Radioimmunotherapy Using Vascular Targeted $^{213}$Bi: The Role of Tumor Necrosis Factor $\alpha$ in the Development of Pulmonary Fibrosis

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Abstract

A monoclonal antibody (201B) specific to murine thrombomodulin, covalently linked to cyclohexyl diethylene-triaminepentaacetic acid, successfully delivers chelated $^{213}$Bi, an $\alpha$-particle emitter, (213-Bi-201B) rapidly to lung vascular endothelium. When injected at doses of 1 MBq/mouse, 213Bi-201B destroyed most of the 100 colonies of EMT-6 mammary carcinomas growing as lung tumors of up to 2000 cells/colony. Some mice were cured of lung tumors, and others had extended life spans compared to untreated control animals but eventually succumbed to tumor recurrence. At injected doses of 4–6 MBq/mouse, 100% of lung tumor colonies were eliminated; however, 3–4 months later, these mice developed pulmonary fibrosis and died. The mechanisms leading to the fibrotic response in other pulmonary irradiation models strongly implicate tumor necrosis factor $\alpha$ (TNF-$\alpha$), released from damaged tissues, as the pivotal inflammatory cytokine in a cascade of events that culminate in fibrosis. Attempts to prevent the development of pulmonary fibrosis, by using antibodies or soluble receptor (rhuTNFR:Fc) as inhibitors of TNF-$\alpha$, were unsuccessful. Additionally, mice genetically deficient for TNF-$\alpha$ production developed pulmonary fibrosis following 213Bi-201B treatment. Interestingly, non-tumor-bearing BALB/c mice receiving rhuTNFR:Fc or mice genetically deficient in TNF-$\alpha$ production and treated with 213Bi-201B, had significantly reduced life spans compared to mice receiving no treatment or 213Bi-201B alone. We speculate that in normal mice, although TNF-$\alpha$ may induce an inflammatory response following $\alpha$-particle radiation mediated tumor clearance and pulmonary damage, its effects in the post-tumor clearance time period may actually retard the development of fibrosis.

Introduction

Inhibition of tumor growth by targeting tumor vasculature is an approach that has therapeutic promise. One such approach to solid tumor therapy is inhibiting angiogenesis. Recent studies have demonstrated tumor growth retardation by blocking the endothelial cell expressed adhesion molecule, $\alpha_v\beta_3$ (1); inhibiting the tumor-derived vascular endothelial growth factor directly (2) or through its receptors (3); inhibiting endothelial cell proliferation with endostatin (4); or by administering the tumor-derived angiogenic factor, angiostatin (5). These methods are specifically directed at limiting tumor proliferation by interfering with blood vessel growth. A related approach is to cause infarction of tumor vessels by destruction of endothelial cells in established vessels with immunotoxins (6) or coagulins (7, 8).

Although approaches to destroy tumor vasculature have retarded tumor growth, they do not address the fate of tumor cells. A method of tumor eradication that offers disruption of blood vessels as well as direct tumor destruction is VT-RAIT3 (9–12). The model system used in this work uses the MAb 210B to murine thrombomodulin, which binds efficiently to normal lung endothelial cells (9, 10). The combination of this MAb and 213Bi (a high-energy $\alpha$-particle emitter) has been successful in treating mice bearing lung tumor colonies of several tumor types. This therapy cured mice of EMT-6 tumor colonies growing in the lung but has, as a side effect, the development of pulmonary fibrosis, which eventually leads to the death of treated mice (12). Thus, 100% cure rates were shadowed by the eventual induction of fatal pulmonary disease. Human and rodent trials studying the effects of externally delivered radiation to the thoracic cavity revealed a sequentially and clinically similar development of pulmonary fibrosis in the chronic stages posttherapy (13, 14). The mechanisms attributed to this often fatal outcome are numerous, and they frequently implicate the inflammatory cytokine, TNF-$\alpha$, released from damaged vasculature and resident lung cells. This cytokine is suspected of being a primary player in the cascade of events initiated to repair radiation-induced tissue damage but leading to chronic inflammation-induced fibrosis (15–17). To evaluate the role of TNF-$\alpha$ in the development of pulmonary fibrosis in the 213Bi VT-RAIT model, we have blocked the activity of this cytokine by various methods in mice receiving 213Bi targeted with MAb 210B to lung vasculature. The results show that in tumor-bearing mice deficient in TNF-$\alpha$, tumor clearance is achieved following 213Bi VT-RAIT tumor therapy; however, pulmonary fibrosis still de-
develops, albeit within a slightly altered time frame. Thus, in this model, the presence of TNF-α is not a primary factor in tumor killing or in the development of pulmonary fibrosis.

Materials and Methods

Cells and Tumor Growth. EMT-6 mammary carcinoma cells from BALB/c mice were recovered from frozen storage, subcultured, and prepared for injection as described previously. Cells were suspended in sterile PBS, and 200 μl were injected into the tail veins of recipient mice.

Mice. Female BALB/c mice were obtained from Taconic Farms (Germantown, NY) and maintained throughout experimentation in filter-top cages in a specific pathogen-free facility at Oak Ridge National Laboratory. When 5–6 weeks of age, mice were injected i.v. with 2.0 × 10^6 EMT-6 mammary carcinoma cells.

TNF-α/LT-α double KO mice (TNF/LT KO) on a 129/sv background (18) of the same age were obtained from the laboratory of Barry T. Rouse (University of Tennessee, Knoxville, TN).

Reagents. A MAb (MAb 201B) to murine thrombo-modulin was generated in rats and purified as previously described (19). MAb 201B localizes rapidly to mouse vascular endothelium (20). An isotype-matched rat, non-specific MAb (MAb 14), was prepared as described elsewhere (21) and used as a control in these experiments.

The “b” isomer of the chelator, CHX-b-DTPA, was provided by Dr. Martin Brechbiel of the NIH (21, 22). ^213^Bi was eluted from an actinium-225 (225Ac) generator as described previously (23). MAbs 201B and 14 (10 mg/ml) were reacted with CHX-b-DTPA at a 1:10 molar ratio (MAb:CHX-b-DTPA) and dialyzed to remove free chelator. ^213^Bi was neutralized and pH adjusted to 4.7 prior to incubating with MAb:CHX-b-DTPA and dialyzed to remove free chelator. ^213^Bi was neutralized and pH adjusted to 4.7 prior to incubating with MAb:CHX-b-DTPA and dialyzed to remove free chelator. ^213^Bi-MAb complex was analyzed by high-performance liquid chromatography to confirm radiolabeling yields of >80%. To determine in vivo stability, the high-performance liquid chromatography-purified complex was injected into mice and, at 1 h postinjection, greater than 30% of the injected dose was recovered in the lungs (10). The immunoreactivity of the MAb conjugate was >90%, and the t1/2 of ^213^Bi retention in the lung was >4 h (10).

TNF-α Inhibition. The dimeric human TNF receptor p80/IgG1 Fc fusion protein (rhuTNFR:Fc) was supplied by Immunex Corp. (Seattle, WA). Seventy-five μg of this preparation was injected i.p. to control or tumor-bearing mice on days 4, 5, and 6 of experimentation, based on information of in vivo use provided by the manufacturer, which represented 24 and 2 h prior to and 24 h after administration of ^213^Bi-MAb.

Twenty μg of purified goat antimouse anti-TNF-α IgG (R & D Systems, Minneapolis, MN), a neutralizing antibody, were injected i.p. into control or tumor-bearing mice on days 5, 6, and 8, which represented 4, 24, and 72 h after administration of ^213^Bi-MAb. This dosage regimen was extrapolated from the manufacturer’s suggested usage of this product in an in vitro neutralization assay.

A nonneutralizing antibody, rat antimouse TNF-α IgG1 (PharMingen, San Diego, CA), was injected at the same concentration into groups of control animals following the same schedule.

Statistics. Mean time to death and body weight data were analyzed by a 2 × 2 repeated measures ANOVA using both genotype (i.e., TNF-α expressing or not) and tumor burden as variables. P values less than 0.05 were considered significant.

Results

Treatment with ^213^Bi-201B Prevents Lung Tumor Growth and Prolongs the Lives of EMT-6 Lung Tumor-Bearing Mice. BALB/c or TNF/LT KO mice receiving tumor cells and subsequently treated with control preparations (^213^Bi chelated with EDTA and mixed with MAb 201B) had gross or histological evidence of tumor growth in all lung lobes. These animals uniformly died by 16 days following tumor inoculation (Table 1) and demonstrated clinical signs of respiratory compromise and weight loss within this time period. BALB/c mice treated with these and other control preparations had a similar disease course, confirming earlier published results (11, 12).

Similarly, all BALB/c mice injected with EMT-6 cells and receiving rhuTNFR:Fc or neutralizing anti-TNF-α treatment with no radioisotope had extensive and ultimately lethal lung tumors. The lungs of all animals bearing tumors had nests of tumor cells (~100 colonies/lung) arranged predominantly around vessels but invading surrounding pulmonary parenchyma as well as along the pleural edges and within alveolar walls.

In contrast, tumor-bearing TNF/LT KO mice receiving ^213^Bi-201B survived beyond 50 days, similar to outcomes in tumor-bearing BALB/c mice treated in the same manner (11, 12). When mice were treated with ^213^Bi-201B 5 days after the EMT-6 cell injection, survival times in TNF/LT KO and BALB/c mice were significantly (P < 0.001) prolonged over that of nontreated tumor-bearing mice (Table 1).

Both groups of mice demonstrated variable degrees of clinical illness, predominantly manifested as a decrease in body weight, within the first 2 weeks post-^213^Bi-201B therapy. These remained relatively healthy, however, until 60–65 days of experimentation and had no recurrence of lung tumor colonies noted grossly or histologically at the time of sacrifice. Histological examination of the lungs of tumor-bearing mice receiving ^213^Bi-201B, which were sacrificed early in the experiment, contained hemorrhagic foci of necrotic tumor cells by 24 h posttreatment. These foci of damage resolved over a period of 7–10 days, and by the time of death, tumor cells had not recurred. Histological evaluation of the lungs of tumor-bearing mice receiving ^213^Bi-201B revealed nests of fibrosis, which appeared to localize to former tumor foci in tumor-cured animals.

Treatment with ^213^Bi-201B Shortens the Lives of Non-tumor-Bearing TNF/LT KO Mice. All non-tumor-bearing BALB/c and TNF/LT KO mice receiving no radiotherapy survived beyond 100 days of experimentation and were sacrificed as healthy animals at days 139 and 120, respectively (Table 1). In contrast, following treatment with ^213^Bi-201B, non-tumor-bearing BALB/c mice had a mean survival time of 135.2 days,
and non-tumor-bearing TNF/LT KO mice had a mean survival time of 69 days, the latter representing a statistically significant reduction in survival (P < 0.05).

**Inactivation of TNF-α Reduced Survival following Treatment with 213Bi-201B.** Three separate approaches were used to block TNF-α activity in this study, and mean time of survival posttherapy and the development of histologically detectable pulmonary damage as a consequence of 213Bi VT-RAFT were evaluated.

Survival of non-tumor-bearing BALB/c mice receiving 213Bi-201B alone was significantly greater (P < 0.05) than that of mice receiving 213Bi-201B/rhuTNFR:Fc (Table 1). Non-tumor-bearing BALB/c mice receiving 213Bi-201B and neutralizing antibody to TNF-α had a reduction in mean survival time when compared to animals treated with 213Bi-201B alone; however, the difference was not statistically significant. A reduction in survival time was also observed, but was not statistically significant, when comparing the effects of combination 213Bi-201B/anti-TNF-α or 213Bi-201B/rhuTNFR:Fc and 213Bi-201B alone in tumor-bearing BALB/c mice. Tumor-bearing and non-tumor-bearing BALB/c mice treated with the nonneutralizing TNF-α antibody alone or in combination with 213Bi-201B had survival times comparable to counterpart control groups, indicating no adverse effects of this antibody (data not shown).

**Treatment with 213Bi-201B Initiated Pulmonary Inflammation.** Within 2 days of treatment, the targeted tumor foci and surrounding interstitial spaces of mice receiving 213Bi-201B contained a predominance of neutrophils that persisted until about 35 days posttherapy, despite the presence of pulmonary macrophages within the necrotic tumor foci and surrounding alveolar spaces. Additionally, both tumor- and non-tumor-bearing BALB/c and TNF/LT KO mice treated with 213Bi-201B had a persistent vasculitis, initially dominated by neutrophils and later by mononuclear cells, throughout the observation period. Non-tumor-bearing mice receiving 213Bi-201B also had histological evidence of vascular damage and an interstitial pneumonitis that persisted until approximately 35 days postinjection, at which point nests of fibrosis began to appear, predominantly around vessels and diffusely within dependent lung areas. Thus, although tumors were eradicated from mice receiving 213Bi-201B, the administration of this therapy to tumor as well as to non-tumor-bearing mice resulted in pneumonitis and vasculitis. Further, the neutrophilic response persisted into the chronic phase posttherapy (30–60 days), which is characterized in our model by the development of pulmonary fibrosis. No assessment of cytokine profiles to determine the nature of the inflammatory response was attempted in any histological sections.

**Table 1** Survival of various treatment groups

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Mean time to death (days)</th>
<th>Body weight at death (% of initial weight)</th>
<th>No. of mice alive at 100 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c: no tumors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No treatment</td>
<td>&gt;139*</td>
<td>100</td>
<td>5/5</td>
</tr>
<tr>
<td>213Bi-201B</td>
<td>135.2 ± 5.5</td>
<td>99.1</td>
<td>5/5</td>
</tr>
<tr>
<td>213Bi-201B + anti-TNF-α</td>
<td>101.6 ± 27.4</td>
<td>100</td>
<td>5/10</td>
</tr>
<tr>
<td>213Bi-201B + rhuTNFR:Fc</td>
<td>74.0 ± 6</td>
<td>76.7</td>
<td>0/5</td>
</tr>
<tr>
<td>BALB/c: tumors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No treatment</td>
<td>15.0 ± 1</td>
<td>77.6</td>
<td>0/5</td>
</tr>
<tr>
<td>213Bi-201B</td>
<td>92.4 ± 25.2</td>
<td>100</td>
<td>2/3*</td>
</tr>
<tr>
<td>213Bi-201B + anti-TNF-α</td>
<td>84.9 ± 30.5</td>
<td>100</td>
<td>2/7*</td>
</tr>
<tr>
<td>213Bi-201B + rhuTNFR:Fc</td>
<td>80.0 ± 5.5</td>
<td>87.1</td>
<td>0/4</td>
</tr>
<tr>
<td>TNF-α null: no tumors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No treatment</td>
<td>&gt;120</td>
<td>100</td>
<td>3/3</td>
</tr>
<tr>
<td>213Bi-201B</td>
<td>69.0 ± 4</td>
<td>72.0</td>
<td>0/3</td>
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<tr>
<td>TNF-α null: tumors</td>
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<tr>
<td>No treatment</td>
<td>12.5 ± 1.5</td>
<td>100</td>
<td>0/2</td>
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<tr>
<td>213Bi-201B</td>
<td>89.0 ± 32</td>
<td>89.6</td>
<td>1/3</td>
</tr>
</tbody>
</table>

* Mice were sacrificed at the termination of the experiments but were healthy.
* Mice were removed from the study due to the development of subcutaneous tumors.

**Discussion**

The efficacy of targeting lung tumors using the short-half-lived α-particle emitter 213Bi complexed to MAB 201B has proven successful, and results of several such experiments have been published elsewhere (10–12). Although this radioimmunoconjugate binds selectively to thrombomodulin expressed on the luminal surfaces of pulmonary vascular endothelial cells, this form of therapy is 100% successful only if tumor foci were less than 10 cells in diameter, which is consistent with the range and penetration of the α-particles emitted from 213Bi (11).

The mechanism of tumor destruction, although dependent on radioisotope, has not been studied in detail. It has been previously shown, however, that nearly 40% of the injected dose accumulates within minutes in normal lung vasculature and microvasculature uniformly throughout the lung (11, 12). Dosimetry calculations indicate that absorbed dose to the lung is predominantly from α-emissions and is approximately 15 Gy/MBq of 213Bi (11). This dose should cause extensive destruction of lung cells; however, acute damage to lung tissue is apparently functionally repaired, because animals survive for months after moderate doses (3–5 MBq/animal) (11). Unfortunately, mice receiving this therapy, whether possessing lung tumors or not, latently develop pulmonary fibrosis, which leads to death. TNF-α has been implicated in both the acute and chronic...
inflammatory events leading to pulmonary fibrosis in humans and animals following thoracic irradiation (13, 14). Thus, this work deals with the role played by TNF-α both in tumor eradication and in the latent development of radiation induced pulmonary fibrosis, which appears to be an inevitable consequence of 213Bi VT-RAIT therapy in mice and a potential consequence of external thoracic irradiation in humans.

As illustrated in Table 1, tumor-bearing BALB/c mice treated with control antibodies died from lung tumor burdens within 16 days, whereas mice treated with 213Bi-201B were cured of lung tumors and had a mean survival time of 92.4 days, which represents a statistically significant prolongation of life. Similarly, treatment of tumor-bearing TNF/LT KO mice with 213Bi-201B was 100% successful in eradicating lung tumor burdens, providing a statistically significant increase in mean survival time from 12.5 days in untreated mice to 89 days for 213Bi-201B treated animals. None of the VT-RAIT-treated animals had lung tumors at the time of sacrifice. Thus, we conclude that treatment with 213Bi-201B eradicates existing lung tumors, prevents lung tumor regrowth, and significantly prolongs the lives of EMT-6 lung tumor-bearing BALB/c and TNF/LT KO mice.

Because TNF-α has been implicated in other models of radiation induced pulmonary fibrosis, an attempt was made to determine the role of TNF-α in the presented VT-RAIT model using three methods of TNF-α deprivation. Two methods were aimed at scavenging circulating TNF-α to prevent receptor triggering and subsequent inflammatory events involved in tumor destruction following 213Bi VT-RAIT in BALB/c mice, and the third used mice genetically incapable of TNF-α synthesis (TNF/LT KO; Ref. 18). However, because the presence of TNF-α has not yet been determined in the lungs of mice receiving 213Bi VT-RAIT, the role of this cytokine in both tumor destruction and the development of fibrosis following VT-RAIT can only be inferred from survival data and evaluations of histological lung damage. Tumor-bearing BALB/c mice treated with 213Bi-201B and rhuTNFR:Fc or neutralizing anti-TNF-α survived 80.0 and 84.9 days, respectively, whereas tumor-bearing TNF/LT KO mice had a mean survival time of 89 days posttherapy. Because these data represent no statistically significant differences, the destruction of tumors in this model appears to be independent of TNF-α status.

As indicated earlier, a consequence of treatment with 213Bi-201B in our model is the development of latent but fatal pulmonary fibrosis, regardless of tumor status. As can be seen in Table 1, treatment with 213Bi-201B reduces survival in non-tumor-bearing BALB/c (not significantly) and TNF/LT KO (significantly) animals by an incompletely understood mechanism but clearly contributed to the development of pulmonary fibrosis. The role played by TNF-α in the development of radiation induced pulmonary fibrosis is uncertain, but mean survival times were significantly reduced in BALB/c mice receiving 213Bi-201B/rhuTNFR:Fc or in TNF/LT KO mice receiving 213Bi-201B (Table 1). Although the use of blocking antibodies may not completely inhibit TNF-α activity, particularly over long periods, the use of gene deleted mice provides assurance of complete TNF-α absence throughout the experiment. Unfortunately, we made no prior assessment of TNF-α activity in the lungs of 213Bi-201B treated mice at any time point before or after VT-RAIT. However, even without this information, following treatment with 213Bi-201B, the absence of TNF-α leads to a significant reduction in survival times, which is histologically attributed to the development of pulmonary fibrosis. A similar effect was seen in models of sepsis-induced acute respiratory distress syndrome following the administration of anti-TNF-α MAbs (23), indicating that interfering with a single cytokine can have profoundly detrimental effects.

The initial insult caused to lung tissues by the administration of 213Bi-201B likely triggers a balanced elevation of inflammatory and protective cytokines. One consequence of this acute response may be the destruction of tumor cells (if present), but another may be damage to resident tissues which elicits an influx of neutrophils. This neutrophilic response, as observed histologically in all mice treated with 213Bi-201B, involves the sequestration and persistence of these cells in vascular and alveolar walls well beyond the time expected in acute phase responses (24). This persistence suggests the perpetuation of a proinflammatory state, of which the development of pulmonary fibrosis is a consequence.

We could not substantiate the damaging effects of the proinflammatory cytokine, TNF-α, in either the eradication of tumors or the subsequent development of pulmonary fibrosis in the presented 213Bi VT-RAIT model. The conclusions that can be drawn from evaluable animals in this study include the following: (a) the lives of all tumor-bearing mice are significantly prolonged following treatment with 213Bi-201B; (b) treatment with 213Bi-201B in non-tumor-bearing mice results in decreased life spans compared to nontreated mice. This effect is significant in genetically deficient TNF-α null (TNF/LT KO) mice; and (c) all mice treated with 213Bi-201B, regardless of genotype or tumor status, develop a fatal pulmonary fibrosis.

Thus, the presence or absence of TNF-α is not of primary importance to either the mechanisms of tumor clearance or the subsequent development of fibrosis in this model of VT-RAIT. Further, although we used a novel and specific method of tumor eradication and have more clearly defined the role of TNF-α, the mechanisms of and the inflammatory mediators involved in the development of latent fatal pulmonary fibrosis following 213Bi-201B therapy remain elusive.

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