Epidermal Growth Factor Receptor Inhibition by a Monoclonal Antibody as Anticancer Therapy

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Abstract
Monoclonal antibody (mAb) 225 against the human epidermal growth factor receptor blocks activation of receptor tyrosine kinase. This retards or arrests cell cycle progression, with accumulation of cells in G1 phase. The mechanism of growth inhibition involves increased levels of p27KIP1 and inhibition of cyclin-dependent kinase-2 activity. mAb in combination with chemotherapy exhibits a synergistic antitumor activity, with successful eradication of well-established tumor xenografts that resist treatment with either mAb or drug alone. A Phase I clinical trial has established the safety of repeated administration of human-mouse chimeric mAb 225 at concentrations that maintain receptor-saturating blood levels for up to 3 months. Phase I trials exploring mAb 225 treatment in combination with doxorubicin, cisplatin, or paclitaxel are ongoing.

Introduction
The overall theme of this symposium in honor of Dr. Emil J Freireich is the importance of pursuing hypothesis-based research to improve the treatment of cancer. It is in this spirit that I review the research that I and my colleagues have carried out in my laboratory, pursuing an understanding of receptor blockade as a potential anticancer therapy.

Hypothesis 1
An antibody that can prevent the binding of a growth factor to its receptor can inhibit proliferation of cultured cells.

This postulate grew from the research of Gordon H. Sato, my colleague and collaborator at the University of California, San Diego, and others who showed during the 1970s that the requirement for serum in cell culture was primarily due to the need for specific polypeptide growth factors and growth promoters (1). The polypeptides best characterized at the time were insulin (now known to substitute for insulin-like growth factor 1), transferrin, and EGF.3 Furthermore, the pioneering work of Stanley Cohen and others had defined the receptor for EGF as a transmembrane tyrosine kinase (2), and Sporn and Todaro (3) and others had shown that ligands such as TGF-α could activate receptors such as the EGF receptor in an autocrine fashion.

Our laboratory produced and screened many hybridomas to identify mAbs that were able to: bind to the receptor with affinity comparable to the natural ligands; compete with binding of the natural ligands; and immunoprecipitate the receptor to facilitate quantitative studies. mAbs 225 IgG2a and 528 IgG1 met these criteria and were able to inhibit cell proliferation (4, 5). Both mAbs were found to prevent the capacity of EGF to activate receptor tyrosine kinase (6, 7). These original studies were performed primarily with the A431 squamous carcinoma cell line, which had been found to express extremely high levels of EGF receptors, in the range of 1–2 million receptors/cell. This is in comparison with 10,000 receptors/cell or fewer on nonmalignant cells in culture.

In subsequent studies, the antibodies have been shown to inhibit EGF receptor tyrosine kinase and proliferation of a variety of cultured malignant epithelial cell lines that express both EGF receptors and TGF-α, including breast (8, 9), colon (10–12), lung (13), kidney (14), and prostate (15, 16). Even more pronounced inhibition of growth was observed when the mAbs were added to cultures of nontransformed cells (5, 17, 18). We postulated that inhibition of proliferation resulted from the blockade of autocrine activation of EGF receptors by TGF-α (19). In support of this interpretation, anti-EGF receptor mAb 455, which was a poor inhibitor of ligand binding, did not inhibit proliferation of the cultured cell lines (5). This interpretation depends on the assumption that for EGF receptor stimulation by the autocrine pathway, receptor activation by ligand (TGF-α) occurs on the cell surface and cannot occur intracellularly following the biosynthesis of these two molecules. We were able to provide evidence that this is indeed the case for EGF receptors (20), in contrast to data from other investigators on the platelet-derived growth factor receptor autocrine activation pathway.

Hypothesis 2
Blockade of EGF receptor activation can inhibit the growth of malignant tumors.

In 1984, research on oncogenes and research on growth factors were linked in an exciting and unanticipated way when it was discovered that the v-erbB oncogene, which had been discovered in chickens, coded for a truncated version of the EGF receptor that was constitutively active (21, 22). Evidence rapidly accumulated that many of the newly discovered oncogenes were, in fact, coding for proteins that mediated the signal transduction pathways that regulate cell growth (reviewed in Ref. 23). Therefore, our mAbs were interfering with a potentially oncogenic pathway, suggesting the possibility of a role in anticancer therapy. Over the next few years, many publications
documented that EGF receptor levels are commonly elevated in pathological specimens of epithelial malignancies (24–28).

In vivo effects of treatment with anti-EGF receptor mAbs were assayed using xenografts of human tumors in athymic mice. Administration of receptor-saturating concentrations of either 225 IgG1 or 528 IgG2a mAb intraperitoneally for 3 weeks, beginning within a few days of tumor cell implantation s.c., caused a dose-dependent, durable inhibition of A431 squamous carcinoma cell growth (29). Experiments with 111In-labeled mAb demonstrated that anti-EGF receptor mAb could selectively image tumor xenografts bearing elevated levels of EGF receptors (30). Successful prevention of tumor growth was subsequently achieved against breast, colon, and prostatic adenocarcinoma cell lines and cervical squamous carcinoma cell lines (10, 19, 31–34). A F(ab')2 225 fragment administered in vivo could produce nearly comparable antitumor activity (35), suggesting that mAb-mediated inhibition of growth did not require an immune mechanism. However, in nude mice treated a week or more after tumor cell inoculation, therapy with mAb 225 failed to eliminate well-established xenografts of most tumor cell lines tested. An exception was the DiFi colorectal adenocarcinoma cell line, which displayed the highest sensitivity to mAb 225 of all cell lines that we have studied. Well-established xenografts of DiFi cells were completely eradicated after mAb treatment initiated a month following tumor cell implantation (10).

Hypothesis 3

The mechanism of mAb-mediated inhibition involves down-regulation of EGF receptors.

Others had demonstrated that binding of natural ligand to EGF receptors causes dimerization of receptors, followed by transphosphorylation of tyrosine residues by the activated tyrosine kinases (36). Receptor dimerization also followed exposure to the bivalent mAb, but this did not cause tyrosine phosphorylation in intact cells (37). Our mAbs were found to recognize human but not murine EGF receptors, whereas EGF from either species could bind equally well to both human and murine receptors. These observations suggested that the mAbs were binding to an epitope on the receptor that was different from the actual binding site for ligand.

We found that the monovalent Fab fragment of mAb 225 was less effective than bivalent F(ab')2 or complete mAb in its capacity to inhibit activation of EGF receptor tyrosine kinase activity. In parallel, antibody bivalency was required to down-regulate receptors from the cell surface into the cytoplasm (37). To explain the requirement of bivalency for optimal inhibition of kinase activation, we noted that mAb and ligand are in a competitive equilibrium for binding to EGF receptors. We reasoned that when bivalent mAb binds to a receptor, the receptor is down-regulated. However, when monovalent mAb fragment binds, both the receptor and the antibody fragment remain on the cell surface, and the monovalent mAb can equilibrate off of the receptor; this leaves the receptor free to bind to ligand, followed by activation and down-regulation.

In additional studies, we demonstrated that TGF-α, which activates EGF receptors in the autocrine stimulation pathway, may have preferential access to receptors, compared with mAb, because the TGF-α is in closer proximity to the receptors. We observed that in cultured tumor cell lines, the majority of TGF-α molecules remain bound to the cell surface membrane in the pro-TGF-α transmembrane form and are not released from this precursor molecule into the surrounding culture medium by proteolytic cleavage (38).

Hypothesis 4

The reduction in cell proliferation that is mediated by anti-EGF receptor mAb is due to modulation of the molecules that regulate cell cycle progression.

Progression through different cell cycle stages is controlled at a series of checkpoints, at which both external and internal signals act to regulate the cell cycle (39). This regulation is mediated by a family of CDKs and their corresponding activating partners, the cyclins (39). In mammalian cells, the main cyclin-CDK complexes regulating movement through G1 and S phases are cyclin D-CDK4/CDK6, cyclin E-CDK2, and cyclin A-CDK2, acting primarily in middle to late G1 phase, the G1/S phase boundary, and S phase, respectively. Progression through G1 requires orderly activation of these different CDKs (40). One of the critical substrates of G1 CDKs is the Rb protein and related proteins, the phosphorylation and subsequent release of which of bound transcription factors such as E2F are required for G1 to S-phase transition (40).

We began our studies of the mechanism of antireceptor mAb activity in experiments with DiFi cells, because of their exquisite sensitivity to EGF receptor blockade. When these cells were exposed to saturating concentrations of mAb 225, the cultures accumulated in G1 (12, 41). Under these conditions, Rb remained hypophosphorylated (12). To explore the mechanism of this inhibition, we assayed the activities of the CDKs associated with Rb phosphorylation during traversal through the G1 phase of the cell cycle. CDK2 activity was assayed with added histone H1 as substrate in immunoprecipitates from DiFi cells formed with anti-CDK2 antibody. We observed that the addition of mAb 225 to cultures produced marked CDK2 kinase inhibition within 24 h. In contrast, there was little change in the activity of CDK6 or CDK4 kinases, measured using glutathione S-transferase-Rb as substrate (41).

To determine the explanation for the reduced CDK2 kinase activity, we first measured the amounts of CDK2, cycle E, and cyclin A by Western blotting and found no decrease in protein levels in cells treated with mAb 225 (41). We then assayed inhibitors of CDK and found that mAb 225 stimulated a 2–3-fold increase in the level of p27KIP1 protein. The increased p27KIP1 was found to be associated with CDK2 but not CDK4/6. Furthermore, removal of p27KIP1 from cell extracts could deplete them of inhibitory activity against CDK2. Changes in the amount of p21WAF1 were not observed.

The observation that mAb 225-induced accumulation of cells in G1 was accompanied by elevated levels of p27KIP1 inhibitor of CDK activity in DiFi colon adenocarcinoma cells was followed by similar findings in studies with A431 squamous carcinoma cells (42), DU145 prostate adenocarcinoma cells (16), and the immortalized but nonmalignant breast cell line MCF10A (18). In the malignant cell lines, except for DiFi, G1 arrest was never complete in response to mAb 225-mediated
EGF receptor blockade, whereas over 98% of mAb-treated nonmalignant MCF10A cells accumulated in G1. In these cell lines, cyclin D levels and CDK6 activity were also found to be modified to varying degrees, which had not been the case for DiFi cells. Thus p27KIP1-mediated inhibition of CDK activity appears to be a generalized response to blockade of EGF-receptor kinase by antireceptor antibody. DiFi cells were found to be uniquely sensitive to deprivation from EGF receptor kinase activity. In fact, receptor blockade in cultures of these cells eventually resulted in activation of programmed cell death. These epithelial cells appeared to be unique auxotrophs for TGF-α/EGF and required receptor activation for survival. All other cells studied either arrested completely in G1 (nonmalignant cells) or slowed growth with accumulation in G1 (malignant cells) in response to EGF receptor blockade but did not respond to receptor blockade with loss of viability.

**Hypothesis 5**

Blockade of EGF receptors may augment the antitumor activity of chemotherapy.

For therapy, we wished to augment the efficacy of anti-EGF receptor mAb so that antitumor activity could be obtained against well-established xenografts and, eventually, human tumors in patients. We noted a report that treatment with cisplatin enhanced the activity of a single dose of an anti-EGF receptor mAb in preventing growth of a tumor xenograft (43). This stimulated us to systematically test the effect of combining mAb 225 treatment with the chemotherapeutic agents most commonly used against epithelial malignancies.

Experiments with well-established (>0.4 cm³) A431 cell xenografts explored the antitumor effects of combined therapy with doxorubicin and anti-EGF receptor mAb (31). Doxorubicin alone at the maximum tolerated dose slowed tumor growth. Treatment with optimal doses of mAb (1 mg twice a week) also reduced the tumor growth rate. In contrast, the combination treatment with doxorubicin and mAb resulted in a major antitumor effect, with tumor elimination in all of the animals. Experiments with xenografts of MDA468 breast adenocarcinoma cells gave similar results. Treatment with a control, nonspecific IgG given in combination with doxorubicin resulted in no change in tumor growth compared with doxorubicin alone.

Parallel studies were carried out using mAb 225 or mAb 528 in combination with the maximum tolerated dose of cisplatin. In these studies against well-established A431 cell xenografts, mAb alone or cisplatin alone had insubstantial effects, but combination therapy again produced cures in tumor-bearing mice that were followed for over 6 months (44).

A third series of experiments was carried out with paclitaxel and mAb 225 against MDA468 breast adenocarcinoma cells. In this case, the tumors were quite sensitive to paclitaxel therapy, and suboptimal drug doses were used to assess the effect of concurrent treatment with mAb 225. The results again demonstrated elimination of well-established xenografts by combined therapy (32).

The mechanisms of these additive effects are under investigation in our laboratory. We favor an interpretation of our findings that implicates checkpoint regulation of the cell cycle as the activator of cell death in this experimental model. When cells are functioning properly, deprivation from the signaling pathways activated by essential growth factors activates the G1 checkpoint known as the restriction point, and the cells arrest in G1. Likewise, cells damaged by chemotherapy arrest typically in G2-M to repair alterations in DNA, tubulin, or other molecules. Malignant cells appear to be able to disobey checkpoints in some situations, without jeopardizing cell survival. This was seen when mAb 225 was added to most tumor cell cultures, which resulted in incomplete G1 arrest. We hypothesize that when tumor cells simultaneously disobey two checkpoint signals (activated by mAb 225 and by chemotherapy), this becomes intolerable and results in cell death. Another way of conceptualizing this is to consider that in the face of chemotherapeutic damage, which signals the cell to pause for repair, the requirement for a growth factor for cell cycle traversal is converted to requirement of the growth factor for cell survival. Thus, malignant epithelial cells damaged by chemotherapy now act like DiFi cells, and when deprived of EGF receptor kinase activity, they can no longer survive. There is ample precedent for growth factors acting as survival factors in hematopoietic cell lines, in cultures of nerve cells, and in epithelial cells driven to proliferate by constitutive expression of myc (45, 46). A corollary of this hypothesis is the prediction that nonmalignant epithelial cells, which obey the checkpoint signals, may be less susceptible to cytotoxicity from this combination therapy.

**Hypothesis 6**

Combination therapy with chemotherapy plus anti-EGF receptor mAb may be effective treatment against the common human epithelial cancers.

We propose a novel method for intensifying the antitumor activity of chemotherapy against epithelial tumors, using concurrent treatment with chemotherapy plus an agent blocking signal transduction from the EGF receptor. This hypothesis is being tested in patients with advanced epithelial cancers. In the initial clinical study, murine mAb 225 was administered in escalating single i.v. doses to successive patients with advanced squamous carcinoma of the lung, a tumor that invariably expresses high levels of EGF receptors (47). The antibody was labeled with 111In to permit pharmacological and nuclear scanning studies. The results showed marked uptake of 225 mAb in the liver, visualization by gamma camera of primary tumors, and all metastases larger than 1 cm in diameter (determined by computed tomographic scan or X-ray) with mAb doses of 40 mg or more. Serum levels of mAb adequate to saturate EGF receptors (20 nM) with mAb doses of 100 mg or more, lack of toxicity, and presence of human anti-mouse antibodies after 2 weeks. This was followed by Phase I studies with a human-chimeric form of the antibody, designated C225. These studies used unlabeled antibody. Escalating doses were administered to different subjects, first as single i.v. infusions, and then as repeated weekly infusions for up to 12 doses. Saturing levels in the blood were again achieved and maintained with the weekly doses of 100 mg/m², without evidence of toxicity. There was stabilization of disease in a number of the patients receiving repeated doses of antibody (48). No anti-human immune responses were detected.

Phase IB/IIA clinical trials have been initiated to test
combination therapy with C225 plus chemotherapy in patients with tumors expressing high levels of EGF receptors. C225 is being combined with doxorubicin for advanced prostate cancer, with cisplatin for advanced head and neck and lung cancer, and with paclitaxel for breast cancer.

Summary

We have demonstrated the capacity of an antireceptor mAb to block activation of signal transduction from EGF receptors. Combination therapy with anti-EGF receptor mAb plus chemotherapy is effective against well-established human tumor xenografts. This novel form of combination therapy may be useful in a large number of patients with epithelial malignancies.

We hypothesize that the mechanism of action of combined treatment with C225 mAb and chemotherapy involves simultaneous activation of two checkpoints regulating cell cycle progression, resulting in activation of cell death in the malignant cells.

References


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