Biodistribution of ^{131}I-labeled Anti-CK8 Monoclonal Antibody in HNSCC in Xenotransplanted SCID Mice

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Abstract. *Background: A new promising approach to improve the outcome of head and neck squamous cell carcinoma (HNSCC) is the application of radio-labeled antibodies directed against tumor-associated antigens. Cytokeratin 8 (CK8), an intermediate filament forming protein, is shown to be de novo expressed in dysplastic lesions as well as in HNSCC. Therefore like the epithelial cell adhesion molecule CK8 seems to be a suitable anchor molecule for targeted radioimmunotherapy (RIT). The aim of this study was to investigate the biodistribution of a radio-labeled Cytokeratin 8-specific monoclonal antibody (mAb) in a SCID (severe combined immunodeficiency disease) mouse model. Materials and Methods: The mAb against CK8 was labeled with ^{131}I and biodistribution was tested in established HNSCC xenografts in SCID mice. 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. Results: Initially, after systemic administration of ^{131}I-anti CK8 monoclonal antibody high activity was seen in all the organs. Over time the general activity decreased, whereas activity accumulated in the tumor. This activity decayed compared to the other tissues with a two- to threefold prolonged radioactive half-life. Conclusion: Specific antibody-antigen-binding is probably responsible for the prolonged radioactive half-life in the tumor and the resulting cumulative activity due to enrichment of the ^{131}I-anti CK8 mAb, so that Cytokeratin 8 seems to be a suitable anchor molecule for radioimmunotherapy in HNSCC.*

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Locoregional control as well as overall survival in head and neck squamous cell carcinoma (HNSCC) remains poor despite progress in locoregional therapy and new adjuvant chemotherapy (1, 2). Although failure of locoregional control causes the majority of deaths related to this cancer, HNSCC has to be regarded as systemic disease because of disseminated tumor cells responsible for the "minimal residual disease" situation and a systemic treatment approach is required (3, 4). Antibody-based therapy already showed good results (5-7). Radioimmunotherapy (RIT) using radio-labeled monoclonal antibodies against tumor associated antigens offers a new therapeutic option. The selective targeting of tumor cells may offer a more effective therapy consisting of the combination of the selective antibody and the therapeutic potential of radioactivity. For hematological malignancies the efficacy of such a systemic therapy has already been shown (8, 9). For HNSCC up to now only tumor-associated antigens with a relatively low tumor specifity have been identified. Despite this fact a CD44v6-directed RIT showed encouraging results (10) as did a SCID (severe combined immunodeficiency disease) mouse model for a ^{131}I-radio-labeled anti-EpCAM (epithelial adhesion molecule) monoclonal antibody (mAb) targeting HNSCC (11).

Cytokeratin 8 (CK8), an intermediate filament forming protein, is mainly located intracellularly and is expressed in many simple epithelia (12). Aberrant expression on the cell surface of some carcinomas such as breast cancer and hepatocellular cancer has been shown (13, 14). By the AMIDA (autoantibody mediated identification of antigens) technology antibodies against CK8 have been identified in the blood of patients with head and neck cancer (15, 16). Both, on squamous cell carcinoma cell lines and tumor biopsies of HNSCC, an aberrant membranous expression of CK8 was additionally demonstrated (17). Thus, CK8 is also regarded as a tumor-associated antigen because of its *de novo* expression on dysplastic squamous epithelium and on HNSCC (18, 19). Therefore it may also be a suitable anchor molecule for RIT in head and neck cancer.

A study was initiated with a radio-labeled anti-CK8 mAb in a SCID mouse model with the major aim of testing the biodistribution of the mAb and prove its usefulness in targeting HNSCC *in vivo*.

Materials and Methods

Antibody. HK-8 is a murine IgG_{2a} mAb against human Cytokeratin 8 (Covance Research Products, Berkeley, CA, USA). Labeling of HK-8 (0.2 mg) with iodine-131 was performed by a simplified and modified iodogen method as described by Weadock *et al*. (20) in coated reaction vials (500 μg Iodogen) in phosphate buffer (0.05 M, pH 7.4) within 10 min. No-carrier-added Iodine-131 was used as NaI in 0.05 M NaOH (7.4 GBq/ml) and was purchased from GE Healthcare Buchler (Braunschweig, Germany). Unbound iodine was removed by anion exchange (Dowex 1X8-100, Sigma-Aldrich, Deisenhofen, Germany) and the solution was subsequently sterile filtered (Acrodisc HT Tuffryn low protein binding, 0.2 μm, Gelman Sciences, Ann Arbor, MI, USA). The labeling efficiency was >90% and after purification the radiochemical purity was >97%. The integrity of all the radio-labeled preparations was assessed by size exclusion high-pressure liquid chromatography (HPLC) and precipitation with trichloroacetic acid. By both methods, the radioactivity was found to be associated with the intact antibody. In HPLC no dimerization or small degradation products were detected.

Cell line. FaDu is a squamous carcinoma cell line derived from a human hypopharyngeal carcinoma (ATTC, Rockville, MD, USA, 1974). The cells were cultivated in Dulbecco's modified Eagle's 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.

Flow cytometric analysis. After excision of the xenotransplanted tumor a cell suspension was assembled. The tumor cells were incubated with a specific first antibody against Cytokeratin 8 in PBS/2% FCS on ice for 30 min. Then the mixture was washed in PBS and incubated with a FITC-labeled secondary goat anti-mouse IgG (Dianova, Hamburg, Germany) for 30 min. Again, the cells were washed in PBS and analyzed with a FACSCalibur and the CellQuest analysis program (Becton Dickinson, Heidelberg, Germany).

Animal model. A total of 6×10^6 FaDu cells resuspended in 0.2 ml DMEM were inoculated subcutaneously into the flank of 12- to 16-week-old female SCID mice (Charles River Inc.). Solid tumors measuring approximately 10 mm in diameter became visible and palpable after three weeks.

Immunohistochemistry. The mice were sacrificed and different organs (heart, lung, liver, spleen, kidney, stomach, bowel) and fresh tumor tissues were excised, embedded immediately in tissue tec (Sakura Finetek, Torrance, CA, USA) and were snap frozen in liquid nitrogen. Ultra-thin tissue sections (4 μm) were mounted on slides and fixed in acetone for 10 min. By treating the sections with hydrogen peroxidase in phosphate-buffered saline for 30 min the endogenous peroxidase was blocked. After washing in PBS, the slides were incubated with HK-8 for 1 h, followed by incubation steps with a biotinylated horse-anti-mouse-IgG secondary antibody and the avidin-peroxidase-complex (Vectastain Burlingame CA, USA), and were finally developed with 0.01% 3-amino-9-ethylcarbazole as chromogen (Sigma, St. Louis, Missouri, USA). After counterstaining with Mayer's hemalaun, the slides were cover-slipped with Kaiser's glycerol gelatin (Merck, Darmstadt, Germany).

Antibody biodistribution. Groups of five mice per time-point bearing HNSCC xenografts were injected with 0.5 MBq ^{131}I-labeled HK-8 via the tail vein. The animals were sacrificed and dissected at 8, 24, 48, 72 or 96 h after injection (Figure 1). The weight and ^{131}I-activity of the blood, the major organs and the tumor were measured. Then the activity expressed as "percentage injected dose per gram tissue" (% ID/g) was calculated (Table I). To calculate the biodistribution the median was used.

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 which received injections of increasing doses of ^{131}I-HK-8 three times per week. The doses used were 7.5, 10, 15 and 17.5 MBq ^{131}I-HK-8.

Results

Antigen expression. An intense, specific binding of the mAb HK-8 to the xenotransplanted FaDu cells was demonstrated by indirect immunofluorescence of the excised xenotransplanted tumor with flow cytometry. The immuno-histochemical staining with HK-8 showed membrane staining of the tumor cells (Figure 2), providing evidence that the xenografts continued to express HK-8 on the cell surface while growing in the SCID mice.

Antibody biodistribution of ^{131}I-HK-8. The organs with high blood flow such as the heart, lung, liver, spleen and kidney showed higher radioactivity 8 h post-injection *(p.i.)* than the tumor. The chronological sequence showed a fast decrease of the radioactivity in these organs and in the blood. The half-life was between 16 h (spleen) and 40 h (blood, kidney,

Table I. *Biodistribution of ^{131}I-HK8 in xenotransplanted SCID mice. Groups of five mice each were injected with ^{131}I-HK8. The tumors and normal organs were excised 8, 24, 48, 72 or 96 h later, weighed and the ^{131}I activity was measured. The median (± quartile) % ID/g at each time-point is shown (ID: injected dose).*

	8 h		24 h		48 h		72 h		96 h	
Blood	23.28	+ 1.33 – 2.83	15.30	+ 3.94 – 1.62	10.99	+ 1.02 – 0.75	8.59	+ 2.89 – 1.68	5.75	+ 1.34 – 1.22
Heart	8.22	+ 0.20 – 0.35	6.77	+ 0.35 – 0.54	3.83	+ 0.15 – 0.37	2.76	+ 0.55 – 0.27	1.98	+ 0.46 – 0.5
Lung	8.88	+ 0.80 – 0.91	6.84	+ 0.41 – 0.40	5.33	+ 0.03 – 0.34	3.92	+ 0.97 – 0.52	2.69	+ 0.31 – 0.37
Liver	13.78	+ 1.21 – 1.10	8.26	+ 0.54 – 0.32	7.19	+ 0.11 – 0.27	4.34	+ 0.22 – 0.11	2.92	+ 0.25 – 0.15
Spleen	105.36	+ 12.50 – 11.01	52.78	+ 3.30 + 2.79	59.92	+ 3.63 – 6.10	30.42	+ 7.48 – 8.43	21.28	+ 2.58 – 2.84
Bowel	9.14	+ 2.02 – 2.22	4.27	+ 1.40 – 1.00	3.65	+ 0.22 – 0.20	2.48	+ 0.67 – 0.34	2.26	+ 2.21 – 0.43
Muscle	1.19	+ 0.13 – 0.15	1.52	+ 0.20 – 0.21	1.34	+ 0.25 – 0.19	1.25	+ 0.18 – 0.20	0.69	+ 0.05 – 0.02
Kidney	7.65	+ 0.91 – 1.04	4.58	+ 0.26 – 0.16	3.24	+ 0.41 – 0.48	3.25	+ 0.48 – 0.66	2.19	+ 0.19 – 0.15
Thyroid	12.32	+ 0.92 – 0.94	13.03	+ 1.47 – 1.51	11.13	+ 0.94 – 0.92	12.22	+ 3.00 – 3.13	12.98	+ 1.25 – 1.36
Stomach	4.02	+ 0.42 – 0.44	3.53	+ 0.47 – 0.27	2.94	+ 0.15 – 0.12	2.21	+ 0.18 – 0.18	1.39	+ 0.16 – 0.18
Tumor	6.53	+ 0.41 – 0.52	7.65	+ 1.08 – 0.15	8.33	+ 0.88 – 1.29	6.72	+ 1.43 – 1.29	3.90	+ 0.10 – 0.12

liver, lung). The radioactivity in these organs decreased over time. The radioactivity in the tumor 8 h *p.i.* was 6.53 % ID/g, rising to 7.65 % ID/g 24 h *p.i.* and reaching 8.33 % ID/g 48 h *p.i.*, resulting in higher radioactivity than the other organs. Not until 72 h *p.i.* did the radioactivity in the tumor decrease and was measured at 3.90 % ID/g at 96 h *p.i.* This activity still remained higher than the activity in the other organs at this time-point. The half-life therefore was longer than 96 h.

The radioactivity in the thyroid was initially 12.32 % ID/g and was therefore twofold the radioactivity in the tumor. The activity then remained constant, and at 96 h *p.i.* was still 12.98 % ID/g. Initially, the activity in the spleen was extremely high showing a value of 105.36 % ID/g. This value decreased to half within 24 h; 96 h *p.i.* only a fifth remained.

Additionally, the tumor/non-tumor ratio (T/NT) was determined, and a high T/NT-ratio for muscle and a low T/NT-ratio for the spleen was shown. The kidney showed a T/NT-ratio of 0.82 8 h *p.i.*, which increases to 1.64 24 h *p.i.* This advantageous ratio rose at 72 h *p.i.* to 2.63 and remained at 1.71 96 h *p.i.* and therefore was still higher than in the other organs.

To obtain a comparative value independent of the blood flow the non-blood/blood ratio (NB/B-ratio) was determined. Figure 3 clearly demonstrates that the NB/B-ratio of the organs such as the liver, lung, kidney, heart and bowel was constant over time. Values were between 0.1 (muscle) up to 0.6 (liver). In contrast, the NB/B-ratio of the tumor showed an increase from 0.28 (8 h *p.i.*) up to 0.68 (48 h *p.i.*) and to a maximum of 0.78 (72 h *p.i.*).

Thus, the course of the NB/B-ratio clearly demonstrated that the changes in the radioactivity of the different organs were due to the changes of the radioactivity in the blood. The different organs showed a straight line over time whereas the NB/B-ratio of the tumor showed a clear ascending line (Figure 3). The decrease of the radioactivity in the tumor was, compared to the other organs, much slower than in the blood. This we expected to be a cumulative effect due to specific binding of the mAb to the tumor associated antigen Cytokeratin 8.

Maximum tolerated dose (MTD). The MTD, defined as the dose resulting in a body weight loss of 15% reversible within three weeks, was determined to be 10 MBq in non-xenograft-bearing mice. No treatment-related death was observed at this dose (Figure 4).

Discussion

Since CK 8 is also only a tumor-associated and not a tumor-specific antigen, ubiquitous activity was shown by the biodistribution data. As expected, the measured radioactivity

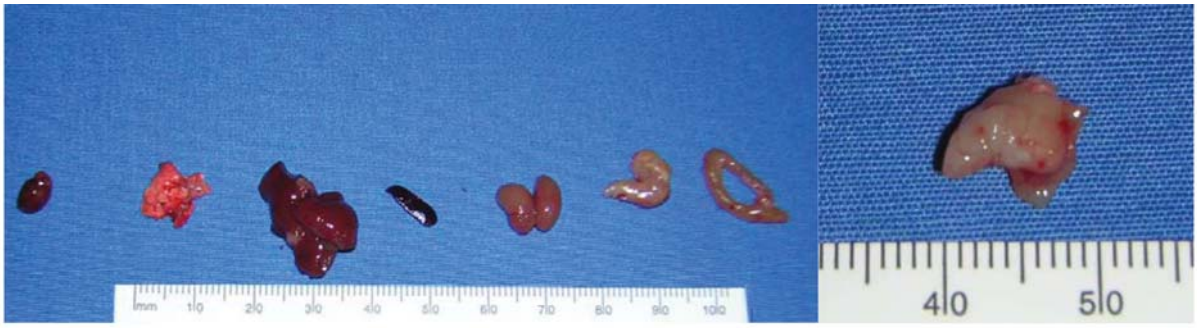

Figure 1. *Left: Organs dissected for biodistribution (left to right): heart, lung, liver, spleen, kidney, stomach, bowel. Not shown are the muscle tissue and the thyroid. Right: The xenotransplanted tumor reached 6 to 10 mm 3 weeks after subcutaneous injection of FaDu cells.*

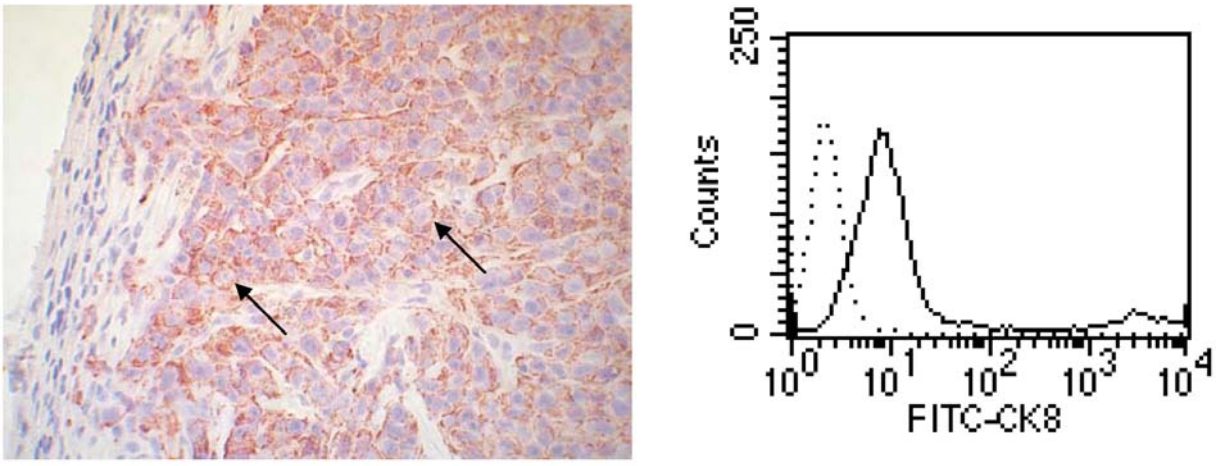

Figure 2. *Left: Immunohistochemical staining of frozen sections of xenotransplanted FaDu cells in SCID mice showing clear staining for Cytokeratin 8 in vivo (arrows). Right: Fluorescent-activated cell sorter analysis of binding of mAb HK-8 to xenotransplanted FaDu cells. Expression of Cytokeratin 8 shown as a histogram; the broken line: isotype control; black line: Cytokeratin 8 staining.*

did not reach the ideal T/NT-ratio as shown for tumor-specific antigens by Tsai *et al.* (21) using an anti-Her2/neu mAb in a nude mice model. Nevertheless, due to the antibody specific binding to CK8, an increase of radioactivity was shown in the tumor, which decreased, in comparison to the other tissues, with a two- to threefold increased half-life. The NB/B-ratio showed a continuous increase over a period of 72 h for both, the present ^{131}I-HK8 and the ^{131}I-C215 used previously (11), indicating that the NB/B-ratio was nearly identically for both radio-labeled mAbs. The NB/B-ratio also showed that improved tumor blood flow, for example due to angioneogenesis, could not be responsible for this fact. Therefore specific antibody-antigen binding appears to be responsible for the prolonged half-life and the accumulation of activity in the tumor.

The biodistribution data showed a high % ID/g regarding the spleen and the liver, which was also shown for ^{131}I-C215 (11). The present authors agree with Reddy *et al.* (22) and Michel *et al.* (23) who hold a specific phenomenon in the SCID mouse model responsible for these findings. SCID mice show fast clearance of IgG2 in the blood, whereas the clearance of IgG1 is normal. The accumulation of radioactivity in immune competent organs like the liver and the spleen is thought to be a consequence of unspecific binding of IgG2 antibodies to the CD64 FCγ receptors of these immune competent organs, which normally bind endogenous IgG2. Due to endogenous IgG2 deficiency the free adhesion positions are occupied by the exogenous IgG2 which leads to the fast clearance from the blood and the consequent high activity in spleen and liver. This phenomenon could be blocked by the co-application of a non-specific antibody of the same isotype as the used antibody (11), which reduces the unspecific activity significantly, but not the uptake of the radio-labeled antibody in the tumor. The

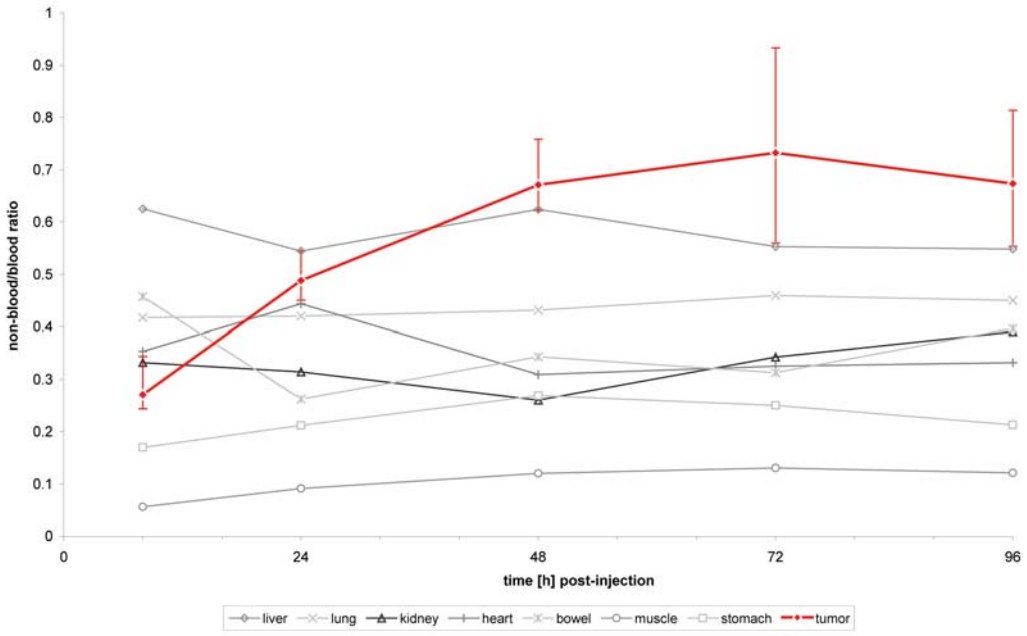

Figure 3. *Non-blood/blood ratio after intravenous administration via tail vein of ^{131}I-HK-8 in SCID mice bearing subcutaneous human HNSCC xenografts. Values are given as median (5 mice per time-point).*

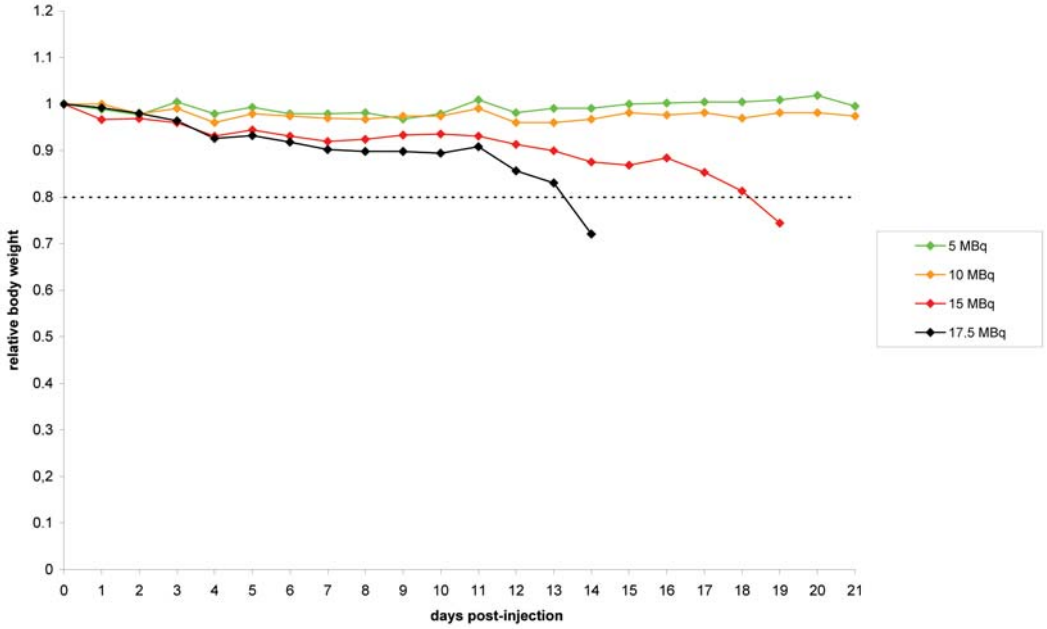

Figure 4. *The maximum tolerated dose of ^{131}I-HK-8 in SCID mice. Shown are the mean values.*

constant high activity in the thyroid, compared to the other organs, using ^{131}I-HK8 was caused by the late blockade of this organ by sodium perchlorate. Early drug based blockage would lead to a low % ID/g as already could shown for ^{131}I-C215 (11). To successfully block the accumulation of iodine in the thyroid, sodium perchlorate should be administered a day before the injection of the radio-labeled antibody and in some cases to be continued during the whole experiment (24).

In comparison to ^{131}I-C215 (11) the MTD was much less in ^{131}I-CK8. This may have been due to the expression of

CK8 in SCID mice (data not shown), leading to a higher toxicity by systemic use than that shown for ^{131}I-C215 (11). A high systemic toxicity may be expected due to the inappropriate T/NT-ratio. Systemic administration necessitating passage through the blood, the vascular wall and the interstitium to reach the tumor cell as well as unspecific or specific antibody binding and metabolisation leads to reduced activity in the target area (25). Therefore locoregional application of the radio-labeled antibody is often recommended to improve the tumor uptake and the therapeutic efficiency (26, 27, 28, 29). However, this model is limited for minimizing the systemic toxicity, since local application is not usable for HNSCC, which is regarded as a systemic disease (3). A promising approach might be fractionated systemic application of the radio-labeled antibodies, leading to a prolonged half-life and high dose in the tumor tissue while inducing low systemic toxicity.

In summary, CK8 is only a tumor associated antigen and therefore systemic is followed by a high level of unspecific binding, causing high toxicity. Further studies are needed to establish whether such toxicity could be reduced by fractionated application since local application is not useful because HNSCC is regarded as a systemic disease. Further studies are also needed to prove the therapeutic efficacy of the ^{131}I-labeled anti-CK8 monoclonal antibody.

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