Design, construction, and *in vitro* analysis of A33scFv::CDy, a recombinant fusion protein for antibody-directed enzyme prodrug therapy in colon cancer

VÂNIA COELHO[1], JENS DERNEDDE[2], ULF PETRAUSCH[1], HOSSEIN PANJIDEH[2], HENDRIK FUCHS[2], CHRISTOPH MENZEL[3], STEFAN DÜBEL[3], ULRICH KEILHOLZ[1], ECKHARD THIEL[1] and P. MARKUS DECKERT[1]

[1]CharitéCentrum für Tumormedizin, Medizinische Klinik mit Schwerpunkt Hämatologie und Onkologie CBF;
[2]CharitéCentrum für Labormedizin, Institut für Klinische Chemie und Pathobiochemie, 12200 Berlin;
[3]Technical University of Braunschweig, Institute of Biochemistry and Biotechnology, D-38106 Braunschweig, Germany

Abstract. Antibody-directed enzyme-prodrug therapy (ADEPT) aims at improving the specificity of conventional chemotherapy by employing artificial antibody-enzyme constructs to convert a non-toxic prodrug into a cytotoxic agent specifically localized to the tumor site. The gpA33 antigen is a promising target for ADEPT in colon cancer, as it is expressed by >95% of human colon cancers, but is absent in all non-gastrointestinal tissues. We designed a recombinant fusion construct of a phage display-generated anti-gpA33 single chain fragment, A33scFv, with cytosine deaminase from yeast (CDy), which converts 5-fluorocytosine (5-FC) into 5-fluorouracil (5-FU). The resulting construct, A33scFv::CDy, was overexpressed in *Pichia pastoris* and secreted into culture supernatant. The fusion protein was purified by affinity chromatography on protein L. Silver-staining after SDS-polyacrylamide gel electrophoresis confirmed molecular mass and purity. Antibody binding and specificity were quantified by flow cytometry. The complete ADEPT system was applied *in vitro* on gpA33-positive LIM1215 cells, assessing cell survival by a fluorescein diacetate assay. Cytotoxicity of the prodrug 5-FC after A33scFv::CDy binding was equimolar to that of 5-FU, and this effect depended specifically on both antibody and enzyme function. These results demonstrate bifunctional activity of the heterogeneous *Pichia*-produced A33scFv::CDy fusion protein and proof of principle for the ADEPT system proposed herein.

Introduction

Monoclonal antibodies have become an accepted modality of cancer therapy. Recombinant antibodies and antibody-based fusion proteins hold the promise of further extending the therapeutic possibilities of this modality. 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 excellent 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).

Several ADEPT systems have shown promising *in vivo* efficacy in a number of tumor models (5) and in several xenograft systems in nude mice (4,6-8), demonstrating in principle that ADEPT can target tumor tissue with high selectivity 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).

An eminent obstacle to the development of scFv-based approaches, however, is posed by the expression of a heterogeneous protein consisting of human antibody sequences

Correspondence to: Dr P. Markus Deckert, Charité - Campus Benjamin Franklin, Hindenburgdamm 30, D-12200 Berlin, Germany
E-mail: markus.deckert@charite.de

and e.g. a bacterial enzyme in a single expression system. Methylotrophic *Pichia* yeasts such as *P. pastoris* combine the simple handling of microbial cultures with the post-translational processing of eukaryotes and thus offer an interesting alternative for large-scale expression of complex proteins (11,12).

In this work we report on the design, cloning, expression and *in vitro* characterization of a new recombinant antibody-enzyme fusion protein for ADEPT, termed A33scFv::CDy.

The A33 monoclonal antibody recognizes a newly characterized cell-surface differentiation antigen of approximately 43 kDa that belongs to the immunoglobulin superfamily. It is expressed on normal human gastrointestinal epithelium and on >95% of primary or metastatic colon cancers, but is absent in most other normal tissues (13).

Radiolabelled humanized A33 antibody has been shown to selectively target primary and metastatic tumor sites of colorectal cancers and to penetrate to the center of large necrotic metastatic lesions, indicating potential therapeutic use of gpA33 as a target for colorectal cancer therapy (14).

As opposed to the quoted work from Senter's and previous results from our group (15), here we used cytosine deaminase from *Saccharomyces cerevisiae* (CDy) rather then *Escherichia coli* (CD), as this isoenzyme promised higher levels of expression yield and catalytic activity.

Materials and methods

Design and cloning of plasmids A33scFv::CDy [pPIC9K] and A33scFv::CDy [pPICZαA]. By PCR cloning, the cDNA sequences encoding A33scFv (16) and CDy were amplified. CDy cDNA was amplified from the commercial vector pORF-Fcy (Invivogen, San Diego, USA) and flanked by unique restriction sites (*Not*I and *Spe*I) with the primer pair Fcy5' (5'-CCTCAACTAGTGGTGGAGGTGGAAGTGTGACA GGGGGAATGGCAAGC-3') and Fcy3' (5'-GGCGAATTA ATTCGCGGCCGCTTACTCACCAATATCTTCAAACCA ATCCTGAGG-3'). Fifty cycles of PCR were performed with incubations for 30 sec at 95˚C, 50 sec at 50˚C and 1 min at 72˚C. After gel extraction and purification using the Qiagen Gel extraction kit, the amplified cDNA was initially subcloned into the pPCR Blunt II vector (Invitrogen) to generate pPCR BluntII CDy. Identity and integrity of the cloned fragment were confirmed by automated DNA sequencing (ABI PRISM 310, Perkin-Elmer, USA). The A33scFv::CDy fragment was excised by digestion with the restriction endonucleases *Avr*II and *Spe*I and ligated with T4 ligase into the pPICZαA or pPIC9K backbones (Invitrogen) to yield the plasmid vectors A33scFv::CDy [pPICZαA] and A33scFv::CDy [pPIC9K], respectively. The common cloning cassette and maps of the resulting vectors are shown in Fig. 1.

Transformation, selection and screening for fusion protein expression. Pichia pastoris KM71 (Invitrogen) was grown in YPD (1% yeast extract, 2% peptone, 2% dextrose) and made electrocompetent following the procedure described by the manufacturer (17). Eighty microliters of electrocompetent *KM71* cells were mixed with 5 μg of either A33scFv::CDy [pPICZαA] or A33scFv::CDy [pPIC9K] linearized by digestion with *Pme*I, and then electroporated by a pulse discharge of
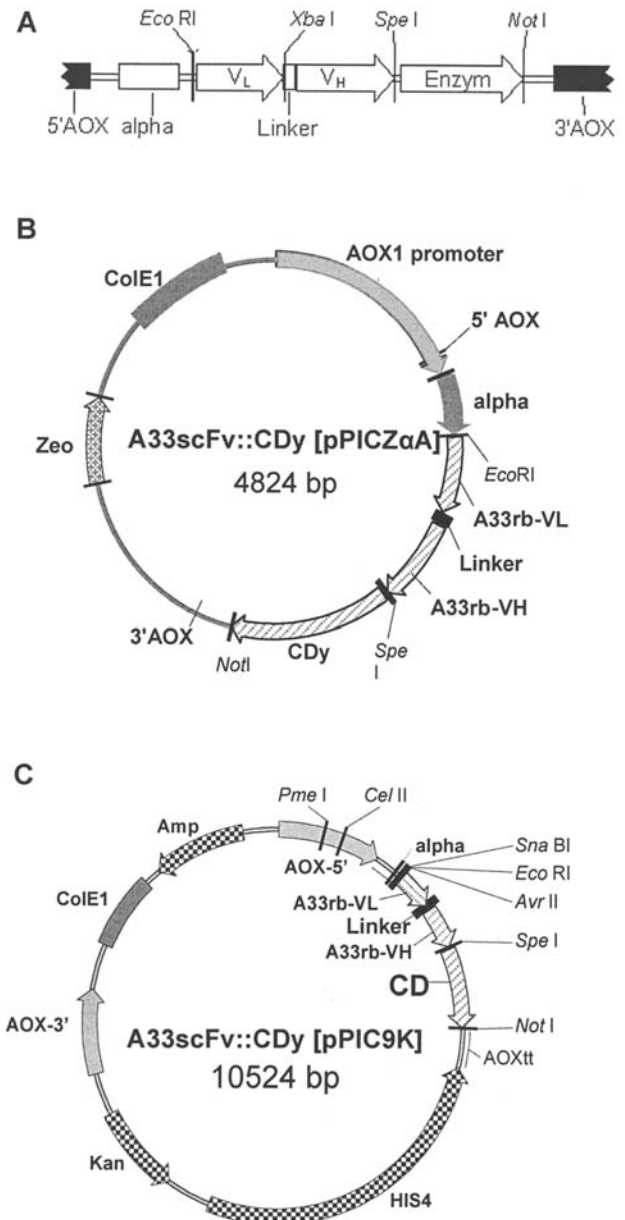

Figure 1. Plasmid design and cloning strategy. A, Common cloning site of pPICZαA and pPIC9K vectors with insertion cassette for the scFv-fusion protein with relevant restriction sites. B and C, Vector constructs based on pPIC9K and pPICZαA. Restriction sites are designated in italics. AOX 5', AOX 3', promotor and terminal region of the native AOX-1 gene, respectively; 5'AOX, 3' AOX, AOX-1 gene flanking primers; A33rbVL, A33rbVH, position of the variable regions of the light and heavy antibody chains, respectively; CDy, cytosine deaminase from yeast; linker, artificial linking sequence; alpha, alpha mating factor secretion signal; Amp, Kan, Zeo, resistance genes against ampicillin, kanamycin, and Zeocin, respectively; His4, histidine deaminase; ColE1, *E. coli* replication factor.

1500 V, 25 μF, and 400 Ω (Bio-Rad Gene Pulser) for 5 min. After pulsing, 500 ml of ice-cold 1 M sorbitol was immediately added to each cuvette. Transformant aliquots of 400 μl each were spread on YPDS plates (1% yeast extract, 2% peptone, 2% glucose,1 M sorbitol, 2% agar), which contained 100 μg/ml of Zeocin for pPICZαA transformants.

PPIC9K-transformed clones were selected for their ability to grow on histidine deficient medium, indicating presence of the HIS4 gene transferred with successful vector trans-

formation. These HIS+ colonies were replica-plated onto plates containing the antibiotic G418 in concentrations from 2 to 10 mg/ml. Finally, in clones resistant to G418 the genomic integration of the transfected gene was checked by PCR (primer sequences available upon request). Clones carrying the A33scFV::CDy sequence were grown in 5 ml BMGY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, $4x10^{-5}$% Biotin, 1% glycerol) in a 50 ml conical tube overnight at 30˚C and 350 rpm agitation. After centrifugation at 3,000 g for 10 min at 20˚C, the cell pellet was resuspended in 5 ml of BMMY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, $4x10^{-5}$% Biotin, 1% methanol) to induce protein expression. The cells were incubated for 3 days at 30˚C and 350 rpm, and fresh methanol was added to a total of 1% every 24 h to maintain induction.

Clones transformed with the pPICZαA-construct grown on YPDS-Zeocin plates were directly inoculated in 500 μl BMGY on a 96-well plate and incubated overnight at 30˚C and 350 rpm. The plate was then centrifuged at 1500 g for 10 min at 20˚C, and cell pellets were resuspended in 500 μl of BMMY to induce protein expression. Induction was maintained for 72 h as described above.

Detection of fusion protein-producing transformants. With pPIC9K-transformants, cleared supernatant samples were obtained from each induced culture, and samples were analyzed by electrophoresis on 10% SDS polyacrylamide gels (SDS-PAGE) under denaturing conditions, followed by electro-blotting onto a nitrocellulose membrane (Amersham Pharmacia Biotech, Freiburg, Germany). CDy was then detected by sequential membrane incubation with a 1:2,000 dilution of polyclonal anti-CDy IgG from sheep (Acris, Hiddenhausen, Germany) and a 1:500 dilution of peroxidase-conjugated anti-sheep IgG (Sigma, St. Louis, USA), developed using the ECL detection kit (Amersham Pharmacia Biotech) and documented photographically. Stained gels and immunoblots were photographed and quantitatively analyzed using the AlphaEase FC software (AlphaInnotech, San Leandro, CA).

To detect expression of the fusion protein in clones transformed with the pPICZαA-based construct, 96-well Nunc Maxisorp plates were coated with 100 ng/well of recombinant IgG-grafted A33 expressed in CHO cells (data not shown) in 100 μl of coating buffer. After washing, the plates were incubated with supernatants from induced *Pichia* clones for 1 h at room temperature, followed by incubation with Protein L-HRP (Pierce, Rockford, USA) diluted 1:5,000 in blocking solution for 1 h at 37˚C. The reaction was stopped with 100 μl/well of 1 N H_2SO_4 and the reaction was quantified colorimetrically at 655 nm using a 96-well plate photometer (Tecan Sunrise, Crailsheim, Germany).

Clones with a signal in the top tenth of the distribution range were selected for inoculation of 20 ml each of BMGY to an $OD_{600\,nm}$ of 0.6 and incubated overnight at 30˚C and 250 rpm. Cells were harvested by centrifugation and resuspended in 10 ml BMMY. Expression was induced as described above, and culture samples were examined for production of the fusion protein by SDS-PAGE and protein staining as with pPIC9K-transformed clones.

Optimization of fusion protein production. Overnight cultures of fusion protein-producing clones were used to inoculate 20 ml BMGY pH 6.0 to an $OD_{600\,nm}$ of 0.6, incubated overnight at 30˚C and 250 rpm, harvested by centrifugation and resuspended in 20 ml BMMY for subsequent methanol induction under various conditions as indicated in the results. Samples were collected from the induced cultures at different time-points and analyzed after purification with protein L (Pierce).

Production of A33scFv::CDy. High-expressing clones selected for protein production were grown in a culture volume of 100 ml each in a 1 l baffled shake flask overnight at 220 rpm and 30˚C. The cells were harvested by centrifugation and were used to inoculate a total of 1 l of BMGY, divided into two baffled shake flasks of 2 l volume to give a starting $OD_{600\,nm}$ of 0.5. After overnight culture at 30˚C and 220 rpm, the cells were harvested at a final $OD_{600\,nm}$ of ~40 by centrifugation at 1500 g and 25˚C for 10 min, resuspended in 1 l BMMY and divided in two baffled 2 l flasks. Expression was then induced at 25˚C and maintained for 60 h by adding 5 ml of 100% methanol every 24 h. Supernatant was subsequently collected by centrifugation as described, and sterile filtered.

Purification of A33scFv::CDy. Five tablets of proteinase inhibitor cocktail (Roche, Mannheim, Germany) were added per liter of *Pichia* culture supernatant to reduce proteolysis. The A33scFv::CDy fusion protein was then purified by affinity chromatography on sepharose-immobilized protein L plus (Pierce) via gravity flow. Each elution was performed with 800 μl of 0.1 M glycin buffer pH 2.5. To each elution fraction 200 μl of 1 M Tris-buffer pH 9.0 was added to adjust the pH to ~6.0.

Flow cytometry. LIM1215 colon cancer cells (provided by the Ludwig Institute for Cancer Research, New York, USA) were harvested and resuspended in 10 ml Dulbecco's PBS. Viable cells were counted after staining an aliquot 1:2 (v/v) with trypan blue. Aliquots of 500,000 cells were entered in 1.5 ml microcentrifuge tubes and incubated with the fusion protein to be tested.

After 40-min incubation at 37˚C with shaking, cells were washed twice with 500 μl Dulbecco's PBS, and resuspended in 250 μl of Dulbecco's PBS. To detect A33scFv::CDy directly, cells were subsequently incubated with FITC-labelled polyclonal anti-CDy (Acris, Hiddenhausen, Germany) in a dilution of 1/160 for 15 min and washed again. For inhibition assays, cells were incubated with A33scFv::GFP for 40 min instead. Physical parameters and fluorescence of 30,000 events each were measured using a FACScalibur instrument (Becton-Dickinson, Heildelberg, Germany).

Cytotoxicity assay. Cytotoxicity was determined *in vitro* using the fluorescein diacetate assay as described by Nygren *et al* (18). Briefly, 2,000 cells each of LIM1215 cells were transferred to a 96-well culture plate and grown to confluence. After washing twice with PBS, the fusion protein to be tested was added to a final volume of 200 μl in RPMI with 10% v/v FCS and incubated at 37˚C with 0.5% CO_2 and incubated for 3 h. Cells were washed twice with PBS, the drug or prodrug

to be tested was added in fresh cell culture medium (RMPI-1640 supplemented with 10% heat inactivated FCS and 1% penicillin/streptavidin) at the concentrations indicated, and the cells were incubated for 48 h at 37°C with 0.5% CO_2. Cells were finally washed twice with PBS and incubated in 10 μl/ml fluorescein diacetate (Sigma) for 45 min. Fluorescence was measured by a Spectra Max Gemini microplate reader (Molecular Devices, Ismaning, Germany) with excitation and emission wavelengths of 485 and 538 nm, respectively. Including the controls *C*, cells without drug, and *B*, sample without cells, the survival index (*SI*) was calculated from the sample fluorescence (*Test*) and expressed in percent using the following formula:

$$SI\,(\%) = \frac{Test - B}{C - B} \times 100$$

Results

Design and construction of A33scFv::CDy. A vector system for the production of scFv::effector fusion proteins comprising two functional cassettes with flanking unique restriction sites that allow for the independent exchange of each component by restriction digest and ligation was designed and cloned. Based on the commercial plasmid backbones pPIC9K and pPICZαA, two transformation vectors were thus generated, leading to identical integrants by omega-insertion into the genome of the transformed *Pichia* strain KM71. Both plasmids put expression of the fusion protein under control of the AOX I promoter located downstream of the α-factor signal sequence to obtain extracellular secretion of the protein (12).

Screening of Pichia transformants for expression. Several sequential assays were necessary to select clones transformed with the pPIC9K-based A33scFv::CDy vector construct. First, exploiting the ability to grow on histidine deficient media conferred by the pPIC9K backbone, KM71 *Pichia* were screened for presence of the insert (18), followed by incubation on media with increasing concentrations of G418 to select for kanamycin resistance. Quantitative differences in kanamycin resistance are conferred by multiple insertions of the *Kan* gene, which was linked to the inserted construct DNA, thus allowing to detect multi-copy integrants.

Finally, the genomic integration of the transfected gene was analyzed and confirmed by PCR (data not shown). Fifty clones revealed genomic integration of the A33scFV::CDy DNA sequence. Six out of these 50 clones displayed high expression of the protein construct in 5-ml screening induction and immunoblot.

The vector backbone pPICZαA allowed for a simplified selection procedure. Conferring Zeocin resistance, this vector allowed the selection of hyper-drug resistant transformants containing multiple copies of the expression cassette in one step. Therefore, all clones growing on YPDS-Zeocin plates were directly analyzed for expression.

Culture supernatants from a total of 93 pPICZαA clones were screened by ELISA, of which 91 were found to exhibit binding to immobilized recombinant gpA33. Clones showing a binding signal in the top tenth of the distribution range were induced in 5-ml pilot cultures, and their supernatants further
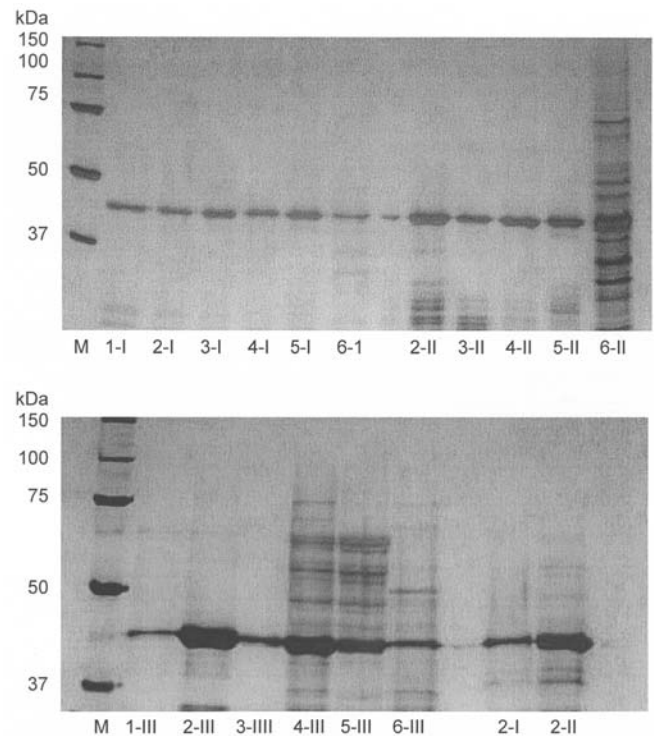

Figure 2. Optimization of expression yield and postexpressional stability. SDS-PAGE. Eluates from protein L affinity chromatography of A33scFv::CDy-containing supernatants induced under the following conditions indicated by Arabic numerals: 1, induction at 30°C; 2, induction at 25°C, both without additives; 3, temperature 30°C and addition of 10 mM EDTA; 4, temperature 30°C and addition of 1.45 μM Pepstatin A + 0.5 mM PMSF; 5, temperature 25°C and addition of 1.45 μM Pepstatin A + 0.5 mM PMSF; 6, temperature 30°C with culture stopped at an $OD_{600\,nm}$ of 6. Samples were taken for analysis at the following time-points indicated by Roman numerals: I, 24 h; II, 48 h; III, 72 h. M, molecular weight marker.

analyzed by SDS-PAGE and silver staining, confirming the expected size of the construct, but also revealing various amounts of degradation products (see also Fig. 3), which prompted optimization studies prior to scaling up of production.

Expression optimization of A33scFv::CDy. Aliquots from a high-producing pPICZαA clone were cultivated in parallel under different conditions and analyzed by silver-stained SDS-PAGE at various time-points as indicated in Fig. 2. At a cultivation temperature of 25°C degradation turned out to be lowest. In addition, adding protease inhibitor further reduced protein degradation.

Production at laboratory scale. A high-producing pPICZαA-transformant *Pichia* clone was selected for further expression experiments based on the data gained from the pilot expressions above. Silver-stained SDS-PAGE revealed a strong uniform band corresponding to the expected size of the fusion protein of 47 kDa. In addition, two weak but distinct bands were consistently visible, which corresponded to degradation products of proteolysis, presumably in the linker region. After purification by centrifugation filtration and protein L affinity chromatography, reducing SDS-PAGE of the eluate reproducibly revealed three remaining contaminant bands (Fig. 3), whose proportion among the total protein, however, was ≤5% as determined by electronic image analysis. The
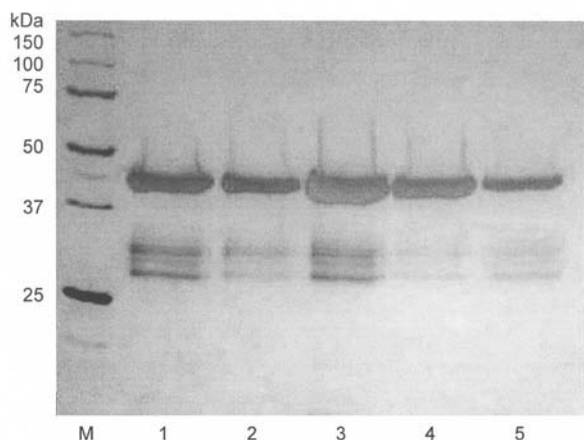
Figure 3. Expression and purification of A33scFv::CDy at the 1 l scale SDS-PAGE. Lanes 1-5 show eluates from different protein L purification runs of 1 l expression culture supernatant each. All lanes show various amounts of degradation products.
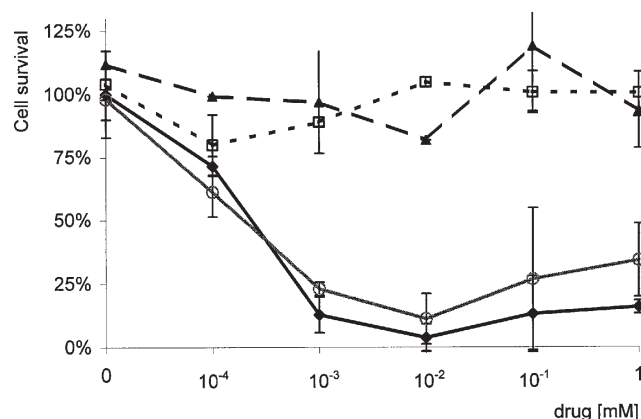

Figure 5. A33scFv::CDy mediates cytotoxicity of 5-FC on gpA33 antigen-positive cells depending on prodrug dose. LIM1215 cells were incubated with 10 ng/ml of fusion protein and, after washing, with a dilution series of the prodrug. Cell survival was determined by fluorescein diacetate assay. 5-FC alone (open squares) and after pre-incubation with the control construct A33scFv::GFP (closed triangels) had no effect on cell survival. After pre-incubation with A33scFv::CDy (open circles), however, 5-FC had approximately the same cytotoxic effect as equimolar amounts of 5-FU (closed carets). Mean and standard deviation of triplicate samples.

total yield after purification was about 500 μg, corresponding to 1.0 mg purified protein per liter of culture.

Antigen-binding activity of A33scFv::CDy. The binding activity and specificity of the expressed recombinant antibody were verified by flow cytometry on gpA33-positive LIM1215 colon cancer cells (19). In a direct assay, binding of A33scFv::CDy was detected by a secondary antibody (Fig. 4A), demonstrating binding activity of both crude supernatant and purified fusion protein. To evaluate binding specificity in an inhibition assay, a fluorescent A33scFv-based fusion protein, A33scFv::GFP, was directly detected, and A33scFv::CDy was used to block its binding to gpA33-positive LIM1215 cells. Here, binding of the fluorescent fusion protein could be quantitatively suppressed by the A33scFv::enzyme fusion protein, demonstrating immunologically specific binding.

ADEPT system in vitro. To test for the dual activity of the A33scFv::CDy fusion protein and to determine its suitability

for the prodrug therapy approach, a fluorescein diacetate cytotoxicity assay of the complete ADEPT system was established.

To establish the difference in cytotoxicity between drug and prodrug, LIM1215 cells were incubated with dilution series of either 5-FU or 5-FC alone, confirming the toxicity of 5-FU as previously reported (15) and the complete lack thereof with 5-FC in the dose range tested (Fig. 5). In the same assay, pre-incubation of 5-FC with the fusion protein reduced the cell survival curve to that of 5-FU. After preincubation with a control fusion protein, however, 5-FC had no effect on cell survival.

The effect of the fusion protein on cell survival was also quantified by dilution series. In a control without prodrug incubation, A33scFv::CDy had no cytotoxic effect of its own. With subsequent 5-FC incubation, 1 ng/ml of fusion
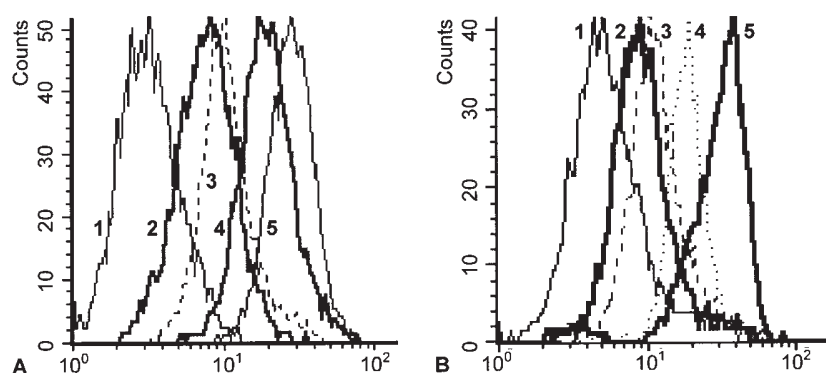

Figure 4. Flow cytometry: A33scFv::CDy binding to gpA33-positive cells. A, LIM1215 single-cell suspension was incubated with A33scFv::CDy and, after washing, with a FITC-labelled anti-CDy antibody. Crude supernatant (4) and purified A33scFv::CDy (5) showed a clear signal over cells alone (1) and incubation with either A33scFv::CDy alone (2) or anti-CDy FITC antibody alone (3). B, Binding competition with A33scFv::GFP. LIM1215 single-cell suspension (negative controls in PBS buffer, 1, and BMMY supernatant medium, 2) was incubated with A33scFv::GFP (positive control, 5) and analyzed by flow cytometry. Pre-incubation of the cells with A33scFv::CDy reduced subsequent A33scFv::GFP binding quantitatively (3, saturation; 4, half-saturation).
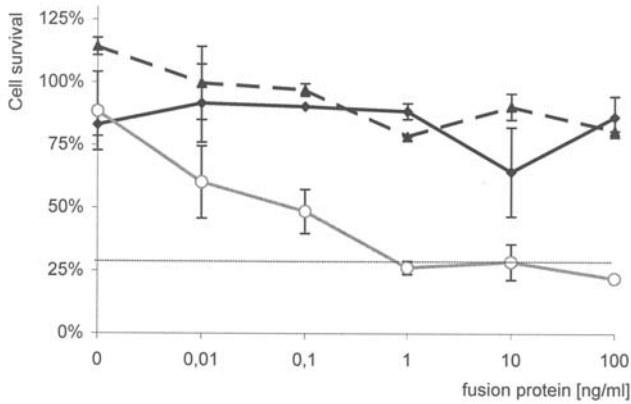
Figure 6. A33scFv::CDy mediates cytotoxicity of 5-FC on gpA33 antigen-positive cells depending on fusion protein dose. LIM1215 cells were incubated with a dilution series of the A33scFv::CDy fusion protein and, after washing, with 100 nM 5-FC prodrug (open circles), demonstrating dose-dependent cytotoxicity. A33scFv::CDy construct alone without subsequent 5-FC incubation (closed triangels) had no cytotoxic effect, nor had pre-incubation with excess control protein A33scFv::GFP prior to A33scFv::CDy and subsequent 5-FC incubation (closed carets). Dotted line, equimolar 5-FU control. Mean and standard deviation of triplicate samples.

protein was sufficient to reach the maximum cytotoxic effect in this assay. Saturation of gpA33 binding sites by excess A33scFv::GFP prior to the incubations with A33scFv::CDy and subsequently with 5-FC completely removed the cytotoxic effect of this ADEPT system.

Discussion

In this report, we describe the design, expression, and *in vitro* characterization of a novel fusion protein for ADEPT, termed A33scFv::CDy. Recombinant fusion proteins promise to overcome the limitations of chemical conjugates for ADEPT, i.e. instability of the chemical linker and the inevitable presence of products with various component ratios. The heterogeneous sources of such fusion proteins, i.e. the antibody's mammalian and the enzyme's microbial origin, however, make it often difficult if not impossible to express a functional fusion protein in a single expression host. This may be due to the presence or absence of suitable chaperones, but also to the intrinsic capacities of a given expression system such as posttranslational processing or susceptibility to intracellular product effects on the host cell.

For their ability to secrete correctly folded and glycosylated proteins into the supernatant of an easy-to-handle microbial culture, thus offering advantages over both bacterial and mammalian expression systems, methylotrophic *Pichia* yeasts have been used to produce a wide range of recombinant proteins including antibody fragments (12,20).

We previously reported the expression of a very similar fusion protein for ADEPT, A33scFv::CD with cytosine deaminase from *E. coli* (15). There, *E. coli* was also the expression host, requiring recovery from inclusion bodies and resulting in a culture yield of about 100 μg/l. Elaborate attempts to reach a higher yield by production in *P. pastoris*, however, failed because bacterial CD could not be expressed or excreted by the yeast (unpublished data). In addition, for yeast CD higher catalytic activity in biological systems had

been reported in comparison to the bacterial isoenzyme (23). These reasons prompted us to redesign an A33scFv fusion protein with cytosine deaminase from yeast.

Producing the new fusion protein in Pichia yeast, we obtained about ten times the expression yield as in bacteria, with approximately 1 mg/l in shake flask cultures. Secretion into culture supernatant greatly facilitated protein retrieval and purification, and the protein was functional in supernatant without further purification. By single-step affinity chromatography on protein L, sufficient purity for pre-clinical experiments was achieved.

However, there was partial degradation of the protein even after purification, indicated by additional bands around a molecular size of 30 kDa. The size suggesting cleavage in the linker region, we tried to narrow down potential specific proteases in *Pichia*, but could not identify a matching cleavage site in public databases. Still, recloning the linker region in future constructs could possibly avoid proteolysis and generate a uniform band under all circumstances. However, for all practical purposes, reducing the culture temperature, adding a protease inhibitor mixture, and speeding the processing procedures solved this problem (21).

Although the transformation method employed led to genomic integration of the gene of interest (as opposed to bacterial expression plasmids), the choice of transformation vector was of influence beyond cloning and selection. To maximize protein expression, integration of multiple copies of the gene of interest is desirable. Of the two transformation vectors employed here, pPICZα appeared to result in a higher number of multi-copy clones. In addition, it has been reported that the large size of the pPIC9K vector may lead to less stable transformants (22), so that we finally selected a pPICZα transformant for production.

The secreted protein was bifunctionally active without any further treatment, i.e., even the uncleared culture supernatant could directly be used for the detection of A33-positive cells in flow cytometry or for *in vitro* ADEPT. Binding specificity was demonstrated by quantitative blocking of A33scFv::GFP binding to gpA33-positive cells after their pre-saturation with A33scFv::CDy in flow cytometry. Finally, the complete ADEPT system was proven in principle by cytotoxicity assays which, in conclusion, demonstrate that 5-FC is specifically toxified by A33scFv::CDy depending both on antibody binding and enzyme activity. With A33scFv::fusion protein in excess, 5-FC was rendered equally toxic to equimolar amounts of 5-FU. While these results largely match those obtained with the previous fusion protein based on bacterial CD (23), our results also appear to confirm the theoretical background for which the yeast isoenzyme was interesting in the first place: with A33scFv::CDy, 1 ng/ml sufficed to induce maximal cytotoxicity, whereas the corresponding value for the bacterial CD-construct was 10 times higher. While we could not compare the two constructs directly because no fresh bacterial fusion protein was available for this study, the order of magnitude of this difference makes it unlikely that this is a mere incidental finding. The improved results with the yeast protein may be attributable both to higher catalytic activity of the homodimerically active yeast enzyme (24) and to higher quality of the *Pichia*-produced protein due to better folding and functional secretion.

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