Biodistribution and Radioimmunotherapy of SCCHN in Xenotransplantated SCID Mice with a ^{131}I-labelled Anti-EpCAM Monoclonal Antibody

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Abstract. *Background: The mortality from squamous cell carcinoma of the head and neck (SCCHN) remains high and almost unchanged throughout the last decades. Therefore, new therapeutic strategies are urgently needed. One promising approach is the application of radio-labeled antibodies directed against tumor-associated antigens. EpCAM is a transmembrane protein, which is overexpressed on almost all SCCHN, making it a suitable anchor molecule for targeted radioimmunotherapy (RIT). The aim of this study was to establish an animal model to investigate the biodistribution and the therapeutic effect of a radio-labeled EpCAM-specific monoclonal antibody (mAb). Materials and Methods: The mAb C215 was labeled with ^{131}I and tested for its antitumor effect against established SCCHN xenografts in SCID mice. Initially, the biodistribution of the mAb in the tumor and different organs was determined with a gamma counter and was calculated as % injected dose/gram tissue. For therapeutic approaches 5, 15 or 25 MBq ^{131}I-labeled mAb was injected as a single bolus into tumor-bearing mice. Control animals received either sodium chloride or the unlabeled mAb. The tumor growth and body weight of the animals were measured at various times after administration of the antibody. Results: Initially, high activity was seen in all organs after systemic administration of ^{131}I-C215. Over time general activity decreased whereas an accumulation of activity was seen in the tumor. Tumor growth was delayed in the groups receiving either 15 MBq or 25 MBq ^{131}I-C215 relative to control groups and the 5 MBq group. However, animals in the high-dose groups suffered from treatment-related toxicity, which led to body weight loss of more than 20%. Conclusion: Our data demonstrate that the EpCAM-specific radio-labeled mAb C215 is a promising tool to target SCCHN leading to significant tumor control. Further studies are necessary to increase efficacy and reduce toxicity of this new therapeutic approach.*

Squamous cell carcinoma of the head and neck (SCCHN) accounts for approximately 5% of all malignant tumors world-wide (1). The clinical prognosis of advanced tumor stages is bad with a 5-year survival rate of only 20%. These patients develop local recurrences in about 40%, and distant metastases in 25% of cases, which are mainly attributable to residual disseminated tumor cells. Consequently, SCCHN has to be regarded as a systemic disease (2).

Despite progress in locoregional therapy, the overall survival of SCCHN patients has not improved for decades, and also new adjuvant chemotherapy has not demonstrated an increase in survival (3, 4). Therefore, the development of an effective adjuvant systemic therapy remains a major challenge. Selective targeting of squamous cancer cells with radio-labeled monoclonal antibodies (mAb) specific for tumor-associated antigens may constitute a more effective therapy of advanced cancer combining the specifity of an antibody with the therapeutic potential of radioactivity. Tumor cells can, thus, be specifically targeted and eliminated. The efficacy of radioimmunotherapy (RIT) has been shown in haematological malignancies (5). In the past decade also CD44v6-directed RIT in head and neck cancer has shown encouraging results (6).

Another suitable target for RIT seems to be EpCAM. EpCAM is a transmembrane glycoprotein that is *de novo*

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expressed on most carcinoma, including SCCHN (7, 8). EpCAM expression correlates with progression and dedifferentiation (9), and has, thus, attracted attention as a diagnostic and therapeutic target molecule for mAb therapeutic trials. The protein has also been used for the detection of disseminated tumor cells in the bone marrow, and as a marker to redefine tumor margins (10-13).

Based on these data the efficacy of a radio-labeled EpCAM-specific antibody for the treatment of SCCHN in a SCID mice model was investigated.

Materials and Methods

Cell line. FaDu is a squamous cell carcinoma cell line derived from a human hypopharyngeal carcinoma (see www.atcc.org for more information). The cells were cultivated in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin and 1 mg/ml glutamine. The cultures were maintained at 37˚C in a humidified atmosphere with 5% CO_2 and were routinely tested for mycoplasma. To establish tumor growth in SCID mice, subconfluent monolayer cells were trypsinized, washed twice in phosphate-buffered saline (PBS), and resuspended in DMEM.

Antibody. C215 is a murine IgG_{2a} mAb directed against one of the epidermal growth factor (EGF)-like extracellular domains of the epithelial cell adhesion molecule (EpCAM) and was a gift of Trion Research Inc. (Martinsried, Germany). The antibody was radio-labeled using the iodogen method described by Weadock *et al.* (14).

Flow cytometry analysis. $1x10^6$ cells were incubated with a specific first antibody in PBS/2% FCS on ice for 30 min. The mixture was then washed in PBS and incubated with a FITC-labeled secondary goat anti-mouse IgG (Dianova, Hamburg, Germany) for 30 min. The cells were washed again in PBS and analyzed with a FACSCalibur and the CellQuest analysis program (Becton Dickinson, Heidelberg, Germany).

Animal model. $6x10^6$ FaDu cells resuspended in 0.2 ml DMEM were inoculated subcutaneously into the flank of female, 12- to 16-week-old SCID mice. After three weeks solid tumors measuring approximately 10 mm in diameter became visible and palpable.

Immunohistochemistry. Mice were sacrificed and fresh tumor tissues were excised, embedded immediately in tissue tec (Sakura Finetek, Torrance, CA, USA) and were snap frozen in liquid nitrogen. 4 μm ultra-thin tissue sections were mounted on slides and fixed in acetone for 10 min. Endogenous peroxidase was then blocked by treating the sections with hydrogen peroxidase in phosphate-buffered saline for 30 min. After washing in PBS, slides were incubated with C215 for 1 hour, followed by incubation steps with a biotinylated horse-anti-mouse-IgG secondary antibody and the avidin-peroxidase-complex (Vectastain Burlingame, CA, USA), and was finally developed with 0.01% 3-amino-9-ethylcarbazole as Chromogen (Sigma, St. Louis, USA). After counterstaining with Mayer's hemalaun, slides were cover-slipped with Kaiser's glycerol gelatine (Merck, Darmstadt, Germany).

Antibody biodistribution. Groups of five mice per time point bearing SCCHN xenografts were injected with 0.5 MBq ^{131}I-labeled C215 *via* the tail vein. Animals were sacrificed and dissected at 1 h, 8 h, 24 h, 48 h, 72 h and 96 h after injection. The weight and activity of the tumor, major organs and blood were measured. The activity expressed as 'percentage injected dose per gram tissue' (%ID/g) was then calculated. The median was used to calculate biodistribution curves for tumors and organs.

Toxicity studies. The maximum tolerated dose (MTD), corresponding to a reversible weight loss of 15% relative to day 0 (start of treatment), was determined by monitoring the weight of non-xenograft-bearing SCID mice given injections of increasing doses of ^{131}I-C215 three times per week. The doses used were 5, 10, 15 and 25 MBq ^{131}I-C215.

Radioimmunotherapy. Mice bearing xenografts were given a single intravenous injection of 5, 15 or 25 MBq ^{131}I-C215. Control groups were injected with sodium chloride or unlabeled C215. Each group consisted of 10 mice. During the following four weeks mice were weighed three times a week and the tumor size was determined daily. The tumor volumes were calculated as length x width^2/2, where length was the longer of the two measurements. Mice were sacrificed when the body weight loss exceeded 20%. The relative tumor volume (RTV) was calculated as the ratio of tumor volume on that day to its value at the start of therapy. The growth curve was plotted as the average of the relative tumor volume within a group *versus* time. Additionally the treatment control ratio (TCR) was determined as the ratio of the RTV of the treated group to that of the control groups.

Results

Antigen expression. Indirect immunofluorescence of the xenotransplanted tumor with flow cytometry demonstrated intense, specific binding of the mAb C215 to xenotransplanted FaDu cells *in vivo* (Figure 1A). Immunohistochemical analysis of EpCAM expression showed membrane staining in approximately 99% of the tumor cells (Figure 1B). These results demonstrate that the xenografts continued to express EpCAM on the cell surface while growing in SCID mice.

Antibody biodistribution. The initial biodistribution studies performed with IgG_{2a} type anti-EpCAM antibody showed a slight accumulation of the antibody in the tumor ranging from 0.99% ID/g at 1h to 0.23%ID/g at 96 h post injection leading to a poor tumor/non tumor ratio (T-NT) (Table I). Nevertheless a longer half life in the tumor tissue of 24 h compared to a half life of 8 h in the other tissues and blood pool resulted in an increasing tumor/blood ratio. The nearly unchanged non blood/blood ratios (NB-B) of the other tissues suggested activity change in the tissues was only due to activity change in the blood pool (Figure 2).

The biodistribution of the IgG_{2a} type anti-EpCAM antibody showed high activity and increasing NB-B ratios in the spleen and liver tissue. To proove specific C215/EpCAM

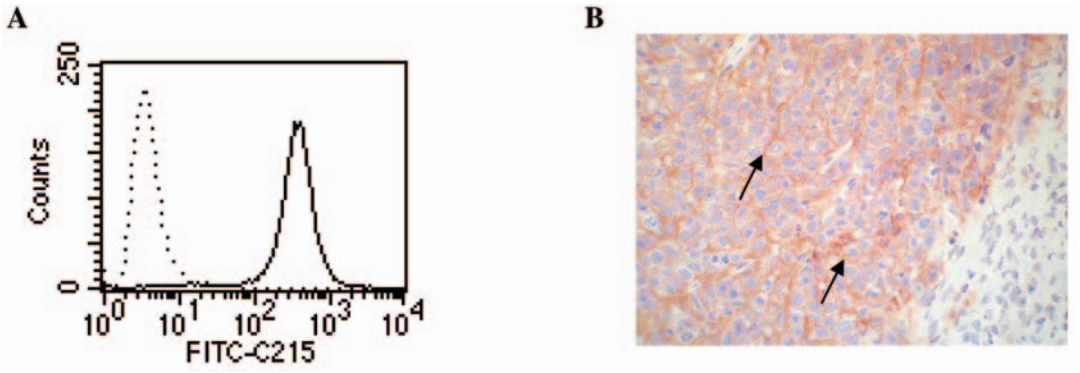

Figure 1. *(A) Fluorescent-activated cell sorter analysis of binding of MAb C215 to xenotransplantated FaDu cells in SCID mice. The expression of EpCAM is shown as histogram, where the broken line represents the isotype control and the black line represents EpCAM staining, respectively. (B) Immunhistochemical staining of frozen sections of xenotransplantated FADU cells in SCID mice showing clear staining for EpCAM in vivo (arrows).*

Table I. *Biodistribution of ^{131}I-C215 in xenotransplantated SCID mice. Groups of five mice each were injected with ^{131}I-C215. The tumors and normal organs were excised 1, 8, 24, 48, 72 and 96 h later, weighed and the ^{131}I activity was measured. The median (± quartile) %ID/g at each time point is shown.*

	1 h		8 h		24 h		48 h		72 h		96 h	
blood	6.76	+0.46 –1.36	2.89	+0.15 –0.15	1.31	+0.04 –0.05	0.53	+0.08 –0.06	0.35	+0.01 –0.05	0.31	+0.03 –0.03
heart	2.33	+0.51 –0.69	1.04	+0.11 –0.11	0.45	+0.02 –0.03	0.19	+0.03 –0.01	0.13	+0.01 –0.01	0.12	+0.01 –0.01
lung	3.58	+0.05 –0.70	1.41	+0.13 –0.13	0.71	+0.02 –0.04	0.36	+0.02 –0.02	0.24	+0.03 –0.03	0.17	+0.02 –0.02
liver	12.26	+0.25 –2.87	5.70	+1.12 –1.04	3.62	+0.31 –0.43	2.02	+0.08 –0.2	2.19	+0.17 –0.13	2.07	+0.32 –0.32
spleen	41.13	+8.12 –14.07	25.57	+3.38 –7.74	16.20	+1.97 –2.79	7.14	+2.12 –2.32	11.20	+1.80 –2.12	11.65	+1.33 –0.70
bowel	3.02	+1.46 –1.61	1.53	+0.37 –0.16	0.78	+0.00 –0.02	0.37	+0.04 –0.05	0.34	+0.10 –0.10	0.22	+0.05 –0.05
muscle	0.38	+0.11 –0.09	0.22	+0.03 –0.02	0.16	+0.02 –0.02	0.09	+0.01 –0.01	0.05	+0.03 –0.01	0.04	+0.00 –0.00
kidney	4.64	+0.21 –0.85	1.82	+0.17 –0.24	0.99	+0.01 –0.02	0.86	+0.05 –0.05	0.41	+0.04 –0.02	0.35	+0.06 –0.05
thyroid	16.21	+4.22 –3.53	2.12	+0.1 –0.13	1.46	+0.16 –0.19	1.51	+0.19 –0.20	0.44	+0.06 –0.04	0.41	+0.03 –0.03
stomach	2.52	+1.55 –0.74	0.70	+0.11 –0.04	0.71	+0.12 –0.08	0.27	+0.02 –0.02	0.23	+0.11 –0.09	0.12	+0.01 –0.00
tumor	0.99	+0.15 –0.21	0.71	+0.06 –0.07	0.59	+0.06 –0.03	0.29	+0.05 –0.05	0.26	+0.06 –0.0	0.23	+0.03 –0.02

ID: injection dose.

binding in the tumor tissue and non-specific IgG_{2a} uptake in the spleen and liver tissue the biodistribution of IgG_{2a} mAb C215 with co-application of 2 μg unlabeled human CD20-specific IgG_{2a} antibody, which does not bind to the murine CD20 molecule was investigated (data not shown). Co-application of the unspecific IgG_{2a} significantly reduced the NB-B of ^{131}I-labeled mAb C215 in the spleen and liver tissue while the tumor/blood ratio remained unchanged. Blocking of the unspecific antibody uptake reduced the spleen/blood ratio to 1.2 at 8 h and 2.0 at 48 h post injection (*p.i.*) (statistically significant with $p<0.001$ *vs.* NB-B of mAb C215 biodistribution without co-application; 8 h *p.i.*: 8.84, 48h *p.i.*: 13.58) and liver/blood ratio to 0.37 at 8 h and 0.37 at 48 h post injection (statistically significant with $p<0.001$ *vs.* NB-B of mAb C215 biodistribution without co-application, 8 h *p.i.*: 1.97, 48 h *p.i.*: 3.85) while tumor/blood ratio did not statistically change due to co-application of the unspecific IgG_{2a} ($p>0.05$; 0.30 *vs.* 0.25 at 8 h; 0.47 *vs.* 0.55 at 48 h).

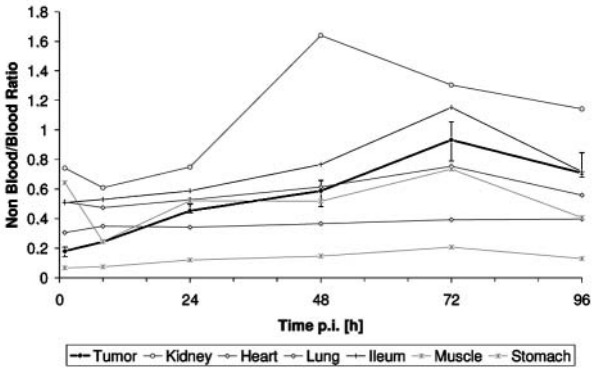

Figure 2. *Non blood/blood ratio after intravenous administration via tail vein of ^{131}I-C215 in SCID mice bearing subcutaneous human SCCHN xenografts. Values are given as median (5 mice per point of time). Over time, there was a specific increase in activity in the tumor. Except for the kidney and bowel, there was no accumulation in the other organs. p.i.: post injection.*

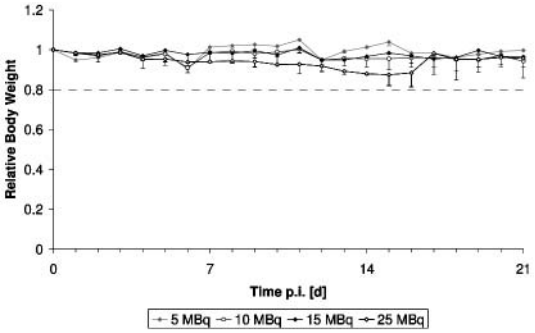

Figure 3. *The maximum tolerated dose of ^{131}I-C215 in SCID-mice. Shown are the mean values. p.i.: post injection.*

Maximum tolerated dose. The MTD, defined as the dose resulting in a body weight loss of 15% reversible within three weeks, was determined to be 25 MBq in non-xenograft-bearing mice. At this dose no treatment-related death was observed (Figure 3).

Radioimmunotherapy. Tumor growth, calculated as the tumor volume at each time point relative to the tumor volume at day 0 of treatment (RTV) is shown in Figure 4. Tumors in the sodium chloride group, the unlabeled mAb group and the 5 MBq group showed exponential tumor growth. The RTV of the unlabeled and the 5 MBq labeled group did not statistically differ from the RTV in the control group ($p>0.05$, log-transformated t-test). In contrast, tumors in animals of the 15 MBq and 25 MBq ^{131}I-C215 groups showed a significantly delayed tumor growth ($p<0.05$) or even regression at days 17, 21 and 23, and days 10 and 17, respectively. At these time points, the RTVs were <1, demonstrating tumor regression. A continuous body weight loss was observed in both the 15 MBq and 25 MBq groups after therapy was initiated. Consequently, since treatment related toxicity continued, mice had to be sacrificed at day 17 (25 MBq group) and day 23 (15 MBq group), when the median body weight loss reached 20% because of diarrhoea. At that time point, a treatment control ratio of 12% in the 15 MBq group and 29% in the 25 MBq group were determined.

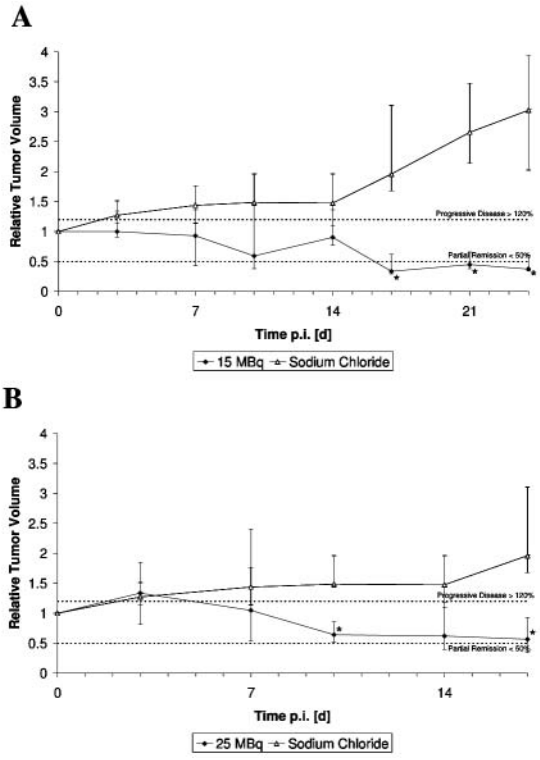

Figure 4. *Radioimmunotherapy of established human SCCHN tumors in SCID mice with ^{131}I-labeled mAb C215. (A) In comparison to the control group, a statistically significant reduction of tumor growth on day 17, 21 and 24 was observed in the therapy group of 15MBq. The tumor volume at the time-point indicated was normalized to the volume at the start of treatment. (B) In comparison to the control group receiving sodium chloride, the application of 25 MBq ^{131}I-C215 induced a reduction in tumor growth in terms of a "stable disease". Values are shown as median ± quartile (*statistically significant).*

Discussion

Disseminated tumor cells are responsible for the "minimal residual disease" situation, which accounts for the poor prognosis of patients with SCCHN (2). Radioimmunotherapy is already well established in patients suffering from non-Hodgkin-lymphoma (15) and holds the promise of being a good systemic treatment option also for other malignant

diseases including SCCHN. In contrast to tumor *specific* antigens in chronic lymphoid disease or breast cancer, only tumor-associated antigens have been identified for SCCHN so far (16). Nevertheless, encouraging results have already been demonstrated using CD44v6 as a target for RIT in SCCHN (6).

Using EpCAM as a target for RIT in SCCHN, similar promising results were shown. Our biodistribution data demonstrate an accumulation of activity in the xenotransplanted tumor. Additionally, there was also an unspecific accumulation seen in other tissues leading to poor tumor/non tumor ratios (T-NT). Nevertheless, it was clearly demonstrated that targeting of the tumor-associated antigen EpCAM also complied with the requirements of RIT. The data show a significantly prolonged half-life in the tumor, while activity in the other tissues decreased in line with blood clearance. The initial accumulation of activity in the liver and spleen probably resulted from the immunoglobulin deficiency of SCID mice, resulting in empty IgG-receptors on immune effector cells (17, 18). Accordingly, co-application of a non-specific antibody of the same isotype as C215 (IgG_{2a}) significantly reduced this unspecific activity. Of interest, the co-application of the unspecific IgG_{2a} did not reduce the uptake of mAb ^{131}I-C215 into the tumor, arguing for a specific binding of C215 to the EpCAM molecule. Similar data has been described by Michel *et al.* (18).

As expected, the unlabeled mAb C215 had no effect on tumor growth since SCID mice are immunodeficient and unable to mount antibody dependant cell toxicity (ADCC). This result is in accordance with that published by Naundorf *et al.*, who showed that the fully humanized anti-EpCAM antibody MT201 induced ADCC only in the presence of human effector cells (19). The radio-labeled C215 antibody, 15 MBq and 25 MBq revealed a significant reduction in tumor growth, whereas 5 MBq had no effect. Tumor volumes decreased significantly by 50% within 17 days in the 15 MBq group. Unfortunately the observed toxicity of the ^{131}I-labeled C215 restricted these promising therapeutic effects to a short observation period in both groups.

In summary, our results demonstrated a therapeutic effect of high doses of ^{131}I-C215. RIT is therefore more effective than the antibody therapy alone. The systemic application was limited by toxic adverse side-effects, even if non-xenograft-bearing mice showed higher MTDs. Thus, the dosage must be appropriately tailored for the human system. Our results were in line with those published in the literature. Although radio-labeled antibodies have demonstrated improvements in the therapy of different malignant haematological diseases, similar results have not been achieved so far for solid tumors. This is probably attributable to the relatively low tumor-specific accumulation of the antibody compared to normal tissues. A limited penetration into solid tumors on one hand and unspecific binding *via* the Fc-part of the therapeutic antibody to normal tissues on the other largely account for this adverse biodistribution. Consequently, many possible improvements are currently being discussed. Goldenberg *et al.* have shown that a repeated fractionated application of the radio-labeled mAb resulted in a more homogenous distribution of the mAb in the tumor (20). Van Dongen *et al.* favoured the application of smaller molecules, such as antibody fragments (21), which, due to their small size, penetrate better, faster and deeper into solid tumors and, therefore, have an improved tumor/non tumor ratio when applied systemically. In contrast, Goldenberg *et al.* have recommended the application of intact immunoglobulins (20) and argue that small molecules are much faster cleared from their target tissues.

Despite the adverse reactions, the principle of the RIT seems to be a promising adjuvant therapy regime for SCCHN. It should be noted, that radio-labeled ligands in animal models show a different pharmacokinetic profile and a different biodistribution than in humans. This is due partly to the different distribution volumes in both organisms. The immunogenicity of humans and animals also differ significantly and the cross reaction with human tissues expressing the homologous antigen must be considered. Future studies should, therefore, focus on the application regime in order to reduce the systemic toxicity.

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