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## Carcinoembryonic antigen antibody inhibits lung metastasis and augments chemotherapy in a human colonic carcinoma xenograft

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**Abstract** Purpose: In addition to its use as a blood marker for many carcinomas, elevated expression of carcinoembryonic antigen (CEA, CD66e, CEACAM5) has been implicated in various biological aspects of neoplasia, especially tumor cell adhesion, metastasis, the blocking of cellular immune mechanisms, and having antiapoptosis functions. However, it is not known if treatment with anti-CEA antibodies can affect tumor metastasis or alter the effects of cytotoxic drugs. Methods: In vitro, human colon cancer cell lines were treated with anti-CEA MAb IgG<sub>1</sub>, hMN-14 (labetuzumab), to assess direct effects on proliferation, as well as antibody-dependent cellular cytotoxicity (ADCC), and complement-dependent cytotoxicity (CDC). In vivo studies were undertaken in nude mice bearing s.c. (local growth) or i.v. (metastatic model) GW-39 and LS174T human colon cancer grafts, to evaluate the MAb alone and in combination with either CPT-11 or 5-fluorouracil (5FU). Results: In vitro, labetuzumab did not induce apoptosis, nor did it affect tumor cell proliferation directly or by CDC, but it did inhibit tumor cell proliferation by ADCC. In vivo, labetuzumab did not increase median survival in the GW-39 metastatic model unless the mice were pretreated with GM-CSF to increase their peripheral WBC counts; GM-CSF alone was ineffective. Also, if GW-39 tumors were pretreated with IFN- $\gamma$  to up-regulate CEA expression threefold prior to i.v. injection, labetuzumab significantly increased median survival of the mice. When nude mice received labetuzumab with CPT-11 or 5FU, median survival increased significantly as compared to the drug or antibody alone. Conclusions: Labetuzumab, a CEA-specific MAb, induces effector-cell function in

vitro against CEA-positive colonic tumor cells, and also inhibits growth of lung metastasis when CEA expression is up-regulated or if peripheral WBCs are increased. The MAb also shows chemosensitizing properties.

**Keywords** ADCC · Apoptosis · CD66e · CPT-11 · 5-Fluorouracil · Immunotherapy

### Abbreviations

ADCC	Antibody-dependent cellular cytotoxicity
CDC	Complement-dependent cytotoxicity
BrdU	Bromodeoxyuridine
CDR	Complementarity-determining region
CEA	Carcinoembryonic antigen
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
5FU	5-Fluorouracil
GM-CSF or rM-GM-CSF	Recombinant murine granulocyte-macrophage colony-stimulating factor
HRP	Horseradish peroxidase
IFN- $\gamma$	Gamma interferon
LAK cells	Lymphokine-activated killer cells
LDH	Lactate dehydrogenase
MAb	Monoclonal antibody
MTD	Maximum tolerated dose
NK cells	Natural killer cells
PBMC	Peripheral blood mononuclear cell
pWBC	Peripheral white blood cell

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### Introduction

Several antibodies have emerged as effective cancer therapeutics, either by affecting the signaling function of their receptor targets, inducing apoptosis or cell-cycle phase disruption, evoking host immune responses,

enhancing chemosensitivity or radiosensitivity, or a combination of these factors. Examples include antibodies to CD20 in non-Hodgkin's lymphoma [18, 65], and to GA733-2 or EpCAM [2, 75], EGFR [52, 72], and HER-2/*neu* [61, 94] in certain solid tumors. The introduction and acceptance of such biological therapies has stimulated an intensive search for other suitable targets for antibody-mediated cancer therapy.

One such candidate target is carcinoembryonic antigen, or CEA (also known as CD66e or CEACAM5) [6, 34], which was first described in 1965 as a gastrointestinal oncofetal antigen [22], but is now known to be overexpressed in a majority of carcinomas, including those of the gastrointestinal tract, the respiratory and genitourinary systems, and of the breast [23, 27, 33, 93, 101].

The CEA gene family is present in two defined clusters on chromosome 19, between 19q13.1 and 19q13.3, represented by 29 gene-like sequences, and is considered to be a subset of the immunoglobulin gene superfamily, with analogous variable and constant regions [34, 78]. CEA is anchored in the cell membrane via a glycosyl phosphatidyl inositol moiety [38], showing temperature- and  $\text{Ca}^{2+}$ -independence typical of classical intercellular adhesion receptors. Further, CEA has been shown to be involved in both homophilic (CEA to CEA) and heterophilic (CEA binding to non-CEA molecules) binding [7, 79, 97], suggesting to some that it is an intercellular adhesion molecule involved in cancer invasion and metastasis [47, 110].

Alternatively, it has been proposed that CEA acts as a signal-transducing protein that repulses cellular contact and increases tumor cell mobility, thus inducing cell migration and metastasis [31, 105]. Supporting this notion is the finding that after transplantation of colorectal tumors into nude mice, the number of liver metastases increased from 2% to 48% following injection of CEA to the mice [41]; however, others have disputed the role of blood CEA in this setting [60]. It has also been reported that CEA overexpression can protect tumor cells from undergoing anoikis, or apoptosis from loss of cell contact with the extracellular matrix [80]. Still other studies have shown that CEA affects expression of various groups of cancer-related genes, especially cell cycle and apoptotic genes, protecting colonic tumor cells from various apoptotic stimuli, such as treatment with 5-fluorouracil (5FU) [95]. Therefore, CEA expression may be a means for cancer cells to overcome apoptosis-inducing therapies. Conversely, inhibition of, or binding to, CEA may result in antitumor effects or even enhance other apoptosis-mediated therapeutic interventions, such as with typical anticancer drugs, or the activation of proapoptotic pathways, as has been proposed for epidermal growth factor receptor (EGFR) antagonists [72]. Thus, after almost 4 decades of research involving CEA, its biological function is still the subject of considerable investigation and debate. In this article, we demonstrate for the first time that CEA-binding antibodies exert antimetastatic effects *in vivo* in appropriate settings of CEA expression, and also have chemosensitizing properties.

## Materials and methods

### Antibody

The MN-14 antibody is a second-generation murine MAb with tenfold higher affinity than the first generation NP-4 antibody [35], directed against a CEA-specific (CEA-CAM5 or CD66e), class III epitope according to Primus et al. [83], and shown subsequently [64] to bind to the A3B3 domain of Gold epitope group 3 of CEA [8]. The CDR-grafted (humanized) MN-14 anti-CEA IgG<sub>1</sub>, hMN-14 or labetuzumab [89], along with the murine MN-14 MAb [35], both supplied by Immunomedics (Morris Plains, NJ, USA), were purified by protein A and ion-exchange chromatography (Q-Sepharose; Pharmacia, Piscataway, NJ, USA). Purity was tested by immunoelectrophoresis, polyacrylamide gel electrophoresis using reducing and nonreducing conditions and size-exclusion high-pressure liquid chromatography. Previous studies have shown that the biodistribution, tumor targeting and pharmacokinetic behavior of labetuzumab was similar to that of murine MN-14 in nude mice [88, 89]. The F(ab)<sub>2</sub> fragment was prepared by pepsin digestion of the intact IgG, followed by passage over a protein A column. The control hA20 anti-CD20 humanized IgG<sub>1</sub> MAb [98] was provided by Immunomedics.

### CEA expression in cell lines

Expression of CEA was assayed in a labetuzumab cell-binding ELISA assay. One hundred thousand cells were added in growth media to wells of a 96-well microtiter plate. Labetuzumab and an isotype-matched negative control, anti-CD20 humanized MAb [98], were diluted in growth media and added to the cells in triplicate at a final concentration of either 1 or 5  $\mu\text{g}/\text{ml}$ . After incubation for 1 h the cells were washed by centrifugation of plates. Secondary antibody, HRP-goat antihuman IgG, Fc-specific, was added, and plates were incubated for 1 h at either 4°C or room temperature. The plates were washed as before, with a final wash in PBS. Bound antibody was detected with a luminescent HRP substrate (LumiGlo; KPL, Gaithersburg, MD, USA). The plates were read in an EnVision plate reader (Perkin-Elmer, Boston, MA, USA), using a luminescence protocol. Cell lines which gave values that were three times the negative controls were considered to be positive.

### Peripheral WBC determination

In some studies, mice were given 1  $\mu\text{g}/\text{day} \times 4$  days *i.p.* of recombinant murine granulocyte-macrophage colony-stimulating growth factor (GM-CSF) (Immunex, Seattle, WA, USA). Immediately after the last treatment with GM-CSF and prior to initiation of labetuzumab therapy, 50  $\mu\text{l}$  of heparinized blood was collected retro-orbitally. Red blood cells were lysed and white blood

cells were counted on a Becton Dickinson FACScan as described previously [11].

#### CEA immunohistochemistry

GW-39sc tumors were removed from nude mice and fixed with buffered formalin. Paraffin-embedded tumor samples were cut into 5.0- $\mu$ m sections. Tumor sections were deparaffinized, rehydrated, blocked, and exposed to 10  $\mu$ g/ml of murine anti-CEA MAb or an irrelevant control murine antibody (Ag-8, raised against murine myelomonocytic leukemia) as previously described [10]. After incubation for 45 min, slides were washed in PBS, and biotinylated goat antimouse secondary antibody applied for 30 min, using the Vectastain ABC Kit (Vector Labs, Burlingame, CA, USA). Endogenous peroxidase was destroyed by flooding the slides with 0.3% H<sub>2</sub>O<sub>2</sub> in methanol. Slides were then incubated with 25  $\mu$ l of avidin-horseradish peroxidase (HRP) conjugate (ABC) under humid conditions for 45 min. Excess ABC was washed off, and the sections were finally covered with 100  $\mu$ l of HRP substrate, 3,3'-diaminobenzidine tetrahydrochloride solution (Sigma; 100  $\mu$ g/ml diaminobenzidine in 0.1 M sodium acetate buffer, pH 6.0, with 0.01% [v/v] H<sub>2</sub>O<sub>2</sub> for 15 min). Slides were then counterstained briefly with hematoxylin.

#### Antibody-dependent cellular cytotoxicity (ADCC) assay

The ADCC activity was determined by an LDH release assay. Tumor cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA). Effector cells were PBMCs obtained from a healthy donor on the day of the experiment, using Lymphoprep lymphocyte separation medium (Accurate Chemical Co., Westbury, NY, USA). Effector cells were resuspended in assay medium (RPMI 1640 medium, 1% fetal bovine serum, penicillin [100 U/ml], streptomycin [100  $\mu$ g/ml]). Target cells were detached from flasks by trypsin-EDTA, washed with assay media, and incubated with antibodies at 1  $\mu$ g/ml for 40 min at 37°C in 96-well microtiter plates. Effector cells were added to the wells, and after 4 h, LDH release was assayed according to the manufacturer's protocol (CytoTox One Homogeneous membrane integrity assay; Promega, Madison, WI, USA). The average absorbance of triplicate determinations was used to calculate the percentage cytotoxicity, calculated as:

$$\% \text{ cytotoxicity} = (\text{experimental-target}_{\text{spontaneous}} - \text{effector}_{\text{spontaneous}}) / (\text{target}_{\text{maximum}} - \text{target}_{\text{spontaneous}}).$$

All statistical analyses were done by Student's *t* test.

#### Complement-dependent cytotoxicity (CDC) assay

The CDC assay was performed using CEA-positive (LS-174T, LoVo, CaPan-1) and CEA-negative cell lines

(HT-29, MCF 7, Calu3, Daudi), and human complement (Quidel, San Diego, CA, USA). Antibodies were diluted in the assay medium (RPMI 1640, 0.1% BSA, penicillin [100 U/ml], streptomycin [100  $\mu$ g/ml]) to 1  $\mu$ g/ml final concentration. Cells were resuspended in media and added to 96-well microtiter plates, and antibodies and complement were added and incubated for 2 h. Cell viability was assayed by the addition of C<sub>12</sub>-resazurin (Molecular Probes, Eugene, OR, USA) to 5  $\mu$ M. After 5 h, the plates were read in an EnVision plate reader (PerkinElmer) at E<sub>x</sub>544 and E<sub>m</sub>590. Dose-response curves were generated from the mean of triplicate determinations, and statistical analyses were performed by Student's *t* test.

#### CD55 and CD59 expression in cell lines

Expression of CD55 and CD59 was measured in a cell-binding ELISA assay. The assay was similar to that described for CEA expression with the following modifications. Phycoerythrin-labeled anti-CD55, anti-CD59 (BD Biosciences, Pharmingen, San Diego, CA, USA), or an isotype-matched negative control, murine IgG (Immunotech, Marseilles, France), were used at a final concentration of 5  $\mu$ g/ml. The cells were incubated on ice for 1 h and washed. Bound antibody was detected by reading in an EnVision fluorescence plate reader at E<sub>x</sub>480 nm and E<sub>m</sub>570 nm. Cell lines giving values that were three times the negative control were considered to be positive.

#### Proliferation assay

Cells were plated into 96-well microtiter plates at 10,000 cells per well, containing RPMI 1640 medium with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100  $\mu$ g/ml). After 24 h, serial dilutions of antibodies (0.039–10  $\mu$ g/ml) in complete growth media were added. A crosslinking antibody, goat antihuman IgG, Fc-specific (Jackson Immunoresearch, West Grove, PA, USA) was added at 20  $\mu$ g/ml. After 4 days, proliferation was assessed in a multiplex metabolic assay (CellTiter Aqueous<sub>96</sub>; Promega, USA) and a DNA synthesis assay (BRDU ELISA; Roche Applied Sciences, Indianapolis, IN, USA), according to the manufacturers' protocols. Dose-response curves were generated from the mean of triplicate determinations, and statistics performed by Student's *t* test.

#### In vivo therapy studies

Female athymic nude mice (6–8 weeks old) were purchased from Taconic (Germantown, NY, USA). Survival therapy studies were performed using our CEA-positive GW-39 intrapulmonary micrometastasis model

(GW-39iv; [9, 26, 89]). The GW-39 tumor has been maintained as a serially transplanted signet-ring cell human colon carcinoma line since 1966 [26], and expresses  $\sim 160 \mu\text{g}$  CEA/g tissue [25]. Subcutaneous GW-39 human colorectal tumors grown in nude mice were used to prepare a 10% or 5% cell suspension. Cells (30  $\mu\text{l}$ ) were injected i.v. into the caudal vein of 5- to 6-week-old female nude mice (Taconic). This model results in approximately 50–100 nodules developing in the lungs with resulting median survival of 7–9 weeks [9, 87]. For studies to assess the effect of higher neutrophil counts on anti-CEA Ab therapy effects, GM-CSF (1  $\mu\text{g}/\text{day} \times 4$  days) was given i.p. to mice on days  $-4$  through  $-1$ . GW-39 cells (30  $\mu\text{l}$  of a 5% suspension) were introduced i.v., and hMN-14 treatments were initiated the same day (100  $\mu\text{g}/\text{day} \times 14$  days and twice weekly for the duration of the study). Labetuzumab was initiated on either day 0 or day 3 after cell implantation, and administered once daily for 14 days and twice weekly thereafter for the duration of the study at a dose of 100  $\mu\text{g}/\text{day}$ . CPT-11 (Camptosar, Florida Infusion, Palm Harbor, FL, USA) was administered i.p. at a dose of 80–160  $\mu\text{g}$  daily for 5 days (10–20% of the MTD based on established doses in the literature [59]), starting on day 0 or day 3 after cell implantation. 5-Fluorouracil (5-FU; Sigma, St Louis, MO, USA) was given at an i.p. dose of 0.4 mg/day  $\times$  5 days (100% of the MTD). For some studies, the GW-39 tumor used for the transplant inoculum came from mice that received 100,000 U of IFN- $\gamma$  (Florida Infusion) twice daily for 4 days to up-regulate CEA expression, as described earlier [30]. Body weight was monitored weekly and animal survival recorded. Results were analyzed by the Kaplan-Meier

estimated survival curves, and significance was determined with the log-rank comparison of survival curves. Median survival time for each treatment group was also determined. For bulky disease studies, GW-39sc (10% tumor cell suspension in 200  $\mu\text{l}$  derived from GW-39 tumors) or LS174Tsc (1e7 cells) was implanted s.c. in nude mice. After 2.5 weeks, when tumor size reached  $\sim 0.3 \text{ cm}^3$ , treatment was initiated. Tumor size was determined weekly by the product of 3 diameters determined by caliper measurements, and change in tumor size was calculated. The percentage growth inhibition (%GI) at 21 days posttreatment was determined: % GI = [(tumor size of untreated group – tumor size of treated group) / (tumor size of untreated tumor group)]  $\times 100\%$ . All studies utilized ten mice per treatment group. Animal studies were performed under protocols approved by the Institutional Animal Care and Use Committee.

## Results

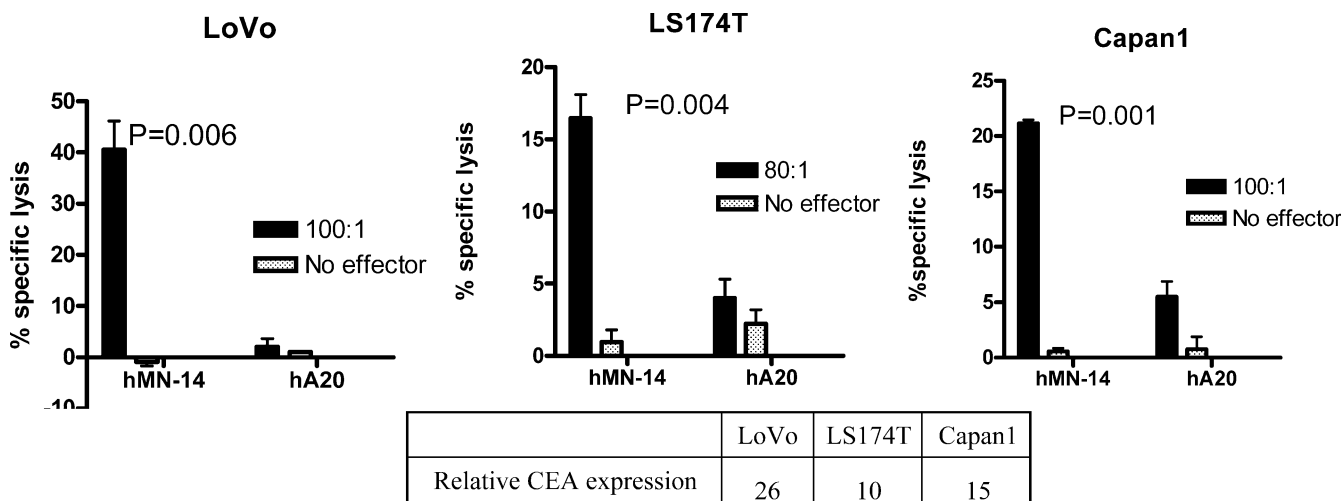
### CEA expression in cell lines

The CEA expression in cell lines was determined in a labetuzumab cell-binding ELISA. The colon cancer cell lines, LS-174T and LoVo, were positive, as was the pancreatic cancer cell line, CaPan-1. HT-29 was classified as CEA-negative in this assay, since binding with labetuzumab was not observed under the conditions tested.

### ADCC activity of labetuzumab

Labetuzumab's potential to mediate killing of tumor cell lines was investigated using PMBCs as effector cells. Labetuzumab was able to induce ADCC activity in both CEA-expressing colon cancer cell lines, LS174T and LoVo, and a CEA-expressing pancreatic tumor cell line, CaPan-1 (Fig. 1). The percentage lysis was significantly

**Fig. 1** ADCC on CEA-positive tumor cell lines. Cells were incubated with labetuzumab (hMN-14) and hA20 (control anti-CD20 humanized MAAb) in the presence of human peripheral blood mononuclear cells. The cells were incubated for 4 h at 37°C. After 4 h, LDH release was assayed by a homogeneous fluorometric assay. Error bars represent standard deviations. *P* values were determined by Student's *t* test





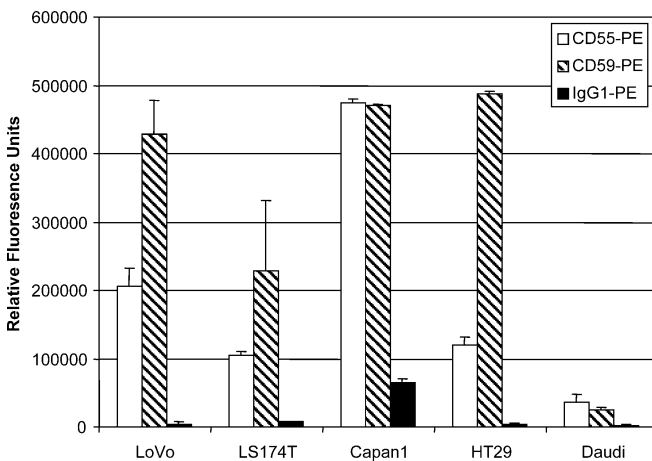
different from an isotype-matched negative control, anti-CD20 MAb, hA20 [37]. No ADCC activity was observed on CEA-negative tumor cell lines, HT-29, Calu3, SKOV4, MCF-7, and Daudi, to which labetuzumab failed to bind.

### CDC activity of labetuzumab

The ability of labetuzumab to mediate complement-dependent lysis was investigated *in vitro* using human complement. A panel of cell lines was tested, including both CEA-positive and CEA-negative cell lines. No CDC activity induced by labetuzumab was found in any cell lines tested, LS-174T, LoVo, and CaPan-1, but in a positive control, CDC activity was confirmed by treating CD20-positive lymphoma cells with anti-CD20 IgG1 MAb [98]. The expression of the complement inhibitory receptors CD55 and CD59 was assayed on the tumor cell lines used in the CDC assay. All CEA-positive tumor cell lines expressed high levels of CD55 and CD59 compared with the low levels of CD55 and CD59 of the CDC-sensitive Daudi lymphoma cell line (Fig. 2).

### Direct effects of labetuzumab on tumor cell growth

No induction of apoptosis was effected by labetuzumab in the absence of effector cells in a 4-h ADCC assay. The direct effect of labetuzumab on tumor cell line growth was investigated in a proliferation assay. Proliferation was assessed both by a metabolic assay, which is proportional to the number of viable cells, and by a BrdU-incorporation assay, which measures cellular DNA synthesis. CEA-positive (LS-174T) and CEA-negative (HT-29) colon cancer cell lines were incubated with labetuzumab in the presence or absence of a cross-linking antibody, goat antihuman IgG, Fc-specific, for 4 days. An isotype-matched negative control antibody,

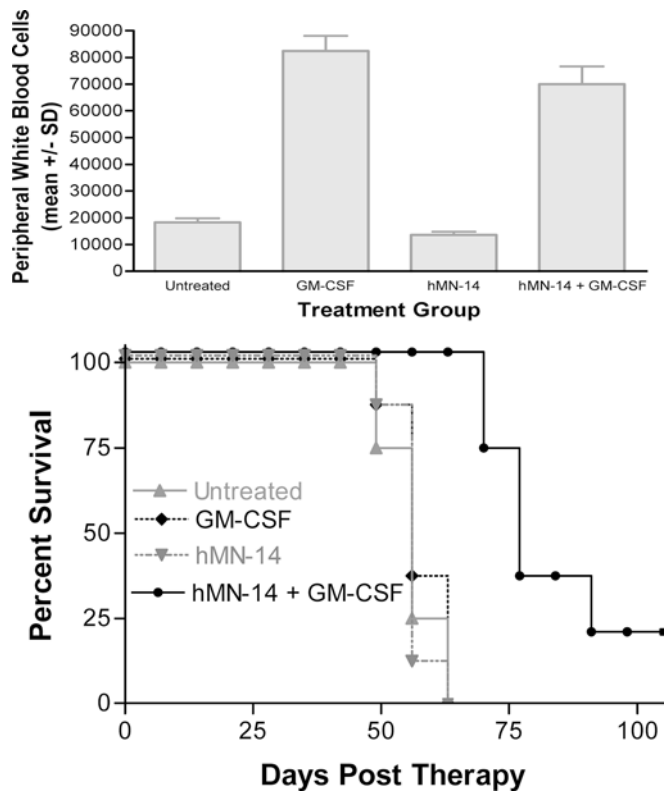


**Fig. 2** Expression of CD55 and CD59 on tumor cell lines. Expression of CD55 and CD59 was determined by direct cell binding ELISA as described in "Materials and methods"

hA20, also was included. There was no difference in the proliferation of any of the cell lines in the presence of labetuzumab, with or without a cross-linking antibody, compared with the negative control antibody (data not shown).

### Effects of labetuzumab alone or in GM-CSF-pretreated mice

The ability of labetuzumab to increase survival of GW-39iv mice under baseline conditions and with increased numbers of effector cells was also evaluated. A 4-day pretreatment of nude mice with rM-GM-CSF (1  $\mu$ g/day) resulted in 4.5- to 5.0-fold increases in total pWBCs ( $P < 0.001$ ; Fig. 3, top panel). A comparison of therapeutic effects of labetuzumab in mice with baseline pWBCs versus mice with GM-CSF-up-regulated pWBC levels is shown in Fig. 3 (bottom panel). GM-CSF treatment alone did not affect survival, nor did repeated dosing with labetuzumab. However, the median survival in the labetuzumab-treated group (7.5 weeks) was increased by 42.7–10.7 weeks in those mice with higher



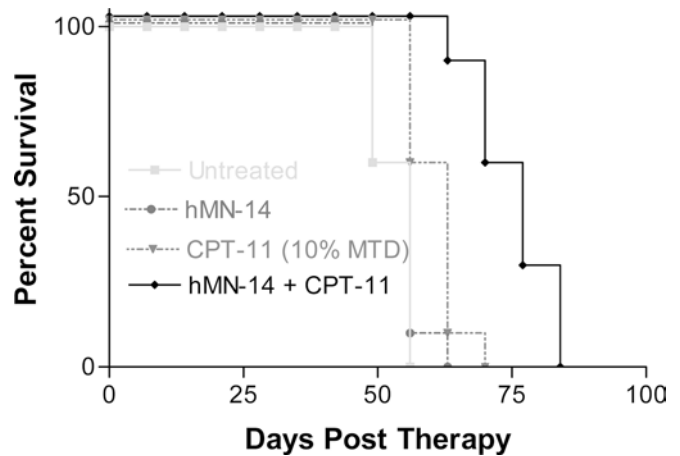
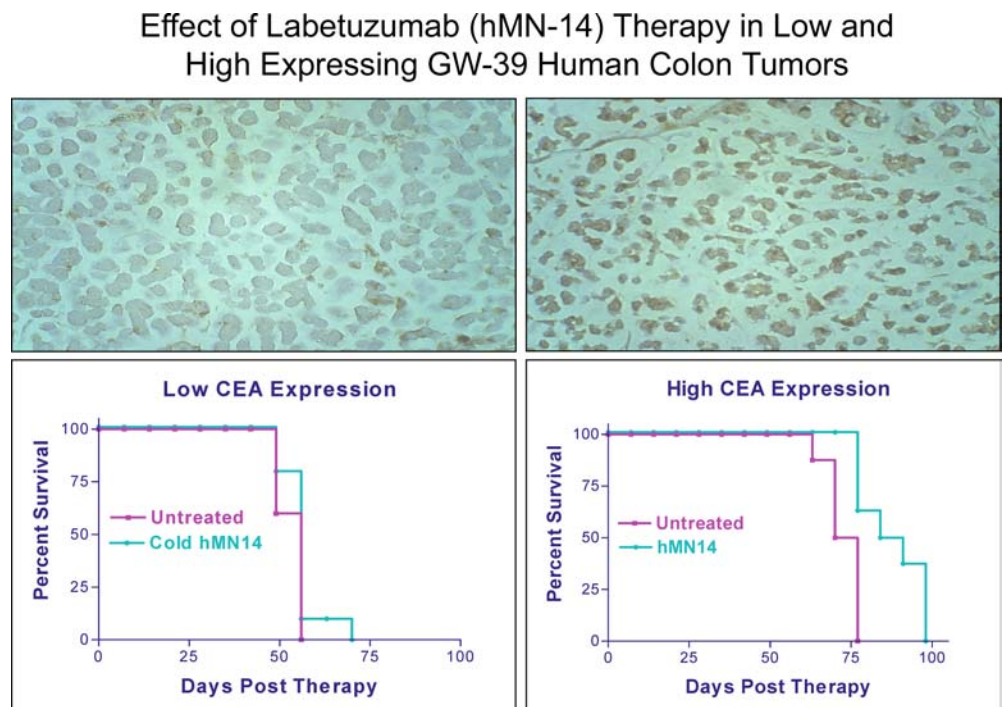
**Fig. 3** Effects of GM-CSF on labetuzumab (hMN-14) therapy in the GW-39 intrapulmonary micrometastasis model. GM-CSF (1  $\mu$ g/day  $\times$  4 days) was given *i.p.* to mice on days  $-4$  through  $-1$ , blood was collected on day 0 of the study, and pWBCs were enumerated by flow cytometry. The mean  $\pm$  SD of each treatment ( $N = 10$ ) was recorded. GW-39 cells (30  $\mu$ l of a 5% suspension) were introduced *i.v.*, and hMN-14 treatments were initiated the same day (100  $\mu$ g/day  $\times$  14 days and twice weekly for the duration of the study). Survival of mice was monitored

pWBC counts resulting from GM-CSF treatment ( $T=9.858$ ,  $P<0.005$ ).

#### Role of CEA expression on the therapeutic effects of labetuzumab

We postulated that the amount of tumor CEA expressed might influence the ability of naked anti-CEA antibody therapy. Stock tumors derived from the s.c. models and then used to establish the GW-39iv model have a heterogeneous CEA expression (160  $\mu\text{g}$  CEA/g tumor by ELISA; [39]), while s.c. tumors derived from mice given a 4-day treatment with IFN- $\gamma$  have much higher and more homogeneous CEA expression (Fig. 4, top panels) that remains  $\sim 3$ -fold elevated for 10–13 days after the 4-day dosing regimen with IFN- $\gamma$  (data not shown). When low CEA-expressing tumors were used to establish the GW-39iv model, labetuzumab did not affect group survival ( $T=0.417$ ;  $P<0.9$ ; Fig. 4, lower left panel). In contrast, when IFN- $\gamma$ -treated tumors with up-regulated CEA expression were used to establish the same model, labetuzumab did improve median survival of mice bearing GW-39iv lung nodules from 10 to 12 weeks ( $T=5.598$ ;  $P<0.025$ ; Fig. 4, lower right panel). In contrast, IFN- $\gamma$ -pretreated tumors without antibody treatment did not show in vivo survival extensions comparable to those subsequently given labetuzumab, indicating that interferon alone does not account for the antimetastatic effects observed with labetuzumab therapy. From the outcome of these two studies, we conclude that although labetuzumab has limited activity in this relatively low CEA-expressing tumor model, it becomes effective when either the number of effector cells

**Fig. 4** Up-regulation of GW-39 CEA expression and therapeutic effects of labetuzumab. Mice bearing stock GW-39 tumors were either left untreated or treated with IFN- $\gamma$  (100,000 IU  $2\times$ /day  $\times$  4 days). Tumor was removed and an aliquot was placed into formalin for immunohistochemistry to measure CEA expression. The remainder was used to prepare a 5% cell suspension that was introduced i.v. into mice. Treatment with labetuzumab (hMN-14) was initiated the same day and survival of mice was monitored in each treatment group ( $N=10$ )

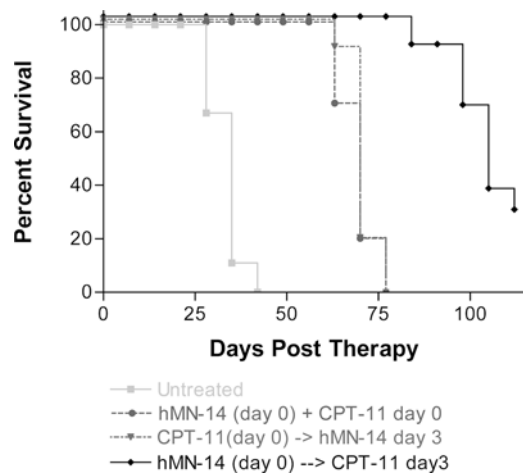


**Fig. 5** Therapeutic effects of labetuzumab (hMN-14) together with CPT-11 in the GW-39 lung micrometastasis model. The tumor model was initiated with 30  $\mu\text{l}$  of a 10% GW-39 cell suspension. Groups of ten mice were either left untreated, treated with labetuzumab (hMN-14 IgG; 100  $\mu\text{g}/\text{day} \times 14$  days and twice weekly thereafter starting on day 1), CPT-11 (10% of the MTD or 80  $\mu\text{g}/\text{day} \times 5$  days on days 1–5), or the combination of labetuzumab + CPT-11. Survival was monitored weekly

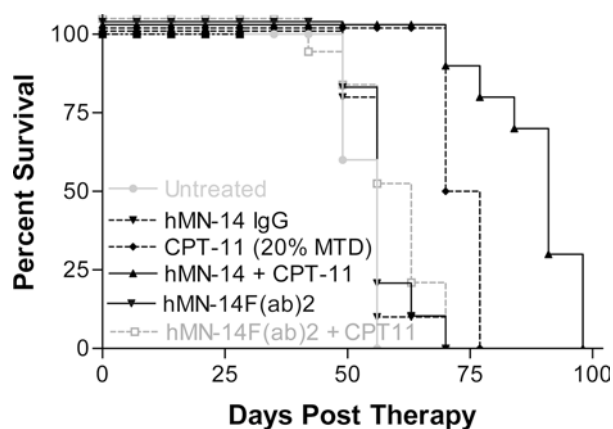
or the amount of target antigen in the tumor is increased.

#### In vivo chemosensitization by labetuzumab

In addition to direct antitumor effects, labetuzumab was found to augment the antigrowth activity of two anti-cancer drugs applied in these models. Figure 5 shows survival curves for the GW-39iv model left untreated, treated with labetuzumab alone, CPT-11 alone, or the



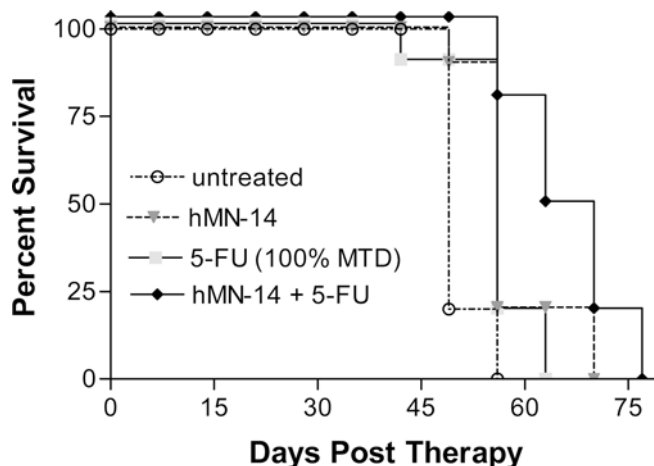
**Fig. 6** Comparison of survival of mice bearing the GW-39 lung micrometastasis model (10% suspension) left untreated, or given labezumab (hMN-14) and CPT-11 (5 days schedule at 10% of the MTD) when both are started on day 0, or CPT-11 is started on day 0 and labezumab is started on day 3, or when labezumab is started on day 0 and CPT-11 is started on day 3



**Fig. 7** Survival curves for mice bearing GW-39 intrapulmonary micrometastases and left untreated or treated with labezumab (hMN-14) alone or labezumab F(ab)<sub>2</sub> (100 µg/day × 14 days and twice weekly for the duration of the study), a 20% MTD of CPT-11 (160 µg/day × 5 days) alone, the IgG and CPT-11, or the F(ab)<sub>2</sub> and CPT-11 together. Antibody treatment was initiated the day of cell implantation (30 µl of a 10% cell suspension) and CPT-11 was started on day 3

combination of antibody and drug, both initiated on the same day. Under baseline conditions (normal pWBC counts and low CEA expression), labezumab did not alter animal survival. Low-dose CPT-11 (10% MTD) increased median survival by 1.4 weeks from 6.8 to 8.2 weeks ( $P < 0.05$ ), whereas CPT-11 + labezumab extended median survival to 10.4 weeks ( $T = 8.02$ ;  $P < 0.005$  compared with CPT-11 group; and  $T = 9.879$ ;  $P < 0.005$  compared with untreated mice).

Since maximal antibody accretion occurs 3 days post injection, we questioned whether initiating labezumab treatments 3 days before CPT-11 dosing would further enhance the therapeutic effect of the



**Fig. 8** Survival of mice with GW-39 intrapulmonary micrometastases left untreated or treated with labezumab (hMN-14; 100 µg/day × 14 days and twice weekly for the duration of the study), or drug alone (5FU; 100% of the MTD or 400 µg/day × 5 days) or a combination of labezumab starting on day 0 and drug starting on day 3. Each treatment group started with ten mice

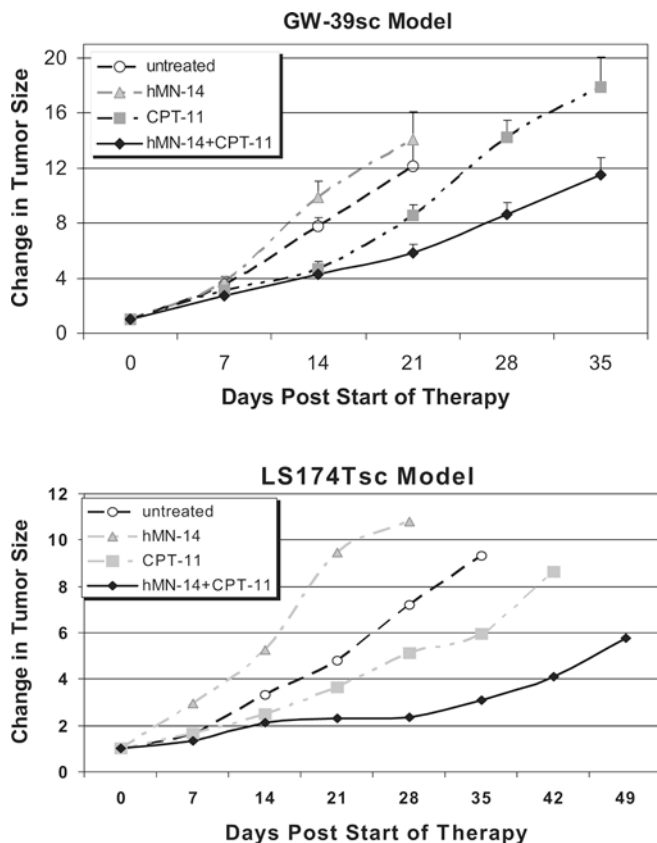
combined modality treatment by allowing high antibody uptake and chemosensitization in vivo. Figure 6 demonstrates that the 3-day pretreatment with labezumab followed by CPT-11 increased median survival by 51.1–14.2 weeks ( $T = 12.22$ ;  $P < 0.001$  compared with labezumab + CPT-11 initiated together or CPT-11 initiated on day 0 and labezumab initiated on day 3, both with a median survival of 9.4 weeks).

The chemosensitizing effect of hMN-14 IgG was not observed in the absence of the Fc portion of the antibody, if the labezumab bivalent F(ab)<sub>2</sub> fragment was used in combination with CPT-11 (Fig. 7). Median survival of CPT-11-treated mice in this study was 11.0 weeks; labezumab + CPT-11, 13.5 weeks; and labezumab F(ab)<sub>2</sub> + CPT-11, 9.3 weeks. The drug-augmenting effect was also absent if a nonspecific intact IgG (Ag8) was used in combination with CPT-11 (data not shown).

5-Fluorouracil, another commonly used chemotherapeutic in the management of colorectal cancer, also was evaluated in combination with labezumab. In the GW-39 xenograft model, this drug is much less efficacious than CPT-11, as evidenced by the need to use a 100% MTD of 5FU to observe any effect on survival, compared with the 10% MTD of CPT-11 which typically resulted in a 20% increase in survival. Labezumab was able to increase the efficacy of 5FU ( $T = 4.376$ ;  $P < 0.05$ ; Fig. 8), improving median survival from 7.4 weeks in the 5FU-treated group to 9 weeks in the 5FU + labezumab-treated group.

In the final experiments, we evaluated whether labezumab could also enhance the effect of CPT-11 in bulky s.c. GW-39 xenografts, as it does for micrometastatic disease in the GW-39iv model, and in a second human colonic s.c. xenograft using LS174T tumors. Figure 9 (upper panel) demonstrates that the percentage growth inhibition (%GI) of labezumab + CPT-11





**Fig. 9** Growth curves for GW-39 tumors (*upper panel*) and LS-174T tumors (*lower panel*) grown s.c. in nude mice. Mice were left untreated, or treated with labetuzumab (hMN-14), CPT-11 (20% MTD), or the combination with antibody initiated 3 days before the 5-day course of CPT-11. Initial tumor size was  $\sim 0.3 \text{ cm}^3$  at the time treatment began. The mean  $\pm$  SD of the change in tumor size from day 0 in each treatment group ( $N = 10$ ) is shown

was 51.8% on day 21 post treatment, compared with CPT-11-treated mice (29.7%) or labetuzumab-treated mice (0%). Similarly, the results with the LS174T model (Fig. 9, lower panel) shows that %GI for labetuzumab + CPT-11 was 67.2% on day 28 post treatment, compared with CPT-11-treated mice (28.9%) or labetuzumab-treated mice (0%). Thus, the chemosensitizing effect of labetuzumab occurs for both minimal, disseminated disease and larger, established, s.c. tumor models.

## Discussion

These investigations contribute several new findings regarding CEA function and the possible role of CEA antibodies in the control of cancer dissemination and the enhancement of chemotherapy. The observation that a specific CEA antibody, targeting the A3B3 (CD66e) epitope [64] that is not shared with other CEA-family members [6, 34], inhibits human colonic cancer spread to the lungs and mortality in nude mice is the first direct support of earlier in vitro studies suggesting a role of CEA in cancer spread and metas-

tasis by pretreating of CEA-expressing metastatic colonic cancer cells with anti-CEA antibody in vitro and then showing a reduction of metastasis in vivo [53]. However, our findings appear to contradict recent experiments on adhesion and differentiation-inhibitory activities of CEA, which indicated that monovalent Fab fragments could reverse tumorigenic effects whereas divalent antibodies, as used in our studies, would be expected to increase the tumorigenic effects of CEA [99].

The alteration of CEA-expressing colonic cancer spread and lethality in a xenograft model by a CEA-specific antibody is supportive of the general view of the role of CEA in tumor biology, despite the mechanisms related thereto still being in dispute. Studies with in vitro and in vivo cell lines, as well as clinical observations, support the view that high CEA production is directly related to malignancy, particularly enhanced metastasis [28, 41, 42, 46–50, 54, 73, 74, 100, 102, 106, 107, 110]. CEA was found to function as an intercellular adhesion molecule promoting cell aggregation by homotypic interactions, because cells transfected with cDNA for CEA formed aggregates, and this aggregation was completely inhibited by CEA antibody treatment of the cells in vitro [7, 79]. The transfectants could have their ability to form liver metastases after splenic injection inhibited by in vitro treatment with CEA antibody [36, 37]. In another line of experimentation, CEA has been found to block cellular differentiation [16], it had tumorigenic effects alone and in combination with other oncogenes [85], and deregulated CEA expression in human colonocyte lines disrupted cell polarization, tissue architecture, and increased tumorigenicity [43].

These past observations on the various mechanisms of CEA are supported by the finding that anti-CEA antibody treatment of CEA-expressing tumor cells can inhibit the homotypic binding in vitro and also the size and number of hepatic metastases [110]. Further, this group found that anti-CEA antibody did not influence tumor cell growth in vitro or the incidence of hepatic metastases or splenic weight, which suggested to them that complement-mediated or antibody-dependent cell-mediated cytotoxicity was not involved by pretreatment of the cells with anti-CEA antibody in vitro, and concluded that the increased metastatic potential to the liver is related to the increased homotypic binding through CEA [110]. However, some CEA MAbs have been reported to have complement-mediated cytotoxicity against tumor cells in vitro [45], contrary to our own experience herein with the GW-39 tumor model, and supported by the high levels measured for the regulatory factors, CD55 and CD59, which are known to inhibit complement-mediated cytotoxicity [21, 40]. These authors also found that their fully human CEA MAb inhibited colon tumor growth in vivo [45], thus agreeing with the results reported here. Using effusion samples from patients with colorectal cancer, it has also been demonstrated that degree of aggregation correlated with CEA expression,



and that *in vitro*, aggregation was completely inhibited by anti-CEA antibody, supporting the notion that CEA on the tumor cell surface mediated this aggregation [55]. Further, since it was shown that the ability to accrete CEA is restricted to Kupffer cells [4] and alveolar macrophages [103], thus being tissue-specific, we speculate that these mechanisms may also explain why CEA antibodies, as shown in our studies, control metastasis to the lungs of mice injected *i.v.* with human colonic tumor cells.

A raised serum CEA level also has been associated with poor cell-mediated immunity and poor survival [54], which is consistent with other evidence that CEA affects lymphocyte function [15, 32, 51, 70, 71, 84] and can interfere with NK and LAK cell activity [81, 82, 86]. These results suggest that CEA may represent an escape mechanism protecting cancer cells from cellular immune attack [70]. In fact, in one study, combining a chimeric mouse–human anti-CEA antibody with LAK cells and IL-2 showed evidence of antitumor activity *in vitro* and *in vivo* [86]. However, these authors failed to report any study of the naked antibody without the use of LAK cells and IL-2, but they did demonstrate that the chimeric antibody mediated ADCC *in vitro*, which is in agreement with the findings reported here as well as other reports that CEA antibodies induce ADCC in cell cultures of colorectal cancer [13, 44, 45, 57, 91, 111]. Finally, our observation that treating mice with GM-CSF enhances the antitumor effects of labetuzumab is consistent with the known effects of this and other cytokines in enhancing antigen expression [20] and ADCC [19].

The *in vivo* studies showing that the humanized anti-CEA antibody, labetuzumab, can enhance the therapeutic effects of two cytotoxic drugs used frequently in colorectal cancer therapy—5-fluorouracil and CPT-11—in both *s.c.* and metastatic human colonic tumor cells propagated in nude mice, are the first to suggest such a role for CEA antibodies, and provoke speculation as to how CEA expression on tumor cells may affect the tumor's sensitivity to such drugs. One explanation may involve a role in blocking cell aggregation, such as by interfering with CEA's adhesion function [111]. The formation of multicellular aggregates of tumor cells has been shown to increase their resistance to chemotherapy agents, so that disruption of tumor spheroids results in increased growth formation and increased chemosensitivity of tumors *in vitro* or *in vivo* [29, 56, 96]. Thus, antibodies that disrupt cell–cell adhesion and prevent the formation of spheroids could increase tumor sensitivity to drugs, as has been shown for the GA733-2 antigen (EpCAM) targeted by the 17.1A antibody, edrecolomab [5]. GA733-2 is, like CEA, a homophilic adhesion receptor that can alter growth, invasion, and metastasis of a colorectal cancer cell line [63].

Another explanation for the chemosensitizing role of CEA antibody may be that inhibition of CEA affects apoptotic stimuli, including the effects of cytotoxic drugs. 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, interferon therapy, and treatment with 5FU [95]. Also, transfection of human colon cancer cells with a CEA antisense-expressing vector resulted in a decrease in CEA expression and low tumorigenicity, but such effects involved primary and not metastatic tumors in mice [3]. It seems likely, therefore, that reducing or blocking CEA by administering antibodies against this receptor may overcome this CEA protection in tumor to apoptotic stimuli, as experienced with antisense oligonucleotides [3]. Thus, certain anticancer drugs could have their apoptotic effects enhanced by blocking CEA production and/or expression.

Both the ADCC and chemosensitization effects observed with labetuzumab are similar to those reported for two other antireceptor antibodies of clinical interest—trastuzumab and cetuximab—and indeed their clinical applications appear to be most effective when combined with anticancer drugs [52, 61, 72, 94]. Common mechanisms of action among these 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, of radiation therapy [72]. In the case of CEA antibodies, such as the CEACAM5 MAb studied here, a contribution in terms of apoptosis enhancement needs to be considered because of the observation that inhibition of endogenous CEA can achieve this when CEA is overexpressed [80, 108], despite our failure to show direct apoptotic effects by labetuzumab in the tumors tested.

These initial observations of the *in vivo* antiproliferative and antimetastatic effects of a humanized CEA MAb, labetuzumab, and its potentiation of two common drugs used in colorectal cancer therapy, need to be confirmed and expanded in other tumor models having various levels of CEA expression and with anticancer drugs having diverse mechanisms of action, and eventually in patients. In a high CEA-expressing human medullary thyroid cancer xenograft, we also have shown that this CEA antibody can inhibit tumor cell growth and also augment the effects of dacarbazine, which is active in this cancer type (Stein et al., *Mol Cancer Ther*, *in press*).

In addition to its well-known role as a serum marker for a number of CEA-expressing malignancies [23, 27, 33], CEA also has gained attention as a target for radiolabeled antibodies [24], drug and toxin immunoconjugates [1, 17, 66, 67, 90, 92], photodynamic immunotherapy [12], cytokine immunoconjugates [62, 109], vaccines [39, 68, 69, 76], and diverse gene therapy strategies [14, 58, 64, 77, 104]. The studies reported here now suggest that CEACAM5-binding antibodies also have antitumor and chemosensitizing functions. Hence, these various lines of investigation attest that after 4 decades [22], CEA continues to be of major interest and focus in oncology.

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