Antiangiogenic and Antitumor Activity of Anti-Epidermal Growth Factor Receptor C225 Monoclonal Antibody in Combination with Vascular Endothelial Growth Factor Antisense Oligonucleotide in Human GEO Colon Cancer Cells

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ABSTRACT

Angiogenesis plays a key role in tumor growth and metastasis. The transforming growth factor α (TGF-α)-epidermal growth factor receptor (EGFR) autocrine pathway controls in part the production of angiogenic factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) in cancer cells. In this study, we have evaluated the antiangiogenic and antitumor activity of monoclonal antibody (MAb) C225, an anti-EGFR chimeric human-mouse MAb, alone and in combination with a human VEGF antisense (AS) 21-mer phosphorothioate oligonucleotide (VEGF-AS) in human GEO colon cancer cells. MAb C225 treatment determined a dose-dependent inhibition of VEGF, bFGF, and TGF-α production by GEO cells in \textit{vitro}. Treatment with VEGF-AS caused a selective inhibition in VEGF expression by GEO cells in \textit{vitro}. Treatment of immunodeficient mice bearing established, palpable GEO xenografts for 3 weeks with VEGF-AS or with MAb C225 determined a cytostatic reversible inhibition of tumor growth. In contrast, a prolonged inhibition of tumor growth was observed in all mice treated with the two agents, in combination with a significant improvement in mice survival compared with controls ($P < .001$), to MAb C225 ($P < .001$), or to VEGF-AS ($P < .001$) treated mice. All mice died within 4, 6, and 8 weeks after tumor cell injection in the control, VEGF-AS and MAb C225 groups, respectively. In contrast, 50% of mice treated with the combination of VEGF-AS and MAb C225 were alive at 13 weeks. Ten % of mice treated with VEGF-AS plus MAb C225 were alive at 20 weeks and had no histological evidence of GEO tumors. Immunohistochemical analysis of GEO tumor xenografts demonstrated a significant reduction of VEGF expression after treatment with VEGF-AS with a parallel reduction in microvessel count. MAb C225 treatment determined a reduction in the expression of VEGF, bFGF, and TGF-α with a reduction in microvessel count. Finally, a significant potentiation in inhibition of VEGF expression and little or no microvessels were observed in GEO tumors after the combined treatment with the two agents.

INTRODUCTION

Growth factors regulate cell proliferation and differentiation and are directly involved in neoplastic transformation (1–2). The TGF-α-EGFR autocrine pathway plays an important role in the development and the progression of human epithelial cancers, including colorectal cancer (3). Enhanced expression of TGF-α and/or EGFR has been detected in the majority of human carcinomas (3) and is generally an indicator of poor prognosis (4). For these reasons, the blockade of the TGF-α-EGFR autocrine pathway has been proposed as anticancer therapy (5–13). Different anti-EGFR blocking MAbs that inhibit the \textit{in vitro} and \textit{in vivo} growth of human cancer cell lines that express TGF-α and EGFR have been developed (14–17). MAb C225 is a chimeric human-mouse IgG1, binds to the EGFR, blocks ligand-induced activation of the EGFR tyrosine kinase, and is currently being evaluated in clinical studies in cancer patients (7, 18, 19).

Angiogenesis, the process leading to the formation of new blood vessels, plays a central role in the survival of cancer cells, in local tumor growth, and in the development of distant metastasis (20). The development of bone vessels within the tumor mass is regulated by the production of several growth factors and growth inhibitors (21). In this respect, different growth factors, such as bFGF, VEGF, and TGF-α, have been identified as positive regulators of angiogenesis and are secreted by cancer.

\textsuperscript{3}The abbreviations used are: TGF, transforming growth factor; EGFR, epidermal growth factor receptor; MAb, monoclonal antibody; MVC, microvessel count; bFGF, basic fibroblast growth factor; AS, antisense; CM, conditioned medium; EGF, epidermal growth factor; VEGF, vascular endothelial growth factor.
cells to stimulate normal endothelial cell growth through paracrine mechanisms (22–24). VEGF is a potent and specific mitogen for endothelial cells, activates the angiogenic switch in vivo, and enhances vascular permeability (24). Enhanced expression of VEGF has been observed in human cancer cell lines and in cancer patients with different malignancies including colorectal, breast, non-small cell lung, and ovarian cancers and is directly correlated with increased neovascularization, as measured by MVC within the tumor (24). The increasing understanding of the biological mechanisms of tumor-induced angiogenesis has stimulated the development of agents able to interfere with the molecules involved in this process (25, 26). Several approaches have been proposed for blocking VEGF-induced endothelial cell proliferation and subsequent tumor angiogenesis. An anti-VEGF MAb that inhibits the growth of a variety of human cancer xenografts in nude mice has been generated (27–29). This MAb has been recently humanized and is under clinical development (30). Another promising approach is the development of MAbs raised against the VEGF-specific flk-1/KDR receptor (31) or of selective inhibitors of the flk-1/KDR tyrosine kinase (32). Finally, experimental evidence has been provided for the potential therapeutic effect of blocking VEGF production by plasmid or viral expression vectors containing VEGF AS mRNA sequences or by VEGF AS oligonucleotides (33–37).

In the present study, we have evaluated the effects of treatment with the blocking anti-EGFR MAb C225 on the production of TGF-α, bFGF, and VEGF in GEO colon cancer cells in vitro and in vivo. We have also determined the ability of an antihuman VEGF phosphorothioate 21-mer antisense oligonucleotide (VEGF-AS; Ref. 37) to interfere with the production of VEGF in these cells. Finally, we have evaluated the antiangiogenic and antitumor activity on GEO tumor xenografts of the combined treatment with VEGF-AS and MAB C225.

MATERIALS AND METHODS

MAbs and Phosphorothioate Oligonucleotides. MAB C225, a human-mouse chimeric anti-EGFR IgG1 class MAB (7, 14, 18), was kindly provided by Dr. H. Waksal (ImClone Systems, New York, NY). The human VEGF-AS had the following sequence: 5′-TGGCTTGAAGATGTACTCGAT-3′ corresponding to nucleotides 261 to 281 of the human VEGF mRNA (37). A phosphorothioate oligonucleotide of the same length with a scramble sequence of oligonucleotides was used as control. The two oligonucleotides have been purchased from Primm (Milan, Italy).

Cell Cultures. GEO human colon cancer cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 20 mM HEPES (pH 7.4), 100 UI/ml penicillin, 100 μg/ml streptomycin, and 4 mM glutamine (ICN, Irvine, United Kingdom) in a humidified atmosphere of 95% air and 5% CO2 at 37°C.
Growth in Soft Agar. Cells (10^4 cells/well) were sus-
pended in 0.5 ml of 0.3% Difco Noble agar (Difco, Detroit, MI) sup-
plemented with complete culture medium. This suspension was laid-
er over 0.5 ml of 0.8% agar-medium base layer in 24-multiwell clus-
ter dishes (Becton Dickinson, Lincoln Park, NJ) and treated with dif-
f erent concentrations of oligonucleotides or of MAb C225. After 10 –14 days, the cells were stained with nitro blue tetrazolium (Sigma Chemical Co., St. Louis, MO), and colonies were counted as described previously (38).

Evaluation of TGF-α, VEGF, and bFGF Secretion. The concentration of TGF-α, VEGF, or bFGF in the CM ob-
tained from GEO cells was measured using commercially avail-
able ELISA kits and according to the manufacturers’ instructions. The ELISA kits for VEGF and for bFGF were purchased from R&D Systems, Inc. (Minneapolis, MN). The ELISA kit for TGF-α was purchased from Oncogene Research Products (Cambridge, MA). GEO cells were plated in 60-mm dishes (Becton Dickinson) and treated for 4 days with different concentrations of MAb C225, VEGF-AS oligonucleotide, or control oligonucleotide. Assays were performed using 24-h-collected, serum-free CM.

Western Blotting. Protein extracts (50 μg of total protein/lane) from GEO cells treated with different concentrations of VEGF-AS or of the control oligonucleotide were separated by SDS-PAGE on 12% precast gels (Bio-Rad Laboratories, Milan, Italy), transferred to nitrocellulose filters, and incubated with a rabbit polyclonal antihuman VEGF antiserum (Santa Cruz Biotechnologies, Inc., Santa Cruz, CA). Immunoreactive proteins were visualized by a chemiluminescence ECL Western blotting kit (Amersham, Milan, Italy).

GEO Xenografts in Nude Mice. Female BALB/c athymic (nu+/nu+) mice, 5–6 weeks of age, were purchased from Charles River Laboratories (Milan, Italy). The research protocol was approved, and mice were maintained in accordance to institutional guidelines of the University of Naples Animal Care and Use Committee. Mice were acclimated to the University of Naples Medical School Animal Facility for 1 week prior to injection of cancer cells. Mice received injections s.c. with 10^7 GEO cells.
GEO cells that had been resuspended in 200 μl of Matrigel (Collaborative Biomedical Products, Bedford, MA). After 7 days, when established tumors of approximately 0.2–0.3 cm³ in diameter were detected, 10 mice/group were treated i.p. with VEGF-AS alone (5 or 10 mg/kg/dose; days 1–5 each week for 3 weeks) or with scramble control oligonucleotide (10 mg/kg/dose; days 1–5 each week for 3 weeks). In a second series of experiments, groups of 10 mice bearing established GEO tumors of approximately 0.2–0.3 cm³ in diameter were treated i.p. with VEGF-AS alone (10 mg/kg/dose; days 1–5 each week for 3 weeks) or with MAb C225 alone (0.5 mg/dose, twice weekly on days 1 and 4 for 3 weeks), or with both drugs. Each group consisted of 10 mice. The experiment was repeated for three times. Data represent the averages on a total of 30 mice for each group; bars, SD.

**Immunohistochemical Analysis.** Immunocytochemistry was performed on cell cultures of GEO cells or on formalin-fixed, paraffin-embedded tissue sections (5 μm) of GEO xenografts processed as reported previously (38, 39). After overnight incubation with the appropriate primary antibody at 4°C, sections were washed and treated with an appropriate secondary biotinylated goat antibody (1:200 dilution; Vectastain ABC kit; Vector Laboratory, Burlingame, CA), washed, reacted with avidin-biotinylated horseradish peroxidase H complex, and incubated in diaminobenzidine and hydrogen peroxide, as described previously (38, 40). The slides were then rinsed in distilled water, counterstained with hematoxylin, and mounted. The following antibodies were used: an anti-Ki67 monoclonal antibody (clone MIB1; DBA, Milan, Italy) used at 1:200 dilution; an anti-VEGF rabbit polyclonal antibody (Santa Cruz) used at 1:50 dilution; an anti-bFGF rabbit polyclonal antibody (Santa Cruz) used at 1:200 dilution; and an antihuman TGF-α mouse MAb (Ab-2; Oncogene Science, Manhasset, NY) used at 1:100 dilution. Novel blood vessels were detected as described by Weidner et al. (41), using a monoclonal antibody raised against the human factor VIII-related antigen (Dako, Milan, Italy) at the dilution of 1:50 and stained with a standard immunoperoxidase method (Vectastain ABC kit). Each slide was scanned at low power (×10–100), and the area with the higher number of new vessels was identified (hot spot). This region was then scanned at ×250 (0.37 mm²). Five fields were analyzed, and for each of them, the number of stained blood vessels was counted. MVC was scored by averaging the five field counts of five individual tumors for each group.

**Statistical Analysis.** The Mantel-Cox log-rank test (42) was used to evaluate the statistical significance of the results. All Ps represent two-sided tests of statistical significance. All analyses were performed with the BMDP New System statistical package version 1.0 for Microsoft Windows (BMDP Statistical Software, Los Angeles, CA).

**RESULTS**

To determine the effects of EGFR blockade on the production of angiogenic growth factors, CM obtained from GEO cells treated for 4 days with different concentrations of MAb C225 were collected and analyzed for the presence of TGF-α, bFGF, and VEGF. As illustrated in Fig. 1, a dose-dependent inhibition in the secretion of these three growth factors was observed. An ~50% reduction in the secretion of TGF-α, bFGF, and VEGF was detected after treatment with MAb C225 (1 μg/ml).

As shown in Fig. 2, a dose-dependent reduction in VEGF expression was observed by Western blotting in GEO cells treated with VEGF-AS as compared with control oligonucleotide-treated cells. Similarly, an immunocytochemical analysis revealed a reduction of both the percentage of GEO cells spe-
We next evaluated the effects of the combination of VEGF-AS and/or MAb C225 on GEO tumor xenografts. Mice bearing GEO tumor xenografts were treated as described in “Materials and Methods.” Both the percentage of specifically stained cells and the intensity of immunostaining were recorded. MVC was scored by averaging the five field counts of five individual tumors for each group as described in “Materials and Methods.”

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Immunohistochemical analysis of GEO colon cancer xenografts after treatment with VEGF-AS and/or MAb C225</th>
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</thead>
<tbody>
<tr>
<td>Tumor size (cm³)</td>
<td>Ki67 (%)</td>
</tr>
<tr>
<td>A. Analysis performed on day 21 after tumor cell injection (2 weeks of treatment)</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.76</td>
</tr>
<tr>
<td>Scramble-AS</td>
<td>0.69</td>
</tr>
<tr>
<td>VEGF-AS</td>
<td>0.34</td>
</tr>
<tr>
<td>MAb C225</td>
<td>0.20</td>
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<tr>
<td>VEGF-AS + MAb C225</td>
<td>0.10</td>
</tr>
<tr>
<td>B. Analysis performed on day 28 after tumor cell injection (3 weeks of treatment)</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.98</td>
</tr>
<tr>
<td>Scramble-AS</td>
<td>1.75</td>
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<tr>
<td>VEGF-AS</td>
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<tr>
<td>MAb C225</td>
<td>0.40</td>
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<tr>
<td>VEGF-AS + MAb C225</td>
<td>0.12</td>
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Specifically stained with an anti-VEGF antibody and the intensity of the cytoplasmic staining (Table 1 and data not shown). Furthermore, an ELISA assay of CM obtained from GEO cells demonstrated a dose-dependent reduction in VEGF protein after VEGF-AS treatment but not after control scramble oligonucleotide treatment (Fig. 3). We have reported previously that GEO cells do not express specific receptors for VEGF (43). In fact, no significant effect of VEGF-AS treatment was observed on GEO cell growth in soft agar (data not shown). In contrast, the anti-EGFR MAb C225 determined a dose-dependent inhibition of GEO growth with an IC₅₀ of ~0.75 µg/ml under the same culture conditions.

We next evaluated whether treatment with the VEGF-AS oligonucleotide could affect GEO tumor growth in immunodeficient mice. We have studied previously the effect of different novel therapeutic agents including MAb C225 in nude mice bearing GEO colon xenografts (39). GEO cells form moderately differentiated adenocarcinomas that express the EGFR and various autocrine and paracrine growth factors including TGF-α, bFGF, and VEGF when injected s.c. in immunodeficient mice (39). When established GEO tumors of approximately 0.2–0.3 cm³ were detectable, mice were treated i.p. with VEGF-AS (5 or 10 mg/kg/dose, days 1–5 each week for 3 weeks) or with scramble control oligonucleotide (10 mg/kg/dose, days 1–5 each week for 3 weeks). As illustrated in Fig. 4, treatment for 3 weeks with the AS oligonucleotide targeting VEGF but not with the scramble control oligonucleotide determined a dose-dependent reduction of GEO tumor growth. This effect was mainly cytostatic and reversible, because shortly after the end of the 3 weeks of treatment, GEO tumors resumed the growth rate of untreated or of control oligonucleotide-treated tumors. We next evaluated the effects of the combination of VEGF-AS and MAb C225 on GEO tumor xenografts. Mice bearing established GEO tumors of approximately 0.2–0.3 cm³ were treated i.p. with VEGF-AS (10 mg/kg/dose, days 1–5 each week for 3 weeks) alone or in combination with MAb C225 (0.5 mg/dose, twice weekly on days 1 and 4 for 3 weeks; Fig. 5). Similarly to VEGF-AS treatment, the anti-EGFR MAb had a mostly cytostatic and reversible growth-inhibitory effect. In contrast, when the two agents were used in combination, an almost complete suppression of tumor growth in all mice was observed. Furthermore, GEO tumor growth was significantly delayed in mice that received MAb C225 plus VEGF-AS treatment. In fact, tumors grew very slowly for ~30 days after the end of treatment when they resumed a growth rate similar to controls (Fig. 5). As shown in Fig. 6, GEO tumors reached a size not compatible with normal life in all untreated mice within 4 weeks. A small increase in survival was observed in the group treated with VEGF-AS alone (P < 0.05). MAb C225 treatment determined a longer survival as compared with the control group (P < 0.01) and with the VEGF-AS-treated group (P < 0.05). The delayed GEO tumor growth in the MAb C225 plus VEGF-AS-treated group was accompanied by a significantly prolonged survival as compared with controls (P < 0.001), with the MAb C225-treated group (P < 0.001), or with the VEGF-AS-treated group (P < 0.001). In fact, all mice died within 4, 6, and 8 weeks after tumor cell injection in the control, VEGF-AS, and MAb C225 groups, respectively. In contrast, 50% of mice treated with the combination of VEGF-AS and MAb C225 were alive at 13 weeks. Moreover, no histological evidence of tumor was found in 3 of 30 (10%) mice that were treated with the combination of VEGF-AS and MAb C225 and that were sacrificed at 20 weeks after tumor cell injection. The combined treatment with MAb C225 and VEGF-AS was well tolerated.
No weight loss or other signs of acute or delayed toxicity were observed in any mouse.

An immunohistochemical analysis of GEO tumors after 2 and 3 weeks of treatment was also performed. As illustrated in Table 2 and Fig. 7, high levels of VEGF-specific staining were detected in 65% of control untreated GEO cells. A strong reduction in VEGF expression was observed after treatment with VEGF-AS (8–10% positive cells with a weaker staining). This effect was accompanied by a reduction in MVC as compared with control tumors. VEGF-AS treatment had no effect on bFGF and TGF-α expression. Control scramble oligonucleotide treatment did not affect VEGF, bFGF, or TGF-α expression and MVC. Treatment with the anti-EGFR blocking MAb inhibited the expression of VEGF, bFGF, and TGF-α and significantly reduced the number of tumor microvessels. The combined treatment with MAb C225 and VEGF-AS determined a further reduction in VEGF expression and an almost complete suppression of neovessel formation (Table 2 and Fig. 7).

DISCUSSION

Various mitogenic stimuli, including an activated ras oncogene, EGF, and TGF-α, can up-regulate the production of VEGF in human cancer cells (20, 24). We have reported previ-
ous that treatment of GEO xenografts with MAb C225 determined a significant reduction of various endogenous growth factors of the EGF family, such as TGF-α, amphiregulin, and CRIPTO (39). In this study, we have extended our previous observations and have demonstrated that MAb C225 treatment significantly inhibits the expression of TGF-α, bFGF, and VEGF in GEO colon cancer cells both in vitro and in vivo. This effect is accompanied by a relevant reduction in angiogenesis in GEO tumor xenografts in nude mice as assessed by MVC. The results of our study are in agreement with recent studies on the effect of EGFR blockade on angiogenesis. Petit et al. (44) reported that MAb C225 treatment inhibited the production of VEGF and neovascularization in A431 human epidermoid carcinoma xenografts. More recently, it has been demonstrated that MAb C225 treatment inhibited angiogenesis in a model of human transitional cell carcinoma grown orthotopically in nude mice by blocking the secretion of various angiogenic factors, including VEGF, bFGF, and interleukin 8 (45). Taken together, these data suggest that MAb C225 treatment has an antitumor effect in vivo that is attributable to direct blockade of the EGFR-dependent mitogenic pathway and, at least in part, to inhibition of the secretion of various paracrine growth factors that are necessary to sustain the proliferation and the functional differentiation of intratumor vessels.

In this study, we have also demonstrated that a 21-mer phosphorothioate oligonucleotide raised against the human VEGF mRNA (37) efficiently blocks the production of VEGF in human GEO colon cancer cells. The inhibition of VEGF production is of biological and potential therapeutic relevance. Treatment of nude mice bearing GEO tumor xenografts for 3 weeks with the VEGF-AS determines a significant inhibition of tumor growth. This effect is probably attributable to the inhibition of the paracrine stimulation of host endothelial cell proliferation and function by VEGF secreted from GEO cancer cells. In fact, reduction of GEO tumor growth in VEGF-AS-treated mice is accompanied by a significant inhibition of tumor angiogenesis as measured by MVC, suggesting that VEGF is a major paracrine angiogenic factor secreted by GEO human colon cancer cells. These data are in agreement with a previous study by Warren et al. (28) that have shown a high level of VEGF expression in several human colon cancer cell lines and in human colorectal liver metastasis.

We have treated nude mice bearing established GEO xenografts with the combination of the VEGF-AS and MAB C225. The combined treatment with these two agents determined an almost complete tumor growth suppression after 3 weeks of treatment. Although this effect was reversible, a sustained inhibition of GEO tumor growth was observed for at least 1 month after the end of treatment. As a result of the combined treatment, mice survival was significantly improved as compared with single agent treatment. The antitumor effect of VEGF-AS and MAB C225 combined treatment was accompanied by an almost complete suppression of tumor angiogenesis. Furthermore, in 10% of this group of mice, no histological evidence of GEO cancer cells was found after 20 weeks from tumor cell injection, suggesting that in these cases tumor eradication of established GEO tumor xenografts was achieved.

The combined inhibition of EGFR mitogenic signaling and VEGF expression has potential therapeutic relevance, because the antitumor activity of this combination could be explored in a clinical setting. In fact, Phase I clinical studies have shown that MAB C225 can be given to patients with advanced cancer at doses that produce receptor-saturating levels in the blood without relevant toxicity (19). Furthermore, Phase II-III clinical studies of MAB C225 in combination with cytotoxic drugs or with radiation therapy are currently ongoing (7, 46, 47). On the other hand, phosphorothioate AS oligonucleotides targeting various genes involved in cancer development and/or progression, such as bel-2, c-raf-1, and protein kinase Cα, are in clinical evaluation in cancer patients (48–50).

The drugs used in the present study have a different mechanism of action and do not antagonize the effects of cytotoxic therapy. Therefore, a combination of anti-EGFR MAB C225 and VEGF-AS after chemotherapy could be investigated in advanced colorectal cancer, a disease which is poorly responsive to cytotoxic drugs.

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