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RGD-avidin–biotin pretargeting to $\alpha_v\beta_3$ integrin enhances the proapoptotic activity of TNFα related apoptosis inducing ligand (TRAIL)

Marc Tarrus · Almer M. van der Sloot · Kai Temming · Marie Lacombe · Frank Opdam · Wim J. Quax · Grietje Molema · Klaas Poelstra · Robbert J. Kok

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Abstract Recombinant TNF-related apoptosis-inducing ligand (TRAIL) is considered a powerful and selective inducer of tumor cell death. We hypothesize that TRAIL's potential as anticancer agent can be enhanced further by promoting its accumulation in tumor tissue. For this purpose, we developed TRAIL complexes that bind to angiogenic endothelial cells. We employed an avidin–biotin pretargeting approach, in which biotinylated TRAIL interacted with RGD-equipped avidin. The assembled complexes killed tumor cells (Jurkat T cells) via apoptosis induction. Furthermore, we demonstrated that the association of the RGD-avidin-TRAIL complex onto endothelial cells enhanced the tumor cell killing activity. Endothelial cells were not killed by TRAIL nor its derived complexes. Our approach can facilitate the enrichment of TRAIL onto angiogenic blood vessels, which may enhance intratumoral accumulation. Furthermore, it offers a versatile technology for the complexation of targeting ligands with therapeutic recombinant proteins and by this a novel way to enhance their specificity and activity.

Keywords Apoptosis · Angiogenesis · Drug targeting · Therapeutic proteins

M. Tarrus · K. Temming · K. Poelstra · R. J. Kok
Department of Pharmacokinetics and Drug Delivery, Groningen University Institute for Drug Exploration, Groningen, The Netherlands

M. Tarrus · K. Temming · M. Lacombe · F. Opdam
Kreatech Biotechnology B.V, Amsterdam, The Netherlands

A. M. van der Sloot · W. J. Quax
Department of Pharmaceutical Biology, Groningen University Institute for Drug Exploration, Groningen, The Netherlands

Present Address:
A. M. van der Sloot
CRG-EMBL Systems Biology Unit, Centre de Regulació Genómica, Barcelona, Spain

G. Molema
Department of Pathology and Laboratory Medicine, Medical Biology Section, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands

R. J. Kok (✉)
Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Sorbonnelaan 16, 3584 CA Utrecht, The Netherlands
e-mail: r.j.kok@uu.nl

Introduction

TNF ligands have strong potential as anti-tumor agents, acting in different ways on either tumor cells or tumor vasculature, or by promoting the induction of tumor-directed immune responses [1]. The broad clinical use of TNF ligands is prohibited however due to lack of tumor selectivity, causing various side effects. A recently identified TNF family member is TNF related apoptosis-inducing ligand (TRAIL) [2]. Many tumor types are sensitive to TRAIL or become sensitive after treatment with chemotherapeutic drugs [3]. Soluble TRAIL variants showed no cytotoxic effects on most primary human cells and were well tolerated in mice and chimpanzees [4, 5]. However, the selectivity of TRAIL for tumor cells over normal cells is lost when TRAIL forms multimeric complexes. For example, it has been observed that cross-linked TRAIL induces apoptosis more efficiently than non-aggregated trimeric TRAIL [6], but such cross-linked TRAIL also caused cell death in normal hepatic cells [7]. It seems therefore attractive to design clustered TRAIL variants with enhanced specificity for tumor tissue. The increased

tissue retention of cellular targeted TRAIL variants may counterbalance or prevent side effects elsewhere in the body. In the present paper, we investigated the development of TRAIL complexes equipped with targeting ligands directed to the tumor vasculature. Enhanced retention of TRAIL complexes at the tumor vasculature will enhance their accumulation in the tumor tissue, thereby favoring the desired therapeutic activity and reducing potential toxicity.

Several recent papers described the development of recombinant TNF family members with enhanced target specificity. One approach is to modify the intrinsic specificity of TRAIL for its target Death-receptor 5 specific by substituting amino-acids located in the receptor binding interface of TRAIL [8]. Development of chimaeras is another successful approach. Linkage of TRAIL to scFv antibody fragments yielded constructs which bind to additional surface-exposed receptors on tumor cell types [9–11]. Similarly, other TNF family members like Fas-ligand and TNFα (have been conjugated to tumor-cell directed targeting ligands, yielding chimeric proteins with full or even enhanced therapeutic activity [11–15].

Although the recombinant approach has been applied successfully for the production of chimeric proteins, low production yields may hamper the feasibility of such a strategy. Especially when multiple targeting ligands are introduced into a protein subunit, which is preferred for high avidity binding, aberrant folding of chimeric proteins can occur. In the current study we therefore pursued a chemical approach, in which TRAIL was biotinylated and complexed to avidin equipped with multiple targeting ligands. Avidin–biotin complexation is a widely accepted approach in diagnostic applications that employ immunodetection steps, and is also considered an attractive strategy for the preparation of tumor targeted complexes, especially in so-called pretargeting approaches in which the complexes are assembled in vivo at the cell surface of tumor cells. Commonly, such strategies employ biotinylated tumor-specific antibodies and a therapeutic or diagnostic cargo coupled to avidin [16, 17].

The approach presented in this paper differs to the above-discussed strategies in several aspects. First, we now equipped TRAIL with targeting ligands directed to angiogenic endothelium rather than to tumor cells, allowing an enhanced binding to the tumor blood vessels. The vascular wall is in direct contact with the systemic circulation, which makes it an easily attainable target for therapeutic proteins. Furthermore, intratumoral pressure, which hampers the penetration of macromolecules into tumor tissue will not affect the homing to of RGD-equipped complexes to the angiogenic endothelium.

Second, we have prepared a different type of targeted avidin that can interact directly via covalently attached RGD-groups with tumor endothelial cells. Thus, the developed avidin–biotin-TRAIL complexes will home to angiogenic endothelium without the need of complexation with a biotinylated antibody, thereby leaving its biotin-binding sites available for complexation with the biotinylated TRAIL. The functionalization of avidin with RGD-homing ligands was carried out using a cyclic RGD-peptide with high affinity and specificity for $\alpha_v\beta_3$ integrin on angiogenic endothelium [18]. We furthermore applied a polyethylenglycol (PEG) linker for coupling of the RGD-ligand, which brings several advantages, such as a reduced non-specific binding of the avidin and reduced immunogenicity [19]. The now developed RGDPEG-avidin conjugates contain the RGD homing ligand attached to the distal end of the PEG groups.

In order to minimize loss of functional TRAIL activity, we have compared two different biotinylation strategies that react at different functional groups in the protein. We used a conventional Biotin-NHS reagent that binds to primary amino groups in the protein, and we employed the Biotin-ULS (ULS: universal linkage system) reagent, which reacts at methionine residues. Figure 1 shows a schematic representation of the developed complexes and their interaction with target cells. The products were evaluated for their association with $\alpha_v\beta_3$ integrin expressing endothelial cells and for their antitumor activity in both endothelium and cultured tumor cells. Our results demonstrate that recombinant TRAIL can be complexed with endothelial specific targeting ligands in such a way that it maintains its extensive cell killing activity either in solution or when displayed on the surface of endothelial cells.

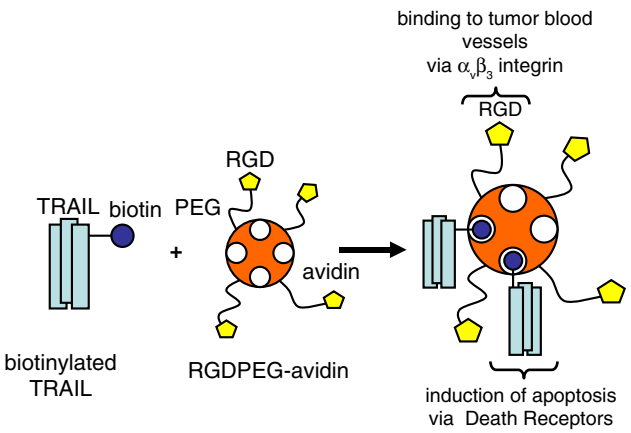

Fig. 1 Schematic representation of RGD-avidin:TRAIL complexes. Soluble recombinant human TRAIL was modified with biotin groups via two different reagents that either react at lysine or methionine residues. Biotinylated TRAIL was complexed to an avidin-based targeting device equipped with approximately 5 RGD-peptide ligands per avidin molecule. A hydrophilic PEG-linker was used to append the RGD-peptides to avidin. The assembled complexes can bind to tumor blood vessels via RGD//$\alpha_v\beta_3$-integrin interactions, while apoptosis can be induced via TRAIL/DR ligation

Materials and methods

Preparation and characterization of RGDPEG-avidin

Avidin was modified with heterobifunctional PEG groups that can react with primary amino groups in avidin, after which RGD-peptide groups can be conjugated to their distal ends. The hydrophilic PEG groups of 3.4 kDa will furthermore reduce non-specific protein interactions.

Briefly, avidin (2 mg, 32 nmol, Sigma) was dissolved in PBS at 10 mg/ml and treated with a 50-fold molar excess of the heterobifunctional vinylsulfone-polyethylenglycol-*N*-hydroxysuccinimide ester (1 mg, 292 nmol, VNS-PEG-NHS; Nektar, Alabama, USA), which had been dissolved in water at a concentration of 20 mg/ml. The PEG linker was added drop wise. The reaction mixture was protected from light with tin foil and incubated for 1 h at room temperature on a spiramix rollerbank. Meanwhile, the RGD peptide c(RGDf(ε-*S*-acetylthioacetyl)K) (Ansynth Service, Roosendaal, The Netherlands) was dissolved at 10 mg/ml in a 1:4 acetonitrile/water mixture. The peptide was added drop wise to the reaction mixture at a molar ratio of 55:1, after which freshly prepared hydroxylamine was added to a final concentration of 50 mM. The reaction was carried out overnight at room temperature while protected from light. Remaining VNS groups were quenched by addition of cysteine (55:1 molar excess over the amount of avidin), after which the product was purified by size exclusion chromatography on a Highload superdex 200 column (Amersham Biosciences), pre-equilibrated in PBS. The final product RGDPEG-avidin was stored in PBS at −20°C.

RGDPEG-avidin conjugates were characterized for protein content by BCA protein assay (Pierce). Coupling of RGDPEG groups to avidin was demonstrated by SDS-PAGE performed on a mini-Protean II system (Bio-Rad) using 12% Ready Gels (Bio-Rad, The Netherlands). Duplicate gels were either stained for protein (Coomassie Brilliant Blue staining) or blotted on a PVDF membrane (Roche, Mannheim, Germany). The membrane was blocked with BSA and subsequently incubated with an in-house raised anti-RGD polyclonal rabbit antiserum specific for the conjugated RGD-peptide [20]. Blots were further developed with goat-anti-rabbit peroxidase (GARPO)/DAB detection. The grade of RGD-pegylation was furthermore determined by MALDI-TOF analysis as described previously [21].

Expression and purification of recombinant human His-tagged TRAIL

A cDNA corresponding to human soluble TRAIL (amino acids 114–281) was cloned downstream of the hexahistidine (His) tag sequence in pET15B (Novagen) using *Nde*I and *BamH*I restriction sites. *Escherichia coli* BL21 (DE3) (Invitrogen) was subsequently transformed with the His-TRAIL construct. The transformed host was grown in shake flasks at four liter scale as described previously [22]. The culture was centrifuged at 2,500 rpm and the supernatant was discarded. The isolated pellet was resuspended in three volumes of extraction buffer (20 mM phosphate buffer pH 8 containing 200 mM NaCl, 10% (v/v) glycerol and 7 mM β-mercaptoethanol).

Cells were disrupted using sonication and extracts were clarified by centrifugation at 40,000 *g*. Subsequently, the supernatant was loaded on a cobalt-charged IMAC TALON column (Clontech) and His-TRAIL was eluted with a step gradient of extraction buffer containing 0.5 M imidazole. Trimeric His-TRAIL was purified by size-exclusion chromatography on a Hiload Superdex 75 column (Amersham Biosciences), to remove impurities. The final product was flash frozen and stored at −80°C in storage buffer (phosphate buffer pH 7.4 containing 150 mM NaCl, 3.5 mM DTT and 20 μM $ZnSO_4$). Purified protein was >95% pure as determined by colloidal Coomassie Brilliant Blue-stained SDS–PAGE gel.

Preparation and characterization of bio-NHS-TRAIL and bio-ULS-TRAIL

We studied two different linkage strategies to introduce biotin into His-TRAIL, by applying either a conventional biotinylation reagent that reacts with primary amino groups in the protein (biotin-NHS), or one that reacts to methionine residues (biotin-ULS™, ULS = universal linkage system). Such an alternative strategy may prove less harmful to the therapeutic activity of TRAIL, depending on the extent to which side chain residues in the active domains are modified. Biotin-ULS was provided by Kreatech Biotechnology (Amsterdam, The Netherlands). All syntheses were performed in a 10:1 molar ratio of biotinylation reagent over TRAIL.

His-TRAIL in storage buffer was extensively dialyzed against PBS containing 10% glycerol at 4°C using Slide-A-Lyzer dialysis cassettes (10,000 MWCO, Pierce) to remove DTT and other components of the storage buffer that can interfere in the reactions. Typically, the purified His-TRAIL (500 ug, 7.6 nmol) at 0.7 mg/ml was mixed either with Sulfo-NHS biotin (Pierce) (10 mg/ml in DMF) or with biotin-ULS (10 mg/ml in 20 mM NaCl), after which the mixtures were incubated for 4 h at 37°C. Unreacted biotinylation reagent was removed by dialysis against PBS at 4°C. The products were sterilized by filtration via disposable 0.2 μm filters and stored at −20°C.

Biotin-TRAIL conjugates were characterized for protein content using BCA protein assay (Pierce). The relative number of coupled biotin molecules was determined by anti-biotin ELISA. Wells were coated with serial dilutions of the biotinylated proteins for 1 h at room temperature, washed with PBS containing 0.05% Tween20 and incubated for 1 h at room temperature with streptavidin-peroxidase complex (Dako, 1:2500 in PBS), followed by a standard incubation with OPD. The protein concentration at which 50% of the maximum absorbance was measured (EC50) was calculated by nonlinear regression (Graphpad Prism), and used to calculate relative biotin:protein ratios in each conjugate. The grade of biotinylation was furthermore assayed by MALDI-TOF analysis as described above for RGDPEG-avidin.

Cells

Jurkat T cells were cultured in RPMI 1640 culture medium (containing 25 mM HEPES and L-glutamine), supplemented with 10% heat-inactivated FCS (Bodinco, Alkmaar, The Netherlands), 1 mM sodium pyruvate, 2 mM L-glutamine, 0.5 mM 2-ME, and 0.1 mg/ml gentamycin sulfate obtained from BioWhittaker, and 0.02 μg/ml fungizone. (Bristol-Meyers, Woerden, The Netherlands)

HUVEC were isolated by the UMCG Endothelial Cell Facility by previously described methods [23]. Primary isolates were cultured in 1% gelatin-coated tissue culture flasks (Corning, Costar, The Netherlands) at 37°C under 5% CO_2/95% air. The culture medium, hereafter referred to as EC medium, consisted of RPMI 1640 (BioWhittaker, Verviers, Belgium) supplemented with 20% heat inactivated fetal calf serum (Integro, Zaandam, The Netherlands), 2 mM L-glutamine (Invitrogen, Breda, The Netherlands), 5 U/ml heparin (Leo Pharmaceutical Products, Weesp, The Netherlands), 100 U/ml penicillin (Yamanouchi Pharma, Leiderdorp, The Netherlands, 100 μg/ml streptomycin (Radiumfarma-Fisiopharma, Italy), and 50 μg/ml endothelial cell growth factor supplement extracted from bovine brain. After attaining confluence, cells were detached from the surface by trypsin EDTA (0.5/0.2 mg/ml in PBS) treatment and split in 1:3 ratio. Cells were used up to passage four.

Binding of RGDPEG-avidin to HUVEC

Binding affinity of RGDPEG-avidin to $\alpha_v\beta_3$-integrin expressed on the surface of HUVEC was determined by competitive displacement of ^{125}I-labeled Echistatin, which is a known ligand for $\alpha_v\beta_3$ [24]. Confluent monolayers of HUVEC in 24-well plates (Costar) were incubated with 100,000 cpm ^{125}I-Echistatin either in the presence of excess of RGDPEG-avidin, free RGD-peptide or avidin as competitors (competitors all in a concentration of 10 μM), or a serial dilution of RGDPEG-avidin and free RGD-peptide. Competition experiments were carried out with divalent cation containing binding buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 1% BSA) at 4°C for 4 h. Cells were washed three times with binding buffer and lysed with 1 M NaOH. Radioactivity was counted in a Packard RIASTAR multiwell gamma counter (GMI, Minnesota, USA). Data were analyzed by non-linear regression using the GraphPad Prism program.

Complexation of biotinylated TRAIL with RGDPEG-avidin

We studied the complexation of biotinylated TRAIL with RGDPEG-avidin in an ELISA-like experimental setup. Wells were coated with 50 ng of avidin or RGDPEG-avidin overnight at room temperature, blocked with BSA (1% in PBS) and incubated with 10 ng of biotinylated TRAIL for 2 h at room temperature. After the wells were washed with PBS/0.05% Tween20, the amount of TRAIL associated with the avidin was detected by anti-TRAIL immunodetection (anti-TRAIL antibody 2E5, Alexis, Breda, The Netherlands) for 1 h, followed by standard detection with GARPO/OPD.

Apoptotic activity of biotinylated TRAIL

The apoptosis inducing activity of the biotinylated products was evaluated on Jurkat T cells and on endothelial cells, using a cell viability assay and a caspase activity assay. Experiments were conducted with the single cell types, Jurkat tumor cells or HUVEC, or with a combination of tumor and endothelial cells.

Assessment of TRAIL activity by MTS cell viability assay

Briefly, cells were seeded in flat-bottom 96-well plates at a density of 3×10^4cells/well (Jurkat) or 1×10^4cells/well (HUVEC) in 100 μl medium supplemented with the indicated compounds. Concentrations of TRAIL, bio-NHS-TRAIL and Bio-ULS-TRAIL ranging from 1 μg/ml to 1 ng/ml were tested on Jurkat. Only one concentration (100 ng/ml) was tested on HUVEC. The MTS cell viability assay (Promega) was performed after 48 h of incubation, according to the manufacturer's recommendations. Experiments were performed in triplicate.

Assessment of TRAIL activity by caspase 3/7 assay

We investigated the induction of apoptosis in Jurkat T cells using a caspase 3/7 activity assay (Promega) employing luminescence detection. Different experimental settings were evaluated either reflecting direct incubation of the products with tumor cells, or settings in which biotinylated TRAIL was complexed to RGDPEG-avidin and/or anchored onto endothelial cells.

First, we determined pro-apoptotic effects of non-complexed TRAIL. About 5×10^3 Jurkat cells were incubated with biotinylated TRAIL at a concentration of 100 ng/ml. Experiments were carried in 96-well plates in 50 µl medium supplemented with the indicated compounds for 4 h, after which 50 µl of caspase 3/7 substrate was added to the cells, followed by luminescence detection with a microplate luminometer according to the provider's instructions.

Next, we determined proapoptotic effects of RGDPEG-avidin:TRAIL complexes in Jurkat cells. These experiments were performed in the presence of endothelial cells, to allow adherence of the RGD-equipped complexes to $\alpha_v\beta_3$ integrin expressing cell layers. HUVEC were preincubated with 10 µg/ml RGDPEG-avidin for 2 h at room temperature after which the excess of non-cell associated RGDPEG-avidin was removed by washing with PBS. Control experiments included RGDPEG-avidin treated HUVEC that were not washed and cells to which no RGDPEG-avidin was added. All wells were supplemented with biotinylated TRAIL (100 ng/ml in culture medium), after which Jurkat cells (5×10^3 cells in 50 µl of medium) were added. Cells were incubated for 4 h at 37°C, after which caspase activity was assayed as described above. Experiments were conducted in triplicate.

Statistical analysis

Statistical analysis was performed using Student's two-tailed *t*-test, assuming equal variances. Difference were considered to be significant when $P < 0.05$ unless otherwise stated.

Results

Preparation and characterization of RGDPEG-avidin

We modified avidin with PEG-linked RGD-peptides to obtain an avidin molecule which could bind to angiogenic endothelial cells. Successful synthesis of RGDPEG-avidin was proven by several analytical techniques. SDS-PAGE analysis (Fig. 2a) showed a single band for avidin of approximately 15 kDa, corresponding to the weight of one

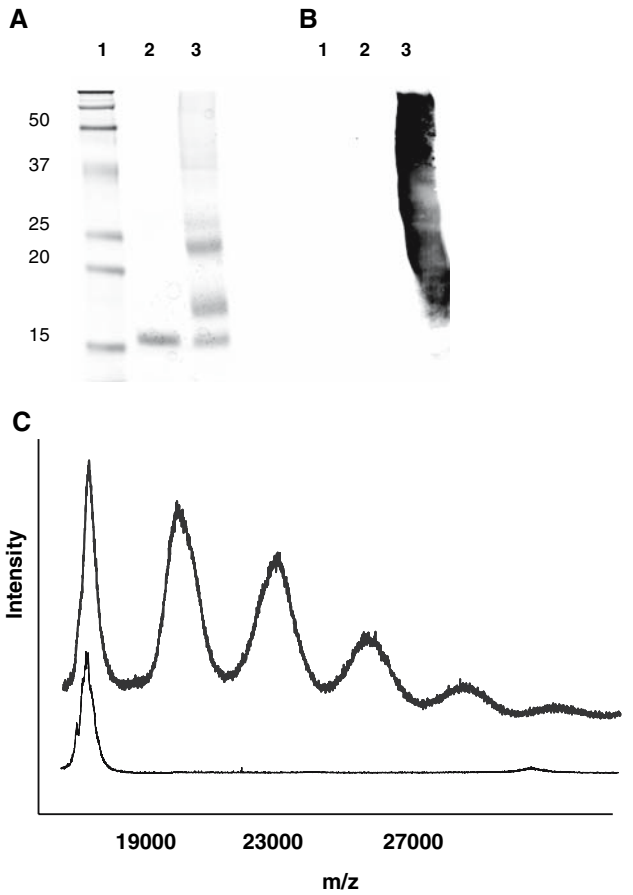

Fig. 2 Characterization of RGDPEG-avidin. Panel A and B: SDS-PAGE separation followed by CBB protein staining (**a**) or Western blotting and anti-RGD detection (**b**). lane 1: MW marker, lane 2: avidin, lane 3: RGDPEG-avidin. Unmodified avidin was separated into its 15 kDa subunits, while RGDPEG-avidin was disassembled into different bands corresponding to subunits with 0, 1, 2 or 3 attached RGDPEG groups. The intrinsic heterogeneity of PEG polymers can be observed in the broadly stained band of the RGDPEG-avidin Western blot. Panel C: MALDI-TOF analysis of RGDPEG-avidin. Upper line: RGDPEG-avidin, bottom line: avidin. During ionization, the tetrameric proteins have disassembled into subunits, yielding a single peak for avidin and multiple peaks for RGDPEG-avidin corresponding to modification degree 0, 1, 2, 3, 4

of its four subunits. Different protein bands were observed for RGDPEG-avidin with molecular weights corresponding to the unmodified subunit and to subunits modified with one or multiple RGDPEG moieties. Second, Western blot of the product showed positive staining of these bands by an anti-RGD-peptide antiserum. Due to the size heterogeneity of the PEG polymer, the RGD-band stained in a broad streak rather than a discrete band (Fig. 2b). Of note, the tetrameric band of avidin was not observed due to the denaturating conditions of SDS-PAGE. Third, we analyzed the product by MALDI-TOF mass spectrometry (Fig. 2c), which showed peaks corresponding to avidin subunits modified with RGDPEG to a different degree. From the

average molecular weights (Mn) we calculated a relative derivatization ratio of 1.4 RGDPEG groups per avidin subunit, or a total of 5.6 RGDPEG groups per tetrameric avidin.

Next, we investigated the interaction of RGDPEG-avidin with HUVEC, which express $\alpha_v\beta_3$-integrin when being at a subconfluent state. To determine binding affinity of RGDPEG-avidin for $\alpha_v\beta_3$-integrin, we studied its capability to compete with the $\alpha_v\beta_3$-integrin ligand ^{125}I-echistatin. While RGDPEG-avidin completely displaced ^{125}I-echistatin from the cells, similar to the free RGD-peptide, avidin did not affect binding of the radiolabeled peptide (Fig. 3a). The sigmoid displacement curve with RGDPEG-avidin (Fig. 3b) demonstrated a 2.9-fold increase in affinity (IC_{50} = 134 nM) as compared to the single RGD-peptide (IC_{50} = 383 nM).

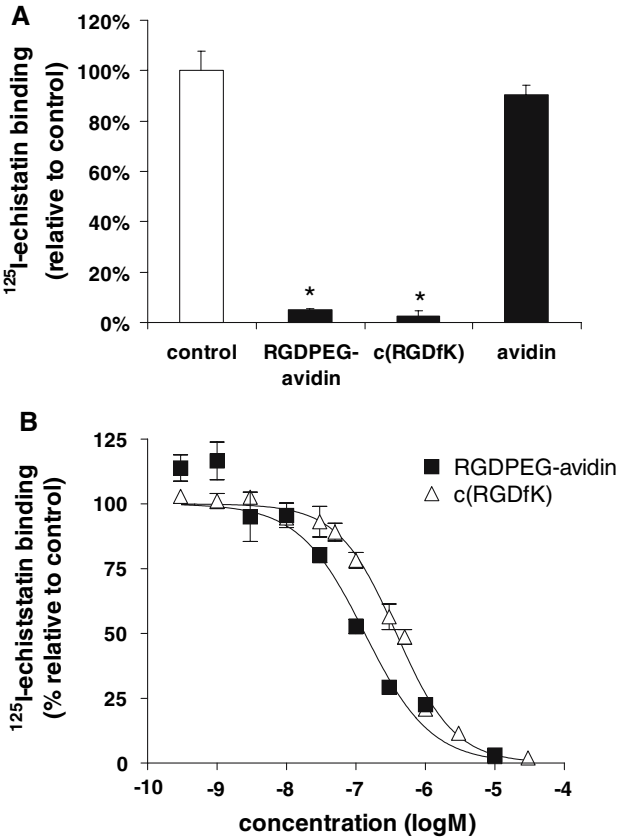

Fig. 3 Association of RGDPEG-avidin with endothelial cells. Subconfluent HUVEC were incubated with radiolabeled echistatin, which is a known ligand for $\alpha_v\beta_3$ integrin, and RGDPEG-avidin, free RGDpeptide or unmodified avidin. Panel A: Binding specificity. Echistatin was displaced by excess of RGDPEG-avidin or free RGD-peptide (both at 10 μM) but not by excess of avidin. *$P < 0.05$. Panel B: Affinity of binding. Echistatin binding was displaced by different concentrations of the added competing ligands, showing the increased affinity of RGPEG-avidin over the single RGDpeptide ligand

Preparation and characterization of biotinylated TRAIL

Since the modification of side-chain residues may diminish the activity of a therapeutic protein, we studied two different biotinylation strategies that target chemical modifications at different amino acid residues, i.e., at lysine groups (biotin-NHS) or methionine residues (biotin-ULS). Such different labeling positions at the surface of the protein may correlate to differences in pharmacological activity of the biotinylated proteins. Anti-biotin ELISA (Fig. 4a) demonstrated a higher biotin/TRAIL modification of biotin-NHS over biotin-ULS, which is in line with the lower abundance of methionine residues as compared to the number of lysine residues. We tried to confirm these data by analysis of the biotinylation extent by MALDI-TOF mass spectrometry, which provided reliable data on the average number of incorporated RGDPEG units in avidin. Although this approach succeeded for biotin-NHS-TRAIL (Fig. 4b), demonstrating the incorporation of about 1 biotin group per subunit of TRAIL, MALDI-TOF analysis failed to detect biotin-ULS modification of TRAIL. Most likely, the mass ionization step induced the dissociation of biotin-ULS from the protein. From the collective results, we concluded that both strategies had yielded biotinylated TRAIL with approximately 1–3 biotin groups per protein (summarized in Table 1).

We studied complexation of biotinylated TRAIL with RGDPEG-avidin that had been coated in 96-well plates. Highest binding was observed for Bio-NHS-TRAIL, probably due to its higher biotin content, while bio-ULS-TRAIL showed approximately 40% less binding (Fig. 5). Unmodified His-TRAIL showed non-specific binding to RGDPEG-avidin as well, despite the absence of biotin groups in this product. Non-specific binding of TRAIL to RGDPEG-avidin was however far lower than binding to unmodified avidin, most likely due to the PEGylation (data not shown).

Cytotoxic and proapoptotic activity of TRAIL complexes

To test whether our products were functionally active, i.e., capable of inducing cell death and apoptosis, we assessed their activity on Jurkat T leukemic cells and HUVEC endothelial cells. Figure 6a shows the dose-dependent cytotoxic effects at Jurkat cells after treatment for 48 h. Both His-TRAIL, Bio-NHS-TRAIL and Bio-ULS-TRAIL effectively killed Jurkat cells with IC_{50} values between 40 ng/ml and 123 ng/ml (Table 1). While the Bio-NHS modification resulted in a considerable reduction in activity compared to the unmodified protein, Bio-ULS-TRAIL displayed almost similar activity as unmodified TRAIL. The pro-apoptotic

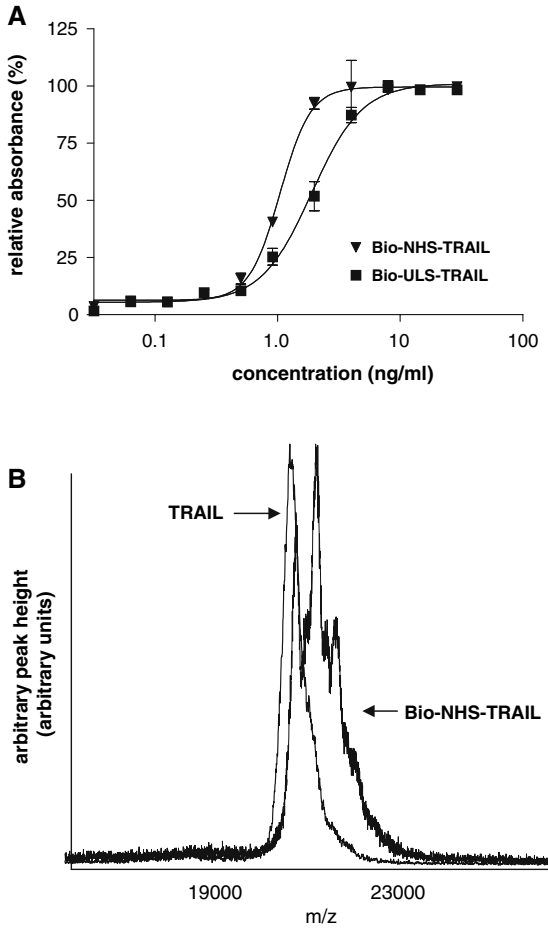

Fig. 4 Characterization of biotinylated TRAILs. Panel A: Anti-biotin ELISA with streptavidin-HRP. Signals were corrected for background and expressed relative to the maximum detected intensity. Panel B: MALDI-TOF analysis of TRAIL and biotin-NHS-TRAIL

activity of His-TRAIL and biotinylated TRAIL derivatives in Jurkat cells was furthermore determined by analyzing caspase 3/7 activity, as shown in Fig. 6b. These experiments confirmed the relative higher activity of bio-ULS-TRAIL versus bio-NHS-TRAIL, and the absence of caspase induction by RGDPEG-avidin (data not shown). In contrast to Jurkat cells, no cytotoxic effects were observed when HUVEC were incubated with the products (Fig. 6c).

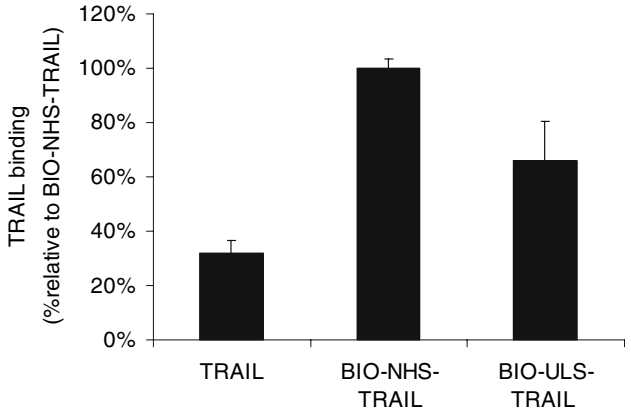

Fig. 5 Association of biotinylated TRAIL with RGDPEG-avidin. 96-well plates were coated overnight with 50 ng RGDPEG-avidin and incubated with indicated compounds for 2 h. TRAIL binding was assessed by anti-TRAIL immunodetection and expressed relative to the highest signal obtained with biotin-NHS-TRAIL. Experiments were performed in triplicate

Finally, we investigated the proapoptotic effects of RGDPEG-avidin:TRAIL complexes in Jurkat cells. These experiments were performed in the presence of endothelial cells, to allow adherence of the RGD-equipped complexes to $\alpha_v\beta_3$ integrin expressing cell layers. First, we determined the effects of avidin:TRAIL complexes formed in the presence of an excess of RGDPEG-avidin. As can be observed in Fig. 7, such complexes with both types of biotinylated TRAIL showed similar activity to the non-complexed biotinylated TRAIL derivatives (striped bars versus white bars, respectively). Furthermore, in agreement with Fig. 6b, Bio-NHS-TRAIL showed a reduced activity as compared to unmodified TRAIL, while Bio-ULS-TRAIL retained its activity.

A striking result was observed when the excess of RGDPEG-avidin was removed before addition of TRAIL and Jurkat cells, leaving only endothelial-associated RGDPEG-avidin available for complexation with biotinylated TRAIL. This procedure significantly potentiated the proapoptotic effect for both Bio-NHS-TRAIL and Bio-ULS-TRAIL, the latter even beyond the activity of unmodified TRAIL.

Table 1 Characteristics of biotinylated TRAIL variants

	Relative biotin content (Biotin/TRAIL)[a]	Activity in cell viability assay (IC_{50} in ng/ml)[b]	Activity in caspase 3/7 assay[c] (%)
TRAIL	–	40	100
Bio-NHS-TRAIL	1.6	123	35
Bio-ULS-TRAIL	0.8	60	126

[a] Expressed as mole biotin bound per mole of trimeric TRAIL. Relative biotin content was determined by MALDI-TOF analysis (bio-NHS-TRAIL) or ELISA (BIO-ULS-TRAIL)

[b] As determined after 48 h of incubation with Jurkat cells

[c] As determined after 4 h of incubation at 10 ng/ml with Jurkat cells

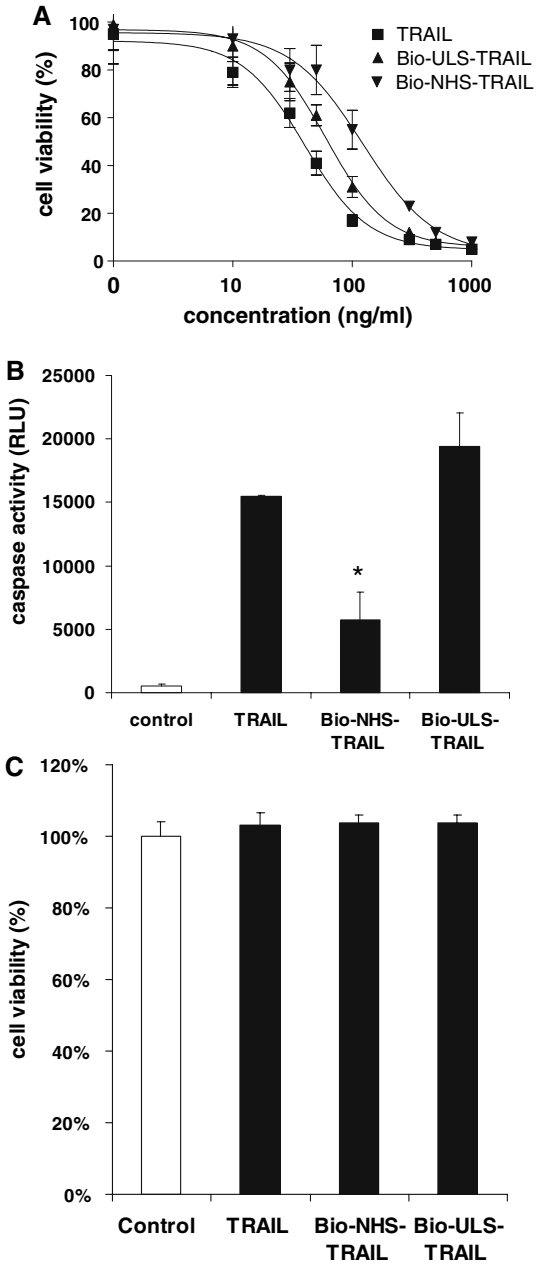

Fig. 6 TRAIL activity assays. Panel A: Cell viability of Jurkat T cells after 48 h of incubation with indicated compounds. Cell viability was assessed by MTS assay. Experiments were performed in triplicate. Panel B: Caspase 3/7 activity in Jurkat T cells after 4 h of incubation with 100 ng/ml of the indicated TRAIL derivatives. RLU: relative light units. Panel C: Cell viability (MTS assay) of HUVEC after 48 h of incubation with 100 ng/ml of indicated compounds

Discussion

In the present study we describe the development and functional evaluation of RGD-avidin:TRAIL complexes. These targeted complexes of TRAIL showed enhanced binding to endothelial cells and maintained their pro-apoptotic activity. As a result of the binding to angiogenic endothelial cells of these complexes, an improved tumor accumulation and enhanced residence within tumor tissue can be expected. Such an improvement in the pharmacokinetic profile of TRAIL may further enhance its therapeutic efficacy and enable a reduction in dosing frequency.

$\alpha_v\beta_3$ integrin is a validated target for drug delivery and imaging approaches directed to angiogenic endothelium. One of the most popular ligands for $\alpha_v\beta_3$ integrin is the cyclic RGD-peptide cyclo(RGDfK). Due to the special constrained conformation of the cyclic RGD-peptide and the D-phenylalanine amino acid, its binding motif adopts a high affinity conformation for α_v-integrins [25]. The cyclo(RGDfK) peptide has been used successfully for the delivery of a wide range of drug or gene carrying systems (e.g., liposomes, polymers, adenoviruses) [18]. Since the cyclopeptide is an unnatural peptide, it can not be encoded by a DNA sequence and requires chemical synthesis and hence chemical coupling techniques to prepare multivalent targeting devices. The present paper describes a versatile approach in which multivalent RGD-avidin is complexed with biotinylated TRAIL. The RGD-peptides are linked to avidin via bifunctional PEG linkers, thereby surrounding the avidin by a hydrophilic PEG shell tethered with targeting ligands. PEGylation is a widely accepted methodology for improvement of therapeutic proteins, and affects both the immunological properties as well as the

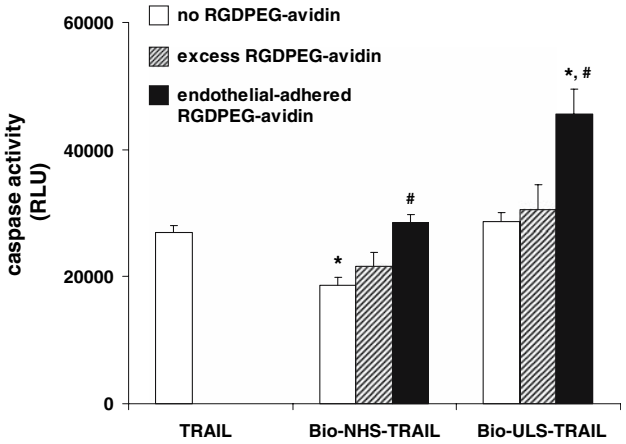

Fig. 7 Proapoptotic activity of RGDPEG-avidin:TRAIL complexes. Assays were performed in 96-well plates seeded with HUVEC that were subsequently incubated at room temperature with RGDPEG-avidin (10 μg/ml) and biotinylated TRAIL (100 ng/ml), to allow assembly of the complexes. Next, Jurkat T cells were added followed by 4 h of incubation at 37°C, after which the activation of caspase 3/7 was determined by a luminescence based assay. Experiments were carried out in the absence of RGDPEG-avidin (control experiment, white bars), in the presence of excess of RGDPEG-avidin (striped bars, 100-fold molar excess over biotinylated TRAIL) or in wells incubated with RGDPEG-avidin that had been washed before addition of biotin-TRAIL and Jurkat cells (black bars). $*P < 0.05$ versus TRAIL; $\#P < 0.05$ versus biotin-TRAIL in the absence of RGDPEG-avidin

renal filtration of proteins [26]. The functionalization of avidin with RGD-PEG groups thus will combine several advantageous properties. RGDPEG-avidin displayed nanomolar affinity for $\alpha_v\beta_3$ integrin expressing endothelial cells, which is in good agreement with the data obtained with other RGD-equipped proteins prepared in our group [27].

We have compared two different biotinylation procedures, which react at different amino acid side chain residues. While biotin-NHS couples at lysine residues, biotin-ULS binds at methionines residues in TRAIL. The methionines in TRAIL are not solvent exposed, as can be derived from the crystal structure of TRAIL [28]. Therefore, the most likely binding site for biotin-ULS is the methionine in the HIS-tag of the recombinant protein. In contrast, most lysine residues are readily accessible, including ones at positions that will interface with the Death receptor. This could explain why Biotin-ULS-TRAIL better maintained its proapoptotic activity than biotin-NHS derivatized TRAIL. Whether the greater loss in activity of biotin-NHS-TRAIL is due to the relative higher extent of derivatization or to modifications of lysine residues in the activity domains of protein can not be concluded from our data.

Upon complexation of biotinylated TRAIL with RGD-PEG-avidin, a bulky structure has been introduced onto the surface of the therapeutic protein. The assembled complexes, however, fully maintained their TRAIL activity indicating that steric hindrance did not interfere in the TRAIL-death receptor ligation. Figure 7 showed similar proapoptotic activities for either biotinylated TRAIL or the complexes formed in the presence of excess of RGDPEG-avidin. Remarkably, we observed an enhancement of caspase activity when the excess of RGDPEG-avidin was removed, leaving only surface-exposed RGDPEG-avidin at the endothelial cell layer. One of the explanations that may account for this is the assembly of multiple TRAIL molecules onto a single avidin, thereby affording a 'mega'TRAIL complex with enhanced death-inducing activity, as was also observed for multimeric forms of Fas-ligand [29]. Such types of complexes would facilitate the close assembly of multiple death receptors, thereby potentiating the induction of death signaling. The presence of an excess of RGDPEG-avidin would avoid the formation of such complexes, as both components were added in a 100:1 molar ratio of avidin:TRAIL. Gently washing of the endothelial cells after RGDPEG-avidin incubation removed the excess of unbound RGDPEG-avidin, leaving only cell-associated avidin after incubation at room termperature. These conditions therefore provided an excess of biotinylated TRAIL over avidin, now favoring the formation of multimeric TRAIL complexes. Alternatively, the exposure of TRAIL via RGD-PEG-avidin onto the surface of endothelial cells may explain its increased activity, as has been observed for chimeric TRAIL variants directed to tumor receptors [10].

HUVEC were not killed by TRAIL or its derivatives, as has been described by others [30]. On the other hand, although TRAIL does not induce apoptosis in endothelial cells, it activates other processes such as production of NO and reactive oxygen species which may alter the tumor responsiveness to TRAIL [31]. This can not explain the results shown in Fig. 7, since endothelial cells were preincubated with RGDPEG-avidin only and preincubations were performed at room temperature rather than at 37°C. Such effects of RGD-equipped TRAIL may occur however in vivo. In addition, tumor derived growth factors might sensitize endothelial cells to TRAIL's apoptosis inducing capacity, similar to the findings reported for TNF-sensitivity [32].

The complexation of biotinylated TRAIL with RGD-PEG-avidin and its association with angiogenic endothelial cells may afford an increased accumulation into the tumor blood vessels, similar to the enhanced tumor accumulation of other RGD-modified proteins [18]. An unresolved issue is whether RGD-avidin/TRAIL complexes bound to the tumor vasculature can interact with tumor cells behind the endothelial lining in vivo. To interact with tumor cells and induce tumor apoptosis, TRAIL complexes bound to the tumor vasculature should dissociate and penetrate into the tumor tissue. Several considerations may be important in this respect, such as the expression of $\alpha_v\beta_3$ integrin on both luminal and abluminal side of the endothelium and the fact that many tumor cells express $\alpha_v\beta_3$ integrin [18]. Both of these will favor the interaction of RGD-avidin complexed TRAIL with tumor cells. Furthermore, RGD-avidin binding to endothelial cells is less tight than the typical antibody binding. This may favor redistribution of the complexes upon their initial binding to $\alpha_v\beta_3$ integrin. Homing to the tumor vasculature will provide elevated concentrations within the tumor microenvironment, which may subsequently provide elevated intratumoral concentrations. Together with the observed enhancement of TRAIL activity, this may favor a more efficient induction of apoptosis in tumor cells.

To summarize, we have prepared a novel type of RGD-targeted TRAIL with binding specificity for angiogenic endothelium and maintenance of strong cytotoxic activity. Whether in vivo application of this product will lead to an enhanced tumor accumulation still needs to be investigated. We now succeeded in coupling the well known homing device RGD to the TRAIL molecule, without losing the apoptosis-inducing activity of TRAIL or the receptor-binding properties of RDG peptides. An enhanced activity could even be demonstrated in vitro. This may, in combination with an improved pharmacokinetic profile

induced by the PEG and RGD moieties, lead to improved effects of TRAIL in vivo. Lastly, the followed synthetic approach offers opportunities for the complexation with alternative targeting ligands that bind directly to tumor cells, such as tumor-specific antibodies or peptide ligands.

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