Biodistribution and efficacy of [^{131}I]A33scFv::CDy, a recombinant antibody-enzyme protein for colon cancer

HOSSEIN PANJIDEH[1], VANIA CASIMIRO DA SILVA COELHO[1], JENS DERNEDDE[2], CHRISTOPHER BACHRAN[2], GREGOR J. FÖRSTER[4], JÜRGEN FRANKE[3], PATRICIA FASOLD[2], HENDRIK FUCHS[2], ECKHARD THIEL[1] and P. MARKUS DECKERT[1]

[1]Medizinische Klinik mit Schwerpunkt Hämatologie und Onkologie CBF, [2]Zentralinstitut für Laboratoriumsmedizin und Pathobiochemie, [3]Klinik für Radiologie und Nuklearmedizin, Charité-Campus Benjamin Franklin, D-12200 Berlin; [4]Klinik für Nuklearmedizin, Charité-Campus Mitte, D-10117 Berlin, Germany

Received November 8, 2007; Accepted January 4, 2008

Abstract. In antibody-directed enzyme-prodrug therapy (ADEPT), an antibody-bound enzyme localizes to tumor tissue, where it selectively converts a subsequently administered non-toxic prodrug into a cytotoxic drug. A33scFv::CDy is a bifunctional fusion construct comprising a single chain antibody against the gpA33 antigen and the prodrug-converting enzyme cytosine deaminase. gpA33 is highly and homogeneously expressed in >95% of all colorectal cancers. Here we describe the biodistribution and tumor-targeting capacity of ^{131}I labeled A33scFv::CDy. ^{131}I labeling of A33scFv::CDy was performed by the chloramine-T method, and the properties of the resulting [^{131}I]A33scFv::CDy conjugate were determined *in vivo* and *in vitro*, including biodistribution studies in nude mice bearing human LIM1215 colon carcinoma xenografts. The [^{131}I]A33scFv::CDy conjugate bound specifically to colorectal cancer cells *in vitro* with KD = 15.8 nM as determined by a saturation assay. *in vivo*, the tumor uptake of [^{131}I]A33scFv::CDy peaked at 87% injected dose/g 47 h post injection. Normal tissue uptake was low, and activity in blood was lower than in tumor at all time-points studied (6-92 h). The tumor-to-blood ratio increased over time with a maximum of 8.1 at 67 h post injection. [^{131}I]A33scFv::CDy thus shows a biodistribution that makes it attractive for both radioimmunotherapy (RIT) and ADEPT. Preliminary therapeutic experiments showed a significant reduction of tumor size in mice treated with the A33scFv::CDy-5-fluorocytosine/5-fluorouracil ADEPT system. This work demonstrates the feasibility of ADEPT and RIT based on the A33scFv::CDy recombinant construct.

Correspondence to: Dr P. Markus Deckert, Charité - Campus Benjamin Franklin, D-12200 Berlin, Germany
E-mail: markus.deckert@charite.de

Key words: tumor targeting, A33 antibody, ADEPT, radioimmunotherapy, colon carcinoma, recombinant fusion proteins

Introduction

Monoclonal antibodies (mAb) have become an accepted modality of cancer therapy. Recombinant antibodies and antibody-based fusion proteins hold the promise of further extending therapeutic possibilities. Single chain variable fragments (scFv) consist of the variable regions of an antibody's heavy and light chains fused together via a flexible linker, whose length determines the quaternary structure. Thus, they carry the complete antigen binding site in a single polypeptide chain of only about 30 kDa. In tumor targeting, scFv have demonstrated good tumor penetration, high ratios of tumor to normal tissue concentration, and low background (1,2). This makes them attractive targeting components of bifunctional fusion proteins such as those needed for antibody-directed enzyme-prodrug therapy (ADEPT). In ADEPT, after binding of an antibody-enzyme construct to the cognate tumor antigen, the enzyme component converts a prodrug into a cytotoxic drug, thus generating drug activity specifically in tumor tissue (3,4). An eminent obstacle to the development of scFv-based approaches, however, is posed by the expression of a heterogeneous protein consisting of mammalian antibody sequences and e.g. a microbial enzyme in a single expression system.

Several ADEPT systems have shown promising *in vivo* efficacy in a number of tumor models (5) and xenograft systems in nude mice (3,6-8), demonstrating in principle that ADEPT can selectively target tumor tissue and deliver chemotherapeutic drugs with high intratumoral concentrations. Senter's group first used bacterial cytosine deaminase for ADEPT to catalyze the deamination of 5-fluorocytosine (5-FC), which is non-toxic in mammals, into 5-fluorouracil (5-FU) (9). Clinical studies on ADEPT have proven the rationale for its application in humans (10).

The glycoprotein A33 (gpA33) is a promising target for antibody-based therapy of metastatic colorectal carcinoma. It is a transmembrane protein (11) of the junctional adhesion molecule family (12,13) that is homogeneously expressed in

>95% of all colorectal carcinomas, with the same level of expression in metastases (14). The expression of the antigen in normal tissue is very limited; almost the only normal tissue expressing the gpA33 antigen is colonic mucosa (14).

After binding to tumor cells, the humanized anti-gpA33 antibody huA33 shows excellent retention in tumor tissue. In clinical studies with radiolabeled murine A33, the radioactive label was still traceable in tumor tissue up to 6 weeks after injection of the antibody, while it cleared from normal tissue within 1 week (15). The same fast clearance from normal colon has been found for huA33, with a clearance rate that corresponds to the turnover time of normal colonocytes (16).

In vitro studies of the murine mAb A33 have shown that after binding to its cognate cellular antigen, the antibody is internalized through cytoplasmic vesicles, transported to perinuclear regions, and subsequently resurfaces intact in a repetitious cycle (17). Internalization of iodinated antibodies usually results in rapid deiodination and release of the radioiodine from the cell. However, this was not seen with ^{125}I-labeled mAb A33, where prolonged retention of radioactivity in tumor sites was observed in a clinical study (15), proving stability of the iodinated antibody under physiological conditions.

While these findings make A33 antibodies attractive targeting agents, yet no *in vivo* data exist on scFv-based gpA33-binding fusion proteins. A33scFv::CDy is a recombinant fusion protein comprising A33scFv, a phage-display-derived anti-gpA33 scFv (18), and cytosine deaminase from *S. cerevisiae*, which efficiently converts non-toxic 5-FC into the cytotoxic drug 5-FU. Here we report on the biodistribution of ^{131}I-labeled A33scFv::CDy and preliminary therapeutic results with this ADEPT concept in mice.

Materials and methods

Fusion protein construct. The recombinant A33scFv::CDy fusion construct was overexpressed and secreted into culture supernatant by Pichia pastoris and purified by affinity chromatography on protein L as previously described (19).

Cell lines. The human colon carcinoma cell lines HT29 (gpA33 negative) and LIM1215 (gpA33-positive, provided by the Ludwig Institute of Cancer Research, New York, NY) were cultivated in Dulbecco's modified Eagle's medium with Glutamax™ (PAA Laboratories GmbH, Pasching, Germany) supplemented with 10% fetal calf serum (BioChrom KG, Berlin, Germany) at 37˚C, 5% CO_2 and 95% humidity and harvested using 0.1% trypsin and 0.02% ethylenediaminetetraacetic acid (Gibco, Grand Island, NY) or, when subsequent immunostaining was required, a cell scraper (Greiner, Munich, Germany).

Radiolabeling of A33scFv::CDy. Iodination of A33scFv::CDy was performed by the chloramine-T method (20). In brief, 400 kBq iodine-131 (GE Healthcare, Freiburg, Germany) in a volume of 40 μl was added to 280 μg A33scFv::CDy in the presence of 100 μl chloramine-T (2.0 mg/ml) to a final volume of 640 μl for 1 min. The reaction was quenched with 100 μl saturated solution of methionine (50 mg/ml). Labeled antibody was separated from free iodine using a NAP 5 column (GE Healthcare, Freiburg, Germany). Specific activity of [^{131}I]A33scFv::CDy was ~400 kBq/mg.

To assess whether the binding capacity of the antibody was impaired by iodination, 2x10^6 LIM1215 or HT29 cells were incubated with 10 μg of [^{131}I]A33scFv::CDy in iodination ratios of 100, 200, or 400 kBq/mg for 1.5 h at 37˚C and subsequently washed three times with serum-free medium by centrifugation at 130 x g at room temperature. Antibody binding to cells was quantified in an automated gamma counter.

In vivo experiments. Four- to six-week-old athymic female (nu/nu) mice (body weight, 20-25 g, Charles River, Sulzfeld, Germany) were housed in groups of three to five animals in an enriched environment under standard conditions of temperature (21-24˚C), humidity (40-60%) and 12-h-light-dark-cycles. Food and water were freely available. All experiments were carried out in compliance with German law under license number G 0303/01 of the Berlin administration for health protection and social matters (Landesamt für Gesundheitsschutz und Soziales, Berlin) and adhering to the United Kingdom Coordinating Committee on Cancer Research 'Guidelines for the Welfare of Animals in Experimental Neoplasia'. After arrival at the facility, the mice were allowed to acclimatize for one week before the experiments were started.

For tumor experiments, mice were injected with 1x10^6 LIM1215 or HT29 cells subcutaneously into the right flank. After 10-14 days, mice bearing tumors of ~15-60 mg (1.3-2.5 mm diameter) were selected for continuation of the experiment.

To determine pharmacokinetics and biodistribution of the recombinant fusion construct, xenograft-bearing mice received 20 kBq of ^{131}I-labeled A33scFv::CDy by intravenous injection into the tail vein, and groups of four animals each were sacrificed at predefined time-points between 6 and 96 h post injection. Tumor, blood, heart, lung, liver, and kidney samples were taken and their activity determined by gamma counting for 90 sec per sample in duplicates. Relative specific activity was calculated as decay-corrected percentage of injected dose per gram of tissue (% ID/g) by reference to standards.

To assess the therapeutic efficacy of the proposed ADEPT concept of eight xenograft-bearing mice, four were intravenously injected 50 μg of A33scFv::CDy fusion protein in three fractions of a total of 100 μl phosphate buffered saline, while the remainder received saline solution as control. After 47 h, all mice received fractioned injections of a total of 15 mg of 5-FC in 200 μl saline solution with 5% (v/v) DMSO prodrug. All animals were observed for another 12 days for tumor growth and adverse effects.

Binding specificity of [^{131}I]A33scFv::CDy in vitro. Detached LIM1215 or HT29 cells (2x10^6) were incubated with 0.21 nmol [^{131}I]A33scFv::CDy in phosphate buffered saline. The cells were incubated for 1.5 h at 37˚C and then washed four times with cold serum-free medium followed by centrifugation (1 min, 130 x g). Radioactivity was determined in a gamma counter for 1.5 min per sample in duplicates.

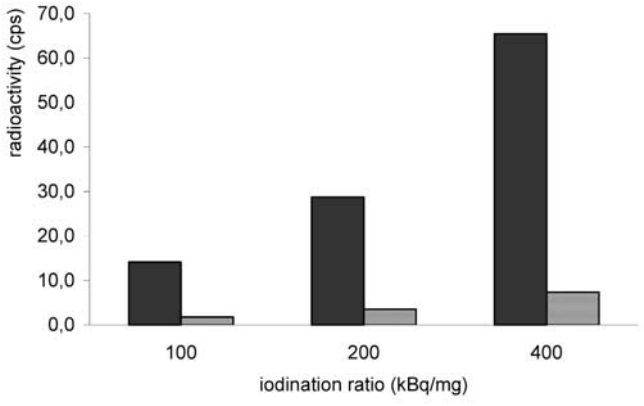

Figure 1. Binding specificity of [^{131}I]A33scFv::CDy *in vitro*. Colorectal LIM1215 cancer cells (black column) and HT29 cells (grey columns) were incubated with 1.47 nmol (10 μg) of A33scFv::CDy radiolabeled with 100, 200 or 400 kBq/mg of ^{131}I. HT29 cells were used as negative control. Activity was measured as counts per second. The 2-fold standard error was between 0.3 and 0.9 counts and thus too small to be usefully depicted in the graph.

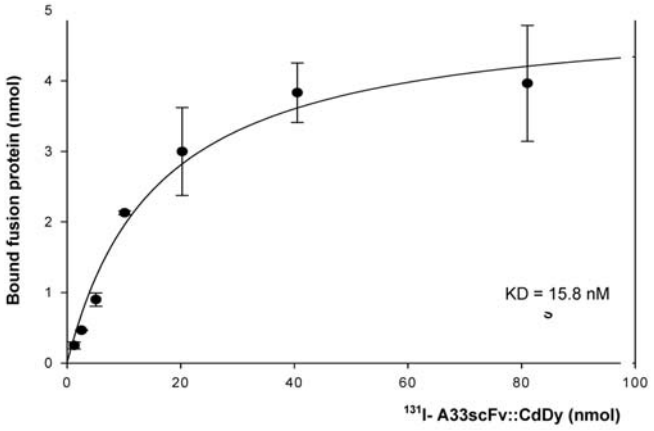

Figure 2. Estimation of the dissociation constant of [^{131}I]A33scFv::CDy. Increasing concentrations of [^{131}I]A33scFv::CDy were added to 2x10^6 LIM1215 cells each. Bound [^{131}I]A33scFv::CDy (nmol) is plotted against an added amount of [^{131}I]A33scFv::CDy (nmol). Error bars represent standard error of the mean.

Binding affinity of [^{131}I]A33scFv::CDy in vitro. The equilibrium dissociation constant (KD) and the binding capacity (Bmax) were used as a measure of binding affinity. The KD and Bmax for [^{131}I]A33scFv::CDy were determined by a saturation assay, where an increasing concentration of [^{131}I]A33scFv::CDy was added to a constant number of cells. LIM1215 cells were seeded at a density of about 2x10^6 cells/fraction tube. Before the experiment, cells were washed once with cold serum-free medium. During the experiment, cells and all solutions were kept at room temperature. A dilution series of [^{131}I]A33scFv::CDy in concentrations ranging from 81 to 1.26 nM was prepared. To all tubes, 1 ml of [^{131}I]A33scFv::CDy solution was added. An excess amount (100 nmol) of unlabeled A33scFv was also added to one tube (blocked) per antibody concentration in order to determine any unspecific binding. The cells were then incubated at room temperature with continuous shaking for 3 h. The cells were washed four times with cold serum-free medium and centrifuged (1 min, 130 x g), counted in all blocked tubes, and the mean was used as cell number for all wells. The amount of radioactivity bound to cells was determined in a gamma counter by comparison to a standard. Results were analyzed by nonlinear regression using Sigma Plot 9.0 to obtain the KD and Bmax values.

Results

Binding properties of [^{131}I]A33scFv::CDy in vitro. A33scFv::CDy was successfully radiolabeled with ^{131}I using the chloramine-T method. Binding activity and specificity were demonstrated by incubating either gpA33-positive LIM1215 cells or HT29 control cells with the radiolabeled fusion protein and determining radioactivity after bound-free separation. Comparing binding after radioiodination with 100-400 kBq/mg, specific binding was demonstrated up to the highest labeling dose, which was used for subsequent experiments (Fig. 1).

Binding affinity of [^{131}I]A33scFv::CDy was determined by a saturation assay, shown in Fig. 2, where the equilibrium dissociation constant (KD) and the binding capacity (Bmax) were determined and used as a measure of affinity. The KD value was calculated to 15.8 nM and the Bmax value was found to be 5.02 nM/2x10^6 cells.

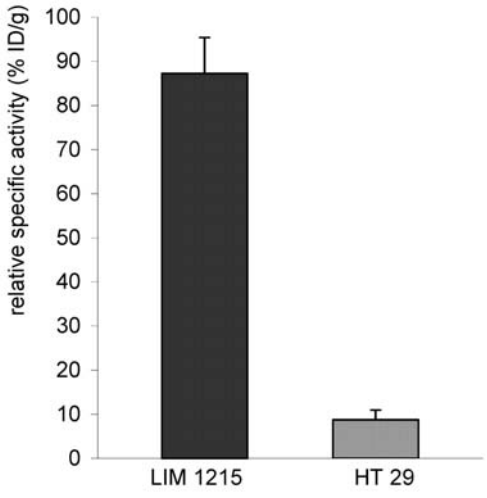

Figure 3. Binding specificity of [^{131}I]A33scFv::CDy *in vivo*. [^{131}I]A33scFv::CDy (50 μg; 20 kBq/mouse) was intravenously injected into the tail vein of nude mice bearing gpA33-positive or gpA33-negative tumor xenografts. Forty-seven hours after administration of [^{131}I]A33scFv::CDy, mice were sacrificed and tumors were collected and measured for radioactivity. LIM1215 (gpA33-positive), black columns; HT29 (gpA33-negative), grey columns. Results are presented as percentage of injected dose per gram of tissue (% ID/g). Error bars represent the standard deviation (n=4).

Binding specificity of [^{131}I]A33scFv::CDy in vivo. Binding specificity *in vivo* was determined by intravenous injection of [^{131}I]A33scFv::CDy into mice bearing colorectal carcinoma xenografts of LIM1215 or HT29 control cells. As Fig. 3 shows, *in vivo* binding was specific for the gpA33-positive LIM1215 xenograft.

Pharmacokinetics and biodistribution. The determined amount of [^{131}I]A33scFv::CDy in colon cancer xenografts and blood

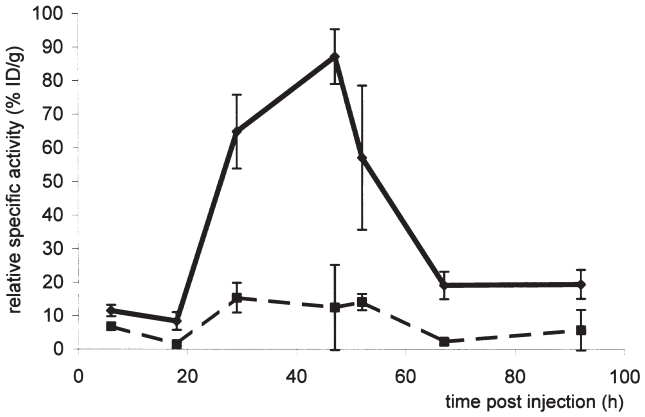

Figure 4. Concentration of [131I]A33scFv::CDy in blood and tumor. [131I]-A33scFv::CDy (50 μg; 20 kBq/mouse) was intravenously injected into the tail vein of nude mice bearing LIM1215 colorectal carcinoma xenografts. At pre-defined time-points, mice were sacrificed and blood and tumors were collected and radioactivity measured. Percentage of injected dose per gram of tissue of tumor (solid line) and blood (dashed line) at 6-96 h post injection. Error bars represent standard deviation (n=4).

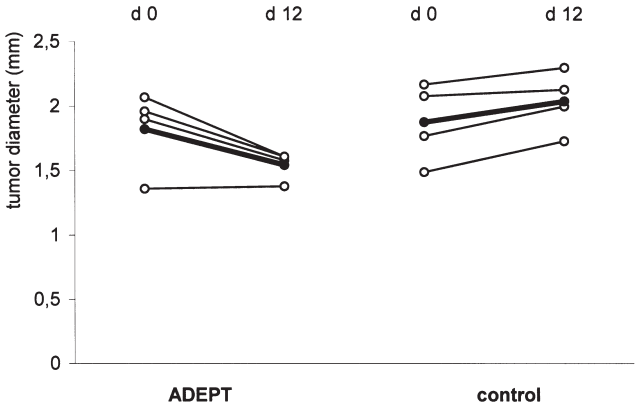

Figure 6. Efficacy of A33scFv::CDy-based ADEPT in xenograft-bearing nude mice. Mice in groups of four received LIM1215 colon carcinoma xenografts as described and were subsequently treated with either 50 μg of A33scFv::CDy per mouse or saline solution for controls, followed 47 h later by 15 mg per mouse of 5-FC prodrug. Tumor diameters at days 0 and 12 after prodrug injection are shown for both groups (thin lines, individual pairs of values; bold lines, mean values).

over time after injection is shown in Fig. 4. The mean uptake of [131I]A33scFv::CDy by antigen-expressing LIM1215 cells reached its maximum with 87.25±8.0% ID/g at 47 h after injection and decreased in two phases thereafter, with a steep slope until 67 h after injection. In contrast, the maximal uptake in the control colon xenograft HT29 was 8.74±2.2% ID/g at 47 h (Fig. 3). The tumor-to-blood ratio of the relative specific activity for LIM1215 tumors increased from 1.7:1 at 6 h to 8.1:1 at 67 h after injection (Fig. 4).

The tissue distribution of [131I]A33scFv::CDy in xenografted mice is shown in Fig. 5. From 47 h post injection on, the tumor contained most of the injected activity at all time-points. During the first three measurements, however, the highest uptake was found in the kidneys, probably due to dissociation of iodine from the protein. Together with the levels in blood and all other organs, this non-specific uptake due to blood-pool activity peaked at 29 h and was well discernable from the maximum tumor uptake at 47 h.

ADEPT in vivo. To test the feasibility of this ADEPT approach *in vivo*, xenograft-bearing mice were first injected the A33scFv::CDy construct and, 47 h later, the 5-FC prodrug. Tumor growth was monitored daily, and the measurements of days 0 and 12 are summarized in Fig. 6. The overall effect is small and no mice were cured of the tumor. However, in the treated group all tumors but one shrank, and this one grew only minimally, whereas the tumors in all untreated mice grew. The differences between both groups were significant

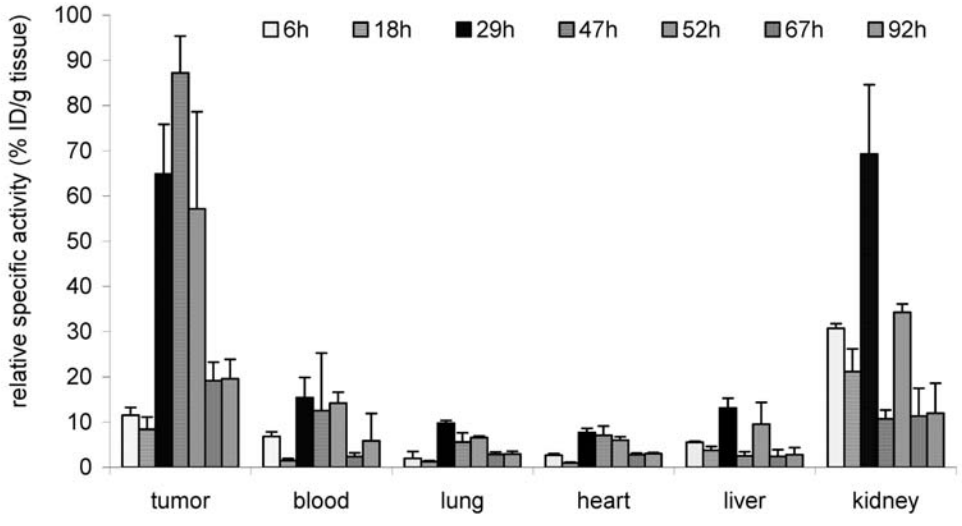

Figure 5. Biodistribution of [131I]A33scFv::CDy in nude mice. The [131I]A33scFv::CDy conjugate (50 μg; 20 kBq/mouse) was intravenously injected into the tail vein of nude mice bearing colorectal tumor xenografts. At the time-points indicated below, mice were sacrificed and organs and blood were collected and radioactivity measured. Results are presented as percentage of injected dose per gram of tissue (% ID/g tissue). Error bars represent standard deviation (n=4). For each organ, seven time-points are shown from left to right: 6, 18, 29, 47, 52, 67, and 92 h post injection.

on day 12 with a p=0.0096 in the two-sided t-test, whereas a pre-existing population difference between the two groups was unlikely with a p=0.8121. Comparing day 0 to day 12 within each group, both the tumor decrease in the treated group (p=0.0376) and the growth in the control group (p=0.0182) were significant. In summary, these results make a genuine therapeutic effect highly more likely than a random effect.

Discussion

The gpA33 antigen is a promising target for radioimmunotherapy (RIT) and ADEPT of metastasized colorectal carcinoma. It is homogeneously expressed in >95% of all colorectal carcinomas with retained expression in metastases. The antigen is absent in most normal tissue apart from colonic and small intestinal mucosa and the excretory ducts of salivary glands (14). Despite its expression in normal colonic mucosa, a clinical study with ^{125}I-labeled murine mAb A33 showed no severe bowel toxicity even at the highest dose (3 GBq/m^2) administered (15), indicating that its expression in normal intestinal tissue does not pose a general obstacle to therapeutic application.

ADEPT has the potential to enhance the efficacy of chemotherapeutic drugs by reducing dose-limiting toxicity. First among the prerequisites to realize this potential is the selective delivery of enzyme to tumor tissue. To overcome the limitations of chemical conjugation such as larger size and heterogeneity of the product (21), we have developed the recombinant A33scFv::CDy fusion protein for use in a combination of RIT and ADEPT (19). The bifunctional fusion protein was produced in Pichia pastoris, which allows for large-scale and high-yield expression of correctly folded and stable, glycosylated (22) protein and its secretion into culture supernatants. Although several antibody-enzyme/prodrug systems have been developed by other groups (5), to our knowledge, A33scFv::CDy and its predecessor, A33scFv::CD, carrying the bacterial isoenzyme (23), are the first single chain antibody fusion proteins against the gpA33-antigen.

In our pharmacokinetic and biodistribution studies in mice with an established xenograft model of gpA33-positive LIM1215 cells, A33scFv::CDy was able to penetrate into tumor tissue and localize in the gpA33-positive, viable regions despite its high molecular weight and the diffusion barriers observed for solid tumors (24,25). The specificity of this localization was demonstrated by lack of radioactivity in gpA33-negative HT29 control tumors. Clearance from blood and solid organs preceded the peak concentration in tumor, leaving a useful therapeutic window. However, decreasing tumor doses are observed even while the blood levels are still at their highest. This may be explained either by actual dissociation of radiolabeled fusion protein from the tumor or by the dissociation of iodine from the fusion protein, e.g. due to de-iodinase activity. The latter might be prevented and the correct answer to this question revealed by using a different radionuclide in future studies. Still, our current results indicate that the fusion construct is specifically bound and retained in tumor tissue, because after blood clearance, a lower, but constant plateau of the tumor-to-blood ratio is reached. This can hardly be explained by passive diffusion kinetics, but rather by a stable fraction within tumor tissue which is not accessible to serum enzymes.

To achieve faster clearance and higher tumor to plasma ratios, three-phase systems using clearance antibodies have successfully been employed (26-30), an approach that might further improve the system proposed here.

Testing the ADEPT system *in vivo*, we found a small but highly significant therapeutic effect in mice bearing LIM1215 xenografts. While no mice were cured, all treated mice had minor responses or stable disease, while in all untreated mice the tumors grew. Both the differences over time within each group and the difference between both groups after treatment were statistically significant. If this indicates a therapeutic effect of the ADEPT system, why was it not stronger? The answer may be an inherent problem of murine xenograft models for ADEPT, as targeting and drug exposure of the tumor happen at two different time-points. Hence, the tumor may outgrow its enzyme-labeled proportion in this interval, giving a false-pessimistic result. To avoid this effect, we chose a relatively slow-growing tumor model. This, in turn, may have the effect that too small a number of cells is sensitive to chemotherapy at any given time. In either case, these findings stress the importance of dose, timing, and repeat applications, which require more detailed investigation in the future.

In addition to ^{131}I (31), the murine A33 antibody has previously been conjugated with 90Y in a preclinical report (32). The different ranges of beta radiation emitted from 90Y and ^{131}I [5.3 and 0.83 mm, respectively (33)] may be exploited for different situations in tumor disease, either alone or in combination (34).

In conclusion, we demonstrated in this study that A33scFv::CDy has favorable pharmacokinetic characteristics for the use in RIT and ADEPT and that its application in ADEPT is feasible and leads to therapeutic effects in a murine model. These results open a perspective towards antibody-targeted combined radio-chemotherapy, which might be dubbed Radio-ADEPT. Future studies of this system will reveal the potential therapeutic effect of A33scFv::CDy in ADEPT and RIT alone, respectively, and in this combination concept, including selection of the optimal radionuclide and optimization of dose and time schedules.

Acknowledgements

This work was supported by an Ernst-von-Leyden Stipendium of the Berliner Krebsgesellschaft to V.C. and by Deutsche Krebshilfe grant no. 1072981 to P.M.D. and H.F.

References

1. Begent RH, Verhaar MJ, Chester KA, Casey JL, Green AJ, Napier MP, Hope-Stone LD, Cushen N, Keep PA, Johnson CJ, Hawkins RE, Hilson AJ and Robson L: Clinical evidence of efficient tumor targeting based on single-chain Fv antibody selected from a combinatorial library. Nat Med 2: 979-984, 1996.
2. Yokota T, Milenic DE, Whitlow M and Schlom J: Rapid tumor penetration of a single-chain Fv and comparison with other immunoglobulin forms. Cancer Res 52: 3402-3408, 1992.
3. Bagshawe KD: Antibody-directed enzyme prodrug therapy for cancer: its theoretical basis and application. Mol Med Today 1: 424-431, 1995.

4. Napier MP, Sharma SK, Springer CJ, Bagshawe KD, Green AJ, Martin J, Stribbling SM, Cushen N, O'Malley D and Begent RH: Antibody-directed enzyme prodrug therapy: efficacy and mechanism of action in colorectal carcinoma. Clin Cancer Res 6: 765-772, 2000.
5. Senter PD and Springer CJ: Selective activation of anticancer prodrugs by monoclonal antibody-enzyme conjugates. Adv Drug Deliv Rev 53: 247-264, 2001.
6. Eccles SA, Court WJ, Box GA, Dean CJ, Melton RG and Springer CJ: Regression of established breast carcinoma xenografts with antibody-directed enzyme prodrug therapy against c-erbB2 p185. Cancer Res 54: 5171-5177, 1994.
7. Sharma SK, Bagshawe KD, Springer CJ, Burke PJ, Rogers GT, Boden JA, Antoniw P, Melton RG and Sherwood RF: Antibody directed enzyme prodrug therapy (ADEPT): a three phase system. Dis Markers 9: 225-231, 1991.
8. Springer CJ, Bagshawe KD, Sharma SK, Searle F, Boden JA, Antoniw P, Burke PJ, Rogers GT, Sherwood RF and Melton RG: Ablation of human choriocarcinoma xenografts in nude mice by antibody-directed enzyme prodrug therapy (ADEPT) with three novel compounds. Eur J Cancer 27: 1361-1366, 1991.
9. Wallace PM, MacMaster JF, Smith VF, Kerr DE, Senter PD and Cosand WL: Intratumoral generation of 5-fluorouracil mediated by an antibody-cytosine deaminase conjugate in combination with 5-fluorocytosine. Cancer Res 54: 2719-2723, 1994.
10. Martin J, Stribbling SM, Poon GK, Begent RH, Napier M, Sharma SK and Springer CJ: Antibody-directed enzyme prodrug therapy: pharmacokinetics and plasma levels of prodrug and drug in a phase I clinical trial. Cancer Chemother Pharmacol 40: 189-201, 1997.
11. Heath JK, White SJ, Johnstone CN, Catimel B, Simpson RJ, Moritz RL, Tu GF, Ji H, Whitehead RH, Groenen LC, Scott AM, Ritter G, Cohen L, Welt S, Old LJ, Nice EC and Burgess AW: The human A33 antigen is a transmembrane glycoprotein and a novel member of the immunoglobulin superfamily. Proc Natl Acad Sci USA 94: 469-474, 1997.
12. Bazzoni G: The JAM family of junctional adhesion molecules. Curr Opin Cell Biol 15: 525-530, 2003.
13. Johnstone CN, Tebbutt NC, Abud HE, White SJ, Stenvers KL, Hall NE, Cody SH, Whitehead RH, Catimel B, Nice EC, Burgess AW and Heath JK: Characterization of mouse A33 antigen, a definitive marker for basolateral surfaces of intestinal epithelial cells. Am J Physiol Gastrointest Liver Physiol 279: G500-G510, 2000.
14. Garin-Chesa P, Sakamoto J, Welt S, Real FX, Rettig WJ and Old LJ: Organ-specific expression of the colon cancer antigen A33, a cell surface target for antibody-based therapy. Int J Oncol 9: 465-471, 1996.
15. Welt S, Scott AM, Divgi CR, Kemeny NE, Finn RD, Daghighian F, Germain JS, Richards EC, Larson SM and Old LJ: Phase I/II study of iodine 125-labeled monoclonal antibody A33 in patients with advanced colon cancer. J Clin Oncol 14: 1787-1797, 1996.
16. Scott AM, Lee FT, Jones R, Hopkins W, MacGregor D, Cebon JS, Hannah A, Chong G, Paul U, Papenfuss A, Rigopoulos A, Sturrock S, Murphy R, Wirth V, Murone C, Smyth FE, Knight S, Welt S, Ritter G, Richards E, Nice EC, Burgess AW and Old LJ: A phase I trial of humanized monoclonal antibody A33 in patients with colorectal carcinoma: biodistribution, pharmacokinetics, and quantitative tumor uptake. Clin Cancer Res 11: 4810-4817, 2005.
17. Daghighian F, Barendswaard E, Welt S, Humm J, Scott A, Willingham MC, McGuffie E, Old LJ and Larson SM: Enhancement of radiation dose to the nucleus by vesicular internalization of iodine-125-labeled A33 monoclonal antibody. J Nucl Med 37: 1052-1057, 1996.
18. Rader C, Ritter G, Nathan S, Elia M, Gout I, Jungbluth AA, Cohen LS, Welt S, Old LJ and Barbas CF III: The rabbit antibody repertoire as a novel source for the generation of therapeutic human antibodies. J Biol Chem 275: 13668-13676, 2000.
19. Coelho V, Dernedde J, Petrausch U, Panjideh H, Fuchs H, Menzel C, Dubel S, Keilholz U, Thiel E and Deckert PM: Design, construction, and *in vitro* analysis of A33scFv::CDy, a recombinant fusion protein for antibody-directed enzyme prodrug therapy in colon cancer. Int J Oncol 31: 951-957, 2007.
20. Barendswaard EC, O'Donoghue JA, Larson SM, Tschmelitsch J, Welt S, Finn RD and Humm JL: ^{131}I radioimmunotherapy and fractionated external beam radiotherapy: comparative effectiveness in a human tumor xenograft. J Nucl Med 40: 1764-1768, 1999.
21. Melton RG: Preparation and purification of antibody-enzyme conjugates for therapeutic applications. Adv Drug Deliv Rev 22: 289-301, 1996.
22. Bretthauer RK and Castellino FJ: Glycosylation of Pichia pastoris-derived proteins. Biotechnol Appl Biochem 30: 193-200, 1999.
23. Deckert PM, Renner C, Cohen LS, Jungbluth A, Ritter G, Bertino JR, Old LJ and Welt S: A33scFv-cytosine deaminase: a recombinant protein construct for antibody-directed enzyme-prodrug therapy. Br J Cancer 88: 937-939, 2003.
24. Jain RK: Delivery of molecular and cellular medicine to solid tumors. Adv Drug Deliv Rev 46: 149-168, 2001.
25. Yuan F, Baxter LT and Jain RK: Pharmacokinetic analysis of two-step approaches using bifunctional and enzyme-conjugated antibodies. Cancer Res 51: 3119-3130, 1991.
26. Cheng TL, Chen BM, Chern JW, Wu MF and Roffler SR: Efficient clearance of poly(ethylene glycol)-modified immunoenzyme with anti-PEG monoclonal antibody for prodrug cancer therapy. Bioconjug Chem 11: 258-266, 2000.
27. Sharma SK, Bagshawe KD, Springer CJ, Burke PJ, Rogers GT, Boden JA, Antoniw P, Melton RG and Sherwood RF: Antibody directed enzyme prodrug therapy (ADEPT): a three phase system. Dis Markers 9: 225-231, 1991.
28. Haisma HJ, van Muijen M, Scheffer G, Scheper RJ, Pinedo HM and Boven E: A monoclonal antibody against human beta-glucuronidase for application in antibody-directed enzyme prodrug therapy. Hybridoma 14: 377-382, 1995.
29. Kerr DE, Garrigues US, Wallace PM, Hellstrom KE, Hellstrom I and Senter PD: Application of monoclonal antibodies against cytosine deaminase for the *in vivo* clearance of a cytosine deaminase immunoconjugate. Bioconjug Chem 4: 353-357, 1993.
30. Sharma SK, Pedley RB, Bhatia J, Boxer GM, El Emir E, Qureshi U, Tolner B, Lowe H, Michael NP, Minton N, Begent RH and Chester KA: Sustained tumor regression of human colorectal cancer xenografts using a multifunctional mannosylated fusion protein in antibody-directed enzyme prodrug therapy. Clin Cancer Res 11: 814-825, 2005.
31. Welt S, Divgi CR, Kemeny N, Finn RD, Scott AM, Graham M, Germain JS, Richards EC, Larson SM, Oettgen HF and Old LJ: Phase I/II study of iodine 131-labeled monoclonal antibody A33 in patients with advanced colon cancer. J Clin Oncol 12: 1561-1571, 1994.
32. King DJ, Antoniw P, Owens RJ, Adair JR, Haines AR, Farnsworth AH, Finney H, Lawson AG, Lyons A, Baker TS, Balsock D, Mackintosh J, Gofton C, Yarranton GT, McWilliams W, Shochat D, Leichner PK, Welt S, Old LJ and Mountain A: Preparation and preclinical evaluation of humanised A33 immunoconjugates for radioimmunotherapy. Br J Cancer 72: 1364-1372, 1995.
33. Mattes MJ: Radionuclide-antibody conjugates for single-cell cytotoxicity. Cancer 94: 1215-1523, 2002.
34. De Jong M, Breeman WA, Bernard BF, Bakker WH, Visser TJ, Kooij PP, Van Gameren A and Krenning EP: Tumor response after [(90)Y-DOTA(0),Tyr(3)]octreotide radionuclide therapy in a transplantable rat tumor model is dependent on tumor size. J Nucl Med 42: 1841-1846, 2001.