Addressing liver fibrosis by TRAIL targetied to hepatic stellate cells

Arabpour, Mohammad

Chapter 3

Targeted elimination of activated hepatic stellate cells by PDGF-TRAIL and EGF-TRAIL (receptor-specific ligand peptide – sTRAIL) fusion proteins

Mohammad Arabpour [1], Klaas Poelstra [2], Hidde J. Haisma [1]

1. Department of Pharmaceutical Gene Modulation, University of Groningen, Groningen, The Netherlands

2. Department of Pharmacokinetics, Toxicology and Targeting, University of Groningen, Groningen, The Netherlands

Abstract

Background Activation and proliferation of HSCs during chronic liver injury cause progressive replacement of hepatic parenchyma with fibrotic tissue; whereas apoptosis appears as a significant event to reduce the number of activated HSCs during the resolution phase of hepatic fibrosis. In nature, Tumor necrosis factor-Related Apoptosis-Inducing Ligand (TRAIL) has the major role in this dynamic process by inducing apoptosis in activated HSCs. Therefore, application of TRAIL is considered a desirable anti-fibrotic therapy. However, the very short half-life of TRAIL *in-vivo* and the dual role of apoptosis in progression and resolution of liver fibrosis demands strategies to increase the selectivity of TRAIL for HSCs while reducing dosage for maximal effect. The present study addresses this issue by targeting TRAIL to activated HSCs via PDGF or EGF receptor specific peptide moieties.

Methods Activated HSCs (LX2 cells) were treated by PDGF receptor specific peptide (pPB) or EGF receptor specific peptide (GE11) moieties linked to the extracellular part of TRAIL in the form of fusion proteins. We then evaluated the viability of affected LX2 cells and extracellular matrix production by the affected LX2 cells.

Results *In vitro* studies showed that treatment of activated HSCs with pPB-TRAIL or GE11-TRAIL did not reduce the viability of activated HSCs to a greater extent than TRAIL alone. Evaluation of TRAIL binding to activated HSCs using confocal microscopy revealed that targeting via anti-EGFR single chain antibody; targeting

via pPB or targeting via GE11 did not increase the binding of TRAIL toward activated HSCs.

Conclusion The application of pPB-TRAIL or GE11-TRAIL fusion proteins did not increase the potency of TRAIL molecule in the targeted elimination of activated HSCs. Our findings indicate that the higher binding capacity of anti-EGFR scFv-TRAIL towards activated HSCs could be accountable for its greater efficiency in eliminating these cells .

Introduction

Gene Therapy using fusion peptide PDGF-sTRAIL and EGF-sTRAIL

Activated HSCs are the major basis cause of liver fibrosis. It has been shown that activated HSCs significantly amplify the hepatic response to liver injury, and depleting fibrotic livers from activated HSCs could ameliorate the fibrotic condition [1]. Therefore, an ideal therapy for liver fibrosis should be able to selectively eliminate activated HSCs in fibrotic liver. We and others have successfully employed TRAIL-receptor agonists to target and eliminate activated HSCs [2,3]. However, the expression of membrane-bound and free TRAIL decoy receptors 1 and 2, that neutralize the effect of TRAIL, impose a hurdle to achieve the efficient application of TRAIL for resolution of liver fibrosis. Moreover, the ubiquitous expression of TRAIL receptors and the complex role of apoptosis in inducing inflammation is a reason to explore more targeted and more efficient TRAIL ligands for the treatment of liver fibrosis [2].

An ideal way to minimize the potential deleterious side effects of TRAIL therapy in liver fibrosis is to channel the apoptotic property of TRAIL specifically to activated HSCs. To address this, a number of moieties may be targeted on the surface of activated HSCs to complement with the effect of TRAIL. Receptors for Platelet Derived Growth Factor (PDGF), Epidermal Growth Factor (EGF) and TGF-β are among the highly expressed receptors on activated HSCs that are suitable for being targeted [2][4-5]. Therefore, monoclonal antibodies (mAbs), single-chain variable fragment (scFv) and receptor specific peptides with high target specificity against these receptors are suitable for targeting application. We have already successfully employed an anti EGFR–TRAIL single chain fusion protein to eliminate activated HSCs [5]. However, antibodies possess a high molecular weight, limited tissue penetration and species-specific recognition that might be disadvantageous for targeting applications if an in vivo model is to be tested. Targeting with peptides may be efficient for a range of cell types (without cross-species barriers in receptor affinity), hence can be applied for targeting the payloads in a range of hosts [6,7]. A small peptide, CSRNLIDC, (pPB) has been successfully employed in a couple of approaches to target therapeutic proteins or vectors to activated HSCs [8][9]. This peptide binds to the PDGF-Rβ, without initiating the signaling cascade [8][9]. The EGFR-specific peptide, YHWYGYTPQNVI, **(GE11)** is a dodecapeptide produced by phage display. Ge11 binds to the EGF receptor (EGFR), yet does not activate the receptor tyrosine kinase activity. The absence of EGFR activation, as well as the specific attachment of to the EGFR confer GE11 with significant advantages for targeting applications [10]. This ligand has been used for targeted delivery of

vectors, lytic peptides and cytotoxic compounds to highly expressing EGFR cancer cells[11,12].

In the present study, we examine the potential application of adenovirally expressed receptor-specific peptide -TRAIL fusion proteins, which may induce apoptosis in activated HSCs. For this purpose, we devised growth factor receptor-specific peptides pPB- or GE11-sTRAIL fusion proteins that specifically target and eliminate activated HSCs.

Material and Methods

Cell lines and cell culture

The cells lines used in this study were NIH/3T3 (mouse fibroblast cell line ATCC® CRL-1658™), HEK293 (Human embryonic Kidney 293 cells, ATCC® CRL-1573™) and LX-2 Immortalized human HSCs (kindly provided by Prof. Scott Friedman,Mount Sinai Hospital, New York, NY, USA). The NIH/3T3 and HEK293 cell lines were grown in Dulbecco's minimum essential medium (DMEM, Gibco) whereas LX2 cells were cultured in Dulbecco's minimum essential medium (DMEM; Glutamax, Gibco, Gaithersburg, MD, USA). Media were supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 ug/ml streptomycin.

Antibodies

The following antibodies (Ab) were used: Mouse anti-EGFR IgG2a, anti-hemagglutinin (HA) mouse IgG1 (InvivoGen, San Diego , California), Anti-mouse IgG polyclonal antibody (Sigma).

Construction of shuttle vector pCMV-TRACK pPB-sTRAIL and pCMV-TRACK GE11-sTRAIL

The vector pCMV-Track-scFv425-sTRAIL [5,13] was used for the construction of a vector, so-called pCMV-Track-pPB-sTRAIL. To construct the new vector DNA encoding pPB was synthesized and amplified via PCR using forward and reverse primers, 5'GGGAGATCTTCCACCATGGAGACAGACACA3' and 5'ACAATCGATGAGGTTCCGCGAGCAACCAGTGGAACC3', respectively. The restriction site for BglII was introduced in the forward primer and for ClaI in the reverse primer. The digested PCR product was introduced into BglII and ClaI-digested vector, and ligated. The resulting construct was designated pCMV-Track-HA-PDGF-sTRAIL. Colony PCR was performed on the transformed PCR to screen for the right size of the insert via primers 5'-(GGGAGATCTTCCACCATGGAGACAGACACA)-3' and 5'-(GAAATTTGTGATGCTATTGC)-3'. For the expression of the EGFR-targeting fusion protein GE11-sTRAIL, a genetic construct was made using the vector pCMV-TRACK-PDGF-sTRAIL as the starting material. Partially complementary (in bold) oligonucleotides 5'-(GGG AGA TCT TCC ACC ATG GAG ACA GAC ACA CTC CTG CTA TGG GTA CTG CTC TG**G GTT CCA GGT TCC**)-3' and 5'-(CCC GCGGCCGC TAT CAC GTT CTG GGG TGT GTA ACC GTA CCA ATG ATA ACC AGT **GGA ACC TGG AACC**)-3' were used to generate the IgK (leader peptide)-GE11 peptide. After digestion the construct was inserted into the pCMV-TRACK-PDGF-sTRAIL vector to generate the final construct pCMV-TRACK-GE11-sTRAIL. The integrity of the cloning procedure was verified by

digestion reactions using the restriction enzymes ClaI, KpnI, PmeI and SalI. Colony PCR was performed on the transformed PCR to evaluate the right size of the insert via primers 5'-(GGGAGATCTTCCACCATGGAGACAGACACA)-3' and 5'-(GAAATTTGTGATGCTATTGC)-3'.

Production of Adenovirally expressed Ad-pPB-sTRAIL and Ad-GE11-sTRAIL

Ad-pPB sTRAIL and Ad-GE11-sTRAIL were generated using the plasmids encoding GE11-sTRAIL or pPB sTRAIL using the Ad-Easy system. In short, the plasmids were co-transformed with the adenoviral genome vector pAdEasy1 in *Escherichia coli* BJ5183 (2). After homologous recombination, pAd- pPB sTRAIL or pAd-GE11-sTRAIL was obtained. Subsequently, the pAd- pPB sTRAIL or pAd-GE11-sTRAIL plasmids were separately transfected into HEK-293 cells. The cells were incubated for 14 days to produce virus, which was visible as GFP-positive viral plaques. The cell lysates and supernatants containing the proteins were screened using Western Blotting with an anti-HA Ab to check for the expression of the desired proteins. The schematic representation of the pPB-sTRAIL or GE11-sTRAIL protein is shown in figure 1a.

Evaluation of pPB-sTRAIL or GE11-sTRAIL effect on activated LX2 cell viability and proliferation

To activate the LX2 cells, cells (1000/well) were seeded and incubated in 96-well, flat-bottomed uncoated plastic plates in media with 1% FBS for up to 7 days as previously described (3). Cells were exposed to various concentrations of pPB-sTRAIL or GE11-sTRAIL or sTRAIL diluted in DMEM medium for 48 hours. Cell

only controls received DMEM 1% FBS**.** The effects of the different constructs on cell viability was assessed in triplicate using MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), in the presence of phenazine methosulfate (PMS)) according to the manufacturers protocol (Sigma, USA).

Western Blot Analysis

For SDS-PAGE and Western blotting, Hek 293 cells were cultured in T75 flasks at 50% confluency. The different viral construct Ad-HA-pPB-TRAIL, Ad-HA-GE11-TRAIL was added to the medium at a concentration of 10 MOI and after 48 hours cell were harvested using 0.05% Trypsin- EDTA in PBS. For control cells only DMEM was added to Activated HEK293T cells. Cells were re-suspended in 300µl of Laemli buffer and the samples were separated on 12.5 % polyacrylamide gels. Gels were stained with Coomassie Brilliant Blue R250 for protein visualization. For Western Blot analysis with Ab against HA tag, gels were blotted on activated PVDF membranes with electrophoretic transfer overnight at 4˚C in blotting buffer. The membrane was then blocked for 1.5 hours with 3% BSA-0.05% Tween in PBS. It was then incubated for 2 hours with an Ab against mouse anti HA tag 1:1000 in PBS with1% BSA and 0.05% Tween. After washing 3X for five minutes with PBS-0.05% Tween, the membrane was incubated for one hour with the second Ab (Rabbit anti-mouse IgG, horseradish peroxidase (HRP) conjugated; Dako, Denmark) diluted 1:1000 in the buffer mentioned above. The membrane was then washed 4X for 15 minutes in the washing solution used above. The blot was developed using an

AEC staining solution system (Sigma, USA) according to the manufacturer's instructions.

HUMAN TRAIL (CD253) ELISA

An ELISA was performed on the supernatants of cells transduced with the adenovectors bearing the TRAIL constructs. Briefly, T293 Hek cells were cultured in T75 flasks at 50% confluency. Different viral constructs, Ad-HA-pPB-TRAIL, Ad-HA-GE11-TRAIL, Ad-HA-sTRAIL or Ad-HA-425scFv-sTRAIL, were added separately to medium at an MOI of 10 and after 48 hours the supernatants were collected and used in the ELISA. During the first incubation, 100 µl of the standards (3000, 1500, 750, 375, 187.5, 93,75 pg/ml TRAIL), samples or blank (DMEM medium) and a biotinylated monoclonal antibody specific for TRAIL are simultaneously incubated in microtiter plates for 2 hours at room temperature. After washing 3X with washing solution, Streptavidin-HRP was added to the micro titer plates to bind to the biotinylated antibody, incubated for another 2 hours and washed 3X. TMB substrate solution, 100 µl, of was added and incubated to develop a colored reaction product. The intensity of this colored product was directly proportional to the concentration of TRAIL present in the samples and was read in a spectrophotometer at 450 nm.

Immunohistochemistry and confocal microscopy

For immunohistochemistry of cultured activated LX2 cells, LX2 cells were cultured on uncoated plastic slides with 1% FCS in DMEM for 5 days. At day 6, cells were treated with an MOI of 10 of control adenovirus, ADTL (Ad5 expressing

EGFP) in DMEM 10%, at day 7 the media were removed and replaced by medium containing 1 μM of HA-scFv425-sTRAIL, HA-pPB-sTRAIL or HA-sTRAIL at intervals of 0, 5 and 15 minutes. After 15 minutes the cell supernatants were removed and cell were briefly washed with PBS, fixed with formaldehyde 10% (Sigma) at 25ºC for 20 minutes, washed 3 time and incubated with PBS for 5 minutes. Next, the cells were incubated with mouse anti-HA diluted 1:100 and 1/10000 Hoechst 33342 in PBS. The cells were washed with PBS 3-times and incubated with Rabbit anti-mouse IgG diluted 1:100 in PBS. Finally, the cells were washed with PBS for 3-times and mounted. Cells were observed with a confocal microscope, Leica sp8, with excitation wavelength 590nm and emission 617nm for Alexa Fluor 594 and excitation wavelength 360nm and emission 460nm for EGFP and blue laser (360nm) for Hoechst 33342. The Images were processed by Imaris (3D viewer) software.

FACS analysis of HA-scFv425-sTRAIL, HA-pPB-sTRAIL or HA-sTRAIL on activated HSCs

Flow cytometer analysis. HSCs were detached using 0.05% Trypsin- EDTA in PBS, washed with ice-cold PBS, and diluted to a concentration of 2 * 10^6 cells/mL using cold PBS. Aliquots of 100 μL (2 * 10^5 cells) were centrifuged at 14,00 rpm for 5 min at 4ºC, the supernatant was discarded, and the cells were separately suspended in 100 μL of HA-scFv425-sTRAIL, HA-pPB-sTRAIL, HA-GE11-sTRAIL or HA-sTRAIL (60 pM/mL). After incubation for 45 min at 4ºC, the cells were washed twice with cold (4ºC) PBS and incubated for an additional 45 min with 1:100 diluted mouse anti–HA secondary antibody under cold conditions. The cells were washed

with PBS 3X and incubated with the Rabbit anti-mouse IgG-FITS conjugate diluted 1:100 in PBS. Finally cells were washed with PBS 3X and suspended in 300 µL PBS (1% BSA). Unlabeled cells and cells labeled with secondary antibody alone served as negative controls. The mean values of the fluorescence intensity of 10,000 cells was determined by fluorescence-activated cell sorting (FACS) analysis (Calibur 1) and analyzed with FlowJo VX software.

Results

pPB-sTRAIL or GE11-TRAIL were produced successfully in Adenovector (Ad5 system)

The genes encoding the fusion proteins pPB-sTRAIL or GE11-TRAIL were successfully cloned into Ad5. The production and secretion of HA-pPB-TRAIL or HA-GE11-TRAIL was confirmed by western blotting. For both proteins, a 20KD monomer was detected using an anti-HA antibody (Figure 1b). In addition, lower molecular weight breakdown products were detected. To evaluate the efficiency of production and secretion of HA-pPB-sTRAIL or HA-sTRAIL, sTRAIL and scFv425-sTRAIL, an ELISA assay was performed on the supernatants of transduced cells. scFv425-sTRAIL was produced most efficiently from adenovirus-infected HEK cells with a concentration of 60 μg / ml. sTRAIL and PDGF-sTRAIL were produced at much a lower concentration, 8 μg / ml and 6 μg / ml respectively. The expression of GE11-sTRAIL was substantially lower: 1 ug/ml (Figure 1c).

pPB-sTRAIL and GE11-TRAIL reduce viability of activated LX2 cells

To evaluate the growth inhibiting effects of adenovirally produced, HA-pPB-sTRAIL, HA-GE11-sTRAIL or HA-sTRAIL, different amounts of supernatants containing the proteins were added to activated or quiescent LX2 cells and the viability of cells was determined 48 hours later by MTS assay. Activated LX2 cells showed a 63% reduction of viability after treatment with HA-pPB-TRAIL at a concentration of 408 ng/ml compared to 65% inhibition by sTRAIL alone (Figure

2B). The inhibition of viability in quiescent HSC was 20% after treatment with HA-pPB-TRAIL and 21% at 408 ng/µl of sTRAIL (Figure 2A). Since 3T3 (mouse fibroblast) cells are known to be expressing high level of PDGFR on their surface. We then evaluated the effect of adenovirally produced, HA-pPB-TRAIL or HA-TRAIL on this cell. Activated or quiescent 3T3 cells were treated with different amount of HA-pPB-TRAIL or HA-TRAIL and the viability of cells was determined 48 hours later by MTS assay. Activated 3T3 cells showed up to 95% reduction of viability after treatment with HA-pPB-TRAIL at a concentration of 408 ng/ml compared to 86% inhibition by sTRAIL (Figure 2D). The inhibition of viability in quiescent HSC was 15% after treatment with 408 ng/ml of HA-pPB-TRAIL and comparable with sTRAIL at the same concentration (Figure 2C). Thus, sTRAIL and PDGF receptor -targeted TRAIL show a relative selectivity towards activated HSCs and 3T3 cells. However, there was no significant difference between targeting TRAIL via PDGF receptor and via its dedicated receptors in activated or quiescent LX2 or 3T3 cells. Comparison between the HA-GE11-TRAIL and HA-TRAIL effect on activated and quiescent HSCs also showed similar pattern as that of pPB-TRAIL (figure 3A, B).

Comparative attachment and internalization of different TRAIL types

Upon ligand binding, TRAIL receptors are internalized via clathrin-dependent endocytosis and clathrin-independent pathways. An increase in TRAIL internalization could reduce the TRAIL efficiency in initiating the caspase cascade. Interestingly, cell surface-bound TRAIL could better initiate the caspase cascade[14]. We therefore further investigated the effect of TRAIL internalization

on its potential for eliminating activated LX2 cells. pPB-sTRAIL, HA-GE11-sTRAIL, sTRAIL and scFv425-sTRAIL were added to activated LX2 cells in equimolar concentrations. The binding and attachment of the constructs was evaluated using flow cytometry analysis. HA-scFv425-sTRAIL binding to activated LX2 cells was substantially greater (100-times) than that of HA-sTRAIL, HA-GE11-sTRAIL or HA-pPB-sTRAIL (Figure 4A). Interestingly, blocking the EGFR by an anti-EGFR antibody reduced the binding of scFv425-sTRAIL substantially (Figure 4B). Internalization of the scFv425-sTRAIL construct into the activated HSCs was faster in comparison to that of sTRAIL and pPB-sTRAIL proteins. scFv425-sTRAIL was completely internalized after 5 minutes of incubation; whereas sTRAIL and pPB-sTRAIL were completely internalized after 15 minutes. Therefore a more stable anchoring of TRAIL to the cell surface receptors could not be accountable for more efficient induction of apoptosis in activated HSCs.

Discussion

Liver fibrosis is considered as end-stage liver diseases and is the primary reason for liver transplantation. The development of liver fibrosis is associated with progressive liver chronic diseases. To date, many specific anti-fibrotic therapies fail due to lack of overall efficiency and the toxicity. The underlying pathophysiology of liver fibrosis revealed that activated HSCs play a central role, both as a causative and as an effector cell [2,15]. Therefore, research focusing on the targeted elimination of activated HSC as the first step in the resolution of liver fibrosis is highly desired. Activation of HSCs is associated with the overexpression of death-

inducing receptors such as TRAIL. Directing TRAIL to activated HSCs has previously been attempted as a means to eliminate liver fibrosis [3,5]. Despite the successful application of TRAIL to eliminate activated HSCs, the very short *in vivo* half-life of TRAIL and simultaneous over expression of its free and membrane-bound decoy receptors (DcR1 and DcR2) impose a hurdle for the clinical application of TRAIL. For this reason a new strategy that could bring more efficient targeting and long term endogenous expression (e.g., gene delivery) of TRAIL could be desirable.

Activated HSCs are highly responsive to a series of growth factors and cytokines such as EGF, Amphiregulin (AR), Beta Cellulin (BTC), PDGF and TGF-β as a result of the higher expression of their corresponding receptors on these cells [2][4-5][16]. We have previously shown that a dual function anti- human EGFR scFv-sTRAIL fusion protein could target and suppress the function of the EGFR and at the same time induce ligand-assisted apoptosis in activated HSCs in vitro[5]. In the current study, we evaluated the replacement of the single chain antibody with specific binding peptides.

We successfully constructed and produced a fusion protein, Ge11-sTRAIL, by genetically fusing GE11 sequence to the N terminal of soluble TRAIL molecule and expressing the protein from a recombinant Adenovector. A reduction in viability of activated human HSCs and mouse fibroblast was observed after exposure to adenovirally-expressed GE11-sTRAIL. This finding is in concordance with previous studies that showed sensitivity of HSCs to TRAIL constructs [3,5]. Whereas

successful anti EGFR scFv, GE11 targeting could not further increase the effects of sTRAIL in eliminating activated or quiescent LX2[5].

In addition, we used a PDGF receptor targeting peptide. The PDGF-receptors consist of two chains, PDGF-Rα and β, that form dimmers. Whereas, PDGF-Rα is equally expressed on quiescent and activated HSCs, PDGF-Rβ is up-regulated during the transition of quiescent cells into activated HSCs [2]. Therefore, the higher expression of the PDGF-Rβ on activated HSCs make it an ideal targeting moiety [15]. pPB, a peptide that exclusively recognizes and binds to PDGF-Rβ, has been previously employed to direct proteins to a number of cell types including HSC that over express PDGF-Rβ [16–20]. We successfully constructed and produced a fusion protein, pPB-sTRAIL, by genetically fusing pPB sequence to the N terminal of soluble TRAIL molecule and expressing the protein from a recombinant Adenovector.

A reduction in viability of activated human HSCs and 3T3 cells was observed after exposure to adenovirally-expressed pPB-sTRAIL. This finding is in concordance with previous studies that showed sensitivity of HSCs to TRAIL constructs [3,5]. However, pPB targeting could not significantly augment the effects of sTRAIL in eliminating activated or quiescent LX2.

TRAIL-induced apoptosis is associated with the initiation of downstream apoptotic signaling upon attachment to its dedicated receptor. Therefore, binding of TRAIL constructs to targeted cell is the first crucial step that determines the outcome of process. We have shown that fusing a single-chain antibody against EGFR with

sTRAIL boosts the efficiency of TRAIL to induce apoptosis in activated HSCs [4,5]. The comparison between the pPB-sTRAIL, sTRAIL and 425scFv-sTRAIL showed the scFv-fusion protein could substantially increase the binding of TRAIL to the surface of activated HSCs. For being selective a fusion protein should be able to out compete with the natural ligand for its target receptor. Whereas natural TRAL with dissociation avidity constant (K_D), of 2nM, PDGFRβ and EGFR receptors peptide dissociation constant are in the order of 22 nM [10] [19]. Therefore the pPB-TRAIL or GE11-TRAIL fusion proteins might not be able to increase the avidity beyond the wt TRAIL affinity. Upon binding to its dedicated receptors, TNF, CD95 and TRAIL initiate receptor aggregation and recruitment of the adaptor protein such as FADD (Fas-associated death domain) to the cytoplasmic tail of the TRAIL receptor. Recruitment of FADD is associated with conversion of procaspase 8 (the upmost compartment of caspase cascade) to caspase-8 in the context of Death-Inducing Signaling Complex (DISC). Caspase-8 and FADD are not recruited to a CD95 or TNF-induced plasma membrane-bound receptor-signaling complex, but instead, are activated elsewhere within the cell. However, unlike TNF and death ligand CD95, TRAIL internalization is not required for TRAIL-induced apoptosis and it may even dampen the apoptotic property of TRAIL for initiating the caspase cascade. Immobilized TRAIL protein induces more efficient apoptosis in target cells than soluble TRAIL [14]. Therefore, it may be speculated that an increased capability of scFv425-TRAIL in reducing cellular viability could be due to decreased internalization of TRAIL. Examination of TRAIL internalization for different TRAIL constructs by confocal laser microscopy tracking of TRAIL into the late

endosome compartment of cells showed that scFv425-sTRAIL internalizes faster than either sTRAIL or pPB-sTRAIL. Hence, a better effectivity of scFv425-TRAIL construct in comparison with sTRAIL and pPB-sTRAIL could not be due to anchoring of the sTRAIL construct on the surface of cellular membrane via scFv425. The higher binding affinity of scFv-sTRAIL rather than the internalization is relevant for inhibition of HSCs. Finally, binding of the scFv moiety to the EGFR could antagonize the effect of this growth factor and its downstream proliferative and anti-apoptotic signaling. The inhibition of HSCs by the scFv-sTRAIL protein is therefore the combined inhibitory effect of receptor inhibition and apoptosis induction.

In conclusion, our findings indicate that application of pPB-TRAIL or GE11-TRAIL fusion proteins could induce apoptosis in activated HSCs. However targeted elimination of activated HSCs via EGFR or PDGFR specific peptides and simultaneous activation of the caspase pathway did not significantly increase the potency of these molecules compared to wt TRAIL.

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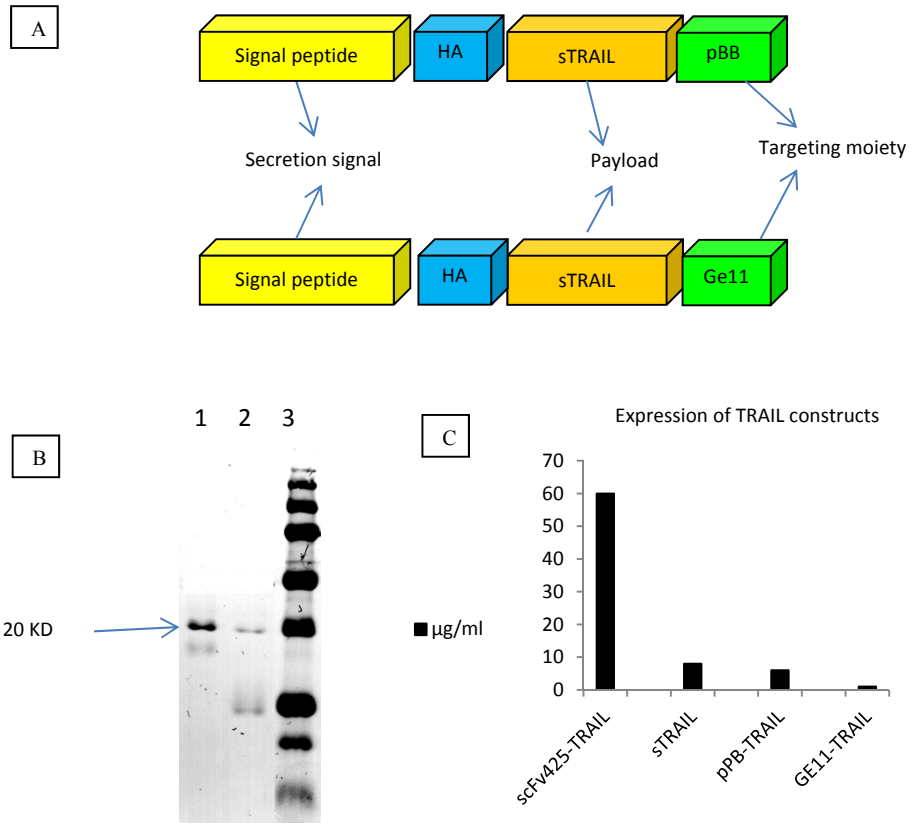


Figure 1: A) Schematic figure of TRAIL-pPB and TRAIL-GE11. B) Western blotting of pPB-TRAIL and GE11-TRAIL showed the band size of 20KD for both proteins lane1: Adenovirally produced PDGF-TRAIL lane 2: Adenovirally produced GE11-TRAIL lane 3: Protein marker. C) Expression of different adenovirally expressed TRAIL constructs by ELISA.

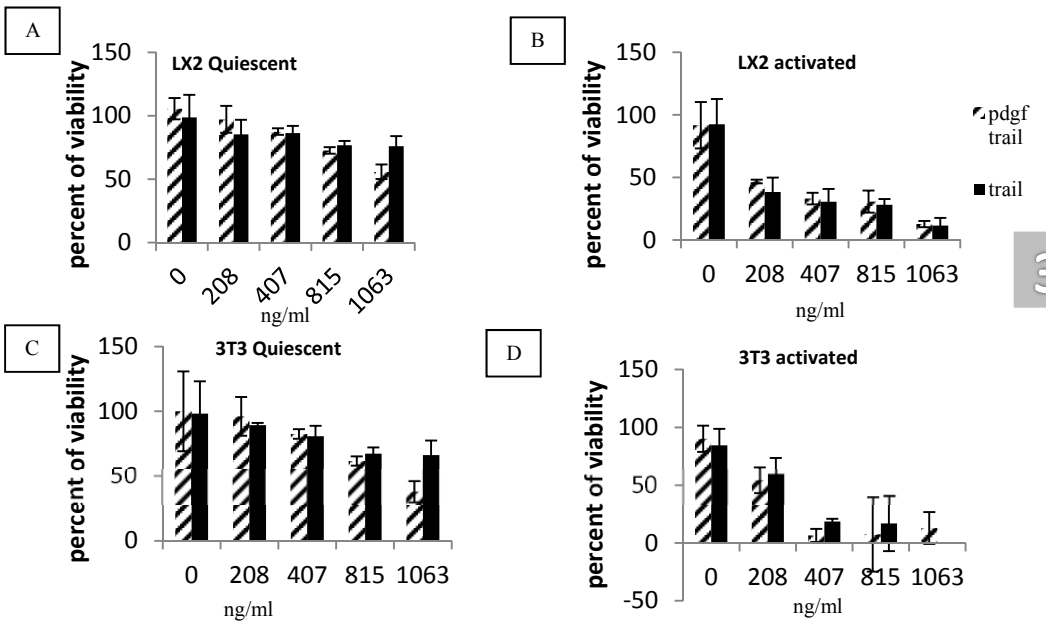


Figure 2: Viability of quiescent (A) and activated (B) LX2 cells treated with different concentration of TRAIL and pPB-TRAIL. Viability of quiescent (C) and activated (D) 3T3 fibroblast cells treated with different concentration of TRAIL and pPB-TRAIL.

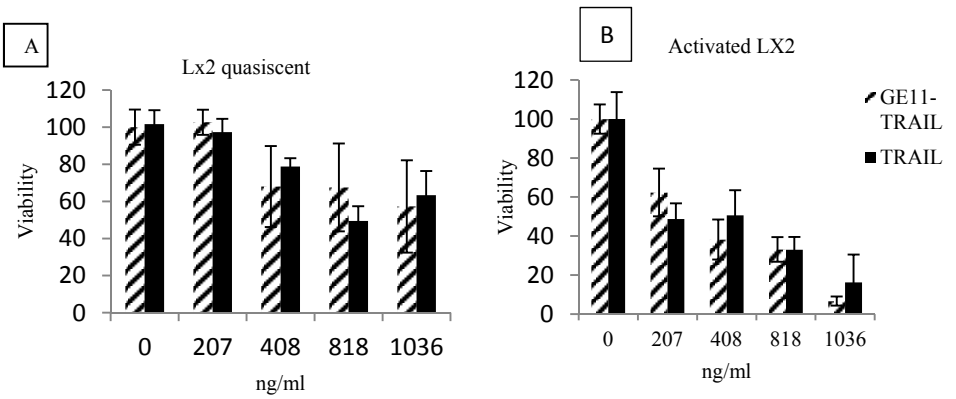


Figure 3: Viability of quiescent (A) and activated (B) LX2 fibroblast cells treated with different concentration of TRAIL and pPB-TRAIL.

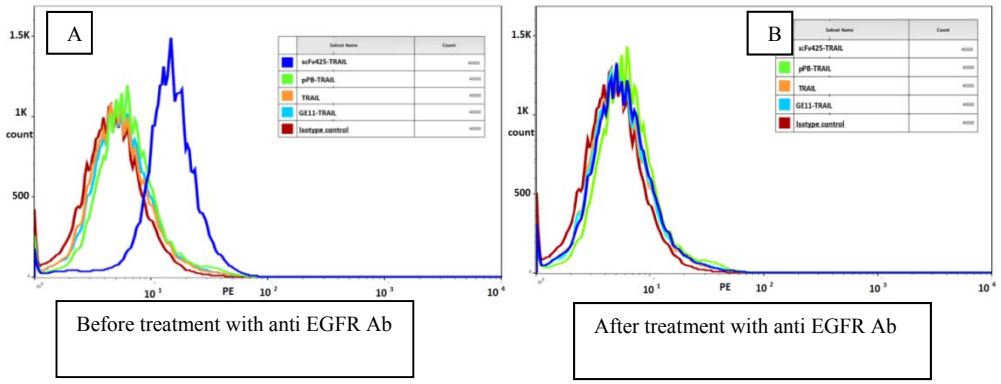
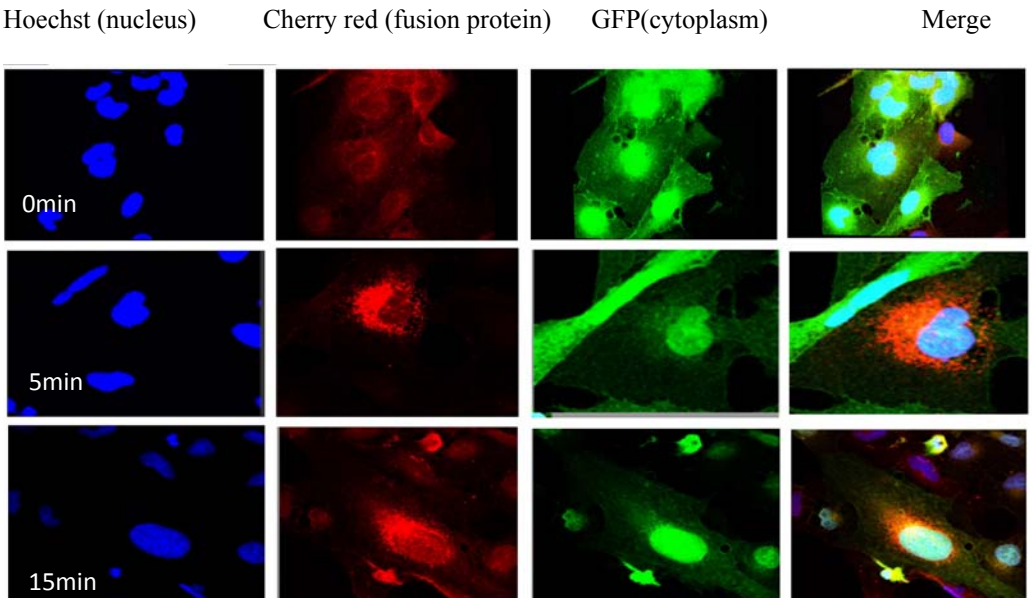
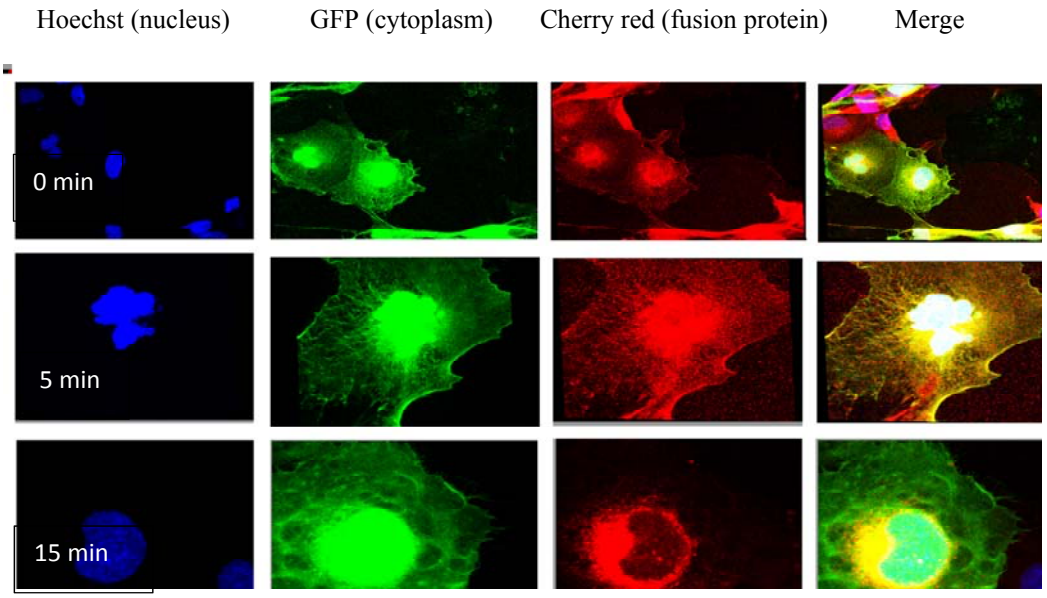


Figure 4: FACS analysis of the binding capacity of TRAIL fusion proteins to activated HSCs (A), Reduction of scFv425-TRAIL binding to LX 2 cells after pretreatment of cells with anti-EGFR (B).

Internalization of TRAIL fusion protein(scFv425-TRAIL) into LX2 cells



Internalization of TRAIL fusion proteins (PDGF-TRAIL) into LX2 cells



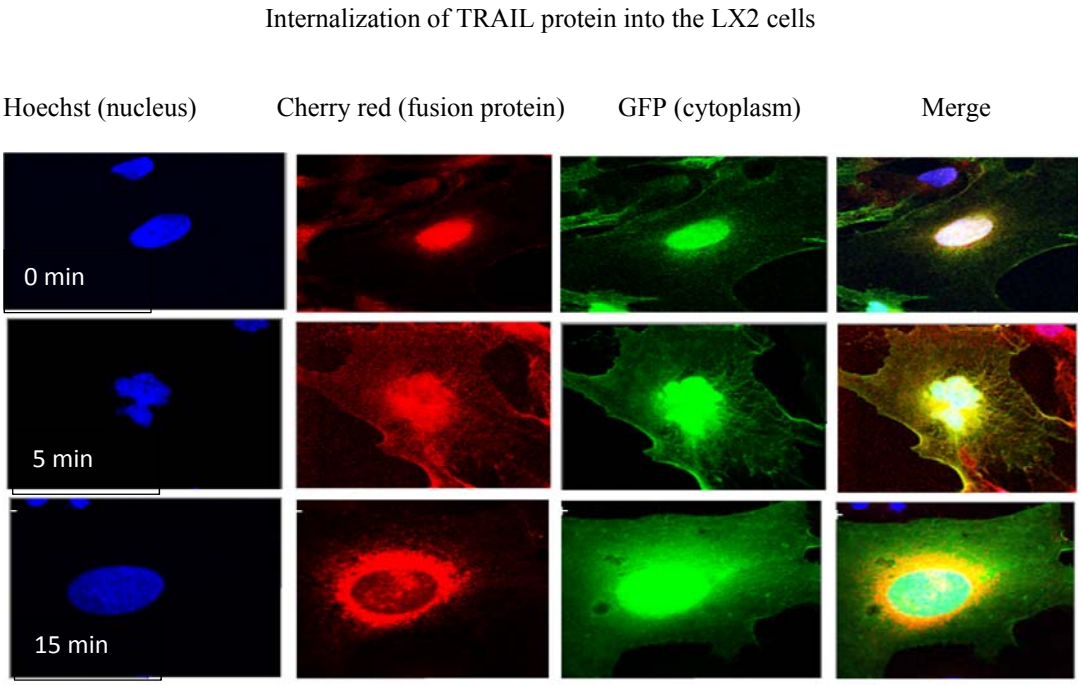


Figure 4: Internalization pattern analysis shows that the internalization of scfv425-TRAIL is faster than that of TRAIL and PDGF-TRAIL. scFv425-TRAIL completely internalized and accumulated around the nucleus after 5 min; whereas TRAIL and PDGF-TRAIL internalized and accumulated around nucleus after 15 min. LX2 cells were transduced with GFP producing Adeasy vectors for 24 hours and incubated with different fusion proteins for different times, fixed with paraformaldehyde and stained for the localization of fusion protein.