Improvement of pharmacokinetics of small recombinant bispecific antibody molecules

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Abbreviations

aa	Amino acid(s)
ABD	Albumin-binding domain
ADCC	Antibody dependent cellular cytotoxicity
AUC	Area under the curve
BiTE	Bispecific T-cell engager
CD	Cluster of differentiation
CDC	Complement dependent cytotoxicity
CDR	Complementarity determining region
CEA	Carcinoembryonic antigen
CTLA-4	Cytotoxic T-lymphocyte antigen 4
dAbs	Human domain antibodies
Db	Diabody
EC_{50}	Half-maximal effective concentration
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
Ep-CAM	Epithelial cell adhesion molecule
EPO	Erythropoietin
EPR	Enhanced permeability and retention effect
Fab	Fragment antigen-binding
Fc	Fragment crystallizable
FcRn	Neonatal Fc-receptor
FcRn hc	Heavy chain of the neonatal Fc-receptor
FDA	US Food and Drug Administration
Fv	Variable fragment
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte macrophage colony-stimulating factor
HAMA	Human anti-mouse antibody
HAP	Homo-amino-acid polymer
HSA	Human serum albumin
IC_{50}	Half-maximal inhibitory concentration
Ig	Immunoglobulin
IL	Interleukin
K_D	Dissociation constant
KO	Knockout
mAb	Monoclonal antibody
MALDI-MS	Matrix-assisted laser desorption/ionization mass spectrometry
MHC	Major histocompatibility complex

MSA	Mouse serum albumin
MW	Molecular weight
NHL	Non-Hodgkin Lymphoma
PBMC	Peripheral blood mononuclear cells
PEG	Polyethylene glycol
PEO	Polyethylene oxide
PSA	Polysialic acid
RSA	Rat serum albumin
scDb	Single-chain Diabody
scDb-ABD	scDb fused to the ABD3 of streptococcal protein G
scFv	Single-chain variable fragment
SEC	Size exclusion chromatography
TAA	Tumor-associated antigen
taFv	Tandem scFv
TNF	Tumor necrosis factor
TNF-R	Tumor necrosis factor receptor
V_H	Heavy chain of the variable domain
V_{HH}	Single V-like domain of camelid single domain antibodies
V_L	Light chain of the variable domain
V-NAR	Single V-like domain of shark single domain antibodies

Summary

The progress in protein engineering of the last two decades led to the development of a variety of small recombinant antibody formats for tumor therapy. These small formats show improved properties compared to the conventional monoclonal antibodies which are commonly IgGs. Small recombinant antibodies penetrate solid tumors more homogenously, cause less side effects for lacking the Fc-region and can be produced more economically in bacteria. However, they exhibit a shorter circulation half-life than IgGs. They are rapidly cleared by renal filtration because of their small size and their lack of the Fc-region, which mediates the recycling after cellular uptake by the neonatal Fc-receptor (FcRn). The short circulation half-life of small antibody formats reduces their anti-tumor efficacy and necessitates frequent infusions, leading to high costs and patient inconvenience. The present study aims at the improvement of pharmacokinetics of a bispecific single-chain diabody (scDb) directed against the tumor-associated antigen CEA and the T-cell receptor complex molecule CD3. Therefore, it is able to retarget cytotoxic T-cells to CEA^+ tumor cells. Three basically different strategies are compared in this study to improve the pharmacokinetics of scDbCEACD3: N-glycosylation, PEGylation and fusion to a bacterial albumin-binding domain (ABD). N-glycosylation and PEGylation of the scDb aim at an increased hydrodynamic radius and thereby reduced renal clearance. The fusion of the scDb to ABD has an additional effect: As well as IgG, albumin is recycled by the FcRn. Bound to albumin, scDb-ABD can exploit this mechanism and be recycled after cellular uptake.

For the N-glycosylation three different derivatives with 3, 6 or 9 N-glycosylation sites (sequons) were engineered, resulting in a moderate enlargement of the scDb, which translated in a moderately prolonged serum half-life. In contrast, PEGylation of the scDb with a large, branched PEG 40 kDa led to a ~ 3-fold enlargement of the hydrodynamic radius, resulting in a drastically increase of the circulation time. The longest circulation time with a ~ 14-fold increased area under the curve (AUC) was observed for scDb-ABD. By comparing its half-life in FcRn heavy chain knockout and in wild-type mice, the contribution of the FcRn-mediated recycling on the prolonged serum half-live of scDb-ABD could be confirmed.

Titration of scDb and its derivatives in flow cytometry on CEA^+ or $CD3^+$ cells revealed no or only marginal influence of the modifications on antigen-binding. However, a 3- to 12-fold decrease was observed in their ability to activate T-cells, as measured by IL-2 release. For scDb-ABD, the reduced potential for T-cell activation was especially observed in presence of albumin, implicating that the large scDb-ABD-albumin complex complicates the target cell -

effector cell interaction. A cytotoxicity assay using CEA^+ target cells and preactivated PBMC revealed that the ability of scDb-ABD to mediate cytotoxicity was also reduced especially in presence of albumin.

Finally, biodistribution studies in tumor bearing nude mice showed that the prolonged half-lives of scDb-ABD and the PEGylated scDb translate in improved accumulation in CEA^+ tumors. Although the serum half-lives of both constructs were similar in this mouse model, tumor accumulation of scDb-ABD was ~ 2-fold higher than tumor accumulation of the PEGylated scDb, making the fusion with ABD a promising approach for the improvement of pharmacokinetics of small recombinant bispecific antibodies. Further studies have to investigate how the improved tumor accumulation translates into anti-tumor efficacy in vivo.

Zusammenfassung

Der Fortschritt im Protein-Engineering der letzten zwei Jahrzehnte führte zur Entwicklung einer Vielzahl von kleinen rekombinanten Antikörperkonstrukten für die Tumortherapie. Die herkömmlichen therapeutisch eingesetzten Antikörper sind meist vom Typ IgG. Kleine Antikörperformate haben gegenüber diesen IgGs verbesserte Eigenschaften. Durch ihre geringe Größe verteilen sie sich schneller und gleichmäßiger in soliden Tumoren. Außerdem haben sie keinen Fc-Teil und verursachen daher auch keine Fc-vermittelten Nebenwirkungen. Zudem lassen sie sich bakteriell und damit ökonomischer herstellen. Ein Nachteil der kleinen Antikörperformate ist, dass sie nur eine kurze Zirkulationsdauer im Organismus aufweisen. Durch ihren geringen hydrodynamischen Radius werden sie schnell über die Niere ausgeschieden. Außerdem werden kleine Antikörperformate schneller abgebaut, da ihnen der Fc-Teil fehlt, über den IgGs nach der Endozytose wieder in den Blutstrom recycelt werden. Die kurze Zirkulationsdauer der kleinen Antikörperformate reduziert ihre Wirksamkeit und macht häufige Infusionen nötig. Das führt zu hohen Kosten und schränkt die Lebensqualität der Patienten ein. Die vorliegende Studie hatte das Ziel, die Pharmakokinetik eines bispezifischen single-chain Diabodys (scDb) zu verbessern, der gegen das tumorassoziierte Antigen CEA und das T-Zellrezeptormolekül CD3 gerichtet ist. Mit der Fähigkeit beide Antigene zu binden, ist der scDb in der Lage, zytotoxische T-Zellen zu CEA positiven Tumorzellen zu rekrutieren. Drei unterschiedliche Strategien zur Verbesserung der Pharmakokinetik werden in dieser Studie verglichen: N-Glykosylierung, PEGylierung und die Fusion mit einer bakteriellen albuminbindenden Domäne (ABD). N-Glykosylierung und PEGylierung haben zum Ziel, den hydrodynamischen Radius des scDb zu vergrößern und damit die renale Ausscheidung zu verringern. Die Fusion mit der ABD soll zusätzlich das FcRn vermittelte Recycling des scDb-ABD-Albumin-Komplexes ermöglichen, denn entsprechend den IgGs, wird auch Albumin nach der Endozytose über den neonatalen Fc-Rezeptor (FcRn) recycelt.

Für die N-Glykosylierungsstrategie wurden drei Derivate des scDb mit 3, 6 oder 9 N-Glycosylierungsstellen (Sequons) produziert. Die N-Glycosylierungen führten zu einer mäßigen Vergrößerung des hydrodynamischen Radius. Die Verbesserung der Zirkulationszeit fiel mit einer 2- bis 3-fach vergrößerten $AUC_{0\text{-}24h}$ moderat aus. Im Gegensatz dazu führte die Konjugation des scDb mit PEG 40kDa zu einer ~ 3-fachen Vergrößerung des hydrodynamischen Radius und resultierte in einer drastisch verlängerten Zirkulationsdauer. Die längste Zirkualtionsdauer mit einer 14-fach vergrößerten $AUC_{0\text{-}24h}$ wurde für den scDb-

ABD beobachtet. Durch den Vergleich seiner Halbwertszeit in Wildtyp- und in FcRn-Knockout-Mäusen konnte der Einfluss des FcRn vermittelten Recyclings auf die verlängerte Zirkulationsdauer des scDb-ABD nachgewiesen werden.

Durchflusszytometrische Messungen von titriertem scDb und seiner modifizierten Derivate auf CEA^+ oder $CD3^+$ Zellen zeigten, dass die Antigenbindung des scDbCEACD3 nicht oder nur marginal durch die Modifikationen beeinflusst wurde. Allerdings ergab ein T-Zell-Aktivierungs-Assay, dass das Potential der Derivate T-Zellen zu aktivieren 3- bis 12-fach reduziert war. Im Fall des scDb-ABD zeigte sich diese Reduktion besonders in Anwesenheit von Albumin, was darauf hinweist, dass der große scDb-ABD-Albumin-Komplex sterisch die Effektorzell-Zielzell-Interaktion behindert. Der negative Einfluss der Albuminbindung auf die Bioaktivität des scDb-ABD spiegelte sich auch in Zytotoxizitäts-Assays wider.

Die Analyse der Organverteilung des scDb und seiner Derivate zeigte, dass die verlängerte Zirkulationsdauer des scDb-ABD und des PEGylierten scDb zu einer verstärkten Akkumulation der Antiköperkonstrukte in CEA^+ Tumoren führt. Dabei weist scDb-ABD eine ~ 2-fach höhere Tumorakkumulation als der PEGylierte scDb auf, obwohl die Serum-Halbwertszeiten der beiden Konstrukte in diesem Mausmodell ähnlich sind.

Die vorliegende Studie zeigt, dass die Fusion mit der ABD ein vielversprechender Ansatz ist, um die Pharmakokinetik von kleinen rekombinanten Antikörperkonstrukten zu verbessern. Weitere Studien müssen zeigen, inwiefern die verbesserte Tumorakkumulation die antitumorale Wirksamkeit der modifizierten Antiköper in vivo verbessert.

Introduction

Antibodies in cancer therapy

The search for an efficient treatment of cancer is burdensome for the fact that this illness has many different phenotypes. A classical approach is to eliminate fast proliferating cells with chemotherapeutics. But this treatment lacks of specificity and the tumor often develops drug resistance. Higher specificity is provided by the use of monoclonal antibodies (mAbs).

A century ago Paul Ehrlich described the potential of antibodies to target cancer cells specifically, but it was not possible to produce mAbs in a sufficient quantity until the development of the hypridoma technology in the year 1975 (Kohler and Milstein, 1975). The first mAbs were produced in mice and therefore showed a strong immunogenic profile. To avoid the formation of human anti-mouse antibodies (HAMA) novel antibodies are chimeric, humanised or completely human.

Since the first mAb has been approved for therapeutic use by the FDA in 1986 (Emmons and Hunsicker, 1987) the availability of mAbs for clinical use is constantly growing. After intravenous (i.v.) injection mAbs distribute fast in the blood stream and are therefore ideal candidates to treat haematological malignancies. Rituximab for example is a FDA approved chimeric antibody for the treatment of Non-Hodgkin's Lymphoma which targets the hematopoietic differentiation marker CD20 on B-cells (James and Dubs, 1997).

The treatment of solid tumors is unequally more complicated. It has to be considered that tumors differ from normal tissue in vasculature, interstitial pressure, cell density, tissue structure and composition and in the composition of the extracellular matrix (ECM) (Jang et al., 2003). Blood vessels in tumors differ from normal vasculature in size, permeability, distribution and enhanced tortuosity leading to a slower blood flow (Tannock and Steel, 1969; Heuser and Miller, 1986; Jain, 1990). Large particles as tumor cells can enter lymphatic capillaries in tumors and impair lymphatic drainages (Jang et al., 2003). That leads, together with an enhanced leakiness of the microvasculature, to a prolonged retention of molecules > 45 kDa. The phenomenon is known as "enhanced permeability and retention effect" (EPR) (Maeda et al., 2000). But for the further penetration of the tumor tissue beyond the vasculature, a large size is adverse. The ECM in tumor tissues is often richer of collagen, which leads to an increased resistance to macromolecular transport (Netti et al., 2000). An important factor for the speed of distribution is the molecular weight (Nugent and Jain, 1984; Clauss and Jain, 1990; Pluen et al., 2001). For example it could be shown that a bivalent anti-

EGFR antibody construct with a molecular weight of ~ 50 kDa exhibited a more homogeneous distribution in a solid tumor than an anti-EGFR-IgG (~ 150 kDa). The total accumulation of antibody in the tumor was similar for both antibody formats, but the IgG remained in the outer layer of the tumor tissue near the vasculature (Tijink et al., 2008). Several attempts have been made to improve tumor penetration properties of antibodies. One possibility is to inject the antibody directly into the tumor (Yokouchi et al., 2008). Another attempt is to make the vasculature leakier by the use of apoptosis-inducing agents (Au et al., 2001). But the most promising approach seems to be the use of small antibody formats.

A simple approach to reduce the size of an IgG is the digest with the protease papain, leading to an antigen-binding fragment (Fab) with a MW of ~50 kDa (Fig. 1). Moreover, the progress in protein engineering of the last two decades provided the opportunity to develop various different small recombinant antibody formats. The smallest antigen-binding unit of a human IgG is the variable fragment (Fv), a combination of the variable domain of the heavy (V_H) and the light chain (V_L). Each of these fragments is stabilized by a disulfide bond. For the generation of small recombinant antibody formats, V_H and V_L are fused by a linker resulting in a single-chain Fv (scFv, ~ 28 kDa). If the linker between V_H and V_L is long, a mixture of

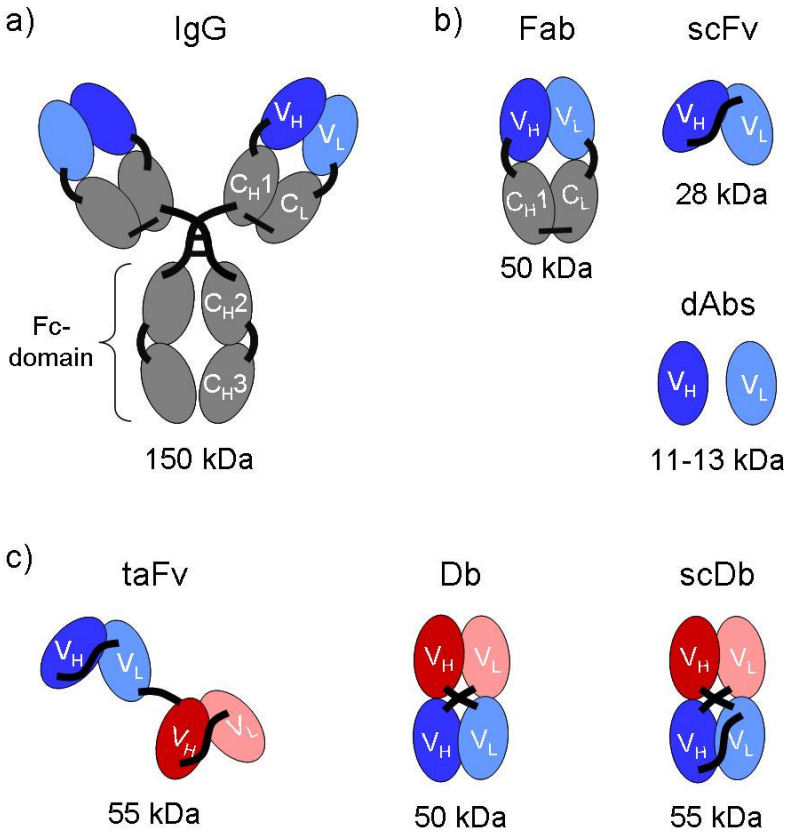

Figure 1. Antibody formats derived from immunoglobulins. A) IgG. B) Monospecific formats: Fab, scFv and human domain antibodies (dAbs). C) Potentially bispecific formats: Tandem scFv (taFv), Diabody (Db) and single-chain Diabody (scDb).

monomeric and dimeric scFvs will be formed. The reduction of the linker below 12-8 amino acids leads to the formation of dimers – so called Diabodies (Db) (Holliger et al., 1993; Kortt et al., 1994; Alfthan et al., 1995). The search for even smaller antigen-binding scaffolds led to the development of single domain antibodies derived from single variable domains (V_L, V_H). The original human domain antibodies (dAbs) exhibit a poor solubility because of their hydrophobic interaction site for the corresponding variable domain. But this problem could be solved by directed mutations in the interface region or by phage display (Colby et al., 2004; Dottorini et al., 2004; Jespers et al., 2004). Single V-like domain antibodies are also derived from heavy chain antibodies of camels or sharks (V_{HH}, V-NAR). The small size and an extended CDR3 of shark and camelid single domain antibodies enable the recognition of difficult accessible epitopes, for example antigens in narrow cavities of the surface of pathogenic viruses (Holliger and Hudson, 2005).

A growing number of publications deals with alternative scaffolds of so-called antibody mimetics (Nygren and Skerra, 2004; Binz and Pluckthun, 2005; Hey et al., 2005). An example for an antibody mimetic in development is the affibody format, a 58 amino acid protein domain derived from one of the IgG binding domains of streptococcal protein A (Tolmachev et al., 2007b). Affibodies, which can be selected with high affinities, are very stable and have a rapid blood clearance in vivo. Therefore, they are promising candidates for cancer imaging (Orlova et al., 2007; Tolmachev et al., 2007a). A disadvantage of the affibody format is its bacterial origin that has a high potential for immunogenicity. Other groups are working with scaffolds derived from fibronectin (Parker et al., 2005), lipocalin (Schlehuber and Skerra, 2005) or ankyrin (Zahnd et al., 2006), which have potentially a lower immunogenicity due to their human origin.

Despite the diversity of antibody mimetics, the immunoglobulins and their derivatives are presently the biggest and the best developed family of binding molecules.

Retargeting of effector cells by bispecific antibodies

Monoclonal antibodies (mAb) mediate complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC) by their Fc-region. For ADCC the Fc-region of IgG molecules can recruit effector cells by binding to FcγRI (CD64) and FcγRIII (CD16). FcγRIII is expressed constitutively on the surface of NK cells. Monocytes and macrophages bear FcγRI and in neutrophiles the expression of FcγRI can be induced by interferon-γ and granulocyte colony-stimulating factor (G-CSF) (van Spriel et al., 2000).

However, normal CDC and ADCC mediated by a therapeutic mAb is often not sufficient to induce a satisfactory effect (Booy et al., 2006). Some efforts have been done to improve ADCC by engineering the Fc-region (Carter, 2006), however, the recruitment of effector cells remains restricted to Fc-receptor expressing cells. The most effective effector cells of the immune system are cytotoxic T-cells. They are very powerful in the elimination of virus-infected cells by releasing granules filled with pore-forming perforin and cell death-inducing granzymes. Cytotoxic T-cells need only to recognize three peptides on a cell to identify it as target cell and to induce its elimination. The formation of a stable immunologic synapse is not necessary (Purbhoo et al., 2004). But T-cells can only identify a target cell if it presents target-specific peptides on the major histocompatibility complex I (MHC I). To receive an effective T-cell response, T-cells, which express the receptor for the MHC I/peptide complex, have to be costimulated by peptide-bearing dentritic cells. This activation leads to the clonal expansion of the specific T-cells. The complexity of the T-cell mediated cytotoxicity facilitates tumor cells to develop immune evasion mechanisms that limit T-cell response to cancer cells (Rabinovich et al., 2007). The aim of many bispecific antibodies is to retarget these powerful cytotoxic T-cells to tumor cells. With one antigen-binding site they bind to a tumor-associated antigen (TAA) and with the other site they bind to CD3, a molecule of the T-cell receptor complex. The direct cross-linking has two major advantages: First, T-cell recruitment is not restricted to the subpopulation of T-cells that express the T-cell receptor recognizing the MHC I/peptide complex. Second, T-cell targeting is independent of MHC I which is often down regulated in tumor cells. It was shown, that a bispecific antibody directed against Ep-CAM and CD3 is able to induce T-cell mediated cytotoxicity of MHC I-negative, Ep-CAM transfected cells (Offner et al., 2006). Beside T-cell recruitment via CD3, it is possible to retarget T-cells by using one antibody binding site for the costimulatory receptor CD28. A bispecific antibody against melanoma-associated proteoglycan and CD28 clearly inhibited the growth of antigen bearing cells in mice (Grosse-Hovest et al., 2005).

The first bispecific antibody was produced in 1985 by the fusion of two hybridoma cell lines, leading to a hybrid-hybridoma cell line (Staerz et al., 1985). This cell line produced bispecific antibodies for T-cell retargeting in the IgG format. Clinical studies using whole IgGs mostly provided disappointing results because the Fc-mediated side effects such as cytokine-release syndrome or thrombocytopenia limited the maximal applicable dose (Segal et al., 1999; van Spriel et al., 2000). Furthermore, the hybrid-hybridoma cell line produced a large quantity of homodimers and inactive heterodimers which had to be extensively removed before usage. Not least, the antibodies were murine and thereby highly immunogenic. The ideal clinically

useful bispecific antibody should be non-immunogenic, it should not have a Fc-region for avoiding side effects, it should be small for an efficient tissue penetration, it should have a long circulation time and must be monovalent for effector cells to avoid systemic effector cell activation (Segal et al., 1999).

A large variety of bispecific formats have been developed and characterized in vitro, in animal models and in clinical trails (Baeuerle et al., 2003; Kontermann, 2005). By the use of human scaffolds the generation of human anti-mouse antibodies could be avoided or at least reduced (Ritter et al., 2001). Common recombinant bispecific antibody formats are tandem scFvs (taFv) and Diabodies, which lacks the Fc-region and can be produced in bacteria (Kontermann, 2005). The use of bispecific taFvs circumvents the problem of homodimer formation because they are expressed in one single chain. The company Micromet is developing bispecific taFvs with one binding site directed against CD3, so-called bispecific T-cell engager (BiTE). For BiTEs it was shown that the mediated T-cell activity is strictly target cell-dependent as demonstrated in mice (Brischwein et al., 2007) and in non-human primates (Baeuerle et al., 2009). A recently published study describes the mode of action of such a BiTE directed against the epithelial cell adhesion molecule (Ep-CAM) (Haas et al., 2009). It was shown that it is able to convert the potential of $CD4^+$ and $CD8^+$ T-cells for serial lysis from basal into a highly accelerated state within several hours. Therefore, the presence of costimulatory molecules is not necessary even if the use of anti-CD28 was able to further increase the potential of the BiTE. The killing of target cells is predominantly mediated by pore forming and pro-apoptotic components released by the granules of the T-cells. Presently, this antibody is in phase I clinical trail with patients with lung or gastrointestinal cancer. The most advanced BiTE Blinatumomab targets the B-cell marker CD19 and is currently undergoing a phase II clinical trail with patients suffering from acute B-cell lymphoblastic leukemia who have shown minimal residual disease after conventional treatment (Micromet, 2008). In another indication for Non-Hodgkin Lymphoma (NHL) treatment of Blinatumomab led to partial and complete tumor regression (Bargou et al., 2008). Presently Rituximab, an IgG directed against CD20, is the leading antibody for the treatment of NHL. For the fact that Rituximab and Blinatumomab bind to different target molecules, a combination of both antibodies is thinkable. First in vitro results showed, that the combination greatly enhanced the activity of Rituximab, in particular at low effector to target cell ratios (d'Argouges et al., 2009).

Bispecific diabodies consist of two different non-covalently associated chains. V_H of one chain forms an active antigen binding-site with V_L of the other chain. It could be shown that

an anti-lysozyme/anti-C1q diabody was able to mediate lysis of lysozyme-coated erythrocytes by recruiting complement (Kontermann et al., 1997b). A shortcoming of the bispecific diabody format is the fractional formation of inactive homodimers which can be drastically reduced by the knob-in-hole method. In case of a diabody directed against HER2 and CD3 a knob was introduced in the anti-HER2-V_H by mutating Val37 to Phe and Leu45 to Trp. The corresponding hole in anti-HER2-V_L was created by the mutation of Phe98 to Met and Tyr87 to Ala. With this change in the V_L-V_H-interface the yield of heterodimers was > 90% (Zhu et al., 1997).

For total prevention of homodimer formation the two chains of a diabody can be connected by a linker (Brüsselbach et al., 1999). These single-chain diabodies (scDb) can be expressed in bacteria in a soluble and active form (Kipriyanov et al., 1999; Völkel et al., 2001; Korn et al., 2004a). A scDb directed against endoglin and the adenovirus fiber knop domain was able to redirect the adenovirus infection to endoglin-positive HUVEC cells (Nettelbeck et al., 2001). The ability to retarget effector cells to target cells could be demonstrated for an anti-endoglin/anti-CD3 scDb. This scDb mediated the killing of endoglin expressing cells by cytotoxic T-cells in the picomolar range (Korn et al., 2004a). In comparison with scDbs, taFvs are often poorly soluble and hence, have to be expressed in eukaryotic cells or have to be refolded (McCall et al., 1999; Kipriyanov, 2003; Grosse-Hovest et al., 2004; Korn et al., 2004b). Furthermore, a direct comparison between a bispecific scDb and its corresponding taFv revealed a higher plasma stability for the scDb format (Korn et al., 2004b).

Meanwhile many animal studies with small recombinant bispecific antibodies are published, in which even curative effects have been demonstrated (De Jonge et al., 1998; Cochlovius et al., 2000a; Cochlovius et al., 2000b; Blanco et al., 2003; Dreier et al., 2003; Ren-Heidenreich et al., 2004).

Improvement of the circulation half-life of small recombinant antibodies

The number and variety of small recombinant antibody formats was constantly rising in the past decade (Holliger and Hudson, 2005; Kim et al., 2005; Kontermann, 2005; Kontermann, 2009a). But small antibodies are rapidly cleared from circulation (Weir et al., 2002; Müller et al., 2007). This can be a benefit for the use in imaging applications but decreases therapeutic efficacy of these molecules. For an efficient cancer treatment with small antibodies patients have to receive infusions in frequent dosing schedules, resulting in high costs and patient

inconvenience. The reason for the short circulation half-life is mainly renal clearance and degradation (Tang et al., 2004). Molecules with a size beneath the renal threshold of about 60 kDa are rapidly filtered out by the kidney. But there is no strict size – half-life correlation. Human IgG1, IgG2 and IgG4 exhibit a half-life of 12-21 d whereas murine IgGs show a much shorter half-life of 2-3 days in humans (Lobo et al., 2004) even if they possess the same size and structure as human IgGs. The reason for this phenomenon is that, when IgGs are pinocytosed by endothelial cells, they are recycled by the neonatal Fc-receptor (FcRn) and thereby rescued from degradation in the lysosome (Chaudhury et al., 2003). According to this, proteins which do not or only weakly bind to the human FcRn such as IgA or murine IgGs, are degraded clearly faster (Chaudhury et al., 2003; Lobo et al., 2004).

In principal there are two strategies to improve the half-life of small antibody molecules: a) enlargement of the molecule above the renal threshold and b) mediation of recycling by the FcRn.

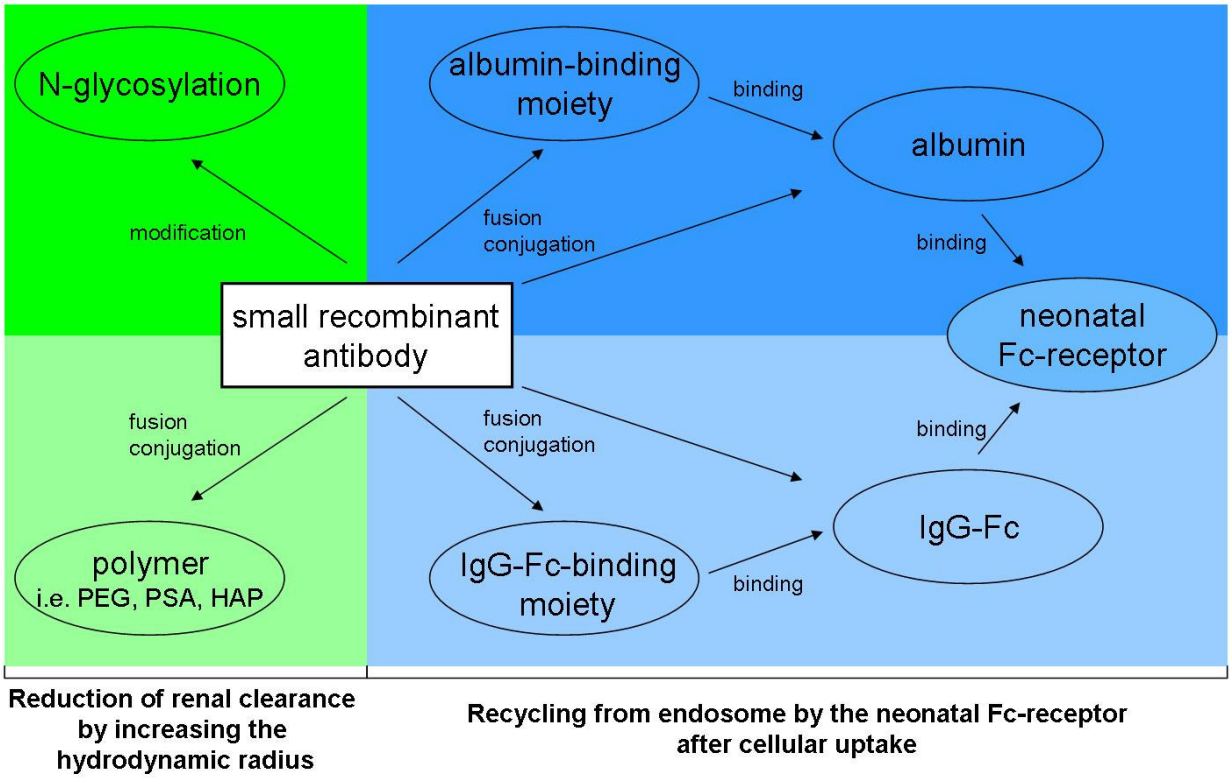

Figure 2. Illustration of different strategies to improve the circulation half-life of small recombinant antibodies. Basically, two different approaches are possible: a) enlargement of the size over the threshold for renal clearance (left) or b) mediation of recycling by the neonatal Fc-receptor via covalent or non-covalent attachment to IgG-Fc or albumin.

Several possibilities are known to increase the size of proteins such as conjugation with polyethylene glycol (PEG), polysialic acid (PSA), hydroxyethyl starch, the insertion of N-glycosylation sites or the fusion to homo-amino-acid polymers (HAP) (Kontermann, 2009b).
For the exploitation of the FcRn-mediated recycling mechanism the protein can be fused or non-covalently bound to the Fc-region of IgG1 or to serum albumin, which binds to the FcRn, too (Fig. 2). Following, the mentioned strategies are described in detail.

PEGylation

Polyethylene glyocol (PEG) is a polymer consisting of ethylene oxide monomers. Polymers with a molecular mass > 100 kDa are usually called polyethylene oxide (PEO) because in these molecules the influence of the terminal hydroxyl group is marginal. PEG chains are very flexible and their dipole character enables every monomer to coordinate 2-3 water molecules resulting in a large hydrodynamic volume. For the coupling to drug molecules, there are different functionalized PEGs available such as amine-reactive PEGs (e.g. succinimidyl esters and p-nitro-phenol carbonates), thiol-reactive PEGs (e.g. maleimide, vinyl sulphone, iodoacetamide), aldehyde-reactive and alcohol-reactive PEGs (Chapman, 2002). Commercial available functionalized PEGs are linear or branched and range in size from 1 kDa up to 50 kDa. An inert methyl-group is often attached to the end of the PEG chain (mPEG), in opposite to the functional group.
The conjugation of drugs with PEG leads to a drastic enlargement of the hydrodynamic diameter, clearly above the threshold for renal clearance, resulting in a prolonged circulation half-life ranging from 4-fold for PEGylated G-CSF to 960-fold for PEGylated IL-6 (Fishburn, 2008). Although, PEGylation influences polarity, structure and surface properties, solubility, stability, immunogenicity, cellular uptake, elimination and tissue localization of the PEGylated drug. PEG is approved by the FDA for clinical use and considered to be safe (Webster et al., 2007). Presently, PEGylation is the most common way to improve the circulation time of protein drugs. Pegasys, interferon alpha-2a conjugated with a branched PEG 40 kDa, is approved by the FDA for the treatment of hepatitis C since 2002 (Iafolla, 2002). Due to the prolonged circulation half-life compared to the non-PEGylated interferon, the dosing schedule could be reduced from thrice- to once-weekly (Rajender Reddy et al., 2002).
Pegfilgrastim is a PEGylated granulocyte colony-stimulating factor (G-CSF), which is also in clinical use. In patients receiving chemotherapy this agent reduces the duration of severe neutropenia and facilitates on-time delivery of scheduled dose of chemotherapy. Compared to

the non-PEGylated G-CSF, which has to be administered daily, Pegfilgrastim has to be used only once per cycle (Gabrilove, 2006). Certulizumab pegol is a TNF-neutralizing Fab conjugated to PEG 40 kDa which is approved for the treatment of Crohn´s disease. Currently it is in development for rheumatoid arthritis and psoriasis (Melmed et al., 2008).

Initially, non-directed PEGylation was performed with short PEG chains and often more than one PEG molecule was coupled to the protein of interest. That frequently led to sterical hindrance for the interaction between the PEGylated drug and the target molecule, resulting in a dramatic loss in bioactivity (Chapman, 2002). Nowadays site-specific PEGylation with larger PEGs in an 1:1 stoichiometry is common, mainly through coupling to a free cysteine, which is often inserted by mutagenesis. By carefully choosing the PEGylation site in the protein, the loss in bioactivity can be decreased and the efficiency of the PEGylation reaction can be optimized (Xiong et al., 2006). But even with a loss in bioactivity, the impressive increase in circulation half-life and stability often leads to a superiority of the PEGylated drug. Site-directed PEGylation with PEG 20 kDa of a scFv directed against HER2 led to a 5-fold loss in affinity. Nevertheless, the PEGylated scFv showed a 8.5-fold improved tumor accumulation compared to the native scFv (Kubetzko et al., 2006). Yang et al. PEGylated an anti-TNF scFv site-specific with PEG 5, 20 and 40 kDa at different positions in the C-terminus or in the linker. Even if the PEGylated scFv lost up to 2 orders of magnitude in affinity as measured by surface plasmon resonance, it had a similar ability to neutralize TNF-alpha in a cellular cytotoxicity assay. The circulation half-lives of these constructs were prolonged in the range of 16- to 144-fold. Therefore, this study demonstrates that PEGylation is a possible strategy for tailoring a favored half-life (Yang et al., 2003). A disadvantage of the PEGylation strategy is that PEG is not biodegradable and therefore accumulates in the body. If high doses of PEGylated drugs are administered frequently, PEG accumulation leads to risks such as renal tubular vacuolation (Bendele et al., 1998). Thus, the generation of anti-PEG antibodies was observed after multiple injections of PEGylated drugs (Cheng et al., 1999; Sroda et al., 2005). Moreover, the production of high-quality PEGylated proteins is expensive and requires additional processing and separation steps (Gaberc-Porekar et al., 2008).

N-glycosylation

All IgGs are N-glycosylated in the C_H2 domain (Asn 297). This N-glycosylation influences the stability and the pharmacokinetic properties of an antibody (Jefferis, 2005). Furthermore,

the glycosylation has a strong impact on the antibodies effector function (Wright and Morrison, 1997). An antibody which was mutated at position 297 and accordingly is aglycosylated lost its ability to bind to the Fcγ-receptor I and to activate the complement system (Tao and Morrison, 1989). The carbohydrate composition plays an important role for the effector function, too. The Fc-associated N-glycans are of the diantenarry complex type and contain one fucose residue. It could be shown that a humanized anti-HER2 IgG1, which lacks fucose in its Fc-associated N-glycan, exhibits an improved ADCC by enhanced binding to Fcγ-receptor III (Shields et al., 2002). Beside the Fc-associated N-glycosylation 20-25 % of the immunglobulins bear an additional N-glycosylation site in the variable domains. It has been reported that these glycosylations have an effect on the pathophysiology of the antibodies (Wright and Morrison, 1993).

Artificial N-glycosylation sites can be created by inserting the sequon Gln-X-Thr/Ser in which X stands for a random amino acid except Pro. The efficiency of the glycosylation is depending on the position of the sequon in the protein, the expressing cell line and its physiological status.

A famous example for the beneficial effect of N-glycosylation on the half-life of protein drugs is erythropoietin which supports the generation of erythrocytes. By inserting 2 additional N-glycosylation sites (5 in total) the half-life of erythropoietin in rats could be prolonged 2- to 3-fold (Egrie and Browne, 2001). The hyperglycosylated erythropoietin, called darbepoetin-α, was approved by the FDA in 2001 for the treatment of patients suffering from anaemia caused by chronic renal failure (Macdougall, 2002).

Furthermore, the half-life of the follicle-stimulating hormone (FSH) could be prolonged by the insertion of additional N-glycosylation sites. Exogenous administered FSH is used in reproduction protocols to stimulate the development and maturation of oocytes. The half-life of FSH in rats could be prolonged from 3.9 h to 7.9 h after i.v. injection by the introduction of two N-glycosylation sites (Weenen et al., 2004).

A contrary effect of N-glycosylation on the circulation half-life was observed for a scFv directed against a melanoma associated proteoglycan that was N-glycosylated at various positions in the linker or at the C-terminus. The N-glycosylated scFvs were produced in Pichia pastoris, leading to N-glycans of the high-mannose type. Half-life of the modified scFvs was reduced ~2-fold. Likely they were eliminated by the mannose-receptor bearing cells as e.g. macrophages (Wang et al., 1998). It was also demonstrated by other groups that high mannose glycoforms are fast eliminated from circulation (Wright and Morrison, 1994; Wright et al., 2000).

To receive an improvement in circulation half-life by N-glycosylation, the glycan pattern has to be similar to that of mammalian cells. Furthermore, it could be observed that the degree of terminal sialic acid residues plays an important role (Cohen et al., 2004). Molecules displaying multiple terminal N-acetylglucosamine residues are more rapidly cleared by the asialoglycoprotein receptor (Meier et al., 1995; Stockert, 1995; Jones et al., 2007). To avoid this mechanism some groups engineered cell lines for the production of glycoproteins with maximal sialic acid content e.g. by high-level expression of α-2,6-sialyltransferase resulting in N-glycans which are more than 90 % sialylated (Weikert et al., 1999; Bragonzi et al., 2000).

Exploitation of the Fc-mediated recycling by the neonatal Fc-receptor

As mentioned above IgG1 antibodies have a long circulation half-life of approximately 23 d in human. This is caused by the pH-dependent recycling of the antibody via the Fc-region by the neonatal Fc-receptor (FcRn). The FcRn consists of the MHC class I-related heavy chain non-covalently associated with β-2-microglobulin. In addition to protect IgGs from degradation, FcRn is also involved in transplacental transport of maternal IgGs to the fetus (Roopenian and Akilesh, 2007). The Fc-region of IgGs can bind two FcRn simultaneously (West and Bjorkman, 2000). In surface plasmon resonance measurements at pH 6.0 an affinity of ~2 µM could be found for the monovalent binding of FcRn to IgG1 whereas no binding occurs at neutral pH (Firan et al., 2001; Dall'Acqua et al., 2006).

The interaction site between the IgG-Fc and the FcRn has been identified by crystallography and mutational analysis (Vaughn et al., 1997; Martin et al., 2001). It could be demonstrated that the histidines located at the junction between C_H2 and C_H3 contribute to the pH-dependent affinity transition of the binding (Raghavan et al., 1995).

The influence of FcRn-mediated recycling on the half-lives of IgGs could be demonstrated by means of FcRn heavy chain knockout mice. The half-life of an IgG in knockout mice was 5-fold shorter (19 h) as its half-life in wild-type mice (95 h) (Chaudhury et al., 2003).

Kenanove et al. mutated the Fc-region of an IgG1 directed against a TAA, resulting in a loss of affinity for the FcRn. This led to a drastic reduction in half-life from 12 d to 8 h (Kenanova et al., 2005). Reciprocally, the mutational improvement of the affinity of an IgG for the FcRn at pH 6.0 led to an 2-fold prolonged circulation half-life without affecting CDC or ADCC (Hinton et al., 2004; Hinton et al., 2006). Thereby it is important to retain the pH-dependency of the binding. An improved affinity of the Fc-FcRn binding at neutral pH resulted in a reduced half-life of the antibody (Dall'Acqua et al., 2002). Beside the undisputable influence

of the FcRn binding for the half-life, it must be remarked that there are other parameters that have also a notable impact on pharmacokinetics. Studies analyzing IgGs with varying affinities for the FcRn showed that biophysical properties as antigen affinity, glycosylation, proteolytic stability and antigen type contribute also to the elimination rate (Gurbaxani et al., 2006; Datta-Mannan et al., 2007).

The positive pharmacokinetic properties of IgGs have been exploited by fusing the Fc-region to protein drugs. For example, this strategy has been applied to extracellular receptor regions (TNF-R2, etanercept; Enbrel; CTLA-4, abatacept, Orenica), growth factors (EPO, FSH, EGF), cytokines (IL-4, IL-15) coagulation factors (FVI, FIX) and also to recombinant antibody fragments such as scFv, domain antibodies and antibody mimetics (Jazayeri and Carroll, 2008). In an intact IgG, the two heavy chains are dimerized in the Fc-region, therefore the Fc-fusion leads to dimerization of the fused protein. For example, an scFv-Fc dimerizes to a molecular weight (MW) of 105-110 kDa and a single-domain-Fc fusion protein to a MW of 80-85 kDa. Due to their bivalency these constructs have a similar avidity as IgGs but show better tumor penetration because of their smaller size (Colcher et al., 1998; Holliger and Hudson, 2005).

Instead of the direct Fc-fusion, it is also possible to fuse the protein of interest to an antibody fragment binding to plasma IgGs. The circulation half-life of a bispecific diabody could be prolonged 5-fold by directing one binding site against IgG (Holliger et al., 1997).

Exploitation of HSA recycling by the neonatal Fc-receptor

As IgG, albumin is recycled by the neonatal Fc-receptor. With a K_D of ~ 5 µM at pH 6.0 human serum albumin (HSA) exhibits a similar affinity for the FcRn as IgG1 that is also pH-dependent (Andersen et al., 2006; Chaudhury et al., 2006). But compared to IgGs, HSA binds to the FcRn in a 1:1 stoichiometry (Chaudhury et al., 2006). IgGs instead bind two FcRn molecules and therefore, binding is increased. HSA consists of 585 amino acids and has a molecular weight of ~ 67 kD. The non-glycosylated protein is structurally divided into three domains, each consisting of two subdomains. With 35-50 g/l it is the most abundant plasma protein. Its physiological function is to maintain the osmotic pressure, to buffer the vascular system and the transport of small molecules such as ions, hormones, fatty acids or bilirubin. The influence of the FcRn-mediated recycling on its half-life could be demonstrated in FcRn heavy chain knockout mice. Here, albumin showed a clearly reduced half-life (24 h) compared to its half-life in wild-type mice (39 h) (Chaudhury et al., 2003). By mutational

analysis it was found that histidine 166 of the FcRn is involved in the albumin-FcRn-binding (Andersen et al., 2006), indicating that the FcRn binding site for albumin is distinct from the binding site for IgGs. This was confirmed by surface plasmon resonance experiments with immobilized FcRn. In these experiments the mixture of albumin and IgG resulted in a higher signal as a saturating concentration of IgG alone, proving that the binding sites for both species are different (Chaudhury et al., 2006). The binding site in albumin is located in domain III which contains several conserved histidines. His464 undergoes a pH-dependent protonation between pH 5.0 and pH 7.0 and may play a major role in the pH dependency of the FcRn-albumin binding (Chaudhury et al., 2006).

The direct fusion of albumin has successfully been applied to agents including interferons, interleukins, insulin, human growth hormone and antibody fragments (Smith et al., 2001; Chuang et al., 2002; Müller et al., 2007). For example, the fusion of albumin to interleukin-2 (albuleukin) led to a prolongation of the plasma half-life to 6-8 h compared to 19-57 min for the unmodified recombinant interleukin-2. In a mouse tumor model albuleukin treatment resulted in an improved survival rate compared to the controls (Melder et al., 2005).

Furthermore, HSA has been fused to a taFv and a scDb (Müller et al., 2007). These molecules were created for the retargeting of cytotoxic T-cells and were directed against CD3 and CEA, a tumor-associated antigen. The fusion to HSA led to a 4- to 5-fold prolongation of the circulation half-life. In their ability to activate T-cells, scDb-ABD was superior over the taFv-ABD and scFv-ABD-scFv formats but less active than the native scDb.

Even if some of the albumin fusion proteins exhibit a reduced bioactivity as for example in the cases of albuferon and albuleukin, the greatly improved half-life leads often to an improved therapeutic efficacy (Sung et al., 2003; Melder et al., 2005).

Instead of directly fusing the protein of interest to albumin several methods have been developed for a non-covalent association. A bispecific $F(ab)_2$ directed against TNF and rat serum albumin revealed a half-life of 43 h in rats compared to 16 h for the control $F(ab)_2$ (Smith et al., 2001). As an alternative for using an anti-albumin antibody fragment it is also possible to use an albumin-binding domain. The best analyzed albumin-binding domain is ABD3 derived from protein G of Streptococcus strain G148. The crystallographic structure (Kraulis et al., 1996) showed a left handed 3-helix bundle consisting of 46 amino acids. Surface plasmon resonance experiments and competition assays revealed an affinity of 1-4 nM for HSA (Johansson et al., 2002; Linhult et al., 2002; Jonsson et al., 2008). The fusion of ABD to an anti-HER2 Fab led to a ~10-fold increased half-life (Schlapschy et al., 2007). Furthermore, ABD was fused to an anti-HER2 affibody. Affibodies are small (~7 kDa)

antibody mimetics derived from the Z-domain of streptococcal protein A with very short half-lives. In a radiotherapy approach in mice the ABD-fused affibody was able to completely prevent tumor formation (Tolmachev et al., 2007b).

Due to its bacterial origin the ABD is potentially immunogenic (Libon et al., 1999). However, it was reported for one case that the fusion with ABD is able to reduce the immunogenicity of the fusion partner (Kontermann, 2009b). The affinity of ABD to albumin could be increased to a range of 50-500 fM (Jonsson et al., 2008). It could be shown that the affinity of an albumin-binding moiety is positively correlated with its half-life. Nguyen et al. produced short albumin-binding peptides with affinities between 36 and 110 nM by phage display (Nguyen et al., 2006). These peptides were fused to an anti-HER2 Fab leading to increased half-lives in the range between 12 and 69 h compared to 6 h for the unmodified Fab. The group found out that the higher the affinity of the peptide for albumin the longer is the half-life of the fusion protein. These findings were confirmed in a similar approach with selected albumin-binding human domain antibodies derived from V_H and V_L by phage display from a synthetic library (Holt et al., 2003). One of these antibodies with an affinity of 1 µM for rat serum albumin (RSA) showed a half-life of 43 h in rat whereas another one with an affinity of 13 nM reached the half-life of RSA with 53 h. The fusion of an anti-albumin human domain antibody to IL-1 receptor antagonist, which is approved for the treatment of rheumatoid arthritis, led to an improved half-life of 4.3 h compared to 2 min for the unmodified IL-1 receptor antagonist. Furthermore, it could be demonstrated, that the prolonged half-life translates in an improved efficacy in a collagen-induced arthritis mouse model (Holt et al., 2008).

The enormous work that has been done to develop the described diversity of the different approaches emphasizes the importance of finding strategies for the prolongation of the circulation half-lives of protein based drugs. Prolonged half-lives lead to less frequent dosing schedules and a smaller amount of drug is needed, resulting in saving costs and more comfort for patients.

Aim of this study

Many studies have been published in the last years that deal with the improvement of pharmacokinetics of small protein-based drugs. One of the biggest challenges in this field is to improve the pharmacokinetics of small antibody formats for the treatment of solid tumors. On the one hand, these molecules should be large enough to prevent renal clearance but on

the other hand, they should be small enough to exhibit a preferably fast and homogeneous tumor penetration. Several studies have been performed to investigate different approaches to overcome this challenge, but these studies have been done with different antibody formats, different affinities, different antigens and in different tumor models. Therefore, a direct comparison between the different approaches is difficult. The present study aims to compare directly three basically different strategies to improve the pharmacokinetics of one certain antibody. In our group, expertise with the scDb and the taFv format is available. The scDb format was chosen for the present study, because it showed a superior solubility and a better stability in human plasma than the taFv format (McCall et al., 1999; Kipriyanov, 2003; Grosse-Hovest et al., 2004; Korn et al., 2004b). Furthermore, previous studies of our group compared directly anti-CEA/anti-CD3 scDb and taFv (Tiegs, 2005; Müller et al., 2007). This comparison revealed, that T-cell activation by scDbCEACD3 was strictly target cell-specific whereas a loss in target cell specificity was observed for taFvCEACD3. Even after the fusion with HSA, scDbCEACD3-HSA was still able to activate T-cells. These positive properties of the scDbCEACD3 led to the decision to use this antibody for the present study (the scDb format is shown in Fig. 3). Moreover soluble CEA, CEA expressing cell-lines and a tumor mouse model were available.

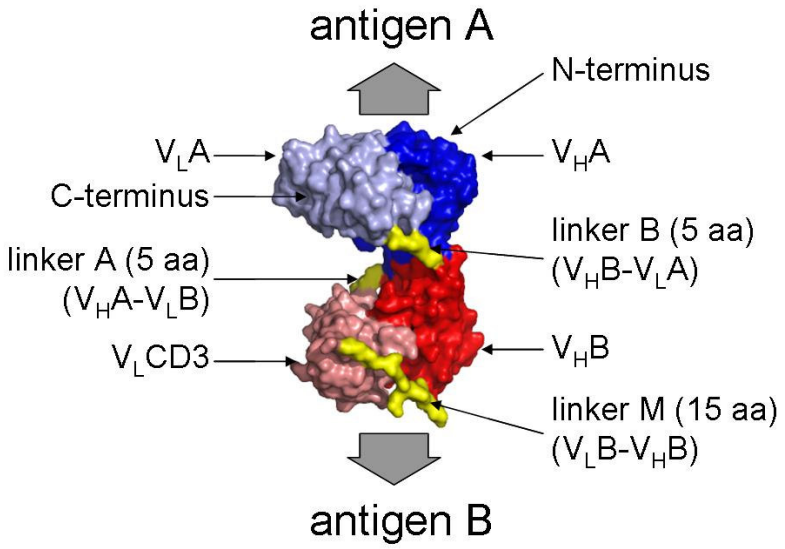

Figure. 3. Model structure of a scDb using the structure of a bivalent diabody (PDB entry 1LMK, (Perisic et al., 1994)). In case of scDbCEACD3, antigen A corresponds to CEA and antigen B to CD3. Visualized with PyMOL Molecular Graphic System (DeLano Scientific, San Carlos, CA, USA).

The carcinoembryonic antigen (CEA) is a 180 kDa GPI-linked glycoprotein which is expressed on the fetal gut or on the luminal surface of the adult colon. During colorectal

oncogenesis CEA is overexpressed and loses its polarity, thus becomes accessible for circulating antibodies. High CEA expression levels were also observed on epithelial, lung, ovarian, breast and thyroid tumors (Hammarstrom, 1999). For its high tumor specificity, CEA has been used successfully as target antigen in several imaging and therapeutic approaches (Alt et al., 1999; Wegener et al., 2000; Korn et al., 2004b; Sharma et al., 2005; Kraeber-Bodere et al., 2006; Cai et al., 2007; Müller et al., 2007). With its two different binding sites scDbCEACD3 is able to retarget cytotoxic T-cells to CEA^+ tumor cells. The ability of bispecific diabodies for T-cell retargeting was shown before for different targets such as CEA and CD19 (Holliger et al., 1999; Cochlovius et al., 2000a; Blanco et al., 2003; Dreier et al., 2003). V_HCEA and V_LCEA of scDbCEACD3 are derived from anti-CEA scFv MFE-23. Chester et al. selected scFv MFE-23 from a murine phage library and determined a dissociation constant (K_D) of 2.5 nM for its affinity for CEA (Chester et al., 1994). Since then scFv MFE-23 was used in various different applications as e.g. adenoviral targeting (Korn et al., 2004b), antibody-directed enzyme prodrug therapy (Brüsselbach et al., 1999), enzyme immunoassays (Kontermann et al., 1997a) and radioimaging (Verhaar et al., 1996). The anti-CD3 binding-site of scDbCEACD3 is derived from variant 9 of humanized UCHT-1 (Zhu and Carter, 1995). This anti-CD3 binding site binds specific human but not murine CD3.

First studies have been made by our group to improve the half-life of scDbCEACD3 (Tiegs, 2005; Müller et al., 2007). It could be shown that fusion of scDbCEACD3 with HSA led to a drastic increase of the AUC from 61 ± 4 to 433 ± 163 h*%. In the present study the influence of N-glycosylation, PEGylation and fusion with an albumin-binding domain (ABD) on the pharmacokinetics and bioactivity of scDbCEACD3 is analysed (Fig. 4). For the N-glycosylation three different variants of scDbCEACD3 with 3, 6 or 9 N-glycosylation sites scDb-ABC_1, scDb-ABC_4, scDb-ABC_7) were produced. The second strategy was a site-directed conjugation of the scDb with a branched mPEG 40 kDa at the V_HCEA-V_LCD3-linker A (scDb-A'-PEG_{40kDa}) or at the C-terminus (scDb-C'-PEG_{40kDa}). Both strategies aim to decrease the renal clearance of the scDb by increasing its size. The third strategy is the fusion of a small streptococcal albumin-binding domain (ABD) to the scDb. The special feature of this strategy is that it aims additionally at the recycling of the scDb-ABD-albumin complex by the neonatal Fc receptor (FcRn).

In vitro characterisations showed that both antigen-binding sites of all scDb variants remained functional. Furthermore, it could be shown that all constructs were still able to activate T-cells target cell-specific and the cytotoxic potential of scDbCEACD3 and scDb-ABD could be verified. The examination of the circulation time of the constructs in mice revealed that the

$AUC_{0\text{-}24h}$ of the N-glycosylated constructs were increased 2- to 3-fold compared to the unmodified scDb. A 10- and 14-fold increase of the ($AUC_{0\text{-}24h}$) could be observed for the PEGylated and the scDb-ABD construct. By comparing the terminal half-lives of the scDb-ABD in FcRn heavy chain knockout and in wild-type mice, it could be demonstrated that the improved half-life of scDb-ABD is partly caused by the recycling via the FcRn. Furthermore, the comparison of the biodistribution of the scDb and its variants in a nude-mice tumor model revealed a superior tumor accumulation of the scDb-ABD.

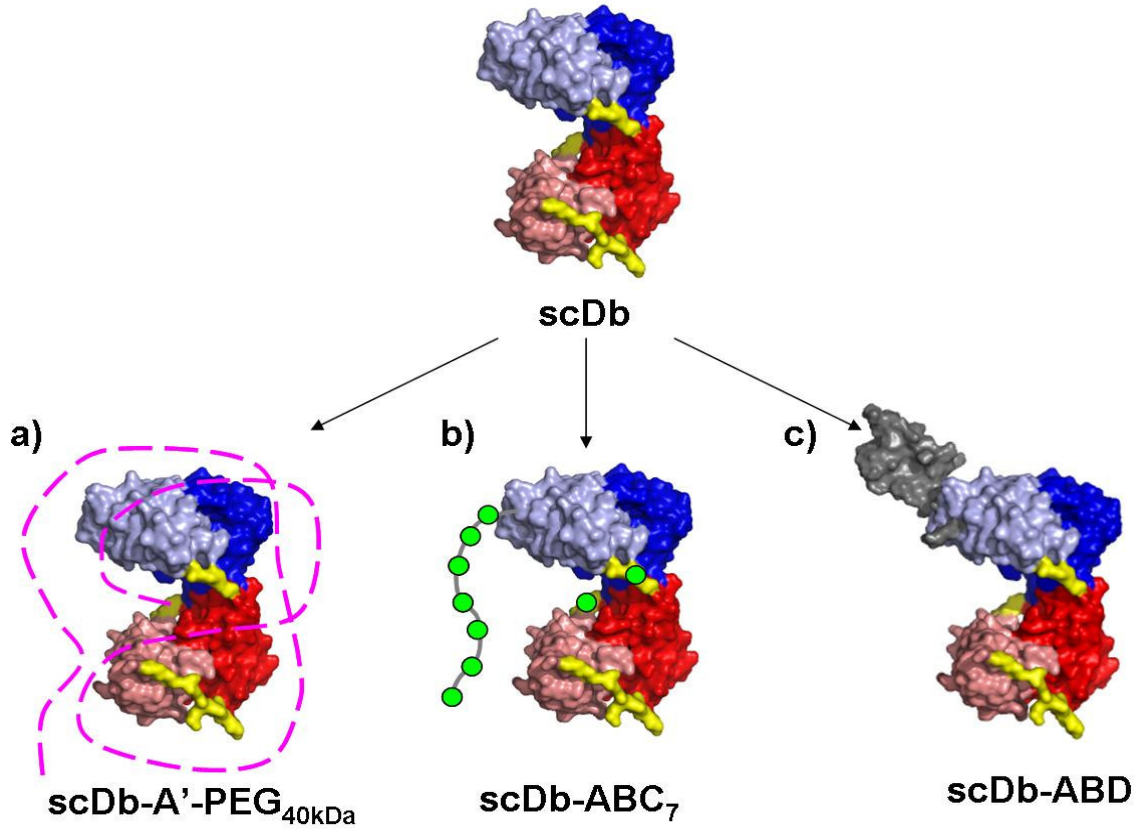

Figure 4. Strategies to improve the pharmacokinetics of a single-chain Diabody (scDb) realized in the present study. A) PEGylation (pink intermitted line symolizes PEG_{40kDa}), b) N-glycosylation (green dots symolize N-glycosylation sites), b) scDb fused to albumin-binding domain 3 of streptococcal protein G. Structures of a bivalent diabody (PDB entry 1LMK, (Perisic et al., 1994)) and ABD3 (PDB 1GJT,(Kraulis et al., 1996)) were used. Visualized with PyMOL Molecular Graphic System (DeLano Scientific, San Carlos, CA, USA).

Manuscripts

Short summaries of the manuscripts

Publication I

"A novel tri-functional antibody fusion protein with improved pharmacokinetic properties by fusing a bispecific single-chain diabody with an albumin-binding domain from streptococcal protein G"

Roland Stork, Dafne Müller and Roland E. Kontermann

Protein Engineering, Design & Selection, VOL. 20, NO. 11, pp. 569-576, 2007.

This publication describes the construction and characterization of the scDbCEACD3 fused to the albumin-binding domain 3 of streptococcal protein G (scDb-ABD). The perspective of this approach is that scDb-ABD binds to albumin that is abundant in the blood, leading to a scDb-ABD-albumin complex that exceeds the threshold for renal clearance and is recycled by the neonatal Fc-receptor (FcRn) after endocytosis. Size exclusion chromatography (SEC) and ELISA confirmed that the binding of ABD to albumin is not affected by the fusion to scDbCEACD3 and remains stable at acidic pH that is typical for the endosome. Flow cytometry on CEA^+ or $CD3^+$ cells as well as ELISA on immobilized CEA revealed that scDb-ABD is able to bind both antigens in presence and absence of albumin. However, the ability of scDb-ABD to activate T-cells, as measured by IL-2 release, was 3-fold decreased compared to scDbCEACD3 and further decreased 4-fold in presence of albumin. ScDb-ABD showed a strongly improved circulation time with a 5- to 6-fold increased terminal half-life, comparable to a scDbCEACD3 fused to HSA. This study shows that the fusion with ABD is a promising approach for the improvement of serum half-life of small recombinant antibodies.

Publication II

"N-glycosylation as novel strategy to improve pharmacokinetic properties of bispecific single-chain diabodies"

Roland Stork, Kirstin A. Zettlitz, Dafne Müller, Miriam Rether, Franz-Georg Hanisch & Roland E. Kontermann

The Journal of Biological Chemistry, VOL. 283, NO. 12, pp. 7804-7812, 2008.

In this publication the construction and characterization of N-glycosylated and PEGylated variants of scDbCEACD3 is described. Both approaches aim at decreasing the renal clearance of the antibody by increasing its size. Three N-glycosylated scDbCEACD3 derivatives with 3, 6 and 9 N-glycosylation sites were produced in HEK-293 cells, bearing glycans comparable to other glycoproteins produced in HEK-293 cells as determined by MALDI-MS.
Even though the antigen-binding of the modified scDbCEACD3 derivatives was similar to that of the parental scDb, their ability to activate T-cells was reduced 3- to 5-fold. The somewhat enlarged hydrodynamic radii of the N-glycosylated constructs translated in a moderately improved circulation half-life. In contrast, PEGylation led to a 3-fold increased hydrodynamic radius, resulting in a 10-fold increase of the $AUC_{0\text{-}24h}$. The direct comparison of the different constructs with a chimeric IgG1 and scDbCEACD3 fused to HSA or ABD revealed that the chimeric IgG1 showed the longest half-life followed by scDb-ABD, scDb-HSA and the PEGylated scDb. N-glycosylation emerged as a possibility to moderately improve the circulation time of scDbCEACD3.

Publication III

"Biodistribution of a bispecific single-chain diabody and its half-life extended derivatives"

Roland Stork, Emmanuelle Campigna, Bruno Robert, Dafne Müller and Roland E. Kontermann

The Journal of Biological Chemistry, in press, 2009

In this study the influence of N-glycosylation, PEGylation and ABD-fusion on the biodistribution of scDbCEACD3 was examined. It was observed that the prolonged half-lives of scDb-ABD and the PEGylated antibody translate in an improved tumor accumulation in CEA^+ tumors. Although both constructs showed a similar circulation time and similar binding to CEA, a 2-fold increased tumor accumulation was observed for scDb-ABD compared to the PEGylated scDb. The hypothesis that the long circulation half-life of scDb-ABD is caused by FcRn interaction could be confirmed by the use of FcRn heavy chain knockout mice. In these knockout mice the half-life of scDb-ABD was halved, whereas no differences could be observed for the PEGylated scDb. Quartz crystal microbalance measurements with immobilized albumin revealed that the ABD-albumin interaction is not decreased by the fusion with scDbCEACD3 or by acidic pH, confirming the ELISA results from publication I. Antibody titration in flow cytometry showed similar binding of scDb-ABD and the unmodified scDb on CEA^+ LS174T and $CD3^+$ Jurkat cells. However, cytotoxicity assays revealed a reduced cytotoxic effect of scDb-ABD, which was further reduced in presence of albumin.

In conclusion the long circulating constructs showed an improved tumor accumulation and the contribution of the FcRn interaction to the improved half-life of scDb-ABD could be confirmed. Furthermore, scDb-ABD was able to mediate target cell-specific cytotoxicity, although this activity was influenced by binding to albumin.

A novel tri-functional antibody fusion protein with improved pharmacokinetic properties generated by fusing a bispecific single-chain diabody with an albumin-binding domain from streptococcal protein G

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Abstract

The therapeutic application of small recombinant antibody molecules is often limited by a short serum half-life. In order to improve the pharmacokinetic properties we have investigated a strategy utilizing fusion with an albumin-binding domain (ABD) from streptococcal protein G. This strategy was applied to a bispecific single-chain diabody (scDb CEACD3) developed for the retargeting of cytotoxic T cells to CEA-expressing tumor cells. This novel tri-functional fusion protein (scDb-ABD) was expressed in mammalian cells and recognized both antigens as well as human and mouse serum albumin. ScDb-ABD was capable to retarget T cells to CEA-expressing target cells in vitro and to activate the effector cells as measured by stimulation of IL-2 release. Although activity was reduced 3-fold compared with scDb and further reduced 4-fold in the presences of human serum albumin, this assay demonstrated that scDb-ABD is active when exposed to all three antigens. Compared with scDb, the circulation time of scDb-ABD in mice was prolonged 5- to 6-fold similar to a previously described scDb-HSA fusion protein. This strategy, which adds only a small protein domain (46 amino acids) and which utilizes high-affinity, non-covalent albumin interaction, should be broadly applicable to improve serum half-lives of small recombinant antibody molecules.

Introduction

Recombinant antibodies have found broad applications in therapy. While engineered whole antibodies, such as chimeric and humanized immunoglobulins, show pharmacokinetic properties similar to that of human antibodies, small recombinant antibody molecules, e.g. single-chain Fv (scFv) or bispecific molecules such as tandem scFv and single-chain diabodies (Müller & Kontermann, 2007), are rapidly cleared from circulation (Weir et al., 2002; Müller et al., 2007). This is mainly due to their small size leading to rapid renal clearance and the lack of recycling processes mediated by the neonatal Fc receptor (FcRn) (Lencer and Blumberg, 2005). Thus, high doses and repeated injections or infusions are necessary to maintain therapeutically effective serum concentrations (Schlereth et al., 2005).

Several strategies have been developed in recent years to extend circulation time of small antibody molecules. One approach utilizes attachment of polyethylene glycol (PEG) chains. PEGylation has been shown to increase serum half-life of scFv and other molecules and to improve diagnostic or therapeutic efficacy (Chapman, 2002). However, this strategy involves a chemical coupling step and can lead to reduced binding and activity of the PEGylated

protein (Kubetzko et al., 2005; Bailon et al., 2001). We have recently applied an albumin fusion strategy (Chuang et al., 2002) to improve the pharmacokinetic properties of small bispecific antibody molecules (single-chain diabody, tandem scFv) (Müller et al., 2007). By this approach, half-lives were increased leading to a 6-7 fold increase of the area-under-the-curve (AUC).

Similar strategies are based on the fusion of an albumin-binding moiety to the therapeutic protein (Makrides et al., 1996; Dennis et al., 2002). For example, albumin-binding antibody fragments have been employed to increase serum half-life of proteins (Smith et al., 2001; Roovers et al., 2007). Furthermore, albumin-binding peptides were isolated by phage or bacterial display and used to improve pharmacokinetics of proteins (Sato et al., 2002; Dennis et al., 2002; Bessette et al., 2004; Nguyen et al., 2006; Dennis et al., 2007).

Natural high-affinity albumin-binding domains are found in protein G of certain Streptococcus strains (Åkerström et al., 1987). For example, protein G of Streptococcus strain G148 contains three homologous albumin-binding domains. Albumin-binding domain 3 (ABD3) has been extensively studied. This domain consists of 46 amino acid residues forming a stable three-helix bundle (Kraulis et al., 1996). ABD3 has broad albumin species specificity and binds to human serum albumin (HSA) with a K_d of approximately 4 nM as determined by Biacore experiments (Johansson et al., 2002; Linhult et al., 2002). Residues involved in binding to HSA have been identified by mutational analysis in the second α-helix of ABD3 (Linhult et al., 2002). Only a few reports have described the use of this domain or larger regions of protein G containing this domain to prolong the circulation time of proteins such as soluble complement receptor 1 (sCR1), CD4, Fab fragments and affibodies (Makrides et al, 1996; Nygren et al., 1991; Schlapschy et al., 2007; Tolmachev et al., 2007).

Here, we applied genetic fusion of the ABD3 domain of streptococcal protein G to a recombinant bispecific single-chain diabody (scDb) to generate a novel tri-functional antibody molecule with improved pharmacokinetic properties. We could show that the scDb-ABD fusion protein is expressed by mammalian cells and that it retains antigen-binding activity for both antigens and binds to human and mouse serum albumin. A comparative analysis with scDb demonstrated a 5- to 6-fold prolonged circulation time in vivo leading to a 14-fold increase of the AUC.

Materials and Methods

Materials

HRP-conjugated anti-His-tag antibody was purchased from Santa Cruz Biotechnology (California, USA), unconjugated anti-His-tag antibody from Dianova (Hamburg, Germany) and anti-mouse IgG-FITC or PE-conjugated antibody from Sigma (Taufkirchen, Germany). Carcinoembryonic antigen was obtained from Europa Bioproducts (Cambridge, UK). HSA and MSA were purchased from Sigma. The human colon adenocarcinoma cell line LS174T was purchased from ECACC (Wiltshire, UK) and cultured in EMEM (Invitrogen, Karlsruhe, Germany) supplemented with 2 mM glutamine, 1% non-essential amino acids and 10% FBS. LoVo cells were provided by Dr. Bruno Robert (Montpellier, France) and cultured in RPMI, 10% FBS. Jurkat and HEK293 were cultured in RPMI, 10% and 5% FBS, respectively. Buffy coat (leukapheresis) from a healthy human donor was kindly provided by Prof. G. Multhoff (Regensburg, Germany). IL-2 was purchased from Immunotools (Friesoythe, Germany) and phytohemagglutinin-L (PHA-L) from Boehringer-Mannheim (Germany). CD1 mice were purchased from Elevage Janvier (Le Genest St. Isle, France).

Construction and expression of a scDb-ABD fusion protein

The construction of scDbCEACD3 has been described previously (Müller et al., 2007). DNA encoding the entire ABD3 domain of protein G of Streptococcus strain G148 was synthesized by GeneArt (Regensburg, Germany) adding a NotI and EcoRI restriction site at the 5' and 3' end, respectively, for cloning into plasmid pAB1 scDbCEACD3. The resulting DNA fragment encoding scDb-ABD was subcloned into mammalian expression vector pSecTagA (Invitrogen, Karlsruhe, Germany). HEK293 cells were stably transfected and scDb-ABD was purified from cell culture supernatant essentially as described previously (Müller et al., 2007).

ELISA

Carcinoembryonic antigen (CEA) was immobilized in 96-well-plates (300 ng/well) overnight at 4°C. After 2 h blocking with 2% (w/v) dry milk/PBS, recombinant antibody fragments were titrated in duplicates and incubated for 1 h at RT. Detection was performed with mouse HRP-conjugated anti-His-tag antibody using TMB substrate (1 mg/ml TMB, sodium acetate buffer pH 6.0, 0.006% H_2O_2). The reaction was stopped with 50 µl of 1 M H_2SO_4.

Absorbance was measured at 450 nm in an ELISA-reader. Binding of scDb-ABD to immobilized human and mouse serum albumin (10 µg/well) was analyzed accordingly. Competition-ELISA was performed according to the method described by Friguet and coworkers (Friguet et al., 1985). ScDb-ABD at a concentration of 3 and 10 nM, respectively, was preincubated with increasing concentrations of HSA or MSA (0.1 nM – 1 µM) for 3 h and subsequently added to microtiter plates coated with MSA. After a 15 min incubation step, plates were washed and bound scDb-ABD was detected as described above. PBS, 2.7% blocking reagent for ELISA (Roche Diagnostics) was used for blocking and incubation in these experiments.

Flow cytometry

$5x10^5$ cells/well were incubated with recombinant antibodies (10 µg/ml) for 2 h at 4°C. After washing, cells were incubated for 1 h at 4°C with mouse anti-His-tag antibody followed by washing and 30 min incubation with PE-labeled anti-mouse IgG. Wash cycles and incubation steps were performed in PBS, 2% FCS, 0.02% azide. Finally, cells were analyzed by flow cytometry using an EPICS XL-MCL (Beckman Coulter, Krefeld, Germany).

Thermal and serum stability

Protein melting points were determined by dynamic light scattering using a Zetasizer Nano ZS (Malvern, UK). Proteins were diluted in PBS to a concentration of 150 µg/ml and analyzed at increasing temperatures (30 to 70°C) using 1°C intervals and an equilibration time of 2 min. Furthermore, stability was determined by incubating scDb-ABD in mouse serum or human plasma at 37°C for up to 18 days. Aliquots were taken at different time points and concentration of active antibody molecules were determined by ELISA as described above.

Size exclusion chromatography

Apparent molecular weights and formation of scDb-ABD albumin complexes were analyzed by HPLC size exclusion chromatography using a BioSep-Sec-3000 column (Phenomenex, Torrance, USA) and a flow rate of 0.5 ml/min. The following standard proteins were used: thyroglobulin, apoferritin, β-amylase, bovine serum albumin, carbonic anhydrase, cytochrome c. Complex formation of scDb-ABD with HSA or MSA was analyzed by incubating

equimolar amounts of scDb-ABD and albumin (8.5 µM) in PBS at room temperature and subsequent analysis by size exclusion chromatography.

IL-2 release assay

Peripheral blood mononuclear cells (PBMC) from a healthy donor were isolated from buffy coat (leukapheresis) as described before (Müller et al., 2007). $1x10^5$ LS174T or HT1080#13.8 cells/100µl/well were seeded in 96-well plates. The next day supernatant was removed and 100 µl of recombinant antibody added. After 1 h preincubation at RT, $2x10^5$ PBMC/100µl/well were added. PBMCs had been thawed the day before and seeded on a culture dish to remove monocytes by attachment to the plastic surface. Only cells that remained in suspension were used for the assay. After addition of PBMCs, the 96-well plate was incubated for 22-24 h at 37°C, 5% CO_2. Plates were centrifuged and cell-free supernatant collected. Concentration of human IL-2 in the supernatant after T-cell retargeting was determined by an IL-2 sandwich-ELISA. Anti-human IL-2 antibodies as well as the standard of recombinant human IL-2 was provided by DuoSet IL-2 ELISA Development System kit (RandD Systems, Nordenstadt, Germany) and the assay was performed following the manufacturer's protocol.

Pharmacokinetics

Animal care and all experiments performed were in accordance with federal guidelines and had been approved by university and state authorities. CD1 mice (female, 15 – 36 weeks, weight between 39-54 g) received i.v. injections of 25 µg scDb, scDb-ABD or scDb-HSA in a total volume of 200 µl. In time intervals of 3, 30, 60, 120, 360 min, 24 h, and 72 h blood samples (100 µl) were taken from the tail and incubated on ice. Clotted blood was centrifuged at 10.000 g for 10 min, 4°C and serum samples stored at -20°C. Serum concentrations of CEA-binding recombinant antibodies were determined by ELISA (as described above), interpolating the corresponding calibration curves. For comparison, the first value (3 min) was set to 100%. Pharmacokinetic parameters AUC, $t_{1/2}\alpha$ and $t_{1/2}\beta$ were calculated with Excel using the first 3 times points to calculate $t_{1/2}\alpha$ and the last 3 time points to calculate $t_{1/2}\beta$. 6 animals were analyzed for scDb-ABD (72 h value measured only for 3 animals). Pharmacokinetics of scDb and scDb-HSA were measured for 3 animals, including data for 72 h, and combined with previously obtained data (Müller et al., 2007). For statistics, Student's t

test was applied.

Results

Production of a scDb-ABD fusion protein

ScDbCEACD3-ABD directed against the carcinoembryonic antigen (CEA) and the T cell receptor complex protein CD3 was generated by fusing the albumin-binding domain ABD3 of streptococcal protein G (composed of 46 aa) to scDbCEACD3 including a C-terminal hexahistidyl tag (Fig. 1a-c). The resulting scDbCEACD3-ABD fusion protein is composed of 550 amino acid residues with a calculated mass of 59.3 kDa. Thus, scDbCEACD3-ABD is approximately 5 kDa larger than scDbCEACD3, which contains also a Myc-tag (505 aa, 54.5 kDa).

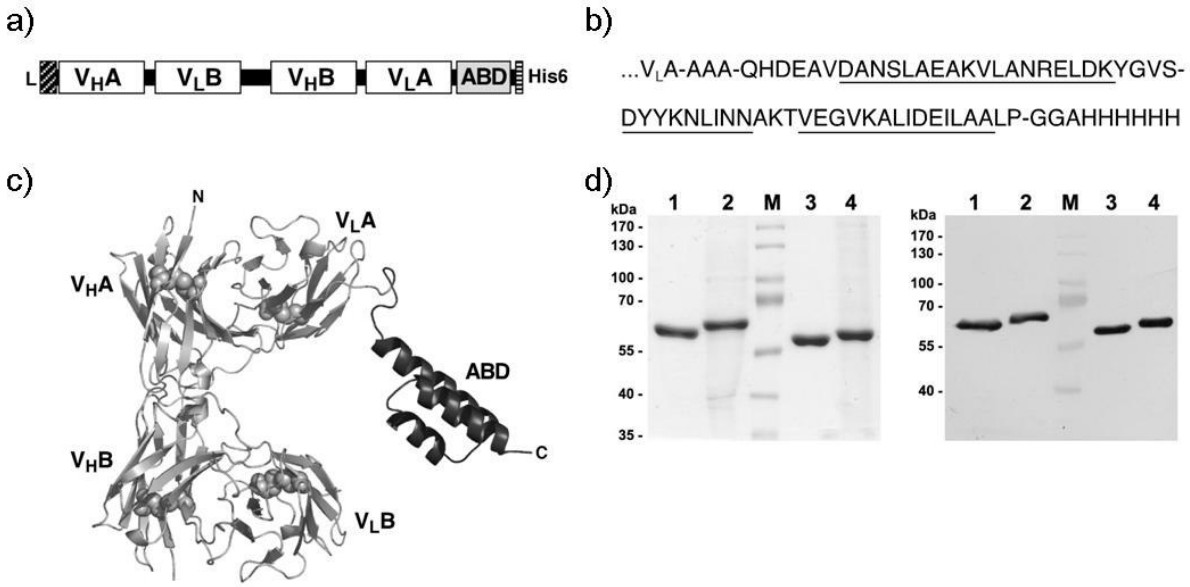

Fig. 1. Composition and production of scDb-ABD. A) Domain structure of scDb-ABD including the N-terminal leader peptide (L) and the C-terminal hexahistidyl-tag (His6) b) Sequence of ABD fused to scDb CEACD3. Alpha-helices are underlined. C) Model structure of a scDb-ABD using the structure of a bivalent diabody (PDB entry 1LMK, Perisic *et al.*, 1994) and the structure of ABD3 of streptococcal protein G (PDB entry 1GJT, Kraulis *et al.*, 1996). Both structures were combined with SwissPDBViewer (Guex and Peitsch, 1997) and visualized with the PyMOL Molecular Graphics System (DeLano Scientific, San Carlos, CA, USA). Cysteine residues are shown space-filled. The middle linker connecting V_LB and V_HA and the C-terminal hexahistidyl-tag is not shown. D) SDS-PAGE and e) immunoblot analysis of purified scDbCEACD3 and scDbCEACD3-ABD (3 µg/lane). scDbCEACD3 (lane 1 and 3) and scDbCEACD3-ABD (lane 2 and 4) were analyzed under non-reducing (lane 1 and 2) or reducing (lane 3 and 4) conditions. Gels were stained with Commassie brilliant blue (d) or immunoblotted with an anti-His-tag antibody (e).

Both, scDbCEACD3 and scDbCEACD3-ABD were purified from the supernatant of stably transfected HEK293 cells with yields of 10 and 15 mg/l culture medium, respectively. SDS-PAGE and immunoblot analysis showed a single protein band for scDbCEACD3 and scDbCEACD3-ABD under non-reducing and reducing conditions (Fig. 1d, e). The apparent molecular masses were 59 kDa for scDbCEACD3 and 62 kDa for scDbCEACD3-ABD.

Antigen binding of scDb-ABD

Both, scDbCEACD3 and scDbCEACD3-ABD recognized CEA in ELISA in the absence or presence of human serum albumin (HSA) (1 mg/ml) (Fig. 2).

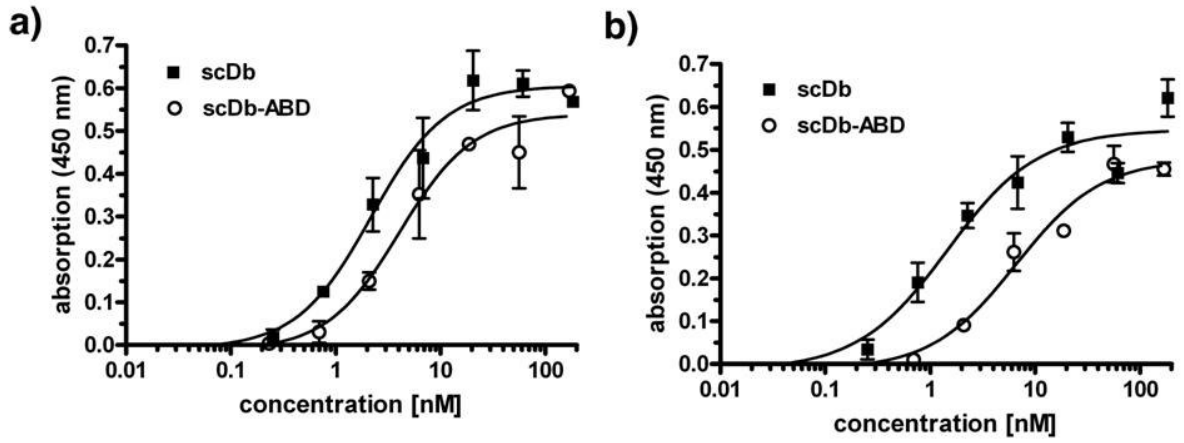

Fig. 2. Binding of scDbCEACD3 and scDbCEACD3-ABD to CEA in ELISA. ELISA plates were coated with CEA (300 ng/well) and binding of scDbCEACD3 and scDbCEACD3-ABD in the absence (a) or presence (b) of HSA (1 mg/ml) was detected via an HRP-conjugated anti His-tag antibody.

Binding of scDbCEACD3 to CEA was not reduced by HSA (half maximal binding at 2.2 nM without HSA and 1.5 nM with HSA), while binding of scDbCEACD3-ABD was slightly reduced (half maximal binding at 4.1 nM without HSA and 6.6 nM with HSA). Both proteins bound with similar efficiency to CEA-expressing tumor cell lines (LS174T, LoVo) and to activated CD3-positive PBMCs. No reduction of binding was observed in the presence of HSA (1 mg/ml) or 50% mouse serum (Fig. 3). No binding was seen with antigen-negative control cells (HEK293). Thus, both antigen-binding sites in scDbCEACD3-ABD are accessible for antigen-binding.

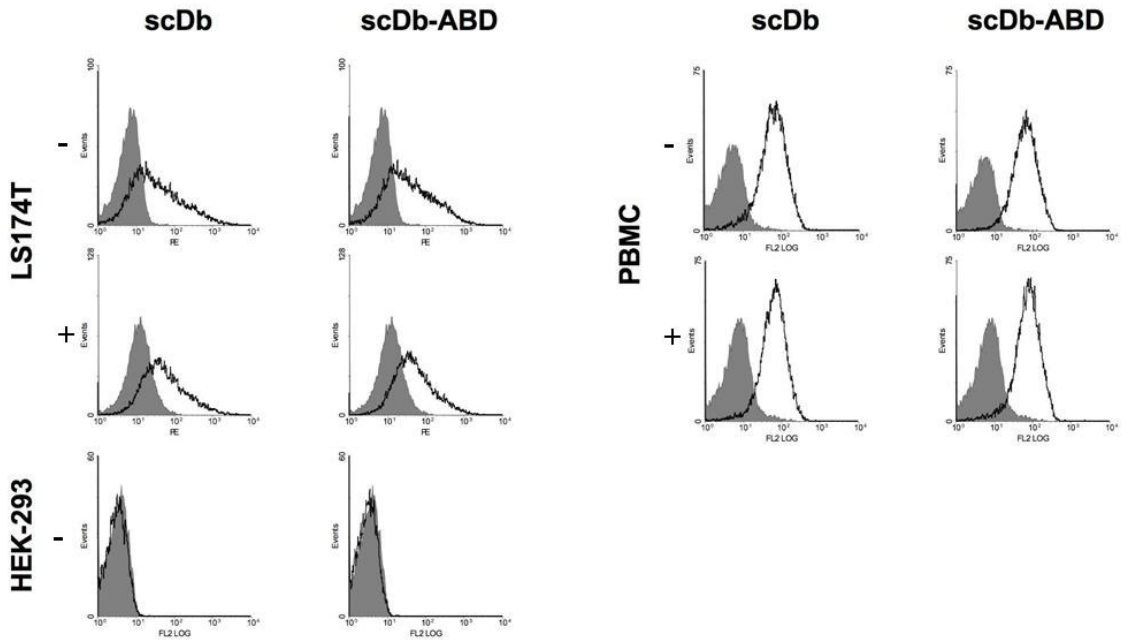

Fig. 3. Binding of scDbCEACD3 and scDbCEACD3-ABD to antigen expressed on cells. Purified scDbCEACD3 and scDbCEACD3-ABD (1 µg/ml) were analyzed by flow cytometry for binding to CD3-expressing PBMCs and CEA-expressing LS174T cells in the presence (+) or absence (-) of 50% mouse serum. HEK293 cells were included as negative control (filled, detection system; line, antibody molecule).

Stability of scDb-ABD

Thermal stability of scDbCEACD3-ABD was found to be similar to that of scDbCEACD3. Melting points were determined to be 48°C for scDbCEACD3-ABD and 49°C for scDbCEACD3. Similar values were measured for scFv CEA (48°C) and scFv CD3 (47°C) (not shown). ScDbCEACD3-ABD was highly stable in human plasma or mouse serum at 37°C with a half-life > 3 days (not shown).

Binding of scDb-ABD to albumin

Size exclusion chromatography (SEC) was applied to demonstrate that scDbCEACD3-ABD interacts with human and mouse serum albumin in solution (Fig. 4). For this study, scDbCEACD3-ABD was incubated with HSA or MSA at equimolar concentrations (8.5 µM) and then separated by SEC. ScDbCEACD3-ABD alone eluted with a major peak (88%) corresponding to an apparent molecular mass of 40 kDa. HSA showed a major peak (92.5%) of 65 kDa and a minor peak of approximately 220 kDa. After incubation with scDbCEACD3-ABD both peaks were shifted to an apparent molecular mass of 150 and 450 kDa, whereas the peak of the unbound scDbCEACD3-ABD was almost completely diminished (approximately

5% left). Similar results were obtained with MSA. MSA alone showed a major peak (50.6%) of 60 kDa and several peaks with higher molecular masses. These peaks were shifted towards the left after incubation with scDbCEACD3-ABD. Only a small fraction (11%) corresponded in size to free scDbCEACD3-ABD.

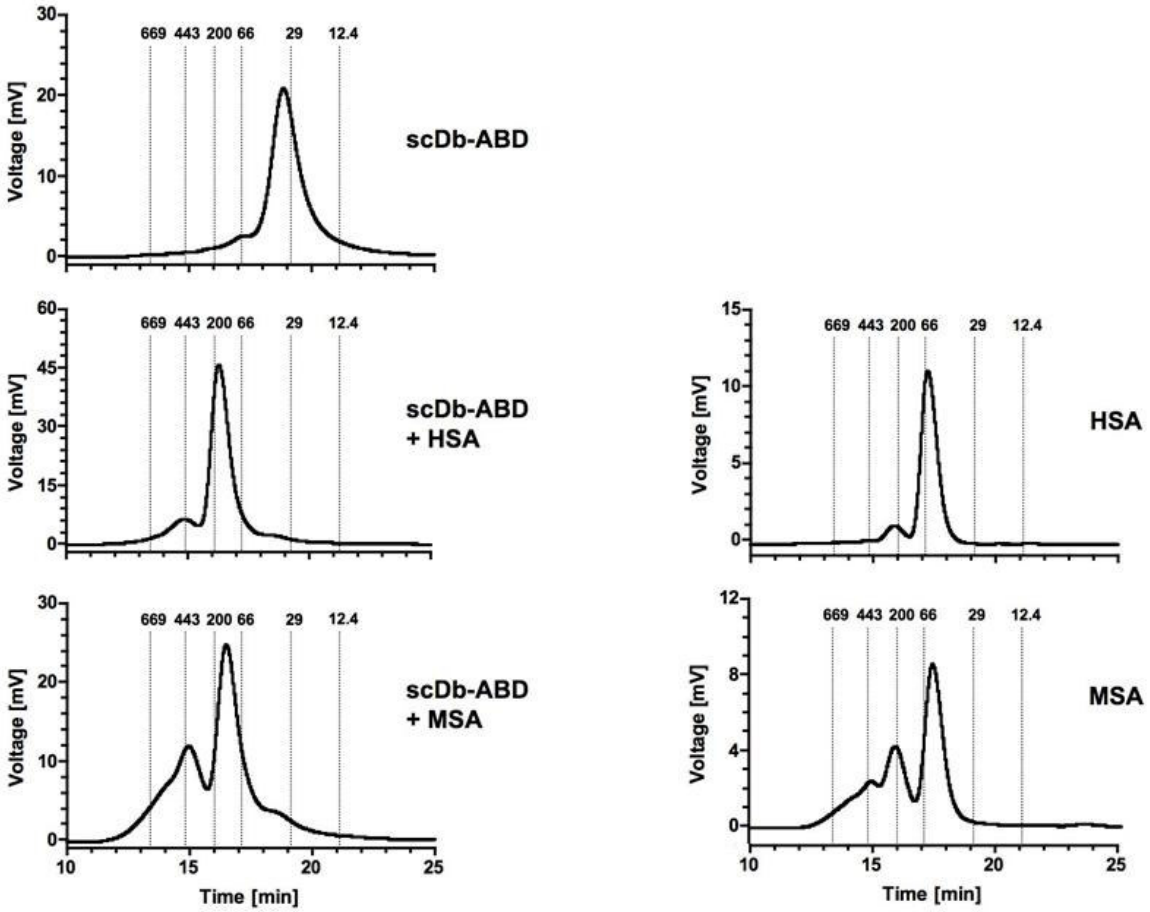

Fig. 4. Formation of scDb-ABD/albumin complexes. ScDbCEACD3-ABD was incubated with HSA or MSA at equimolar concentrations and analyzed by size exclusion chromatography. scDb-ABD, HSA and MSA alone were included. Peak positions of marker proteins are indicated.

A control experiment with scDbCEACD3 incubated with HSA showed no apparent interaction of these molecules (not shown). In a further experiment, we analyzed inhibition of binding of scDbCEACD3-ABD to immobilized MSA by soluble HSA or MSA. In this competition-ELISA we determined IC_{50} values of 4.4 nM for MSA and 3.4 nM for HSA, respectively (Fig. 5a). Next, we compared binding of scDbCEACD3-ABD to MSA at pH 7.4 and pH 6.0 in ELISA in order to investigate if scDbCEACD3-ABD albumin complexes are stable in an acidic environment. Binding of scDbCEACD3-ABD to MSA at pH 6.0 was not affected (using 10 µg/ml scDb-ABD) or slightly increased (1.2 to 1.9-fold at 3 and 1 µg/ml, respectively) compared to binding at pH 7.4 (Fig. 5b).

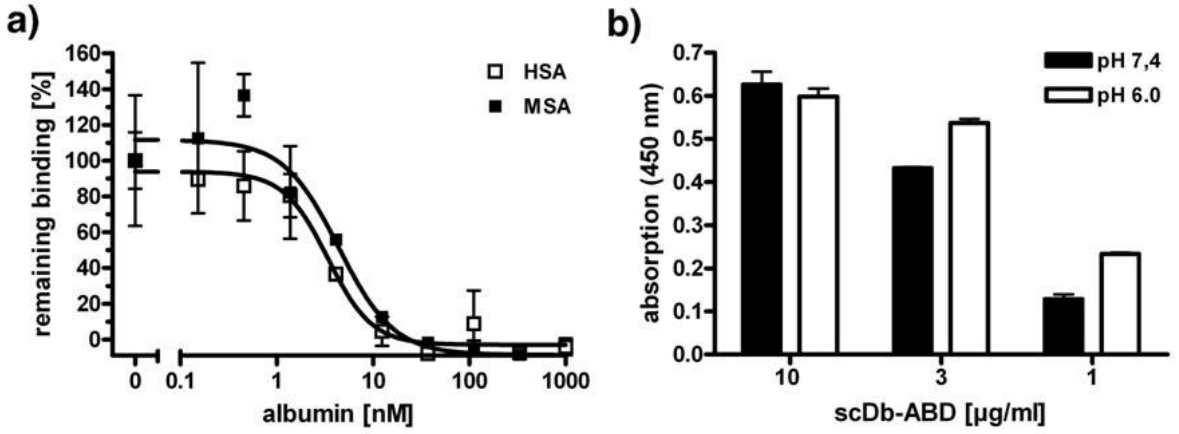

Fig. 5. Binding of scDbCEACD3-ABD to albumin. A) Competition-ELISA analyzing binding of scDbCEACD3-ABD to immobilized MSA in the presence of increasing concentrations of HSA or MSA. B) Binding of scDbCEACD3-ABD to immobilized MSA in ELISA at neutral (pH 7.4) or acidic pH (pH 6.0).

T cell stimulatory activity of scDb-ABD

An IL-2 release assay was performed to demonstrate simultaneous binding of scDbCEACD3-ABD to CEA-expressing target cells and CD3-positive effector cells leading to activation of the effector cells. Both molecules, scDbCEACD3 and scDbCEACD3-ABD, activated human effector cells in a concentration-dependent manner in the presence or absence of HSA (Fig. 6a). For scDbCEACD3 maximal IL-2 release was induced between 2-10 nM (EC_{50} = 0.5 nM). In the absence of HSA, activation induced by scDbCEACD3-ABD was approximately 3-fold reduced compared with scDbCEACD3 (EC_{50} = 1.5 nM). The presence of HSA in the cell culture medium (1 mg/ml) had no or only a marginal effect on activation by scDbCEACD3, while activation by scDbCEACD3-ABD was further reduced 4-fold (EC_{50} = 6 nM). Selectivity of activation in this target cell-based assay was demonstrated with a control scDb directed against fibroblast activation protein and CD3 (scDb33CD3), which compared with scDbCEACD3 and scDbCEACD3-ABD induced only low IL-2 release at a concentration of 25 nM (Fig. 6b).

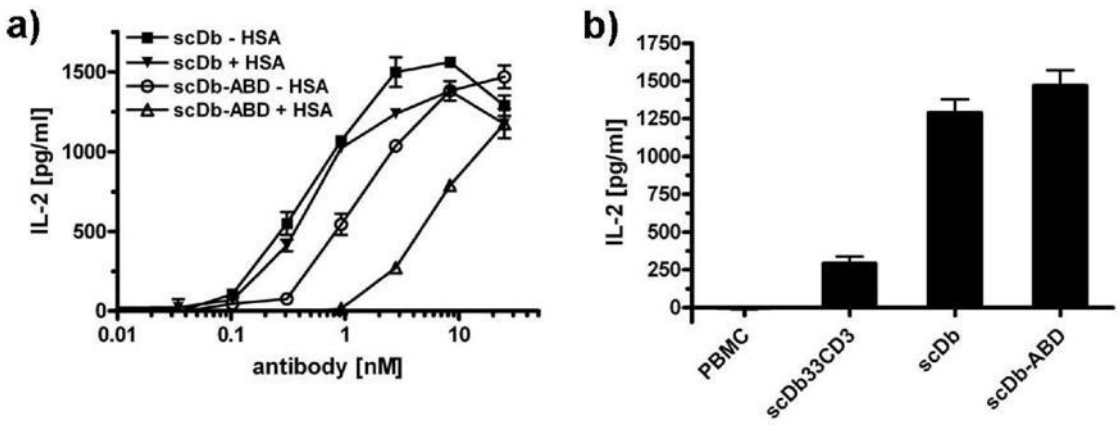

Fig. 6. Antibody-induced IL-2 release. A) LS174T were preincubated for 1 h with scDbCEACD3 and scDbCEACD3-ABD at varying concentrations before adding human PBMCs. IL-2 release was measured after 24 h in ELISA. B) PBMCs were incubated in presence of LS174T together with 25 nM antibodies (scDbCEACD3, scDbCEACD3-ABD or a control scDb (scDb33CD3 directed against fibroblast activation protein and CD3)). IL-2 release was measured after 24 h by ELISA.

Pharmacokinetic properties of scDb-ABD

Serum concentrations of scDbCEACD3 and scDbCEACD3-ABD were determined at different time points after a single dose i.v. injection into CD1 mice. 25 µg were used per animal in accordance with doses applied in studies of other recombinant bispecific antibodies in mice (Cochlovius et al., 2000; Kipriyanov et al., 2002). Initial serum concentrations were found to be between 11.8 – 65.7 µg/ml for scDb and between 4.1 to 27.0 µg/ml for scDb-ABD.

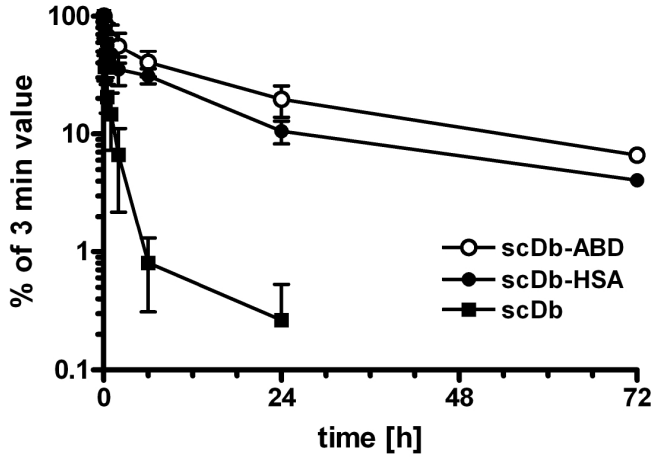

Fig. 7. Pharmacokinetic properties. 25 µg scDbCEACD3, scDbCEACD3-ABD or scDbCEACD3-HSA were injected i.v. into CD1 mice (n = 6). Serum concentrations of the antibody molecules were determined at different time points by ELISA. Data were normalized considering maximal concentration at the first time point (3 min).

Compared with scDbCEACD3, scDbCEACD3-ABD showed a prolonged circulation time, with an increase in initial and terminal half-lives by a factor of 5-6 and the $AUC_{(0-24)}$ by a factor of 14 (Fig. 7) (Table 1). Half-lives determined for scDbCEACD3-ABD were similar to those of a scDbCEACD3-HSA fusion protein (Müller et al., 2007). The $AUC_{(0-24)}$ of scDbCEACD3-ABD was significantly increased by a factor of 1.4 ($p < 0.05$) compared with scDbCEACD3-HSA.

Table 1. Pharmacokinetic properties

construct	M_r (kDa)	$t_{1/2}\alpha$ (min)	$t_{1/2}\beta$ (h)	$AUC_{(0-24)}$ (%*h)
scDb	54.5	10.2 ± 3.6	5.6 ± 1.8	61 ± 22
scDb-ABD	59.3	64.2 ± 37.2	27.6 ± 6.0	861 ± 210
scDb-HSA	119.6	63.6 ± 31.8	25.0 ± 2.4	608 ± 83

Parameters were determined from data obtained for the first 24 h.

Discussion

Fusion of the albumin-binding domain 3 (ABD3) of streptococcal protein G to scDb CEACD3 prolonged circulation half-lives in mice by a factor of 5-6 and the AUC increased by a factor of 14 compared with scDb. This finding indicates that scDb-ABD utilizes binding to mouse albumin to achieve prolongation of circulation time most likely caused by reduced renal clearance and FcRn-mediated recycling effects of scDb-ABD albumin complexes formed in vivo. This assumption is supported by the observation that scDb-ABD formed complexes with MSA and HSA in vitro when incubated at equimolar concentrations. Furthermore, binding of scDbCEACD3-ABD to MSA in ELISA was not reduced at pH 6.0 indicating that scDb-ABD albumin complexes are stable at low pH, e.g. found in endosomes. Using proteolytically and chemically produced fragments of HSA the binding site(s) of streptococcal protein G was found to reside in a segment spanning residues 330-446, indicating that protein G binds to domain II and III of HSA (Falkenberg et al., 1992). Crystallographic studies of a complex of HSA and the ABD of protein PAB of Finegoldia magna, which is homologous to the ABD3 of streptococcal strain G148 (60% identity), revealed that the ABD binding site is located in domain II of albumin involving albumin residues 212, 309, 318, 321, 326, and 329 (Lejon et al., 2004). The exact FcRn binding site on albumin is not know but has been suggested to be contained within domain III of albumin (Anderson et al., 2006; Chaudhury et al., 2006). Thus, most likely the ABD and FcRn binding

sites on albumin are located on separate regions of albumin. Therefore, FcRn-mediated recycling should not be impaired by binding of ABD to albumin. However, currently we do not have direct evidence that FcRn-mediated recycling is involved in prolongation of circulation time in mice.

Prolonged circulation time was also observed for a fusion protein consisting of two fragments of streptococcal protein G fused N- and C-terminally of CD4. This bacterially expressed fusion protein showed a half-life of 15-24 h after single-dose injection into mice similar to a CD4-Fc fusion protein (Nygren et al., 1991). A CD4-HSA fusion protein showed after a single-dose injection into rabbits a terminal half-life of 34 ± 4 h compared with 47 ± 6 h for HSA and 0.25 ± 0.1 h for soluble CD4 (Yeh et al., 1992). In another study, fusion of soluble complement receptor 1 (sCR1) to an albumin-binding domain of protein G increased terminal half-life by a factor of 2.9 to 5 h (Makrides et al., 1996). Recently, this albumin-binding domain was fused to a monovalent or bivalent affibody directed against HER2 and used to generate radiolabeled conjugates (Tolmachev et al., 2007). Compared with the nonfused affibody dimer half-life was prolonged from 0.64 h to 35.8 h. Furthermore, a ^{177}Lu-labeled ABD-affibody dimer showed high and specific tumor uptake and completely prevented growth of SKOV-3 microxenografts in mice. These studies together with the present work clearly demonstrate that fusion of one or more albumin-binding domains of protein G is able to increase circulation time in animals.

A reduced tumor cell binding was described for a monomeric affibody ABD fusion protein indicating that binding to albumin can influence additional binding to target cells probably due to a sterical hindrance of binding (Tolmachev et al., 2007). We found that HSA or mouse serum had no effect on binding of scDbCEACD3-ABD to CEA-expressing tumor cells or IL-2 stimulated CD3-positive PBMCs, although binding of scDbCEACD3-ABD to CEA in ELISA was reduced in the presence of HSA. Further experiments showed that activation of effector cells by target cell-bound scDb-ABD is reduced in the presence of albumin by a factor of 4. This finding demonstrated that scDbCEACD3-ABD is still active when exposed to effector and target cells in the presence of albumin but also showed that overall activity of scDbCEACD3-ABD is influenced by albumin.

The approach of using an albumin-binding domain derived from protein G is similar to approaches based on albumin-binding peptides and other small binding domains such as single-domain antibodies, i.e. are utilizing complexation with naturally occurring albumin. A 20mer albumin-binding peptide with the sequence QRLMEDICLPRWGCLWEDDF was isolated from a phage display library (Dennis et al., 2002). This peptide was applied to

prolong circulation time of an anti-HER2 Fab fragment derived from antibody 4D5 leading to rapid accumulation and retention in tumors and favorable tumor to normal tissue ratios (Dennis et al., 2007). This peptide has an affinity for MSA of 44 nM and for HSA of 556 nM. In mice, AUC of the fusion protein (AB.Fab4D5-H) was increased by a factor of 114 compared with Fab4D5 and terminal half-life increased from 1.28 h to 19.7 h (Nguyen et al., 2006). Furthermore, using shorter sequences of this albumin-binding peptide with lower affinity for albumin it was found that reduced affinity correlates with reduced half-life. We have measured the affinity of scDbCEACD3-ABD for HSA and MSA by competition ELISA (Friguet et al., 1985). The IC_{50} values of 3.4 nM for HSA and 4.4 nM for MSA are close to previously determined IC_{50} values for the recombinant ABD3 domain (1.6 nM for HSA and 10.2 nM for MSA) (Johannsson et al., 2002) and a K_d of 3.86 nM for HSA as measured by surface plasmon resonance (Linhult et al., 2002). This finding indicates that fusion of ABD to scDb does not reduce affinity for albumin. Thus, ABD fusion proteins exhibit a high affinity for HSA and should be highly suitable to improve pharmacokinetics of proteins in human.

Concerns of using a streptococcal ABD domain for improvement of pharmacokinetics may arise from the risk of being immunogenic in human. Indeed immunogenicity of the minimal albumin-binding domain (aa 254-299) of protein G was found in various mice strains (Sjölander et al., 1997). Furthermore, an increased antibody response was observed for a fusion protein of the albumin-binding region (BB; aa 113-326) of protein G and part of an RSV-A antigen (Libon et al., 1999). For therapeutic applications, especially involving repeated injections, it is essential that immunogenicity of the ABD is reduced or ideally eliminated. It was speculated that the BB domain is a T cell-dependent protein acting as a carrier and supplying additional T cell epitopes. Hence, deimmunization strategies should be applicable to reduce immunogenicity of the ABD (De Groot et al., 2005; Baker and Jones, 2007).

In summary, we found that scDbCEACD3-ABD recruited effector cells to target cells in vitro even in the presence of excess amounts of HSA, leading to activation of the effector cells as measured by IL-2 release. These experiments also showed that activation of effector cells is influenced by the ABD moiety and interaction with albumin, resulting in a 3- to 12-fold reduced activity compared with scDb. Nevertheless, this data clearly demonstrate that scDb-ABD is active when exposed to effector and target cells in the presence of albumin. Further in vivo studies have to demonstrate potency of this novel tri-functional scDb-ABD fusion proteins in respect to effector cell retargeting and the effects of prolonged circulation time on induction of anti-tumor responses.

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Abbreviations: ABD, albumin-binding domain; AUC, area under the curve; CEA, carcinoembryonic antigen; HSA, human serum albumin; MSA, mouse serum albumin; PBMC, peripheral blood mononuclear cells; scDb, single-chain diabody

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N-glycosylation as novel strategy to improve pharmacokinetic properties of bispecific single-chain diabodies

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Abstract

The therapeutic efficacy of recombinant antibodies such as single-chain Fv fragments and small bispecific or bifunctional molecules is often limited by rapid elimination from the circulation because of their small size. Here, we have investigated the effects of N-glycosylation on the activity and pharmacokinetics of a small bispecific single-chain diabody (scDb CEACD3) developed for the retargeting of cytotoxic T cells to CEA-expressing tumor cells. We could show that the introduction of N-glycoslyation sequons into the flanking linker and a C-terminal extension results in the production of N-glycosylated molecules after expression in transfected HEK293 cells. N-glycosylated scDb variants possessing 3, 6 or 9 N-glycosylation sites, respectively, retained antigen-binding activity and bispecificity for target and effector cells as shown in a target cell-dependent IL-2 release assay, although activity was reduced approximately 3- to 5-fold compared to the unmodified scDb. All N-glycosylated scDb variants exhibited a prolonged circulation time compared to scDb, leading to a 2- to 3-fold increase of the area under the curve (AUC). In comparison, conjugation of a branched 40 kDa PEG chain increased AUC by a factor of 10.6, while a chimeric anti-CEA IgG1 molecule had the longest circulation time with a 17-fold increase in AUC. Thus, N-glycosylation complements the repertoire of strategies to modulate pharmacokinetics of small recombinant antibody molecules by an approach that moderately prolongs circulation time.

Introduction

Whole antibodies, especially chimeric, humanized or fully human IgG molecules, exhibit a long circulation time in the human body, which can reach a half-life of 27 days (1, 2). In contrast, antibody fragments, e.g. Fab fragments, or recombinant formats, such as single-chain Fv fragments (scFv) or bispecific derivatives thereof (tandem scFv, diabodies, single-chain diabodies), are rapidly cleared from circulation (2-4). This is mainly due to the small size leading to rapid renal clearance and the lack of recycling processes mediated by the neonatal Fc receptor (FcRn). Thus, repeated injections or infusions are required to maintain a therapeutically effective dose in the body (5).

Several strategies to improve pharmacokinetic properties and thus dosing and therapeutic efficacy of recombinant antibodies have been developed in recent years. These strategies can be divided into 1) those based on reducing renal clearance by increasing the apparent size of the therapeutic molecule, and 2) those that in addition implement FcRn-mediated recycling processes (6-8).

PEGylation of proteins is a well-established strategy to improve pharmacokinetic properties by increasing the molecular mass and the hydrodynamic radius (9-11). Several PEGylated proteins are in clinical use, e.g. PEGylated interferon α-2a (PegIntron, PEGASYS) and PEGylated granulocyte colony-stimulating factor (Neulasta), or are under clinical development, e.g. a PEGylated anti-TNF Fab fragment (certolizumab pegol) (9, 12) Furthermore, direct fusion to albumin or an albumin binding moiety, such as albumin-binding peptides or bacterial albumin-binding domains, was applied to improve pharmacokinetics of therapeutic proteins including interferon-α, interleukin-2, insulin, Fab fragments and recombinant bispecific antibody molecules (4, 8, 13-15). This strategy is based on the observation that albumin has a similar half-life as IgG and also utilizes FcRn-mediated recycling processes. One albumin fusion protein, albumin-interferon-α (Albuferon alpha), is currently in clinical development (16).

The introduction of N-glycosylation sites is another approach successfully applied to improve pharmacokinetic and pharmacodynamic properties of a few therapeutic proteins (17, 18). Darbepoetin alpha (Aranesp) is a recombinant form of human erythropoietin possessing 2 additional N-glycosylation sites resulting in a hyperglycosylated protein (5 N-glycosylation sites in total) with an approximately 2-fold prolonged half-life and an improved in vivo activity (19). In other studies, glycosylated analogs of follicle-stimulating hormone (FSH)

with prolonged circulation time and increased activity have been generated by adding N-glycans through an N-terminal extension of the a subunit (20) or through a peptide linker joining the β and the α subunit (21), demonstrating the feasibility of this approach.

As yet, no studies have been described that successfully applied N-glycosylation to prolong the circulation time of small recombinant antibodies. Here, we performed a comparative analysis of N-glycosylated bispecific single-chain diabody (scDb) molecules with other modifications including PEGylation. N-glycosylation at 3, 6 or 9 Asn-X-Thr sites introduced at the flanking linker sequences and C-terminal extensions prolonged half-life approximately 2-fold leading to a 2- to 3-fold increase of the AUC. In contrast, site-directed chemical coupling of a branched PEG molecule of 40 kDa to a cysteine residue introduced in one of the linker sequences prolonged circulation time approximately 5-fold resulting in a 11-fold increase of the AUC, similar to fusion of the scDb molecule with human serum albumin or a albumin binding domain of streptococcal protein G.

Materials & Methods

Materials

Horseradish peroxidase-conjugated anti-His-tag antibody was purchased from Santa Cruz Biotechnology (California, USA), unconjugated anti-His-tag antibody from Dianova (Hamburg, Germany) and anti-mouse IgG-FITC or PE-conjugated antibody as well as goat anti-rabbit IgG-FITC-conjugated antibody from Sigma (Taufkirchen, Germany). Rabbit antiserum recognizing PEGylated scDb was produced by Pineda Antikörper-Service (Berlin, Germany). HRP-conjugated anti-rabbit IgG was purchased from Roche Applied Sciences (Mannheim, Germany). Carcinoembryonic antigen was obtained from Europa Bioproducts (Cambridge, UK). Branched $mPEG_2$-Mal was purchased from Nectar Therapeutics (Birmingham, USA). The human colon adenocarcinoma cell line LS174T was purchased from ECACC (Wiltshire, UK) and cultured in RPMI 1640 supplemented with 5% FBS. HEK293 were cultured in RPMI 1640, 5% FBS. Buffy coat from a healthy human donor was kindly provided by Prof. G. Multhoff (München, Germany). IL-2 was purchased from Immunotools (Friesoythe, Germany) and phytohemagglutinin-L (PHA-L) from Roche Applied Sciences (Germany). CD1 mice were purchased from Elevage Janvier (Le Genest St. Isle, France).

Construction of scDb molecules

scDbCEACD3 (4) was used as starting material for the construction of scDb variants. Cysteine residues were introduced into linker A or the C-terminus by PCR (scDb-A', scDb-C'). Similarly, N-glycosylation sites were introduced into the linkers A and B as well as at the C-terminus (scDbABC$_1$, scDbABC$_4$, scDbABC$_7$) (see also Fig. 1). The N-glycosylation sites for scDbABC$_4$ and scDbABC$_7$ were first optimized using the NetNglyc-Software (22), which allowed an in silico prediction of N-glycosylation efficiency. All constructs were cloned into pSecTagA (Invitrogen, Karlsruhe, Germany) via SfiI/EcoRI for eukaryotic expression.

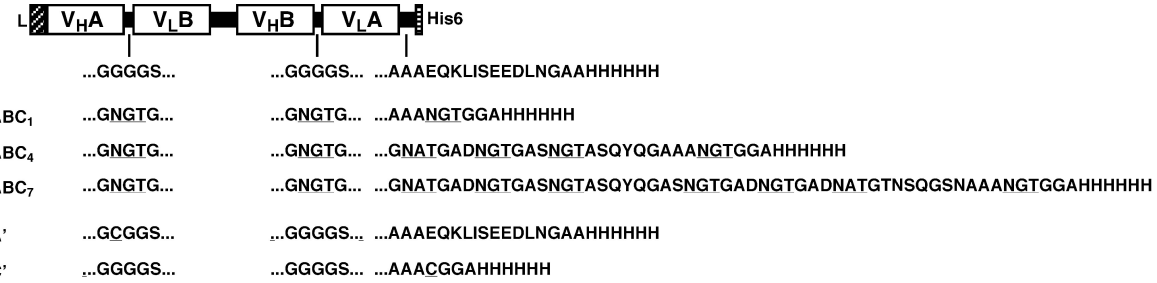

Fig. 1. Composition of N-glycosylated scDbs and PEGylated scDbs. Schematic arrangement of variable domains in a single-chain diabody as well as sequences of linkers A and B and the C-terminal extensions of the modified scDb molecules are shown (A, linker A; M, linker M; B, linker B; L, leader peptide; His6, hexahistidyl-tag).

Protein expression and purification

Plasmid DNA encoding the respective recombinant antibody was transfected with Lipofectamine^{TM}2000 (Invitrogen, Karlsruhe, Germany) into HEK293 cells. Stable transfectants were generated by selection with zeocin (300 µg/ml). For protein production, cells were first expanded and grown in RPMI 1640, 5% FBS to 90% confluence and subsequently cultured in Opti-MEM$^{®}$I (Invitrogen, Karlsruhe, Germany) replacing media every 3 days for 3-4 times. Supernatants were pooled and proteins were concentrated by ammonium sulfate precipitation (60% saturation), before loading onto a Ni-NTA column (Qiagen, Hilden, Germany). Purification by immobilized metal ion affinity chromatography (IMAC) was performed as described elsewhere (4).

PEGylation of scDb molecules

scDb-A' and scDb-C' were reduced with 5 mM Tris(2-carboxyethyl)-phosphine (TCEP) (Pierce, Rockford) for 2 hours at room temperature. TCEP was removed by dialyzing the

solution overnight in an airtight bottle with oxygen free phosphate buffer (10 mM Na_2HPO_4/NaH_2PO_4, 0.2 mM EDTA, 30 mM NaCl, pH 6.7). Subsequently, a 10-fold molar excess of $mPEG_2$-Mal (40 kDa) was added and incubated overnight at 4 °C under non-oxygen conditions. The unbound $mPEG_2$-Mal was removed by IMAC purification. PEGylation was visualized by barium iodide staining of SDS-polyacrylamide gels as described (23).

Chimeric anti-CEA IgG1

The regions encoding the human γ1 heavy chain constant region and the human C_κ domain were amplified from cDNA generated from RNA isolated from human PBMCs and combined with DNA encoding the V_H and V_L domain of anti-CEA antibody MFE23 (24) cloned previously into vector pSecTagA. DNA encoding the complete heavy and light chain was then cloned into pEE6.4 or pEE12.4, respectively (Lonza Biologics, Berkshire, UK). The two plasmids were then combined according to the manusfacturer's protocol resulting in a bicistronic expression plasmid (pEE12.4-IgG1-CEA). The linearized plasmid was transfected into CHO-K1 cells and stably transfected clones were selected in glutamine-free RPMI 1640 medium (Invitrogen, Karlsruhe, Germany) in the presence of 25 µM methionine sulphoximine (MSX). IgG1 was purified from cell culture supernatant by protein A chromatography. In brief, proteins were precipitated from supernatant with 60% saturated ammonium sulfate, resuspended in PBS, pH 8 and loaded onto a protein A-sepharose CL-4B column (Sigma, Taufkirchen, Germany). Bound protein was eluted with 100 mM glycine pH 3.0 and protein-containing fractions were dialyzed against PBS.

ELISA

Carcinoembryonic antigen (CEA) (300 ng/well) was coated overnight at 4°C. After 2 h blocking with 2% (w/v) dry milk/PBS, recombinant antibody fragments were titrated in duplicates and incubated for 1 h at RT. Detection was performed either with mouse HRP-conjugated anti-His-tag antibody or rabbit antiserum and HRP-conjugated goat anti-rabbit antibody using TMB substrate (1 mg/ml TMB, sodium acetate buffer pH 6.0, 0.006% H_2O_2). The reaction was stopped with 50 µl of 1 M H_2SO_4. Absorbance was measured at 450 nm in an ELISA-reader.

Flow cytometry

$5x10^5$ cells/well were incubated with recombinant antibodies (10 µg/ml) for 2 h at 4°C. After washing, cells were incubated for 1 h at 4°C with mouse anti-His-tag antibody or rabbit antiserum followed by washing and 30 min incubation with PE-labeled anti-mouse IgG or FITC-conjugated goat anti-rabbit antibody. Wash cycles and incubation steps were performed with PBS, 2% FBS, 0.02% azide. Finally, cells were analyzed by flow cytometry using an EPICS XL-MCL (Beckman Coulter, Krefeld, Germany).

Size exclusion chromatography

Apparent molecular masses (M_r) of the native proteins were determined by HPLC size exclusion chromatography using a BioSep-Sec-3000 or BioSep-Sec-2000 column (Phenomenex, Torrance, USA) and PBS as mobile phase at a flow rate of 0.5 ml/min. The following standard proteins were used: thyroglobulin (669 kDa, R_S 8.5 nm), apoferritin (443 kDa, R_S 6.1 nm), β-amylase (200 kDa, R_S 5.4 nm), bovine serum albumin (67 kDa, R_S 3.55 nm), carbonic anhydrase (29 kDa, R_S 2.35 nm), cytochrome c (12.4 kDa, R_S 1.77 nm).

Carbohydrate analysis

Glycoproteins (10 – 30 µg) were denatured in the presence of SDS and 2-mercaptoethanol prior to addition of NP40 and 100 U of PNGaseF (NEB Biolabs) according to the supplier's instructions. After incubation for 18 h the sample was desalted on graphitized carbon columns (Carbograph, Alltech) (25). The samples were dried by vacuum centrifugation and in a desiccator in the presence of P_2O_5/KOH. To the dry sample 50 µl of base (NaOH/DMSO) was added and incubated for 30 min at RT with occasional shaking. Finally, an aliquot of 25 µl methyl iodide was added to the reaction mixture followed by incubation for further 30 min at RT. After neutralization with dilute acetic acid the methylated glycans were extracted with chloroform-water. The chloroform phase was dried under nitrogen and the glycans solubilized in methanol. Matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) was performed on a Bruker Reflex IV instrument (Bruker Daltonics, Bremen, Germany). The methylated glycan samples (approx. 500 pmol per µl) in methanol were applied to the stainless steel target by mixing a 0.5 µl aliquot with 1.0 µl of matrix (saturated solution of 2,5-dihydroxy benzoic acid in acetonitrile / 0.1% TFA, 1:2). Analyses were performed by positive ion detection in the reflectron mode as described previously (26).

IL-2 release assay

Peripheral blood mononuclear cells (PBMC) were prepared from a leukapheresis as previously described (4). $1x10^5$ LS174T or HT1080#13.8 cells/100µl/well were seeded in 96-well plates. The next day supernatant was removed and 100 µl of recombinant antibody in RPMI, 10%FBS added. After 1 h preincubation at RT, $2x10^5$ PBMC/100µl/well were added. PBMCs had been thawed the day before and seeded in RPMI, 10%FBS on a culture dish, to remove monocytes by attachment to the plastic surface. Only cells that remained in suspension were used for the assay. After addition of PBMCs, the 96-well-plate was incubated for 24 h at 37°C, 5% CO_2. Plates were centrifuged and cell-free supernatant collected. Concentration of human IL-2 in the supernatant after T-cell retargeting was determined by an IL-2-Sandwich-ELISA. Anti-human IL-2 antibodies as well as the standard of recombinant human IL-2 was provided by DuoSet IL-2 ELISA Development System kit (R&D Systems, Nordenstadt, Germany) and the assay was performed following the manufacturer's protocol.

Pharmacokinetics

Animal care and all experiments performed were in accordance with federal guidelines and have been approved by university and state authorities. CD1 mice (female, 18-30 weeks, weight between 31-44 g, 3-6 mice/group) received an i.v. injection of 25 µg recombinant antibody in a total volume of 200 µl. In time intervals of 3, 10, 30, 60, 120, 360 min and 24 h blood samples (100 µl) were taken from the tail and incubated on ice. Clotted blood was centrifuged at 10,000 g for 10 min, 4°C and serum samples stored at -20°C. Serum concentrations of CEA-binding recombinant antibodies were determined by ELISA (as described above), interpolating the corresponding calibration curves. For comparison, the first value (3 min) was set to 100%. Pharmacokinetic parameters AUC, $t_{1/2}\alpha$ and $t_{1/2}\beta$ were calculated with Excel using the first 3 times points to calculate $t_{1/2}\alpha$ and the last 3 time points to calculate $t_{1/2}\beta$. For statistics, Student's T-test was applied.

Results

N-glycosylated scDb molecules

To generate N-glycosylated scDb molecules 2 N-glycosylation sequons (NGT) were introduced in the flanking linkers (A, B) and 1 to 7 sequons at C-terminal extensions (Fig. 1). Thus, scDb-ABC_1 contains 3 sequons, scDb-ABC_4 6 sequons, and scDb-ABC_7 9 sequons. These modified scDb molecules were expressed in stably transfected HEK293 cells and purified by IMAC with yields of 8 – 17 mg/L supernatant. SDS-PAGE analysis revealed reduced mobility of these three molecules compared to unmodified scDb depending on the number of sequons (Fig. 2a, Table 1).

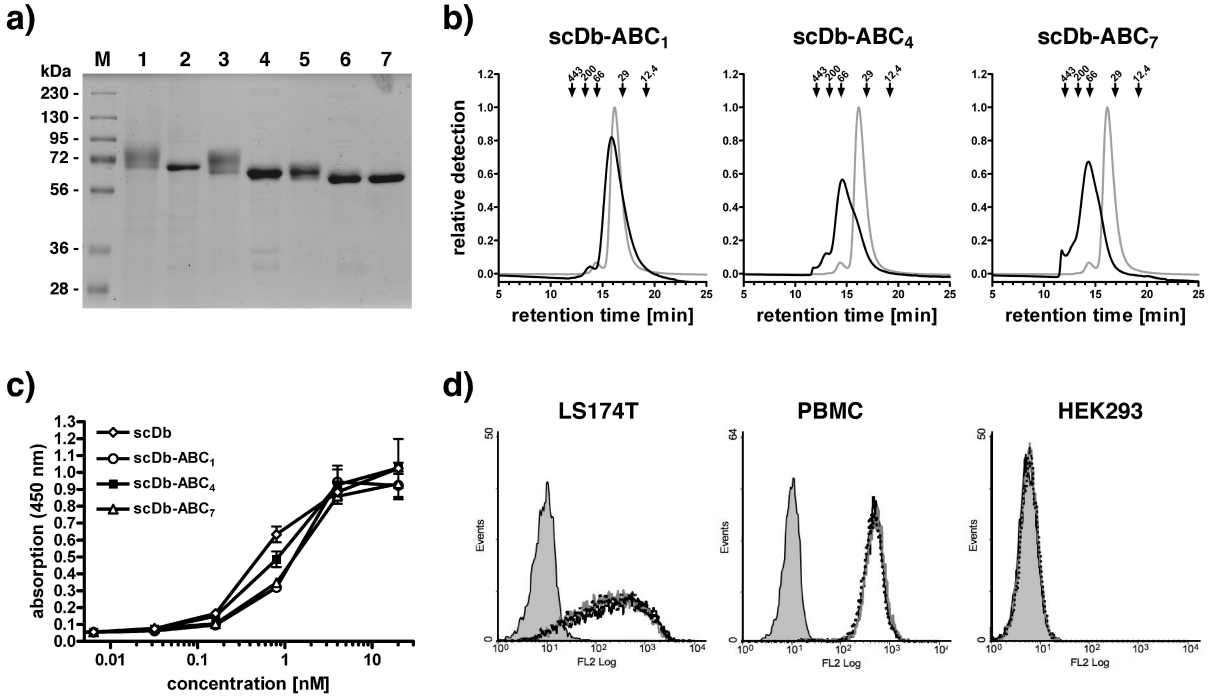

Fig. 2. N-glycosylated scDbs. A) SDS-PAGE analysis of N-glycosylated scDb molecules (1+2 scDb-ABC_7; 3+4, scDb-ABC_4; 5+6, scDb-ABC_1; 7, scDb) before (1, 3, 5, 7) and after (2, 4, 6) treatment with PNGase F. 3 µg purified protein was loaded per lane and the gel was stained with Coomassie b) Size exclusion chromatography of unmodified (gray line) and N-glycosylated scDb molecules (black lines). C) ELISA of binding of unmodified and N-glycosylated scDb variants to immobilized CEA (n=2). D) Flow cytometry analysis of binding of unmodified and N-glycosylated scDb variants to CEA-expressing tumor cells (LS174T), CD3-positive PBMCs, or HEK293 cells included as negative control. Gray filled, cells incubated with secondary antibody alone; black line, scDb-ABC_1; black dotted line, scDb-ABC_4; gray line, scDb-ABC_7.

N-glycosylation of these proteins was confirmed by enzymatic deglycosylation with PNGase F, which reduced the apparent molecular mass to that calculated for the unmodified protein (Fig. 2a). Some heterogeneity in the degree of N-glycosylation was observed. In size exclusion chromatography (SEC) proteins eluted with a major peak corresponding in size to

apparent molecular masses of 36 kDa for unmodified scDb, 38 kDa for scDb-ABC_1, 65 kDa for scDb-ABC_4, and 78 kDa for scDb-ABC_7 (Fig. 2b; Table 1).

Table I. scDb constructs

Construct	length	calc. M_r [a]	apparent M_r (kDa)		Hydrodynamic
			SDS-PAGE	SEC	radius
	aa	*kDa*	*kDa*	*kDa*	*nm*
scDb	505	54.5	62	36	2.7
scDb-ABC_1	497	53.6	62 – 68	38	2.9
scDb-ABC_4	519	55.6	64 – 80	65	3.5
scDb-ABC_7	544	57.8	68 – 87	78	3.8
scDb-A'-PEG_{40k}	505	54.5	230	650	7.9
chimeric IgG	1382	145.1	~ 280	280	5.7
HSA	585	66.5	67	66	3.5
scDb-HSA [c]	1080	119.6	121	90	3.9
scDb-ABD [b]	550	59.3	64	32	2.5
scDb-ABD + HSA [b]	-	125.8	-	150	4.8

[a] calculated from the amino acid sequence without posttranslational modifications.
[b] Data from Ref. 15.
[c] Data from Ref. 4.

As expected from SDS-PAGE analysis scDb-ABC_4 and scDb-ABC_7 eluted over a broad range further indicating size heterogeneity of these proteins. Binding to CEA was not or only marginally impaired by N-glycosylation as shown in ELISA with immobilized CEA and flow cytometry with CEA-expressing cell line LS174T as well as CD3-positive PBMCs (Fig. 2c, d). MALDI-MS further revealed the structural heterogeneity of attached N-glycans, which were of the bi-, tri- and tetra-antennary complex type (Table 2). No high-mannose or hybrid N-glycans were detected. The degree of sialylation of the N-glycans was found to be 34% for scDb-ABC_1, 23% for scDb-ABC_4 and 22% for scDb-ABC_7 as deduced from a semi-quantitative analysis of the MALDI-MS data.

Table 2: N-Glycans of scDbs variants expressed in HEK293 cells

Mass	Glycan structure	Content[a] scDb-ABC_1	scDb-ABC_4	scDb-ABC_7
(m/z)		%	%	%
1836	FH3N4[b]	1.5	0.7	6.8
2040	FH4N4	1.3	6.3	7.8
2081	FH3N5	1.5	3.9	9.5
2244	FH5N4	4.7	8.6	6.4
2285	FH4N5	11.1	13.1	13.5
2326	FH3N6	1.8	1.1	3.6
2418	F2H5N4	5.6	5.6	2.4
2459	F2H4N5	18.5	20.8	5.7
2489	FH5N5	13.6	15.3	20
2500	F2H3N6	6.6	-	0.8

2592	F3H5N4	-	-	-
2605	SFH5N4	7.4	7.5	7.9
2646	SFH4N5	14.2	2.2	-
2693	FH6N5	-	1.2	1.1
2821	SF2H4N5	7	5.2	5.2
2851	SFH5N5	2.2	1.7	5.4
2869	F2H6N5	-	-	-
2966	S2FH5N4	2.3	3.9	1.9
3042	F3H6N5	-	-	-
3212	S2FH5N5	0.7	0.9	1
3216	F4H6N5	-	-	-
3390	F5H6N5	-	-	-
3415	S2FH6N5	-	0.5	-
3504	SFH7N6	-	0.6	0.5
3750	SFH7N7	-	0.9	0.5

[a] Semi-quantitative analysis of MALDI-MS data
[b] F, fucose (desoxyhexose); H, hexose; N, N-acetylhexosamine; S, sialic acid (NeuAc)

PEGylated scDb molecules

For site-directed PEGylation we generated 2 scDb variants having an additional cysteine residue either in linker A (scDb-A') or at the C-terminus (scDb-C') (Fig. 1). Both variants were expressed in stably transfected HEK293 cells and purified by IMAC with yields of 8 and 10 mg/L supernatant, respectively. SDS-PAGE analysis showed under reducing conditions a single band with an apparent molecular mass of 59 kDa and under non-reducing conditions two bands corresponding to monomeric protein (apparent molecular mass 57 kDa) and disulfide-linked proteins (apparent molecular mass of 170 kDa for scDb-A' and 112 kDa for scDb-C') (Fig. 3a). Both proteins were PEGylated by chemical conjugation of a branched 40 kDa mPEG chain ($mPEG_2$-Mal) to reduced scDb-A' and scDb-C' (Fig. 3b). PEGylation resulted in an increase of the molecular mass to approximately 230 kDa as shown by SDS-PAGE analysis. Coupling efficiency were 94% for scDb-A' and 79% for scDb-C' as determined by SEC (Fig. 3c). Both proteins eluted with a major peak corresponding to an apparent molecular mass of approximately 650 kDa and a hydrodynamic radius of 7.9 nm (Table 1). Interestingly, PEGylated scDb-A' (scDb-A'-PEG_{40k}) and scDb-C' (scDb-C'-PEG_{40k}) were not or only weakly recognized by an anti-His-tag antibody (Fig. 3b). Thus, for further analysis a polyclonal rabbit antiserum was produced by immunization with scDb-A'-PEG_{40k}, which recognized both unPEGylated and PEGylated scDb variants in ELISA (not shown). Using this antiserum similar binding of scDb and scDb-A'-PEG_{40k} to immobilized CEA was found (Fig. 3d). Furthermore, PEGylation did not influence binding to CEA-

expressing cell line LS174T or CD3-positive PBMCs as shown by flow cytometry (Fig. 4e). Because of the higher PEGylation efficiency all further studies were performed with scDb-A'-PEG_{40k}.

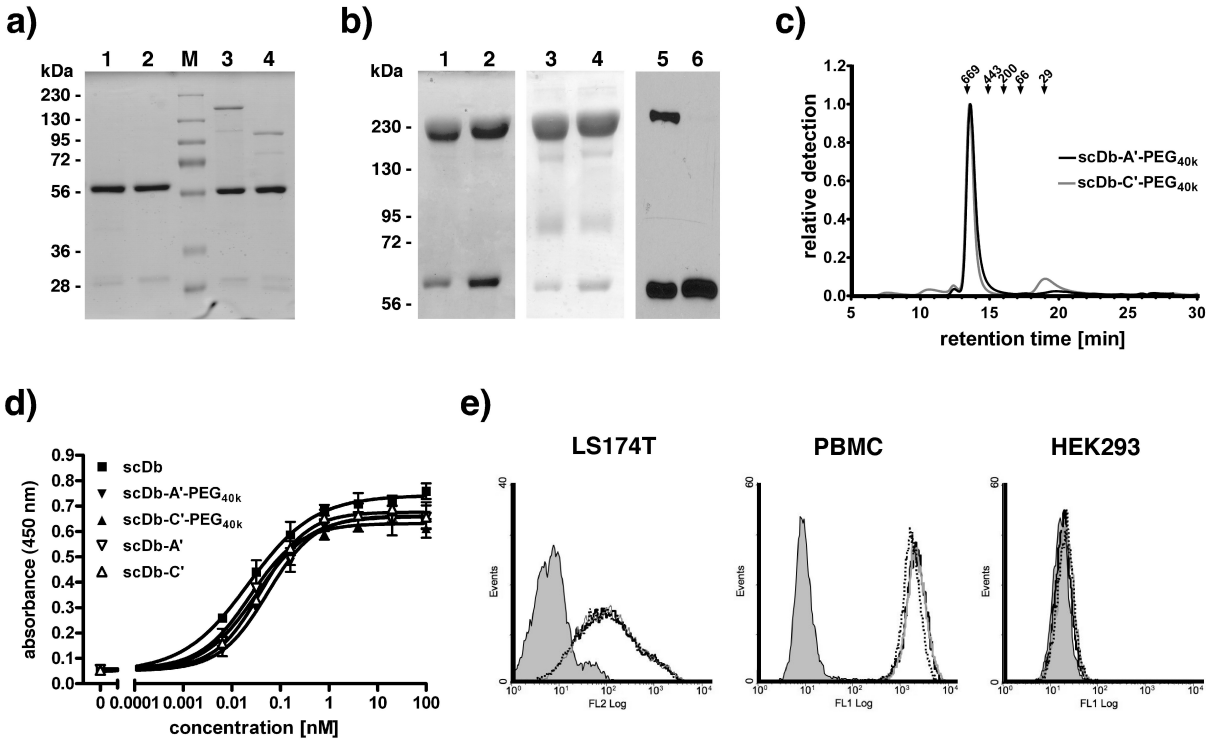

Fig. 3. *PEGylated scDbs.* A) SDS-PAGE analysis of purified scDb-A' (1, 3) and scDb-C' (2, 4) under reducing (1, 2) or non-reducing (3, 4) conditions. 3 µg purified protein was loaded per lane and the gel was stained with Coomassie b) SDS-PAGE analysis of PEGylated scDb-A'-PEG_{40k} (1, 3, 5) and scDb-C'-PEG_{40k} (2, 4, 6). Gels were stained with Coomassie (1, 2), barium iodide (3, 4), or immunoblotted with an anti-His-tag antibody (5, 6). C) Size exclusion chromatography of scDb-A'-PEG_{40k} and scDb-C'-PEG_{40k}. d) ELISA of binding of unmodified and PEGylated scDb molecules to immobilized CEA (n=2). E) Flow cytometry analysis of binding of unmodified and PEGylated scDb molecules to CEA-expressing tumor cells (LS174T), CD3-positive PBMCs, or HEK293 cells included as negative control. Gray filled, cells incubated with immune serum and secondary antibody alone; black line, scDb; black dotted line, scDb-A'-PEG_{40k}; gray line, scDb-C'-PEG_{40k}.

Chimeric anti-CEA IgG1

A chimeric anti-CEA IgG1 was generated by fusing murine anti-CEA V_H and V_L (24) to human constant regions ($C_{\gamma 1}$, C_κ). The antibody was purified from supernatant of stably transfected CHO-K1 cells with yields of 3.7 mg/L supernatant. In SDS-PAGE analysis a single band with an apparent molecular mass of > 250 kDa was observed under non-reducing conditions (Fig. 4a). After reduction two bands with apparent molecular masses of 56 kDa and 29 kDa were observed. By size exclusion chromatography an apparent molecular mass of 280 kDa and a hydrodynamic radius of 5.7 nm was determined (Fig. 4b) (Table 1). The discrepancy between calculated and apparent molecular mass of the intact IgG molecules was

also observed for other recombinant and natural whole antibodies using an established set of standard proteins (see Materials and Methods) as molecular weight markers. This discrepancy is best explained by the Y-shaped structure of IgG molecules, which discerns it from other globular proteins. The purified antibody recognized CEA in ELISA (Fig. 4c) and on CEA-expressing tumor cell line LS174T in flow cytometry (Fig. 4d).

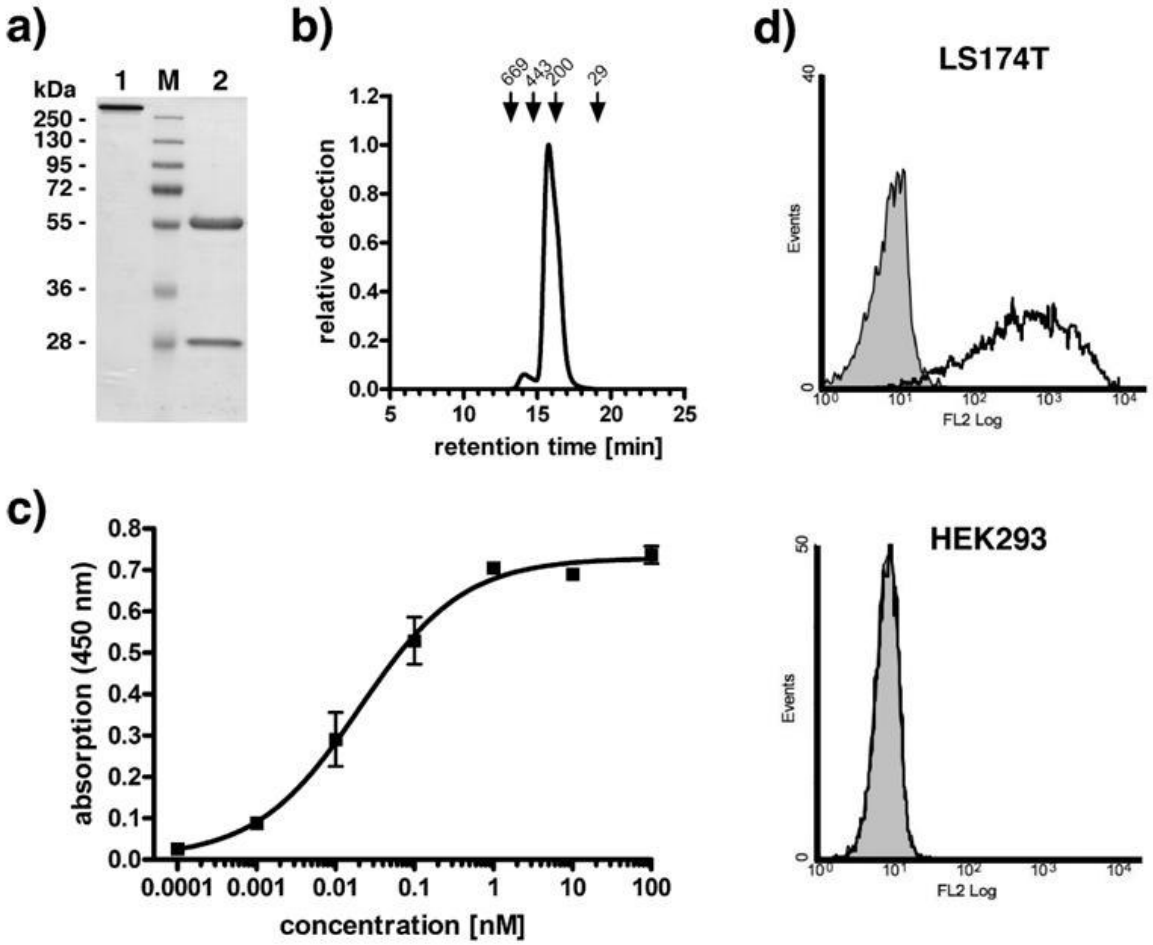

Fig. 4. Chimeric anti-CEA IgG1. a) SDS-PAGE analysis of purified anti-CEA IgG1 under non-reducing (1) or reducing (2) conditions. 5 µg purified protein was loaded per lane and the gel was stained with Coomassie b) Size exclusion chromatography. C) ELISA of binding of anti-CEA IgG1 to immobilized CEA (n=2). D) Flow cytometry analysis of binding of anti-CEA IgG1 to CEA-expressing tumor cells (LS174T) or HEK293 cells included as negative control. Gray filled, cells incubated with secondary antibody alone; black line, anti-CEA IgG1.

Target cell-dependent effector cell activation

The potential of the glycosylated and PEGylated bispecific anti-CEACD3 scDb to activate effector cells (PBMCs) in a target cell-specific manner was determined by an IL-2 release assay. In this assay CEA-expressing cells are incubated with PBMCs in the presence of increasing concentrations of bispecific antibody and stimulation of PBMCs is determined by

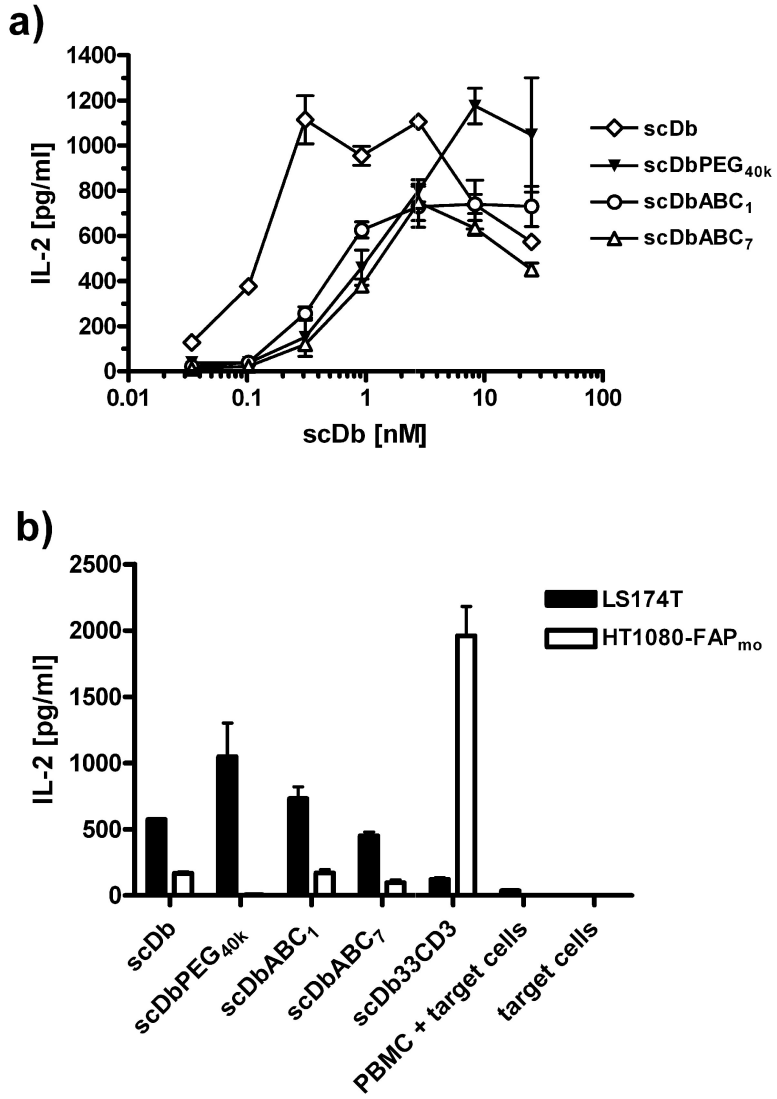

Fig. 5. IL-2 release assays. A) CEA-expressing LS174T were preincubated for 1 h with anti-CEA x anti-CD3 specific scDb, scDb-ABC_1, scDb-ABC_7 or scDb-A'-PEG_{40k} at varying concentrations before adding human PBMCs. IL-2 release was measured after 24 h by ELISA (n=2). B) PBMCs were incubated in presence of LS174T or HT1080-FAP together with 25 nM bispecific anti-CEA x anti-CD3 antibodies scDb, scDb-ABC_1, scDb-ABC_7 and scDb-A'-PEG_{40k} or a control scDb (scDb33CD3) directed against fibroblast activation protein and CD3. IL-2 release was measured after 24 h by ELISA.

Measuring IL-2 release after 24 h. This assay showed that glycosylated scDb (determined for scDb-ABC_1 and scDb-ABC_7) as well as scDb-A'-PEG_{40k} are able to stimulate PBMCs. However, both modifications reduced stimulatory efficacy of the scDb (Fig. 5a). Unmodified scDb mediated a maximal release of approximately 1.2 ng/ml IL-2 with an EC_{50} of 0.14 nM and an optimum between 0.3 – 3 nM. ScDb-A'-PEG_{40k} reached a similar maximal release although with an EC_{50} of 1.3 nM. ScDb-ABC_1 and scDb-ABC_7 reached only a maximal IL-2 release of approximately 0.8 ng/ml with an EC_{50} of 0.4 and 0.9 nM, respectively. Selectivity of target cell-dependent stimulation was shown with control experiments using CEA-negative

HT1080 cells stably transfected to express fibroblast activation protein (FAP). All scDb CEACD3 molecules (at a concentration of 25 nM) mediated no or only marginal stimulation of PBMCs, while a control scDb33CD3 directed against FAP and CD3 showed strong stimulation of PBMCs (Fig. 5b).

Pharmacokinetic properties

Pharmacokinetics was determined after a single dose (25 µg) i.v. injection into CD1 mice (Fig. 6; Table 3).

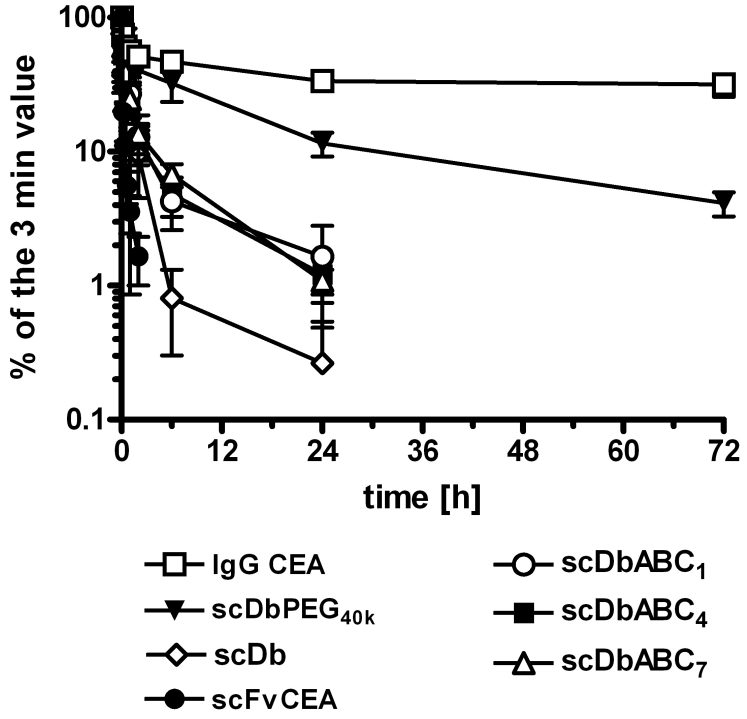

Fig. 6. Pharmacokinetics. 25 µg of antibody molecules were injected i.v. into CD1 mice. Serum concentrations of the antibody molecules were determined at different time points by ELISA. Data were normalized considering maximal concentration at the first time point (3 min).

All constructs showed a biphasic elimination from circulation. The glycosylated scDb molecules were all cleared similarly from circulation with an approximately 2-fold increased initial half-life ($t_{1/2}\alpha$) compared to unmodified scDb, while terminal half-life ($t_{1/2}\beta$) was increased by a factor 1.1 to 1.6. Compared to scDb, the area under the curve ($AUC_{0\text{-}24h}$) was increased 2.3-fold for scDb-ABC_1 and scDb-ABC_4, and 2.9-fold for scDb-ABC_7 (Table 2). ScDb-A'-PEG_{40k} showed markedly improved pharmacokinetics. Compared to unmodified scDb, half-lives were increased 2.3- to 5-fold and AUC increased by a factor of 10.6.

Chimeric anti-CEA IgG1 showed the strongest improvement of pharmacokinetics with 7-fold increase of the terminal half-life and a 17-fold increase of the AUC (Table 3).

Table 3. Pharmacokinetic properties of antibody constructs

Construct	$t_{1/2}\alpha$	$t_{1/2}\beta$	AUC(0-24h)
	min	*h*	*%*h*
scFv [2]	5.6 ± 0.7	0.6 ± 0.2[a]	16 ± 4 [a]
scDb	10.2 ± 3.6	5.6 ± 1.8	61 ± 22
scDb-ABC_1	19.4 ± 3.6	8.9 ± 3.0	142 ± 28
scDb-ABC_4	22.1 ± 4.7	7.2 ± 0.9	142 ± 36
scDb-ABC_7	19.7 ± 5.4	6.2 ± 1.0	174 ± 20
scDb-A'-PEG_{40k}	31 ± 19.2	13.1 ± 3.3	646 ± 316
scDb-HSA[b]	63.6 ± 31.8	25 ± 2.4	608 ± 83
scDb-ABD[c]	64.2 ± 37.2	27.6 ± 6.0	861 ± 210
chimeric IgG	43.8 ± 10.9	39 ± 13.7	1038 ± 345

[a] determined for up to 2 h only.
[b] Data from Ref. 4
[c] Data from Ref. 15

Discussion

We have generated N-glycosylated scDb by genetically introducing several N-glycosylation sites into linker sequences connecting the variable domains as well as a C-terminal extension of varying length. All scDb variants containing 3, 6 or 9 sequons were expressed in glycosylated form in HEK293 cells. Yields were similar to that obtained for the unmodified scDb indicating that the modifications do not interfere with translation and secretion into the cell culture supernatant. SDS-PAGE analysis revealed heterogeneity in the degree of N-glycosylation. This finding indicates that not all sites are glycosylated with the same efficiency, especially in the scDb-ABC_7 construct containing 7 sequons in the C-terminal extension in close proximity to each other.

All N-glycosylated constructs were biologically active and recognized CEA and CD3 similar to unmodified scDb as shown by ELISA and flow cytometry analysis. Furthermore, a target cell-dependent IL-2 release assay demonstrated the capability to simultaneously bind both antigens on cells and thus to stimulate effector cells. However, compared to unmodified scDb the N-glycosylated scDb variants showed an approximately 3- to 5-fold reduced stimulatory activity. Similar observations were made with a PEGylated scDb as well as for fusion proteins of scDb and human serum albumin or an albumin-binding domain of streptococcal protein G

(4, 15). These findings indicate that modifications of the scDb structure and size have a negative effect on stimulation of PBMCs, which might be caused by interfering with the formation of close contacts between target and effector cells. In order to investigate if this is a general phenomenon we have initiated studies investigating other scDb molecules with different target cell specificity.

Glycosylation of scDb led to a prolonged circulation with a 2.3 to 2.9-fold increase of the AUC. Interestingly, pharmacokinetic properties of the three N-glycosylated scDb variants did not differ significantly. This finding indicates that the addition of 3 N-glyosylation sites is sufficient to prolong circulation time. These three sites increase the molecular mass by approximately 6 kDa as determined by SDS-PAGE analysis leading to a small increase of the Stokes radius from 2.7 to 2.9 nm. For hyperglycosylated erythropoietin (darbepoetin alpha) with a molecular mass of 37 kDa an approximately 2-fold increase of the terminal half-life was observed after i.v. injection into rats and a 3-fold increase of the terminal half-life from 8.5 h for normally glycosylated epoetin alpha to 26.3 h for darbepoetin alpha in dialysis patients (19). Glycosylated FSH analogs containing 2 or 4 N-glycosylation sites and a molecular mass of approximately 55 kDa exhibited a 2- to 4-fold increase of the AUC after i.v. injection into rats (20, 21). Thus, for hyperglycosylated erythropoietin and glycosylated FSH a similar increase in half-lives was observed as for our glycosylated scDb variants. These findings indicate that the addition of a few N-glycans improves the pharmacokinetic properties of a protein leading to a moderately increase in circulation time by a factor of 2 to 4.

Comparison of an anti-CEA Fab' molecule with a natural N-glycosylation site in the V_L domain obtained from hybridoma-produced IgG (thus being glycosylated) and a bacterially expressed non-glycosylated Fab' revealed faster elimination from circulation and reduced accumulation in the tumor of the non-glycosylated Fab' (27). Accelerated clearance was also described for the non-glycosylated forms of various other glycoproteins, including antithrombin, lymphotoxin, interferon-γ, and granulocyte macrophage colony-stimulating factor (GM-CSF), further underlying the importance of N-glycosylation for pharmacokinetics (28-31).

The type of N-glycosylation plays an important role in determining the circulation time of glycoproteins. Thus, an engineered N-glycosylated scFv containing high-mannose carbohydrate chains at a C-terminal extension was more rapidly cleared from circulation than the unmodified scFv (32). Similar observations were made for interferon-γ (29). We found that the three N-glycosylated scDb variants all contained N-glycans of the complex type (bi-,

tri- and tetra-antennary). No hybrid or high-mannose N-glycans could be detected. These complex type N-glycans were composed of a mixture of sialylated and non-sialylated chains. The predominant N-glycans found in all three N-glycosylated scDb variants are FH4N5, F2H4N5 and FH5N5, which account for approximately 40-50% of the N-glycans. These N-glycans differ from those found in the Fc-regions of antibodies, which bear mainly FH3N4 (G0F) oligosaccharides (33). The N-glycosylation pattern of the scDb variants is, however, similar to that found in other recombinant glycoproteins expressed in HEK293 cells (34). Glycosylation of recombinant antibodies and other proteins is influenced by the cell line and conditions used for expression (18, 35). Thus, it was found that expression of glycoproteins in CHO, NSO, and Sp2/0 cells can produce abnormally glycosylated antibodies that lack potency and are potentially immunogenic (36). For example, murine NSO cells express more heterogeneous oligosaccharide patterns, e.g. than CHO cells, with a tendency for a-linked galactose residues and N-glycolylneuraminic acid absent in glycoproteins from primate cell lines (37). Furthermore, experimental data indicate that N-glycosylation pattern are also protein dependent, e.g. as shown by studies of the glycosylation pattern of recombinant osteonectin, TSC-36 and bone sialoprotein expressed in HEK293 cells (34, 38, 39).

In vivo experiments have shown that the extent and type of sialylation influences serum half-lives of recombinant proteins (32, 40). Hence, the pharmacokinetic properties of our N-glycosylated scDb molecules might be further improved using expression systems leading to increased production of fully sialylated N-glycans with α2,6-linked terminal sialic acid residues (41).

PEGylation of scDb strongly improved pharmacokinetics of scDb, similarly to fusion of scDb to serum albumin or an albumin-binding domain as shown in previous studies (see also Table 1 and 3) (4, 15). We found that conjugation of a 40 kDa branched mPEG chain strongly increased the hydrodynamic radius of the protein from 2.7 to 7.9 nm as determined by SEC. Similar observations were made for other PEGylated recombinant antibodies (42). Thus, the increased circulation time is likely caused by a reduced renal clearance. The introduction of a cysteine residue into one of the flanking linkers or at the C-terminus allowed for a site-directed and efficient coupling of a single PEG molecule. Several studies showed that random PEGylation of whole antibodies or Fab fragments can reduce antigen-binding activity depending on number and length of conjugated PEG chains (summarized in 10). However, reduced antigen-binding was also described for PEGylated antibody molecules generated by site-directed PEGylation mainly caused by reduced target association rates (43). We could not observe a reduced binding of the modified scDb molecules to each antigen separately as

shown in ELISA and flow cytometry experiments, although for all modified scDb reduced activity was observed in IL-2 release assays where simultaneous binding is required.

None of the applied strategies reached the pharmacokinetics observed for a chimeric IgG1, which exhibited a 17-fold higher AUC than scDb. Similar to the albumin-based strategies the long circulation time of IgG is based on a reduced renal clearance and on FcRn-mediated recycling processes (44). However, currently we do not know to which extent the latter contributes to pharmacokinetics of IgG and the albumin-utilizing modifications. A comparison of the pharmacokinetics in wild-type and FcRn knock-out mice revealed a reduction of terminal half-life of albumin (from 39 h to 24 h) and IgG (from 95 h to 19 h), while the half-life of IgA was not affected (45). This finding indicates that FcRn-mediated recycling contributes to different extents to pharmacokinetics of albumin and IgG in mice.

In summary, we have completed our comparative analysis of various strategies to prolong circulation time of a bispecific single-chain diabody including fusion to albumin (4), fusion to an albumin-binding domain (ABD) of streptococcal protein G (15), PEGylation and N-glycosylation. These studies established that in mice longest circulation time is obtained by fusion of scDb to an ABD (14-fold increase of AUC), followed by a scDb-HSA fusion protein and PEGylated scDb (approximately 10-fold increase of AUC), while N-glycosylation in HEK293 cells resulted only in a moderate increase in circulation time (2- to 3-fold increase of AUC) (summarized in Table 3). Further studies have now to demonstrate how these increases in circulation time translate into improved efficacy in vivo.

Footnotes

Abbreviations: HSA, human serum albumin; ABD, albumin-binding domain. We would like to thank Sabine Münkel (IZI, Stuttgart) for help with HPLC analysis and Prof. Dr. Arnd G. Heyer (University of Stuttgart) for initial help in glycoanalysis. This work was supported by a grant from the Deutsche Forschungsgemeinschaft (Ko1461/2). # present address: multimmune GmbH, c/o Klinikum Rechts der Isar der TU München, Ismaninger Straße 22, 81675 München, Germany.

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Biodistribution of a bispecific single-chain diabody and its half-life extended derivatives

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Abstract

Small recombinant antibody molecules such as bispecific single-chain diabodies (scDb) possessing a molecular mass of approximately 55 kDa are rapidly cleared from circulation. We have recently extended the plasma half-life of scDb applying various strategies including PEGylation, N-glycosylation and fusion to an albumin-binding domain (ABD) from streptococcal protein G. Here, we further analyzed the influence of these modifications on the biodistribution of a scDb directed against carcinoembryonic antigen (CEA) and CD3 capable of retargeting T cells to CEA-expressing tumor cells. We show that a prolonged circulation time results in an increased accumulation in CEA^+ tumors, which was most pronounced for scDb-ABD and PEGylated scDb. Interestingly, tumor accumulation of the scDb-ABD fusion protein was approximately 2-fold higher compared to PEGylated scDb, although both molecules exhibit similar plasma half-lives and similar affinities for CEA. Comparing half-lives in neonatal Fc receptor (FcRn) wild-type and FcRn heavy chain knockout mice the contribution of the FcRn to the long plasma half-life of scDb-ABD was confirmed. Half-life of scDb-ABD was approximately two-fold lower in the knockout mice, while no differences were observed for PEGylated scDb. Binding of the scDb derivatives to target and effector cells was not or only marginally affected by the modifications, although, compared to scDb, a

reduced cytotoxic activity was observed for scDb-ABD, which was further reduced in the presence of albumin. In summary, these findings demonstrate that the extended half-life of a bispecific scDb translates into improved accumulation in antigen-positive tumors but that modifications might also affect scDb-mediated cytotoxicity.

Introduction

Bispecific single-chain diabodies (scDb) are recombinant molecules composed of the variable heavy and light chain domains of two antibodies connected by three linkers in the order V_HA-V_LB-V_HB-V_LA (1). These domains assemble into molecules with a compact structure and molecular masses of approximately 55 kDa. Bispecific single-chain diabodies have been developed for various applications including the retargeting of cytotoxic T lymphocytes to tumor cells for cellular cancer therapy (2).

Although scDb are capable of efficiently retargeting effector cells to tumor cells the small size leads to their rapid elimination after i.v. injection. The terminal half-life of these molecules in mice is only in the range of 5-6 h, compared to several days for whole IgG molecules (3,4). The fast clearance of such small molecules from circulation hampers therapeutic applications, e.g. requiring infusions or repeated injections to maintain a therapeutically effective dose over a prolonged period of time (5). For example, a bispecific tandem scFv directed against CD19 and CD3 (blinatumomab) having a similar size as an scDb molecule had to be given as an 8 week infusion (maximum dose 60 $\mu g/m^2$ per day) in a clinical phase I trial for the treatment of B cell lymphoma patients (6).

In order to extend plasma half-lives of therapeutic proteins and thus to improve pharmacokinetics and pharmacodynamics, several strategies can be applied (7). Strategies such as conjugation of polyethylene glycol chains (PEGylation) or production of hyperglycosylated variants through introduction of additional N-glycosylation sites primarily aim at increasing the hydrodynamic volume of the molecule, thus reducing renal filtration and degradation. Some of these strategies further implement FcRn-mediated recycling processes, e.g. fusion to the IgG Fc region and fusion or binding to serum albumin.

We recently applied several of these strategies to improve the plasma half-life of a scDb molecule. These strategies included site-directed conjugation of a 40 kDa PEG chain (PEGylated scDb, scDb-A'-PEG_{40k}), production of N-glycosylated scDb variants possessing 3, 6, or 9 N-glycosylation sites (scDb-ABC_{1-7}), a scDb-human serum albumin fusion protein (scDb-HSA), and a scDb fused to an albumin-binding domain from streptococcal protein G

(scDb-ABD) (3,4,8). In these studies we showed that N-glycosylation only moderately increased half-life, while a strong improvement was observed for the PEGylated scDb, scDb-HSA and scDb-ABD.

In the present study we further analyzed the biodistribution of unmodified scDb as well as three of the scDb derivatives (PEGylated scDb, N-glycosylated scDb, scDb-ABD) in tumor-bearing mice. We show that the modified scDb molecules exhibit a reduced renal clearance and that an extended half-life leads to an increased accumulation in antigen-positive tumors. The strongest improvement was observed for scDb-ABD. Using FcRn knockout mice we confirmed that FcRn-mediated recycling contributes to the long half-life of scDb-ABD. Affinities of the scDb derivatives for target and effector cells were not or only marginally affected by the modifications, although, compared to scDb, a reduced cytotoxic activity was observed for scDb-ABD, which was further reduced in the presence of albumin. These findings demonstrate that half-life extension of scDb results in increased tumor accumulation but that modifications might also affect scDb-mediated cytotoxicity.

Experimental Procedure

Materials

HRP-conjugated anti-His-tag antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, USA), unconjugated mouse anti-His-tag antibody from Dianova (Hamburg, Germany) and anti-rabbit IgG-FITC or anti-mouse IgG-PE-conjugated antibody from Sigma (Taufkirchen, Germany). Carcinoembryonic antigen was obtained from Europa Bioproducts (Cambridge, UK). HSA was purchased from Sigma. The human colon adenocarcinoma cell line LS174T was purchased from ECACC (Wiltshire, UK) and cultured in RPMI, 5% FBS, 2 mM glutamine (Invitrogen, Karlsruhe, Germany). The stably transfected human fibrobast activation protein (FAP)-expressing fibrosarcoma cell line HT1080 FAP_{hu} (kindly provided by W. Rettig, Boehringer Ingelheim Pharma, Vienna, Austria) were grown in RPMI, 5% FBS, 2 mM glutamine. Buffy coat from healthy human donors were obtained from the blood bank (Ulm, Germany). IL-2 was purchased from Immunotools (Friesoythe, Germany) and phytohemagglutinin-L (PHA-L) from Boehringer-Mannheim (Mannheim, Germany). FcRn heavy chain knockout mice (strain B6.129X1-*Fcgrt*^{tm1Dcr}/Dcr) were purchased from Jackson Laboratories (Bar Harbor, USA). Athymic female nude mice were purchased from Harlan Laboratories (Indianapolis, USA). For iodination ^{125}I and ^{131}I from Perkin Elmer (Boston,

USA) was used.

Biodistribution

ScDb and the various scDb derivatives were prepared as described previously (3,4,8). Antibody constructs were iodinated by the Iodogen method (Pierce Chemical Co., Rockford, USA) yielding a specific activity of 1.4 – 2.2 µCi/µg. 6 weeks old athymic nude mice were xenografted s.c. with 8 x 10^5 MC-38 murine colon carcinoma cells on the right flank and 10^6 LS-174T human colon carcinoma cells on the left flank. When the tumors reached a volume of approximately 500 mm^3, lugol iodine solution was added to the drinking water one day before the injection of the radiolabeled biomolecules. 4 groups of 3 mice were injected i.v. with a mix of 5 µg of ^{125}I-scDb and 5 µg of ^{131}I-scDb-ABD or ^{125}I-scDb-A'-PEG_{40k} and ^{131}I-scDb-ABC_7. Groups of mice were sacrificed at 2, 24, 48 or 96 h after i.v. injection. Blood, tumors and tissues were weighed, and the radioactivity counted in a dual channel scintillation counter (CobraTM II, Packard).

ELISA

Carcinoembryonic antigen (CEA) (300 ng/well) was coated overnight at 4 °C and remaining binding sites were blocked with 2% (w/v) skimmed milk powder/PBS. Purified recombinant antibodies and serum samples were titrated in duplicates and incubated for 1 h at RT. Detection was performed either with mouse HRP-conjugated anti-His-tag antibody or rabbit antiserum against PEGylated scDb and HRP-conjugated goat anti-rabbit antibody (8) using TMB substrate (1 mg/ml TMB, sodium acetate buffer pH 6.0, 0.006% H_2O_2). The reaction was stopped with 50 µl of 1 M H_2SO_4. Absorbance was measured at 450 nm in an ELISA-reader.

Affinity measurements

Affinities of scDb-ABD for HSA at different pH were determined by quartz crystal microbalance measurements (A-100, Attana). HSA was chemically immobilized on a carboxyl sensor chip according to the manufacturer's protocol at a density resulting in a signal increase of 120 Hz. Binding experiments were performed in PBS pH 7.4 or 50 mM sodium phosphate buffer, 150 mM NaCl, pH 6.0 at a flow rate of 25 µl/min. The chip was regenerated with 6.3 µl 10 mM HCl. Before every measurement a baseline was measured which was subtracted from the binding curve. A mass transport model (9) was fitted to the data.

Flow cytometry

Binding to CEA- and CD3-expressing cells were determined by flow cytometry (10). LS174T or Jurkat-CD3 cells (2 x 10^5) were incubated with dilution series of antibodies for 3 h at 4 °C. Cells were then washed with PBS and bound antibodies were detected with mouse anti-His-tag antibody and PE-labeled goat anti-mouse antibody or, in case of the PEGylated construct, with rabbit antiserum (8) and FITC-labeled goat anti-rabbit IgG antibody. Data were fitted with GraphPrism software (La Jolla, USA) from 3 independent binding curves. From these 3 individual EC_{50} the mean and standard error was calculated.

Pharmacokinetics

Animal care and all experiments performed were in accordance with federal guidelines and have been approved by university and state authorities. C57BL/6 and BL/6 FcRn heavy chain knockout mice (20-33 weeks, weight between 21-39 g) received an i.v. injection of 25 µg scDb-ABD or PEGylated scDb in a total volume of 200 µl. In time intervals of 3 min, 3 h, 1, 2, 3, and 4 days blood samples (100 µl) were taken from the tail and incubated on ice. Clotted blood was centrifuged at 10 000 g for 10 min, 4 °C and serum samples stored at -20 °C. Serum concentrations of CEA-binding recombinant antibodies were determined by ELISA (as described above), interpolating the corresponding calibration curves. For comparison, the first value (3 min) was set to 100%. Terminal half-lives ($t_{1/2}\beta$) and AUC were calculated with Excel. For statistics, Student's T-test was applied.

In vitro cytotoxicity

Cytotoxicity assays were performed according to Asano et al. (11). 15.000 LS174T or 5.000 HT1080 FAP_{hu} cells per well were seeded into 96-well plates grown over night. Dilution series of antibodies were then added to the target cells. Peripheral blood mononuclear cells (PBMCs) from a healthy donor were isolated from buffy coat as described before (3). PBMCs were thawed the day before and seeded on a cell culture dish to remove monocytes by the attachment to the plastic surface. Cells that remained in suspension were preactivated with 1 µg/ml PHA-L and 100 U/ml IL-2 (3) for at least 3 days. These preactivated PBMCs were added to the target cells in an E:T ratio of 3:1 and incubated for 24 h. After the wells were washed 3 times with PBS, 100 µl medium with 50 µg/ml MTT (methylthiazolyldiphenyl-tetrazolium bromide) (Sigma, Taufkirchen, Germany) was added and cells were incubated for

2 h. Then 100 µl of lysis buffer (10% SDS, 50% N,N-dimethylformamid, pH 4.7) was added and wells were incubated over night. $Abs_{595\ nm}$ - $Abs_{655\ nm}$ was measured in an ELISA reader and data were normalized to the values of the untreated cell.

Results

Biodistribution

Single-chain diabody scDb CEACD3 and three derivatives thereof (PEGylated scDb, scDb-A'-PEG_{40k}; N-glycosylated scDb possessing 9 NXT sequons, scDb-ABC_7; scDb fused to an albumin-binding domain, scDb-ABD) were labeled with ^{125}I or ^{131}I and injected into nude mice bearing CEA^+ LS174T and CEA^- MC38 tumors. Tissue distribution was monitored over a period of 4 days (Fig. 1; Table 1).

Table 1. AUC of the various scDb constructs in different tissues (%ID/g * h).

tissue	scDb	scDb-ABC_7	scDb-A'-PEG_{40k}	scDb-ABD
blood	77.7 ± 3.1	110.0 ± 2.9	924.9 ± 34.3	767.8 ± 18.3
CEA^+ tumor	167.7 ± 19.8	149.9 ± 6.4	450.0 ± 13.8	752.5 ± 46.6
CEA^- tumor	66.5 ± 14.3	45.7 ± 3.8	170.9 ± 8.7	225.8 ± 23.3
liver	50.0 ± 2.9	85.3 ± 2.1	251.9 ± 11.3	202.6 ± 12.8
kidney	334.0 ± 28.6	81.2 ± 5.4	262.7 ± 8.3	217.0 ± 7.5
lung	64.9 ± 14.3	66.7 ± 2.2	354.8 ± 15.6	377.1 ± 4.3
spleen	39.6 ± 0.6	56.0 ± 3.4	145.7 ± 4.6	119.4 ± 6.2
heart	33.2 ± 2.4	44.9 ± 1.5	227.6 ± 15.6	239.8 ± 13.2
muscle	16.7 ± 0.5	24.5 ± 1.2	67.7 ± 4.2	70.6 ± 9.7
bone	25.5 ± 3.7	32.1 ± 1.8	81.3 ± 1.4	89.2 ± 5.1
skin	58.7 ± 1.1	58.1 ± 4.4	147.6 ± 4.2	172.0 ± 9.7
intestine	32.9 ± 6.5	38.3 ± 3.5	95.0 ± 20.8	78.7 ± 2.2
colon	23.6 ± 2.9	27.5 ± 1.3	63.1 ± 5.9	53.8 ± 3.1

Unmodified scDb was rapidly cleared from the blood and showed strong kidney accumulation after 2 h (Fig. 1a).

A selective accumulation within the CEA^+ tumor was observed with the highest value (approximately 7 %ID/g) after 2 h. Similar values were found for the N-glycosylated scDb (scDb-ABC_7), which, however, showed after 2 h as compared to scDb a strongly reduced accumulation in the kindey (Fig. 1b). PEGylated scDb and scDb-ABD had a strongly increased residence in the blood, which resulted also in increased values in all the other organs (Fig. 1c, d). Both modified scDb variants exhibited an increased and selective accumulation in the CEA^+ tumors reaching maximum values after 24 to 48 hours, while concentrations in the CEA^- tumors gradually dropped similar to the other organs and tissues

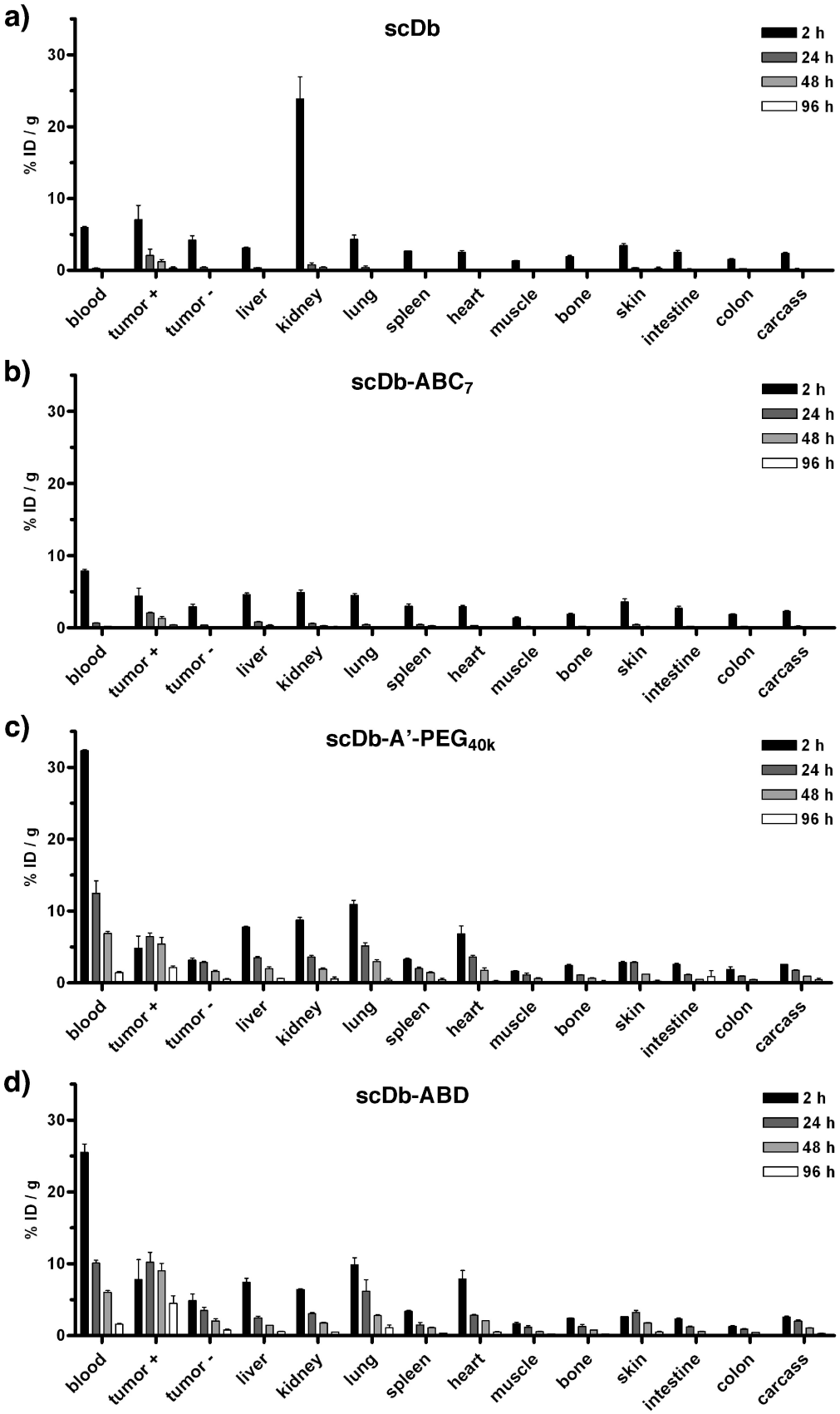

Fig. 1. Organ distribution of ^{131}I-labeled scDb (a), ^{131}I-labeled glycosylated scDb-ABC$_7$ (b), ^{125}I-labeled scDb-A'-PEG$_{40k}$ (c), and ^{125}I-labeled scDb-ABD (d) in nude mice bearing subcutaneous CEA$^+$ (LS174T) and CEA$^-$ (MC38) tumors.

analyzed. Enrichment in CEA^+ tumors was also evident from increased tumor-to-blood ratios in comparison to CEA^- tumors (Fig. 2). Over the period of 4 days scDb reached a maximal tumor-to-blood ratio in CEA^+ tumors of approximately 13 after 2 days, while that of scDb-ABC_7 reached only a value of approximately 8 after 4 days (Fig. 2a). Tumor-to-blood ratios of scDb-A'-PEG_{40k} and scDb-ABD in CEA^+ tumors were weaker and gradually increased to a value of 1.5 and 3, respectively, at day 4 (Fig. 2b). The tumor-to-blood ratio in CEA^- tumors did not change over the period of 4 days, except for scDb, which was slightly higher than that of scDb-ABC_7 reaching a value of approximately 4 after 4 days.

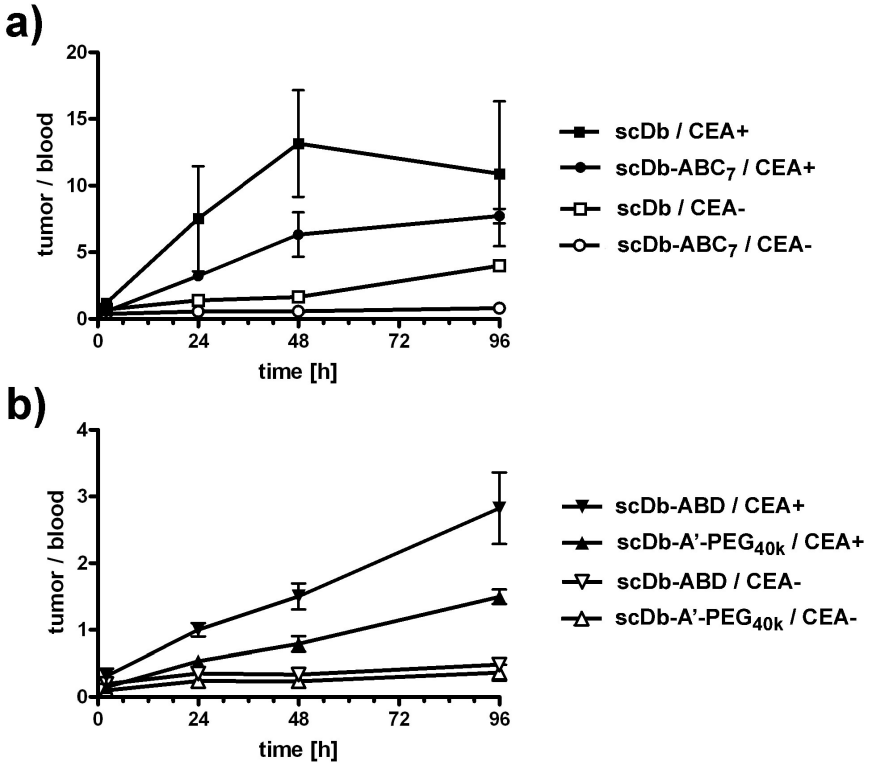

Fig. 2. Tumor-to-blood ratios over the period of 4 days of a) ^{131}I-labeled scDb and ^{131}I-labeled glycosylated scDb-ABC_7, and b) ^{125}I-labeled scDb-A'-PEG_{40k} and ^{125}I-labeled scDb-ABD in CEA^+ (LS174T) and CEA^- (MC38) tumors.

Over the period of 4 days, PEGylated scDb showed as compared to scDb a 12-fold increase in AUC in the blood, while that of scDb-ABD was increased 10-fold. The blood AUC of scDb-ABC_7 was only increased 1.4-fold. The strongest accumulation in the CEA^+ tumor was found for scDb-ABD with a 4.5-fold increase in AUC as compared to scDb. The AUC of the PEGylated scDb in the CEA^+ tumor was increased 2.7-fold. Comparing the AUC between CEA^+ and CEA^- tumors we observed for all constructs 2.5 to 3.3-fold higher values in the CEA^+ tumors. Tumor-to-blood ratios for CEA^+ tumors were 2.2, 1.4, 0.5, and 1.0 for scDb,

scDb-ABC_7, PEGylated scDb, and scDb-ABD, respectively, as determined from the AUC. Tumor-to-blood ratios for CEA^- tumors were 0.9, 0.4, 0.2, and 0.3 for scDb, scDb-ABC_7, PEGylated scDb, and scDb-ABD, respectively, as determined from the AUC.

Binding to CEA- and CD3-expressing cells

Binding of scDb and the half-life extended derivatives to CEA-expressing LS174 cells and CD3-expressing Jurkat cells was determined by flow cytometry measurements (Fig. 3, Table 2). All molecules exhibited a similar EC_{50} for CEA^+ LS174T (in the range of 0.7 to 1.2 nM). Also, EC_{50} values of scDb, scDb-ABD and PEGylated scDb for $CD3^+$ Jurkat cells were in a similar range (between 0.4 to 0.8 nM). ScDb-ABC_7 showed a somewhat reduced binding for $CD3^+$ cells (EC_{50} = 3.4 nM), however, this difference was statistically not significant (p = 0.13). EC_{50} values of scDb and scDb-ABD for both antigens were not significantly affected by the presence of 1 mg/ml HSA (scDb ± HSA: p = 0.73 for binding to LS174T and p = 0.16 for binding to Jurkat; scDb-ABD ± HSA: p = 0.99 for binding to LS174T and p = 0.5 for binding to Jurkat).

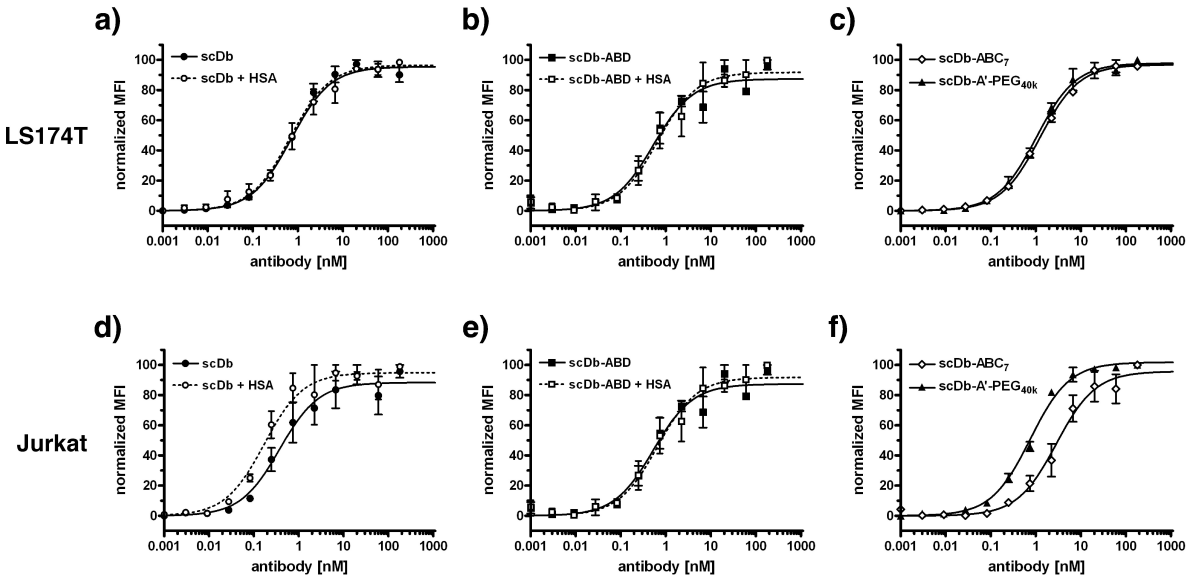

Fig. 3: Flow cytometry analysis of binding of scDb and its half-life extended derivatives to CEA-expressing LS174T (a-c) and CD3-positive Jurkat cells (d-f). (n = 3). For scDb and scDb-ABD the EC_{50} was determined in the absence or presence of HSA (1 mg/ml).

Table 2. Binding of scDb and its derivatives to cell surface-expressed CEA and CD3

construct	HSA	EC_{50} for LS174T	EC_{50} for Jurkat
		nM	*nM*
scDb	-	0.7 ± 0.1	0.4 ± 0.1
scDb	+	0.8 ± 0.2	0.2 ± 0.03
scDb-ABD	-	0.9 ± 0.4	0.6 ± 0.2
scDb-ABD	+	0.9 ± 0.4	1.3 ± 0.8
scDb-A'-PEG_{40k}	-	0.7 ± 0.1	0.8 ± 0.04
scDb-ABC_7	-	1.2 ± 0.01	3.4 ± 1.6

Affinity of scDb-ABD for HSA

The affinity of scDb-ABD for HSA was determined by quartz crystal microbalance measurements (Fig. 4; Table 3). At neutral pH (pH 7.4) scDb-ABD exhibited an affinity of 1.7 nM for HSA. At acidic pH (pH 6.0) affinity was 0.9 nM for HSA. These findings confirm that scDb-ABD is capable of binding to serum albumin at both neutral and acidic pH (4).

Table 3. Binding of scDb-ABD to HSA at pH 7.4 and 6.0 analyzed by QCM

pH	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	K_D (nM)
7.4	2.0×10^5 ± 3.0×10^3	3.5×10^{-4} ± 4.5×10^{-6}	1.7 ± 0.04
6.0	4.3×10^5 ± 1.1×10^4	4.1×10^{-4} ± 8.7×10^{-6}	0.9 ± 0.05

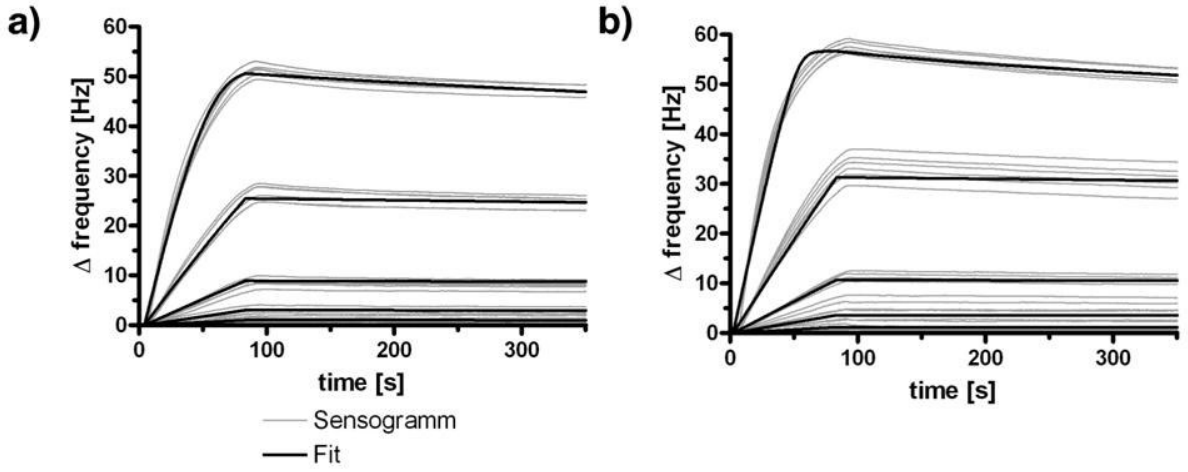

Fig. 4. QCM affinity measurements of scDb-ABD binding to immobilized HSA at pH 7.4 (a) and pH 6.0 (b) (n = 6). Data were fitted (bold lines) assuming mass-limited transport.

Pharmacokinetics of scDb-ABD in FcRn heavy chain knock-out mice

Next, we analyzed clearance of scDb-ABD in C57BL/6 wild-type and FcRn heavy chain knockout mice. After a single dose i.v. injection into the tail vein blood samples were taken and analyzed by ELISA for the presence of active molecules (Fig. 5). A terminal half-life of 53.0 ± 10.0 h was determined for scDb-ABD in wt mice (n=10), while half-life was reduced to 24.8 ± 2.2 h in the FcRn knockout mice (n=10). In contrast, no differences of the terminal half-lives were observed for scDb-A'-PEG_{40k}, included as control, with 47.9 ± 2.7 h in wt mice (n=2) and 51.4 ± 4.3 h in FcRn heavy chain knockout mice (n=3).

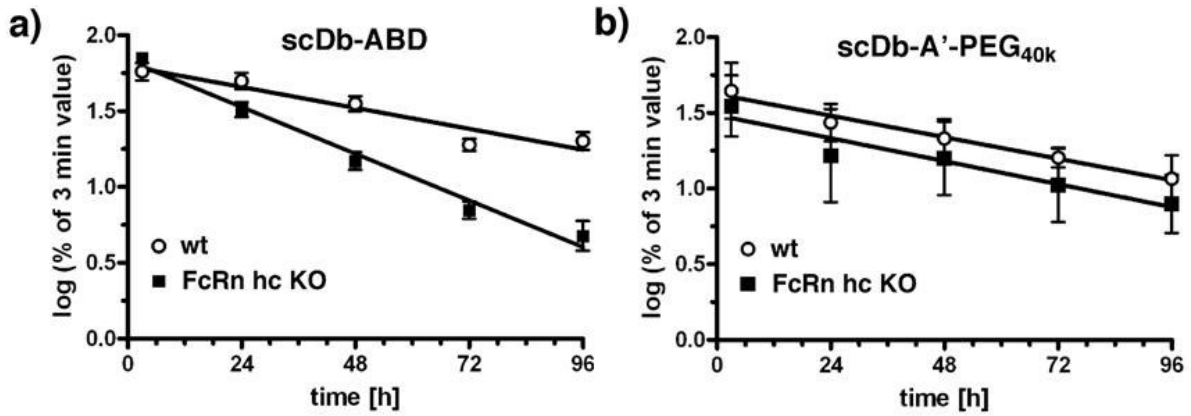

Fig. 5. Plasma clearance of scDb-ABD (a) or scDb-A'-PEG40k (b) in C57BL/6 wild-type (wt) and FcRn heavy chain knockout (FcRn hc KO) mice after a single i.v. dose (25 µg) of antibody molecules.

In vitro cytotoxicity of scDb and scDb-ABD

Using pre-activated human PBMCs we observed a concentration-dependent killing of CEA^+ LS174T tumor cells by scDbCEACD3 and scDbCEACD3-ABD (Fig. 6a). At an effector to target (E:T) ratio of 3, half-maximal killing (EC_{50}) was reached at 50 pM scDb and 100 pM scDb-ABD, respectively. The addition of 1 mg/ml HSA had no effect on the EC_{50} of scDb. In contrast, the EC_{50} of scDb-ABD was reduced 5-fold to 500 pM. No cytotoxicity of scDb and scDb-ABD was observed towards CEA^- HT1080 cells (Fig. 6b).

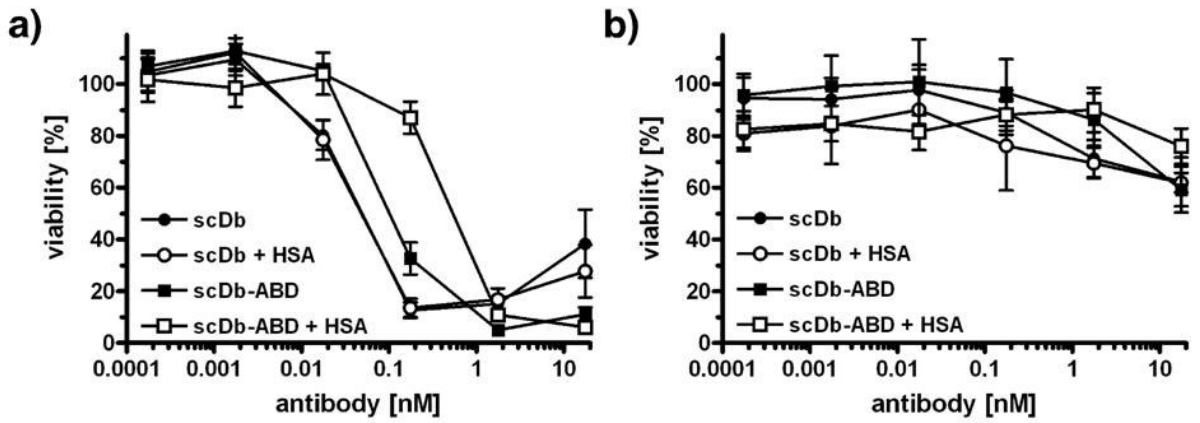

Fig. 6. ScDb-mediated cytotoxicity. CEA^+ LS174T cells (a) or CEA^- HT1080 cells (b) were incubated with preactivated PBMCs at a ratio of 1:3 and varying concentrations of scDbCEACD3 and scDbCEACD3-ABD in the presence or absence of HSA (1 mg/ml). Remaining viable target cells were determined by MTT assay after 24 h (n=3).

Discussion

In the present study we determined the biodistribution of an anti-CEA x anti-CD3 bispecific single-chain diabody and showed that a prolonged half-life translates into an increased accumulation in CEA-positive tumors. All modified scDb molecules exhibited similar EC_{50} values for binding to CEA- and CD3-expressing cell lines indicating that modifications do not interfere with binding to the cell surface-exposed antigens. Thus, the reduced accumulation of scDb and scDb-ABC_7 as compared to PEGylated scDb and scDb-ABD is not caused by a reduced affinity for the CEA-expressing tumor cells. Furthermore, binding of scDb and scDb-ABD to these cell lines was not affected in the presence of serum albumin. This finding is in accordance with results obtained for a half-life-extended anti-HER2 Fab 4D5, which was either fused to the same ABD or an albumin-binding peptide (AB.Fab4D5) (12,13). In contrast, different results were described for PEGylated antibody fragments. Thus, C-terminal conjugation of the scFv 4D5 with a 20 kDa PEG resulted in a 5-fold loss of affinity (14), while, for example, a PEGylated tandem scFv directed against MUC-1 showed a similar binding to MUC-1 as the wild-type tandem scFv (15). A recent study of polysialylated anti-CEA scFv MFE-23 molecules revealed that the conjugation chemistry has a strong effect on immunoreactivity (16). Random polysialylation of in average 1.4 11 kDa PSA chains per scFv resulted as compared to the unmodified scFv in a 20-fold reduction of binding in ELISA. In contrast, no reduction was observed after site-directed conjugation of 1 PSA polymer to a C-terminal cysteine residue, which is similar to our approach of generating PEGylated scDb.

Although binding of scDb to CEA^+ tumor cells and $CD3^+$ effector cells was not affected by

the various modifications, previous studies showed that they reduce the bispecific antibody-mediated stimulatory activity on PBMCs in a target cell-dependent assay (4,8). In these studies, a 3- to 12-fold reduction was observed for scDb-ABC_7, PEGylated scDb and scDb-ABD. The T cell-stimulating activity of scDb-ABD was 3-fold reduced as compared to the unmodified scDb and was further reduced 4-fold in the presence of HSA, while HSA had no effect on the stimulating activity of unmodified scDb. As shown in the present study, this resulted also in a comparable reduction of the cytotoxic effects using preactivated human PBMCs as effector cells. Presumably, these modifications lead to a sterical hindrance of the formation of a close contact between target and effector cells and/or the ability to efficiently activate the T cell receptor through binding to CD3. This reduction might also be influenced by the choice of target antigen and the location of the epitope. Further studies are, therefore, required to investigate the effects of half-life extending modifications on the bioactivity of other bispecific antibodies.

The modifications leading to the longest half-life extension, i.e. PEGylation and fusion to an ABD, resulted in increased accumulation in CEA^+ tumors. The highest tumor accumulation was found for the scDb-ABD fusion protein, which was approximately fivefold increased as compared to the unmodified scDb. Interestingly, PEGylated scDb showed an approximately two-fold lower accumulation in CEA^+ tumors as compared to scDb-ABD, although possessing a slightly higher plasma half-life and a similar cell binding activity. In a previous study we showed that the hydrodynamic radius of scDb-ABD bound to HSA is 4.8 nm compared to 7.9 nm of the PEGylated scDb, which might explain the better tumor penetration of scDb-ABD (8). Increased tumor accumulation was also observed for various other half-life-extended recombinant antibody molecules, such as PEGylated anti-HER2 scFv 4D5 and anti-HER2 AB.Fab4D5 (13,14). The AB.Fab showed a 5-6 fold higher tumor accumulation than the Fab without the binding peptide and the accumulation was much faster in case of the AB.Fab than in case of the parental IgG (13). The latter finding also indicates that the size of the molecule (the MW of AB.Fab bound to albumin is approximately 130 kDa, that of IgG 150 kDa) influences tumor accumulation. Furthermore, the noncovalent binding of scDb-ABD (and AB.Fab) to albumin, allowing the dissociation of the antibody moiety from albumin, might facilitate tumor penetration. The site-directed conjugation of an scFv directed against HER2 with PEG 20 kDa caused a 8.5-fold better tumor accumulation (14). Recently an anti-EGFR tandem nanobody was fused to an anti-albumin nanobody. The nanobody construct revealed a similar circulation half-life (~ 48 h) in mice as our scDb-ABD construct. Compared to the anti-EGFR IgG cetuximab, the nanobody construct showed a similar tumor

accumulation, but distribution within the tumor tissue was more homogeneous (17). This might be due to the smaller size of the nanobody construct (~ 50 kDa), which is similar to the size of the scDb-ABD, as compared to 150 kDa for the IgG molecule. Site-directed polysialylation of anti-CEA scFv MFE-23 (MFE-23-Cys-PSA) with an apparent molecular mass of > 300 kDa as determined by size exclusion chromatography showed a 10-fold increased tumor accumulation as compared to unmodified MFE-23, leading to a maximum accumulation of approximately 10% ID/g tumor (16). These data compare very well with our own data obtained for PEGylated scDb and scDb scDb-ABD. Thus, these experiments further confirm that an extended half-life translates into increased tumor accumulation.

The long circulation time of scDb-ABD is caused by an increased hydrodynamic radius of the scDb-ABD albumin complex, which is approximately 2-fold increased as compared to scDb (4.8 nm versus 2.7 nm) (8), as well as recycling by the FcRn. In the present study we confirmed that recycling by FcRn contributes to the long half-life of scDb-ABD. In FcRn heavy chain knockout mice half-life of scDb-ABD was reduced approximately 2-fold. Similar values were reported for the half-life of radioiodinated mouse albumin, which was reduced from 39 h in wild-type mice to 24 h in heavy chain knockout mice (18). HSA binds pH-dependent to the FcRn with an affinity of approximately 5 µM at pH 6.0 (19,20). A prerequisite of the recycling of scDb-ABD via the FcRn is that the complex between scDb-ABD, albumin and FcRn remains stable in the acidic environment of the endosome. In QCM studies we found that the affinity of scDb-ABD is not decreased at pH 6.0 and that the measured affinities are similar to those determined by others for the binding of the single ABD domain to HSA at neutral pH (21-23).

Our results and data from others clearly demonstrate that PEGylation, N-glycosylation and fusion to an ABD can improve pharmacokinetic properties of recombinant antibodies to various extent. However, these improvements might not only be dependent on the applied half-life extension strategy but also from the antibody format itself, which directly affects protein size and stability (24, 25). Furthermore, it is likely that antibody affinity and valency but also other factors such as tumor vascularization, vessel permeability, and the kind of the target antigen as well as its density on target cell and within the tumor plays a determinant role in biodistribution (26-30). Thus, different results might be obtained for scDb recognizing other target cell antigens. In this context it is noteworthy that no experimental data are currently available to which extend binding to CD3-positive T cells affects plasma half-life and biodistribution. A physiological-based pharmacokinetic model suggests that rather than guiding the T cells to the target tissue, the bispecific antibody is being dragged around the

system by the T cell (31). Experimental data of a bispecific tandem scFv directed against CD19 and CD3 analyzed in chimpanzees revealed a half-life of 2 h, indicating that the binding to T-cells has no beneficial impact on the circulation time (32). The scDb CEACD3 used in our study is human-specific and, therefore, does not bind to mouse T cells complicating the analysis of this aspect, for instance in mice. This question can be answered using a bispecific scDb directed against mouse CD3. A respective scFv (2C11) is available (33) and has been already used by others to study antitumor effects of bispecific tandem scFv molecules possessing a similar size as scDb molecules (34). In an immunocompetent mouse model the antitumor effect of these small bispecific molecules on solid tumors could be demonstrated (35). Further studies are therefore planned to convert our scDb and its derivatives into bispecific anti-CEA x anti-mouse CD3 molecules.

In summary, we showed for our scDb that an extended half-life translates into increased tumor accumulation and that fusion of an albumin-binding domain to a scDb is superior compared to a PEGylated scDb prepared by site-directed conjugation of a 40 kDa branched PEG chain. Further studies in immunocompetent mice are now planned to investigate the antitumor activity of these bispecific T cell-recruiting antibody derivatives in order to elucidate if the prolonged circulation time compensates for the reduced bioactivity observed for the target cell-dependent activation of cellular cytotoxicity in vitro.

Footnotes

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The abbreviations used are: ABD, albumin-binding domain; AUC, area under the curve; CEA, carcinoembryonic antigen; FcRn, neonatal Fc receptor; HSA, human serum albumin; PBMC, peripheral blood mononuclear cells; PEG, polyethylene glycol, QCM, quartz crystal microbalance; scDb, single-chain diabody; scFv, single-chain fragment variable.

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Discussion & Perspectives

The rise of small antibody formats reinforced the need for strategies to improve their circulation half-life. The rapid clearance of small antibodies leads to reduced therapeutic efficacy and necessitates frequent dosing schedules, causing high costs and patient inconvenience (Tang et al., 2004; Mahmood and Green, 2005; van de Weert et al., 2005). Various different approaches have been examined to prolong the circulation time of small antibodies with different impact on half-life, tumor accumulation and antibody activity. The comparison of different strategies that can be found in literature is difficult because they are applied to different antibody formats with different antigens and they are analyzed in varying in vitro and in vivo models. This work compares directly three basically different modifications of an antibody: N-glycosylation, PEGylation and fusion to an albumin-binding domain. All three strategies are applied to the same single-chain diabody, the scDbCEACD3.

Production of the modified scDb constructs and effects of the modifications on circulation half-life

For the N-glycosylation of scDbCEACD3 three different constructs were produced, each with one N-glycosylation site in the V_HCEA-V_LCD3-linker A, one in the V_HCD3-V_LCEA-linker B and with 1, 4 or 7 sequons at the C-terminus (scDb-ABC_1, scDb-ABC_4, scDb-ABC_7). N-glycosylation of proteins is a stochastic process, resulting in the observed heterogeneity in the SDS-PAGE analysis. This could be seen especially for the scDb-ABC_7 construct in which seven N-glycosylation sites are in close proximity at the C-terminus. The heterogeneity of the degree of N-glycosylation in a single production batch have been found for various other N-gylcosylated proteins such as recombinant human interferon-α2b (Ceaglio et al., 2007), recombinant follicle stimulating hormone (Perlman et al., 2003) and recombinant human acetylcholinesterase (Chitlaru et al., 1998). N-glycosylation of the scDbCEACD3 led to a moderate enlargement of its hydrodynamic radius from 2.7 nm to 2.8 – 3.8 nm. The circulation times of these molecules were moderately improved with a 2- to 3-fold increased $AUC_{0\text{-}24h}$ compared to the unmodified scDb. Even though the sizes of the N-glycosylated constructs were correlated with the number of N-glycosylation sites, a correlation between the circulation half-lives and the number of N-glycosylation sites could not be found. Limitations in prolonging the half-life of proteins by adding additional N-glycosylation sites have been observed before. The insertion of two additional N-glycosylation sites in recombinant follicle-

stimulating hormone (rFSH) increased the AUC after i.v. injection in mice from 11 to 32 mIU-h/ml, whereas a further addition of 2 sequons led even to a slight decrease of the AUC (Weenen et al., 2004). Also, the increase from 3 to 4 N-glycosylation sites in recombinant human acetylcholinesterase did not further prolong the half-life of the enzyme in rhesus macaques (Cohen et al., 2004).

The moderate increased circulation time of the N-glycosylated scDbs is typical for the prolonged half-life of hyperglycosylated proteins. For example, hyperglycosylated erythropoietin with two additional N-glycosylation sites revealed a 3-fold prolonged circulation half-life in human (Egrie and Browne, 2001). The C-terminal addition of two additional N-glycosylation sites to rFSH led to a 3- to 4-fold increased half-life in rats (Perlman et al., 2003).

A possibility to further prolong the half-life of the N-glycosylated scDbs could be to exert influence on the glycan composition. Mass spectroscopy analysis revealed that the glycan pattern of the N-glycosylated scDbCEACD3 variants were similar to that published for other recombinant proteins expressed in HEK-293 cells (Kaufmann et al., 2004). The degree of sialic acid-capped glycans was between 22 – 34 %, decreasing with the growing number of N-glycosylation sites. Glycans produced in mammalian cells, which are not capped with sialic acid, mostly exhibit a terminal N-acetylglucosamine. It is known that proteoglycans displaying a high degree of terminal N-acetylglucosamine are more rapidly cleared from circulation by the asialoglycoprotein receptor (Meier et al., 1995; Stockert, 1995; Jones et al., 2007). By increasing the degree of sialylation, the elimination through the asialoglycoprotein receptor can be minimized. Several groups already created expression cell lines which produces more than 90 % sialylated glycoproteins (Weikert et al., 1999; Bragonzi et al., 2000). Rhesus acetylcholinesterase produced in genetically engineered HEK-293 cells, which co-expressed a high level of heterologous α-2,6-sialyltransferase, was fully sialylated (Cohen et al., 2004). Compared to partially sialylated acetylcholinesterase expressed in unmodified HEK-293 cells, the fully sialylated acetylcholinesterase showed a prolongation of the terminal half-life from 72 h to 113 h in rhesus macaques and from 71 h to 118 h in mice.

The strongest increase in size, measured by SEC, was observed for the PEGylated constructs. ScDbCEACD3 was site-directed PEGylated in the V_HCEA-V_LCD3-linker or C-terminal with a branched mPEG 40 kDa. In both cases the PEGylation led to a ~ 3-fold increase in the hydrodynamic radius corresponding to a protein with 650 kDa. The dramatic increase in size of the conjugates, which is much more than assumed from the sum of the molecular mass, is caused by 2-3 water molecules that bind to PEG per ethylene oxide unit (Roberts et al., 2002).

A drastic enlargement of the hydrodynamic radius was also observed in SEC analysis for PEGylated anti-HER2 scFv4D5. While the unmodified scFv4D5 showed an apparent MW of 32 kDa the scFv4D5 conjugated with PEG 20 kDa revealed an apparent MW > 200 kDa (Kubetzko et al., 2006). The strong enlargement of the PEGylated scDbCEACD3 led to a drastically prolonged circulation with a 10-fold increase of the $AUC_{0\text{-}24h}$ compared to the unmodified construct, likely through the prevention of renal clearance and very similar to the improvement of the same scDbCEACD3 fused to HSA (Müller et al., 2007). The positive effect of PEGylation on the circulation time of proteins is studied well. For example, a scFv directed against granulocyte macrophage colony-stimulating factor (GM-CSF) showed a 30-fold increase in half-life after conjugation to PEG 40 kDa (Krinner et al., 2006). By conjugating an antibody fragment to PEG chains of different lengths, the circulation half-life can be modulated over a wide range (Yang et al., 2003).

The increase in size of the scDb caused by the fusion to the ABD was not visible in SEC, but bound to albumin the hydrodynamic radius of the scDb-ABD nearly duplicated, resulting in a size well above the threshold for renal clearance. ScDb-ABD showed the longest circulation time of the scDbCEACD3 variants with a 14-fold increased $AUC_{0\text{-}24h}$ compared to the unmodified scDb and an increase in terminal half-life from 5.6 h to 27.6 h. Further experiments with FcRn heavy chain knockout (FcRn hc KO) mice revealed that the drastic prolongation of circulation time of the scDb-ABD-albumin complex is not simply caused by enlargement of the molecule, but by effective recycling via the FcRn. A 2-fold decreased terminal half-life for scDb-ABD was observed in FcRn hc KO mice, compared to wild-type mice (24.8 h vs. 53 h), whereas a PEGylated scDb as negative control showed similar half-lives in the different mouse strains (47.9 h vs. 51.4 h). The interaction site between albumin and an albumin-binding domain of Finegoldia magna, which is homologous to ABD3 of streptococcus strain G148, could be located in domain II of albumin (Lejon et al., 2004). Hence, binding of albumin to FcRn, which interacts with albumin domain III (Chaudhury et al., 2006), should not be interfered by ABD-albumin binding. A prerequisite for the recycling of the scDb-ABD is the stability of the scDb-ABD-albumin-FcRn complex at the acidic pH of the endosome. The affinity of human FcRn to HSA was determined to be ~ 5 µM at pH 6.0 (Andersen et al., 2006; Chaudhury et al., 2006). On the basis of quartz crystal microbalance measurements, the present study reveals that the binding between scDb-ABD and albumin remained stable at pH 6.0. The determined K_D for the binding at neutral pH was according to the K_D measured by others for the binding of ABD to albumin (Johansson et al., 2002; Linhult et al., 2002; Jonsson et al., 2008). Similar results have been published for the recycling of

albumin before (Chaudhury et al., 2003). Radiolabeled mouse albumin was injected i.v. in FcRn hc KO and in wild-type mice. The half-life of albumin was reduced from 39 h to 24 h in mice lacking the functional FcRn. The same study showed that the half-life of IgGs is reduced from 95 h to 19 h in FcRn hc KO mice, revealing that IgGs are more efficiently recycled than albumin. That may be a reason why the half-life of an IgG could not be reached by the scDb-ABD fusion strategy. A similar impact on the circulation half-life as for scDb-ABD was found for an anti-HER2 Fab4D5-ABD fusion protein (Schlapschy et al., 2007). The unmodified Fab4D5 showed a terminal half-life of 2.1 h in mice whereas 20.9 h were observed for Fab4D5-ABD. Several other studies confirmed that the fusion to an albumin-binding moiety is an effective strategy to improve the circulation half-life of Fab molecules in mice, rats and rabbits (Dennis et al., 2002; Nguyen et al., 2006).

Impact of the modifications on the bioactivity of scDbCEACD3

Functional analysis of the scDbCEACD3 variants were made by examination of their antigen-binding, their ability to activate T-cells and their potential to mediate cytotoxicity. Titration of scDbCEACD3 and its variants in flow cytometry on CEA^+ LS174T cells or $CD3^+$ Jurkat cells revealed similar binding of all constructs, hence, modifications did not interfere with antigen-binding. In contrast, an ELISA, in which scDb and scDb-ABD was titrated on immobilized CEA in presence of albumin showed a ~ 4-fold decrease in binding of scDb-ABD. A possible explanation of the contrary results could be that in ELISA the access to the epitope on immobilized CEA was more complicated for large antibodies such as the scDb-ABD-albumin-complex compared to the small scDb. In contrast, in flow cytometry assays on cells, CEA is attached only with its C-terminus to the cell surface. The epitope for the anti-CEA binding site of the antibody is located in the N-terminal domain of the long CEA molecule (Sainz-Pastor et al., 2006), making the epitope more accessible for large antibody molecules. The results of the antibody titration analyzed by flow cytometry appear to be more relevant because the assay conditions are more similar to the conditions in the tumor.

Differences in the binding of antibodies to immobilized antigen and the antibody activity in cellular assays have been described before. Yang et al. conjugated an anti-TNF scFv with PEG 40 kDa. In surface plasmon resonance measurements on immobilized TNF a 7.6-fold decrease in affinity could be observed for the PEGylated scFv. As opposed to that, in a cytotoxicity assay in which the scFv inhibited the effect of TNF in solution, no differences could be found between the IC_{50} of the PEGylated and the unmodified scFv (Yang et al.,

2003).

Even if antigen-binding on cells of the scDbCEACD3 was not influenced by the modifications, a 3- to 12-fold decrease was observed for the different variants in their ability to stimulate effector cells (PBMC) as determined by measuring IL-2 release after 24 h. ScDb-ABD showed a 3-fold decreased EC_{50} compared to scDb in absence of HSA and a further 4-fold decrease of the EC_{50} in presence of HSA. The reduced ability for T-cell activation translates in a comparable reduced cytotoxicity in an assay using preactivated PBMC. ScDb-ABD and the PEGylated scDb, the molecules with the strongest gain in size, showed a stronger reduction in activity than the N-glycosylated scDb, implicating that the reduced activity of the modified constructs is caused by sterical hindrance of the effector cell – target cell interaction. If the reduction is also influenced by the choose of target antigen, has to be investigated in further studies. Nevertheless, all constructs were able to activate T-cells target cell-specific and for the scDb-ABD construct it could be demonstrated that the construct is able to mediate cytotoxicity in presence and absence of HSA.

Effects of the modifications on tumor accumulation of scDbCEACD3

The intention in improving the half-life of therapeutic antibodies for the treatment of solid tumors is to receive an enhanced accumulation in the tumor where the antibody can fulfil its therapeutic effect. Biodistribution studies of the scDbCEACD3 variants were performed in nude mice each bearing a CEA^+ LS174T and a CEA^- MC-38 tumor. Both long-lasting constructs, scDb-ABD and scDb-A'-PEG_{40kDa}, translated their prolonged half-lives in an improved tumor accumulation. Even if the PEGylated scDb showed a ~ 20 % higher AUC in the blood than scDb-ABD, tumor accumulation of scDb-ABD was 4.5-fold increased compared to the unmodified construct whereas tumor accumulation of scDb-A'-PEG_{40kDa} was increased only 2.7-fold. Since both antibodies did not show a difference in antigen-binding, presumably the smaller hydrodynamic radius of the scDb-ABD-albumin complex compared to that of the PEGylated scDb facilitates tumor penetration. The effect might be further enhanced through the non-covalent character of the binding between scDb-ABD and albumin, allowing the dissociation. Furthermore, transcytosis mechanisms are known which help albumin to overcome the endothelial barrier (Minshall et al., 2002). For myeloperoxidase that binds to albumin it could be shown that albumin mediates its transcellular transport (Tiruppathi et al., 2004). Possibly, small antibodies fused to albumin-binding moieties benefit

from the same mechanism. Higher accumulation of scDb-ABD in the tumor leads to an increased clearance of the antibody from the blood. This may explain the smaller AUC of scDb-ABD in the blood compared to scDb-A'-PEG_{40kDa}, which stands in contrast to the results from the tumor-free mouse model.

The observed beneficial effects of PEGylation and antibody fusion to an albumin-binding moiety on tumor accumulation are in accordance to studies published by other groups. Site-directed conjugation of anti-HER2 scFv4D5 with PEG 20 kDa led to 8.5-fold improved tumor accumulation after 48 h, even though the modification caused a 5-fold loss in affinity. The maximal tumor accumulation of ~ 10 %ID/g was measured 24 h after i.v. injection (Kubetzko et al., 2006). Similar results were obtained for anti-CEA scFvMFE-23 conjugated with polysialic acid (PSA). The construct showed a large apparent size (> 300 kDa) in SEC, resulting in a 10-fold increased AUC in the tumor with a maximal tumor uptake of ~ 10 %ID/g after 24 h (Constantinou et al., 2009). The maximal tumor accumulation of scDb-A'-PEG_{40kDa} was measured after 24 h, too, but maximal tumor uptake was 6.5 %ID/g. The smaller tumor accumulation of scDb-A'-PEG_{40kDa} might be caused by different antigen densities of the implanted tumor cells. Furthermore, scDbCEACD3 was conjugated with PEG 40 kDa, resulting in a larger hydrodynamic radius compared to scFv4D5 conjugated with PEG 20 kDa, leading to an enhanced hindrance of diffusion in tumor tissue.

ScDb-ABD showed the best tumor accumulation compared to the other scDbCEACD3 variants. Maximal tumor accumulation of 10 %ID/g was measured 24 h after injection. Increased tumor accumulation could be observed, too, for anti-HER2 Fab4D5 fused to an albumin-binding peptide (AB.Fab4D5) (Dennis et al., 2007). 24 h after injection AB.Fab4D5 showed a 5- to 6-fold higher tumor accumulation than Fab4D5. AB.Fab4D5 accumulated in the tumor similar to the parental antibody Trastuzumab but penetrated the tumor tissue more efficiently. Similar results were also found for a nanobody-trimer (~ 50 kDa) with two binding sites for EGFR and one for albumin (Tijink et al., 2008). Tumor accumulation of the construct was comparable with anti-EGFR IgG Cetuximab, but the nanobody-trimer distributed more homogenously in the tumor tissue. These results implicate that the smaller size of the albumin-binding antibody constructs facilitates tumor penetration.

The bacterial origin of the streptococcal ABD may causes concerns about its immunogenic potential. For a minimal albumin-binding domain of streptococcal protein G (aa 254-299) immunogenicity was found in various mouse strains (Sjolander et al., 1997). Furthermore, increased immunogenicity was found for the albumin-binding region (aa 113-326) of streptococcus protein G (Libon et al., 1999). In contrast, observations have been reported that

the ABD used in the present study might be able to reduce the immunogenicity of its fusion partner (Kontermann, 2009b). Nevertheless, if immunogenicity would be observed for scDb-ABD, deimmunization strategies could be applied which are aimed mainly at the reduction of T-cell epitopes in the protein sequence (De Groot et al., 2005; Baker and Jones, 2007; Holgate and Baker, 2009; Jones et al., 2009).

Perspectives

In the present work the organ distribution study was focused on the influence of the modifications of scDbCEACD3, not complicating the analysis by regarding the T-cell interaction of the antibodies. The anti-CD3 binding-site of scDbCEACD3 is human specific and does not bind to mouse CD3. To what extent the binding to CD3 does influence the pharmacokinetics of an antibody is presently not known. A physiological whole body computer model predicts that bispecific anti-CD3 antibodies are dragged around the system by T-cells instead of guiding the T-cells to the target tissue (Friedrich et al., 2002). In contrast, the direct comparison of the circulation time of two BiTE antibodies in mice, with one BiTE that binds to murine CD3 and another one that did not, revealed similar circulation profiles for both constructs. The BiTE that did not bind to murine CD3 showed a 34 % increased terminal half-life whereas the AUC did not differ significantly between the constructs (Schlereth et al., 2006).

To analyse the biodistribution of scDbCEACD3 and its derivatives under regard of antibody-CD3 interaction, the anti-human-CD3 binding site of scDbCEACD3 can be exchanged by an anti-murine-CD3 binding site. The distribution of the resulting scDbCEACD3$_{mu}$ could be examined in a syngeneic immunocompetent mouse model. The respective anti-murine-CD3 scFv2C11 is available (Liao et al., 2000) and has been used successfully for the construction of BiTE molecules which showed antitumor effects in mice (Schlereth et al., 2006; Amann et al., 2009). A CEA^{+} cell line, derived from MC-38 cells, which forms syngeneic tumors in C57BL/6 mice, is also available. This tumor model could be used to study how the increased tumor accumulation and decreased activity of the scDbCEACD3$_{mu}$ and its derivatives translate into antitumor efficacy.

A further challenge will be to find half-life extended derivatives of scDbCEACD3$_{mu}$ without or only marginal changes in bioactivity. The loss in bioactivity of the examined derivatives, despite a nearly unchanged binding for both antigens, led to the assumption that the gain in size hinders the T-cell – target cell interaction sterically. A loss in bioactivity was also

observed for a scDbCEACD3 fused to HSA (Tiegs, 2005; Müller et al., 2007). An interesting approach would be the fusion of the scDb with that domain of albumin, which is responsible for the FcRn interaction. Affinity measurements of the different albumin domains on immobilized FcRn revealed that albumin domain III contributes to the albumin-FcRn interaction (Chaudhury et al., 2006). The fusion protein of albumin domain III and scDbCEACD3$_{mu}$ would have a smaller size than scDb-HSA (MW ~ 76 kDa vs. ~ 120 kDa), therefore it should hinder the T-cell – target cell interaction to a lesser extent. Its circulation half-life should remain prolonged through the recycling by the neonatal Fc-receptor. Preliminary results of circulation half-life experiments in mice revealed that scDbCEACD3 fused to albumin domain III showed a significant prolonged half-life compared to the unmodified scDbCEACD3 (data not shown).

The comparison of the circulation time of the half-life extended scDbCEACD3 derivatives and the chimeric IgG showed that the IgG exhibits clearly the longest terminal half-life, implicating that the possibilities for half-life extension are not yet exhausted. By fusing anti-HER2 Fab4D5 to albumin-binding peptides with different affinities for albumin, the terminal half-life of the antibody could be modulated between 12 and 69 h. A positive correlation between the affinity of the fused albumin-binding peptide for albumin and the serum half-life could be observed (Nguyen et al., 2006). A recently published study showed that the affinity of ABD for HSA could be improved more than 1000-fold by a combination of combinatorial protein engineering, in vitro selection via phage display and rational design (Jonsson et al., 2008). Presumably, the circulation half-life of scDb-ABD could be further improved by exchanging the native ABD by a high-affinity one.

Another approach could be to enhance the scDb-HSA interaction with FcRn. It was observed before that the serum half-life of IgGs could be improved by increasing the affinity of IgG-Fc for FcRn (Hinton et al., 2004; Hinton et al., 2006). Importantly, the binding must remain pH-dependent, otherwise the effect reverses (Dall'Acqua et al., 2002). Probably, the fusion of the scDb with mutagenized HSA, phage display selected for improved pH-dependent binding to FcRn, would result in a further prolonged circulation half-life.

Beyond the small recombinant antibodies, the efficacy of a variety of protein-based drugs is also limited by their short circulation half-lives. Further studies have to investigate, how these drugs can benefit from the described strategies.

Conclusion

In conclusion, three strategies for improved pharmacokinetic properties were applied to scDbCEACD3. In all approaches the derivatives retained the functionality of their binding sites and were able to mediate target cell-specific T-cell activation, however, with a 3- to 12-fold decreased efficiency. Also, it could be shown that scDb-ABD was still able to mediate cytotoxicity, even if a reduction of the cytotoxic potential of scDb-ABD was observed which was amplified in presence of albumin. The circulation half-life of scDbCEACD3 could be modulated over a broad range between the half-life of the unmodified scDb and that of a chimeric IgG1. N-glycosylation led to a moderate improvement whereas PEGylation and ABD-fusion resulted in drastically increased half-lives. Moreover, it could be demonstrated that the interaction of scDb-ABD and FcRn contributes to the prolonged half-life of scDb-ABD. The prolonged half-lives of scDb-ABD and the PEGylated scDb translated in an improved tumor accumulation of these molecules. Highest tumor accumulation was found for scDb-ABD, making the fusion with ABD a promising approach for the improvement of pharmacokinetics of small recombinant bispecific antibodies. Further studies will reveal, how the improved tumor accumulation of the modified constructs translates into anti-tumor efficacy in vivo.

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