Target cell-restricted apoptosis induction by 528scFv-TRAIL fusion protein specific for human EGFR and expressed in *Escherichia coli*

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Abstract. We report the preparation and functional characterization of an *Escherichia coli*-expressed recombinant fusion protein, 528scFv-TRAIL, specific for the human epidermal growth factor receptor (EGFR) and empowered by the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). The 528scFv-TRAIL, expressed as insoluble inclusion bodies in *E. coli*, was solubilized and then refolded by using a modified stepwise dialysis method. Treatment with 528scFv-TRAIL resulted in the specific binding to the cell surface of EGFR-positive cells with concomitant deployment of the TRAIL moiety to DR-5 receptor in a manner comparable to a commercially available form of recombinant TRAIL (cTRAIL). 528scFv-TRAIL, prepared by either of three refolding processes described herein, showed potent cytotoxic activity against EGFR-positive TFK-1 cell line and was superior to its parental 528scFv; a recombinant variable fragment with single specificity against human EGFR. Narrow variations in the cytotoxic potential of 528scFv-TRAIL were ascribed to manipulation of redox conditions during the refolding process. Together, our findings point to the potential value of 528scFv-TRAIL for treatment of EGFR-expressing cancers. Furthermore, preparation of 528scFv-TRAIL from insoluble aggregates in a prokaryotic cell based expression system by means of *in vitro* refolding introduces a feasible cost-benefit, time-efficient approach for industrial-scale production.

Introduction

Antibodies (Abs) are some of the most powerful tools in therapy and diagnostics and are currently one of the fastest growing classes of therapeutic molecules. Recombinant antibody (rAb) fragments are becoming popular therapeutic alternatives to full length monoclonal Abs since they are smaller, possess different properties that are advantageous in certain medical applications, can be produced more economically and are easily amendable to genetic manipulation. Single-chain variable fragment (scFv) Abs are one of the most versatile rAb formats as they have been engineered into larger, multivalent, bi-specific and conjugated forms for many clinical applications (1).

Induction of apoptosis has been established as an important approach in the development of novel anti-cancer therapeutics. Cells can undergo apoptosis via two different pathways: the intrinsic and extrinsic. The intrinsic pathway is preferentially triggered by intracellular proteins such as p53 in response to a wide range of damaging influences, for example radiation and chemotherapy. Intrinsically triggered apoptosis is mainly regulated by proteins of the Bcl-2 family that control the release of pro-apoptotic factors from the mitochondrial intermembrane space. In contrast, the extrinsic pathway is triggered by activation of death receptors which belong to the tumor necrosis factor (TNF) receptor superfamily. These cell surface receptors are activated when crosslinked by their ligands. Ligand-induced death receptor crosslinking then initiates a downstream intracellular signal transduction cascade culminating in apoptosis.

Within the TNF superfamily of cytokines, the TNF-related apoptosis-inducing ligand (TRAIL) has emerged as the most promising for death receptor targeted cancer therapy due to its remarkable feature of selectively inducing apoptosis in tumors *in vivo* without causing toxicity to normal cells (2). TRAIL was first identified on the basis of its high sequence homology to CD95L and TNF (3,4). Among all death-inducing ligands, the TRAIL pathway is the most complex with five different TRAIL receptors being reported. However, only two of them, TRAIL-R1 (DR4) and TRAIL-R2 (DR5), are able to execute a full-fledged apoptotic cell death (5-7).

TRAIL-R1 and -R2 harbor a death domain (DD) in their intracellular portion, a six-seven α-helices motif which can

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bind to other DDs via homotypic interactions (8). TRAIL binds to TRAIL-R1 or -R2 which leads to receptor homo- or heteromerical trimerisation (9) and subsequent recruitment of Fas associated death domain protein (FADD/Mort1) to their intracellular DDs. FADD acts as adaptor protein by itself having both a DD and a death effector domain (DED) which enables caspase-8 and -10 as well as cellular FLICE inhibitory protein (cFLIP) to bind via their respective DDs. The resulting multiprotein complex is referred to as the DISC (10-12). The formation of the TRAIL DISC promotes conversion of pro-caspases into their active forms which can then activate downstream caspases. Following their activation at the DISC, caspase-8 and -10 are cleaved in an autocatalytic process, generating large and small subunits. These form active hetero-tetramers which activate downstream effector caspases, e.g., caspase-3 and -7 (13).

EGFR is a well-established target antigen featuring oncogenic mutations, overexpression and aberrant signaling in various human carcinomas such as colorectal, bile duct, breast and lung carcinomas (14-18). Signaling through EGFR is considered to regulate tumor cell functions, such as cell-cycle progression, inhibition of apoptosis, angiogenesis, tumor cell motility, adhesion, and invasion (19-25).

Here we introduce an efficient cost-benefit strategy based on an *E. coli* expression system and *in vitro* refolding for the production of a recombinant fusion protein, 528scFv-TRAIL, that combines EGFR-signaling inhibition with TRAIL-mediated target cell-restricted apoptosis induction.

Materials and methods

Construction and cloning of 528scFv-TRAIL. 528scFv-TRAIL was constructed in the T7 promoter-based pUT plasmid encoding an N-terminal His tag upstream of the cloning sites. The 528 mAb is a murine IgG2a directed to the human EGFR antigen (26). The V-region genes of 528 hybridoma cells were cloned by reverse transcription (RT)-PCR as described previously (27). The heavy chain (VH) and light chain (VL) genes were then amplified by PCR using the primer mix previously reported by Krebber *et al* (28). The VH and VL domains were genetically linked via a flexible peptide linker $(Gly4Ser)_3$ in the orientation VH-$(Gly4Ser)_3$-VL. The antibody variable fragment 528scFv, encoding VH-$(Gly4Ser)_3$-VL, was directionally inserted using restriction enzymes *Nco*I and *Xho*I. A second $(Gly4Ser)_3$ linker and the *Xho*I restriction site were added to the carboxyl terminus of VH-$(Gly4Ser)_3$-VL fragment via PCR amplification. A PCR-truncated DNA fragment encoding amino acids 114-281 of the extracellular domain of human TRAIL (29) was then cloned in frame using restriction enzymes *Xho*I and *Bam*HI. The N terminus of the TRAIL fragment was thus linked to 528scFv via the second $(Gly4Ser)_3$ linker, yielding plasmid pUT-528scFv-TRAIL (Fig. 1).

Expression of 528scFv-TRAIL in E-coli. 528scFv-TRAIL fusion protein was prepared from inclusion bodies expressed in *E. coli* as described previously (27,31). In brief, *E. coli* strain BL21 (DE3) harboring the bacterial expression vector pUT-528scFv-TRAIL was grown at 28˚C in Luria-Bertani broth. In order to induce protein production, 1 mM isopropyl-1-thio-ß-D-galactopyranoside (IPTG) was added to the culture and the cells were grown overnight. Cells were harvested by centrifugation (2000 x g, 35 min), resuspended in 10 ml phosphate-buffered saline (PBS), ultrasonicated at 150 W for 15 min and centrifuged at 4500 x g for 20 min. The precipitating intracellular insoluble fraction was separated and the pellet was solubilized overnight at 4˚C in 10 ml of 6 M guanidinium hydrochloride in PBS (Gu-HCl/PBS). Solubilized proteins were purified through a 2 ml Talon Metal Affinity Resin column (Clontech, Palo Alto, CA, USA), followed by elution with 6 M Gu-HCl/PBS containing 50, 150 and 500 mM imidazole respectively.

Refolding of 528scFv-TRAIL. We applied either of three refolding processes to prepare 528scFv-TRAIL from the above purified sample. In refolding process I, solubilized 528scFv-TRAIL was diluted to 7.5 μM with 6 M Gu-HCl/PBS, the redox reagent ß-ME was then added at a final concentration of 375 μM. The denatured 528scFv-TRAIL (5 ml) underwent step-wise dialysis into PBS through solutions of 6, 3, 2 and 1 M Gu-HCl/PBS with buffer exchange every 6 h, thereafter through solutions of 0.5 and 0.0 M Gu-HCl/PBS with buffer exchange at 12-h interval. At the dialysis steps of 1 and 0.5 M Gu-HCl/PBS we added each of 400 mM L-arginine and 375 μM oxidized glutathione (30), Fig. 2 shows a schematic depiction of the refolding steps. In process II, PBS was replaced by 50 mM Tris-HCl containing 200 mM NaCl and 1 mM EDTA. Step-wise dialysis was then carried out essentially as described in process I. In process III, removal of guanidine was carried out through one-step dialysis into 0.0 M Gu-HCl/PBS containing 400 mM L-arginine over a period of 12 h. L-arginine was then removed by dialysis against PBS for another 12 h. In process III, neither pre-treatment with ß-ME nor oxidized glutathione conditioning was performed. In all three refolding processes, the external buffer solution was continually adjusted to pH 8.0 at 4˚C.

Gel filtration chromatography. Gel filtration analysis with a Hiload Superdex 200-pg column (10/300; GE Healthcare Bio-Science Corp., Piscatway, NJ, USA) was used to evaluate the molecular mass and structure of the refolded fusion protein. The column was pre-equilibrated with PBS, and then 250 μl of the refolded recombinant protein was applied to the column at a flow rate of 0.5 ml/min.

Flow cytometric analyses. EGFR and DR5 specific binding by 528scFv-TRAIL was assessed by flow cytometry using the EGFR-positive, DR5-positive tumor cell line TFK-1 (human bile duct carcinoma) and the EGFR-negative, DR5-positive tumor cell line Jurkat (human T cell leukemia). In short, $1x10^6$ cells were first incubated on ice with equal molar concentrations of either 528scFv-TRAIL or cTRAIL (commercial recombinant TRAIL: TRAIL extracellular domain amino acids 114-281 with N-terminus His tag, Funakoshi, Japan). After being washed with PBS + 0.1% NaN_3, cells were incubated with anti-TRAIL rabbit polyclonal IgG (CosmoBio, Japan) and then washed and incubated with FITC-conjugated anti-rabbit IgG (Santa Cruz, USA). Incubations were carried out for 30 min at 0˚C. The fluorescence-activated cells were analyzed by flow cytometry (FACSCalibur, BD Biosciences).

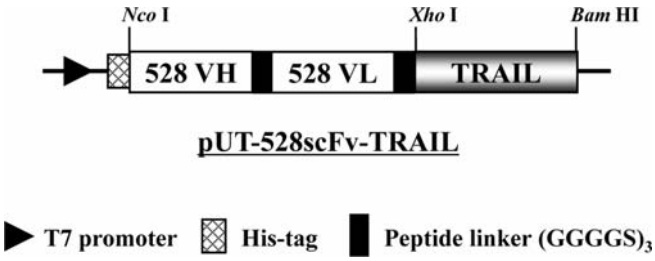

Figure 1. Construction of 528scFv-TRAIL expression vector. Heavy and light chain region genes of 528 are designated as 528 VH and 528 VL respectively. His-tag, a sequence encoding six N-terminal histidine residues. Peptide linker, a triple repeat of four glycine and one serine residues.

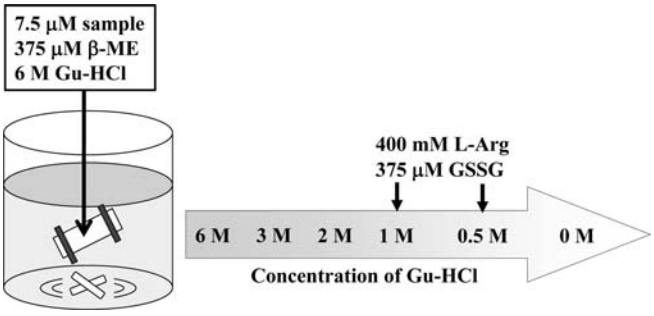

Figure 2. Procedure for refolding of *E. coli*-expressed recombinant 528scFv-TRAIL. The buffer solution during refolding is either PBS or 50 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA (pH 8.0, 4˚C). Each dialysis step lasts 6-12 h. ß-ME, ß-mercaptoethanol; Gu-HCl, guanidinium hydrochloride; GSSH, oxidized form of glutathione; L-arg, L-arginine.

In vitro growth inhibition assay. In vitro growth inhibition of TFK-1 cells (a human bile duct carcinoma line) was assayed with a 3-(4,5-dimethylthiazole-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium inner salt (MTS) assay kit (CellTiter 96 AQueous Non-Radioactive Cell Proliferation assay; Promega, Madison, WI, USA). Cell suspensions (5000 cells in 100 μl of culture medium) were plated in 96-well, flat-bottomed plates (Costar, Cambridge, MA, USA), and the plated cells were cultured overnight to allow them to adhere to the well. After removal of the culture medium by aspiration, increasing concentrations of the recombinant fusion proteins, each in 100 μl medium, were added to each well. After incubation of the cells for 48 h at 37˚C, each well was washed with PBS three times, and then 95 μl of culture medium containing 5 μl of a fresh mixture of MTS-phenazine methosulfate solution (Promega) was added to each well. The plates were incubated for 1 h at 37˚C and then read on a microplate reader (model 3550; Bio-Rad, Hercules, CA, USA) at a wavelength of 490 nm. Growth inhibition of target cells was calculated according to the following equation: percentage growth inhibition of target cells = [1-(A490 of experiment-A490 of background)/(A490 of control-A490 of background)] x 100 (31).

Results

Expression of 528scFv-TRAIL in E. coli. The gene encoding 528scFv-TRAIL was inserted into the pUT vector as explained previously. 528scFv-TRAIL recombinant fusion protein was then produced using *E. coli* strain BL21 (DE3) harboring the plasmid pUT-528scFv-TRAIL. The expressed fusion protein primarily existed in the intracellular fraction as inclusion bodies. As shown in Fig. 3, SDS-PAGE analysis revealed that target-size fusion protein could be retrieved with high purity from all sequential elution fractions flowing through the immobilized metal-affinity column (IMAC) under denaturing conditions. The total yield of scFv fusion protein was 21.4 mg per one liter of bacterial culture.

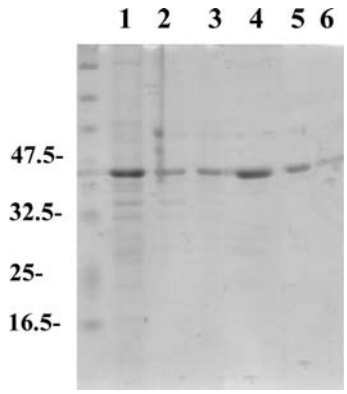

Figure 3. SDS-PAGE of fractions separated by immobilized metal affinity chromatography (IMAC) under denaturing conditions. Molecular mass markers (in kilodaltons) are shown on the left. Lane 1, total proteins in the insoluble fraction; lane 2, flow-through fraction; lane 3, wash fraction; lanes 4-6, 50, 150 and 500 mM imidazole elution fractions.

Refolding and determination of molecular structure. To obtain soluble, functional recombinant fusion protein we optimized the refolding system previously reported by Tsumoto *et al* (30). We applied three parallel approaches depending on the dialysis buffer employed and whether or not adjustments of redox conditions, via treatment with thiol reagents, were performed. The recombinant protein eluted from the affinity column was diluted with 6 M Gu-HCL/PBS. This denatured, purified and reduced fusion protein was then renatured by dialysis with Gu-HCl at sequentially decreased concentrations as described in Materials and methods. Gel filtration chromatography of the refolded 528scFv-TRAIL (refolding process II) revealed a major peak corresponding to the estimated molecular weight of the monomeric form of 528scFv-TRAIL (Fig. 4A). A much lesser fraction of 528scFv-TRAIL spontaneously adopted a dimeric form yielding a smaller peak (Fig. 4A). SDS PAGE of the eluted monomeric fraction is shown in Fig. 4B.

Binding properties of 528scFv-TRAIL. Binding of refolded 528scFv-TRAIL to the targeted antigens was confirmed by flow cytometry (Fig. 5). 528scFv-TRAIL bound with the EGFR-negative, DR5-positive Jurkat cells in a manner almost identical to that of commercial recombinant TRAIL. This specific reactivity was markedly enhanced with the EGFR-positive, DR5-positive TFK-1 cells indicating a strong cumulative effect endowed by the bispecific conformation of 528scFv-TRAIL on its overall binding affinity to target antigens.

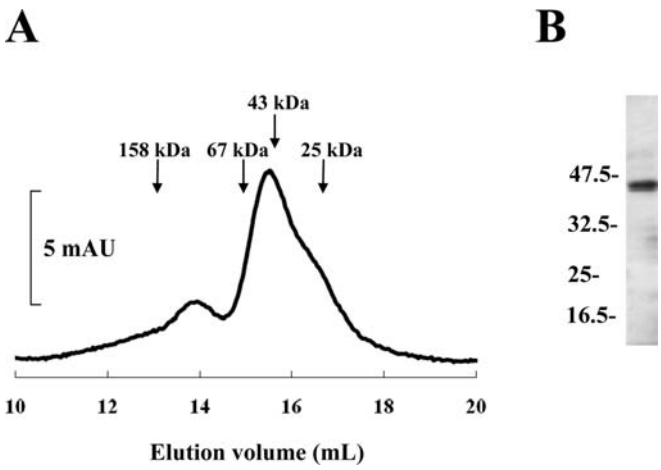

Figure 4. Gel filtration of 528scFv-TRAIL (refolding process II). (A) Chromatograph of gel filtration. Refolded 528scFv-TRAIL was subjected to gel filtration on Hiload Superdex 200-pg column (10/300). The column was pre-equilibrated with PBS, and 250 μl of refolded 528scFv-TRAIL was applied at a flow rate of 0.5 ml/min. (B) SDS-PAGE of the eluted monomeric fraction.

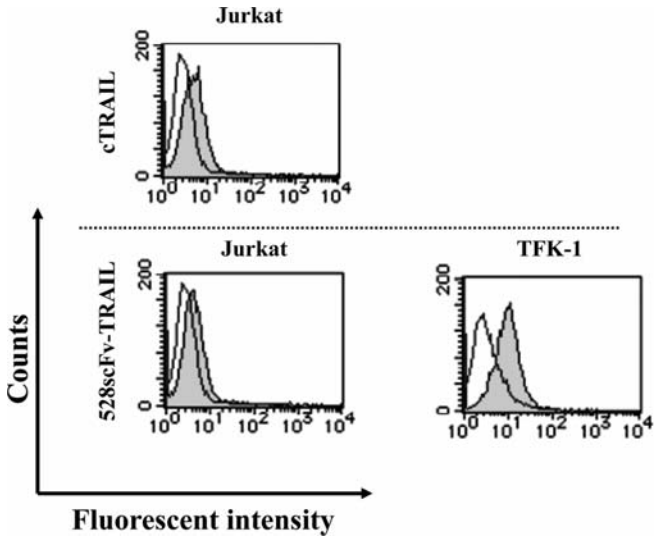

Figure 5. Flow cytometric analysis of reactivity of constructed 528scFv-TRAIL (refolding process I) with Jurkat and TFK-1 cells. Cells were incubated with PBS as a negative control (open areas) and with commercial recombinant TRAIL (cTRAIL) as a positive control. Cells were incubated with cTRAIL or 528scFv-TRAIL, followed by incubation with anti-TRAIL rabbit polyclonal IgG and then stained with an FITC-conjugated anti-rabbit IgG (shaded areas).

Tumoricidal activity of 528scFv-TRAIL. We evaluated the anti-tumor effect of 528scFv-TRAIL, refolded under each of the three refolding processes mentioned in Materials and methods, against the human bile duct carcinoma TFK-1 cell line. 528scFv-TRAIL (refolding process I) showed stronger tumoricidal activity when compared to its parental anti-EGFR mono-specific 528scFv (Fig. 6A). This enhancement in cytotoxicity over its parental form is ascribed to summation of the apoptosis-inducing effect exerted by the TRAIL moiety in 528scFv-TRAIL. Under similar redox conditions, alteration of the refolding buffering system in refolding

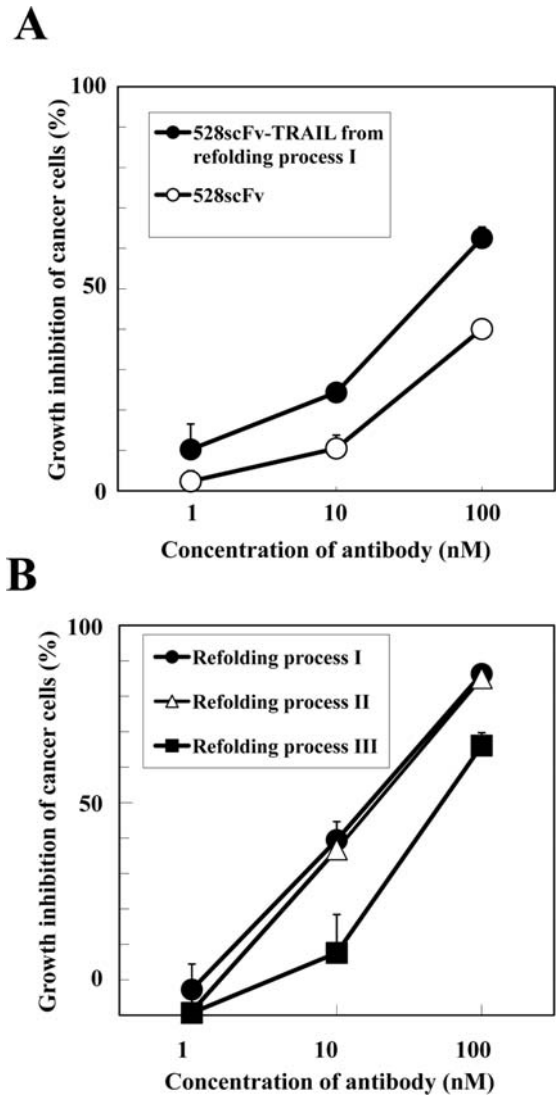

Figure 6. Growth inhibition assay of human bile duct carcinoma cell line TFK-1. Growth inhibition indices were determined by a 48-h MTS assay. (A) Cells were incubated with increasing concentrations of 528scFv-TRAIL (refolding process I) or parental 528scFv. (B) Cells were incubated with increasing concentrations of 528scFv-TRAIL prepared by refolding process I, II or III. Data are mean values from at least triplicate determinations.

processes I and II did not have any significant effect on the overall recovery of biological activity. On the other hand, 528scFv-TRAIL prepared by refolding process III, in which treatment with neither ß-ME nor oxidized glutathione was performed, did show a small, but statistically significant, drop in anti-tumor activity (Fig. 6B).

Discussion

In this study, we describe an immunotherapeutic approach in which EGFR-signaling inhibition is combined with target-cell restricted apoptosis induction using the novel fusion protein 528scFv-TRAIL. We also show that recombinant 528scFv-TRAIL, retaining competitive binding properties and potent anti-tumor activity, can be obtained by *in vitro* refolding under systematically constructed conditions. This modified refolding scheme is based on a previously reported

refolding system developed in our laboratory (30) that allows the recovery of functional recombinant proteins from insoluble fractions of *E. coli* cultures in a highly efficient cost-benefit manner.

528scFv-TRAIL showed an enhanced cumulative binding affinity to the EGFR-positive, DR5-positive TFK-1 cells. The affinity of 528scFv for EGFR has been thoroughly investigated and is well established (32). On the other hand, given the monomeric structure of 528scFv-TRAIL, binding of its TRAIL moiety to DR5 receptor, however, should be interpreted in view of the incompletely characterized complexity of TRAIL receptor biology. Several groups have found experimental evidence that TRAIL receptors do not only exist in a homo-trimeric form but can also form heterodimeric complexes (12,33). Other reports have demonstrated strong cytotoxic activity of monomeric recombinant TRAIL refolded *in vitro* (34-36). Together, these reports offer the ground to explain the robust biological activity of 528scFv-TRAIL despite the fact that it existed in a predominantly monomeric form, and not as homotrimers, as was shown by gel chromatography.

Delicate control of disulfide bond formation is a crucial factor in the refolding of insoluble antibody variable fragments and a major problem with refolding is the formation of incorrect inter- and intra-molecular disulfide bridges (37,38). Indeed, artificial control of the redox conditions was found to be the most effective means for optimizing the conditions for proper disulfide bond formation (30). Previous studies further suggest that the correct formation of disulfide bonds at the appropriate refolding stage may be critical for obtaining sufficient yield as well as for the efficient recovery of biological activity of the refolded recombinant protein. Pretreatment with a powerful reducing thiol reagent, such as ß-ME, allows for the initial disruption of incorrect disulfide bonds, whereas adding an oxidizing thiol reagent, such as glutathione in its oxidized form, to the refolding buffer has been reported to result in the restoration of correct disulfide bonding (39). With this in mind, we kept a steady control of the concentrations of redox reagents added to the refolding system, combined with the stepwise removal of Gu-HCl. We evaluated the effects of these controlled redox conditions, as well as any potential cross-talk between redox conditions and different buffers used in dialysis, particularly on the recovery of biological activity. In our study, we found that under similar redox conditions, altering the buffering system did not result in any significant change in biological activity. However, 528scFv-TRAIL refolded by the refolding process III, in which neither ß-ME nor oxidized glutathione was added, did show a small drop in biological activity compared to refolding processes I and II in which both thiol reagents were involved. These results are in line with previous reports and further emphasize the crucial role of optimized redox conditions for recovering the biological activity of denatured recombinant proteins.

In conclusion, we managed to establish an affordable cost-benefit, time-efficient approach for the production of a bispecific recombinant fusion protein, capable of executing target cell-restricted, TRAIL-mediated apoptosis in EGFR positive tumor cells. We further claim that the refolding strategies described in this study should be applicable, with minor adjustments, to the refolding of various single-chain Fv fragments and recombinant proteins expressed in inclusion bodies from bacterial cultures.

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