Intravesical α-Radioimmunotherapy with ^{213}Bi-Anti-EGFR-mAb Defeats Human Bladder Carcinoma in Xenografted Nude Mice

Birgit Pfost[1], Christof Seidl[1], Michael Autenrieth[2], Dieter Saur[3], Frank Bruchertseifer[4], Alfred Morgenstern[4], Markus Schwaiger[1], and Reingard Senekowitsch-Schmidtke[1]

[1]Department of Nuclear Medicine, Technische Universität München, Munich, Germany; [2]Department of Urology, Technische Universität München, Munich, Germany; [3]Department of Internal Medicine II, Technische Universität München, Munich, Germany; and [4]European Commission, Joint Research Centre, Institute for Transuranium Elements, Karlsruhe, Germany

Transurethral resection of urothelial carcinoma often results in tumor recurrence due to disseminated tumor cells. Therefore, new therapeutic strategies are urgently needed. The aim of this study was to establish an orthotopic human bladder carcinoma mouse model using the epidermal growth factor receptor (EGFR)–overexpressing bladder carcinoma cell line EJ28 and to compare therapeutic efficacy of intravesically instilled α-particle–emitting ^{213}Bi-anti-EGFR-monoclonal antibody (mAb) with mitomycin C. **Methods:** Female Swiss *nu/nu* mice were intravesically inoculated with luciferase-transfected EJ28 human bladder carcinoma cells after the induction of urothelial lesions by electrocautery. At different time points after cell inoculation, mice were treated intravesically with ^{213}Bi-anti-EGFR-mAb, mitomycin C, or unlabeled anti-EGFR-mAb. Tumor development and therapeutic response were evaluated via bioluminescence imaging. **Results:** Mice without therapy and those treated with unlabeled anti-EGFR-mAb reached a median survival of 41 d and 89 d, respectively. Mice that underwent therapy with 0.925 MBq of ^{213}Bi-anti-EGFR-mAb 1 h, 7 d, or 14 d after cell instillation survived more than 300 d in 90%, 80%, and 40% of the cases, respectively. Therapy with 0.37 MBq 1 h or 7 d after tumor cell inoculation resulted in survival of more than 300 d in 90% and 50% of mice, respectively. Mitomycin C treatment after 1 h and 7 d prolonged survival to more than 300 d in 40% and 50%, respectively; however, treatment turned out to be nephrotoxic. In contrast, no signs of nephrotoxicity could be observed after ^{213}Bi-anti-EGFR-mAb treatment. **Conclusion:** The study suggests that radioimmunotherapy using intravesically instilled ^{213}Bi-anti-EGFR-mAb is a promising option for treatment of bladder cancer in patients.

Key Words: α-emitter ^{213}Bi; bladder cancer; locoregional therapy; orthotopic mouse model; bioluminescence imaging

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Received May 8, 2009; revision accepted Jun. 30, 2009.
For correspondence or reprints contact: Reingard Senekowitsch-Schmidtke, Department of Nuclear Medicine, Technische Universität München, Ismaninger Strasse 22, 81675 Munich, Germany.
E-mail: senekowitsch@lrz.tum.de

Bladder cancer is the most common malignancy of the urinary tract. A total of 116,000 new cases of bladder cancer are diagnosed every year in Europe. About 32% of the patients die because of the consequences of the disease (*1*). Most (95%) bladder tumors are pathogenetically assigned to urothelial cell carcinoma, according to the new World Health Organization classification (*2*); the remaining 5% belong to squamous cell carcinomas and adenocarcinomas. Seventy percent of bladder urothelial cell carcinomas are not muscle-invasive. The initial treatment of non–muscle-invasive bladder cancer is transurethral resection (TUR) of the tumor. Risk of recurrence is up to 80% because of free-floating tumor cells released during TUR. The immediate instillation of mitomycin C or other chemotherapeutics eradicates remaining tumor cells that normally adhere to the lesions caused by TUR or ablatively affects residual tumor cells (chemoresection) and thus reduces recurrence risk by 39% (*3*). Yet 15%−40% of patients treated with adjuvant intravesical chemotherapy have tumor recurrence within 5 y. Therefore, new adjuvant therapeutic strategies after TUR are urgently needed.

To investigate therapeutic efficacy of new treatment concepts, orthotopic xenograft models are indispensable. In the case of bladder cancer, this means the inoculation of human bladder cancer cells into the bladder of immunodeficient mice (*4–6*). An orthotopic xenograft bladder tumor model is close to the clinical situation, allowing a reliable transfer of data. To monitor tumor growth and therapeutic efficacy, noninvasive imaging concepts are preferable. For that purpose, tumor cells are stably transfected with genes coding for fluorescent proteins (*7*) or enzymes catalyzing bioluminescence (*8*), allowing for the continuous visualization of tumor development after intravesical instillation of tumor cells.

The first aim of our study, therefore, was to establish an orthotopic human urothelial carcinoma mouse model using

the bladder carcinoma cell line EJ28-luc, stably transfected with the gene coding for firefly luciferase. EJ28 cells show overexpression of the epidermal growth factor receptor (EGFR), a phenomenon that has been observed in up to 86% of all urothelial carcinomas analyzed (*9,10*). The grade of EGFR overexpression is closely related to tumor progression and prognosis (*11,12*). On binding of the ligand epidermal growth factor (EGF), EGFR triggers intracellular signaling cascades regulating cell proliferation, prevention of apoptosis, angiogenesis, cell motility, and metastasis (*13*). Therefore, EGFR is a promising molecule for targeted therapy of bladder cancer.

The eradication of single tumor cells or small tumor cell clusters is efficiently achieved via high-linear-energy-transfer α-particle–emitting radionuclides. The coupling of α-emitters to appropriate carrier molecules targeting tumor cells because of overexpression or exclusive expression of cell surface molecules creates powerful weapons for the elimination of disseminated tumor cells, as has been demonstrated in numerous experimental and clinical studies (*14*). Radioimmunoconjugates composed of the α-emitter ^{213}Bi and a tumor-specific antibody targeting mutant d9-E-cadherin have been successfully applied for the eradication of disseminated gastric tumor cells in vitro and in vivo (*15–18*). Thus, the coupling of ^{213}Bi to antibodies specifically binding to EGFR should produce a promising radiopharmaceutical for the elimination of bladder cancer cells.

The second aim of our study was to evaluate bladder retention and the therapeutic efficacy of intravesically applied ^{213}Bi-anti-EGFR–monoclonal antibody (mAb) at different time points after tumor cell instillation in comparison to the unlabeled antibody and chemotherapy using mitomycin C. Tumor development and therapeutic efficacy were monitored noninvasively via bioluminescence imaging.

MATERIALS AND METHODS

Cell Line and Transfection

The human urothelial carcinoma cell line EJ28, isolated from a primary bladder carcinoma, was stably transfected with plasmid pcDNA3.1 containing the coding sequence of firefly (*Photinus pyralis*) luciferase under the control of the cytomegalovirus promoter (*19*). Transfected EJ28-luc cells were grown in RPMI medium supplemented with 10% fetal calf serum, 1% nonessential amino acids, a 1% penicillin/streptomycin solution (Biochrom), and geniticin (0.5 mg/mL; Gibco) in a humidified atmosphere containing 5% CO_2. Cells were harvested after rinsing the monolayer with 1 mM ethylenediaminetetraacetic acid in phosphate-buffered saline (PBS).

Coupling of ^{213}Bi to Anti-EGFR-mAb

Anti-EGFR-mAb (matuzumab; Merck) was conjugated with the ^{213}Bi chelating agent SCN-CHX-A″-diethylenetriaminepentaacetic acid (DTPA) (Macrocyclics) before radiolabeling was performed as previously described (*20*). The α-emitter ^{213}Bi was eluted from an ^{225}Ac/^{213}Bi generator system provided by the Institute for Transuranium Elements (*21*). CHX-A″-DTPA–chelated anti-EGFR-mAb (50–100 μg) was incubated with the ^{213}Bi eluate (37–148 MBq) in 0.4 M ammonium acetate buffer at pH 5.3 for 7 min at room temperature. Unbound ^{213}Bi was separated via size-exclusion chromatography, and purity of ^{213}Bi-anti-EGFR conjugates was controlled as described earlier (*15*).

Binding of ^{213}Bi-Anti-EGFR-mAb to EJ28-Luc Cells

For binding studies, 3×10^6 EJ28-luc cells in 0.5 mL of PBS were incubated with ^{213}Bi-anti-EGFR-mAb (0.25 μg) or unspecific ^{213}Bi-anti-MUC1 (0.82 μg) for 30 min on ice. After the addition of 0.5 mL of PBS, cells were centrifuged (1,200 rpm, 3 min) and the cellular pellet was washed with 0.5 mL of PBS. ^{213}Bi activity in the cellular pellet and supernatants was quantified in a γ-counter (1480 Wizard TM3; Wallac) and expressed as percentage of total activity bound to cells (*22*).

Quantification of EFGR via Scatchard Analysis

EJ28-luc cells (1×10^6) were incubated with ^{213}Bi-anti-EGFR conjugates at concentrations from 5 to 320 ng/100 μL in a total volume of 200 μL for 20 min at 37°C. After centrifugation (3 min, 1,200 rpm), cells were washed twice in PBS and ^{213}Bi activities of cellular pellets and pooled supernatants were quantified using a γ-counter. The amount of antibody conjugates bound to the cell surface was determined depending on antibody concentration, resulting in a linear Scatchard plot. Finally, the maximum capacity of bound antibody corresponding to the number of binding sites per cell was calculated by linear fitting.

Analysis of Cell Survival via Clonogenic Assay

To assess the cytotoxicity of ^{213}Bi-anti-EGFR-mAb, EJ28-luc cells were seeded in 24-well plates (100/well) and allowed to adhere for 24 h. Cells were treated with 0.37, 0.74, and 1.48 MBq of the ^{213}Bi immunoconjugate per milliliter; controls received PBS only. Seven days after treatment, the number of clones comprising more than 50 cells was determined and expressed as percentage of controls.

Animals and Tumor Model

Female Swiss *nu/nu* mice (age, 6–8 wk; Charles River) were kept at our animal care facility for at least 2 wk before tumor cell instillation. Mice were housed 5 per cage in isolated ventilated cages at a mean temperature of 26°C and a humidity of 50%−60%; food and water were given ad libitum. All animal experiments were approved by the government of Upper Bavaria and performed in accordance with the guidelines for the use of living animals in scientific studies and the German Law for the protection of animals. The human urothelial carcinoma xenograft was established orthotopically by intravesical instillation of EJ28-luc cells after gentle electrocautery of the murine bladder, according to the method of Günther et al. (*23*), with slight modifications. Cauterization of the bladder mimics TUR and therefore should facilitate adherence of instilled tumor cells to the bladder wall. Mice were anesthetized using medetomidine/midazolam/fentanyl (500/5/50 μg/kg). A 26-gauge Teflon (DuPont) intravenous catheter without needle (Insyte-W; Becton Dickinson) was inserted transurethrally into the bladder using a lubricant (Vidisic; Dr. Mann Pharma). A guiding wire was inserted into the bladder of a mouse positioned dorsally on the ground plate of the cautery unit (Erbotom T 71 D; ERBE) via the Teflon catheter and carefully pushed forward to the bladder wall. The wire was attached to the cautery unit, and a monopolar coagulation mode was applied for 2 s at the lowest level (7 W). After removal of the guiding wire, EJ28-luc cells (2×10^6/100 μL of

RPMI) were instilled via the catheter and allowed to settle in the bladder wall for approximately 2 h, the time span of anesthesia. Mice were observed daily for signs of illness and alterations in body weight. Intravesical tumor growth was monitored weekly by bioluminescence imaging.

Immunohistochemistry

EGFR-overexpressing urothelial tumors derived from intravesical instillation of EJ28-luc cells were verified postmortem by immunohistochemical staining of the EGFR. For that purpose, tumor cryosections (4 μm) were fixed in acetone and blocked with 5% mouse serum in PBS–bovine serum albumin for 30 min. Sections were then incubated with the primary antibody (human anti-EGFR-mAb, 1:40 dilution) for 1 h at room temperature. After the blocking of endogenous peroxidases with methanol/H_2O/H_2O_2 (200/45/2) for 10 min, sections were incubated with the secondary antibody (polyclonal rabbit antihuman IgG, conjugated with horseradish-peroxidase, 1:80 dilution) (Dako) for 30 min at room temperature. Sections were then washed with PBS, and the peroxidase substrate 3,3′-diaminobenzidine tetrahydrochloride (Vector) was applied according to the manufacturer's instructions to produce a brown precipitate. Sections were evaluated using a light microscope.

Biodistribution of ^{213}Bi-Anti-EGFR-mAb

The biodistribution of ^{213}Bi-anti-EGFR-mAb was assayed in tumor-free mice after intravesical application. Animals were intravesically injected with 6.66 MBq of ^{213}Bi-anti-EGFR-mAb in 100 μL of PBS. Forty-five and 90 min after injection, mice were sacrificed and organs were prepared for measurement of ^{213}Bi-anti-EGFR-mAb accumulation in a γ-counter. Uptake was expressed as percentage of injected activity per gram of tissue.

Radioimmunotherapy with ^{213}Bi-Anti-EGFR-mAb and Chemotherapy with Mitomycin C

Mice that had been intravesically instilled with EJ28-luc cells were divided into 9 groups consisting of 10 animals each. These groups received 0.925 MBq of ^{213}Bi-anti-EGFR-mAb in 100 μL of PBS intravesically at 1 h, 7 d, or 14 d after cell inoculation, or 0.37 MBq of ^{213}Bi-anti-EGFR-mAb in 100 μL of PBS at 1 h or 7 d after cell instillation, or 40 μg mitomycin C in 40 μL of 0.9% NaCl at 1 h or 7 d, after cell inoculation, or 2 μg of unlabeled anti-EGFR-mAb at 1 h after instillation of cells. The control group received PBS intravesically at 1 h after tumor cell inoculation. During therapy, mice were anesthetized (90 min). Free-floating tumor cells in the murine bladder 1 h after cell inoculation correspond to tumor cell dissemination in the bladder of patients after TUR. Tumor development 7 and 14 d after cell inoculation mimics advanced tumor cell clusters, as observed in urothelial carcinoma in situ.

Mice were observed daily for signs of illness and monitored weekly by bioluminescence imaging to evaluate therapeutic efficacy. Survival was observed up to 300 d. Mice were sacrificed as soon as a large tumor burden, nephropathy, ascites, or cachexia had developed. Survival was calculated by the Kaplan–Meier method.

Bioluminescence Imaging

Bioluminescence imaging was performed with a cooled charge-coupled device camera attached to a light intensifier unit (Hamamatsu). Mice were intraperitoneally injected with 300 μL of D-luciferin (15 mg/mL) (SYNCHEM) and 180 μL of anesthetic (82% saline, 10% ketamine [Ketavet; Pharmacia & Upjohn], and 8% xylazine [Rompun; Bayer]). After 10 min, mice were positioned dorsally on a gel cushion in a dark box, and images were processed using Simple PCI software (Hamamatsu). Photographic images were taken under dimmed light. Then bioluminescence images were recorded with an exposure time of 180 s. Dimmed-light images were merged with the corresponding bioluminescence images, and the light emissions were transformed into pseudocolors. Starting 7 d after cell inoculation, bioluminescence imaging was performed every 7 d to monitor tumor growth before and after therapy. For quantification of light emissions, mean gray-level intensities were measured in regions of interest (ROIs) over the urogenital region of the mice and corrected for background.

Statistical Methods

Statistical analyses were conducted using SigmaPlot (Systat) and SPSS software (version 16.0, SPSS Inc.). Survival rates were calculated and displayed by the Kaplan–Meier method. Survival curves were compared by log-rank test. Because of the lack of normal distribution, median and interquartile ranges (from the 25th to the 75th percentiles) of survival times were reported. Because the follow-up period was set to 300 d per mouse, mice that survived longer than 300 d were censored and defined as >300 in the statistical analysis. The Student t test was used to compare means between 2 independent samples. P values less than 0.05 were considered statistically significant.

RESULTS

Characterization of ^{213}Bi-Anti-EGFR-mAb In Vitro

After a 7-min incubation of the ^{213}Bi eluate with anti-EGFR-mAb, the labeling yield varied between 95% and 97% of ^{213}Bi bound to anti-EGFR-mAb, resulting in specific activities of 0.35–1.4 MBq of ^{213}Bi per μg of antibody. The removal of unbound ^{213}Bi via size-exclusion chromatography resulted in a purity of ^{213}Bi-anti-EGFR-mAb of greater than or equal to 99%. The in vitro stability of ^{213}Bi-anti EGFR-mAb exceeded 4 half-lives of ^{213}Bi (3 h). Scatchard analysis using ^{213}Bi-anti-EGFR-mAb revealed 5.1×10^5 molecules of EGF receptor expressed per EJ28-luc cell, with a dissociation constant of 0.95 nM. Binding of ^{213}Bi-anti-EGFR conjugates to EJ28-luc cells appeared highly specific (39%), compared with unspecific ^{213}Bi-anti-MUC1-mAb targeting MUC1 mucin not expressed on EJ28-luc cells (1.5%).

Analysis of cytotoxicity of ^{213}Bi-anti-EGFR-mAb via the clonogenic assay revealed dependence on ^{213}Bi activity concentration. The seeding of 100 cells per well produced 53 colonies in untreated controls. The treatment of cells with ^{213}Bi-anti-EGFR-mAb (0.37, 0.74, and 1.48 MBq/mL) reduced colony formation to 13 (25%), 3 (6%), and 0.2 (0.4%) on average, respectively.

Orthotopic Xenograft Mouse Model

Intravesical instillation of 2×10^6 EJ28-luc cells after gentle electrocautery of the murine bladder caused tumor development in the bladder in 80% of the animals. Xenografted animals had to be sacrificed because of progressive tumor burden between 20 and 69 d after cell instillation. Median survival time of tumor-bearing mice ($n = 10$) was 41 d. Postmortem sections of the bladder

revealed that the severity of illness was less dependent on tumor dimensions than on the localization of the urothelial carcinoma in the murine bladder. If a small tumor was close to the urethra, urethral obstruction occurred, followed by renal failure due to the accumulation of urine in the kidneys. In contrast, signs of illness because of tumor growth at the bladder apex or the lateral bladder wall became evident only after the development of a massive tumor burden (not shown). These tumors could be easily detected by abdominal palpation. Microhematuria was observed continuously in the case of tumor development in the bladder and ceased 7 d after cell inoculation in the case of xenotransplantation failure (not shown).

Tumor growth was monitored weekly via noninvasive bioluminescence imaging. Because of the anatomic location of the bladder close to the abdominal wall, tumor burden was detected already at day 7 after cell inoculation (Fig. 1). At that stage, macroscopic investigation of the bladder after sacrifice revealed no pathologic findings. Therefore, bioluminescence imaging is an efficient tool for the monitoring of tumor development and also for the evaluation of therapeutic efficacy.

Immunohistochemical Staining

The overexpression of EGFR on EJ28-luc cells could be verified immunohistochemically on xenografted urothelial carcinoma in the mouse bladder. For that purpose, cryosections of bladders of mice that had been intravesically instilled with EJ28-luc cells were immunostained. EJ28-luc tumor cells attached to the urothelium displayed intense brown color, indicative of a strong EGFR overexpression (Fig. 2). Counterstaining with hematoxylin denotes normal urothelium (blue). Thus, EGFR overexpression of EJ28-luc cells is still present during tumor proliferation in the murine bladder.

Biodistribution of ^{213}Bi-Anti-EGFR-mAb

Forty-five and 90 min after intravesical instillation of ^{213}Bi-anti-EGFR-mAb (6.66 MBq in 100 μL), the uptake of the radioimmunoconjugate in the different organs was analyzed via quantification of ^{213}Bi activity. As presumed, locoregional intravesical application of ^{213}Bi-anti-EGFR-mAb ensured excellent retention of the therapeutic compound in the bladder with negligible systemic activity (Fig. 3). These data suggest low systemic toxicity, as confirmed after the sacrifice of animals surviving more than 300 d without any signs of disease. Slight kidney uptake of ^{213}Bi-anti-EGFR-mAb is probably due to the ascension of ^{213}Bi immunoconjugates via the ureters caused by pressure during application, which should be easily controllable in a future therapeutic application in patients.

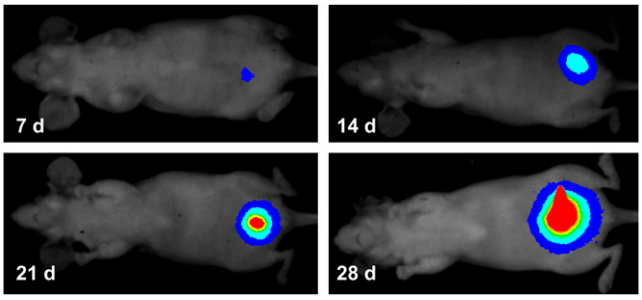

FIGURE 1. Monitoring of tumor growth via bioluminescence imaging, 7, 14, 21, and 28 d after intravesical instillation of 2×10^6 EJ28-luc cells.

Noninvasive Visualization of Therapeutic Efficacy Using Bioluminescence Imaging

To monitor therapeutic response and efficacy after intravesical ^{213}Bi-anti-EGFR-mAb treatment, bioluminescence images of tumors were recorded at different time points before and after therapy. Figure 4A illustrates the complete eradication of a tumor 14 d after cell instillation after treatment with 0.925 MBq of ^{213}Bi-anti-EGFR-mAb at day 7. After the application of ^{213}Bi-anti-EGFR-mAb 14 d after cell instillation, both complete eradication and decrease of tumor burden could be observed. In the case of tumor decrease, as observed at day 21 after cell instillation, however, tumor progression occurred at later time points, as shown in Figure 4B. Additionally, light emissions from tumors of selected mice were quantified over ROIs before and after therapy using Simple PCI software. Light emissions of intravesical tumors of mice treated with ^{213}Bi-anti-EGFR-mAb (0.925 MBq) at 7 d (Fig. 5A) and 14 d (Fig. 5B) after tumor cell instillation indicate complete or partial remission of intravesical tumors.

Therapeutic Efficacy of ^{213}Bi-Anti-EGFR-mAb

Mice that were treated with PBS or unlabeled anti-EGFR-mAb 1 h after tumor cell instillation reached a median survival of 41 and 89 d, respectively. Groups that underwent ^{213}Bi-anti-EGFR-mAb therapy with 0.37 or 0.925 MBq 1 h after cell instillation both showed a significantly longer median survival of more than 300 d ($P < 0.001$) and did not develop any tumor. A disease-free survival was observed in 90% of the animals. Two mice had to be sacrificed because of an infectious disease on days 289 and 298. After the intravesical instillation of ^{213}Bi-anti-EGFR-mAb 7 d after the inoculation of tumor cells, median survival was 49 d in the case of 0.37 MBq and more than 300 d in the case of 0.925 MBq, with 50% and 80%, respectively, of mice reaching a disease-free survival of more than 300 d. Therapy 14 d after intravesical cell instillation with 0.925 MBq of ^{213}Bi-anti-EGFR-mAb was less successful, resulting in a median survival of 55 d ($P = 0.096$), with 40% of mice surviving more than 300 d without tumor recurrence (Table 1; Fig. 6). In all cases, macroscopic urothelial lesions caused by α-particle irradiation could not be observed. Complete responders to therapy showed no tumor regrowth during their life span, whereas partial responders showed tumor recurrence 14 d after therapy. All mice surviving more than 300 d were free of tumors at sacrifice.

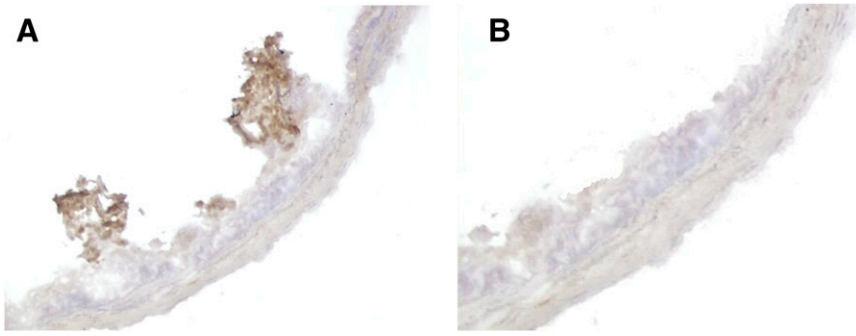

FIGURE 2. Immunohistochemical verification of EGFR overexpression in xenografted urothelial carcinoma. (A) Human EGFR-positive (brown) urothelial carcinoma after intravesical instillation of EJ28-luc cells. (B) Normal murine urothelium (blue).

Therapeutic Efficacy of Mitomycin C

The intravesical treatment of mice with mitomycin C (40 μg), compared with no treatment, also resulted in a significant prolongation of survival ($P < 0.05$), both 1 h and 7 d after cell instillation. Surprisingly, the benefit from mitomycin C therapy was independent of the time point of therapy: median survival was 289 and 251 d, and the percentage of animals that survived longer than 300 d was 40% and 50% after therapy 1 h and 7 d after instillation, respectively. The postmortem examination of sacrificed mice revealed pathologic disorders in the kidneys, for example, renal fibrosis. Therapy with mitomycin C instilled 1 h after tumor cell inoculation was significantly less effective ($P < 0.05$) than treatment with both 0.37 MBq and 0.925 MBq of ^{213}Bi-anti-EGFR-mAb (Table 1; Fig. 6).

DISCUSSION

Standard therapy of urothelial carcinoma involves TUR of the primary tumor, followed by intravesical instillation of mitomycin C. However, intravesical instillation of mitomycin C induced nephrotoxic side effects in mice in our study. In general, mitomycin C is known for its necrotizing properties, resulting also in severe complications in the kidneys such as ureteral inflammation and stenosis (*24*). Studies on intravesical instillation of mitomycin C revealed a transurothelial resorption of 1%−5% of mitomycin C due to its molecular weight of 334 Da (*25,26*). Moreover, in vitro studies of pharmacodynamics of mitomycin C on surgical bladder tumor samples have documented heterogeneity in the response of bladder tumors (*27*). Although yielding a reduction of recurrence of 14%−17%, the application of mitomycin C after TUR generally has limited benefits against disease progression (*28*). Because current intravesical treatment strategies using chemotherapy for urothelial carcinoma after TUR are of limited efficacy (*24,29*), the aim of this study was to develop a new therapeutic concept and to evaluate it in an appropriate orthotopic model for bladder carcinoma.

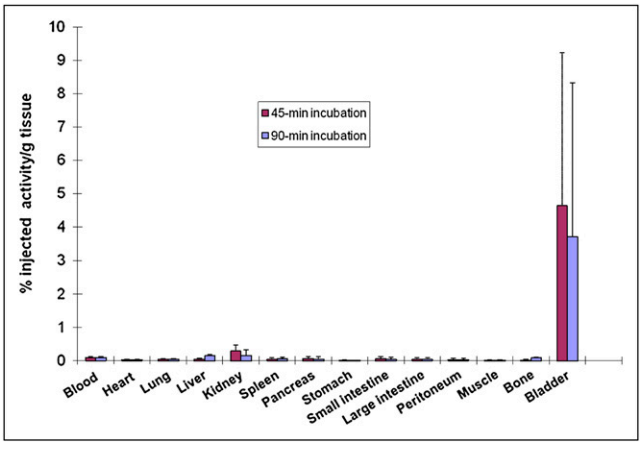

FIGURE 3. Biodistribution of ^{213}Bi-anti-EGFR-mAb (6.66 MBq) at 45 and 90 min after intravesical instillation in bladders of tumor-free mice.

Orthotopic xenografts of human urothelial carcinoma, though difficult to establish, are considered the best model for the evaluation of new therapeutic approaches against recurrent bladder cancer after TUR. Gentle cauterization of the murine urothelium before cell instillation mimics the clinical situation after TUR and offers the chance to study therapeutic efficacy on the local human tumor. The success rate of tumor establishment after intravesical instillation has been described to be cell line–dependent and to be less reliable than after intramural injection (*30*). The EJ28-luc cells used in this study have turned out as highly tumorigenic, because intravesical instillation of cells after cauterization resulted in tumor growth in 80% of immunodeficient mice. Because we could not detect any abdominal spread of tumor cells via bioluminescence imaging, bladder perforation due to cauterization could be excluded at any time. Abdominal tumor spread has been observed in another orthotopic xenograft model not using cauterization (*8*).

Noninvasive bioluminescence imaging has turned out to be an excellent technique for the longitudinal surveillance of tumor development and detection of extravesical tumor spread or metastasis at an early stage (*31*). Using luciferase-transfected EJ28-luc cells, we established an animal model of human orthotopic urothelial carcinoma for the noninvasive monitoring of tumor development and therapeutic efficacy. EJ28 has previously been investigated in a subcutaneous xenograft mouse model (*32*).

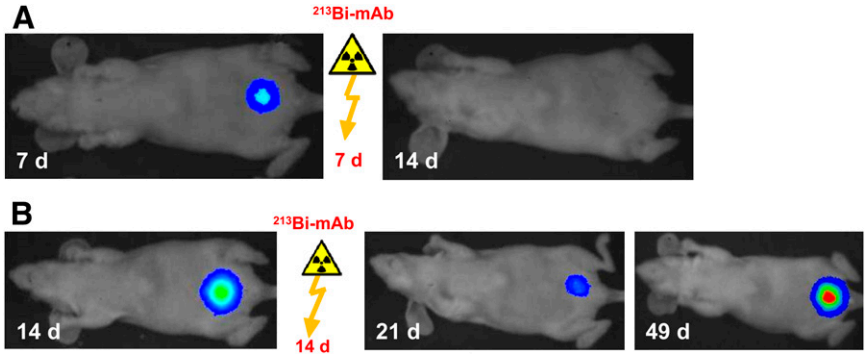

FIGURE 4. Monitoring of therapeutic efficacy via bioluminescence imaging after treatment with 0.925 MBq of ^{213}Bi-anti-EGFR-mAb. (A) Complete eradication of urothelial carcinoma as observed 14 d after cell instillation after therapy at day 7. (B) Partial eradication of urothelial carcinoma after therapy 14 d after cell instillation as observed at day 21 and subsequent tumor progression (day 49).

Earlier studies revealed an overexpression of the MUC1-glycoprotein in various bladder carcinoma cell lines (*33*). Tumor uptake of ^{67}Cu-anti-C595-mAb targeting MUC1 could be demonstrated preclinically (*34*). In a first clinical study, intravesical instillation of ^{67}Cu-anti-C595-mAb successfully targeted superficial bladder cancer in 12 of 15 patients (*35*). However, we observed a low binding of ^{213}Bi-anti-MUC1-mAb to EJ28-luc-bladder carcinoma cells, suggesting weak expression of MUC1.

In contrast, analysis of EGFR expression on EJ28-luc cells revealed overexpression, with approximately 5×10^5 EGF receptors per cell. Thus, the EGFR expression of EJ28-luc is up to 16-fold higher than the EGFR expression in the human urothelial carcinoma cell lines RT4 and J28, which have been determined to express $3–4 \times 10^4$ EGFR molecules per cell (*36*). Generally, EGFR is an interesting target for use in therapy of tumor cells showing EGFR overexpression. Therefore, antibodies targeting EGFR are promising tools, especially as carriers for cytotoxic compounds.

Native humanized anti-EGFR-mAb (matuzumab) has been reported to competitively inhibit the binding of natural ligands to EGFR (*37*) and to block ligand-induced receptor activation (*38*). Therefore, matuzumab is investigated in clinical phase I and II trials in patients with EGFR-positive non–small cell lung cancer (*39*), ovarian cancer, and primary peritoneal malignancies (*37*). Although antibodies targeting EGFR have been shown to induce apoptosis in subsets of tumor cells, the effects of these antibodies are largely cytostatic rather than cytotoxic. Phase I dose-escalating studies using matuzumab revealed an antitumor effect without cumulative toxicity after the intravenous application of the native antibody (400–1,600 mg/wk) (*40*). However, as has been announced recently by the companies in charge, the results of phase II clinical trials on non–small cell lung and gastric cancers did not meet the predefined clinical endpoints.

Matuzumab, however, can be used as a carrier for cytotoxic radionuclides to selectively eradicate tumor cells. As has been demonstrated in clinical phase I studies, α-particle–emitting radionuclides coupled to antibodies have shown high efficacy in therapy of acute myeloid leukemia, melanoma, and malignant glioma (*41–43*).

^{213}Bi immunoconjugates have proven to be effective in the treatment of intraperitoneal tumor cell dissemination after locoregional intraperitoneal injection in mouse models (*16,18*).

In the present study, we showed that the intravesical application of ^{213}Bi-anti-EGFR-mAb at different time points after instillation of bladder tumor cells was highly efficient in preventing tumor development and reducing tumor size in the bladder. As demonstrated via the moni-

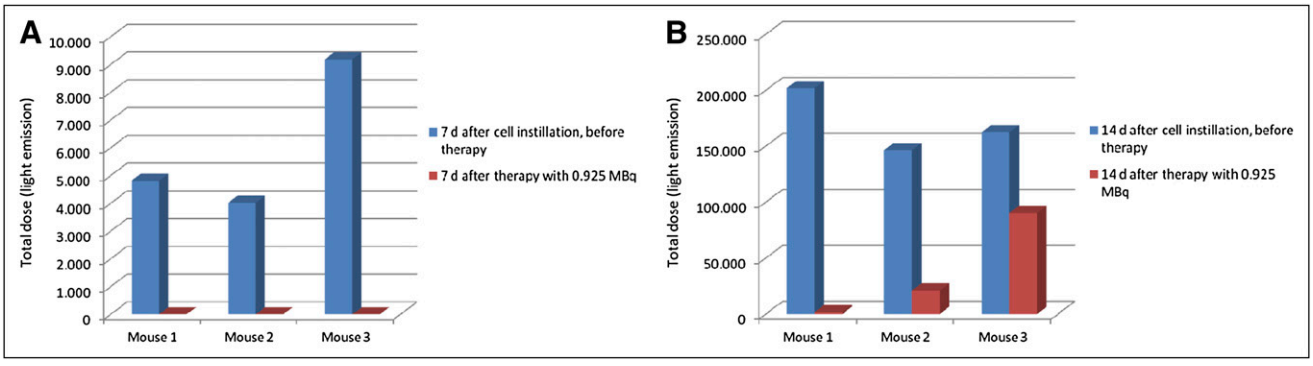

FIGURE 5. Light emissions over ROIs of intravesical tumors before and after therapy with 0.925 MBq of ^{213}Bi-anti-EGFR-mAb. (A) Seven days after tumor cell instillation before therapy and 7 d after intravesical therapy, complete eradication of tumor cells is seen. (B) Fourteen days after tumor cell instillation before therapy and 14 d after intravesical therapy, complete (mouse 1) and partial eradication of tumor cells (mice 2 and 3) is seen.

TABLE 1. Median Survival of Mice Intravesically Instilled with EJ28-Luc Tumor Cells After Intravesical Therapy with ^{213}Bi-Anti-EGFR, Mitomycin C, or Unlabeled Anti-EGFR-mAb at Different Time Points After Cell Instillation

Therapy (time after cell inoculation)	Median survival after intravesical instillation of...			
	^{213}Bi-anti-EGFR-mAb		Mitomycin C	Unlabeled anti-EGFR-mAb
	0.37 MBq	0.925 MBq	40 μg	2 μg
1 h	>300 d (1 mouse died on day 297)	>300 d (1 mouse died on day 286)	289 d (IQR, 110 to >300	89 d (IQR, 53 to 125)
7 d	49 d (IQR, 34 to >300)	>300 d (2 mice died on days 173 and 257)	251 d (IQR, 138 to >300)	n.d.
14 d	n.d.	55 d (IQR, 23 to > 300)	n.d.	n.d.

Median survival of controls (n =10) was 41 d (IQR, 25–59). IQR = interquartile range; n.d. = not determined.

toring of survival depicted in Kaplan–Meier plots, the therapeutic efficacy of ^{213}Bi-anti-EGFR-mAb was superior to mitomycin C but decreased with increasing time span between tumor cell instillation and application of ^{213}Bi radioimmunotherapy (Fig. 6). Tumor mass 14 d after cell instillation turned out to be too large for efficient therapy with the short-range α-emitter ^{213}Bi, and thus complete remissions could not be observed after therapy with ^{213}Bi-anti-EGFR-mAb. The improvement of therapeutic efficacy at advanced tumor stages 14 d after cell instillation could probably be achieved by a fractionated regimen, that is, the repeated weekly application of ^{213}Bi-anti-EGFR-mAb therapy. Fractionated ^{213}Bi-mAb therapy has turned out to be superior to a single application also in the intraperitoneal treatment of diffuse-type gastric cancer (*44*).

In addition, native anti-EGFR antibody (matuzumab) revealed an antitumor effect as reported for the treatment of patients with EGFR-positive non–small cell lung cancer or ovarian cancer (*38*). However, elimination of tumor cells in these patients could be observed only after the intravenous injection of relatively high amounts of the native antibody ranging from 400 to 1,600 mg per week. These amounts would correspond to 0.125–0.5 mg per mouse and thus are approximately 100-fold higher than the ^{213}Bi-anti-EGFR-mAb intravesically instilled in our studies. Therefore, the observed antitumor effect of ^{213}Bi-anti-EGFR-mAb must be due to ^{213}Bi and not to the anti-EGFR-mAb. As we have shown (Fig. 6), the treatment of mice with unlabeled anti-EGFR-mAb (2 μg) 1 h after cell instillation, compared with the treatment of controls with PBS, significantly

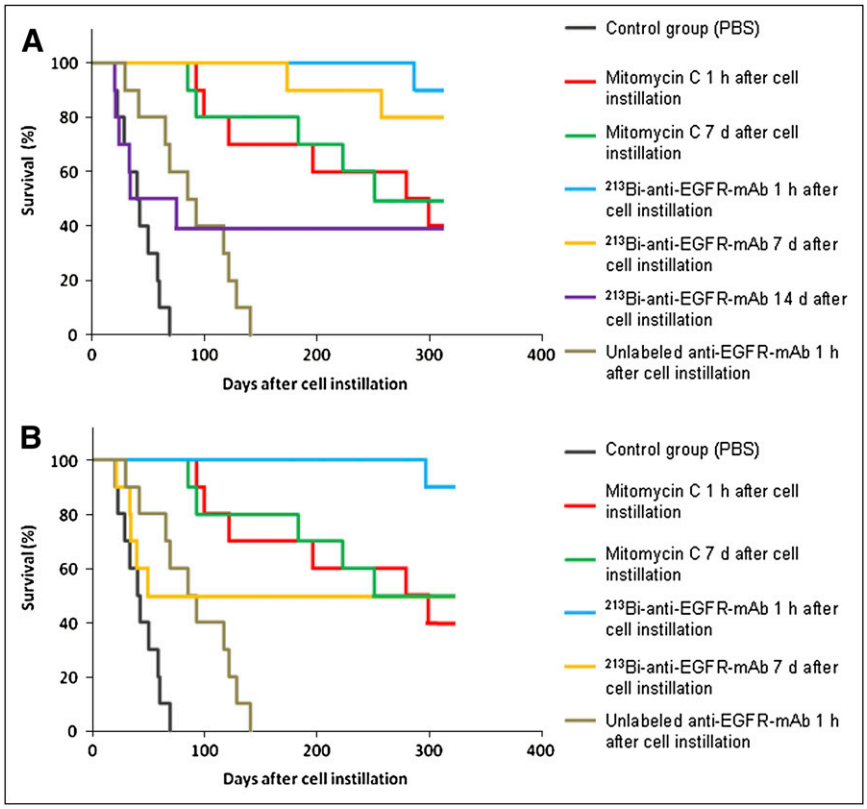

FIGURE 6. Survival of mice (Kaplan–Meier plot) after intravesical therapy with PBS (mock treatment), unlabeled anti-EGFR-mAb (2 μg), mitomycin C (40 μg), or ^{213}Bi-anti-EGFR-mAb at different time points after intravesical cell instillation. (A) 0.925 MBq of ^{213}Bi-anti-EGFR-mAb. (B) 0.37 MBq of ^{213}Bi-anti-EGFR-mAb.

prolonged survival ($P < 0.005$). However, the therapeutic efficacy of both 0.37 MBq and 0.925 MBq of ^{213}Bi-anti-EGFR was significantly superior to the unlabeled mAb, again indicating the efficacy of ^{213}Bi coupled to the anti-EGFR-mAb.

After the intraperitoneal injection of ^{213}Bi immunoconjugates, ^{213}Bi has been shown to accumulate in the renal cortex because of systemic resorption of the ^{213}Bi immunoconjugates. Histopathologic alterations in the kidneys were observed only at ^{213}Bi activities of at least 22 MBq (*16*). The intravesical application of ^{213}Bi-anti-EGFR-mAb targeting tumor cells in the bladder should minimize systemic toxicity, as has been demonstrated via biodistribution showing excellent retention of the ^{213}Bi immunoconjugate in the bladder. Nevertheless, a low ^{213}Bi activity could also be detected in the kidneys, probably due to ascension via the ureters caused by pressure during intravesical instillation of ^{213}Bi-anti-EGFR-mAb. Such low activities of ^{213}Bi are not nephrotoxic (*16*), and accordingly pathologic alterations in the kidneys of mice after intravesical therapy with ^{213}Bi immunoconjugates have not yet been observed. Furthermore, a future injection of ^{213}Bi-anti-EGFR-mAb in the bladder of patients should be feasible because of extensive experience in the intravesical application of compounds used for chemotherapy.

CONCLUSION

Intravesically instilled α-radioimmunotherapy using ^{213}Bi-anti-EGFR-mAb effectively eradicated disseminated tumor cells and superficial tumors, as demonstrated by the significantly prolonged survival of the animals. Moreover, this preclinical study showed a striking 100% response rate of the tumor to ^{213}Bi-anti-EGFR-mAb therapy. We are convinced that this new therapeutic approach that emphasizes the advantage of early instilled ^{213}Bi-mAb combined with minimized toxic side effects will promote the development of α-radioimmunotherapy to provide urothelial carcinoma patients with a promising treatment option after TUR.

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