

# The epidermal growth factor receptor as a target for cancer therapy

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## Abstract

Epidermal growth factor (EGF) receptors are expressed at high levels in about one third of epithelial cancers, and autocrine activation of EGF receptors appears to be critical for the growth of many tumors. We hypothesized that blockade of the binding sites for EGF and transforming growth factor- $\alpha$  on EGF receptors with an antireceptor monoclonal antibody (mAb) might be an effective anti-cancer therapy. We produced murine mAb 225 against EGF receptors and demonstrated blockade of receptor function, as well as inhibition of cell growth in cultures and in nude mouse xenografts. mAb C225 is the human:mouse chimeric version of mAb 225. Cell cycle inhibition occurred in G<sub>1</sub> phase, and was due to upregulation of p27<sup>Kip1</sup>, resulting in inhibition of cyclin E/cyclin dependent kinase-2 activity and hypophosphorylation of Rb. In addition, the amount and/or activities of a number of proapoptotic molecules were enhanced. The antitumor activity *in vivo* against xenografts was at least partly attributable to reduced vascularization, resulting from decreased vascular endothelial growth factor and basic fibroblast growth factor production by the tumor cells. Metastasis of xenografts was curtailed with mAb C225 treatment, accompanied by a decrease in tumor production of MMP-9. Further studies showed that mAbs 225 and C225 enhanced the cytotoxicity of chemotherapy against xenografts of a variety of human cancer cell lines. Well established xenografts resistant to either mAb or drug treatment alone were eradicated by the combination therapy. Drugs for which this has been demonstrated include doxorubicin, paclitaxel, cisplatin, and topotecan. Antibody treatment also potentiated the responsiveness of human tumor xenografts to radiation therapy. These findings led to clinical trials of human:mouse chimeric mAb C225 in combination with chemotherapy or radiotherapy. Results from phase I and II trials involving more than 500 patients are quite promising, in particular in advanced head and neck cancer treated with C225 plus cisplatin or radiation, in advanced colon cancer treated with C225 plus CPT-11, and in advanced pancreatic cancer treated with C225 plus gemcitabine. Phase III trials are now underway.

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## Background

The design of anti-cancer therapy employing an inhibitor of epidermal growth factor (EGF) receptor function was hypothesis-driven, based on knowledge available in the early 1980s. (1) The EGF receptor and its ligands, EGF and transforming growth factor- $\alpha$  (TGF- $\alpha$ ), had been identified, primarily through the pioneering research of Nobel Laureate Dr Stanley Cohen (Cohen 1962, Cohen *et al.* 1980). (2) The autocrine mechanism, involving autostimulation of EGF receptors on the surface of cancer cells by TGF- $\alpha$  which they produced, was described in a landmark paper by Drs Michael Sporn and George Todaro (1980). (3) Dr Gordon Sato and others had defined the role of growth factors and transferrin in replacing the requirement for serum in cell cultures (Barnes & Sato 1980). (4) EGF receptors and the src oncogene product were shown to have the novel enzymatic activity of a tyrosine

kinase (Chinkers & Cohen 1981, Cooper & Hunter 1981, Erickson *et al.* 1981). (5) Subsequent studies showed the homology of EGF receptors and the v-erbB oncogene, establishing the receptor as a cellular oncogene (Downward *et al.* 1984, Lin *et al.* 1984, Ullrich *et al.* 1984, Xu *et al.* 1984). (6) Reports extending into the late 1980s demonstrated that many human solid tumors express high levels of EGF receptors, which often correlated with a poorer prognosis (Ozanne *et al.* 1986; reviewed in Salomon *et al.* 1995).

The approach of selecting a monoclonal antibody (mAb) as the agent for inhibiting EGF receptor function was suggested to us by 'experiments of nature,' in which circulating antibodies against receptors were known to produce stable physiological changes (diseases) in patients: myasthenia gravis, where the antibody target is the acetylcholine receptor; rare forms of insulin resistant diabetes, where the antibody target is the insulin receptor;

and forms of thyroid dysfunction, where the antibody target is the thyrotropin receptor.

## Laboratory investigations

In 1983–84, we reported production of murine mAbs 225 and 528 with the following properties (Kawamoto *et al.* 1983, Sato *et al.* 1983, Gill *et al.* 1984). (1) Binding to EGF receptors with a  $K_d$  similar to the natural ligands (1–2 nM). (2) Stoichiometric inhibition of EGF binding to its target site on the receptor. (3) Inhibition of the capacity of EGF/TGF- $\alpha$  to stimulate activation of receptor tyrosine kinase activity. (4) Increased rate of receptor internalization.

The antibodies inhibited proliferation of cultured tumor cells, both in culture and in nude mouse xenografts (Kawamoto *et al.* 1983, Sato *et al.* 1983, Gill *et al.* 1984, Masui *et al.* 1984). These effects were initially demonstrated with the A431 vulvar squamous carcinoma cell line, and were subsequently extended to a variety of epithelial human tumor cell lines originating from breast, prostate, lung, colon, head and neck, pancreas, and bladder (reviewed in Mendelsohn 1997, 2000).

In most studies, the proliferation rate of cancer cell lines was reduced but not totally inhibited, with exceptions to be discussed below. In contrast, the proliferation of cultured non-transformed cells in culture was completely arrested (Sato *et al.* 1983, Markowitz *et al.* 1990, Chou *et al.* 1999). Cells cultured in the presence of control mAbs which bind to other epitopes on the EGF receptor and do not block binding of ligand had no effect on cell proliferation (Sato *et al.* 1983, Markowitz *et al.* 1990, Wu *et al.* 1995).

A human:mouse chimeric version of murine mAb 225 was produced and was found to have improved binding and enhanced anti-tumor activity against human tumor xenografts, with elimination of well established tumors (Goldstein *et al.* 1995). This antibody, known as C225, is undergoing extensive evaluation in clinical trials.

The explanations for the anti-tumor activity of mAb 225/C225 have been the subject of intensive study in our laboratory and in others. A number of mechanisms are called into play when EGF receptor activity is inhibited by this antibody, some of which can be contributing to efficacy against human cancer cells in culture and *in vivo*. (1) Blockade of cell cycle traversal by induction of increased levels of p27, resulting in inhibition of cyclin dependent kinase-2 (CDK2) activity. (2) Increased levels and activities of pro-apoptotic molecules. (3) Enhancement of the cytotoxic activities of a number of chemotherapeutic agents, as well as radiation therapy.

In addition, other mechanisms are active only in the *in vivo* setting, and may be relevant to efficacy against human cancers in the clinical setting. (1) Inhibition of

angiogenesis by inhibition of tumor cell production of pro-angiogenic agents, and by direct cytotoxic activity on endothelial cells in tumor neovasculature. (2) Inhibition of invasion and metastasis. (3) Potential cytotoxicity mediated by immunological mechanisms.

These mechanisms will be presented in more detail.

## Inhibition of cell cycle traversal

When nontransformed human cells growing in culture are exposed continuously to saturating concentrations of mAb 225, cell cycle traversal is arrested in  $G_1$  phase. This has been shown with human foreskin fibroblasts, a colon adenoma cell line, and MCF10A immortalized mammary cells (Sato *et al.* 1983, Markowitz *et al.* 1990, Chou *et al.* 1999). The response of cultured malignant cell lines to mAb 225 varies from slowing of the proliferation rate to complete arrest in the  $G_1$  phase of the cell cycle (Kawamoto *et al.* 1984, Wu *et al.* 1995, Peng *et al.* 1996).

In studies with three different cell lines, A431 squamous carcinoma, DiFi colon adenocarcinoma and DU-145 prostatic adenocarcinoma, the accumulation of cells in  $G_1$  phase could be attributed to a rise in the p27<sup>Kip1</sup> inhibitor, with increased binding of p27<sup>Kip1</sup> to CDK2 and reduced CDK2 activity (Peng *et al.* 1996, Wu *et al.* 1996, Fan *et al.* 1997). *In vivo* studies with nude mouse xenografts confirmed that treatment of tumored animals with C225 resulted in a decline in proliferating cell nuclear antigen (PCNA) accompanied by increased p27<sup>Kip1</sup> (Perrotte *et al.* 1999).

## Programmed cell death

The DiFi colon adenocarcinoma cell line is unusual in that these cells in culture appear to be auxotrophs for EGF/TGF- $\alpha$ . When EGF receptor activity is blocked by addition of mAb 225 to cultured DiFi cells, apoptosis is observed following cell cycle arrest, and all cells are dead within 48 h (Wu *et al.* 1995). DiFi cells lack Bcl-2 and respond to C225 treatment with a rise in Bax (Mandal *et al.* 1998). In addition, C225 induces an increase in caspase 8 activity, followed by a rise in caspases 9 and 3 to levels comparable to those causing cell death in cultures exposed to ultraviolet radiation (Liu *et al.* 2000). Studies with other cell lines have shown a fall in Bcl-2 levels or phosphorylation (inactivation) of Bcl-2 (Huang *et al.* 1999, Tortora *et al.* 1999), and mAb 225 has also been found to sensitize cells to tumor necrosis factor (Fong *et al.* 1992).

Thus, it is evident that inhibition of EGF receptor activity activates a number of pro-apoptotic pathways and deactivates anti-apoptotic pathways in cultured cells. The observation that apoptosis is seen in only a few cell lines exposed to mAb C225 in culture suggests that, in most

situations, the pro-apoptotic activity may be at a level inadequate for induction of programmed cell death. Further studies (see below) suggest that this apoptotic activity can be enhanced when cells are exposed simultaneously to both C225 and chemotherapy or radiation.

### Enhanced cytotoxicity of chemotherapy and radiotherapy

Our studies demonstrating synergistic antitumor activity when C225 is combined with chemotherapy were stimulated by two reports in the literature. In the original observation from the laboratory of Dr Michael Sela, cisplatin in combination with another anti-EGF receptor mAb showed augmented activity against a human tumor xenograft (Aboud-Pirak *et al.* 1988). A similar observation was made soon afterwards using cisplatin in combination with a mAb against HER-2 (Hancock *et al.* 1991). We decided to pursue these observations aggressively. We were able to demonstrate synergistic antitumor activity against well-established human tumor xenografts of both breast adenocarcinoma and vulvar squamous carcinoma cell lines when murine mAb 225 treatment was combined with the maximum tolerated doses of either doxorubicin or cisplatin (Baselga *et al.* 1993, Fan *et al.* 1993a). Treatment either with drug alone or with antibody alone merely reduced tumor growth, whereas combined therapy eradicated the well-established xenografts. Curative antitumor activity against a mammary adenocarcinoma xenograft was also observed when mAb 225 treatment was combined with paclitaxel, this time with suboptimal doses of the chemotherapeutic agent (Baselga *et al.* 1994). Another study showed synergistic antitumor activity of mAb C225 when given in combination with topotecan against a colon adenocarcinoma xenograft (Ciardiello *et al.* 1999). These data demonstrate that blockade of EGF receptors by mAb 225 or mAb C225 can potentiate the antitumor activities of a variety of chemotherapeutic agents which have widely different mechanisms of action.

Recent experiments have also demonstrated synergistic antitumor activity when mAb C225 was combined with radiation therapy, both with cultured tumor cells (Huang *et al.* 1999) and with tumor cell xenografts (Milas *et al.* 2000).

The mechanism of the synergy observed with these combination therapeutic regimens is under study. The net result of two concurrent insults (e.g. mAb C225 and a chemotherapeutic agent) appears to be augmented activation of pro-apoptotic pathways to the point where programmed cell death occurs. In this situation, as in others described in the literature, a growth factor can actually become a survival factor that is required for maintaining

survival of the cell (Evan *et al.* 1992, Meikrantz & Schlegel 1995).

### Inhibition of angiogenesis

Cell growth inhibition by anti-EGF receptor mAb 225 is incomplete for most cultured human tumor cell lines, whereas the effects against xenografts are often more pronounced, especially with human:mouse chimeric mAb C225. A possible explanation for these observations was provided by studies demonstrating anti-angiogenesis effects resulting from EGF receptor blockade (Petit *et al.* 1997, Perrotte *et al.* 1999, Bruns *et al.* 2000). Cultured bladder cancer cells were found to secrete high levels of vascular endothelial growth factor (VEGF), interleukin 8 (IL-8), and basic fibroblast growth factor (FGF) into the culture medium, and production of these angiogenesis factors was reduced by the addition of mAb C225. When orthotopic xenografts of these bladder cancer cells were excised and examined histologically following three weeks of treatment with C225, a marked decrease in the presence of new blood vessels was observed, as shown by staining with anti-CD31, and there were marked reductions in the amounts of VEGF, IL-8, and basic FGF present in the tumor cells as compared with controls (Perrotte *et al.* 1999). Similar observations were made with xenografted A431 cervical squamous carcinoma cells (Petit *et al.* 1997) and a pancreatic adenocarcinoma cell line (Bruns *et al.* 2000).

These findings suggest that one function of the EGF-receptor signal transduction pathway is to stimulate production of angiogenesis factors, and that inhibition of this signaling pathway results in indirect antitumor activity due to inhibition of angiogenesis.

This concept is strengthened by a recent observation with a human pancreatic tumor xenograft that treatment with mAb C225 produced apoptosis of vascular endothelial cells, in addition to apoptosis of the cancer cells (Bruns *et al.* 2000). This finding suggests that the angiogenic agents produced in response to EGF receptor activation serve as survival factors for new endothelial cells, or that the mAb has a selective direct effect upon these endothelial cells.

### Inhibition of invasion and metastasis

In an orthotopic bladder carcinoma xenograft model it has been shown that treatment of tumor-bearing mice, beginning 28 days after tumor cell implantation, results in prevention of metastases to the regional lymph nodes and lungs (Perrotte *et al.* 1999). Histological staining of tumors from treated animals showed a marked decrease in the amount of metalloproteinase-9 present. Additional experiments in culture showed that treatment of tumor cells

with mAb C225 reduced tumor cell migration. These studies provide evidence that cellular activities potentiating metastasis can also be regulated by the EGF receptor signaling pathway.

### Potential immunological mechanisms

We were able to show that antitumor activity against xenografts of human cancer cells is retained by the F(ab')<sub>2</sub> portion of mAb 225, indicating that the efficacy *in vivo* is not dependent on immune mechanisms (Fan *et al.* 1993b). The primary activity of C225 appears to be delivered through inhibition of EGF receptor-mediated signaling pathways. However, immune mechanisms could be contributing to the anti-tumor effects observed in patients treated with the human:mouse chimeric mAb C225, which has a human IgG1 backbone. C225 has the capacity to mediate antibody-dependent cellular cytotoxicity against a cultured melanoma cell line, using human peripheral blood mononuclear cells as the effector cells (Naramura *et al.* 1993).

### Clinical trials with anti-EGF receptor mAbs

A series of phase I and I/II trials with mAb C225 has been completed and the results were recently reported. Pharmacological studies showed dose-dependent saturable pharmacokinetics with no change in behavior of mAb C225 after repeated doses, confirming the lack of significant anti-C225 antibody formation. The recommended phase II and III dose is a 400 mg/m<sup>2</sup> loading dose, followed by a 250 mg/m<sup>2</sup> weekly maintenance dose (Baselga *et al.* 2000).

Phase I trials with mAb C225 and other anti-EGF receptor mAbs have shown antibody binding to tumor EGF receptors and demonstrated that saturation of receptors can be achieved (Perez-Soler *et al.* 1994, 1998, Modjtahedi *et al.* 1996).

A phase Ib/IIa trial in advanced head and neck cancer involved combined treatment with weekly C225 plus 60 Gy of local radiotherapy given as 2 Gy/day over 6 weeks (Ezekiel *et al.* 1998). The response rate was 100%, and 13 of 15 patients achieved a complete remission as evidenced by endoscopy and computed axial tomography scans. The expected complete plus partial response rate to radiation alone is 50–60%, based on the literature. A year later over 50% of patients remained in complete remission (Bonner *et al.* 2000).

A second phase Ib/IIa trial involved treatment with 100 mg/m<sup>2</sup> cisplatin monthly plus C225 weekly, with dose escalation in groups of three or four patients (Mendelsohn *et al.* 1999). Nine of twelve patients were able to be evaluated for clinical response. There were two complete responses and four partial responses, for an overall

response rate of 67%, and only one patient had disease progression during therapy. Of special significance was the fact that three of the six responders, including a complete responder, had received cisplatin in combination with other therapy prior to the study, with disease progression.

Two pivotal phase III randomized multicenter trials are under way, assessing the efficacy of cisplatin with or without mAb C225, and radiotherapy with or without mAb C225, for treatment of advanced head and neck cancer.

Additional pivotal clinical trials are exploring a novel design where chemotherapy is given and responders are followed on treatment until progression of disease is observed; at that point mAb C225 is added to the chemotherapy and patients are observed for clinical responses. This approach is being evaluated with cisplatin in advanced head and neck cancer, and with irinotecan in metastatic colorectal cancer. In addition, a trial of gemcitabine in combination with mAb C225 is ongoing in pancreatic cancer.

Over 500 patients have been treated with mAb C225. There has been little evidence of immunogenicity, with only 4% of patients developing antibodies against C225. None of these patients developed allergic symptoms (Khazaeli *et al.* 2000). Seven percent of patients experienced mAb-related allergic reactions during the first infusion, and in two percent there were grade 4 anaphylactic reactions, readily reversed using standard treatments (Cohen *et al.* 2000). The major toxicity was a common acneform rash, which occurred at a grade 3/4 level in 9% of patients and was reversible once treatment was completed. In general, treatment with chemotherapy or radiation therapy plus mAb C225 was well tolerated (Baselga *et al.* 2000).

### Inhibitors of EGF receptor tyrosine kinase acting intracellularly

Over the last decade drug discovery efforts have produced a variety of compounds, primarily quinolone derivatives, which inhibit the EGF receptor tyrosine kinase (Levitzki & Gazit 1995, Klohs *et al.* 1997, Shawver 1999). These inhibitors differ among themselves in their potency, specificity against the EGF receptor, reversibility of action, and bioavailability. Oral compounds are generally preferred for clinical development. The inhibitors appear to block tyrosine kinase activity by attaching to the ATP binding site on the intracellular portion of the receptor. PD153035 (Fry *et al.* 1994, Bos *et al.* 1997), CP-358,774 (Moyer *et al.* 1997), AG1478 (Busse *et al.* 2000) and ZD1839 (Budillon *et al.* 2000) inhibit receptor tyrosine kinase activity and proliferation of tumor cells bearing high levels of EGF receptors. In cases where it has been tested, these agents produce G<sub>1</sub> arrest preceded by accumulation of p27<sup>Kip1</sup>, confirming and extending the previous observations

made with mAb C225. Two other related drugs, PD168393 (Fry *et al.* 1998) and CI-1033 (Smaill *et al.* 1999) act against both the EGF (ErbB1) receptor and the closely related ErbB2 receptor. A number of these agents have been moved into clinical trials, with ZD1839 being the most advanced (Ferry *et al.* 2000). A phase III trial is exploring the efficacy of ZD1839 for augmenting the response to chemotherapy in patients with advanced squamous carcinoma of the lung.

The relative merits of antireceptor mAbs and chemical tyrosine kinase inhibitors will be determined through clinical trials. The concentration of mAb needed for receptor inhibition in intact cells is generally lower than the concentration of the chemical inhibitors, and the specificity of the mAb for EGF receptors is greater. Furthermore, mAb treatment results in receptor down-regulation, which might provide an added effect. Differences in the toxicity profiles are beginning to appear. Whether or not immune mechanisms are contributing to the efficacy of mAb-mediated treatment remains to be determined. It may be that there will be a place for both types of therapy, in some cases taking advantage of the specificity and different mechanism of action of the mAb, while capitalizing on the availability of an active oral agent in other situations.

## Conclusion

The results of these preclinical studies clearly establish the basis for current clinical trials evaluating the efficacy of anti-EGF receptor mAb C225 in combination with chemotherapy or radiation therapy. Chemical inhibitors of the receptor are also under study in the clinic. Since approximately one third of all human epithelial cancers are known to express high levels of EGF receptors, and production of TGF- $\alpha$  is common in these tumors, there is hope for broad applicability of this new form of therapy to a variety of malignancies.

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Dr Mendelsohn is on the Board and holds stock options in ImClone Systems, Inc., New York, which is carrying out the clinical trials with mAb C225.

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