apoptosis stimulated by chemotherapy, limited proteolysis of the receptors, or partial inhibition of enzymatic activity can also lead to an apparent reduction in EGFr kinase activity following treatment with C225 and cisplatin. Therefore, different or perhaps more convincing ways of measuring EGFr/C225 association in clinical samples were investigated.

**Table 3** Maximum toxic effects (*n* = 12)

<table>
<thead>
<tr>
<th>Toxic effects</th>
<th>Number of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CTC a CTC a CTC a</td>
</tr>
<tr>
<td></td>
<td>grade 2 grade 3 grade 4</td>
</tr>
<tr>
<td>Hematologic</td>
<td></td>
</tr>
<tr>
<td>Neutropenia</td>
<td>2 3 1</td>
</tr>
<tr>
<td>Neutropenic fever</td>
<td>1 0 0</td>
</tr>
<tr>
<td>Thrombocytopenia</td>
<td>1 0 0</td>
</tr>
<tr>
<td>Anemia</td>
<td>3 1 0</td>
</tr>
<tr>
<td>Nonhematologic</td>
<td></td>
</tr>
<tr>
<td>Fatigue</td>
<td>3 2 0</td>
</tr>
<tr>
<td>Nausea/vomiting</td>
<td>4 0 0</td>
</tr>
<tr>
<td>Peripheral neuropathy</td>
<td>1 1 0</td>
</tr>
<tr>
<td>Creatinine elevation</td>
<td>0 0 0</td>
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<tr>
<td>Orthostatic hypotension</td>
<td>0 2 0</td>
</tr>
<tr>
<td>Allergic reaction b</td>
<td>1 e 1 d 0</td>
</tr>
<tr>
<td>Skin reaction c (folliculitis-like reaction)</td>
<td>0 2 0</td>
</tr>
</tbody>
</table>

a CTC, National Cancer Institute Common Toxicity Criteria.
b Related to C225 infusion.
c,d Dose of C225: c 100 mg/m²; d 500 mg/m².

One patient had received a loading dose of 100 mg/m²; one had received 500 mg/m².

To maximize evaluation of clinical tissues for EGFr/C225 association, the primary antihuman IgG immunoprecipitate was subjected to EGFr immunoblotting and compared (in some specimens) with the results obtained for kinase activity assessments of EGFr recovery. As shown in Fig. 3, EGFr was not immunoprecipitable with antihuman antibody without prior C225 infusion. In specimens analyzed by both the kinase activity assay and EGFr immunoblotting, loss of kinase activity from the supernatant fraction (after immunodepletion of antihuman immune complexes) correlated with an increase in EGFr in the antihuman IgG/C225 immunoprecipitate (see Fig. 3). In samples taken after initial C225 therapy, an increase in EGFr recovery was noted in five of six patient samples. (Acc 8 may have undergone tumor necrosis and proteolysis.) Further treatment with C225 and cisplatin (posttherapy 2) resulted in reduced recovery of EGFr in this fraction. This reduced recovery of EGFr/C225 complexes may be attributable to several factors, including the possibility for C225-induced down-regulation of EGFr. However, tumor apoptosis and necrosis brought about through the combination of C225 with cisplatin therapy cannot be ruled out as a contributor to this apparent loss of detectable C225/EGFr complexes. Although these possibilities cannot be differentiated by the current study, the results provided by C225/EGFr complex studies and immunohistochemical studies suggest that treatment with C225/cisplatin results in a high-affinity interaction between EGFr and C225 that affects EGFr expression or recovery in patients with head and neck cancer and potentiates tumor regression (19, 29).

Although the primary objectives of this study were assessment of tumor EGFr saturation and determination of the biological dose of C225, we evaluated antitumor activity in the participating patients. All patients had failed primary therapy, including surgical resection and/or postoperative radiotherapy. Six of the 12 patients had been treated with systemic chemotherapy or gene therapy after disease recurrence. Among nine patients who could be assessed for disease response, six (67%) had major responses, including two complete responses. Among these six cases, three patients had received prior cisplatin-containing combination chemotherapy. Two of these three patients had resistance to cisplatin; they experienced disease progression while receiving cisplatin-based chemotherapy just prior to enrollment on this protocol. Such antitumor activity is highly encouraging. In second-line therapy with single-agent cisplatin for recurrent head and neck cancer, one may expect to see a response rate <10% (32, 33). Therefore, C225 therapy in combination with cisplatin appears to have a synergistic effect in recurrent head and neck cancer as predicted in xenograft models. The detailed mechanism of this synergistic effect must be more fully addressed in future studies.

Combined C225 and cisplatin therapy does not appear to induce overlapping toxic effects. Bone marrow suppression, emesis, and peripheral neuropathy in association with orthostatic hypotension have been associated with cisplatin (32, 33), whereas folliculitis-like skin rashes and allergic reaction may be associated with C225 infusion as described previously (24, 34). This study particularly emphasizes that biological end points (i.e., EGFr tumor saturation, tumor EGFr tyrosine kinase activity, and degree of C225/EGFr complex formation) are far more relevant than the traditional end point (i.e., determination of
maximum tolerated dose or dose-limiting toxic effects) in Phase I studies when biologicals are given alone or in combination with chemotherapeutic agents.

In summary, a C225 loading dose of 400 mg/m² with a maintenance C225 dose of 250 mg/m² achieved nearly complete saturation of EGFR in tumor tissue, and the combination of C225 with cisplatin achieved a high percentage of tumor responses. There is also a suggestion of synergism between C225 and cisplatin in recurrent head and neck cancer. On the basis of this EGFR saturation study, a Phase II study with large sample size is being conducted using 400 mg/m² C225 for the loading dose and 250 mg/m² for the maintenance dose in combination with cisplatin for refractory patients with recurrent head and neck cancer. A Phase III study is also being conducted through the Eastern Cooperative Oncology Group comparing C225 plus cisplatin with placebo plus cisplatin in recurrent head and neck cancer. A multi-institutional randomized Phase III trial is also underway to determine the role of C225 in combination of radiation therapy versus radiation therapy alone in locally advanced head and neck cancer. The doses of C225 in this study are 400 mg/m² for loading and 250 mg/m² weekly for maintenance during the radiation therapy. These studies should ultimately assist in determining the potential impact of targeted EGFR biological therapy in combination with cytotoxic chemotherapeutic agents or with radiation therapy for the treatment of EGFR-expressing epithelial cancers.

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Epidermal Growth Factor Receptor-targeted Therapy with C225 and Cisplatin in Patients with Head and Neck Cancer

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