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Effects of combination anti-vascular endothelial growth factor receptor and anti-epidermal growth factor receptor therapies on the growth of gastric cancer in a nude mouse model

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Abstract

We hypothesised that the combination of anti-angiogenic and anti-epidermal growth factor (EFG)-receptor (R) therapies would more effectively inhibit gastric cancer growth than single-agent therapy. TMK-1 gastric cancer cells were injected into the gastric wall of nude mice to generate tumours. After 4 days, mice were randomly assigned to the following groups: control, DC101 ([vascular endothelial growth factor (VEGF)-receptor (R)-2 antibody], C225 (EGF-R antibody), or a combination of DC101 and C225. The combination therapy significantly inhibited gastric tumour growth compared with the control group, whereas the decrease in tumour growth in mice treated with DC101 or C225 alone did not reach statistical significance. All mice administered DC101 demonstrated decreased tumour vascularity and increased endothelial cell apoptosis. C225 alone did not affect angiogenesis, but inhibited tumour cell proliferation. The combination therapy led to a further decrease in tumour cell proliferation. The combination of anti-VEGF-R and anti-EGF-R therapies was effective in inhibiting gastric cancer growth. These findings support the hypothesis that inhibiting multiple biological pathways that mediate tumour growth may be an effective therapeutic strategy. © 2002 Published by Elsevier Science Ltd.

Keywords: Angiogenesis; Growth factors; VEGF; EGF; Gastric cancer

1. Introduction

Gastric cancer is one of the most common causes of cancer death worldwide [1]. Radiation therapy or chemotherapy does not significantly affect the length or quality of life of patients with advanced gastric cancer [2]. Thus, novel therapies are needed to target the molecular alterations that lead to gastric cancer development and progression.

Tumour angiogenesis is essential for the growth and metastasis of solid tumours, and its induction is mediated by numerous angiogenic factors [3]. Among these factors, vascular endothelial growth factor (VEGF) is the one most commonly associated with aggressive disease in most tumour types. There are at least four VEGF receptors, the most important of which is VEGF-receptor-2 (R2, *KDR/flk-1*). Inhibition of VEGF-R2 activity has been reported to suppress both primary tumour growth and metastasis [4,5]. Since expression of VEGF is associated with the progression and prognosis of gastric carcinoma, then inhibiting the activity of the VEGF-receptor–ligand system in gastric cancer may be a means of inhibiting tumour growth. In previous studies of a murine model of colon cancer (a tumour in which angiogenesis is primarily driven by VEGF), inhibition of VEGF activity led to significant decreases in tumour angiogenesis, growth and metastasis formation [4].

Experimental and clinical studies have provided evidence that the epidermal growth factor (EGF) acts as a growth factor in gastrointestinal tract malignancies and

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other tumours [6,7]. EGF and its receptor have been found to correlate with prognostic factors in patients with gastric cancer, suggesting that EGF and EGF-R might play a crucial role in the growth regulation of gastric cancer [8]. Thus, inhibition of EGF-R activity by the use of either anti-EGF-R monoclonal antibody (MAb) or inhibitors of the EGF-R-specific tyrosine kinase has been proposed as a potential therapeutic modality in gastric cancer.

In the present study, we evaluated the potential anti-angiogenic activity of the anti-VEGFR MAb DC101 and the antiproliferative activity of the anti-EGFR MAb C225, separately and in combination, on gastric cancer growth in an orthotopic model in nude mice.

2. Materials and methods

2.1. Reagents and antibodies

Reagents used were obtained as follows: minimal essential medium (MEM), fetal bovine serum (FBS), penicillin-streptomycin, vitamins, sodium pyruvate, L-glutamine, non-essential amino acids, trypsin-ethylene diamine tetra acetic acid (EDTA), Hanks balanced salt solution (HBSS), and Trypan blue from Life Technologies, Inc. (Grand Island, NY, USA); ketamine, xylazine and metallic abdominal wall Autoclips from Clay Adams (Parsippany, NJ, USA); polyethylene glycol (PEG)-300, Tween-80, and Gill 3 haematoxylin from Sigma Chemical Company (St. Louis, MO, USA); sodium monophosphate and diphosphate salts from EM Science (Gibbstown, NJ, USA); optimum cutting temperature (OCT) compound from Miles Inc. (Elkhart, IN, USA); diaminobenzidine substrate (DAB) and Universal Mount from Research Genetics (Huntsville, AL, USA); Superfrost slides from Fisher Scientific Co. (Houston, TX, USA); terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nicked-end labelling (TUNEL) kits from Promega (Madison, WI, USA); and 4,6-diamidinon-2-phenylindole dihydrochloride (DAPI) mount from Vector Laboratories, Inc. (Burlingame, CA, USA).

Antibodies for immunohistochemical analysis were obtained as follows: rat anti-mouse CD31/PECAM-1 antibody from Pharmingen (San Diego, CA, USA); mouse antiproliferating cell nuclear antigen (PCNA) clone PC10 DAKO A/S from DAKO Corp. (Carpinteria, CA, USA); peroxidase-conjugated goat anti-rat immunoglobulin G (IgG) [H + L] and Texas red- and fluorescein-conjugated goat anti-rat IgG from Jackson Research Laboratories (West Grove, PA, USA); and peroxidase-conjugated rat anti-mouse IgG_{2a} from Serotec Harlan Bioproducts for Science, Inc. (Indianapolis, IN, USA).

Anti-EGF-R monoclonal antibody (C225) and anti-mouse VEGF-R2 monoclonal antibody (DC101) were provided by ImClone Systems (New York, USA) [9,10]. Previous studies have shown that non-specific-IgG AB developed in a similar fashion had no effect on tumour growth and its effect was no different than that of solvent (phosphate-buffered saline (PBS)) [11].

2.2. Cell culture

TMK-1 cells (a poorly differentiated human gastric adenocarcinoma cell line) were kindly provided by Dr Eiichi Tahara (Hiroshima University, Hiroshima, Japan) [12] and were cultured and maintained in Dulbeccos MEM supplemented with 10% FBS. Cells for injection were harvested from subconfluent cultures. Trypan-blue exclusion was used to ensure a cell viability of more than 90%.

2.3. Animal care, tumour implantation and therapy

Six-week-old male athymic nude mice (The National Cancer Institute's Animal Production Area, Frederick, MD, USA) were acclimated for 1 week, caged in groups of four. Each mouse was anaesthetised by intraperitoneal (i.p.) injection of 100 μl of ketamine (80 mg/kg) and xylazine (16 mg/kg), and underwent an upper midline laparotomy. TMK-1 cells (10^6) in HBSS were injected into the wall of the mid-stomach. Four days after the injection of tumour cells, mice were randomly assigned to one of four groups and injected i.p. every third day (in the morning) with 1 ml of control vehicle (PBS), DC101 (800 μg/mouse), C225 (1 mg/mouse), or a combination of DC101 and C225. For the later group, the agents were mixed together for the injection. All animal studies were conducted according to institutional guidelines approved by the Animal Care and Use Committee of The University of Texas M. D. Anderson Cancer Center.

2.4. Necropsy and tissue preparation

The mice were killed by cervical dislocation 31 days after tumour-cell implantation due to the fact that mice in the control group were looking moribund. The gastric tumours were excised (the tumour was easily dissected from surrounding tissues as there was a clear and distinct margin), weighed, and sectioned, and the tumour sections were either embedded in OCT solution and frozen at −70 °C or formalin-fixed and paraffin-embedded.

2.5. Immunohistochemical analysis of tissues

Tumour vascularity was determined by staining frozen tumor sections with antibody to CD31 (1:400). Tumour cell proliferation was assessed by staining paraffin-embedded tissues with antibody to PCNA (1:100). Immunohistochemical analyses then were performed as

previously described in Ref. [4]. Briefly, paraffin-embedded sections were treated by standard deparaffinisation, and sections frozen in OCT were treated by fixation in acetone and chloroform. Endogenous peroxidases were blocked with 3% (w/v) hydrogen peroxidase (H_2O_2) in methanol and the slides were washed in PBS, incubated for 20 min with protein-blocking solution (PBS supplemented with 1% (w/v) normal goat serum and 5% (w/v) normal horse serum), incubated overnight at 4 °C with primary antibodies directed against CD31 or PCNA, washed, incubated with protein-blocking solution, incubated for 1 h at room temperature with peroxidase-conjugated secondary antibodies (1:200), washed, incubated with DAB, washed, counterstained with haematoxylin, washed, and mounted with Universal Mount and dried on a 56 °C hot plate. Negative controls involved the same procedure with the omission of the primary antibody.

2.6. Immunofluorescent staining for tumour vascularity (CD31) and apoptosis (TUNEL)

Frozen sections were stained for CD31 by immunofluorescence according to the above protocol with the following modifications. After sections were incubated overnight at 4 °C with the primary antibody, washed and incubated with protein-blocking solution, they were incubated for 1 h at room temperature with a secondary antibody which was conjugated to either Texas red (red fluorescence) or fluorescein (green fluorescence) as previously described in Ref. [4]. TUNEL staining was performed according to the manufacturer's protocol. Briefly, the sections were fixed with 4% (w/v) methanol-free paraformaldehyde, washed, permeabilised with 0.2% (w/v) Triton X-100, washed, incubated with the kit's equilibration buffer, incubated with a reaction mix containing equilibration buffer, nucleotide mix, and the TdT enzyme at 37 °C for 1 h, incubated for 15 min at room temperature with 2× standard saline citrate in order to stop the TdT reaction, washed, stained with DAPI mount (nuclei), and covered with glass coverslips [4].

2.7. Quantification of tumour vascularity, cell proliferation, apoptosis and endothelial cell apoptosis

Tumour vessels and PCNA-positive cells were evaluated by light microscopy, counted in five random 0.159-mm^2 fields at 100× magnification, imaged digitally, and processed with Optimas Image Analysis software (Biscan, Edmond, WA, USA). Apoptosis was quantified using immunofluorescence by imaging sections digitally and processing them with Adobe Photoshop software (Adobe Systems, Mountain View, CA, USA). CD31-positive endothelial cells were detected by localised red fluorescence with a rhodamine filter. The mean value for each tumour was then used for further statistical analysis.

Tumour cell apoptosis was determined by localised green fluorescence with a fluorescein filter. Endothelial cell apoptosis was determined by co-localisation of green (TUNEL-positive cells) and red (endothelial cells) fluorescence yielding a yellow/orange colour. Nuclei were detected by blue fluorescence of the DAPI with its respective filter. Cell counts were obtained in five random 0.011-mm^2 fields per slide at 400× magnification. The percentages of apoptotic tumour and endothelial cells were calculated as (number of apoptotic cells/total number of cells)×100).

2.8. Statistical analysis

Statistical comparisons among groups were made with the Mann–Whitney U-test (InStat Statistical Software, San Diego, CA, USA) at the 95% Confidence Level (CL); a P value of less than 0.05 was considered to indicate statistical significance.

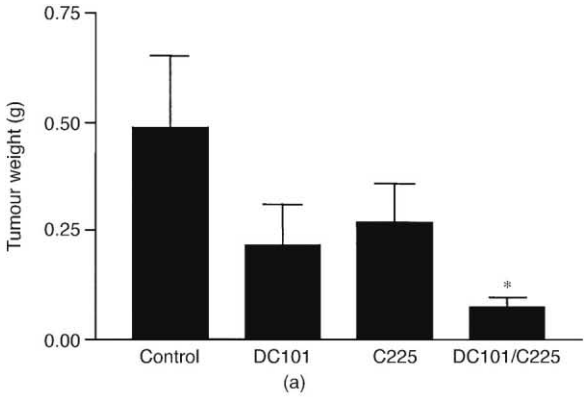
3. Results

3.1. Growth of TMK-1 cells in vivo

Mice treated with DC101, C225, or the combination of both demonstrated no difference in body weight or detectable toxic effects. With regard to tumour mass, tumour growth seemed to decrease in the DC101 alone and C225 alone groups, but this trend did not reach statistical significance ($P=0.068$, and $P=0.092$, respectively). However, combination therapy led to significant inhibition of gastric tumour volume over that of the control group ($P=0.023$) (Fig. 1a and b). No significant differences in gastric tumorigenicity were observed between treatment groups (80–90% tumorigenicity for all groups).

3.2. Tumour angiogenesis and tumour cell proliferation

Mice treated with DC101 alone or DC101 in combination with C225 had 50% and 45%, respectively, fewer tumour vessels than did the control group ($P<0.001$) (Fig. 2a). C225 alone had no effect on tumour vascularity. Tumours from mice treated with C225 alone had fewer proliferating tumour cells than did those of controls ($P=0.035$). There was no clear association between PCNA-positive cells and vascular structures. Although tumour cell proliferation seemed to decrease in mice treated with DC101, this trend did not reach statistical significance ($P=0.073$). However, the combination of DC101 and C225 led to a greater decrease in tumour cell proliferation than that produced by either drug alone ($P<0.001$ versus control, $P<0.05$ versus DC101 or C225 groups) (Fig. 2b).



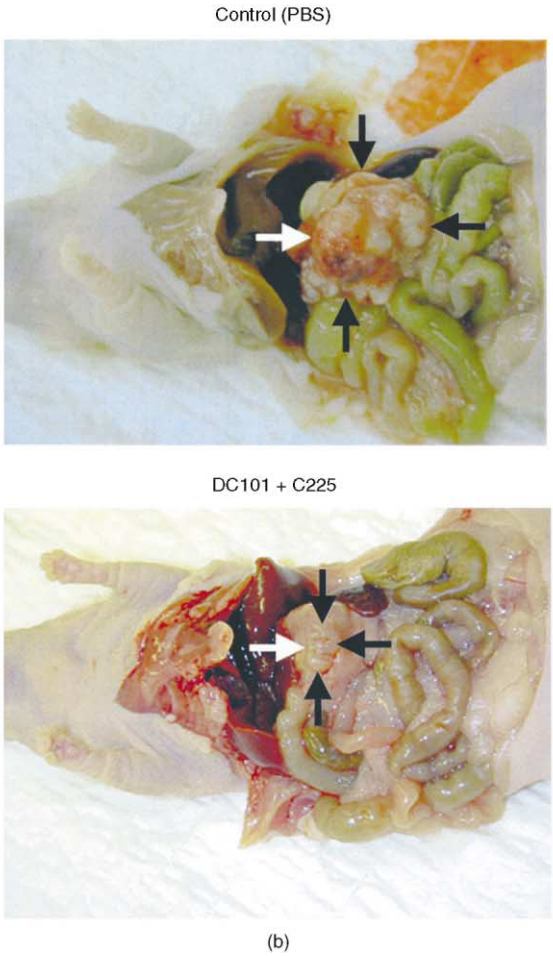


Fig. 1. Effects of DC101 and C225 on gastric tumour growth. (a) Gastric tumours in the DC101 + C225 treatment group were significantly smaller than those of the control mice. Bars indicate standard error. $*P < 0.05$ (10 mice per group). (b) Photographs demonstrating the effect of the combination of DC101 + C225 therapy on gastric tumour growth compared with controls.

3.3. Apoptosis of tumour and endothelial cells

Tumour sections from mice treated with DC101 alone or C225 alone had 1.9 and 1.7 times as many TUNEL-positive cells as did tumours from the control group, respectively. The combination of DC101 and C225 led to a further (2.5×) increase in tumour cell apoptosis over that caused by single-agent therapy ($P < 0.001$, versus control) (Fig. 3b). Concurrent immunofluorescent CD31 and TUNEL staining, done to quantify the endothelial apoptosis in the tumour sections [4], revealed that DC101 alone or in combination with C225 produced a 1.9- and 2.0-fold increase, respectively, in endothelial cell apoptosis over that of the controls (Fig. 3a). There was no clear association between TUNEL-positive cells and vascular structures as TUNEL-positive staining was diffuse throughout the tumour. C225 alone did not affect endothelial cell apoptosis. Representative images illustrating microvessel density, tumour cell proliferation, and endothelial cell apoptosis in tumours from the various groups are shown in Fig. 4.

4. Discussion

The present study was undertaken to assess the antitumour effect of an anti-VEGF-R-2 MAb (DC101) and an anti-EGF-R receptor MAb (C225), given either alone or in combination, on human gastric cancer growth, vascularity, cell proliferation and apoptosis in an orthotopic nude-mouse model. We found that the combination of these two antibodies led to significant suppression of tumour growth, proliferation, and vascularity and significant enhancement of tumour cell apoptosis in this model. Angiogenesis is essential for the growth and metastasis of solid malignancies and of all the angiogenic factors identified, VEGF is the factor most often associated with vascularity and tumour metastases. Several studies in gastric cancer demonstrate an association between VEGF expression and tumour progression and metastasis [13,14]. Blockade of the VEGF ligand-receptor system has also results in preclinical models, where tumour growth inhibition led to a prolongation in survival [15]. In clinical trials, the combination of anti-VEGF therapies, when combined with standard chemotherapeutic regimens, have resulted in enhanced response rates and increased time to tumour progression [16]. Previous reports have also suggested that interfering with VEGF-dependent signalling is a particularly promising way to inhibit tumour-induced angiogenesis in gastric cancer [17]. In the present study, we found that the combination of the two antibodies (DC101 and C225) led to a significant suppression of tumour growth, proliferation and vascularity and a significant enhancement of tumour-cell apoptosis in our mouse model.

In addition to being an angiogenic factor, VEGF has recently been shown to function as a survival factor for tumour endothelial cells [4]. *In vitro* studies demonstrate that VEGF may prevent endothelial cell apoptosis through various mediators including Bcl-2, A1, and the

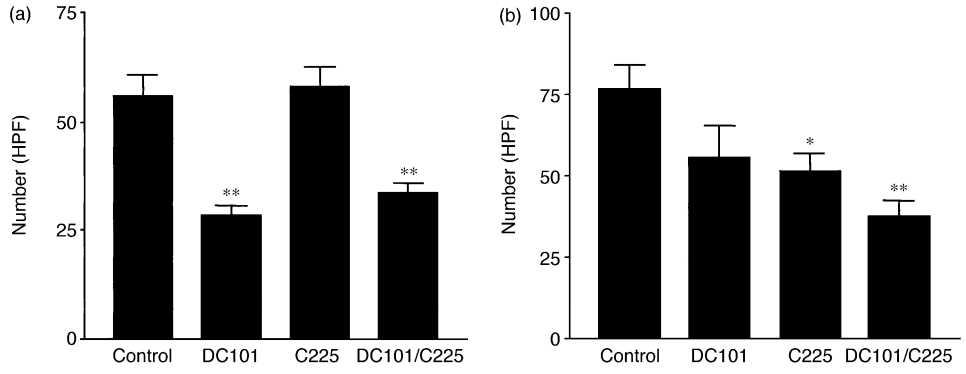


Fig. 2. Effects of DC101 and C225 on tumour vascularity (a) and tumour cell proliferation (b). Immunohistochemical staining of tumour sections for CD31 and proliferating cell nuclear antigen (PCNA) was used to quantify tumour vessels and tumour cell proliferation. $*P < 0.05$, $**P < 0.001$ (10 mice per group). HPF, high-power field.

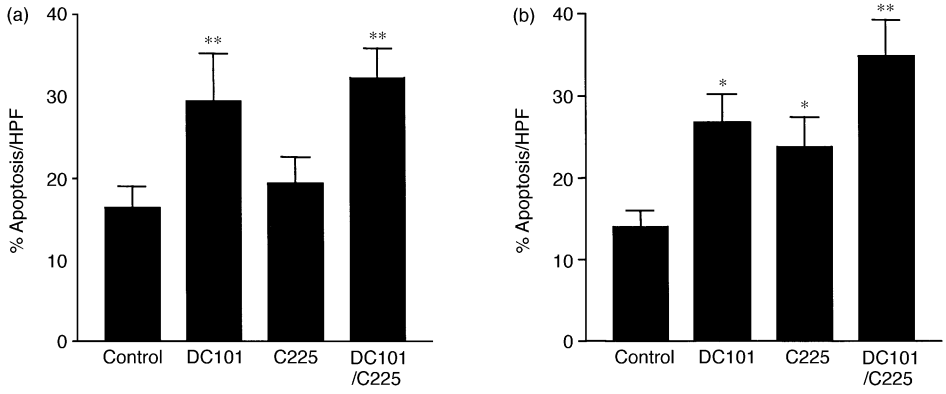


Fig. 3. Effects of DC101 and C225 on the apoptosis of endothelial cells (a) and tumour cells (b). Immunofluorescent double staining of tumour sections for CD31 and terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick-end labelling (TUNEL) was performed to quantify the percentages of endothelial and tumour cells undergoing apoptosis. HPF, high-power field. Bars indicate standard error. $*P < 0.05$, $**P < 0.001$ (10 mice per group).

phosphatidyl inositol 3 (PI3)-kinase/Akt signal transduction pathway [18]. These signals may be mediated through the VEGF-R-2 receptor. Thus, inhibition of VEGF activity could not only prevent further angiogenesis, but also could interfere with endothelial-cell survival signals that themselves could led to endothelial cell apoptosis. DC101, a monoclonal rat anti-mouse Flk-1 (VEGF-R2) antibody, potently blocks ligand binding and inhibits VEGF-induced signalling *in vitro*, as well as strongly inhibiting angiogenesis and diminishing tumour growth in mice [11].

EGF-R and its ligands are commonly overexpressed in gastric cancer, and their expression is associated with a poor prognosis [12,19]. This co-expression of both the EGF-R and its ligands (EGF and transforming growth factor-alpha (TGF-α)) in gastric cancer cell lines implies the existence of an autocrine mitogenic loop [19]. *In vitro,* blockade of EGF-R activity with anti-EGFR monoclonal antibody resulted in the inhibition of tumour cell proliferation [20]. These results suggest that EGF-R is a potential therapeutic target in gastric carcinoma. In preliminary studies, we have demonstrated that the TMK-1 cell line, the human gastric cancer cell line used in these studies, expresses the EGF-R (data not shown). In addition, EGF can stimulate TMK-1 cell proliferation in *in vitro* assays after 72 h, and pretreatment of these cells with C225 can block this effect (data not shown).

C225, an anti-EGFR monoclonal antibody, binds to the EGF receptor with a Kd similar to that of EGF and blocks EGF binding and EGF-mediated activation of the receptor [21]. Blockade of EGF-R signalling pathways results in a cell cycle arrest at the G1-S checkpoint [22]. The induction of a G1 arrest by C225 is associated with the inhibition of the cyclin-dependent kinase inhibitor $p27^{kip1}$ [23]. Furthermore, C225 has recently been shown to augment the antitumour activity of several chemotherapeutic agents and irradiation in different various mouse xenograft models [24]. The molecular pathways for these effects are unclear, but may be mediated by several different mechanisms, including those affecting DNA repair, cell cycle checkpoint

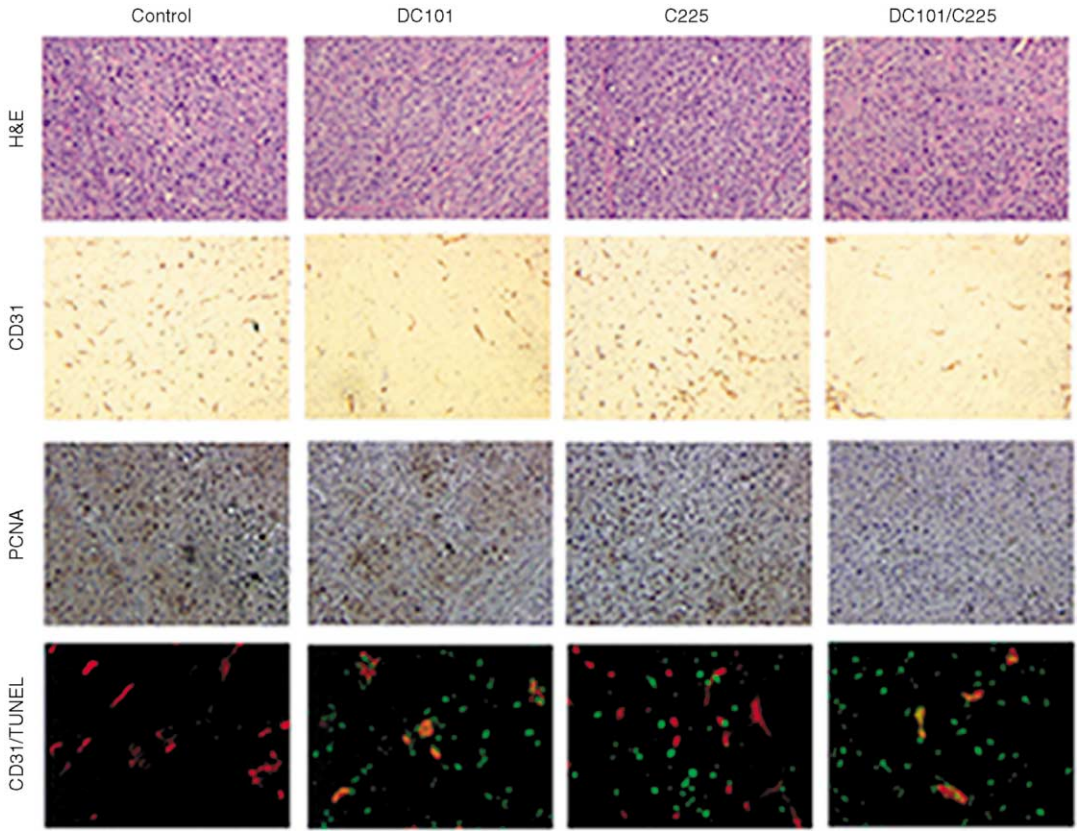


Fig. 4. Representative images of tumour sections stained with haematoxylin and eosin (row 1; 40× magnification), stained immunohistochemically for CD31 (row 2; 100×) or proliferating cell nuclear antigen (PCNA) (row 3; 100×), and or stained sequentially for CD31 (red) and terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick-end labelling (TUNEL) (green) (row 4, 400×).

control, metastasis and/or angiogenesis [25]. Recently, Radinsky and associates [26] have reported that C225 could modulate angiogenesis through downregulation of the angiogenic factors VEGF and Interleukin-8 (IL-8). In the TMK-1 cell line, we did not find that the addition of EGF led to an increase in VEGF or IL-8 mRNA expression, and the addition of C225 *in vitro* did not decrease expression of mRNA for these angiogenic factors (data not shown). This suggests that the EGF-R pathway can induce angiogenic factors in some cancer cells, but not all.

Given the importance of both the VEGF and EGF ligand receptor systems in gastric cancer progression, we hypothesised that using a combination therapy of these agents that inhibited the activity of both these factors would inhibit tumour growth to a greater degree than inhibiting either factor alone. We performed western blot analysis on cell lysates and showed that the EGF-R was expressed on TMK-1 cells, but its expression was relatively low compared with cells known to overexpress EGF-R, such as A431 cells (data not shown).

In the present study, we have demonstrated that the combination of anti-angiogenic and antigrowth factor therapy resulted in a marked inhibition of the growth, vascularity and proliferation of human gastric cancer xenografts. We did not observe toxic effects at the doses administered, as evidenced by no changes in body weight or grooming habits. To clarify the mechanism involved in the growth inhibition resulting from the combination therapy, we also evaluated endothelial and tumour cell apoptosis by immunohistochemical double staining for CD31 (vessels) and TUNEL (apoptosis). With this approach, we found significantly greater induction of endothelial apoptosis relative to tumour cell apoptosis in DC101-treated groups than in the control group. Because VEGF receptors, with rare exceptions, are expressed exclusively on endothelial cells, it is unlikely that DC101 directly induces tumour cell apoptosis. In addition, DC101 only reacts with the murine isoform of VEGF-R2. Therefore, it is possible that inhibiting the action of VEGF may lead to endothelial apoptosis, which could then lead to a subsequent increase in tumour cell apoptosis. The results of the present study are in accordance with previous observations [4]. We observed a significant increase in apoptotic death of endothelial cells after treatment with DC101, but not with C225 therapy. However, C225 treatment did lead to an increase in apoptosis in the

tumour cells. C225 is known to have differing effects on the cell cycle and the induction of apoptosis, depending on the cell line studied and culture conditions [27,28]. C225 is most efficacious when administered in combination with cytotoxic therapy, such as chemotherapy or irradiation. The finding that C225 directly led to *in vivo* tumour cell apoptosis is consistent with previous reports [15].

In summary, the combination of targeting both the EGF-R and VEGF-R was effective in significantly inhibiting tumour growth of gastric cancer in nude mice. Targeting multiple pathways in tumour progression may be a potential treatment strategy for gastric cancer or other solid tumours that are dependent on EGF-R and VEGF for tumour cell proliferation, survival and angiogenesis.

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