Targeted induction of apoptosis for cancer therapy

Bremer, Edwin

chapter 6

Target cell-restricted apoptosis induction of acute leukemic T-cells by a recombinant TRAIL fusion protein with specificity for human CD7.

Edwin Bremer[1], Douwe F. Samplonius[1], Matthias Peipp[2], Linda van Genne[1], Bart-Jan Kroesen[1], Georg H. Fey[3], Martin Gramatzki[4], Lou F.M.H. de Leij[1], Wijnand Helfrich[1]

[1]Groningen University Institute for Drug Exploration (GUIDE), Department of Pathology & Laboratory Medicine, Section Medical Biology, Laboratory for Tumor Immunology, University Medical Center Groningen, University of Groningen, The Netherlands.

[2]Division of Nephrology, University Hospital Schleswig-Holstein, Campus Kiel, Kiel, Germany;

[3]Chair of Genetics, University of Erlangen, Erlangen/Nuremberg, Germany;

[4]Division of Stem Cell and Immunotherapy, 2nd Medical Department, University Clinic Schleswig-Holstein, Kiel, Germany

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Abstract

Current treatment of human T-cell Leukemia and lymphoma is predominantly limited to conventional cytotoxic therapy and is associated with limited therapeutic response and significant morbidity. Therefore, more potent and leukemia-specific therapies with favourable toxicity profiles are urgently needed. Here, we report on the construction of a novel therapeutic fusion protein, scFvCD7:sTRAIL, designed to induce target antigen-restricted apoptosis in human T-cell tumours. ScFvCD7:sTRAIL consists of the death inducing ligand TRAIL genetically linked to an scFv antibody fragment specific for the T-cell surface antigen CD7. Treatment with scFvCD7:sTRAIL induced potent CD7-restricted apoptosis in a series of malignant T-cell lines, while normal resting leukocytes, activated T-cells, and vascular endothelial cells (HUVECs) showed no detectable apoptosis. The apoptosis-inducing activity of scFvCD7:sTRAIL was stronger than that of the immunotoxin scFvCD7:ETA. In mixed-culture experiments with CD7-positive and CD7-negative tumour cells, scFvCD7:sTRAIL induced very potent bystander apoptosis of CD7-negative tumour cells. *In vitro* treatment of blood cells freshly derived from T-acute lymphoblastic leukemia (ALL) patients resulted in marked apoptosis of the malignant T-cells that was strongly augmented by vincristin. In conclusion, scFvCD7:sTRAIL is a novel recombinant protein causing restricted apoptosis in human leukemic T-cells with low toxicity for normal human blood and endothelial cells.

Introduction

In the last few decades, the treatment outcome of patients with leukemia and lymphoma has significantly improved. Nonetheless, only a minority of patients with T-cell ALL or peripheral T-cell lymphoma (PTCL) achieve long-term tumour-free survival[1]. Conventional cytotoxic therapy in these diseases is usually associated with substantial side-effects and limited response. Therefore, more potent targeted therapies with greater specificity and favourable toxicity profiles are needed in order to increase the so-far unsatisfactory treatment success of human T-cell tumours.

Recently, several leukemia-targeted therapeutic agents have been developed, including naked monoclonal antibodies (MAb), MAb-toxin conjugates, radioimmunoconjugates, and small molecules inhibiting key cellular functions such as tyrosine kinases. The research on many of these agents is still in early phases. Clinical experience with therapeutic antibodies in T-ALL is limited to the anti-CD3 MAb OKT3[2], which produced only a transient anti-tumour effect, while in more mature T-cell lymphoma, antibodies to CD52 (CAMPATH-1H)[3] and to CD25[4] have been used with considerable efficacy.

Currently, several CD7 MAb-toxin conjugates are evaluated in pre-clinical studies and

clinical trials, some with promising results[5-8]. However, targeted strategies using MAb-toxin conjugates can be severely hampered by toxin-related side effects, such as severe hepatic veno-occlusive disease frequently observed during treatment of AML with the anti-CD33 MAb-calicheamicin conjugate gemtuzumab-ozogamicin (GO, Mylotarg™)[9,10]. Thus, the development of antibody-derived therapeutic agents with strongly improved toxicity profiles is urgently needed.

A promising candidate for safe and cancer-restricted induction of apoptosis is the death-inducing ligand TRAIL. TRAIL is a recently identified member of the TNF family of death inducing ligands and shows selective apoptotic activity towards a variety of tumour cell types without toxicity for normal cells[11,12]. Native TRAIL is expressed as a homotrimeric type II transmembrane protein (memTRAIL) that can be proteolytically cleaved into soluble homotrimeric TRAIL (sTRAIL). Various forms of sTRAIL have been generated by recombinant DNA technology all showing potent *in vitro* and *in vivo* anti-tumour effects[13-15]. TRAIL binds to an elaborate receptor system comprising at least two agonistic receptors, TRAIL-R1 and TRAIL-R2[16-18], and two antagonistic receptors, TRAIL-R3 and TRAIL-R4[19-21]. The various TRAIL receptors are widely expressed on a variety of normal tissues and malignant cell types. Initially, TRAIL-R3 and –R4 were thought to act as decoy receptors, protecting normal and TRAIL-resistant tumour cells from apoptosis. However, recent reports show no correlation between TRAIL-sensitivity and expression of either TRAIL-R3 or TRAIL-R4[22,23]. Consequently, the mechanism for the tumour-selective activity of TRAIL remains elusive.

Clustering of TRAIL-R1 and -R2 by TRAIL leads to formation of the death-inducing signalling complex (DISC)[24-26]. The DISC includes the adaptor protein FADD and the initiator pro-caspases-8 or -10[24,27-29]. Efficient DISC assembly results in concomitant activation of initiator and effector caspases (e.g. caspases-3, -6, -7) and ultimately leads to apoptotic cell death.

Recently, it was shown that TRAIL receptors 1 and 2 have quite distinct crosslinking requirements for the initiation of apoptosis[30]. TRAIL-R2 signals apoptosis only after efficient receptor crosslinking by either native memTRAIL, aggregated sTRAIL variants, or by sTRAIL preparations secondarily crosslinked by antibodies. Apoptosis signalling by TRAIL-R1 was largely independent of the receptor crosslinking characteristics of a particular form of sTRAIL[30]. Furthermore, TRAIL-R2 had superior binding affinity for TRAIL[31], resulting in predominant binding of sTRAIL to TRAIL-R2 over TRAIL-R1.

Differential expression of TRAIL-R1 and -R2 has been described for various tumour types, usually with TRAIL-R2 being the most prevalent. Consequently, tumour cells predominantly expressing TRAIL-R2 are relatively insensitive to treatment with homogeneous trimeric sTRAIL preparations.

The tumour-selective activity of the various sTRAIL preparations was shown to critically

rely on their respective state of aggregation. High molecular weight sTRAIL aggregates in solution generated significant apoptotic activity to certain normal cell types[32]. Preparations containing only homogeneous non-aggregated homotrimeric forms of sTRAIL showed more authentic tumour-selective pro-apoptotic activity[33].

Therefore, to fully exploit the therapeutic potential of TRAIL, several characteristics of both the TRAIL receptor system and sTRAIL should be taken into account. First, the wide spread expression of the various TRAIL receptors throughout the human body. Second, the differential binding affinities and crosslinking requirements of the agonistic receptors TRAIL-R1 and TRAIL-R2, and third, the solution behavior of the sTRAIL preparation. Previously, we and others[34,35] demonstrated that these criteria can be largely met by genetically fusing sTRAIL to a tumour-specific recombinant antibody fragment. This fused sTRAIL, scFv:sTRAIL, was selectively directed to a pre-determined target antigen and was deposited on the cell surface of target cells only. Target antigen bound scFv:sTRAIL acquired TRAIL-receptor activating properties resembling that of native memTRAIL. Local accumulation and activation of this TRAIL construct and the corresponding receptors greatly improved its therapeutic potential.

Here we describe a novel fusion protein, designated scFvCD7:sTRAIL, that contains a scFv antibody fragment specific for human CD7, a cell surface glycoprotein abundantly expressed on most T cell malignancies and approximately 10% of acute myeloid leukemias (AML)[36-39]. Expression of CD7 on normal blood cells is limited to T- and myeloid cells in early hematopoietic cell ontogeny, thymocytes, NK cells, and a large distinct subset of peripheral blood T-cells[40-44]. Fusion protein scFvCD7:sTRAIL shows enhanced and target antigen-restricted apoptotic activity towards human T-ALL cells with no toxicity to normal human blood and endothelial cells.

Material and methods

Monoclonal antibodies, scFv antibody fragment, and scFvCD7:ETA

MAb TH69 is a murine IgG1 with specificity for human CD7[45]. DNA encoding the anti-CD7 scFv 3A1F[46] was kindly provided by Dr. Chris Pennell, Department of Laboratory Medicine and Pathology, University of Minnesota. MAb TH69 and scFvCD7 compete for binding to the same epitope on the extracellular domain of human CD7. The immunotoxin scFvCD7:ETA comprises an anti-CD7 scFv genetically linked to pseudomonas Exotoxin-A (ETA)[5]. TRAIL-neutralizing MAb 2E5 was purchased from Alexis (Kordia Life Sciences, Leiden, The Netherlands).

Chemotherapeutics

Vincristin (USPC Inc, Rockville, MD, USA), 1 mg/ml in PBS. UCN01 (provided by Kyowa Hakko Europe GmbH, Düsseldorf, Germany), 10 mM in DMSO. Cycloheximide (CHX),

(Sigma), 100 mg/ml in DMSO. Actinomycin D (Sigma), 2 mg/ml in ethanol. Final working concentrations were prepared by serial dilutions of stocks solutions in serum free medium.

Cell lines

Human T-ALL cell lines Jurkat, CEM, MOLT-16 (all CD7-positive) and the human B-cell lymphoma Ramos (CD7-negative), were purchased from the ATCC (Manassas, USA). CD7-positive Ramos cells were generated by transfection of Ramos cells with plasmid pSecTag/HygroC-CD7 and selection of CD7-positive transfectants using Hygromycin B (500 µg/ml) followed by flowcytometric cell sorting. Transfection of Ramos with CD7 did not alter TRAIL-receptor or c-FLIP expression. All cell lines were cultured in RPMI (Cambrex, New Jersey, New Hampshire, USA) supplemented with 15% FCS, at 37ºC in humidified 5% CO_2 containing atmosphere.

Isolation of leukocytes, PBLs, activated T-cells, and HUVECs

Leukocytes were isolated from whole blood of healthy donors using the Ammonium Chloride method[47]. Peripheral blood lymphocytes (PBLs) were isolated from whole blood of healthy donors by standard density gradient centrifugation procedures (Lymphoprep, Axis-Shield PoC As., Oslo, Norway). Freshly isolated resting PBLs were resuspended at $2.0x10^6$ cell/ml in RPMI, supplemented with 10% human pooled serum. Activated T-cells were generated by incubation of freshly isolated PBLs with anti-CD3 MAb (0.5 µg/ml) for 72 h, followed by IL2 stimulation (100 ng/ml) for 48 h. Human umbilical vein endothelial cells (HUVEC) were isolated as previously described[48]. HUVEC cells were used before culture passage number four and, for experiments, were pre-cultured in 6 well plates at 60% confluency.

Construction of scFvCD7:sTRAIL

Eukaryotic expression plasmid pEE14scFv:sTRAIL was generated for the rapid construction, evaluation and stable expression of scFv:sTRAIL fusion proteins in CHO-K1 cells[35]. Plasmid pEE14scFv:sTRAIL is based on a vector previously described[49]. Important features are the murine kappa light-chain leader peptide encoded upstream of 2 multiple cloning sites (MCSs) that are separated by a 26 residue in-frame linker sequence, and the glutamine synthetase selectable marker gene, which allows for amplified expression of the recombinant protein in production cell line CHO-K1. The vector exploits the strong CMV promoter to drive recombinant protein expression, while the leader peptide directs the fusion protein through the ER and Golgi complex, resulting in excretion of correctly folded fusion protein into the culture supernatant[35]. In the first MCS, a 45 bp DNA fragment encoding scFvCD7 derived from phagemid pCANTAB5E/scFv3A1F was

directionally inserted using unique SfiI and NotI restriction enzyme sites. The second MCS contains a PCR-truncated 593 bp DNA fragment encoding the extracellular domain of human TRAIL (sTRAIL).

Production and characterization of scFvCD7:sTRAIL

Fusion protein scFvCD7:sTRAIL was eukaryotically expressed in CHO-K1 cells essentially as described[35]. In short, CHO-K1 cells were transfected with plasmid pEE14scFvCD7:sTRAIL using Fugene-6 reagent (Roche). Stable transfectants with amplified expression were generated by the glutamine synthetase selection method[50]. Individual clones, obtained after single cell sorting using the Moflo high-speed cell sorter (Cytomation, Fort Collins, USA), were analyzed for stable and high expression of scFvCD7:sTRAIL in the absence of MSX selection reagent using a solid-phase sandwich TRAIL ELISA according to manufacturer's recommendations (Diaclone SAS, Besançon, France). The procedure identified CHO-K1 production cell line 10F1, which stably secreted scFvCD7:sTRAIL (7.3 µg/ml) into the medium. Large-scale production of scFvCD7:sTRAIL was performed using roller bottles (Greiner Bio-One, Frickenhausen, Germany) at 37°C in serum-free CHO-S SFM II suspension medium (Gibco, Life Technologies, Breda, The Netherlands) to a density of $5.0 \cdot 10^6$ cells/ml, after which supernatant was harvested (1500g, 10 min) and stored at -80°C until further use.

Size-Exclusion FPLC of scFvCD7:sTRAIL

Solution behaviour of scFvCD7:sTRAIL was analyzed by size-exclusion (SE) FPLC using a calibrated HiLoad 16/60 Superdex 200 Prep-grade column (Amersham Biosciences, Upsala, Sweden) with a bed volume of 120 ml; 5 ml supernatant derived from CHO-K1 cell line 10F1 was loaded onto the column, after which individual samples were collected at 3-min intervals. All samples were analyzed for their capacity to induce apoptosis using TRAIL-sensitive Jurkat cells. Furthermore, all samples were subjected to a sensitive TRAIL-specific ELISA to quantitate individual scFvCD7:sTRAIL content.

CD7-specific binding of scFvCD7:sTRAIL

CD7-specific binding of scFvCD7:sTRAIL was assessed using Ramos and Ramos.CD7 cells. In short, 1.0×10^6 cells were incubated with scFvCD7:sTRAIL in the presence or absence of CD7 MAb TH69 (5 µg/ml). CD7-specific binding of scFvCD7:sTRAIL to the cell surface of Ramos.CD7 cells was analyzed by flow cytometry using a PE-conjugated anti-TRAIL MAb (Diaclone SAS, Besancon, France). Incubations were carried out for 45 min at 0°C and were followed by two washes with serum free medium.

CD7-restricted induction of apoptosis by scFvCD7:sTRAIL

CD7-positive tumour cells were seeded at $0.5 \cdot 10^6$ cells/well in a 24-well plate and treated for 16 h with 100 ng/ml scFvCD7:sTRAIL (unless indicated otherwise), in the presence or absence of MAb TH69 (2 µg/ml) or MAb 2E5 (1 µg/ml). Induction of apoptosis was assessed using one of the following apoptosis assays: Assessment of apoptosis by monitoring exposure of phoshatidylserine; The early apoptotic feature of exposure of phosphatidyl serine on the outer membrane was analyzed by flow cytometry using an AnnexinV-FITC/PI kit (NeXins Research, Kattendijke, The Netherlands) according to manufacturer's instructions. Assessment of apoptosis by monitoring loss of mitochondrial membrane potential (Δψ); Δψ was analyzed by flow cytometry using the cell-permeant green-fluorescent lipophilic dye DiOC6 (Molecular Probes, Eugene, USA) actively taken up by intact mitochondria of living cells only. After treatment, cells were harvested by centrifugation (300xg; 5 min), incubated for 30 min at 37°C with fresh medium containing 0.1 µM DiOC6, washed once with PBS, and analyzed by FACS. Immunoblot analysis of caspase activation and PARP cleavage; Induction of apoptosis evidenced by activation of caspase-8 and caspase-3, and PARP cleavage, was assessed by immunoblot analysis using anti-caspase-8 (Cell signalling technology, Beverly, MA, USA), anti-active caspase-3 (BD biosciences, San Jose, CA, USA), and anti-PARP (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Appropriate secondary PO-conjugated antibodies were from DAKO Cytomation (Glostrup, Denmark). Cells were seeded in 6 well plates at a final concentration of $2.0 \cdot 10^6$ cells/ml and treated as indicated. Cell lysates were prepared and immunoblot analysis was performed essentially as described[35]. *Detection of apoptotic DNA fragmentation;* Apoptotic DNA-fragmentation was analyzed using MAb F7-26 (Alexis, Kordia Life Sciences) according to manufacturer's recommendations. MAb F7-26 specifically detects DNA fragmented by apoptosis without reactivity for otherwise fragmented double-stranded DNA[51].

Differential quantification of apoptosis in target and bystander cells during mixed culture experiments

For mixed culture experiments, differential cell membrane labelling of target and bystander cells was achieved using the Vibrant Multicolour Cell-Labelling kit (Molecular probes). Briefly, CD7-positive Jurkat cells were labelled with the red fluorescent dye DiI, while CD7-negative Ramos bystander tumour cells or 'innocent' bystander leukocytes were not labelled. Labelling was performed by incubation of Jurkat cells ($1.0 \cdot 10^6$ cells/ml in serum free medium) with 5 µM DiI (37°C, 5 min), followed by three washes with medium. DiI-labelled target and non-labelled bystander cells were mixed at the indicated ratios at a final concentration of $0.5 \cdot 10^6$ cells/well of a 24-well plate. After treatment, differential fluorescent characteristics of target cells and bystander cells were used to separately evaluate induction of apoptosis in both populations by Δψ or AnnexinV staining.

CD7-restricted apoptosis induction in patient-derived leukemic cells

Blood cells derived from T-ALL patients, containing >90% leukemic T-cells, were briefly cultured and subsequently analyzed for sensitivity to apoptosis induction by scFvCD7:sTRAIL. Cells were treated for 16 h with scFvCD7:sTRAIL (1 µg/ml) in the presence or absence of MAb TH69 or MAb 2E5. Alternatively, cells were treated with scFvCD7:sTRAIL or vincristin (10 ng/ml) alone or in combination. Apoptosis was assessed by AnnexinV/PI staining as described above.

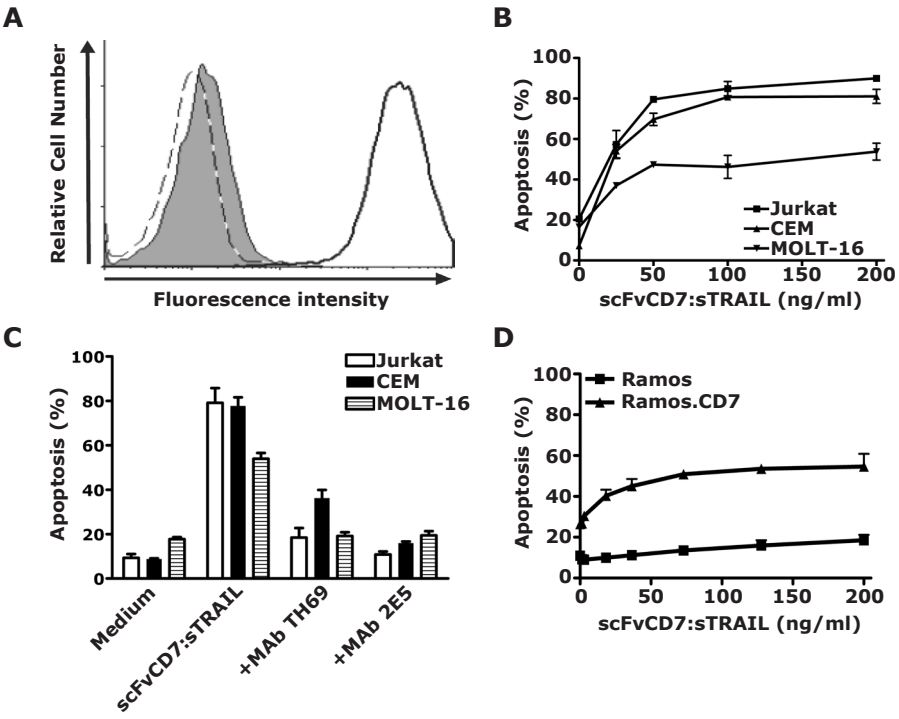

Fig.1. CD7-specific binding and apoptosis induction by scFvCD7:sTRAIL. A; Binding of scFvCD7:sTRAIL was analyzed by flow cytometry using CD7-negative Ramos and the transfectant cell line Ramos.CD7, ectopically over-expressing CD7. Ramos (solid fill) or Ramos.CD7 (solid line) were incubated with scFvCD7:sTRAIL. Additionally, Ramos.CD7 was pre-incubated with MAb TH69 followed by incubation with scFvCD7:sTRAIL (dashed line). **B;** CD7-positive T-ALL cell lines Jurkat, CEM, and MOLT-16 were treated for 16 h with increasing concentrations of scFvCD7:sTRAIL. **C;** Jurkat, CEM, and MOLT-16 were treated with scFvCD7:sTRAIL (100 ng/ml) in the presence or absence of MAb TH69 or TRAIL-neutralizing MAb 2E5. **D;** Ramos and Ramos.CD7 cells were treated for 16 h with increasing concentrations of scFvCD7:sTRAIL. In all of the above-described experiments, apoptosis was assessed by Δψ. Indicated values are mean + standard error of the mean of three independent experiments.

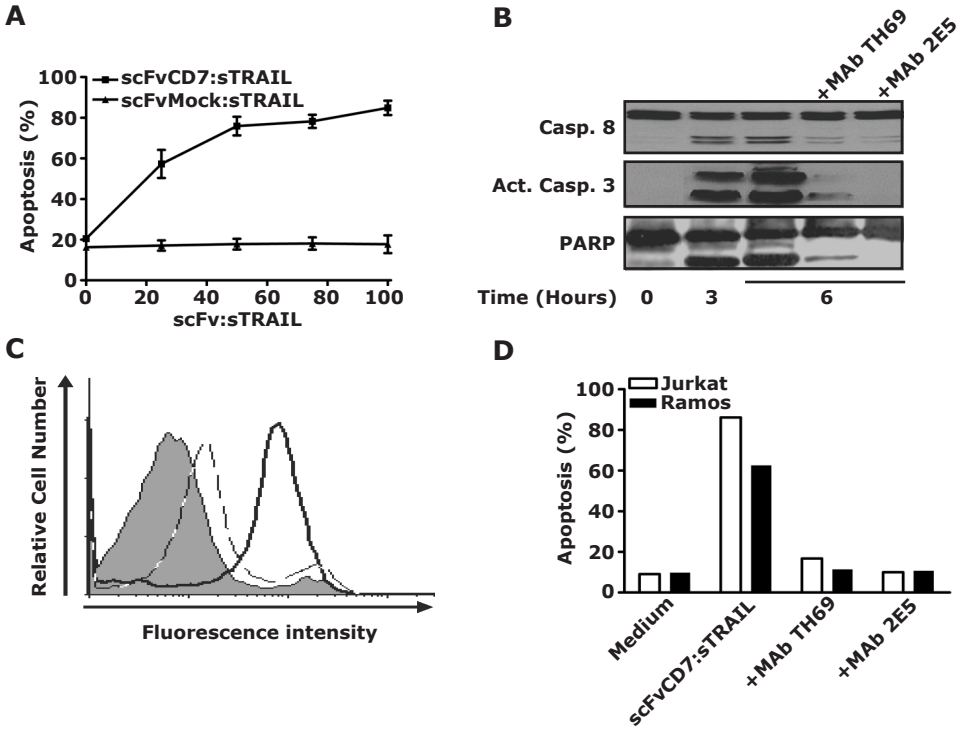

Fig.2. CD7-specific apoptosis induction by scFvCD7:sTRAIL. A; Jurkat cells were treated for 16 h with increasing concentrations of scFvCD7:sTRAIL or with MOCK-scFv:sTRAIL, containing an antibody fragment of irrelevant specificity, after which apoptosis was assessed by Δψ. Indicated values are representatives of three independent experiments **B;** Jurkat cells were treated with scFvCD7:sTRAIL (100 ng/ml) for the indicated time-points. For the 6 h incubation time, cells were additionally incubated with MAb TH69 or MAb 2E5. Cell lysates were assessed for the characteristic TRAIL-associated apoptotic features of caspase-8 activation, caspase-3 activation and PARP cleavage by immunoblot. **C:** Jurkat cells were treated for 24 h with 100 ng/ml scFvCD7:sTRAIL (solid line) in the presence or absence of MAb TH69 (dashed line). Apoptotic DNA fragmentation was assessed using MAb F7-26 as described in M&M section. Fluorescent intensity of conjugate control is shown as solid fill. **D;** Mixed cultures of Jurkat target cells and Ramos bystander cells (ratio 7:3) were treated for 16 h with scFvCD7:sTRAIL (300 ng/ml) in the presence or absence of MAb TH69 or MAb 2E5. Differential fluorescent labeling of the target and bystander population was used to separately assess apoptosis induction by Δψ. Indicated values are representatives of three independent experiments.

Results

Solution behavior of scFvCD7:sTRAIL

Supernatant of CHO-K1 clone 10F1 containing scFvCD7:sTRAIL was fractionated by SE-FPLC. Induction of apoptosis of the TRAIL-sensitive cell line Jurkat was restricted to individual samples collected after 97-114 min. The chromatographic mobility of

scFvCD7:sTRAIL corresponded to a molecular weight of approximately 160 kDa, in close agreement with the 154 kDa calculated for trimeric scFvCD7:sTRAIL. A sensitive TRAIL specific ELISA subsequently confirmed that only these fractions contained scFvCD7:sTRAIL, indicating that scFvCD7:sTRAIL was produced as homogenous trimers in the absence of high molecular weight aggregates (data not shown).

CD7-restricted binding and induction of apoptosis by scFvCD7:sTRAIL

Incubation of Ramos.CD7 cells with scFvCD7:sTRAIL, resulted in specific and abundant binding (Fig.1A, shaded peak). Binding to Ramos.CD7 cells was specifically inhibited by pre-incubation with CD7-competing MAb TH69 (Fig.1A, dashed line) to levels observed for CD7-negative Ramos cells (Fig.1A, solid fill) and, therefore, was CD7-specific. Binding of soluble scFvCD7:sTRAIL via its TRAIL domain to cell surface-expressed TRAIL receptors was also assessed by FACS. To this end, a MAb specific for the HA-tag present at the N-terminus of scFvCD7:sTRAIL was used. Binding of scFvCD7:sTRAIL to CD7-negative Ramos cells was barely detectable (data not shown).

Treatment of CD7-positive Jurkat, CEM, MOLT-16, and Ramos.CD7 cells with scFvCD7:sTRAIL for 16h resulted in pronounced induction of apoptosis at concentrations as low as 50 ng/ml (Fig.1B). Apoptosis was specifically inhibited by pre-treatment with MAb TH69 and co-treatment with TRAIL-neutralizing MAb 2E5 (Fig.1C). Parental Ramos cells were fully resistant to induction of apoptosis by scFvCD7:sTRAIL at all concentrations tested (Fig.1D). Treatment of CD7-positive Jurkat cells with a MOCK-scFv:sTRAIL fusion protein containing an scFv antibody fragment of irrelevant specificity, targeted at the carcinoma-associated antigen EGP2[35], did not induce apoptosis (Fig.2A). ScFvCD7:sTRAIL-mediated apoptosis was characterized by the activation of initiator caspase-8 and effector caspase-3, PARP cleavage (Fig.2B), and apoptotic DNA fragmentation (Fig.2C).

Induction of apoptosis in malignant bystander cells by scFvCD7:sTRAIL

In mixed culture experiments of CD7-positive target cells (Jurkat) and CD7-negative bystander cells (Ramos) (ratio 1:1), a potent anti-tumour bystander effect of up to 61% apoptosis was detected in Ramos bystander cells (Fig.2D). Apoptosis was abrogated in both target and bystander cells when treatment was preceded by incubation with CD7 MAb TH69.

scFvCD7:sTRAIL does not induce apoptosis in normal human leukocytes, activated T-cells and resting/TNF-α activated HUVEC

Incubation of freshly isolated leukocytes showed strong and specific binding of scFvCD7:sTRAIL to the cell surface of T-cells and NK-cells, but not to B-cells (Fig.3A).

Again, binding of scFvCD7:sTRAIL was specifically inhibited by treatment with MAb TH69 (Fig.3B). Treatment of freshly isolated leukocytes, containing both T-cells and NK cells, with scFvCD7:sTRAIL did not induce apoptosis in any of the blood cell types analyzed, even when treatment was prolonged to 8 days (Fig.3C). Also CD3/IL-2-activated T-cells were fully resistant to prolonged treatment with scFvCD7:sTRAIL (Fig.3D). Thus, the pro-apoptotic effect of scFvCD7:sTRAIL was restricted to CD7-positive malignant cells.

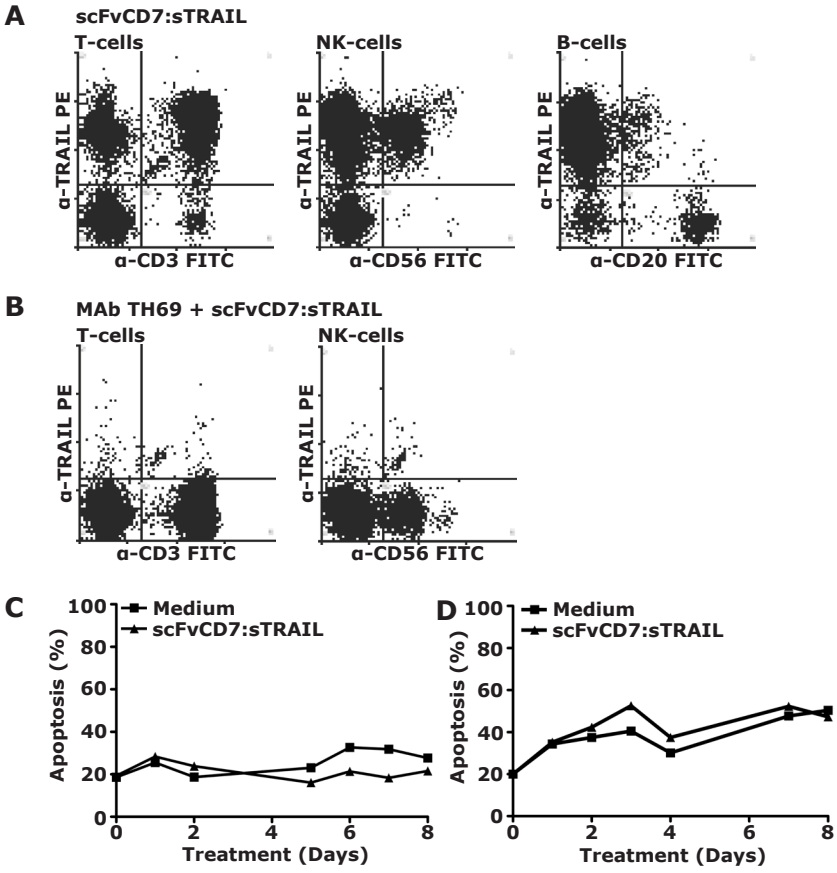

Fig.3. No apoptosis induction in normal human leukocytes and activated T-cells. A; Binding of scFvCD7:sTRAIL to freshly isolated leukocytes was analyzed by double staining using PE-conjugated anti-TRAIL and either a T-cell specific marker (anti-CD3 FITC), an NK-cell specific marker (anti-CD56 FITC), or a B-cell specific marker (anti-CD20 FITC). **B;** Leukocytes were co-incubated with scFvCD7:sTRAIL and MAb TH69, whereupon specific binding was determined. **C;** Resting PBLs and **D;** activated T-cells were subjected to treatment with scFvCD7:sTRAIL (1,5 μg/ml) for up to 8 days. Apoptosis induction was assessed by AnnexinV/PI staining.

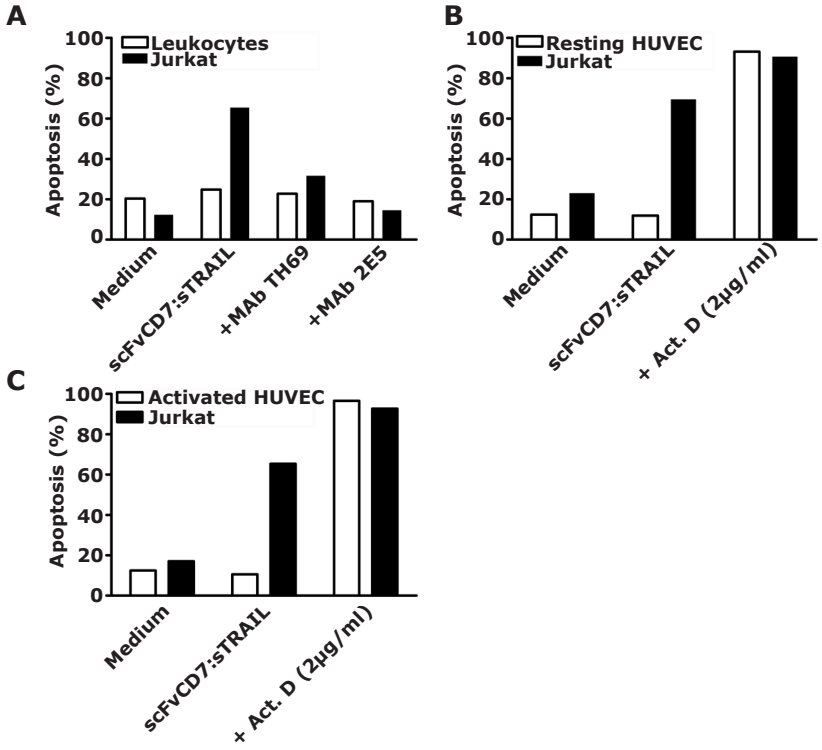

Fig.4. No apoptosis induction in normal human leukocytes and HUVEC. A; Isolated leukocytes were mixed at a ratio of 1:10 with DiI-labeled Jurkat cells. Mixed cultures were treated for 24h with scFvCD7:sTRAIL in the presence or absence of MAb TH69 or MAb 2E5. **B;** Resting HUVEC were mixed with Jurkat (ratio of 1:1) and subsequently treated with scFvCD7:sTRAIL or Actinomycin D (2 µg/ml) for 24 h. **C;** HUVEC were activated with TNF-α for 4 h and then treated with scFvCD7:sTRAIL or Actinomycin D (100 ng/ml) for 24 h. In all mixed culture experiments, the differential fluorescent labeling of Jurkat target and innocent bystander cells was used to separately evaluate apoptosis by AnnexinV staining. Indicated values are representatives of three independent experiments.

Next we assessed whether membrane bound scFvCD7:sTRAIL deposited on CD7-positive Jurkat cells could exert an 'innocent' bystander effect towards normal leukocytes by treatment of mixed cultures of Jurkat tumour cells and freshly isolated leukocytes (ratio 10:1) with scFvCD7:sTRAIL. Separate analysis of target cells and bystander cells showed no increase in apoptosis in leukocyte bystander cells, while induction of apoptosis in Jurkat target cells reached up to 65% (Fig.4A).

To assess the apoptotic activity of scFvCD7:sTRAIL deposited on CD7-positive Jurkat cells towards innocent vascular endothelial bystander cells, Jurkat cells were co-cultured with resting - or TNF-α activated HUVEC cells at a ratio of 4:1. No increase in apoptosis

was observed, neither in resting (Fig.4B) nor in activated HUVEC cells (Fig.4C), while induction of apoptosis in Jurkat cells reached up to 69%.

scFvCD7:sTRAIL induces more potent apoptosis than the immunotoxin scFvCD7:ETA

The pro-apoptotic activities of scFvCD7:sTRAIL and the immunotoxin scFvCD7:ETA were compared by treating Jurkat and CEM cells with equimolar concentrations of either fusion protein. After treatment for 24 h the apoptotic activity of scFvCD7:sTRAIL was clearly stronger than that of scFvCD7:ETA (Fig.5A). Stronger induction of apoptosis by scFvCD7:sTRAIL was maintained when treatment was prolonged to 72 h (data not shown).

As stated above, both freshly isolated leukocytes and activated-T cells were resistant to prolonged treatment with scFvCD7:sTRAIL. However, when resting leukocytes or activated T-cells were treated with equimolar amounts of scFvCD7:ETA, a marked induction of apoptosis of up to 46% after 72 h was observed in resting leukocytes (Fig.5C), while approximately 76% apoptosis induction was observed in activated T-cells (Fig.5D).

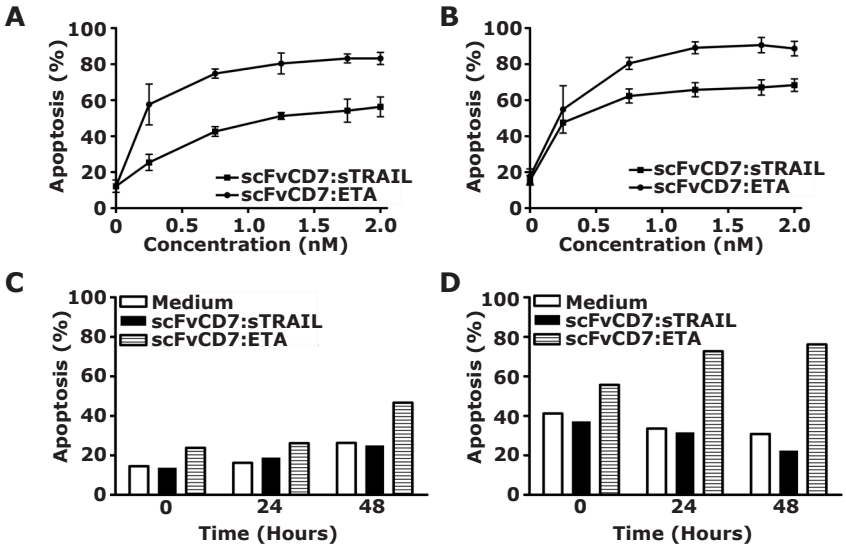

Fig.5. Activity of scFvCD7:sTRAIL compared to scFvCD7:ETA A; CEM and **B;** Jurkat cells were treated for 24h with increasing equimolar concentrations of scFvCD7:sTRAIL and scFvCD7:ETA. Indicated values are mean + standard error of the mean of three independent experiments **C;** resting leukocytes and **D;** activated T-cells were treated for 24, 48 and 72 h with an equimolar concentration (1.75 nM) of either scFvCD7:sTRAIL or scFvCD7:ETA. Indicated values are representatives of three independent experiments. Apoptosis induction was assessed by AnnexinV/PI staining.

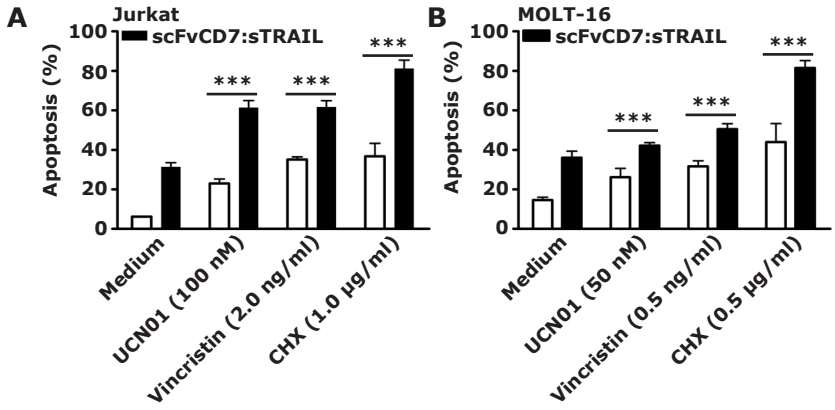

Fig.6. Potentiation of scFvCD7:sTRAIL treatment by several classes of chemotherapeutics. A; Jurkat and **B;** MOLT-16 were treated for 16 h either alone or simultaneously with scFvCD7:sTRAIL (15 ng/ml) and Vincristin, UCN01, and CHX, at the concentrations indicated. Apoptosis induction was assessed by Δψ.

Augmentation of scFvCD7:sTRAIL activity by several classes of chemotherapeutics

Jurkat and MOLT-16 cells were treated with scFvCD7:sTRAIL in the presence or absence of established - and recently developed therapeutics (Fig.6A and B, respectively). Co-treatment with scFvCD7:sTRAIL and the microtubule inhibitor Vincristin resulted in significant additive induction of apoptosis of 27% and 19% in Jurkat and MOLT-16 cells, respectively. Co-treatment with the protein synthesis inhibitor CHX resulted in an additive induction of apoptosis of 44.5% and 37.5%, while co-treatment with the staurosporin analog UCN01 increased the induction of apoptosis with 39% and 15% in Jurkat and MOLT-16 cells, respectively. On normal human PBLs, activated T-cells, and HUVECs, combination treatment with either of the drugs and scFvCD7:sTRAIL did not result in increased apoptosis (data not shown).

Synergistic induction of apoptosis in patient-derived T-ALL cells by scFvCD7:sTRAIL and vincristin

Blood cells freshly derived from three T-ALL patients were subjected to treatment with scFvCD7:sTRAIL (1 µg/ml) for 16 h, after which induction of apoptosis was visualized by AnnexinV/PI staining (Fig.7A). In two out of three T-ALL patients, treatment with scFvCD7:sTRAIL markedly induced apoptosis (25% and 16%, respectively), whereas one patient sample was resistant to treatment. Apoptosis induction by scFvCD7:sTRAIL was specifically inhibited by pre-incubation with CD7 MAb TH69 or TRAIL-neutralizing MAb 2E5 (Fig.7B). Combination treatment of primary T-ALL patient material with scFvCD7:sTRAIL and vincristin resulted in over 50% apoptosis (Fig.7C), whereas single

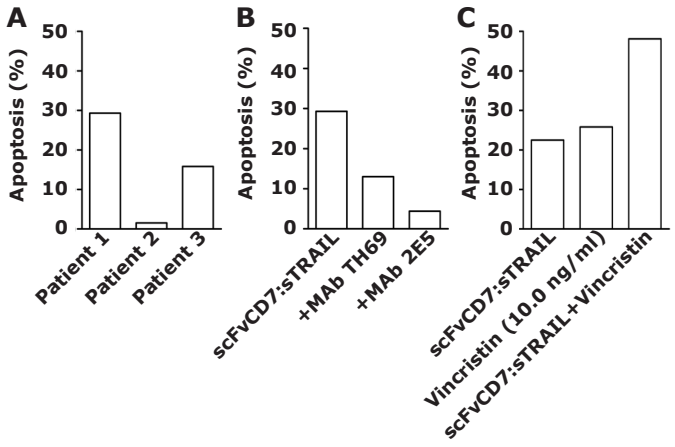

Fig.5. Apoptosis induction by scFvCD7:sTRAIL in patient derived T-ALL cells. A: Blood cells directly derived from T-ALL patients, containing >90% leukemic T-cells, were subjected to treatment with scFvCD7:sTRAIL (1 µg/ml). **B;** Primary T-ALL patient material was subjected to scFvCD7:sTRAIL (1 µg/ml) in the presence of MAb TH69 or MAb 2E5. **C;** T-ALL patient material was subjected to single agent treatment with scFvCD7:sTRAIL, vincristin (10 ng/ml), or to combination treatment with scFvCD7:sTRAIL and vincristin. In all experiments, apoptosis was assessed by AnnexinV/PI staining.

agent treatment induced approximately 20% apoptosis. Induction of apoptosis was inhibited when treatment was performed in the presence of CD7 MAb TH69 or TRAIL-neutralizing MAb 2E5 (data not shown).

Discussion

In recent years an increased understanding of pathogenic mechanisms has provided new targets and strategies for anti-leukemic therapy. These range from novel chemotherapeutic agents, therapeutic antibodies, bispecific antibodies, immunotoxins and radioimmunoconjugates, to targeted therapy with small molecules interfering with key cellular components such as tyrosine kinases. Here we describe a novel promising approach for the therapy of CD7-positive T-cell leukemia by induction of target antigen-restricted apoptosis using a recombinant scFvCD7:sTRAIL fusion protein with specificity for CD7.

Our experiments demonstrate specific binding of scFvCD7:sTRAIL to the cell surface of CD7-positive cells only. Binding of scFvCD7:sTRAIL to TRAIL-receptors on CD7-negative tumour cells via its TRAIL domains was often below detectable levels, which might be explained by the fact that polypeptide ligands, such as TRAIL, have typical fast-on/fast off binding rates. In contrast, antibody fragments, such as scFvs, usually retain the fast-on/slow-off rates typical for antibody-mediated binding. Stable trimeric scFvCD7:sTRAIL contains 3 identical scFv domains, which potentially enhances binding

to CD7-positive cells by the associated avidity effect. Enhanced avidity has been shown to be beneficial for *in vivo* tumour targeting in many antibody-based therapeutic strategies[52,53]. Moreover, the CD7 target antigen was selected for its specific and abundant surface expression on human T-cell leukemia and lymphoma. Although not examined in detail here, we have indications that the number of CD7 molecules on the surface of T-ALL cells greatly exceeds that of TRAIL receptors. Taken together, these arguments explain why scFvCD7:sTRAIL predominantly binds to target cells via its scFv domain.

CD7-selective binding increases the local concentration on the target cell surface which allows the sTRAIL domain of scFvCD7:sTRAIL to bind to proximal TRAIL receptors more frequently, thereby, enhancing the pro-apoptotic signalling. As previously shown, target antigen-bound scFv:sTRAIL acquires TRAIL-receptor activating properties resembling that of native memTRAIL. Similarly, scFvCD7:sTRAIL can fully activate not only TRAIL-R1 but also TRAIL-R2 upon specific CD7-mediated immobilization to the cell surface of targeted cells. Treatment of a series of CD7-positive T-ALL cell lines with scFvCD7:sTRAIL potently induced apoptosis, evidenced by activation of caspase-8, caspase-3, PARP cleavage, and apoptotic DNA fragmentation. When treatment was performed in the presence of CD7-blocking MAb TH69, apoptosis was strongly inhibited, which clearly demonstrated that scFvCD7:sTRAIL performed its pro-apoptotic action in an antigen-restricted manner. FACS analysis provided evidence that selective binding to CD7 led to the exclusive deposition of scFvCD7:sTRAIL on the cell surface of targeted cells. As a consequence, a surplus of CD7-bound scFvCD7:sTRAIL becomes available on the cell surface for binding and crosslinking of agonistic TRAIL receptors on neighbouring tumour cells. When neighbouring cells are also CD7-positive, a strong reciprocal 'fratricide' apoptosis of tumour cells is induced.

The function of CD7 and its possible ligands are still largely unknown. Recent reports indicate that Galectin-1 mediated crosslinking of CD7 induces apoptosis in activated T-cells and T-ALL cells[54]. We asked whether crosslinking of CD7 on T-ALL cells by soluble trimeric scFvCD7:sTRAIL would be sufficient to induce apoptosis. Therefore, T-ALL cells were treated with scFvCD7:sTRAIL in the presence of a TRAIL-neutralizing MAb. As a result, apoptotic activity was almost completely abrogated, demonstrating that apoptosis by scFvCD7:sTRAIL was pre-dominantly TRAIL-mediated.

As CD7 is expressed on a large subset of normal human T-cells and NK-cells, the potential for unwanted apoptosis by CD7-specific binding of scFvCD7:sTRAIL was examined in resting and activated normal blood cells. Both resting leukocytes and activated T-cells were resistant to treatment with scFvCD7:sTRAIL for up to 8 days, with no increase in apoptosis compared to control experiments. The striking preferential pro-apoptotic activity of TRAIL and TRAIL-fusion proteins for tumour cells over normal cells has been reported by other authors, but the underlying molecular mechanism remains unclear.

Subsequently, the possibility of a so-called 'innocent bystander' effect of cell surface

deposition of scFvCD7:sTRAIL on CD7-positive leukemia cells towards neighbouring normal blood cells or HUVEC cells was assessed. In an *in vitro* model, Jurkat cells were co-cultured with normal human leukocytes. Separate analysis of these leukocytes showed no increased apoptosis in leukocyte bystander cells. Similarly, no innocent bystander apoptosis was observed towards resting and TNF-α activated HUVEC cells.

Bystander activity towards target antigen-negative tumour cells may be of great value in cases where heterogeneous or lost target antigen expression allows tumour cells to escape from therapy. Antibody-based therapy of leukemia has been associated with target antigen-negative recurrences after treatment with Rituximab in B-cell lymphoma[55,56] and CAMPATH-1H in T-cell prolymphocytic leukemia[57]. The bystander effect of scFvCD7:sTRAIL is based on the principle that targeted tumour cells are not only eliminated, but are also exploited to crosslink agonistic TRAIL receptors on neighbouring tumour cells lacking the target antigen. Recently, we reported on an exceptionally potent anti-tumour bystander effect of an analogous scFv:sTRAIL fusion protein with specificity for a carcinoma-associated cell surface antigen[58]. In the current study we made similar observations when mixed cultures of CD7-negative Ramos cells and CD7-positive Jurkat cells were treated with scFvCD7:sTRAIL. Potent bystander apoptosis towards the CD7-negative Ramos cells was observed, which was fully dependent on CD7-specific binding of scFvCD7:sTRAIL to the surface of Jurkat cells.

Currently, various MAb-toxin conjugates and a small number of scFv:toxin fusion proteins are being evaluated for their therapeutic application in human cancer. However, targeted therapy using toxin-based conjugates has imperative drawbacks. In order to specifically kill a target cell, binding of a toxin-based molecule must be followed by efficient cellular internalization. Subsequently, the toxin must be delivered to the appropriate intracellular compartment in order to exert its full cytotoxic effect. These features limit the choice of target antigens on malignant cells to those known to rapidly internalize after binding. Importantly, most if not all currently used toxins are equally toxic to both normal and malignant cells. Therefore, the safety and efficacy of MAb-toxins and scFv:toxins solely relies on the tumour-selectivity of the antibody used. In contrast, targeted therapy using scFvCD7:sTRAIL does not require internalization, intracellular enzymatic conversion or trafficking to exert its pro-apoptotic effect. Furthermore, TRAIL was previously shown to have a surprising intrinsic tumour-selective activity. Consequently, the safety and efficacy of scFv:sTRAIL fusion proteins is determined by both the tumour-selective activity of TRAIL and the tumour-selectivity of the antibody fragment used. Moreover, a broad array of cell surface molecules can be used as target antigens, even those that are not strictly cancer-associated such as CD7, which is abundantly expressed on normal T-cells and NK cells.

The tumour selectivity of our TRAIL-based fusion protein scFvCD7:sTRAIL was directly

compared with the ETA-based immunotoxin scFvCD7:ETA. At equimolar concentrations, scFvCD7:sTRAIL was significantly more potent than scFvCD7:ETA. Furthermore, in contrast to treatment with scFvCD7:sTRAIL, resting peripheral blood lymphocytes were sensitive to treatment with scFvCD7:ETA. After treatment for 72 h, a marked increase in apoptosis was noted. Activated normal T-cells showed an even more pronounced apoptotic response to scFvCD7:ETA.

Recently, pro-apoptotic effects of certain sTRAIL preparations towards HUVEC and other normal cell types were reported[59-63]. Differences reported for TRAIL–related toxicity might be due to solution behaviour of the various sTRAIL preparations used. It was shown that prokaryotically produced HIS-tagged sTRAIL preparations can contain high molecular weight aggregates that cause toxicity towards hepatocytes[32]. We chose to produce scFvCD7:sTRAIL using CHO-K1 cells, an established industry-grade eukaryotic production system for recombinant therapeutic protein drugs. Previously, this system was shown to produce homogenous and biologically active scFv:sTRAIL trimers in the absence of high molecular weight aggregates[35]. In our experiments, even cell-surface bound scFvCD7:sTRAIL produced no apoptosis in neighbouring normal cells. Apparently, crosslinking of agonistic TRAIL receptors by cell surface-bound TRAIL significantly differs from receptor crosslinking by aggregated TRAIL species. Nevertheless, further *in vivo* research is needed to exclude unwanted apoptotic activity of scFvCD7:sTRAIL towards normal human cells and tissues.

ScFvCD7:sTRAIL treatment was combined with a number of chemotherapeutic agents to evaluate whether apoptotic activity could be significantly enhanced. Treatment with scFvCD7:sTRAIL and the microtubule inhibitor vincristin, a chemotherapeutic agent long part of clinical practice for T-ALL, strongly enhanced apoptosis. Co-treatment with PKC/cyclin inhibitor UCN01, a recently developed anti-leukemic agent, also significantly enhanced apoptosis. Additionally, inhibition of protein synthesis by CHX strongly potentiated apoptosis. Most likely other cytotoxic regimes can be identified that significantly enhance the target-cell restricted apoptotic activity of scFvCD7:sTRAIL to further improve its anti-leukemic effect with no or reduced overlapping toxicities.

Previous reports on targeted leukemia therapy indicated that leukemic cells freshly derived from patients responded more poorly to treatment when compared to the leukemia-derived cell lines. When blood cells derived from T-ALL patients, containing >90% T-ALL cells, were treated with scFvCD7:sTRAIL a marked CD7-restricted and TRAIL-mediated induction of apoptosis was observed in two out of three patients. When treatment was performed in the presence of vincristin, induction of apoptosis was strongly enhanced in a synergistic manner.

In conclusion, scFvCD7:sTRAIL is a representative of a novel class of immunotherapeutic molecules, which acts by inducing apoptosis in an antigen-restricted manner, but avoids

undesirable side-effects of known immunotoxins. The potent and highly selective anti-leukemic activity of scFvCD7:sTRAIL, either alone or in combination with chemotherapeutic agents, holds great promise for the treatment of human T-cell tumours.

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