TARGET CELL-RESTRICTED AND -ENHANCED APOPTOSIS INDUCTION BY A scFv:sTRAIL FUSION PROTEIN WITH SPECIFICITY FOR THE PANCARCINOMA-ASSOCIATED ANTIGEN EGP2

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The apparent tumor selective apoptosis-inducing activity of recombinant soluble TNF-related apoptosis-inducing ligand (TRAIL) has aroused much interest for use in clinical application. However, to exploit fully its therapeutic potential, the characteristics of both the TRAIL receptor system and soluble TRAIL (sTRAIL) should be taken into account: first, the widespread 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 particular sTRAIL preparations. Therefore, we constructed a novel TRAIL fusion protein, designated scFvC54:sTRAIL, comprising the human scFv antibody fragment C54 genetically linked to the N-terminus of human sTRAIL. The scFvC54:sTRAIL fusion protein was designed to induce apoptosis by crosslinking of agonistic TRAIL receptors only after specific binding of scFvC54:sTRAIL to the abundantly expressed carcinoma-associated cell surface antigen EGP2 (alias EpCAM). Target antigen-restricted apoptosis induction was demonstrated for various EGP2-positive tumor cells and could be inhibited by an EGP2 competing antibody. Target antigen binding converted soluble scFvC54:sTRAIL into a membrane-bound form of TRAIL that was capable of signaling apoptosis not only through TRAIL-R1 but also through TRAIL-R2. Size-exclusion fast protein liquid chromatography (FPLC) indicated that scFvC54:sTRAIL was produced as stable and homogeneous trimers in the absence of detectable TRAIL aggregates. The favorable characteristics of the scFvC54:sTRAIL fusion protein potentially reduce the amount of sTRAIL required for antitumor activity and may be of value for the treatment of various human carcinomas.

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The specific susceptibility of tumor cells to the proapoptotic activity of TNF-related apoptosis-inducing ligand (TRAIL), and the apparent lack of susceptibility of normal cells, makes this molecule a promising anticancer therapeutic agent. Native TRAIL is expressed as a homotrimeric type II transmembrane protein (memTRAIL), but can also be proteolytically cleaved to form a soluble trimer (sTRAIL).1,2 To date, various forms of soluble recombinant TRAIL have been generated, including FLAG, HIS and nontagged sTRAIL variants, all of which induce apoptosis in a wide range of human tumor cell lines.3 Potent antitumor activity of various sTRAIL variants has been demonstrated in several mouse xenograft models of human cancers, including colorectal cancer,4,5 glioblastoma4 and breast cancer.5

Both memTRAIL and sTRAIL can interact with the agonistic TRAIL receptors TRAIL-R1/DR4 and TRAIL-R2/DR5, which initiate apoptosis via their intracellular death domains.6–9 TRAIL also binds, albeit with lower affinity,10 to TRAIL-R3/DCR1 and TRAIL-R4/DCR2,7,11–13 both of which lack a functional death domain. TRAIL-R3 and TRAIL-R4 are considered to act as receptors that potentially modulate TRAIL activity. Expression of the different TRAIL receptors has been demonstrated not only on various tumors, but also on a wide variety of normal human tissues, indicating that apoptosis induction by TRAIL is a delicately regulated mechanism, much of which is still elusive.

Clustering of TRAIL-R1 and -R2 by TRAIL leads to formation of the death-inducing signaling complex (DISC)14–17 by recruitment of the adapter protein FADD and resultant binding and activation of initiator caspases-8 and -10.16,18–21 Activated caspase-8 and -10 subsequently activate downstream effector caspses, including caspase-3, -6 and -7, which cleave cytoskeletal and nuclear proteins essential for cell survival such as PARP, α-fodrin, DNA fragmentation factor (DFF) and lamin A, resulting in apoptosis.

Formation of the TRAIL receptor DISC is strongly enhanced when aggregated or complexed TRAIL binds to TRAIL-R1 or TRAIL-R2. Furthermore, TRAIL-R1 and -R2 were shown to have rather distinct crosslinking requirements for the initiation of apoptosis.2 Both sTRAIL and memTRAIL can efficiently activate TRAIL-R1 even at low concentrations, whereas TRAIL-R2 can only be activated by memTRAIL or recombinant sTRAIL that is secondarily crosslinked by antibodies. Previously, Wajant et al.23 demonstrated that signaling capacity of sTRAIL for TRAIL-R2 could be restored by genetic fusion to a recombinant antibody fragment (single-chain variable fragment, or scFv) recognizing the tumor stroma marker fibroblast activation protein (FAP).

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Independently, we developed a versatile expression system in CHO cells for the rapid construction and evaluation of scFv: sTRAIL fusion protein variants. Here we present a detailed characterization of a novel scFv:sTRAIL fusion protein that selectively targets the pancarcinoma-associated membrane antigen EGP2 (epithelial glycoprotein 2, also known as GA733-2, EpCAM or CO17-1A antigen). EGP2 is a well-established target antigen that is overexpressed on the cell surface of various human carcinomas such as colorectal, breast and small cell lung carcinoma.

In normal epithelia, EGP2 expression is limited to the basolateral membrane. Furthermore, EGP2 is not shed into the circulation and has been extensively studied in antibody-mediated imaging and immunotherapy of human carcinomas. This is the first report on the construction and detailed characterization of an scFv:sTRAIL fusion protein with specificity for the therapeutically relevant pancarcinoma-associated target antigen EGP2/EpCAM.

MATERIAL AND METHODS

Monoclonal antibodies and scFv antibody fragment

MAb MOC31 is a murine IgG1 with high affinity for human EGP2. The anti-EGP2 scFvC54 has been previously selected from a large semisynthetic phage display library with random human variable heavy-chain domain (VH)-variable light-chain domain (VL) pairings and has a VH-(G4S)3-VLx format. MAb MOC31 and scFvC54 compete for binding to the same epitope on the extracellular domain of EGP2. TRAIL activity-neutralizing MAB 2ES was purchased from Alexis (Kordia Life Sciences, Leiden, The Netherlands). MAB 2ES neutralizes TRAIL activity by binding to an epitope on the extracellular domain of TRAIL that inhibits binding to the various TRAIL receptors. A multimeric form of the extracellular domain of EGP2 (sEGP2) was produced and purified as described previously. Multimeric sEGP2 was used to crosslink scFvC54/TRAIL secondarily.

Cell lines

The following cell lines were purchased from the ATCC (Rockville, MD): A172 and U87MG (both astrocytoma grade IV), SW948 (colorectal adenocarcinoma), Chinese hamster ovary (CHO-K1) and Jurkat (human ALL T-cell line). Jurkat cells express high levels of TRAIL-R2 on the cell surface but do not express detectable amounts of TRAIL-R1. As a result, Jurkat cells are only sensitive to crosslinked or aggregated sTRAIL preparations. All cell lines were cultured in their respective media supplemented with 10% FCS at 37°C in humidified 5% CO₂ atmosphere unless indicated otherwise.

EGP2-transduced cell lines

EGP2-transduced cell lines A172.EGP2, U87MG.EGP2 and Jurkat.EGP2 were generated by infection of the respective parental cell lines with retroviral particles encoding both EGP2 and enhanced green fluorescent protein (EGFP). In short, EGP2 cDNA was cloned into a retroviral vector derivative of LZR5-pBMN-lacZ kindly provided by Dr. G. Nolan (Stanford University School of Medicine, San Francisco, CA), yielding LZR5-EGP2-IRES-EGFP. To produce retroviral particles, LZR5-EGP2-IRES-EGFP was transfected into the amphotrophic packaging cell line Phoenix using Fugene-6 transfection reagent according to manufacturer’s recommendations (Roche Diagnostics, Almere, The Netherlands). Transfected cells were selected by culturing in the presence of 1 µg/ml puromycin, 300 µg/ml hygromycin and 1 µg/ml diphertheria toxin (BD Biosciences Clontech, Palo Alto, CA). Viral particle-containing supernatant was harvested after 3 days and stored at −80°C until further use. Jurkat, A172 and U87MG cells (0.5 × 10⁶ cells) were transduced with 1 ml supernatant containing 1 × 10⁶ retroviral particles. Supernatants were removed after overnight incubation, after which cells were transferred to fresh medium. Transduced cells were selected both for EGFP fluorescence and EGFP expression (MAB MOC31-PE) using the MoFlo high-speed cell sorter (Cytometry, Fort Collins, CO).

Analysis of membrane expression of TRAIL receptors and EGP2

Differential expression of TRAIL-R1, -R2, -R3 and -R4 on the various cell lines was assessed by flow cytometry using TRAIL receptor-specific MAbs (Alexis). In short, cells were harvested and washed with serum-free medium and resuspended at a concentration of 5 × 10⁶ cells in 100 µl fresh medium containing the respective anti-TRAIL-R MAbs. Specific binding was detected using secondary PE-conjugated antibodies (DakoCytomation, Glostrup, Denmark). EGP2 expression on the tumor cell surface was analyzed by incubation with MOC31-PE. All antibody incubations were carried out for 45 min at 0°C and were followed by 2 washes with serum-free medium.

Construction of scFvC54: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. Plasmid pEE14scFv:sTRAIL is based on a vector we described earlier. Important features of this novel vector are the presence of the murine kappa light-chain leader peptide encoded upstream of 2 in-frame cloning sites, a multiple cloning site, a 26 residue in-frame linker sequence, and the glutamine synthetase selectable marker gene, which allows for amplified expression of the recombinant protein in the production cell line CHO-K1. The vector exploits the strong CMV promoter to drive recombinant protein expression, while the leader peptide directs the produced fusion protein through the ER and Golgi complex, resulting in excretion of fusion protein into the culture supernatant. In the first MCS, a 730 bp DNA fragment encoding scFvC54 derived from phagemid pHENscFvC54 was directionally inserted using the unique SfiI and Nol restriction enzyme sites. In the second MCS, a PCR-truncated 593 bp DNA fragment encoding the extracellular domain of human TRAIL (sTRAIL) was cloned in frame using restriction enzymes XhoI and HindIII. TRAIL cDNA truncation was performed by PCR with proofread DNA polymerase according to standard protocol using primers T1, 5′-ATCTCGAGTTAGCTCTAACCTCCTGAGGAAAACCAATTT3′ (XhoI site is underlined), and T2, 5′-CCTTACAGGTCTGAGTTACAGCTTAAAGGCAACTAAAAAG3′ (HindIII site is underlined). Figure 1(a) depicts a schematic presentation of the monomeric form of the scFvC54:sTRAIL fusion protein.

Production of scFvC54:sTRAIL in CHO-K1 cells

CHO-K1 cells were transfected with plasmid pEE14scFvC54: sTRAIL using the Fugene-6 reagent (Roche). Stable transfectants were generated by the glutamine synthetase selection method, essentially as described previously. Briefly, pEE14scFvC54: sTRAIL-transfected CHO-K1 cells were cultured in GMEM medium (First Link, West Midlands, U.K.) supplemented with 5% dialyzed fetal calf serum (Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands) and 100 µM L-methionine-sulfoximine (MSX; Sigma). Individual clones, obtained after single cell sorting using the MoFlo high-speed cell sorter, were analyzed for stable and high expression of scFvC54:sTRAIL in the absence of MSX using a solid-phase sandwich TRAIL ELISA kit according to manufacturer’s recommendations (Diacline SAS, Besançon, France). The procedure identified a (MCS) that CHO-K1 production cell line clone designated 70C1, which stably secreted scFvC54:sTRAIL into the medium at a concentration of 3.44 µg/ml. Large-scale production of scFvC54:sTRAIL fusion protein was performed by culturing cell line 70C1 in 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 × 10⁶ cells/ml, after which supernatant was harvested (1,500g, 10 min) and stored at −80°C until further use.

Immunoblot analysis of scFvC54:sTRAIL fusion protein

 Supernatant derived from scFvC54:sTRAIL production cell line 70C1 was separated by nondeducing SDS-PAGE (12% acryl-
Control at a final concentration of 1 ng/ml. Soluble Flag-tagged TRAIL (Alexis) was used as a positive control after which specific binding was visualized using chemoluminescence (Roche). Soluble Flag-tagged TRAIL (Alexis) was used as a positive control after which specific binding was visualized using chemoluminescence (Roche). Detection of scFvC54:sTRAIL content.

Subjected to a sensitive TRAIL-specific ELISA to quantitate the scFvC54:sTRAIL content. Furthermore, all samples were analyzed for their capacity to induce apoptosis using the TRAIL-sensitive cell line SW948. Additionally, scFv:sTRAIL can induce apoptosis in a bicellular fashion in which specific binding to one cell results in the crosslinking of TRAIL receptors on a neighboring tumor cell.

**Figure 1**

(a) Schematic presentation of a monomeric scFv:sTRAIL fusion protein in which a recombinant antibody fragment (scFv) is genetically fused to human sTRAIL via a linker sequence of 26 amino acid residues. (b) Target cell-restricted crosslinking of TRAIL receptors by an scFv:sTRAIL fusion protein. In principle, by binding to the target antigen (triangle), scFv:sTRAIL can crosslink agonistic TRAIL receptors and induce apoptosis in a monocellular fashion. (c) Additionally, scFv:sTRAIL can induce apoptosis in a bicellular fashion in which specific binding to one cell results in the crosslinking of TRAIL receptors on a neighboring tumor cell.

**Size-exclusion FPLC of scFvC54:sTRAIL**

The solution behavior of scFvC54: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 cell line 70C1 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 the TRAIL-sensitive cell line SW948. Furthermore, all samples were subjected to a sensitive TRAIL-specific ELISA to quantitate the scFvC54:sTRAIL content.

**Target antigen-restricted cell surface binding of scFvC54:sTRAIL**

Target antigen-restricted binding of scFvC54:sTRAIL to the cell surface was assessed by flow cytometry using cell lines SW948 (EGP2-positive) and Jurkat (EGP2-negative). In short, cells were harvested and washed with serum-free medium. Subsequently, cells were incubated with scFvC54:sTRAIL (300 ng/ml) in the presence or absence of either the EGP2 competing antibody MOC31 (7.15 µg/ml) or the TRAIL activity-neutralizing MAb 2E5 (1 µg/ml). Detection of cell surface-bound scFvC54:sTRAIL was performed using anti-TRAIL-PE (Diaclone SAS). All antibody incubations were carried out for 45 min at 0°C and were followed by 2 washes with serum-free medium.

**Target antigen-restricted apoptosis induction by scFvC54:sTRAIL**

Target antigen-restricted apoptosis induction by scFvC54:sTRAIL (300 ng/ml) was assessed by analysis of the following apoptosis-related cellular phenomena: tumor cell viability, phosphatidyl serine exposure on the outer cell membrane, caspase-8 and -3 activation, DFF degradation by activated caspase-3 and DNA fragmentation. The different analyses were performed in the presence or absence of either MOC31 (7.15 µg/ml) or 2E5 (1 µg/ml) and are described in more detail below.

**Viability assay**

Tumor cell viability was assessed by MTS assay (Promega Benelux, Leiden, The Netherlands). Briefly, cells were seeded in 96-well microculture plates at a density of 3 x 10^4 cells/well in 100 µl medium. After overnight culture, spent medium was removed and replaced by 200 µl medium containing the various experimental conditions. After 16 hr, MTS assay was performed according to manufacturer’s recommendations. Each experimental and control group consisted of 6 independent wells.

**Immunoblot analysis of apoptosis**

After treatment of SW948 cells with scFvC54:sTRAIL, intracellular apoptotic features were detected by incubation with antibodies against active caspase-8, active caspase-3 and DFF (Pharmingen, San Diego, CA). Briefly, 2.5 x 10^6 tumor cells were seeded in 6-well plates and treated for 1, 2, 3, 5, 6, 12 and 24 hr, respectively, with the various experimental conditions indicated in Figure 4. Cells were collected by centrifugation (2,000g; 10 min), lysed in lysis buffer (20 mM Tris-HCl, 5.0 mM EDTA, 2.0 mM EGTA, 100 mM NaCl, 0.5% SDS, 0.50% NP-40, 1 mM PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin) and sonicated on ice for 2 x 5 sec. Subsequently, cleared supernatants were collected by centrifugation (15,000g, 10 min) and separated by SDS-PAGE (12% AA) under reducing conditions and transferred to NC by electroblotting. Blots were incubated with the appropriate primary monoclonal antibodies and HRPO-conjugated secondary antibody. Specific binding was visualized by chemoluminescence (Roche). All antibody incubations were carried out for 1.5 hr in PBS/5% skim milk and were followed by 3 washes with PBS/0.1% Tween.

**Flow cytometric analysis of apoptosis induction by scFvC54:sTRAIL**

Cells were harvested and resuspended in fresh medium at 1 x 10^6 cells/ml and incubated for 16 hr with the various experimental conditions. After 16 hr, cells were harvested by centrifugation (1,500g, 5 min) and analyzed with the AnnexinV-FITC/PI kit (IQ Products, Groningen, The Netherlands), the caspaseflow cytometry apoptosis detection kit (Biovision, Mountain View, CA) and the single-strand DNA-specific MAB F7-26 (Alexis). AnnexinV-FITC/PI staining was performed according to manufacturer’s recommendations and identifies early apoptotic cells with phosphatidyl serine exposure on the outer cell membrane and late apoptotic AnnexinV-FITC/PI double-positive cells.
Caspase activation by scFvC54:sTRAIL was analyzed using the Caspscreen flow cytometry apoptosis detection kit that detects cleavage of the nonfluorescent substrate (aspartyl)2-rhodamine 110 (D2R) into fluorescent rhodamine 110 by activated caspases.

DNA fragmentation was analyzed with MAb F7-26 according to manufacturer’s recommendations. MAb F7-26 specifically detects apoptotic DNA fragmentation and is based on the high sensitivity of DNA to thermal denaturation in condensed chromatin of apoptotic cells. MAb F7-26 specifically detects deoxycytidine in ssDNA of at least 25–30 bases in length in the absence of any reactivity to double-stranded DNA.

RESULTS

Construction of scFvC54:sTRAIL

DNA encoding the extracellular domain of TRAIL (sTRAIL) was generated by PCR using proofread DNA polymerase. The resulting 593 bp PCR product encoded amino acids 95–281 of TRAIL, including cysteine residue number 230 (original position in memTRAIL), which is essential for the structure and activity of TRAIL. The DNA fragment encoding scFvC54 and the sTRAIL PCR product were subsequently inserted in the first and second MCS of eukaryotic expression vector pEE14, yielding plasmid pEE14-scFvC54:sTRAIL. Sequence analysis confirmed the correct and in-frame fusion of the scFvC54 and sTRAIL encoding DNAs (data not shown).

Eukaryotic production of scFvC54:sTRAIL

CHO-K1 cells transfected with pEE14-scFvC54:sTRAIL were selected for stable and amplified secretion of scFvC54:sTRAIL using the GS selection method and a TRAIL-specific ELISA. This procedure identified the producer CHO-K1 clone designated 70C1 that secreted 3.44 μg/ml fusion protein into the culture medium. Immunoblot analysis of a 70C1 culture medium sample identified scFvC54:sTRAIL as a fusion protein with an apparent molecular weight of approximately 52 kDa (Fig. 2, lane 1). This is in close proximity of the molecular weight of 51,180 Da calculated for monomeric scFvC54:sTRAIL. Flag-tagged sTRAIL was detected as a 25 kDa monomer (Fig. 2, lane 2). The observed molecular weight of scFvC54:sTRAIL is the result of the genetic fusion of scFvC54 (26.6 kDa), 26 amino acid linker (2.6 kDa) and sTRAIL (22 kDa). In addition to monomeric scFvC54:sTRAIL, 2 other bands were detected corresponding to dimeric (102 kDa) and trimeric (154 kDa) forms of scFvC54:sTRAIL. The latter bands were not observed when SDS-PAGE was performed under reducing conditions with sample boiling (data not shown).

Target antigen-restricted binding of scFvC54:sTRAIL

Incubation of SW948 cells (EGP2-positive) with scFvC54: sTRAIL resulted in strong and specific binding of scFvC54:sTRAIL to the cell surface (Fig. 2b). The observed binding was target antigen-specific since preincubation with the EG2 blocking MAb MOC31 completely inhibited scFvC54:sTRAIL binding (Fig. 2b). Cell surface binding of scFvC54:TRAIL via its TRAIL domain to tumor cells was minimal, as exemplified for Jurkat cells (EGP2-negative), to which weak TRAIL domain-mediated binding was observed (Fig. 2c, solid line). This binding could be completely inhibited by coinoculation with MAb 2E5 (Fig. 2c, dashed line).

Target antigen-restricted apoptosis induction by scFvC54:sTRAIL

Treatment with scFvC54:sTRAIL resulted in prominent apoptosis induction (as measured by a strong reduction of cell viability) in all the EGP2-positive tumor cell lines tested (Fig. 3a), whereas viability was only minimally reduced in the EGP2-negative cell lines (Fig. 3b). Induction of apoptosis by scFvC54:sTRAIL was target antigen-dependent since preincubation with saturating amounts of MAb MOC31 restored cell viability to medium control.
in all EGP2-positive cell lines (Fig. 3a). Coincubation of SW948 cells with a fixed concentration of scFvC54:sTRAIL and increasing concentrations of MAb MOC31 resulted in a dose-dependent reduction in apoptosis (Fig. 3c). Similar results were obtained for all other EGP2-positive tumor cell lines (data not shown). Furthermore, incubation of EGP2-positive tumor cells with an scFv:sTRAIL fusion protein containing an scFv of irrelevant target specificity did not induce apoptosis (Fig. 3d). Apoptosis induction by scFvC54:sTRAIL was abrogated by coincubation with TRAIL-neutralizing MAb 2E5 (Fig. 3c), which was observed for all cell lines tested (data not shown).

**Apoptotic features induced by scFvC54:sTRAIL**

Apoptotic features induced by scFvC54:sTRAIL were analyzed at elapsed time points of 1, 2, 3, 6, 12 and 24 hr. For the initiator caspase-8 and the effector caspase-3, the following pattern was observed. Activation was detectable after 3 hr, which increased up to 6 hr (Fig. 4a). Activation levels of both caspase-8 and -3
respectively). Treatment with culture medium containing no
completely inhibited apoptosis induction (Fig. 4b). Coincubation of SW948 cells with scFvC54:sTRAIL and MAb MOC31 or MAb 2E5 completely inhibited apoptosis induction (Fig. 4b, lanes 3 and 4, respectively). Treatment with culture medium containing no

**Figure 4** — (a) Immunoblot analysis of intracellular apoptotic features induced by scFvC54:sTRAIL (300 ng/ml) in SW948 cells. Activation of initiator caspase-8 (43 kDa protein band) and effector caspase-3 (29 kDa protein band) was detectable after 3 hr of treatment. Maximum activation levels were reached after 6 hr, which decreased after 12 hr, and were undetectable after 24 hr of treatment. A marked decrease of the caspase-3 substrate DFF (45 kDa protein band) was observed after 3-hr treatment. No intact DFF was detectable after 24 hr of treatment. A marked activation levels were reached after 6 hr, which decreased after 12 hr, and were undetectable after 24 hr of treatment. A marked decrease of the caspase-3 substrate DFF (45 kDa protein band) was observed after 3-hr treatment. No intact DFF was detectable after 24 hr of treatment. A marked decrease of the caspase-3 substrate DFF (45 kDa protein band) was observed after 3-hr treatment. No intact DFF was detectable after 24 hr of treatment.

(b) Immunoblot analysis of target antigen-restricted apoptosis induction after 12-hr treatment with scFvC54:sTRAIL (300 ng/ml). Lane 1, no signs of apoptosis in the absence of scFvC54:sTRAIL; lane 2, treatment with scFvC54:sTRAIL results in cleavage of caspase-8, caspase-3 and complete degradation of DFF; lanes 3 and 4, pretreatment with MAb MOC31 (7.4 μg/ml) or 2E5 (1 μg/ml) inhibits cleavage of caspase-8, caspase-3 and abrogates degradation of DFF. In lanes 3 and 4, the addition of the murine MAb resulted in the appearance of an additional antibody-derived protein band detected by the rabbit antimouse HRPO-conjugated antibody.

decreased after 12 hr. After 24 hr, no active caspase-8 or -3 could be detected. Increasing levels of active caspase-3 corresponded closely with declining DFF levels after 3 and 6 hr, with no DFF detectable after 12 and 24 hr (Fig. 4a). Coincubation of SW948 cells with scFvC54:sTRAIL and MAB MOC31 or MAB 2E5 completely inhibited apoptosis induction (Fig. 4b, lanes 3 and 4, respectively). Treatment with culture medium containing no

**TRAIL-R2 activation by scFvC54:sTRAIL**

Jurkat cells (EGP2-negative, TRAIL-R1-negative, TRAIL-R2-positive) were used to assess the specific activation of TRAIL-R2 by scFvC54:sTRAIL (Fig. 5a). Jurkat cells proved to be insensitive to incubation with scFvC54:sTRAIL even for prolonged periods of time (Fig. 5b and c; 6 and 16 hr). However, when Jurkat cells were incubated with scFvC54:sTRAIL in the presence of a multimeric form of the extracellular domain of EGP2 (sEGP2), strong induction of apoptosis was observed (Fig. 5b and c; 6 and 16 hr). When increasing amounts of multimeric sEGP2 were added in the presence of a fixed concentration scFvC54:sTRAIL (350 ng/ml), apoptosis was induced in a dose-dependent manner (Fig. 5d). Jurkat cells cultured in the presence of the highest concentrationmultimeric sEGP2 alone showed no signs of apoptosis (data not shown). Furthermore, only treatment with scFvC54:sTRAIL secondarily crosslinked with sEGP2 resulted in the activation of caspase-3 (Fig. 5e) and complete DNA fragmentation after 16 hr (Fig. 5f).

**Solution behavior of scFvC54:sTRAIL**

Supernatant containing scFvC54:sTRAIL was subjected to SE FPLC. As indicated in Figure 6(a), apoptosis induction of the TRAIL-sensitive cell line SW948 was restricted to the individual samples collected after 95–115 min. TRAIL ELISA subsequently confirmed that only these fractions contained scFvC54:sTRAIL (Fig. 6b). The elution peak of scFvC54:sTRAIL corresponded to a molecular weight of approximately 160 kDa, which is in close proximity of the 154 kDa calculated for trimeric scFvC54:sTRAIL.

**DISCUSSION**

Human sTRAIL appears to be a promising new anticancer agent. However, the widespread expression of TRAIL receptors throughout the human body and the recently reported possible TRAIL-related toxicity toward certain normal cells, at least of certain recombinant forms of this cytokine, might hamper its clinical development. Augmentation of the therapeutic value of sTRAIL can be achieved by increasing its tumor selective binding properties through the genetic fusion to a tumor selective antibody fragment.23 Here we demonstrated that specific targeting of sTRAIL to EGP2-positive cancer cells can be attained by the scFvC54:sTRAIL fusion protein, in which the carcinoma-specific antibody fragment scFvC54 is genetically linked to the N-terminus of human sTRAIL. The high-affinity scFvC54 domain specifically recognizes EGP2, an established cell surface target antigen that is highly overexpressed on a variety of human carcinomas.29–41 Specific binding of scFvC54:sTRAIL to EGP2-positive tumor cells was readily demonstrated by flow cytometry. Preincubation with a competing anti-EGP2 MAb MOC31 selectively blocked the binding in a dose-dependent manner. Binding to EGP2-negative cell lines was below detectable levels, except for Jurkat cells, to which a weak extracellular binding was observed that could be blocked by a TRAIL-neutralizing MAb. Together, this demonstrated that the scFvC54 targeting domain strongly enhanced the tumor selective binding of scFvC54:sTRAIL to EGP2-positive tumor cells only.

Fluorescence-activated cell sorting (FACS) data further indicated that EGP2-specific binding converted soluble scFvC54:sTRAIL into an artificial membrane-bound form of TRAIL. Since the number of EGP2 target molecules greatly exceeded that of the TRAIL receptors on the same cell, a surplus of sTRAIL domains was available for subsequent crosslinking of agonistic TRAIL receptors on neighboring tumor cells. When EGP2-positive tumor cells (Jurkat.EGP2, A172.EGP2 and U87MG.EGP2) were subjected to treatment with scFvC54:sTRAIL, an efficient induction
FIGURE 5 – (a) Schematic representation of treatment of Jurkat cells (TRAIL-R1-negative, TRAIL-R2-positive) with (1) scFvC54:sTRAIL alone and (2) scFvC54:sTRAIL that is secondarily crosslinked with a multimeric form of the extracellular domain of EGP2 (sEGP2). AnnexinV-FITC/PI staining identified early apoptotic cells with phosphatidyl serine exposure on the outer cell membrane and late apoptotic AnnexinV-FITC/PI double-positive cells after treatment of Jurkat cells with scFvC54:sTRAIL (300 ng/ml) in the absence or presence of sEGP2 (200 ng/ml). Jurkat cells treated with scFvC54:sTRAIL secondarily crosslinked with sEGP2 showed a dramatic increase in early (white part of bar) and late apoptotic cells (black part of bar) after 6 hr (b) and 16 hr (c). Incubation with scFvC54:sTRAIL or sEGP2 alone had no effect on apoptosis induction. (d) Addition of increasing amounts of multimeric sEGP2 dose-dependently increased the level of apoptosis induction by a fixed concentration of scFvC54:sTRAIL (350 ng/ml) in Jurkat cells. In the absence of multimeric sEGP2, only background level of apoptosis was observed. Increasing amounts of sEGP2 resulted in a dose-dependent 5-fold increase in apoptosis induction at 200 ng sEGP2. (e) Flow cytometric analysis of caspase activation in Jurkat cells by sEGP2 crosslinked scFvC54:sTRAIL. Caspase activation by scFvC54:sTRAIL (300 ng/ml) in the presence or absence of multimeric sEGP2 (200 ng/ml) was analyzed using the caspscreen flow cytometry apoptosis detection kit that detects cleavage of the nonfluorescent substrate (aspartyl)-rhodamine 110 (D,R) into fluorescent rhodamine 110 by activated caspases. Treatment of Jurkat cells with scFvC54:sTRAIL secondarily crosslinked with sEGP2 resulted in a marked increase in fluorescence intensity (solid line). Treatment with scFvC54:sTRAIL alone (dashed line) or with unconditioned medium (bold line) did not result in caspase activations. (f) Flow cytometric analysis of DNA fragmentation in Jurkat cells by sEGP2 crosslinked scFvC54:sTRAIL. DNA fragmentation by scFvC54:sTRAIL (300 ng/ml) in the presence or absence of multimeric sEGP2 (200 ng/ml) was analyzed using MAb F7-26 that specifically binds to ssDNA that has been fragmented by the apoptotic process. Treatment of Jurkat cells with scFvC54:sTRAIL secondarily crosslinked with sEGP2 for 16 hr resulted in a marked increase in fluorescence intensity (solid line), indicating strong and complete DNA fragmentation (solid line). Treatment with scFvC54:sTRAIL alone (dashed line) or with unconditioned medium (bold line) did not result in apoptotic DNA fragmentation.
TRAIL-sensitive cell line SW948 and (were analyzed (Superose 200 column. Individual samples collected at 3-min intervals confirmed that only these fractions contained scFvC54:sTRAIL. The restricted to samples collected after 95–115 min. TRAIL ELISA tent using a sensitive TRAIL-specific ELISA. Apoptosis induction was calculated for trimeric scFvC54:sTRAIL. The retention time of the elution peak of scFvC54:sTRAIL corresponded to a molecular weight of approximately 160 kDa, which is in close proximity of 154 kDa present on the same tumor cell.

Recently, it was shown that TRAIL receptors 1 and 2 have quite distinct crosslinking requirements for the initiation of apoptosis.22 TRAIL-R2 appears to signal apoptosis only after efficient receptor crosslinking by either native memTRAIL, aggregated sTRAIL variants, or by sTRAIL preparations secondarily crosslinked by antibodies. Apoptosis signaling by TRAIL-R1 appears to be relatively independent of the receptor crosslinking characteristics of a particular form of sTRAIL. Furthermore, it was shown that TRAIL-R2 had superior binding affinity for TRAIL, resulting in predominant binding of sTRAIL to TRAIL-R2 over TRAIL-R1.10

To analyze the TRAIL receptor crosslinking effects of scFvC54:sTRAIL, we exploited Jurkat cells, a cell line that expresses TRAIL-R2 but no detectable levels of TRAIL-R1, resistant to relatively high concentrations of nonaggregated sTRAIL. When Jurkat.EGP2 transfectant cells were subjected to scFvC54:sTRAIL treatment, efficient induction of apoptosis of up to 60% was achieved, indicating that target antigen-restricted apoptosis induction in these cells is initiated via TRAIL-R2 crosslinking. Furthermore, when parental Jurkat cells were subjected to a fixed concentration of scFvC54:sTRAIL, in the presence of increasing amounts of a multimeric form of sEGP2, a dramatic increase in apoptosis induction was observed, which directly correlated to the concentration of crosslinking multimeric sEGP2 added. From this it can be concluded that the target antigen-restricted apoptosis-inducing capacity of scFvC54:sTRAIL is directly proportional to the degree of TRAIL-R2 receptor crosslinking. Moreover, the data indicate that scFvC54:sTRAIL can overcome TRAIL resistance related to the differential expression of TRAIL-R2 over TRAIL-R1, as is observed for many different TRAIL-resistant cell lines. The parental cell lines Jurkat, A172 and U87MG, used in the present study, exemplify the preferential expression of TRAIL-R2 over TRAIL-R1 and the subsequent insensitivity to treatment with scFvC54:sTRAIL. Interestingly, SW948 cells (EGP2-positive colon carcinoma cells) expressing both TRAIL-R1 and TRAIL-R2 were almost completely rescued from apoptosis by preincubation with blocking MAb MOC31 (Fig. 3c). This implies that, even in the presence of TRAIL-R1, scFvC54:sTRAIL-mediated apoptosis appears to be predominantly initiated by TRAIL-R2. Although not formally proven here, it appears that this possibly is the result of the preferential crosslinking capacity of scFvC54:sTRAIL for the high-affinity TRAIL-R2 receptor over TRAIL-R1, which binds TRAIL with lower affinity.10

The current data independently corroborate with the results previously published by Wajant et al.,42 who demonstrated that the restricted signaling capacity of sTRAIL could be converted into a TRAIL-R2 stimulating ligand after genetic fusion to an scFv antibody fragment specific for the tumor stroma marker FAP.

Recently, several studies reported on the apoptosis inducting potential of certain recombinant sTRAIL preparations toward primary human cells such as normal human hepatocytes, keratinocytes, prostate epithelial cells and brain tissue. It has been suggested that this potential toxicity is related to high-molecular-weight sTRAIL aggregates present in certain sTRAIL preparations.47 Purified his-tagged sTRAIL, refolded from bacterial expression systems, appeared to contain TRAIL aggregates that might be directly responsible for the hepatocyte toxicity observed for this particular preparation. Thus, the production of nonaggre-
gated sTRAIL derivatives appears to be important in order to avoid organ-specific or systemic toxicity.

In the present study, we aimed at producing biologically active and correctly folded scFvC54:sTRAIL by directing it through the endoplasmic reticulum of eukaryotic CHO-K1 cells, thus taking advantage of the associated stringent quality control mechanisms associated with conventional nontargeting sTRAIL preparations. The favorable characteristics of scFvC54:sTRAIL potentially reduce the amount of TRAIL required for antitumor activity and may thereby reduce the risk of potential toxicity associated with conventional nontargeting sTRAIL preparations.

In conclusion, to exploit fully the therapeutic potential of sTRAIL, characteristics of both the TRAIL receptor system and sTRAIL should be taken into account: first, the widespread 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 particular sTRAIL preparations. The fusion protein scFvC54:sTRAIL complies with these notions and is the first example of an sTRAIL variant with enhanced tumor selective apoptosis induction toward EGP2-positive tumor cells. The favorable characteristics of scFvC54:sTRAIL potentially reduce the amount of TRAIL required for antitumor activity and may thereby reduce the risk of potential toxicity associated with conventional nontargeting sTRAIL preparations.

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