Fractionated Locoregional Low-Dose Radioimmunotherapy Improves Survival in a Mouse Model of Diffuse-Type Gastric Cancer Using a $^{213}$Bi-Conjugated Monoclonal Antibody

Stefanie Bloechl, Roswitha Beck, Christof Seidl, Alfred Morgenstern, Markus Schwaiger, and Reingard Senekowitsch-Schmidtke

**Abstract**

**Purpose:** Locoregional radioimmunotherapy of i.p. tumor cell dissemination of diffuse-type gastric cancer using the $\alpha$-emitter $^{213}$Bi displayed good therapeutic results after a single application depending on the time interval between tumor cell inoculation and injection of the $^{213}$Bi-immunoconjugate. The aim of the present study was to compare single versus double i.p. injection of a tumor-specific antibody (d9MAb) conjugated with low activities of $^{213}$Bi in terms of therapeutic efficacy and toxicity.

**Experimental Design:** Nude mice were inoculated i.p. with $1 \times 10^7$ human gastric cancer cells (HSC45-M2) expressing tumor-specific mutant d9-E-cadherin (d9-E-cad). After tumor cell inoculation, the mice were injected i.p. with a single injection at day 1 or 8, or double injections at days 1 and 8 or days 8 and 15 with 0.37, 0.74, or 1.48 MBq $^{213}$Bi-d9MAb. Therapeutic efficacy was determined by median survival, and toxicity was evaluated by leukocyte and platelet counts. The development of i.p. carcinomatosis was monitored by carcinoembryonic antigen concentrations in the serum of the mice.

**Results:** The median survival of treated animals increased, depending on the time interval (days) between tumor cell inoculation and therapy, and the injected activity, from 22 days of untreated mice to 48 days (0.37 MBq, 1 day), 84 days (0.37 MBq, 1 and 8 days), 46 days (0.37 MBq, 8 days), 46 days (0.74 MBq, 8 days), 78 days (0.74 MBq, 8 and 15 days), and 44 days (1.48 MBq, 8 days). The injected activities did not reduce leukocyte and platelet counts. Carcinoembryonic antigen, which was not detectable in the serum of tumor-free mice, increased after tumor cell inoculation and tumor proliferation and decreased after each therapeutic application of $^{213}$Bi-d9MAb.

**Conclusions:** Double application of only 0.37 MBq of $^{213}$Bi-d9MAb at days 1 and 8 after tumor cell inoculation significantly prolonged median survival in nude mice suffering from i.p. tumor cell dissemination compared with a single injection. Even in an advanced stage of the disease, double injection of 0.74 MBq at days 8 and 15 was superior to a single injection of 1.48 MBq at day 8 without any sign of toxicity.

Dissemination of single tumor cells into the peritoneal cavity is the major cause of tumor recurrence even after R0 resection of the primary solid tumor. Currently, there is no effective treatment for peritoneal carcinomatosis. A promising therapy concept has been established in a mouse model applying i.p. the $\alpha$-emitter $^{213}$Bi conjugated to a monoclonal antibody (d9MAb) which binds to a tumor-specific mutant E-cadherin (d9-E-cad) found in human diffuse-type gastric cancer (1, 2).

The use of the high-linear energy transfer $\alpha$-emitter $^{213}$Bi coupled to an appropriate carrier seems an attractive approach for therapy of i.p. disseminated gastric cancer (3). The specific binding of the labeled carrier to single tumor cells or tumor cell clusters and the short path length of $\alpha$-particles emitted by $^{213}$Bi of only several cell diameters result in a high cytotoxic potential (4, 5). $^{213}$Bi ($t_{1/2} = 46$ minutes) is eluted from a $^{225}$Ac/$^{213}$Bi generator and rapidly coupled to mAbs by the chelator CIIX-$\alpha$-diethylentriaminetetraacetic acid (6, 7). After i.p. injection of the $^{213}$Bi-immunoconjugate, most of the activity of $^{213}$Bi decays within the peritoneal cavity thus preventing systemic toxicity.

In previous studies, we could show that by optimization of the applied activity of $^{213}$Bi-d9MAb, animals with i.p. diffuse-type gastric cancer cells (HSC45-M2) were cured without any signs of long-term toxicity when the time interval between tumor cell inoculation and $^{213}$Bi injection was 1 day (8).

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With increasing time intervals between tumor cell inoculation and $^{213}$Bi-therapy, however, survival of the animals decreased.

Therefore, the aim of the present study was to compare the therapeutic efficacy of single versus double application of $^{213}$Bi-d9MAb starting with low activities at different time points after tumor cell inoculation.

Because the HSC45-M2 tumor cells were reported to secrete carcinoembryonic antigen (CEA), we evaluated if CEA serum levels could be used as a tool for monitoring the development of the tumor mass in this mouse model (9).

Materials and Methods

**Antibodies and labeling.** A rat mAb reacting with mutant E-cadherin lacking exon 9 (d9-E-cad) was generated as described previously (1). The d9MAb (IgG2a, clone 6H8) specifically binds to HSC45-M2 gastric cancer cells due to expression of mutant d9-E-cad.

The α-emitter $^{213}$Bi, eluted from a $^{225}$Ac/$^{213}$Bi generator system (provided by the Institute for Transuranium Elements, Karlsruhe, Germany) was coupled to the antibody via the chelate SCN-CHX-AAc-diethylenetriaminepentaacetic acid as described previously (6). Briefly, mAbs conjugated to the chelate were incubated with BiI$_4$ from the the $^{225}$Ac/$^{213}$Bi generator system for 5 to 10 minutes in 0.4 mol/L ammonium acetate buffer at pH 5.3 (10). The $^{213}$Bi-immunoconjugates were purified by size exclusion chromatography. Binding of $^{213}$Bi-d9MAb to HSC45-M2 cells was assayed as described previously (8).

**Carcinomatosis model.** The model for i.p. tumor cell dissemination of diffuse-type gastric cancer expressing d9-E-cad has already been described (3, 8). Briefly, 6-week-old female nude mice were i.p. inoculated with $1 \times 10^7$ HSC45-M2 cells in 0.5 mL of supplemented DMEM. The cell line expressing d9-E-cad was established from a patient and kindly provided by Dr. Kazuyoshi Yanagihara from the National Cancer Center Research Institute, Chuo-ku, Tokyo (9, 11).

One day after tumor cell inoculation, tumor cells were still in the peritoneal cavity as single tumor cells or tumor cell clusters as assessed histologically. At day 8, however, macroscopically detectable small tumor deposits could be found on the serosa, especially in the region of pancreas and liver.

All animal studies were done in accordance with the guidelines for the use of living animals in scientific studies and the German Law for the Protection of Animals.

**Radioimmunotherapy studies.** Mice were treated in groups of $\sim 10$, either with single or double i.p. injection of $^{213}$Bi-d9MAb with different activities in a volume of 0.5 mL at different time intervals (days) after tumor cell inoculation as follows: 0.37 MBq (1 day), 0.37 MBq (1 and 8 days), 0.37 MBq (8 days), 0.37 MBq (8 and 15 days), 0.74 MBq (8 days), 0.74 MBq (8 and 15 days), and 1.48 MBq (8 days). Controls were treated with saline only, because the naked d9MAb has no therapeutic effect, as has been shown earlier (8). Mice were observed up to day 245 after tumor cell inoculation or sacrificed as soon as tumor cachexia or ascites, as determined sonographically (11 MHz), had developed. The survival curves were estimated by the Kaplan-Meier method and compared by using the log-rank test with $P < 0.05$ considered significant. For the survival curves, the median survival times with 95% confidence intervals were calculated.

**Carcinoembryonic antigen concentration in serum.** For quantitative measurement of CEA in the serum of mice, an automated chemiluminescence system CEA assay (ASC:180 CEA, Bayer, Leverkusen, Germany) was used according to the manufacturers’ instructions. The ACS:180 CEA assay is a two-site sandwich immunoassay. The assay measures CEA concentrations up to 100 ng/mL with a minimum detectable concentration of 0.5 ng/mL. CEA levels in the serum of mice were determined before tumor cell inoculation, before therapy at day 8, and afterwards in 7-day intervals and at sacrifice of the mice.

**Toxicity of $^{213}$Bi-d9MAb.** Toxicity was evaluated by determination of WBC and platelet counts. Approximately 50 μL of blood were taken from the jugular vein before therapy, 2 days after radioimmunotherapy and afterwards in 7-day intervals up to 51 days after activity application. After dilution of the blood with a shedding reagent (Cellpack, Sysmex, Weisbaden, Germany), analysis was done using a blood analyzer (SE 9000, Sysmex).

**Histologic examination of kidneys.** Mice were sacrificed at day 100 after single or double i.p. $^{213}$Bi-d9MAb application. Kidneys were fixed in 10% formalin and embedded in paraffin, cut at 5-μm slices, and stained with H&E.

Results

$^{213}$Bi-labeling of d9MAb and HSC45-M2 cell binding of $^{213}$Bi-immunoconjugates. Labeling of d9MAb with $^{213}$Bi resulted in maximal specific activities of 1.48 GBq/mg mAb. Following size exclusion chromatography, the radiochemical purity of the labeled antibody fractions varied between 95% and 99%. The in vitro stability of $^{213}$Bi-d9MAb at room temperature exceeded four half-lives (3 hours) of $^{213}$Bi. As described earlier, $\sim 25\%$ of $^{213}$Bi-d9MAb bound to HSC45-M2 cells compared with only 2% of nontargeting $^{213}$Bi-labeled mAb.

Survival after $^{213}$Bi immunoimmunotherapy. Survival of nude mice treated i.p. with single or double injections of $^{213}$Bi-d9MAb with different activities was significantly prolonged for all treatment groups compared with untreated controls ($P < 0.01$). The median survival of all groups is given in Table 1. Untreated mice showed a median survival of 22 days after i.p. injection of tumor cells. A single injection of only 0.37 MBq at day 1 after tumor cell inoculation increased the median survival to 48 days. Double injections of 0.37 MBq at days 1 and 8 led to a significantly increased survival of 84 days compared with a single injection ($P < 0.01$). Figure 1 shows the survival curves of these two therapy groups compared with untreated controls.

Even in an advanced stage of the disease at day 8 after tumor cell inoculation, therapy with a single injection of 0.37 MBq $^{213}$Bi-d9MAb increased survival to 37 days. Double injections of 0.37 MBq at days 8 and 15 after tumor cell inoculation increased median survival to 46 days.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Median survival (95% confidence interval), d</th>
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</thead>
<tbody>
<tr>
<td>Untreated control</td>
<td>22 (12-32)</td>
</tr>
<tr>
<td>0.37 MBq (1 d)</td>
<td>48 (36-60)</td>
</tr>
<tr>
<td>0.37 MBq (1 and 8 d)</td>
<td>84 (49-119)</td>
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<tr>
<td>0.37 MBq (8 d)</td>
<td>37 (28-46)</td>
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<tr>
<td>0.37 MBq (8 and 15 d)</td>
<td>46 (34-58)</td>
</tr>
<tr>
<td>0.74 MBq (8 d)</td>
<td>42 (34-50)</td>
</tr>
<tr>
<td>0.74 MBq (8 and 15 d)</td>
<td>78 (37-109)</td>
</tr>
<tr>
<td>1.48 MBq (8 d)</td>
<td>44 (13-75)</td>
</tr>
</tbody>
</table>

NOTE: Injected activity (time between tumor cell inoculation and activity application).

Table 1. Median survival (d) and 95% confidence interval of mice after i.p. inoculation of tumor cells (HSC45-M2) and single or double injections of different $^{213}$Bi-d9MAb activities
A single injection of 0.74 MBq at day 8 led to a median survival of 42 days. Double injections of 0.74 MBq at days 8 and 15 increased survival significantly to 78 days ($P < 0.05$). A single injection of 1.48 MBq at day 8 was less efficient than a double injection of 0.74 MBq at days 8 and 15 with a median survival of 44 days ($P = 0.05$). These results are shown in Fig. 2.

**Time course of carcinoembryonic antigen levels.** CEA was not detectable in the serum of tumor-free mice. After tumor cell inoculation, CEA increased differently in the individual animals due to tumor cell proliferation depending on the development of the i.p. tumor mass. The curve in Fig. 3A shows a slow increase to 4.76 ng/mL in the first 8 days after tumor cell inoculation. The animal represented by Fig. 3B showed a rapid increase to 26.04 ng/mL, 8 days after tumor cell inoculation. In some animals, however, the increase of CEA was delayed beginning to increase not before day 8 after tumor cell inoculation. These results indicate that tumor development on one hand can be monitored via CEA concentrations in the serum and on the other hand varies considerably in the individual animals observed.

After each therapy, CEA declined in the serum of all animals investigated. Figure 3B shows the CEA time course in the serum of a mouse sacrificed at day 40 after tumor cell inoculation, with an early rapid CEA increase, a decrease to zero after the first application of 0.37 MBq of $^{213}$Bi-d9MAb, and again an increase within 14 days after the second injection of 0.37 MBq. Figure 3C also shows a CEA increase after tumor cell inoculation. CEA decreased to zero after double injection of 0.74 MBq at days 8 and 15 after tumor cell inoculation and did not show an increase until day 49 after tumor cell inoculation. This animal survived up to 245 days without any sign of tumor in the peritoneal cavity.

**Toxicity of $^{213}$Bi-d9MAb.** Toxicity was evaluated by determination of leucocyte and platelet counts. In all treatment groups, no change in leucocyte and platelet counts compared with controls could be observed. Macroscopic inspection of animals after sacrifice at day 245 as well as histologic kidney examination did not show any sign of toxic effects of the injected $^{213}$Bi-d9MAb activities.

**Discussion**

Several investigations have provided evidence that $\alpha$-emitters due to high-linear energy transfer have therapeutic advantages in radioimmunotherapy compared with $\beta$-emitters (12–15). For example, $^{211}$Bi (linear energy transfer $\sim$ 100 keV/\mu m) is an ideal cytotoxic agent for elimination of single tumor cells or small tumor cell aggregates after coupling to an appropriate carrier. Rapid targeting of the immunoconjugate to tumor cells is essential for an effective therapy using short half-lived isotopes such as $^{213}$Bi with a half-life of 46 minutes. This requires fast coupling of $^{213}$Bi, eluted from an $^{225}$Ac/$^{213}$Bi generator system, to antibodies via bifunctional CHX-AEC-diethylenetriaminepenta-acetic acid (\approx 20 minutes). Effective targeting of the tumor cells can be attained following locoregional application of the immunoconjugate. Selective binding increases the radiation absorbed dose to tumor cells and tumor cell clusters while minimizing the irradiation of adjacent normal tissue.

In a series of preclinical studies using $\alpha$-emitter immunoconjugates, relatively high activities ranging from 5.5 to 40.7 MBq have been applied in i.p. or s.c. tumor models. In these studies, a growth inhibition or regression of the tumor with increasing activity is described. However, the observation period in all these studies was too short to evaluate long-term radiation-induced toxicity (16–24).

Our previous studies have shown that $^{213}$Bi activities exceeding 14.8 MBq result in radiation induced death of the animals 150 to 200 days after i.p. application of $^{213}$Bi-immunoconjugates.

Therefore, the aim of our study was to evaluate the therapeutic efficiency of low $^{213}$Bi activities coupled to a tumor-specific antibody applied as single or double injection in an i.p. model of disseminated tumor cells of diffuse-type gastric cancer.
The results show that double injection of low activities was superior to single injection in terms of survival at different stages of tumor cell dissemination. Even double injections of 0.74 MBq in a time interval of 7 days did not cause any toxic effects. The therapeutic concept of multiple injections of low $^{213}$Bi activities seems appropriate also for therapy of patients with microscopic i.p. tumor burden. The rationales for multiple application of radioimmunoconjugates versus single application have been discussed in detail by DeNardo et al. (25).

The evaluation of the time course of i.p. tumor development with different imaging modalities currently available is still insufficient. Because the tumor cells used in our i.p. tumor model have been described to secrete CEA, we investigated changes in CEA levels in the serum following tumor cell inoculation and $^{213}$Bi immunotherapy. We could reveal that CEA serum levels increased following inoculation of HSC45-M2 tumor cells and decreased after each therapy with $^{213}$Bi-d9MAb. However, increase as well as decrease of CEA concentrations varied individually in all animals examined. This suggests that following inoculation of a defined number of tumor cells, tumor development is individually different. Nevertheless, CEA levels in the serum can be used to estimate tumor progression/regression in the HSC45-M2 tumor model.

In summary the data of our study support locoregional multiple applications of low activities of $^{213}$Bi-radioimmunoconjugates for therapy in patients, especially with respect to the limited availability of the $\alpha$-emitter $^{213}$Bi at present.

**Acknowledgments**

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**Fig. 3.** CEA concentration depending on time after tumor cell inoculation. A, without $^{213}$Bi-d9MAb. B, after double injection of 0.37 MBq $^{213}$Bi-d9MAb (8 and 15 days). C, after double injection of 0.74 MBq $^{213}$Bi-d9MAb (8 and 15 days).
References


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