Vascular Endothelial Growth Factor Receptor-2-blocking Antibody Potentiates Radiation-induced Long-Term Control of Human Tumor Xenografts

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Abstract

Antiangiogenic therapy can enhance radiation-induced tumor growth inhibition. However, the effects of combined antiangiogenic and radiation therapy on long-term tumor control and normal tissue response have not been reported. We treated mice bearing two different human tumor xenografts with anti-vascular endothelial growth factor receptor-2 antibody (DC101) and five dose fractions of local radiation and followed them for at least 6 months. DC101 significantly decreased the dose of radiation necessary to control 50% of tumors locally. The decrease was 1.7- and 1.3-fold for the moderately radioresistant small cell lung carcinoma 54A and the highly radioresistant glioblastoma multiforme U87, respectively. In contrast to tumors, no increase in skin radiation reaction by the antibody was detected. Surprisingly, 44% of mice bearing 54A tumor developed clear ascites after DC101 treatment at its highest dose; this was fatal to 20% of mice. This adverse effect was seen only in mice that received whole-body irradiation 1 day before tumor implantation. The encouraging results on two human tumor xenografts suggest that vascular endothelial growth factor receptor-2 blockade merits further investigation to assess its potential as an enhancer of radiation therapy in the clinic.

Introduction

Since the seminal studies of Teicher and coworkers (1, 2), several groups have shown an enhancement of chemo- and radiation therapy by antiangiogenic agents in tumor-bearing mice (e.g., Refs. 3–8). The combined effect of radiation with different antiangiogenic compounds (angiostatin, anti-VEGF antibodies, TNP-470 alone or in combination with minocycline) on tumor growth delay has been synergistic, additive, or less than additive (1–5, 8). Mechanisms underlying the enhancement of radiation response by antiangiogenic agents include an increase in tumor oxygenation (1, 2), a decrease in vascular density (8), and possibly, radiosensitization of endothelial cells (4, 5). An increase in tumor oxygenation (1, 2, 8), a decrease in vascular density and killing of both endothelial and parenchymal cells in tumors, mainly by apoptosis (e.g., Refs. 8, 10, 13–15). In particular, DC101 has been effective against several murine and human tumors grafted into mice, alone (10, 14) or combined with chemotherapy (7). However, DC101 has not been used in combination with radiation. Here DC101 and fractionated radiation were administered to two human tumor xenografts growing in the legs of nude mice. These two tumors were the moderately radiosensitive small cell lung carcinoma 54A, and the highly radioresistant glioblastoma multiforme U87. Both tumor and normal tissue responses were evaluated. Because of the dependence of radiation sensitivity on pO2 and previous work with TNP-470 (3), we evaluated changes in tumor oxygenation after DC101 administration. We also investigated any potential interaction between DC101 and WBI of mice.

Materials and Methods

Animals and Tumors. Athymic NCr/Sed nude (nu/nu) male mice, 8–9 weeks of age, bred and maintained in our defined flora- and specific-pathogen-free animal colony were used. In all but one set of experiments, the mice were further immunosuppressed by WBI at a dose of 5 Gy (0.7 Gy/min; Gamma-cell-40 137Cs unit; Atomic Energy of Canada, Ltd., Ottawa, Canada) 1 day before tumor implantation. Such an irradiation procedure has been used in our laboratory for many years in similar experiments with nude mice and can significantly increase the TCD50 values (16). To determine a potential interaction between DC101 and WBI, in one experiment non-WBI mice were also used for tumor growth. Two human tumor lines were used in the study: small cell lung carcinoma 54A and glioblastoma multiforme U87. Third- to sixth-generation source tumors were excised; viable tumor tissue was cut into small chunks (1–1.5 mm) and then transplanted s.c. into the calf area of the right hindlegs of the animals. Treatment began —3–4 weeks later when individual tumors reached a mean diameter of 8 mm (day 0).

Treatment of Tumors by Fractionated Radiation and DC101. Starting on day 0, DC101 was given i.p. every 3 days, as determined previously (10), for a total of six injections; the dose/injection was 20 or 40 mg/kg. Irradiation of tumors commenced on day 0 for radiation-alone groups or the day after the first DC101 injection for combined-treatment groups, and continued on a daily basis. Five equal fractions (graded from 5 to 24 Gy each) were given at a dose rate of 5.4 Gy/min, using a specially designed 137Cs irradiator (17). During irradiation, mice were immobilized on a brass plate such that the tumor was held in the center of a 3-cm diameter radiation field. In combined treatment on day 3, radiation was given immediately prior to DC101 injection.

Evaluation of Tumor Response to Therapy. Tumor size was measured with a caliper three times a week. The time taken for a tumor to triple its initial volume (from ~260 to 780 mm3) was determined. The difference between mean values of this parameter for individual tumors in treatment and control...
groups was defined as the tumor growth delay achieved as a result of therapy. The mice were sacrificed when a tumor reached 12 mm in diameter. The TCD_{50} was evaluated for radiation alone or in combination with DC101. The mice with locally controlled tumors were followed for 90 days after the last tumor recurrence observed in any group. The TCD_{50} values were calculated by regression analysis on a log-dose grid, correcting for censored animals (18).

**Evaluation of Skin Response.** Acute skin reaction in the field of tumor irradiation was evaluated using a slightly modified standard scoring scale (19). A skin reaction score of 0.5–1 corresponded to minor dry abnormalities (reddening, white scales), and 1.5–3 corresponded to increasing severity of moist desquamation. The normal tissue response was evaluated in parallel with tumor size measurements during the first 30 days after the beginning of radiotherapy (with or without DC101), and the maximum scores of individual mice of a group were then averaged. Because adjacent irradiated tumors complicated the scoring of skin reaction, especially minor ones at lower radiation doses, the same treatments were also performed in non-WBI mice not bearing any transplanted tumors. In this case, using the same scoring scale, we evaluated the reactions every 1–2 days over 9–30 days after the start of leg irradiation. Average skin reaction over this period of time was calculated and plotted as a function of radiation dose.

**Single-Dose Irradiation under Different Tumor pO_{2}**. To examine for possible interactions between radiation and DC101-induced effects in tumors, including antibody-induced changes in tumor oxygenation, an additional study was performed with 54A xenografts (in WBI mice). We compared their response to single-dose irradiation performed under normal blood flow and respiration versus clamp hypoxia tumor conditions. In the latter case, the animals were anesthetized with ketamine/xylazine (100/10 mg/kg, i.m.), and a heavy clamp was placed across the thigh above the tumor beginning 2 min prior to and continuing during irradiation. A single injection of DC101 was administered 2 days before or immediately after irradiation under normoxic or hypoxic conditions.

**Polarographic Measurements of Tumor pO_{2}**. pO_{2} in U87 tumors was measured with the Eppendorf-pO_{2} Histogram (model no. 6650; Eppendorf, Hamburg, Germany; Ref. 20) in anesthetized mice (ketamine/xylazine, as above). The pO_{2} probe was inserted into the tumor to a depth of 1–1.5 mm through a puncture of the skin made by a 23-gauge needle. An electrocardiogram patch was attached to the abdominal wall as a reference electrode. The measurements were performed in four different regions of the tumor; 11 readings were taken per track in 0.4-mm steps.

**Western Blot Analysis of Flk-1 Expression in Tumors.** Untreated U87 and 54A tumors, four of each type, were solubilized in 500 μl of TNT lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton]. Tumor lysates were clarified by centrifugation at 14,000 × g for 10 min and incubated overnight at 4°C with DC101. Immune complexes were precipitated with protein G-Sepharose beads (Pharmacia-Amersham, Piscataway, NJ) and separated on 12% SDS-PAGE gels (Novex, Carlsbad, CA). Proteins were transferred onto polyvinylidene difluoride membranes and probed with anti-Flk-1 polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Membranes were incubated with antirabbit IgG-horseradish peroxidase (BioSource, Camarillo, CA) and developed by an enhanced chemiluminescence detection system (ECL; Pharmacia-Amersham).

**Results and Discussion**

**Tumor Growth Delay Induced by DC101 Alone and in Combination with Radiation.** DC101 (administered as six injections with a 3-day interval) significantly inhibited the growth of both 54A and U87 tumors in a dose-dependent manner (Fig. 1). This effect was tumor dependent. For the high dose of DC101 (40 mg/kg per injection), tumor growth delays were 17.7 ± 1.9 and 7.7 ± 1.0 days (mean ± SE) for 54A and U87, respectively. Because the tumor...
volume doubling time in control groups was 1.3 times greater in U87 than in 54A xenografts, the difference in specific (normalized to the doubling times) growth delays for these tumors was 3-fold. It should be mentioned that the U87 used here was one of the least susceptible to DC101 treatment among several tumor types that have been studied to date (7, 10, 14).

Treatments of patients by radiation and nearly all systemic modalities are based on multiple dose protocols. Therefore, we used a fractionated dose schedule for both DC101 and radiation. An additional argument for fractionation was that each modality might influence the efficacy of the other. Radiation increases VEGF production by tumor cells \textit{in vitro} and \textit{in vivo} (5), thus presumably decreasing the capacity of DC101 to compete with VEGF for VEGFR2 binding. On the other hand, VEGF signaling may protect endothelial cells from radiation killing, as shown \textit{in vitro} (5), and therefore DC101 is a possible sensitizer of these cells to radiation.

Fig. 1, \textit{C} and \textit{D}, shows the tumor growth delay for DC101 combined with relatively small doses of fractionated radiation (five equal daily fractions, of 5–6 Gy each, on days 1–5). Similar to antibody alone, 54A xenografts responded better than U87 to both radiation and the combined therapy. In both tumor models, the combined effect of radiation and DC101 in terms of growth delay was not significantly different from additive.

It should be noted that the dynamics of tumor volume response after irradiation, with or without DC101, differed considerably between the two xenografts (Fig. 2, \textit{A} and \textit{B}). U87 tumors rarely shrank below the initial size (at the beginning of treatment), and then only after 1.5–2 months. In contrast, the response of 54A was much faster, and in the case of combined treatment, the median tumor volume decreased below the initial level on day 5, was 3-fold lower on day 15, and then usually continued to decrease.

DC101 Significantly Decreased TCD\textsubscript{50} Values for Both Xenografts. The main finding of the present study was that the use of DC101 in combination with radiation significantly enhanced the tumor control probability. Fig. 2, \textit{A} and \textit{B}, demonstrates the increasing number of locally controlled tumors of both lines with dose of

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**Fig. 2.** \textit{A} and \textit{B}, dynamics of size changes in individual 54A and U87 tumors treated by fractionated radiation and/or DC101 (40 mg/kg per injection). The increase in radiation dose (from \textit{top} to \textit{bottom}) increased the tumor growth delay and eventually led to local control (LC) of four 54A tumors and two U87 tumors in the case of combined therapy. Two animals in these groups were found dead (FD) 150 and 120 days after the beginning of therapy, with no obvious cause of death. Stars near some curves for 54A tumors treated with DC101 (with or without radiation) indicate those mice that developed ascites (see the text). X coordinates of the stars do not exactly correspond to the time of ascites. \textit{C} and \textit{D}, tumor control as a function of total dose of radiation alone and combined with DC101 (5–12 animals per point). The data points, fitted lines and TCD\textsubscript{50} values with their 95% confidence intervals were determined with correction for censored mice (e.g., FD animals in panels \textit{A} and \textit{B}, bottom; see Ref. 18). In 54A xenografts, as determined 185–200 days after the beginning of treatment, the TCD\textsubscript{50} values (with 95% confidence intervals) were as follows: 66.2 (59.6–73.6) Gy for RT alone; 54.8 (45.1–66.6) Gy for RT + 1/2DC; and 39.1 (31.7–48.1) Gy for RT + DC. The corresponding values for U87 (on days 220–235) were 97.8 (85.3–112.0) Gy, 86.3 (74.6–99.8) Gy, and 74.8 (63.7–87.7) Gy, respectively. DC, 40 mg/kg, every 3 days, × six injections; 1/2DC, 20 mg/kg, every 3 days, × six injections.
radiation was independent of their sequence. The results show independence of curve shifts (a) to the right, as a result of the radioprotective effect of clamp hypoxia, and (b) up, as a result of additional DC101-induced tumor growth inhibition. The combined effect of DC101 and radiation was independent of their sequence.

DC101 Did Not Modify Tumor Radiosensitivity and Oxygenation. To reveal possible interactions between radiation and DC101, in particular to assess DC101-induced changes in tumor pO2, additional studies were performed based on a standard radiobiological “paired-curve” analysis (21, 22). 54A tumors received a single irradiation at different doses under normoxic or clamp hypoxia conditions. Tumor growth delays after the following six treatments were compared: radiation alone (group I); radiation 1–2 min before (group II) or 2 days after (group III) a single DC101 injection (40 mg/kg); and the same three regimens but with clamp hypoxia (groups IV–VI) of tumors during irradiation.

The results obtained are presented in Fig. 3 and suggest two conclusions. First, regardless of tumor oxygen status during irradiation, DC101 did not significantly change the shape of the dose-response curves, rather it only shifted them up. Furthermore, no effect of sequencing of agents on tumor growth delay was observed (DC101 prior to versus after radiation). Hence, the combined effect of single-dose radiation and DC101 was additive and not associated with a detectable DC101-induced radiosensitization of tumors. The latter is not consistent with the substantial role of VEGF signaling in radiosensitivity of endothelial cells in vivo, as would be expected on the basis of in vitro experiments (5). It may be that DC101-induced sensitization of the endothelial cells was not large enough to be of considerable therapeutic importance.

Second, it is unlikely that such radiosensitization, if it occurred, was masked by an opposite radioprotective effect by DC101-induced tumor hypoxia. As seen in Fig. 3, clamp hypoxia equally displaced all three dose-response curves for radiation, without or with DC101, horizontally to the right. This suggests that the antibody did not significantly modify the fraction of radiobiologically hypoxic tumor cells 2 days after a single injection.

Because of the lesser effect of DC101 on U87, a similar experiment with this tumor model would have had a smaller resolution and therefore was not performed. Using the Eppendorf technique showed no significant effect of the antibody on oxygenation of this tumor model. Two days after DC101 injection (40 mg/kg), the median pO2 value did not differ significantly from that in the control group (4.0 ± 0.3 versus 4.3 ± 0.4 mm Hg, respectively, mean of medians ± SE from 15 mice in each group).

These results for tumor pO2, together with the data on TCD50, are very encouraging because they show no significant decrease of tumor oxygenation by 1–2 doses of DC101, despite the antivasculogenic effect of the antibody (7, 10, 14). The comparison of our findings with those using TNP-470 (3) suggests that different antiangiogenic agents may have different effects on tumor oxygenation. Thus, inhibitors of the VEGF pathway that either block VEGFR2 (as described in this study) or neutralize VEGF (8) may be preferable to angiogenesis inhibitors such as TNP-470 in this respect. However, the changes in oxygenation may also depend on tumor type (8, 14) as well as duration and dose fractionation of both antiangiogenic and radiation treatments. Tumor cell apoptosis by DC101 (7, 12) could also contribute to the improved tumor response resulting from combined DC101 and radiation. Further studies on oxygenation and apoptosis in various tumors during combined treatments with different antiangiogenic agents and radiation are now needed to optimize this approach.

Anti-VEGFR2 Therapy Did Not Modify Radiation Response of Skin. Acute moist skin desquamation in the field of tumor irradiation was always observed after fractionated doses ≥60 Gy. Typically starting 11–14 days after the beginning of irradiation, the reaction peaked several days later and then gradually decreased. Importantly, and in contrast to tumor response, DC101 did not significantly change the radiation-induced skin damage (Fig. 4A). In an experiment on non-WBI mice without tumors, complete radiation dose-response curves were obtained for such treatments (Fig. 4B). Here there also was no effect of DC101 on the acute skin reaction to radiation.

Why this normal tissue damage induced by radiation was not influenced by DC101 treatment is not clear. Despite its presence in mice during the course of irradiation and throughout the period of developing skin moist desquamation, the antibody did not affect the severity of damage or the time course of healing. This is somewhat surprising because VEGF and its receptors are thought to play an important role in healing mechanical wounds of normal tissues (23, 24). Virtually nothing is known, however, about the importance of these molecules for normal tissue radiation response. Regardless of the underlying mechanisms, our observation of an unchanged skin radiation response is novel and of potential clinical significance. These results imply clinical gain by use of a similar strategy to enhance the efficacy of radiation therapy.
Fig. 4. Adverse effects of the combined antiangiogenic and radiation therapy. A, data from experiment on U87 xenografts showing that at a dose of 70 Gy, DC101 did not modulate maximum radiation reaction of the skin adjacent to irradiated tumors. Columns and bars are mean ± SE (six animals in each group). B, radiation dose-dependent curves for the skin reaction of nude mice that did not have tumors. The data document more comprehensively the absence of radio-modifying effects of DC101 in skin. Data points and bars are mean ± SE of individual skin reactions averaged from the 9th to 30th day after the beginning of leg irradiation (five mice per data point). RT, fractionated radiation; DC, 40 mg/kg, every 3 days, × six injections; 1/2DC, 20 mg/kg, every 3 days, × six injections. C, histology of the colon in a mouse that developed ascites after treatment of the 54A tumor with DC101 at a dose of 40 mg/kg per injection. Note the large separation between the external muscularis and mucosa. The mucosa forms large folds that project into the lumen. There is an obvious atrophy of the mucosa, the epithelial layer is stretched, and large separations can be seen between the crypts. Bar, 200 μm.

Animal Weight Changes. Importantly, the treatments only minimally affected the weight of the animals. Weight temporally decreased under the influence of both fractionated radiation and DC101, typically toward the end of treatment or shortly thereafter. The antibody and radiation alone decreased body weight by ~2–4% and 6%, respectively, and their combined effect on weight loss was approximately additive (8–10%).

Tumor- and Dose-dependent Intestinal Toxicity. Unexpectedly, a tumor type-dependent toxicity at the high dose of DC101 (without or with fractionated radiation) was observed in mice bearing 54A xenografts: 44% of the 80 animals developed ascites, with the maximum response occurring typically 15–25 days after the last DC101 injection at a dose of 40 mg/kg. In Fig. 2A, such mice are identified by stars. Twenty-four percent of mice recovered, whereas the remaining 20% eventually died. At necropsy, 5 ml or more of clear ascites fluid were aspirated from the abdominal cavity. Portions of the colon and small intestine of affected animals were 2–3-fold larger in diameter than normal. Histological analysis of the colon and small intestine demonstrated an enlargement of the lamina propria and submucosa, most likely because of accumulation of edema fluid. Modifications to tissue structures were more striking in the wall of the colon, with large separations between the mucosa and external muscularis and large folds in the mucosa that projected into the lumen (Fig. 4C). The mucosa was atrophic with obvious stretching of the columnar epithelium. In animals that recovered from the ascites, the colon wall had an almost normal appearance but the separation between the mucosa and external muscularis could still be observed in discrete areas. In contrast, no apparent toxicity was observed in the same experiments at the low dose (20 mg/kg per injection) of DC101. In addition, only 4% of 47 mice bearing U87 tumors and treated with the high dose of antibody developed ascites (which was eventually resorbed), and no such effect was observed in 20 non-WBI mice without tumors in the skin-reaction experiment. Under comparable experimental conditions, DC101 did not induce delayed toxicity in SCID mice with a grafted neuroblastoma (7).

Role of WBI in DC101-induced Toxicity. To examine whether this adverse effect of the high dose of DC101 in mice bearing the 54A tumor was a result of their WBI at a dose of 5 Gy 1 day before tumor transplantation, we performed the following experiment. Three groups of nude mice were compared: WBI (5 Gy) animals with (group I) or without (group II) implanted 54A tumors, and non-WBI mice grafted with the same tumor (group III). WBI did not influence the time necessary for tumors to grow to 8 mm in diameter (when treatment was started). The animals were subjected to the standard fractionated treatment with DC101 (40 mg/kg × six injections) combined with radiation (5 Gy × five fractions); in group II, DC101 alone was also used. Results for group I were consistent with our previous findings: 5 of 10 mice developed ascites. Virtually the same effect was observed in group II: 9 of 20 mice had ascites. However, none of 11 animals in group III showed such adverse effect.

Collectively, the results obtained suggest that the whole-body irradiation of mice at dose of 5 Gy almost 1 month before DC101 treatment was responsible for the observed complications in experiments with 54A tumor. The mechanisms of interaction between
DC101 and radiation-induced changes in the intestine remain to be elucidated. In addition, the reasons that DC101 interfered with radiation damage in this normal tissue but not in skin need to be determined.

No less puzzling is why the presence of U87 tumors, in contrast to 54A, alleviated the combined intestinal toxicity of DC101 and WBI. We speculate that U87 tumors were a significantly more capacious “sink,” eliminating DC101 from the mouse body, than 54A. Two facts seem to support this idea. First, as shown by our Western blot analysis, Flk-1 expression was on average at least 3–5-fold higher in U87 than in 54A untreated tumors (data not shown). Second, the dynamics of tumor size changes under the influence of DC101 (with or without radiation) were very different between these two xenografts. The volume of U87 tumors was larger than the initial volume for the entire DC101 treatment period as well as subsequently (Fig. 2B). In contrast, 54A tumors shrank very fast under the influence of combined treatment (Fig. 2A), thus likely decreasing the amount of DC101 extraction from the organism. When DC101 was used without local irradiation, the development of ascites was observed only among those mice in which 54A tumors responded well to the antibody. Additional experiments on plasma pharmacokinetics and tissue distribution of DC101 in WBI and non-WBI animals are needed to test this hypothesis.

Conclusion

Anti-VEGFR2 antibody in combination with fractionated radiotherapy considerably (1.3–1.7-fold) decreased the dose of radiation necessary to locally control 50% of two different human tumor xenografts. In contrast, anti-VEGFR2 therapy did not modulate radiation-induced skin damage. However, DC101 exacerbated a residual damage in the intestine from the WBI. In the meantime, the results obtained serve as a basis for future preclinical studies with orthotopic and spontaneous tumors, and clinical trials using VEGFR2-blocking antibodies with radiotherapy.

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References
