A humanized monoclonal antibody to carcinoembryonic antigen, labetuzumab, inhibits tumor growth and sensitizes human medullary thyroid cancer xenografts to dacarbazine chemotherapy

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Abstract
A variety of observations have shown that carcinoembryonic antigen (CEA) is associated with growth and metastasis of cancers, including correlation of CEA serum levels with poor clinical outcome, mediation of cell-cell adhesion by CEA, and involvement of CEA in the immune recognition of tumors and apoptotic pathways. The purpose of this study was to investigate the effect that an anti-CEA monoclonal antibody (MAb) may have on the growth of medullary thyroid cancer (MTC), a CEA-expressing tumor, alone and in combination with chemotherapy. Antitumor effects were evaluated in a nude mouse-human MTC xenograft model. Using the TT MTC cell line grown s.c., we compared tumor growth in untreated mice with that of mice given the humanized anti-CEA MAb labetuzumab or an isotype-matched control MAb. The effects of time of administration post-tumor injection, MAb dose response, specificity of response, and combination with dacarbazine (DTIC) chemotherapy were studied. The humanized anti-CEA MAb, labetuzumab, has direct, specific, antitumor effects in this model, without conjugation to a cytotoxic agent. In addition, labetuzumab sensitizes these tumor cells to chemotherapy with an effective drug in this model, DTIC, without increased toxicity. Significant delays in tumor growth were caused by the MAb therapy or chemotherapy alone; however, the combination of these agents was significantly more effective than either agent given as a monotherapy or use of an irrelevant MAb in this model. The superiority of the combined modality treatment argues for the integration of CEA MAb therapy into chemotherapeutic regimens for MTC management and possibly other CEA-expressing neoplasms. [Mol Cancer Ther 2004;3(12):1559 – 64]

Introduction
Although medullary thyroid cancer (MTC) confined to the thyroid gland is potentially curable by total thyroidectomy and central lymph node dissection, disease recurs in ∼ 30% to 50% of these patients. The 5-year survival rate for all types of MTC is between 78% and 91%, and the 10-year survival rate is between 61% and 75% (1). These facts, as well as the limited value shown by chemotherapy and radiation therapy in disseminated disease, have led to the search for novel treatment modalities. Alternative strategies have been explored in recent years including gene therapy approaches (2), dendritic cell vaccination (3), and monoclonal antibody (MAb) therapies (4–6).

The expression of carcinoembryonic antigen (CEA) in MTC has been well documented for over 25 years and this has led to the exploitation of radiolabeled anti-CEA MAbs for therapy of this disease. Numerous reports on the association of CEA with MTC have been published, including detection of CEA in serum, immunohistologic detection in paraffin sections of MTC biopsies, and scintigraphic detection of MTC lesions using radiolabeled anti-CEA antibodies (7–10). The availability of a human MTC cell line, TT (11), has allowed examination of treatment options in a preclinical setting. TT cells express a high level of CEA, and 131I- and 90Y-labeled anti-CEA MAbs specifically target TT tumors and cause significant antitumor effects in nude mice bearing these xenografts (4). In addition, the combination of radioimmunotherapy and chemotherapy augments the antitumor effects of either treatment alone in the xenograft model, without a significant increase in toxicity (12, 13). Clinically, excellent targeting of MTC has been found with radiolabeled anti-CEA antibodies (5, 14), and antitumor effects have been achieved with 131I-labeled anti-CEA antibodies (6).

Based on these observations and the large body of literature indicating that CEA is associated with growth and metastasis of cancers (15–17), we investigated the effect that an unlabeled anti-CEA MAb may have on the growth of a CEA-expressing MTC tumor alone and in combination with chemotherapy with dacarbazine (DTIC). Here, we show that the unlabeled humanized anti-CEA MAb, labetuzumab, has antitumor efficacy in MTC, without conjugation to a cytotoxic agent. However, combined therapy of the anti-CEA MAb with DTIC augments the antitumor effects of antibody or chemotherapy alone, without increased toxicity to the host.
Materials and Methods

Monoclonal Antibodies, Cell Lines, and Chemotherapy

TT, a human MTC cell line, was purchased from the American Type Culture Collection (Rockville, MD). The cells were grown as monolayers in DMEM (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum, penicillin (100 units/mL), streptomycin (100 μg/mL), and l-glutamine (2 mmol/L). The cells were routinely passaged after detachment with trypsin-0.2% EDTA.

MN-14 is a class III anti-CEA (CD66e, CEACAM-5) MAb, reacting with CEA and unreactive with the normal cross-reactive antigen, biliary antigen, and meconium antigen (16). The construction and characterization of the humanized forms of MN-14 (labetuzumab) and LL2 (epratuzumab), the anti-CD22 MAb used here as a negative isotype-matched control, have been described previously (15, 18). Both antibodies were provided by Immunomedics, Inc. (Morris Plains, NJ). The antibodies were purified by protein A chromatography.

The choice of DTIC as the chemotherapeutic agent for these studies and the dose schedule used were based on results of our earlier work (12). DTIC was purchased from Florida Infusion, Inc. (Palm Harbor, FL). In our previous study, the maximum given dose of DTIC was selected based on the dose of the drug given clinically to humans on a mg/m² basis (300 mg/m², days 0, 1, and 2; equivalent to 1.08 mg per dose in a 0.0036 m² mouse). The present study was designed using submaximal doses of the drug, so that any putative advantage due to the combination of agents would not be masked by the general sensitivity of this tumor model to DTIC. DTIC was given at 75 μg per dose, on days 2 to 4 post-tumor cell injection, by i.p. injection.

In vivo Studies

Tumors were propagated in female nu/nu mice (Taconic Farms, Germantown, NY) at 6 to 8 weeks of age by s.c. injection of 2 × 10⁸ washed TT cells, which had been propagated in tissue culture. Antibodies were injected i.v., via the lateral tail vein, into the tumor-bearing animals. Details on the quantities of antibodies injected and the time of administration are indicated in the Results section for each study. Results are given as the mean ± SE. Tumor size was monitored by weekly measurements of the length, width, and depth of the tumor using a caliper. Tumor volume was calculated as the product of the three measurements. Statistical comparisons were made using Student’s t test to compare tumor volumes and area under the growth curves. Toxicity was evaluated by weekly measurement of body weights. Animal studies were done under protocols approved by the Institutional Animal Care and Use Committee.

Results

To study the effect of labetuzumab on the growth of TT tumors in nude mice, the MAB was given either as a single i.v. injection (0.5 mg labetuzumab per dose) given 1 or 11 days post-tumor cell injection or as weekly i.v. injections (0.25 mg labetuzumab per dose) initiated either 1, 3, or 7 days after inoculation of TT cells. Figure 1A shows the tumor growth curves of animals treated with a single injection of 0.5 mg labetuzumab 1 or 11 days later (●, untreated; ■, day 1 treated; ▲, day 11 treated) or (B) given weekly i.v. injections (0.25 mg labetuzumab/dose) initiated either 1, 3, or 7 days after inoculation of TT cells (●, untreated; ●, day 1 treated; ▲, day 3 treated; ■, day 7 treated). Points, means of respective treatment groups; bars, SE.
of seven to eight animals were studied. Significant differences in mean tumor sizes ($P < 0.05$) between the untreated group and all three treatment groups were observed. However, the difference in mean tumor size between the untreated mice and the day 7 treatment group was only significant at one time point, day 28. Day 1–treated mice yielded significant differences from 21 to 77 days, and day 3–treated mice yielded significant differences from 21 to 70 days. *t* Test analysis of the area under the growth curves indicated significant growth inhibition for the groups treated with labetuzumab either 1 or 3 days after TT cell administration compared with the untreated group. However, this analysis did not reach the 95% confidence limit for a difference between the untreated group and the group treated on day 7 ($P = 0.057$ at 5 weeks). No significant differences in body weights were caused by any of the treatments.

Figure 2 summarizes the results of a study on the specificity of the antitumor response. The effect of unlabeled labetuzumab on the growth of TT tumors in nude mice was compared with that of an isotype-matched control humanized MAb, epratuzumab (anti-CD22 IgG1), and the murine anti-CEA MAb MN-14. MAbs (0.5 mg per mouse) were given i.v. 1 day after inoculation of the TT cells followed by three additional weekly doses of 0.5 mg per mouse. Groups of 15 animals were studied. The growth inhibition, without effect on body weight, shown in the experiment summarized in Fig. 1 was confirmed in this study. Significant differences in mean tumor sizes ($P < 0.05$) between the labetuzumab and the untreated groups were observed starting at day 23. At day 37, the mean tumor volume in the group treated with labetuzumab was 42.7% of the untreated control animals. Treatment with murine MN-14 yielded results similar to the labetuzumab. Treatment with epratuzumab (anti-CD22 humanized MAb) did not slow tumor growth; instead, there was a small (insignificant) increase in growth rate. For example, at day 37, 87% of the tumors treated with labetuzumab were <0.5 cm$^3$ compared with 40% of the untreated group and 29% of the epratuzumab-treated group. *t* Test analysis of the area under the growth curves showed significant differences ($P < 0.05$) between the untreated group and the groups treated with either labetuzumab or murine MN-14 but not the group treated with epratuzumab. In addition, the labetuzumab group was significantly different from the epratuzumab group but not from the murine MN-14-treated animals.

The effect of dose of labetuzumab on the growth of TT tumors in nude mice was also evaluated. Antibody doses were given 1 day after the TT cells, then weekly until the termination of the study. Weekly doses ranged from 0.125 to 2.0 mg labetuzumab per mouse in groups of six mice. Significant differences in mean tumor sizes and area under the growth curves between the untreated group and all treatment groups were observed (Fig. 3), again without significant changes in body weight. For example, between days 21 and 49, mean tumor volume in the two lowest labetuzumab treatment groups were 27% to 40% of the size of tumors in the untreated animals. Treatment with the lower doses, 0.125 and 0.25 mg, seemed to be more effective than treatment with the higher doses, although the difference did not reach statistical significance. Thus, dose-dependent results were not observed in this study. Indeed, dose-dependent results have not been commonly observed in antibody treatment studies, even clinically, so this should not be unexpected.

Combining naked anti-CEA MAb therapy and chemotherapy was evaluated by comparing the growth of TT in untreated mice with those treated with DTIC alone, labetuzumab alone, or a combined treatment of DTIC with...
labetuzumab. Animals were given i.p. injections of labetuzumab at 100 μg per dose on days 2 to 5, 7 to 11, and 15 and then every 7 days. Groups of 10 animals were studied. DTIC was given at 75 μg per dose on days 2 to 4. In the combined modality group, treatment with the anti-CEA MAb was initiated on the same day as the first dose of DTIC. Growth curves of TT tumors in mice given these treatment regimens are shown in Fig. 4. All of the treatment groups had significant improvement in efficacy compared with the untreated animals, without increased toxicity. At 7 weeks, P values for t test of area under curve were 0.017, 0.037, and 0.0002 for DTIC-only, labetuzumab-only, and DTIC + labetuzumab, respectively, compared with untreated animals. Combined therapy of the naked anti-CEA MAb with DTIC augments the antitumor effects of either antibody or chemotherapy alone. At 7 weeks, P values for t test of area under curve of the combined treatment were 0.0026 and 0.0002 compared with the DTIC-only and labetuzumab-only treatment groups, respectively. Eight of 10 mice treated with DTIC plus labetuzumab exhibited no palpable tumor at 7 weeks post-treatment compared with only 1 of 10 in the DTIC-only group and none in the labetuzumab-only and untreated groups. Mean tumor volumes at 7 weeks were 0.018 ± 0.012, 0.284 ± 0.062, 0.899 ± 0.172, and 1.578 ± 0.503 cm³ for the DTIC plus labetuzumab, DTIC, labetuzumab, and untreated groups, respectively. No significant differences in body weights were caused by any of the treatments. Thus, labetuzumab yields antitumor efficacy in MTC, without conjugation to a cytotoxic agent. However, combined therapy of the naked anti-CEA MAb with DTIC augments the antitumor effects of antibody or chemotherapy alone, without increased host toxicity.

**Figure 4.** Effect of combination treatment, DTIC + labetuzumab. Animals were given s.c. injections of TT cells and either left untreated ( ), or given an i.p. injection of labetuzumab at 100 μg per dose on days 2–5, 7–11, and 15 and then every 7 days ( ); DTIC given at 75 μg per dose on days 2–4 ( ▲ ); or combined modality treatment consisting of the same labetuzumab and DTIC schedules with the labetuzumab treatment initiated on the same day as the first dose of DTIC ( △ ). Points, means of respective treatment groups; bars, SE.

### Discussion

CEA, a member of the immunoglobulin supergene family (19), was first described as a tumor marker by Gold and Freedman (20). CEA was originally thought to be a tumorspecific antigen of colorectal cancer. However, it was later found to be present in a diverse number of carcinomas, benign tumors, and diseased tissues as well as in the normal human colon (21). CEA has been shown to mediate cell-cell adhesion through homotypic and heterotypic interactions, which in turn have implicated a role for CEA in various aspects of tumorigenesis (22). In one report, CEA levels have been indicated to have prognostic significance with a more favorable prognosis in CEA-positive tumors compared with that of CEA-negative undifferentiated tumors (23). However, other articles report the opposite, a more aggressive phenotype with increased metastases was associated with high levels of CEA in preclinical models (24, 25). The role of CEA in hepatic metastasis may be based on the ability of circulating CEA to induce Kupffer cells to release cytokines. These cytokines in turn may activate liver endothelial cells, enabling them to bind to circulating CEA-positive tumor cells (26). CEA has also been implicated in the immune recognition of tumors, possibly by interfering in natural killer cell-tumor cell adhesion (27), as well as having an effect on cellular differentiation, probably by interfering with normal intercellular adhesion forces (28).

The many roles attributed to CEA in neoplasia suggest that targeting CEA with an anti-CEA MAb may affect the growth of CEA-expressing tumors by interfering with its function(s). Indeed, antibody binding to receptor molecules on tumor cells has been shown to affect the behavior and proliferation of these tumor cells. For example, antitumor efficacy of unlabeled MAbs reacting with tumor-associated antigens has been reported in non-Hodgkin’s lymphoma with anti-CD20 (rituximab; ref. 29) and anti-CD22 (epratuzumab; ref. 30) MAbs as well as in colon cancer using the anti-epithelial glycoprotein-2 MAb, 17-1A (31), and A33 (32), in breast cancer using anti-HER-2 (33), and in colonic and other tumors with anti–epidermal growth factor receptor (34) MAbs. These unconjugated MAbs may inhibit tumor growth by blocking intracellular signaling or other biological activities of their respective antigens or by stimulating natural immunologic functions, such as antibody-dependent cell-mediated cytoxicity (ADCC) or complement-mediated lysis. It is also interesting that most of these antibodies seem to have their greatest effect in combination with anticancer drugs (32–35). Common mechanisms of action among the various antibodies given with drugs include cell cycle arrest, potentiation of apoptosis, and inhibition of angiogenesis, resulting in augmentation of the antitumor effects of chemotherapy and, in some cases, radiation therapy (34). In the case of CEA antibodies, such as labetuzumab studied here, a contribution in terms of apoptosis enhancement needs to be considered because of the observation that CEA plays a regulatory role in apoptosis (36). Using a CEA-targeted ribozyme in human colon cancer cells to regulate CEA...
levels, it has been reported that CEA does not affect cell cycle or proliferation but does protect the cells from undergoing apoptosis under various conditions, including confluent growth, UV light, IFN therapy, and treatment with 5-fluorouracil (36).

In this study, we show that labetuzumab, an antibody specific to CEA, has antitumor efficacy in MTC without conjugation to a cytotoxic agent and can sensitize these tumor cells to chemotherapy with an effective drug in this model. The current experiments show that CEA-binding antibodies have direct antitumor effects and can also chemosensitize human tumor cells in vivo. In nude mice bearing human MTC xenografts, labetuzumab was shown to significantly delay tumor growth. Differences in mean tumor sizes between the anti-CEA MAb–treated and the untreated groups were observed beginning at 3 weeks and lasting at least 2 months. The effect of delaying the treatment with naked anti-CEA MAB was studied up to 11 days after tumor cell implantation. The antibody yielded statistically significant inhibition of tumor growth when given up to 3 days post-tumor cell inoculation but not when given 7 or 11 days after the tumor cells were transplanted. Treatment with an isotype-matched non–CEA-binding control MAb, epratuzumab (anti-CD22 humanized MAb), did not slow tumor growth, demonstrating that the effect of labetuzumab on tumor growth is antigen specific. The observation that the effect is greatest when treatment is given at an early time point after injection of tumor suggests that the antibody is most active during early spread of the tumor cells. Although this remains to be proven, we find this possibility intriguing and of possible significance in terms of blocking the establishment of new metastatic sites. In addition, the anti-CEA MAB was able to enhance the effect of chemotherapy; the combination of chemotherapy with DTIC and anti-CEA MAB treatment was significantly more effective than either treatment alone. Of the mice given the combined modality treatment, 80% had complete responses compared with 10% in the chemotherapy-only group and none in the untreated and MAB-only groups. Thus, unlabeled labetuzumab has shown antitumor efficacy in MTC without conjugation to a cytotoxic agent, and combined therapy of the naked anti-CEA MAB with DTIC augments the antitumor effects of antibody or chemotherapy alone, without increased host toxicity.

These studies corroborate a recent report of Blumenthal et al. (17), in which in vivo antiproliferative and antimetastatic effects of labetuzumab were observed in CEA-expressing human colonic carcinoma models. Significant survival increases were obtained when the animals were given granulocyte-macrophage colony-stimulating factor to increase their peripheral WBC counts or when the tumors were treated with IFN to up-regulate CEA expression. In addition, labetuzumab potentiated the effect of two common drugs used in colorectal cancer, 5-fluorouracil and CPT-11. Blumenthal et al. (17) did not observe induction of apoptosis or complement-mediated cytotoxicity by labetuzumab in in vitro studies in colon cancer cell lines. However, similar to anti-HER-2 and anti–epidermal growth factor receptor MAbs (37, 38), labetuzumab was shown to inhibit tumor cell proliferation by ADCC (17). The lack of complement-mediated cytotoxicity against tumor cells in vitro may be explained by the high levels of complement regulatory factors, CD55 and CD59, in these colon cancer cell lines (17), which are known to inhibit complement-mediated cytotoxicity (39, 40). Thus, the work of Blumenthal et al. (17) suggests that labetuzumab induces effector-cell function against CEA-positive human colonic tumor cells as shown by the in vitro ADCC results and the importance of WBCs for in vivo activity.

In contrast, other anti-CEA MAbs have been reported to induce complement-mediated cytotoxicity as well as ADCC activity against a CEA-expressing gastric cancer cell line (41). However, CD55 and CD59 levels were not measured in this cell line. These authors also found that their fully human anti-CEA MAB inhibited tumor growth in vivo in human gastric cancer xenografts grown in severe combined immunodeficient mice (41), thus agreeing with the results reported here and by Blumenthal et al. (17).

An important role of CEA in tumorigenic potential was also shown by transfection of human colon cancer cells with a CEA antisense-expressing vector (42). Parental cells with high CEA expression were highly tumorigenic, whereas clones with decreased CEA expression showed considerably lower tumorigenicity. It seems likely, therefore, that in addition to functioning by stimulating natural immunologic functions (ADCC and/or complement-mediated cytotoxicity), anti-CEA MAbs may have antitumorigenic effects because they reduce or block CEA and thus may overcome the putative role of CEA in protecting tumor cells from apoptotic stimuli, as experienced with antisense oligonucleotides (42). It is possible that the chemosensitization we observed for the anti-CEA antibody may also be caused by overcoming inhibition of apoptosis by CEA, including apoptosis induced by certain cytotoxic drugs.

Thus, the observations of the antiproliferative effects of CEA MAB and its potentiation of chemotherapy has been shown in MTC as well as in other cancer model systems with various levels of CEA expression and with anticancer drugs with diverse mechanisms of action. These effects on tumor growth and metastasis are supportive of the general view of the role of CEA in tumor biology, despite the need for additional studies to fully understand the mechanisms underlying these observations. The superiority of the combined modality treatment argues for the integration of CEA MAB therapy into chemotherapeutic regimens for MTC management. Clinical trials are needed to assess these principles in patients with MTC and other CEA-expressing cancers.

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References