Anti-integrin monoclonal antibody CNTO 95 enhances the therapeutic efficacy of fractionated radiation therapy in vivo

Shoucheng Ning,1 Jeffrey A. Nemeth,2 Rebecca L. Hanson,2 Kevin Forsythe,1 and Susan J. Knox1

1Department of Radiation Oncology, Stanford University Medical Center, Stanford, California; and 2Centocor, Inc., Radnor, Pennsylvania

Abstract
Selective targeting of up-regulated integrins on tumor cells is a novel antiangiogenesis strategy for treating solid tumors. CNTO 95 is a fully human anti-αv integrin monoclonal antibody and has shown antitumor activity when used as a single agent in preclinical studies. We previously showed that radiation combined with an integrin αvβ3 antagonist cRGD peptide increased the therapeutic efficacy of radiation in preclinical tumor models. We hypothesized that the combination of radiation and CNTO 95 would synergistically enhance the efficacy of radiation therapy. The in vitro studies showed that CNTO 95 radiosensitized and induced apoptosis in M21 cells in vitronectin-coated dishes. In mice bearing established human cancer xenograft tumors, CNTO 95 alone had only a moderate effect on tumor growth. The combined therapy of CNTO 95 and fractionated radiation significantly inhibited tumor growth and produced the longer tumor growth delay time in multiple tumor models. Maintenance dosing of CNTO 95 following irradiation contributed to efficacy and was important for continued inhibition of tumor regrowth. Immunohistochemistry studies showed that the combined use of CNTO 95 and radiation reduced the αv integrin and vascular endothelial growth factor receptor expression and the microvessel density and increased apoptosis in tumor cells and the tumor microenvironment. CNTO 95 alone and in combination with radiation did not produce any obvious signs of systemic toxicity. These results show that CNTO 95 can potentiate the efficacy of fractionated radiation therapy in a variety of human cancer xenograft tumor types in nude mice. These findings are very promising and may have high translational relevance for the treatment of patients with solid tumors. [Mol Cancer Ther 2008;7(6):1569–78]

Introduction
Radiation therapy is widely used to treat cancer, with more than 50% of all cancer patients receiving radiation therapy during the course of their disease. Radiation therapy can play a pivotal role in the local and regional control of malignant diseases. It is used both alone as a single modality, and as a component of multimodality therapy. Despite recent technical advances in the precise delivery of radiation therapy, many patients treated with radiation therapy are not cured or adequately palliated. The efficacy of radiation therapy is limited by the inability to deliver a high enough dose to kill all of the tumor cells in the field while maintaining acceptable toxicity to the surrounding normal tissues. Another factor limiting the efficacy of radiation therapy is the occurrence of radiation resistance. Chemotherapy has been used with radiotherapy to treat the radioresistant tumors and improve the therapeutic index, but is often associated with significantly increased toxicity. The use of more specific agents to target tumor cells and the tumor microenvironment in combination with radiotherapy is a promising area of investigation. For example, preclinical and clinical studies have shown that the use of antiangiogenic agents with radiation significantly enhances the therapeutic index of radiotherapy (1–3).

CNTO 95 is a fully human anti-αv integrin monoclonal antibody that binds human αv integrins with high affinity (Kd ~200 pmol/L; ref. 4). Preclinical studies have shown that CNTO 95 exhibits antitumor and antiangiogenic activity in human cancer xenograft models when used as a single agent with few adverse effects (4, 5). Recently, a phase I study of CNTO 95 in patients with advanced solid tumors has shown that CNTO 95 was well tolerated (6). Biopsy data from an angiosarcoma patient in the phase I trial showed tumor penetration and localization of CNTO 95 antibody in the targeted tumor. CNTO 95 is now in phase II clinical trials for the treatment of a variety of malignancies. Although the specific mechanism of action accounting for the antitumor activity of CNTO 95 has been not fully elucidated, inhibition of tumor angiogenesis and proliferation of tumor cells via inhibitory effects on targeted integrins are thought to play an important role in the inhibition of tumor growth.

In an effort to increase the therapeutic index of radiation therapy, we designed experiments to study the efficacy of fractionated radiation therapy combined with CNTO 95 for the treatment of human xenograft tumors in nude mice. Based on our previous studies (7–9), we hypothesized that CNTO 95 would potentiate the efficacy of fractionated radiation therapy by blocking the
proangiogenic prosurvival signal transduction pathways of integrins in tumor cells and tumor vascular endothelial cells, which may be up-regulated by radiation while not increasing the adverse effects of radiation therapy.

Materials and Methods

Antibodies

CNTO 95 was provided by Centocor, Inc. CNTO 95 was diluted in physiologic saline (0.9% sodium chloride) at a concentration of 1.0 mg/mL for i.p. injection. The mouse IgG1 antihuman integrin αv monoclonal antibody, antihuman integrin αvβ3 monoclonal antibody (MAB1976Z), antihuman integrin αvβ5 monoclonal antibody (MAB1961Z), mouse IgG2a antihuman integrin αvβ6 (MAB2077Z), and mouse IgG1 antihuman integrin β3 (MAB1951Z) were received from Chemicon. Mouse IgG1 isotype control and mouse IgG2a isotype control were purchased from BD Biosciences. The rabbit anti–FLK-1 polyclonal antibody, anti-αv integrin polyclonal antibody, and an anti-CD31 polyclonal antibody (platelet/endothelial cell adhesion molecule 1) were purchased from Santa Cruz Biotechnology. Secondary antibody, R-phycoerythrin–conjugated AffinityPure F(ab’)2 fragment goat anti-mouse IgG + IgM (H+L) was purchased from Jackson Immuno Research Laboratories.

Cell Culture

Human tumor cell lines M21 melanoma, UMSCC-22B oral squamous carcinoma, U87 glioblastoma, and HT29 colon adenocarcinoma cells were maintained in DMEM (Invitrogen) supplemented with 10% FCS, 100 units/mL penicillin, and 100 μg/mL streptomycin in a 37°C humidified incubator with a mixture of 95% air and 5% CO2. All experiments were done on exponentially growing cells with a cell population doubling time of ~20 to 36 h.

Integrin Screening Assay

The expression of αv integrins was characterized on four tumor cell lines of M21, 22B, U87, and HT29. Cells were harvested and washed twice with serum-free medium (RPMI 1640 supplemented with 1× NEAA). Cells were counted and resuspended at a concentration of 5 × 10^6/mL in serum-free medium. One hundred microliters of cells per well were added to U-bottomed 96-well plates, nine wells for each cell line. Plates were centrifuged at 1,400 rpm for 4 min. Supernatant was decanted and cells were resuspended in 10 μg/mL of primary antibody or medium only for controls. Primary antibodies tested include αv, αvβ3, αvβ5, αvβ6, β1, IgG1 isotype control, and IgG2a isotype control. Plates were incubated on ice for 1 h. Plates were centrifuged at 1,400 rpm for 4 min and the antibody was removed by decanting. Cell pellets were washed thrice by resuspending in fluorescence-activated cell sorting (FACS) buffer (200 μL/well) and centrifuging. Secondary antibody (1:150 dilution in serum-free medium) was added in 100 μL/well (or 100 μL/well of serum-free medium for medium only control) for 1 h on ice. Cells were washed thrice as previously mentioned. Cells were resuspended in 400 μL of FACS buffer and analyzed on a FACS machine (Becton Dickinson).

In vitro Clonogenic Assay

Radiation survival curves were generated using an in vitro clonogenic assay as described previously (10). Briefly, M21 cells were plated in triplicate in 60-mm Petri dishes (Becton Dickinson) and incubated overnight before treatment. Following addition of CNTO 95, cells were irradiated with 0 to 10 Gy at room temperature using a 137Cs source with a dose rate of 276 cGy/min. After incubation for 14 d, dishes were stained with 0.25% crystal violet and colonies containing ≥50 cells were counted. Results were expressed as a surviving fraction of the plating efficiency of the untreated control cells.

Cell Cycle and Apoptosis Analysis

The cell cycle distribution after exposure to CNTO 95 and radiation was analyzed by using a flow cytometer as previously described (11). The level of apoptosis was quantitated by measuring the number of subdiploid (sub-G1) cells. Briefly, M21 cells were grown in 60-mm dishes and treated with radiation and CNTO 95 alone or in combination. At 8, 12, 24, 36, 48, 72, and 96 h, cells were collected with trypsin-EDTA solution and fixed in 100% ethanol. Cells were washed twice with PBS-EDTA solution, treated with 100 μg/mL of RNase A for 30 min at room temperature, and stained with propidium iodide (25 μg/mL). The DNA content was analyzed with a FACScalibur flow cytometry (Becton Dickinson). The percentage of cells in the apoptotic sub-G1, G1, S, and G2-M phases was calculated.

Mouse Tumor Model and Therapy

Male nude mice, 8-wk-old and 20 to 25 g in body weight, were purchased from Taconic Laboratory. Mice were tested and found to be negative for specific pathogens. The mice were normally bred and maintained under specific pathogen-free conditions, and sterilized food and water were available ad libitum. Mice were injected s.c. in the right flank with 5 × 10^6 tumor cells in a suspension volume of 100 μL. One tumor per mouse was inoculated. When tumors reached an average size of 120 mm^3 (80-200 mm^3), mice were randomly assigned to the different treatment groups. Six to 8 mice were used in each group. CNTO 95 was injected i.p. at a dose of 10 mg/kg body weight, thrice a week for 1 to 4 wk, as specified in each experiment. For irradiation, the unanesthetized tumor-bearing mice were placed in individual lead boxes with tumors protruding through a cutout window at the rear of each box. The radiation was delivered using a Philips RT-250 200 kVp X-ray unit (12.5 mA; Half Value Layer, 1.0-mm Cu) at a dose rate of 140 cGy/min. Tumors were locally irradiated with a dose of 250 cGy per fraction daily for 5 to 10 consecutive days as specified in each experiment. The length and width of the tumors were measured with calipers before treatment and thrice a week thereafter until the tumor volume reached at least four times the pretreatment volume. The tumor volume was calculated using the following formula: tumor volume (mm^3) = π/6 × length × width^2. The tumor volume quadrupling (4×) time was determined by a
best-fit regression analysis. The tumor growth delay time (in days) is the difference between the tumor volume quadrupling time of treated tumors compared with that of untreated control tumors. Both the tumor volume quadrupling time and tumor growth delay time was calculated for each individual animal and then averaged for each group. In some experiments, a complete response of tumors was recorded if a tumor completely disappeared and was not palpable at the end of the experiment. Body weight was measured twice a week. The mouse experiments described herein were approved by the Stanford University Administrative Panel for Laboratory Animal Care.

**Immunohistochemistry Analysis**

For histologic analyses of integrin $\alpha_v$ and vascular endothelial growth factor (VEGF) receptor (VEGFR) expression, microvessels, apoptosis, and general histopathologic changes in tumors that were treated with CNTO 95 and radiation, tumor-bearing mice were sacrificed 3 d after the last fraction of 5 d of radiation, with three mice per treatment regimen. Tumors were removed, immediately frozen in optimum cutting temperature compound, and stored in $-80^\circ$C until processing. Frozen tumors were sectioned at 6-μm thickness and stained with H&E for general histologic analysis, with an anti–Flk-1 polyclonal antibody for VEGF receptor expression, an anti-$\alpha_v$ integrin polyclonal antibody for $\alpha_v$ integrin expression, and an anti-CD31 polyclonal antibody for microvessel density counts, and with an ApopTag peroxidase in situ oligo ligation apoptosis detection kit (Chemicon International) for detecting apoptosis following the manufacturer’s standard protocol. Slices that were stained with Flk-1 and $\alpha_v$ integrin primary antibodies and green fluorescence secondary antibody were counterstained with 4',6-diamidino-2-phenylindole. Slides that were stained with anti-CD31 antibody or apoptosis assay kit were briefly counterstained with hematoxylin for background nuclear visualization. Slides were visualized and photographed using a Leica DM6000B fluorescence/light microscope (Leica Microsystems) connected to a Leica DFC420 and a Spot digital camera (Diagnostic Instruments). For quantification of VEGFR and integrin $\alpha_v$ expression, tissue sections from three tumors were analyzed at $\times200$ magnification (10 × 20) and fluorescence intensities were measured using Spot3 program. For quantification of microvessels, tissue sections from three tumors were analyzed at $\times100$ magnification and CD31-positive vessels were counted. CD31-positive vessels that were separated from an adjacent vessel were counted as one vessel. Eight fields per section were randomly analyzed and data are presented as a mean ± SD.

**Statistics**

Data were statistically analyzed using a two-tailed Student’s $t$ test.

**Results**

**In vitro Radiation Survival**

Initially, experiments were done to determine the effect of CNTO 95 on radiation survival in vitro. M21 melanoma cells were grown in either vitronectin, fibronectin, or soft agar precoated tissue culture dishes. CNTO 95 at a final concentration of 13 μg/mL was added and dishes were irradiated. Figure 1 shows the radiation dose-response survival curves of M21 cells in the presence or absence of CNTO 95. The effect of CNTO 95 on radiation survival was dependent on the coating matrix. CNTO 95 alone reduced the survival of M21 cells by 3% in vitronectin-coated dishes, but had no effect on survival in fibronectin- or soft agar-coated dishes. Radiation alone at doses of 0 to 10 Gy reduced the clonogenic survival in a dose-dependent manner. In vitronectin-coated cell culture dishes, the surviving fraction at 2 Gy (SF2) was 0.82 ± 0.04. When combined with CNTO 95, the survival curves shifted downward with a SF2 of 0.71 ± 0.08. The survival at 10 Gy radiation was decreased from 0.063 ± 0.01 for radiation alone to 0.011 ± 0.003 for radiation plus CNTO 95. In fibronectin-coated dishes, the survival curves for radiation alone and radiation plus CNTO 95 were over-

![Figure 1](https://example.com/mct1571fig1.png)
lapping with each other, suggesting that CNTO 95 did not affect the radiation response of M21 cells. In dishes coated with soft agar, radiation alone produced a dose-dependent decrease in survival. The addition of CNTO 95 further reduced the radiation survival and the SF2 from 0.64 ± 0.02 for radiation alone to 0.47 ± 0.1 for the combination of radiation and CNTO 95. These data indicate that CNTO 95 was able to block the interaction between precoated vitronectin and αv integrin receptors on M21 cells and enhance the radiation sensitivity of the melanoma cells under these culture conditions.

Cell Cycle Distribution and Apoptosis Induced by CNTO 95 and Radiation

The cell cycle profile after exposure to CNTO 95 and radiation was analyzed using a flow cytometer. The level of apoptosis was quantitated by measuring the number of sub-G1 cells. Figure 2 shows the time course (8–96 hours) of cell cycle distribution of M21 cells and the level of apoptotic sub-G1 cells at 96 hours following exposure to CNTO 95 and radiation. Following 5 to 10 Gy irradiation, M21 cells were arrested at G2-M phase for 24 hours, with a peak level of G2-M population of 57% to 62% at 8 hours compared with 28% for untreated cells at same time point. Cells were gradually recovered from G2-M phase arrest at 48 hours. Treatment with CNTO 95 did not significantly affect cell cycle redistribution of M21 cells. The population of apoptotic sub-G1 cells following 5 or 10 Gy radiation increased over 96 hours. At 96 hours, 5 Gy radiation induced 3.1 ± 0.9% sub-G1 cells. When combined with 20 μg/mL CNTO 95, the sub-G1 population was increased to 8.6 ± 2.3% (P < 0.01 versus 5Gy alone). At same time point, 10-Gy radiation alone or in combination with CNTO

![Figure 2. A time course of cell cycle distribution of M21 cells after treatment with 5 to 10 Gy radiation and 20 μg/mL CNTO 95. Cells were collected at designated time points, stained with propidium iodide, and analyzed for DNA content using FACS Calibur flow cytometry. Bottom, the apoptotic sub-G1 cells at 96 h after treatment. Columns, mean from four independent experiments; bars, SD.](image-url)
induced even higher levels of apoptosis, with sub-G1 populations measured at 7.3 ± 1.0% and 12.7 ± 1.4% for radiation alone and radiation plus CNTO 95 (P < 0.01), respectively. CNTO 95 alone did not significantly induce apoptosis in M21 cells, as the sub-G1 population was 2.4 ± 0.5% for cells treated with CNTO 95 alone and 1.5 ± 0.4% for untreated control cells.

Table 1. Analysis of integrin staining intensity in four tumor cell lines by FACS

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Iso IgG1</th>
<th>Iso IgG2α</th>
<th>αv</th>
<th>αv/β3</th>
<th>αv/β5</th>
<th>αv/β6</th>
</tr>
</thead>
<tbody>
<tr>
<td>M21</td>
<td>1.3</td>
<td>3.9</td>
<td>1.1</td>
<td>3.4</td>
<td>98.8</td>
<td>361.9</td>
</tr>
<tr>
<td>22B</td>
<td>1.2</td>
<td>3.3</td>
<td>1.1</td>
<td>2.8</td>
<td>99.6</td>
<td>330.1</td>
</tr>
<tr>
<td>U87</td>
<td>1.1</td>
<td>3.0</td>
<td>1.1</td>
<td>2.8</td>
<td>99.4</td>
<td>261.8</td>
</tr>
<tr>
<td>HT29</td>
<td>1.0</td>
<td>3.3</td>
<td>1.1</td>
<td>3.0</td>
<td>91.5</td>
<td>469.8</td>
</tr>
</tbody>
</table>

NOTE: ‘% Positive’ is the percentage of positive cells compared with isotype control. Abbreviation: MFI, mean fluorescence intensity.

Integrin Expression of Tumor Cells
Because CNTO 95 binds and inhibits multiple members of the αv integrin family, it was of interest to characterize the integrin expression patterns of the tumor cell lines to be used in in vivo studies. As seen in Table 1, all four tumor cell lines stained intensely with an antibody against αv. M21 and U87 cells stained intensely for integrin αv/β3, with

Figure 3. Therapeutic efficacy of combination therapy of CNTO 95 and fractionated radiation therapy in M21 melanoma, 22B head and neck cancer, U87 glioblastoma, and HT29 colon cancer xenograft tumor models in nude mice. There were four groups: untreated control; CNTO 95 10 mg/kg, three doses per week for 1 wk; 250 cGy local tumor radiation daily for 5 d; and combination of CNTO 95 and radiation. Six to eight animals were used in each group. Points, average tumor volume of each group (mean) versus time from start of treatment; bars, SD.
Table 2. Tumor growth delay time of xenograft tumors in nude mice treated with CNTO 95 and fractionated radiation alone or in combination

<table>
<thead>
<tr>
<th>Treatment regimen</th>
<th>TGD time ± SD (d)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>M21</td>
</tr>
<tr>
<td>Untreated control</td>
<td>—</td>
</tr>
<tr>
<td>CNTO 95 alone</td>
<td>2.1 ± 3.8</td>
</tr>
<tr>
<td>Radiation alone</td>
<td>5.7 ± 3.4</td>
</tr>
<tr>
<td>CNTO 95 + radiation</td>
<td>14.8 ± 2.8</td>
</tr>
<tr>
<td>Enhancement ratio</td>
<td>2.6</td>
</tr>
</tbody>
</table>

Abbreviation: TGD, tumor growth delay.

The tumor volume quadrupling times of untreated control tumors were 10.3 ± 1.9 d for M21, 9.8 ± 4.4 d for 22B tumors, 5.0 ± 0.6 d for U87, and 6.9 ± 1.3 d for HT29.


Comparison of tumor growth delay times between groups of CNTO 95 + radiation and radiation alone.

Weaker staining in 22B cells, and negligible staining in HT29 cells. Conversely, the intensity of αvβ5 was highest for 22B and HT29 cells, with moderate staining seen for M21 and U87. Moderate staining was also seen for integrin αvβ6 in M21 cells having the weakest staining. Thus, the four tumor cell lines present a variety of integrin profiles.

In vivo Therapeutic Efficacy

Groups of nude mice with established human melanoma (M21), head and neck squamous carcinoma (22B), glioblastoma (U87), and colon adenocarcinoma (HT29) xenograft tumors were treated with (a) 10 mg/kg CNTO 95, thrice per week; (b) 250 cGy/fraction local tumor irradiation daily for 5 consecutive days; (c) a combination of CNTO 95 and local tumor irradiation as above; or (d) an i.p. injection of normal saline (0.01 mL/g body weight) thrice weekly as an untreated control. In combination therapy, CNTO 95 was injected 1 hour after irradiation in experiments with M21, U87, and HT29 tumor models and 1 hour before irradiation in experiments with the 22B tumor model. Data are shown in Fig. 3 (tumor growth curves) and Table 2 (tumor growth delay time). CNTO 95 alone at a dose of 10 mg/kg did not significantly inhibit tumor growth in any of the tumor models. The 4× tumor growth delay time ranged from 3.2 ± 2.3 days for 22B tumors to 1.1 ± 1.1 days for HT29 tumor model and was not statistically significantly different compared with the relevant control groups (P = 0.1–0.3). Fractionated local tumor irradiation alone inhibited initial tumor growth and produced tumor growth delay times of 5.7 ± 3.4 days for M21, 16.4 ± 2.4 days for 22B, 7.2 ± 4.6 days for U87, and 12.0 ± 1.7 days for HT29 tumors, respectively (P < 0.01 compared with the untreated control group and CNTO 95 alone). The combination therapy of CNTO 95 and radiation effectively inhibited tumor growth and prolonged the tumor growth delay times to 14.8 ± 2.8 days for M21, 30.4 ± 5.8 days for 22B, 11.4 ± 4.1 days for U87, and 15.2 ± 2.1 days for HT29 tumors, respectively. CNTO 95 enhanced radiation-induced tumor growth delay in all four tumor models studied, with the magnitude of relative enhancement ratio in an order of M21 > 22B > U87 > HT29 (Table 2). Furthermore, the combined treatment of CNTO 95 and radiation resulted in one complete regression of 6 mice with 22B tumors, without tumor regrowth up to 3 months after the experiment was terminated.

The body weight of tumor-bearing mice was also measured in the therapeutic studies described above. Radiotherapy alone caused a 5% to 13% decrease in body weight on days 2 to 5 and the body weight was recovered thereafter. CNTO 95 alone or in combination with radiation did not cause any significant decrease in body weight compared with mice that were treated with radiation alone. Also, there was no notable change in the general appearance, skin reaction, or daily activity of tumor-bearing mice treated with CNTO 95 with or without fractionated radiation therapy.

Optimization of the Sequence of Administration of CNTO 95 and Radiation

Experiments were done to study the effect of the sequence and timing of administration of CNTO 95 relative to the time of irradiation on M21 tumor growth. First, we studied the sequence and timing effect with a single-dose combination. Nude mice with established M21 tumors were treated with a single dose of 500 cGy radiation at 0 hour, and CNTO 95 at a single dose of 10 mg/kg was administered i.p. either 24, 4, 2, and 0 hour before radiation or 2, 4, and 24 hours after radiation. As shown in Table 3A, there was a sequence- and timing-dependent effect on M21 tumor growth delay in the combined treatment groups. The longest tumor growth delay occurred when CNTO 95 was administered 2 to 4 hours before radiation (9.0–8.8 days). However, there were no statistically significant differences between all the groups studied (P > 0.05).

Table 3. Sequence and timing effect of administration of CNTO 95 in combination with radiation on M21 tumor growth delay

<table>
<thead>
<tr>
<th>CNTO 95 schedule</th>
<th>TGD ± SD (d)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 h before</td>
<td>5.5 ± 2.9</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Radiation alone</td>
<td>8.8 ± 3.0</td>
<td></td>
</tr>
<tr>
<td>0 h before</td>
<td>9.0 ± 4.4</td>
<td></td>
</tr>
<tr>
<td>2 h after</td>
<td>7.9 ± 3.5</td>
<td></td>
</tr>
<tr>
<td>4 h after</td>
<td>6.7 ± 4.3</td>
<td></td>
</tr>
<tr>
<td>24 h after</td>
<td>6.3 ± 3.5</td>
<td></td>
</tr>
</tbody>
</table>

B. CNTO 95 given 1 h before versus 1 h after fractionated radiation

<table>
<thead>
<tr>
<th>Treatment regimen</th>
<th>TGD ± SD (d)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT-250 cGy x 10</td>
<td>5.1 ± 3.1</td>
<td></td>
</tr>
<tr>
<td>CNTO 95 1 h before</td>
<td>23.4 ± 8.5</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>CNTO 95 1 h after</td>
<td>13.5 ± 8.5</td>
<td></td>
</tr>
</tbody>
</table>
We next studied the sequence effect of fractionated radiation therapy combined with CNTO 95. Mice with M21 tumors were treated with 250 cGy local tumor radiation daily for 5 consecutive days (Monday–Friday) a week for 2 weeks. CNTO 95 was injected i.p. at 10 mg/kg, three doses per week (Monday, Wednesday, Friday) for 2 weeks, either 1 hour before or 1 hour after radiation. Groups of mice treated with radiation only or normal saline were included as the radiation and untreated controls. Data are shown in Table 3B. Administration of CNTO 95 1 hour before radiation produced a tumor growth delay time of 23.4 ± 8.5 days, compared with 13.5 ± 8.5 days when CNTO 95 was administered 1 hour after radiation (P < 0.05), suggesting that there is a sequence- and timing-dependent effect in the M21 tumor model for the combined treatment with fractionated radiation and CNTO 95 in a multiple dose treatment regimen.

### CNTO 95 Administered as a Maintenance Therapy Following Combination Therapy

To study the hypothesis that CNTO 95 enhances the efficacy of fractionated radiation therapy by blocking the radiation–up-regulated proangiogenic prosurvival signals of integrins in tumor cells and tumor vascular endothelial cells, experiments were done to continuously administer CNTO 95 following the completion of combination therapy regimen as a maintenance therapy. Nude mice with M21 xenograft tumors were treated with two different regimens:

<table>
<thead>
<tr>
<th>Treatment regimen</th>
<th>TGD ± SD (d)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. CNTO 95 1 wk concomitant therapy with or without maintenance therapy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RT 250 cGy x5 + CNTO 95 3 doses/wk x 1 wk</td>
<td>5.7 ± 2.2</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>RT 250 cGy x5 + CNTO 95 with maintenance</td>
<td>8.1 ± 1.7</td>
<td></td>
</tr>
<tr>
<td>B. CNTO 95 2 wk concomitant therapy with or without maintenance therapy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RT 250 cGy x10 + CNTO 95 3 doses/wk x 2 wk</td>
<td>13.5 ± 8.5</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>RT 250 cGy x10 + CNTO 95 with maintenance</td>
<td>23.6 ± 6.6</td>
<td></td>
</tr>
</tbody>
</table>

Figure 4. Immunohistochemical analysis of αv, VEGFR expression, vessel density, apoptosis, and general histopathologic changes in treated M21 tumors. Tumor-bearing mice were treated as described in Fig. 3. Tumors were harvested 3 d after the last fraction of radiation and stained with an anti-αv, Flk-1, and CD31 antibodies; apoptosis assay kit; and H&E staining. A, digital photographs were taken at ×200 or ×400 magnification with a Leica microscope equipped with a digital camera. B, quantification of αv, VEGFR, CD31, and terminal deoxyribonucleotide transferase–mediated nick-end labeling-positive staining. Eight fields per section per tumor were randomly analyzed. Columns, mean of eight fields; bars, SD.
(a) mice were treated with 250 cGy radiation daily for 5 consecutive days and CNTO 95 10 mg/kg, 3 doses a week for 1 week; or (b) mice were treated for 1 week as above, followed by continuing administration of CNTO 95, 3 doses per week as a maintenance therapy until tumors reached the 4× volume. An untreated control group of tumor-bearing mice was included in the study. Results from this study are shown in Table 4A. As can be seen, CNTO 95 used as a maintenance therapy following the 1–week CNTO 95–radiation combination therapy more efficiently inhibited tumor growth and produced a longer tumor growth delay time (8.1 ± 1.7 days) when compared with the treatment regimen without maintenance therapy of CNTO 95 (5.7 ± 2.2 days, P < 0.05).

We next studied the 2-week combination therapy with or without maintenance dosing with CNTO 95. Mice bearing M21 tumors were divided into two groups: (a) mice were treated with 250 cGy radiation daily for 5 consecutive days per week for a total of 2 weeks and CNTO 95 at 10 mg/kg, 3 doses per week for 2 weeks; or (b) mice were treated as above for 2 weeks, followed by continuing treatment with CNTO 95, 3 doses per week until the completion of the studies. An untreated control group of tumor-bearing mice was also included in the study. As shown in Table 4B, the combined treatment with maintenance therapy of CNTO 95 (group 2) was superior to the regimen without maintenance of CNTO 95, and the tumor growth delay time was 23.6 ± 6.6 days for treatment regimen with maintenance therapy versus 13.5 ± 8.5 days for the regimen without the maintenance therapy (P < 0.05), suggesting that the maintenance dosing of CNTO 95 following initial combined therapy was effective and important for continued tumor growth inhibition.

**Immunohistochemical Study of Angiogenesis and Apoptosis In vivo**

To understand the mechanism of action by which CNTO 95 enhances the efficacy of radiation therapy, we studied angiogenesis and apoptosis in treated M21 tumors using immunohistochemistry staining. Mice with M21 tumors were treated with (a) CNTO 95 10 mg/kg, 3 doses total on Monday, Wednesday, and Friday; (b) 250 cGy local tumor irradiation daily for 5 consecutive days (Monday–Friday); (c) a combination of CNTO 95 and fractionated radiation as described in the single modality regimen above; or (d) an i.p. injection of normal saline (0.01 mL/g body weight), 3 doses, as an untreated control. Flk-1, integrin αv, and CD31 staining was used for detecting the VEGF receptor and αv integrin expression and microvessels, respectively. The ApopTag peroxidase in situ oligo ligation–positive apoptotic cells were much more evident in tumors treated with the combination regimen than that with other regimens (P < 0.01 combination versus CNTO 95 alone; P < 0.05 versus radiation alone). H&E staining revealed many more large and diffuse area of necrosis in tumors treated with radiation, either alone or in combination with CNTO 95. There was no apparent difference in the H&E staining of CNTO 95-treated tumors and untreated control tumors.

**Discussion**

Here, we report data demonstrating that CNTO 95, a fully human anti-αv, integrin monoclonal antibody, significantly enhanced the responses of a variety of human cancer xenograft tumors to fractionated radiation therapy in vivo. We found that CNTO 95 could radiosensitize human melanoma cells in vitronectin-coated culture dishes and in mice bearing established human tumor xenografts. CNTO 95 had a limited effect on tumor growth by itself, but effectively inhibited the tumor growth and prolonged the tumor growth delay time when used in combination with radiation therapy. The synergistic interaction of CNTO 95 and fractionated radiation was dependent on the sequence and timing of administration. The greatest extent of radiosensitization was achieved when CNTO 95 was given 1 to 4 hours before radiation. Furthermore, CNTO 95 could be used as a maintenance therapy following the initial combination treatment. Immunohistochemical studies revealed that the combined use of CNTO 95 and radiation therapy increased antiangiogenic effects and apoptosis in tumor cells and the tumor microenvironment. All four tumor cell lines express high levels of αv integrin, and different levels of integrins αvβ3, αvβ5, and αvβ8. There was no definite relationship between the αv integrin expression profile and radiation enhancement in the four xenograft tumor models studied. CNTO 95 was well tolerated and did not cause obvious systemic toxicity in terms of body weight in tumor-bearing mice. These
findings suggest that the combined use of CNTO 95 and fractionated radiation therapy has potential to be a safe and effective treatment regimen for a variety of solid tumors in patients.

Integrins are a large family of cell surface receptors that bind to extracellular matrix proteins, organize the cytoskeleton, and mediate the outside-in and inside-out signaling of cells. Integrins play an important role in modulating responses to antitumor therapies and in promoting the survival and proliferation of both tumor cells and tumor vascular endothelial cells through the regulation of the cell-cell and cell-extracellular matrix interactions (12). The integrins αvβ3 and αvβ5 are two well-studied integrins. They are highly overexpressed on tumor cells and tumor-associated neovascular endothelial cells compared with their expression on endothelial cells in normal tissues (13). Studies have shown that ligation of αvβ3 and other integrins activates downstream signaling proteins, such as focal adhesion kinase, integrin-linked kinase, and protein kinase B (Akt), resulting in cell proliferation, migration, and adhesion of tumor cells and tumor vascular endothelial cells (14–16). However, unligated integrin αvβ3 activates an apoptotic death pathway to inhibit tumor angiogenesis (17). The differential expression of integrins in tumors and mature endothelial cells in normal tissues makes them a promising therapeutic target for antitumor antiangiogenic therapy.

CNTO 95 is a fully human anti-αv integrin antibody and does not cross-react with mouse integrins and has limited cross-reactivity with rat integrins. In the experiments described here, the CNTO 95 antibody could only bind to human tumor cells but not mouse endothelial cells, and therefore did not have a direct effect on angiogenesis in the host microenvironment. As expected, CNTO 95 alone had very limited antitumor activity in nude mice bearing well-established human tumor xenografts. This is consistent with the results from the previous studies. In those studies, CNTO 95 could inhibit tumor development and growth in nude mice with freshly inoculated human tumor cells, independent of an antiangiogenic mechanism (4). However, the inhibitory effect of CNTO 95 on tumor growth was greater in nude rats because the antibody could inhibit proliferation of both tumor cells and tumor-associated vascular endothelial cells (4). As reported here, CNTO 95 potentiated the cytotoxic effects of radiation therapy on tumor cells as well as vascular endothelial cells in tumors, because the VEGFR and αv integrin expression and the density of microvessels was significantly reduced in tumors treated with radiation plus CNTO 95. These results suggest that CNTO 95 had an indirect effect on tumor angiogenesis in mice. It is likely that the ligation of CNTO 95 antibody with integrin receptors on tumor cells blocked or reduced the transmission of signals between tumor cells and the extracellular matrix, thereby inhibiting angiogenesis in tumors, as supported by the observation that CNTO 95 alone could inhibit the expression of angiogenic markers VEGFR and αv integrin. More importantly, radiation not only kills tumor cells and vascular endothelial cells in tumors but also induces and activates the expression of a variety of proangiogenic and prosurvival factors, such as VEGF, fibroblast growth factor, platelet-derived growth factor and their tyrosine kinase receptors, integrin receptors, cyclooxygenase 2, and phosphatidylinositol 3-kinase/Akt (18–20). These overexpressed growth factors function as autocrine and paracrine signals to protect tumor cells and endothelial cells against radiation cell killing by inhibiting apoptotic signaling and promoting neovascular formation in tumors (18, 19, 21–26). Studies have shown that there is a cross-talk between integrins and tyrosine kinase receptors during tumor angiogenesis and in response to antitumor therapies (16, 27). The blocking of the cross-talk could inhibit, at least in part, the radiation-induced overexpression of the proangiogenic and prosurvival factors, or make tumor cells less able to respond to these radiation-induced and overexpressed growth factors.

The use of antiangiogenic therapy in combination with radiation could also have additional complementary actions, because antiangiogenic therapy only inhibits or affects the formation of new blood vessels, with limited or no effect on preexisting vasculature in well-established tumors, whereas radiation exerts its antitumor effects by killing both tumor cells and preexisting vascular endothelial cells in tumors, with no effect on postradiation angiogenesis. The lack of responsiveness of established tumors to the treatment with CNTO 95 alone suggests that established tumors are able to use preexisting blood vasculature to support their growth for at least a limited period of time (7, 8). The beneficial effect of CNTO 95, when used as a maintenance therapy, may be secondary to the ability of CNTO 95 to block proangiogenic signaling from surviving tumor cells and to thereby inhibit tumor growth via the inhibition of new blood vessel formation.

Recent studies on brain tumor stem cells have indicated that tumor stem cells may rely on the interaction with a perivascular niche to maintain their stem cell self-renewal properties, as well as their ability to promote the tumor growth, recurrence, metastasis, and radioresistance by preferential activation of the DNA damage response (28, 29). Indeed, antiangiogenic therapy could disrupt such microvascular niches for tumor stem cells and result in the loss of resistance to cytotoxic therapy (30). Therefore, we hypothesize that combined use of antiangiogenic therapy and radiation could synergistically target and kill both tumor stem cell and nonstem cells, and consequently enhance the therapeutic efficacy of radiation therapy.

It is also possible that CNTO 95 may result in the improvement of tumor oxygenation in irradiated tumors because previous studies have shown that antiangiogenic agents can increase tumor oxygenation in mice (31). Increased tumor Po2 could enhance the sensitivity of tumor cells to radiation and contribute to the efficacy of this combined treatment. Another study has shown that integrin αvβ3 antagonist cilengitide could increase the radiolabeled antibody uptake in tumors by 50%, suggesting that antiintegrin therapy might increase the tumor vascular permeability, with a secondary effect on oxygenation (32).
This effect, at least in part, may explain why the radio-sensitization effect of CNTO 95 was time and sequence dependent. Preadministration of CNTO 95 could therefore increase the vascular permeability and oxygen levels in tumors and make tumor cells more sensitive to radiation therapy.

In summary, our results reported here provide further evidence of a beneficial interaction between radiation therapy and anti-integrin antiangiogenic therapy. Because CNTO 95 is a fully human monoclonal antibody and reacts with multiple αv integrin heterodimers, rather than against a single specific integrin, and is well tolerated in patients, it is reasonable to speculate that CNTO 95 will be potent and effective when used in combination with radiation therapy in a variety of cancer patients.

Disclosure of Potential Conflicts of Interest

J.A. Nemeth and R.L. Hanson: Centocor, Inc., employees. The other authors disclosed no potential conflicts of interest.

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Mol Cancer Ther 2008;7:1569-1578.