Addressing liver fibrosis by TRAIL targetied to hepatic stellate cells

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Chapter 2

Targeted elimination of activated hepatic stellate cells by an anti-epidermal growth factor-receptor single chain fragment variable antibody-tumor necrosis factor-related apoptosis-inducing ligand (scFv425-sTRAIL)

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Abstract

Background Progressive liver fibrosis is the result of chronic liver injury and is characterized by the excessive accumulation of extracellular matrix that may result in liver failure. Activated hepatic stellate cells are known to play a central role in this process and their elimination is a crucial step towards the resolution and reversion of liver fibrosis. In the present study, we investigated the potential application of an anti-epidermal growth factor receptor single chain fragment variable antibody-tumor necrosis factor-related apoptosis inducing ligand (scFv425-sTRAIL) fusion protein in the targeted elimination of activated hepatic stellate cells.

Methods Activated hepatic stellate cells (LX2 cells) were treated by adenovirus-derived scFv425-sTRAIL to evaluate its effect on the viability and extracellular matrix production.

Results *In vitro* treatment of activated hepatic stellate cells with scFv425-sTRAIL induced a significant reduction in viability (up to 100% reduction) and extracellular matrix production (60% reduction), yet no significant effect was observed on hepatic parenchymal cells. Blockage of the epidermal growth factor receptor (EGFR) by a monoclonal antibody significantly reduced the effectiveness of scFv425-sTRAIL in activated hepatic stellate cells, whereas a reduced effectivity was also observed after inhibition of the caspase pathway.

Conclusions Evidence is presented for the successful application of the scFv425-sTRAIL fusion protein in the targeted elimination of activated hepatic stellate cells via EGFR and simultaneous activation of the caspase pathway. scFv425-sTRAIL may thus represent a new therapeutic compound against liver fibrosis.

Keywords EGFR; liver fibrosis; targeted therapy; TRAIL

Introduction

Following chronic injury, the liver may develop into a pathologic state referred to as fibrosis. The key factor in this process is the hepatic stellate cell (HSC). Although the HSC is dedicated as a retinoid storage cell in its original quiescent form, upon activation, it starts the secretion of extracellular matrix (ECM), mainly collagens I and III, which may accumulate over time and affect normal liver functions [1]. Despite the advances that have been made with the aim of understanding the molecular mechanisms and the pathophysiology underlying liver fibrosis, an effective therapeutic approach still remains elusive. The central role of HSCs during liver fibrogenesis makes this cell type an ideal target to stop and even reverse liver fibrosis[2,3]. To achieve this, several conventional drugs such as transforming growth factor (TGF-β) inhibitors, as well as more targeted unconventional methods, such as gene therapy [2,4][5], have been introduced and brought some progress to the field. Induction of apoptosis in HSC as the nexus of liver fibrosis gave also this approach major attention [3,6].

Although some drugs such as IDN-6556A, gliotoxin and sulfasalazine induced apoptosis and elimination of HSCs *in vitro*, the lack of overall efficiency and the cytotoxicity associated with these treatments form a barrier for its use in the clinic

[7,8]. Activation of HSCs is associated with the overexpression of death-inducing receptors such as tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptors, including DR4 and DR5, and hence render HSCs susceptible to the apoptotic effects of TRAIL agonists [6,9]. The application of TRAIL agonists has thus been described as a potential strategy to eliminate activated HSCs [9]. However, survival factors such as epidermal growth factor (EGF) may interfere in this process by blocking the downstream pathway of apoptosis and supporting the growth and proliferation of active HSC. Interestingly, it was shown that the presence of TRAIL ligands in itself can cause the shedding of growth actors that adds to the protection and survival of active HSC [10]. On the other hand, the ubiquitous expression of TRAIL receptors and the complex role of apoptosis in inducing inflammation make it even more difficult to explore TRAIL ligands for the treatment of liver fibrosis .[3,6] A problem that is usually associated with inefficient TRAIL treatment is over responsiveness of activated HSCs to growth factors including EGF [11–13] that could amplify activated HSC proliferation and protect against TRAIL apoptosis by interfering with downstream caspase cascade signaling. In the present study, we demonstrate an increase of EGF receptor (EGFR) expression associated with progressive activation of HSCs. EGF ligands are found in free and membrane bound forms. During the activation process, membrane metalloproteinases could release membrane bound EGFs into the environment through a process called shedding. Interestingly TRAIL molecules itself can also up-regulate the EGF shedding and contribute to TRAIL resistance. We previously showed that a dual function targeted fusion protein could suppress the function of

the EGFR at the same time as inducing ligand-assisted apoptosis[14,15]. This fusion protein is well characterized and size exclusion chromatography data showed that eukaryotic expressed single chain fragment variable (scFv)-TRAIL fusion proteins are present as active thermostable homotrimers, with no detectable inactive monomers or dimers present [16]and could successfully eradicate tumor cells carrying both EGF and TRAIL receptors [14,15]. Employment of an adenovector as a carrier for transferring therapeutic genes has already been explored for a numbers of gene therapy applications. An interesting feature of this vector is its inherent property for homing to hepatic cells through its specific so called coxsackie adeno receptor. Our approach for employing adenovirally produced 425-TRAIL is particularly beneficial because TRAIL has very short half-life (30 min) and needs high dosages and frequencies of administration to be able to show clinical effect [16,17].Therefore, we consider that targeted, stable and in situ production of scFv425-TRAIL should result in the *in vitro* elimination of activated HSCs and a reduction of collagen production. This property could lead to more efficient targeting and further elimination of active HSCs. Therefore, we assessed the effect of an adenovirally expressed soluble TRAIL ligand-anti-EGFR antibody (scFv425) fusion protein for its potential therapeutic application in the targeted resolution of liver fibrosis. A schematic representation of the proposed active mechanism of scFv425-sTRAIL is shown in Figure 1. The results obtained in the present study show that the scFv425-sTRAIL bi-functional fusion protein efficiently eliminates *in vitro* activated HSC and reduces collagen production and α-smooth muscle actin (α-SMA) expression in treated cells.

Materials and methods

Cell line and culture

LX-2 Immortalized human hepatic stellate cell line were kindly provided by Prof. Scott Friedman (Mount Sinai Hospital, New York, NY, USA) and were cultured in Dulbecco's minimum essential medium (DMEM; Glutamax, Gibco, Gaithersburg, MD, USA) containing 10% fetal bovine serum, 100 U/ml penicillin, 100 g/ml streptomycin, 50 g/ml gentamicin and 100 nmol/l insulin. HepG-2 human hepatoma cells (ATCC#HB-8065), Huh-7 human hepatoma cells and HEK-293 human embryonic kidney cells (ATCC# CRL-1573) were cultured in DMEM containing 10% fetal bovine serum.

Antibodies and inhibitors

The following antibodies (Ab) were used: anti-TRAIL monoclonal Ab anti-Human CD253 (TRAIL) (eBioscience Affymetrics Co., Carlsbad, CA, USA), mouse anti-EGFR immunoglobulin (Ig)G2a, Mouse anti-α-SMA IgG, Goat anti-collagen-I IgG Ab (Sigma, St Louis, MO, USA), anti-hemagglutinin (HA) mouse IgG1 (InvivoGen, San Diego, CA, USA) anti-ß-actin mouse IgG (Sigma). For inhibition of the caspase pathway the pan-caspase inhibitor FAMVAD-fmk carboxyfluoresceinVAD-fmk (Bachem, Bubendorf, Switzerland) was used.

Production of adenovirally expressed scFv425-sTRAIL and TRAIL

scFv425-sTRAIL was produced using transduction of 293 T cells with a multiplicity of infection (MOI) of 5 from the Ad easy1-scFv425-sTRAIL recombinant vector and harvested 72 h later as described previously [15]. The supernatants containing the fusion protein were screened using western blotting with an anti-HA Ab to check

for expression of the 53-kDa fused protein. For evaluation of efficient trimerization of the adenovirally produced scFv425-TRAIL a western blot was performed under non-denaturing conditions (Figure 2). Ad-sTRAIL was generated by introducing the gene encoding sTRAIL into E1- and E3-deleted replication-incompetent recombinant Ad-5 adenovirus using the Ad-Easy system (Agilent Technologies Inc., Santa Clara, CA, USA). In short, polymerase chain reaction (PCR) amplification of sTRAIL gene from scFv425-sTRAIL included adenovirus [14]using forward primer (AGGCCCAGCCGGCCACCTCTGAGGAAACCAT) and reverse primer for SV40 poly adenylation signal (GAAATTTGTGATGCTATTGC) and DNA was inserted using SfiI and EcoRV unique sites of pAdTRACK-CMVscFv425: sTRAIL [15]The resulting vector was co-transformed with the adenoviral genome vector pAdEasy1 in Escherichia coli BJ5183[18]. After homologous recombination, pAd-sTRAIL was obtained. Subsequently, pAd-sTRAIL was transfected into HEK-293 cells. The cell lysate and supernatant containing the protein were screened
using western blotting with an anti-HA Ab to check for the expression of the 26-kDa protein. The control virus AdTL is an E1- and E3-deleted recombinant serotype 5 adenovirus that contains a green fluorescent protein (GFP) and luciferase gene expression cassette, each under the control of a cytomegalovirus promoter, has been described previously [19].

Evaluation of scFv425-sTRAIL effect on activated LX2 cell viability and proliferation

To activate the LX2 cells, cells (1000 per well) were seeded and incubated in 96-well, flat-bottomed uncoated plastic plates in DMEM, Glutamax (Invitrogen)media with 1% fetal bovine serum (FBS) for up to 7 days as described previously [9]. Cells

were exposed to various concentrations of scFv425-sTRAIL or sTRAIL diluted in DMEM medium for 48 h. Cell only controls received DMEM 1% FBS and virus controls received different concentrations of control adenovector (AdTL). The effect of different constructs and agents on cell viability was assessed using MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2- (4-sulfophenyl)-2H-tetrazolium), in the presence of phenazine methosulfate) in accordance with the manufacturer's instructions (Sigma) in triplicate. For evaluating the effect of caspase inhibition on preventing the apoptosis induced by scFv425-sTRAIL, commercially supplied lyophilized FAM-VAD-fmk pan caspase inhibitor was reconstituted to a final concentration 2 μg/ml in DMEM. The media of activated LX2 cells were replaced by FAM-VAD-fmk DMEM 24 h prior to adding 1.6 nM of adenovirally expressed scFv425-sTRAIL fusion protein. Induced apoptosis was evaluated by measuring the viability after 48 h by the MTS assay. For evaluation of the role of EGFR in scFv425-sTRAIL apoptosis, 1 μg/ml of anti-EGFR IgG2a Ab was added 24 h prior to adding scFv425-sTRAIL fusion protein to block the EGFR on the cells.

Western blotting

For SDS-PAGE and western blotting, LX2 cells were cultured in T75 flasks at 50% confluency. TGF-ß (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added to medium at a concentration of 2 ng/ml and, after 48 h, scFv425-sTRAIL was added to a final concentration a 0.3 nM or 1.6 nM. For control cells, only DMEM was added to activated LX2 cells. Forty-eight hours later, the supernatants were removed and cells were washed with phosphate-buffered saline (PBS). Some 500 μl of Laemli buffer was added and the samples were separated on 12.5% polyacrylamide

gels. For natural view of protein trimerization proteins are prepared in a non-reducing non-denaturing sample buffer, without adding 2- mercaptoethanol and boiling. Gels were stained with Coomassie Brilliant Blue R250 for protein visualization. For western blot analysis with Ab against collagen I, α-SMA and β-actin, gels were blotted on activated polyvinylidene fluoride membranes with electrophoretic transfer overnight at 4 °C in blotting buffer. The membrane was then blocked for 1.5 h with 3% bovine serum albumin (BSA)–0.05% Tween in PBS. It was then incubated for 2 h with an Ab against human collagen I diluted 1 : 1000, α-SMA diluted 1 : 1000 or β-actin diluted 1 : 5000 separately in PBS with 1% BSA and 0.05% Tween. After washing three times for 5 min with PBS–0.05% tween, the membrane was incubated for 1 h with the second Ab [rabbit anti-mouse IgG, horseradish peroxidase (HRP) conjugated (Dako, Glostrup, Denmark) for β-actin and α-SMA and mouse anti-goat IgG-HRP (Dako) in the case of collagen I], all diluted 1 : 1000 in the buffer described above. The membrane was then washed four times for 15min in the washing solution used above. The blot was developed using an AEC staining solution system (Sigma) in accordance with the manufacturer's instructions.

Immunohistochemistry

For immunohistochemistry of cultured activated LX2 cells, first, LX 2 cells were cultured on uncoated plastic 96-well plates with 1% FBS in DMEM for 7 days, and were then treated with different amounts of scFv425-s TRAIL. After 48 h, the medium was removed and the cells were washed three times with PBS, fixed with absolute methanol (Sigma) at –20 °C for 20min, air dried for 30 min and re-hydrated

with PBS for 5min. Next, the cells were incubated with anti-collagen I diluted 1:75 or anti-α-SMA diluted 1:600 in PBS. Finally the cells were washed with PBS three times and incubated with the second Ab [rabbit anti-mouse IgG-HRP (Dako) for α-SMA and mouse-anti Goat IgG-HRP (Dako) for collagen I, all diluted 1:100 in PBS. Finally, cells were washed with PBS three times and stained using the acetylcholinesterase (Sigma) staining solution system in accordance with the manufacturer's instructions.

Fluorescence-activated cell sorting (FACS) analysis of DR4, DR5, DecoyR1 and DecoyR2 expression on quiescent and activated HSCs

For flow cytometer analysis, HSCs were detached using 0.05% Trypsin-EDTA in PBS, washed with ice-cold PBS, and diluted to a concentration of 2×106 cells/ml using cold PBS. Aliquots of 100 µl (2×105 cells) were centrifuged at 18 g. for 5min at 4 °C, the supernatant was discarded, and the cells were suspended in either 100 µl of rabbit anti-DR4 (10µg/ml), rabbit anti-DR5 (10 µg/ml) rabbit anti-DecoyR1 (10 µg/ml) or rabbit anti-DecoyR2 (10 µg/ml) antibodies for TRAIL receptors and anti-human EGFR (5 µg/ml). After incubation for 45min at 4 °C, the cells were washed twice with PBS and were incubated for an additional 45 min with 1:100 diluted anti-rabbit PE-conjugated secondary antibody under cold and dark conditions. After two final washings, cells were suspended in 300 µl of PBS (1% BSA). Unlabeled cells and cells labeled with secondary antibody alone served as negative controls. The mean values of fluorescence intensity of 10 000 cells were determined by FACS analysis (Calibur 1; Becton-Dickinson Biosciences, Franklin Lakes, NJ, USA) and analyzed with FlowJo VX software (Tree Star Inc., Ashland, OR, USA).

Caspase 3/7 assay

Caspase 3/7 activities were assayed by using Apo-ONE™ Homogeneous Caspase-3/7 Assay kit (Promega. Madison, WI, USA). Briefly, 2×104 cells from 425-TRAIL, TRAIL treated quiescent and activated HSCs, 2×104 control cells and blank (no cells) were transferred into a 96-well plate and 100 µl of Homogeneous Caspase-3/7 Reagent was added. The plate was covered with a plate seal. After incubation for 48 h, fluorescence of each well was measured at an excitation wavelength of 485nm and an emission wavelength of 530 nm.

RNA isolation and real-time reverse transcriptase (RT)-PCR analysis

Total RNA from cultured LX2 cells was isolated using the SV total RNA isolation system (Promega Z3100) (all tests carried out in triplicate) in accordance with the manufacturer's instructions. The amount of Runaways measured by Nano Drop ND-1000 spectrophotometer (Nano Drop Technologies, Wilmington, DE, USA) and analyzed qualitatively by gel electrophoresis. Subsequently, synthesis of first-strand cDNA from total RNA was performed with the Reverse Transcription System (Promega A3500) in a volume of 20µl containing 250 ng of oligo dT (Promega). The cDNA obtained was diluted with Millipore water (Millipore Corporation, Billerica, MA, USA) to a concentration of 10 ng/µl and1µlwas applied for each PCR reaction. α-SMA primer was ordered as Assays by- Design (Applied Biosystems, Foster City, CA, USA) (4331348/assay name ACT-R-ACT2). GAPDH was used as a housekeeping gene (Rodent GAPDH Control Reagent; Applied Biosystems). The PCR reaction was carried out in TaqMan PCR Master Mix (Applied Biosystems) with a final concentration of 200 nM for GAPDH primers and 250 nM for primers of

the other genes under investigation. The amplification reaction was performed in an ABI PRISM 7900HT sequence detector (Applied Biosystems) with the cycling conditions: 2 min at 50 °C, 10min at 95 °C and 40 two-step cycles of 15 s at 95 °C and 60 s at 60 °C. For each sample, the real-time PCR reaction was performed in triplicate, and the averages of the obtained threshold cycle (Ct) values were processed for further calculations in accordance with the comparative Ct method described in the ABI manual (http://www.appliedbiosystems.com). In brief, gene expression levels were normalized to the expression of the housekeeping gene GAPDH, giving the ΔCt value. Then, the average value of ΔCt obtained from day 0 culture LX2 was subtracted from the average of the ΔCt value of each sample, yielding the ΔΔCt value. Finally, the gene expression level was calculated as $2^{-\Delta\Delta Ct}$, giving the final value that is normalized to the housekeeping gene and relative to the control sample values of the studied α-SMA gene.

Protein quantification

Quantitation of protein expression in western blots was carried out by scanning the blots using a GS-710 calibrated imaging densitometer (Bio-Rad, Hercules, CA, USA) and Image J, version 1. 46 (NIH, Bethesda, MD, USA) by putting a frame around the desired band and calculating the occupied area for each band in comparison with the standard band.

Results

Progressive activation of LX2 cells in cell culture

To characterize the activation stat of LX2 cells, we cultured cells for 9 days on plastic. This model of progressive activation in culture has already been established and well characterized for LX2 cells for *in vitro* study of liver fibrosis [9].LX2 cells were harvested and screened for signs of activation via mRNA expression of α-SMA, an indicative marker of activation, during different time points. An increase of five-fold in mRNA expression of α-SMA was associated with the progressive culture (Figure 3). This model was further used to evaluate the effect of different TRAIL constructs on activated HSCs.

scFv425-sTRAIL reduces viability of activated LX2 cells

To evaluate the effect of adenovirally produced scFv425- sTRAIL, different amounts of supernatant containing scFv425-sTRAIL or sTRAIL were added to activated LX2 stellate cells and the viability of cells was determined 48 h later by the MTS assay. Activated LX2 cells showed a reduction of viability of up to 90% after treatment with scFv 425-sTRAIL at a concentration of 0.6 nM compared to only 50% inhibition by sTRAIL alone (Figure 4A). The inhibition of viability in quiescent HSC was 60% after treatment with 1.6 nM scFv425-sTRAIL and 20% with 1.6 nM sTRAIL (Figure 4B). Thus, the EGFR-targeted TRAIL shows a relative selectivity towards activated stellate cells. To exclude the role of adenovirus transduction in decreased proliferation of LX2 cells both activated and quiescent LX2 cells were transduced by a MOI of 50 of a control GFP-expressing adenovirus. The results showed no effect on the viability of LX2 cells (data not shown). Finally, to examine the specificity of scFv425-sTRAIL, hepatic parenchymal cells (huh7 and Hep-G2) were treated with scFv425-sTRAIL. Both cell lines showed no detectable

difference in viability after application of scFv425-sTRAIL (Figure 4C), confirming the selectivity of the fusion protein towards EGFR expressing cells.

scFv425-sTRAIL employs both EGFR signaling and the apoptosis pathway

To evaluate the contributions of the EGFR and apoptosis pathways, we used selective inhibitors during the treatment of LX2 cells with scFv425-sTRAIL fusion protein. Activated LX2 cells were exposed to scFv425-sTRAIL and an EGFR specific Ab or a caspase inhibitor. At a concentration where scFv425-sTRAIL induced no survival at all, significant viability (30%±4%) was observed in the presence of the anti-EGFR Ab (Figure 5). Similarly, inhibiting the caspase cascade by addition of the global caspase inhibitor (FAM-VAD-fmk) was associated with a significant inhibition of the effect of scFv425-sTRAIL (20%±5% viability). These results suggest that the 425-sTRAIL fusion protein reduced viability in activated stellate cells by blocking both the EGFR and caspase-associated apoptotic pathways. An increase in susceptibility to TRAIL is associated with the expression of TRAIL-R1/DR4 and TRAIL-R2/DR5 receptors, yet the resistance to TRAIL is considered partially a result of the presence of decoy receptors TRAIL-R3/DcR1 and TRAILQ8R4/DcR2 therefore we assessed the expression of these receptors using FACS analysis (Figure 6). In accordance with the observed activation profile for α-SMA expression in activated HSCs, there was increased DR4 and DR5 receptor expression. TRAIL-R1/DR4 expression increased by 9.0% and TRAILR2 / DR5 expression increased by 5.6%. However, the rate of TRAIL-R2/DR5 expression in comparison with DR4 was 10-fold higher, which means that DR5 plays the major role in TRAIL-mediated apoptosis. The expression of both TRAIL-R3/DcR1 and

TRAILR4/DcR2 also increased in HSCs after activation, yet TRAILR4/DcR2 increased to a greater extent. EGFR expression increased by 5% on the surface of activated HSCs. We next evaluated the contribution of TRAIL receptor expression with respect to sensitivity towards our TRAIL constructs. Measurement of caspase activity in HSCs treated with scFv425-TRAIL showed an increase of 22% and 26% in caspase3/7 activity in quiescent and activated HSCs compared to TRAIL-treated cells. Caspase activity increased by 60% in activated HSCs treated with scFv425-TRAIL compared to quiescent HSCs treated with scFv425-TRAIL. This is almost two-fold higher than caspase elevation as a result of TRAIL treatment of activated HSCs (Figure 7).

scFv425-sTRAIL reduces collagen I and α-SMA expression

One of the characteristic of activated HSCs is the increased expression of fibrotic markers such as α-SMA and collagen I. To evaluate the potential effect of scFv 425-sTRAIL on α-SMA and collagen I expression, LX2 cells were cultured on a plastic surface for 5 days and different amounts of scFv425-sTRAIL were added to cell cultures. After 48 h, cells were stained with anti-collagen I and anti-α-SMA. We observed a reduction in the expression of α-SMA and collagen in activated LX2 cells in response to a sub-lethal dose of scFv425-sTRAIL (0.32 nM). Quantification of staining indicated a reduction in expression of collagen I and α-SMA of up to 46% and 60%, respectively (Figure 8). Thus, the addition of scFv425-sTRAIL to activated HSCs at a sub-lethal dose could reduce collagen production and HSC activation.

Discussion

Persistent liver injury initiates a cascade of events including secretion of a number of cytokines such as TGF-β, platelet-derived growth factor (PDGF) and endothelin-1. These cytokines activate HSCs and this leads to exponentiation of ECM production and accumulation, a condition referred to as liver fibrosis. HSCs have a central role in this process, which makes them an ideal target in the treatment of liver fibrosis. Although liver fibrosis is a serious situation that may eventually develop into a life-threatening condition, a cure still remains elusive [2,4]. It has been shown that activated HSCs significantly amplify the hepatic response to liver injury, and depleting fibrotic livers from activated HSCs may ameliorate the fibrotic condition [8]. TNF, CD95L (Fas) and TRAIL are among the most studied factors in TNF family members that could induce death and apoptosis in cells. The ligands are employed by a number of immune cells specially natural killer and cytotoxic T lymphocyte cells to induce controlled apoptosis in tumor or infected cells [20]. However, depending on the modulation and the signaling pathway that they initiate, their corresponding target cells can experience different and even contradictory consequences [21]. HSC cells have the receptors for all three types of ligands. CD95L may induce cell death in activated HSCs through JNK-assisted tyrosine phosphorylation of CD95, whereas it could block the apoptotic pathway via CD95 tyrosine nitration and even have a thriving effect on quiescent HSC via EGFR phosphorylation [22]. TNF is secreted from mononuclear cells and damaged hepatic cells and exerts effect via TNF-receptor-1 or 2 (TNFR1 or 2). Upon binding to its dedicated receptor, TNFR2 could induce cell death in active HSC via the Fas-

associated protein death domain, yet, as a result of interaction with TNFR1, it can cause proliferation and activation in HSCs, and hence enhance liver fibrogenesis [23]. TRAIL, either soluble or membrane-attached, is primarily produced by activated natural killer cells and Kupffer cells to induce apoptosis in their target cells. Two types of TRAIL-binding receptors have been identified: TRAIL-R1 (also referred to as DR4) and TRAIL-R2 (also called DR5/killer/TRICK2) that are expressed on the surface of HSCs, However, DR5 is expressed to a higher extent on the surface of the activated HSC and is responsible for the induction of death through the extrinsic caspase pathway and caspase-8- dependent activation [24]. One of the appealing features

of TRAIL as a pro-apoptotic receptor ligand is that it does not appear to have the liver toxicity precluding the testing *in vivo* of related death-inducing ligands such as CD95 ligand and TNF-α, which both cause massive hemorrhagic necrosis of various tissues including the liver [25]. Previously, TRAIL-induced apoptosis was successfully used to eliminate active HSC [9]; however, simultaneous overexpression of EGF-like ligands in fibrotic and cirrhotic livers [11,13] and its downstream signaling and protection of activated HSCs represents a hurdle to achieving effective TRAIL-based treatment [11,22,26]. In the present study, we therefore developed a selective treatment for the elimination of activated HSCs via EGFR targeted TRAIL to enhance the selectivity TRAIL and thus improve the remedy. TRAIL receptors overexpress in activated HSC and are therefore an ideal targets for TRAIL agonists. We showed a reduction in viability of 20% and 50% in quiescent and activated stellate cells, respectively, via exposure to adenovirally

expressed sTRAIL at a concentration 0.6 nM. This finding is in concordance with previous studies that showed sensitivity of HSCs to TRAIL [9]. ECM-producing cells such as HSC are highly responsive to a series of growth factors such as EGF, amphiregulin, β cellulin and PDGFand their dedicated receptors which may trigger survival and/or proliferation signaling through the EGFR and/or PDGFR on their surface [11–13]. Indeed, growth factors such as amphiregulin have been proven to have a pivotal role in the development of liver fibrosis via different mechanisms; involving extracellular Kinas½(ERK1/2), transcription factor c-fos and TRAIL [12]. Meanwhile, the contribution of TRAIL ligands in accelerating ectodomain shedding of EGF and heterodimer activation of PDGF could add to the complex protection layer and survival of activated HSCs [10] . An enhanced apoptosis may be achieved by combining the TRAIL treatment with compounds that block the protective mechanisms, giving reason for us to couple trail with anti-EGFR antibodies. This has the additional benefits of directing soluble TRAIL to EGFR-expressing cells, that is, activated HSC. Indeed our previous studies have already confirmed the potent anti-tumor [23,25]effect of such a compound in mouse models with EGFR-expressing tumors [14,15]. In the present study, we show that an adenovirus derived fusion protein scFv425-sTRAIL can induce a 100% viability loss in activated versus 50% in quiescent HSCs. This finding is in accordance with the elevation in caspase 3/7 signaling in affected HSCs. Blocking the EGFR on HSCs through a mouse monoclonal Ab to eliminate the EGFR signaling pathway reduced the efficiency of scFv425-sTRAIL and increased viability by up to 30%. This finding highlights the importance of a simultaneous approach for coupling death signaling and survival

inhibition for eliminating activated HSCs. On the other hand, inhibition of the caspase cascade via a global inhibitor also attenuated the effect of scFv425-sTRAIL indicating that both caspase-based and EGFR-based mechanism are involved in the apoptosis induction by the fusion protein. An interesting observation in the present study is the role of scFv425-sTRAIL in reducing collagen I expression in activated HSC. Collagen I is a major component of ECM. Inhibition of EGF receptor signaling [27]may result in the attenuation of fibrosis reduction via inhibition of collagen deposition and HSC activation) (Figure 4) [17], next to the induction of apoptosis. A direct relation has already been established between a reduction fibrogenesis and the number of α-SMA positive cells [4]. A reduction in the amount of α-SMA has been demonstrated in the presence of some inhibitors of EGFR signaling, such as Gefitinib and Erlotinib. Treatment of pulmonary artery smooth muscle cells with these drugs reduced α-SMA expression by 10% [28]. Because the reduction in fibrilar collagens in the present study was accompanied by a reduction in the number of α-SMA positive cells, it may be speculated that decreased expression of ECM proteins is caused by a reduction in HSC activation. The later could be attributed to inhibitory effect of trimeric form of anti-EGFR in the fusion protein against EGFR in activated HSC [27]. Because fibrosis is considered as rather a long-term and persistent problem, a durable and efficient approach is needed if an objective and non-causative cure is to be pursued. Our current strategy involves treating activated HSCs with an adenovirally produced scFv425-sTRAIL protein. The next step is the in vivo production of this fusion protein by adenovirus-transfected hepatocyte cells. The natural homing property of adenovirus towards the

liver and the HSC-specific nature of the expressed molecule add to the specificity of our designed strategy. The present study is the first to show a significant anti-fibrotic effect of this compound in HSC, which warrants further studies in vivo.

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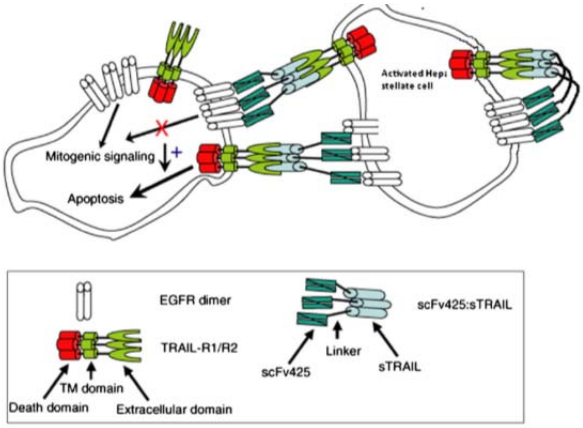

Figure 1. Schematic representation of the fusion protein and its cellular interactions. Binding of the anti-EGFR antibody to activated HSCs in the fusion protein (scFv425-sTRAIL) causes inhibition of cell proliferation and provides a scaffold that facilitates the binding of TRAIL to its receptor on HSC in Cis and Trans form.

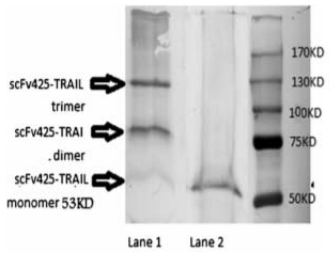

Figure 2. scFv 425-TRAIL trimerization western blot analysis of adenovirally expressed scFv425-TRAIL under nondenaturing (lane 1) and reducing (lane 2) conditions showed efficient trimerization of the scFv425-TRAIL fusion construct under non-reducing conditions.

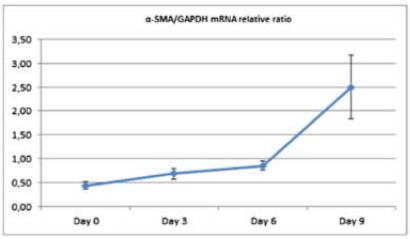

Figure 3. ALpha-SMA mRNA expression increases following replating on a plastic surface. Human LX2 cells were grown to confluence and then lifted and replaced on the plastic surface of six-well plates for 9 days. At day 9, cells were harvested. α-SMA mRNA was extracted and quantified by real-time RT-PCR as described in the Materials and methods. An increase in α-SMA mRNA is associated with the length of time in culture.

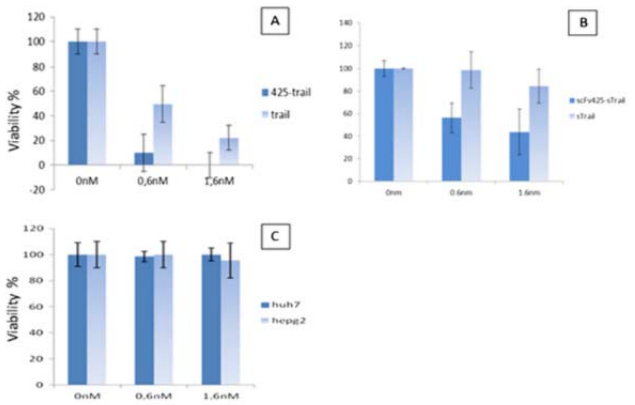

Figure 4. scFv425-sTRAIL efficiently kills activated HSCs. (A) MTS viability assay for activated LX2 cells. (B) MTS viability assay for quiescent LX2 cells. Dark bar, scFv425-sTRAIL; light bar, sTRAIL. (C) parenchymal hepatic cells (Hep-G2 and Huh7). Dark bar, huh7; light bar, HEPG2. The effects of scFv425-sTRAIL were most prominent in activated LX2 cells and absent in parenchymal hepatic cells.

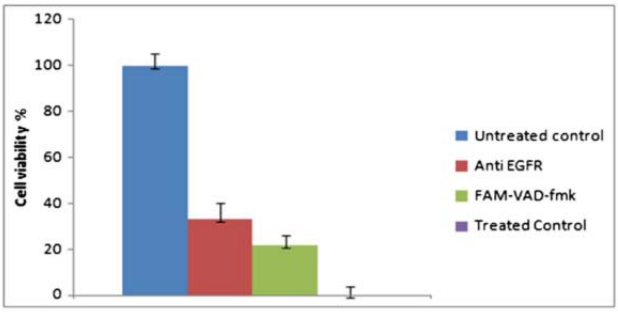

Figure 5. Simultaneous interaction with EGFR and caspase cascade signaling pathways is crucial for efficient killing of active HSC by scFv425-sTRAIL. Activated LX2 cells incubated with Ad scFv425-sTRAIL (violet) or left untreated (blue). scFv425-sTRAIL-treated cells were subsequently treated with 50 μM FAM-VAD-fmk (green) or with 1 μg of anti-EGFR Ab (blue) to block the effects of the caspase cascade and EGFR, respectively. It can be seen that both inhibitors affected the activity of scFv425-sTRAIL.

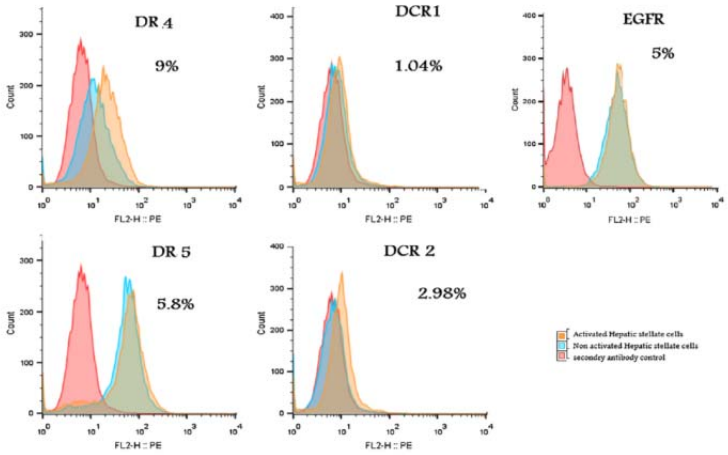

Figure 6. FACS analysis profiling of different TRAIL and EGF receptors and their expression on the surface of quiescent and activated forms of human HSCs. An increase in the expression of surface receptors is associated with the activation of HSCs during culture-induced activation. DR5 is expressed almost ten-fold more than DR4 on the surface of human HSCs. Expression of decoy receptors (DCRs) increases with activation of HSCs; this increase is higher for DCR2.

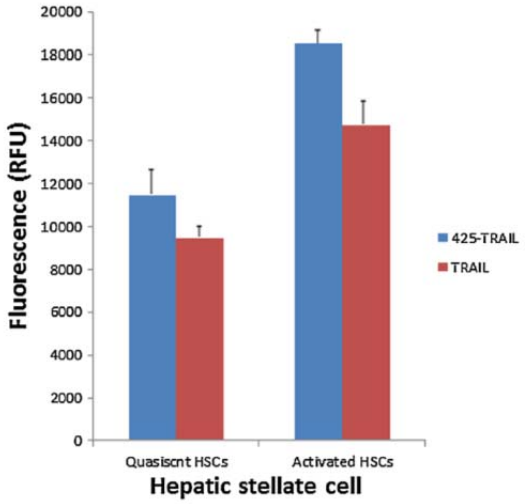

Figure 7. Comparison of caspase 3/7 enzyme activity in quiescent and activated HSCs treated with adenovirally produced 425-sTRAIL or sTRAIL. An increase in the amount of caspase-3 activity in LX2 cells treated with 425-sTRAIL or sTRAIL is concurrent with the activation state of cells. 425-sTRAIL could induce more caspase activation in quiescent and activated hepatic stellate cells compared to sTRAIL.

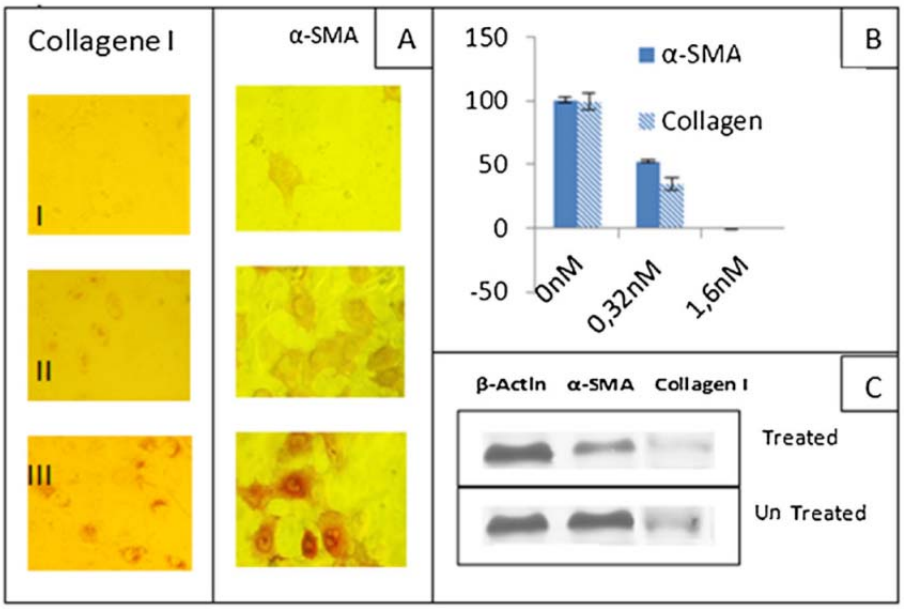

Figure 8. scFv425-sTRAIL reduces the expression of extracellular matrix proteins in activated HSCs. (A) LX2 cells were treated with (I) 1.6 nM, (II) 0.3 nM and (III) 0 nM of scFv425-sTRAIL and stained for collagen (left) or α-SMA (right). (B) Bar graph of collagen (light bar) and α-SMA (dark bar) expression as determined by western blotting before and after treatment with 0.32 nM scFv425- sTRAIL. (C).Representative western blot staining for β-actin, α-SMA and collagen I of cultures of activated LX2 cells with (upper) and without 0.32 nM scFv425-sTRAIL (lower).