Immune Activation by Epidermal Growth Factor Receptor–Specific Monoclonal Antibody Therapy for Head and Neck Cancer

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Objective: To determine if the epidermal growth factor receptor (EGFR)-specific monoclonal antibodies (mAbs) cetuximab or panitumumab mediate in vitro immune activation against squamous cell carcinoma of the head and neck (SCCHN) cell lines.

Design: In vitro study.

Setting: Basic science research laboratory.

Intervention: Squamous cell carcinoma of the head and neck cell lines were treated with the Food and Drug Administration–approved EGFR-specific mAbs cetuximab or panitumumab in the presence or absence of peripheral blood mononuclear cells from healthy donors.

Main Outcome Measures: Cetuximab and panitumumab were compared in terms of their cytotoxic effects, ability to induce apoptosis, bind to EGFR, and block phosphorylation of this receptor in SCCHN cell lines.

Results: We demonstrate that both cetuximab and panitumumab have similar levels of EGFR binding, induction of apoptosis, cell lysis, and inhibition of phospho-EGFR in SCCHN cell lines, suggesting similar direct effects. However, neither of these mAbs demonstrated in vitro antitumor activity when used alone. In contrast, in the presence of peripheral blood lymphocytes, either of them can mediate antibody-dependent cell cytotoxicity in vitro when used in doses similar to those found in patients receiving them clinically.

Conclusion: We propose that antibody-dependent cell cytotoxicity may constitute an important antitumor mechanism that could contribute to overall clinical effectiveness of EGFR-specific antibodies.

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The epidermal growth factor receptor (EGFR) has become one of the most promising therapeutic targets in the treatment of various solid tumors. Of these, squamous cell carcinoma of the head and neck (SCCHN) represents an appealing candidate for this targeted therapy because of its poor outcome, high rate of metastasis, toxicity of current therapies, and its prevalence of EGFR overexpression, the highest of all solid tumors.

Two approaches for EGFR inhibition have been developed, one based on the use of inhibitors blocking intracellular receptor phosphorylation and the other based on monoclonal antibodies (mAbs) specific for the receptor’s extracellular domain. Two EGFR-specific mAbs are currently being evaluated for SCCHN: the human-mouse chimeric mAb cetuximab (IgG1 isotype) and the fully human IgG2 panitumumab (IgG2 isotype). Despite the clinical data supporting their effectiveness,1,2 their exact antitumor mechanism has not been fully elucidated. However, it is likely that reduction of EGFR ligand binding and receptor activation is enhanced by a mAb-mediated cytotoxic immune response against EGFR-expressing tumor cells. This latter mechanism has been demonstrated to play an important role in other mAb therapies such as rituximab,1 a CD20-specific chimeric IgG1 mAb used for the treatment of lymphoproliferative diseases, and the murine anti-GD2 3F84,5 mAb used in the treatment of neuroblastomas. In both of these cases, mAbs owe their clinical efficacy at least partly to antibody-dependent cell cytotoxicity (ADCC). Antibodies opsonize cells that will be eventually phagocytosed or lysed through ADCC mainly by natural killer (NK) lymphocytes and macrophages and other effector cells bearing Fcγ receptors (FcγRs). Effector activation occurs through the binding of the Fc portion of an IgG mAb to the FcγRs present on the effector cell surface, leading to degranulation, target cell lysis, and phagocytosis.

Even though, to our knowledge, the role of ADCC in therapy with these antibodies has not yet been studied, in vitro studies have shown that EGFR-specific mAbs are potent inducers of ADCC against...
adherent melanoma⁶ and SCCHN cell lines.⁷ Furthermore, a dimer of the variable portion of the anti-EGFR mAb C225, which is missing the Fc domain that mediates immune cell binding, possesses somewhat reduced in vivo anti-A431 xenograft tumor activity compared with the native monoclonal mAb, despite equivalent EGFR blockade.⁸ This shows the potential importance of an immune-mediated mechanism in the overall clinical response seen with these mAbs.

We demonstrate herein that the 2 Food and Drug Administration–approved EGFR-specific mAbs cetuximab and panitumumab mediate immune activation through ADCC in SCCHN cell lines in vitro at doses similar to those found in vivo in patients receiving them.

METHODS

CELL LINES AND FLOW CYTOMETRY

The previously characterized EGFR<sup>high</sup> SCCHN cell line PCI-15B<sup>11,12</sup> was used for all of the experiments. This cell line was grown using 10% fetal bovine serum–Dulbecco modified Eagle medium at 37°C (5% carbon dioxide). Cultures were tested every 30 days and were free of Mycoplasma contamination. Cells were incubated with cetuximab or panitumumab for 60 minutes at 4°C and stained with goat antihuman fluorescein isothiocyanate–labeled Fc-specific mAb, and analyzed by flow cytometry. The graph shows the mean fluorescence intensity (MFI) obtained at each mAb. The error bars indicate SE.

RESULTS

BINDING OF CETUXIMAB OR PANITUMUMAB TO THE SCCHN CELL LINE

To determine the binding capacity of cetuximab (IgG1 isotype) or panitumumab (IgG2 isotype), we incubated the EGFR<sup>high</sup> SCCHN cell line PCI-15B with either of these mAbs for 1 hour at 4°C and analyzed the treated cells by flow cytometry. The results of these experiments are shown in Figure 1. Even though binding of both EGFR-specific mAb to the EGFR<sup>high</sup> PCI-15B cell line is similar, panitumumab exhibited slightly higher binding activity than did negative controls. PCI-13B cells treated with UV light for 5 minutes were used as a positive control for annexin binding.

REAGENTS AND mAbs

Epidermal growth factor was purchased from Sigma-Aldrich Corp, and antiphospho-EGFR (Tyr 1068) was purchased from Cell Signaling, Boston, Massachusetts. For protein blotting, an EGFR-specific polyclonal mAb (SC Biotechnologies, Santa Cruz, California) was used. The human antimouse β-actin mAb was purchased from Sigma-Aldrich Corp. Cetuximab and panitumumab were purchased from the manufacturers (Imclone [Branchburg, New Jersey] and Amgen [Thousand Oaks, California], respectively).

IMMUNOBLOT ANALYSIS

Briefly, 70% to 80% of confluent cells were serum starved for 72 hours. Cells were then treated with either cetuximab or panitumumab or control mAb at 37°C for 2 hours, followed by stimulation with 20 ng/mL of recombinant human epidermal growth factor (Sigma-Aldrich Corp) at 37°C for 15 minutes. After treatment, cells were harvested in lysis buffer (10 mM Tris hydrochloride [pH 7.6], 50 mM sodium diphosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 1% Triton X-100, 1:30 protease inhibitor, and protein tyrosine phosphatase inhibitor), vortexed overnight, and centrifuged at 16 300 rpm for 30 minutes at 4°C. The supernatant protein was quantitated and normalized. Fifty grams of protein were size fractionated through sodium dodecyl sulfate–polyacrylamide gel (10%) electrophoresis, transferred to a nitrocellulose membrane, and immunoblotted with the indicated mAbs.

CYTOTOXICITY ASSAYS

The lactate dehydrogenase–based cytotoxicity detection kit (Roche Diagnostics, Mannheim, Germany) was used. Briefly, cells were plated at a concentration of 1.5 × 10⁵ cells/mL on 96-well plates on complete RPMI (Roswell Park Memorial Institute)–10 medium with 25 mM HEPES buffer. Cetuximab and panitumumab were added at the specified concentration, and peripheral blood mononuclear cells (PBMCs) were added at a concentration of 1.5 × 10⁶/mL and incubated for 18 hours at 37°C (5% carbon dioxide) for 18 hours. Controls for spontaneous (cells only) and maximal lysis (cells treated with 1% Triton X) and irrelevant antibodies (IgG1 or IgG2 isotype controls) were included. Each reaction was done in triplicate. The supernatants were collected and analyzed for lactate dehydrogenase content using the aforementioned kit. Results were normalized with the following formula and plotted on a graph:

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\text{Lysis} = \left( \frac{\text{Experimental Lysis} - \text{Spontaneous Lysis}}{\text{Experimental Lysis} - \text{Maximal Lysis}} \right) \times 100.
\]
cetuximab at concentrations of 0.1 µg/mL or lower, with no differences seen at higher mAb concentrations.

**EGFR-SPECIFIC mAbs HAVE NO CYTOTOXIC EFFECTS IN VITRO**

To determine the cytotoxic effects of EGFR-specific mAbs on the EGFR

SCCHN cell line, we treated these cells with 10 µg/mL of cetuximab or panitumumab for 18 hours and tested them for annexin binding as a marker of early apoptosis. Neither cetuximab nor panitumumab induced significant amounts of apoptosis with this moderately high mAb concentration. To determine the cytolytic effects of EGFR-specific mAb, we treated the EGFR

SCCHN cells for 18 hours with cetuximab or panitumumab and analyzed the supernatant for lactate dehydrogenase release as a measure of cytotoxicity. None of the mAbs induced significant tumor cell lysis compared with an irrelevant mAb (P < .05, 1-tailed permutation test) (Figure 3). Antibody-dependent cell cytotoxicity activity appeared to be dose dependent, with variation between each of these donors. All 4 donors mediated ADCC with the highest dose of panitumumab (10 µg/mL) (Figure 3) and 3 of them with 1 µg/mL of panitumumab (Figure 3B-D). Antibody-dependent cell cytotoxicity activity on all of these donors is statistically significant compared with an isotype-matched irrelevant specificity control mAb (P < .05, 1-tailed permutation test).

**SIMILAR DOSES ARE REQUIRED TO MEDIATE ADCC AND BLOCKING pEGFR**

To determine the dose of EGFR-specific mAb required to block the phosphorylated EGFR (pEGFR) on the PCI-15B cell line, we treated this cell line with the same mAb concentrations used in the cytotoxicity assays and pulsed them with recombinant human epidermal growth factor. Cells were serum starved for 48 hours and then treated for 2 hours with cetuximab or panitumumab at the indicated concentrations (Figure 4). After this treatment, cells were pulsed with 20 ng/mL of recombinant human epidermal growth factor for 15 minutes and analyzed using Western blot for pEGFR level of expression. Both panitumumab and cetuximab completely blocked EGFR phosphorylation in doses greater than 1 µg/mL, with only partial blockade at lower doses. These find-
ings demonstrate that the dose of cetuximab or panitumumab required for complete pEGFR blockade is similar to (or higher) than the one required to mediate ADCC.

COMMENT

Despite the clinical efficacy of EGFR-specific mAbs, the precise antitumor mechanism responsible for the responses seen in patients receiving them is unknown. As we have shown, treatment with the EGFR-specific mAbs cetuximab or panitumumab does not induce significant apoptosis in vitro even when used at high concentrations. However, both cetuximab and panitumumab mediate immune effects in vitro that are likely to contribute to their overall clinical efficacy. While cetuximab mediated ADCC with doses much lower than those required to block pEGFR, panitumumab-mediated ADCC with doses similar to those required for full pharmacologic blockade of pEGFR (1-10 µg/mL). Since the concentration of these mAbs in the serum of patients receiving them has been shown to be higher than this\(^\text{11}\) (>0.01 µg/mL in the case of cetuximab and >01 µg/mL in the case of panitumumab), it is very likely that the observed cytotoxic effects seen in vitro occur in vivo and contribute to the clinical responses seen with the use of these mAbs.

It is of interest that variability of ADCC mediated by these mAbs is seen between individuals. These variations may be explained by functional polymorphisms of the FcyRs present on the surface of immune effector cells. These polymorphisms have been shown to play a crucial role in the use of the chimeric IgG1 mAb rituximab in the treatment of lymphoproliferative diseases. This antibody not only induces apoptosis of CD20\(^+\) cells but also owes much of its efficacy to ADCC. Differences seen in the clinical response of patients to this mAb are a consequence of polymorphisms in the FcyRs. These functional polymorphisms lead to different affinities and avidities of FcyRs for each mAb isotype. Thus, we presume that the differences we have observed in ADCC with EGFR-specific mAbs are likely related to these polymorphisms. Determining how these polymorphisms interact with each of these antibodies and the immune status of treated patients with cancer

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**Figure 3.** Determination of antibody-dependent cell cytotoxicity by immune effector cells to squamous cell carcinoma of the head and neck cell lines treated with the epidermal growth factor receptor–specific monoclonal antibodies (mAbs) in 4 donors (A, donor 1; B, donor 2; C, donor 3; and D, donor 4). The PCI-15B cell line was incubated with different concentrations of cetuximab or panitumumab and with immune effector cells at an effector to target ratio of 3:1 for 18 hours. Supernatants were analyzed for lactate dehydrogenase as a measure of cytotoxicity, and the results were plotted in a graph where the x-axis represents the mAb concentration and the y-axis represents percentage of lysis. The error bars indicate SE.
may allow clinicians to obtain the best possible response from each patient receiving them.

The first successful, randomized clinical trial using cetuximab in patients with SCCHN showed that patients who received previous radiotherapy had improved clinical responses compared with those who had not. Even though it has been demonstrated that cetuximab enhances radiosensitivity of tumor cells, it has also been demonstrated that tumor lysis by chemotherapies improves immune responses and that lysed tumor cells bound to mAbs are processed and presented more efficiently and generate stronger immune responses. Perhaps patients receiving radiotherapy have better overall responses owing to the interaction of cetuximab with the immune system, allowing patients to mount stronger immune-mediated responses, a fact that has been implicated in better prognosis and survival of tumor-bearing patients.

Our findings need to be confirmed in SCCHN specimens. Patients with SCCHN have lower absolute lymphocyte counts, down-regulation of cytokine and chemokine serum levels, and impaired NK cell activity secondary to SCCHN cells. Thus, these patients, particularly those with advanced-stage disease, are likely to have impaired ADCC responses, a hurdle that might be overcome with strategies aimed to strengthen the immune system.

It will be important to determine whether patients with SCCHN generate immune activation during EGFR-specific mAb therapy and to identify host factors, such as disease stage and FcγR polymorphisms that influence the immune response to these targeted immunotherapies. Determination of these will provide the information necessary to enhance the responses to these therapies and improve the prognosis and survival of patients with SCCHN.

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REFERENCES