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Receptor-specific TRAIL as a means to achieve targeted elimination of activated hepatic stellate cells

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ABSTRACT

Activated hepatic stellate cells (HSCs) are known to play a central role in liver fibrosis and their elimination is a crucial step toward the resolution and reversion of liver fibrosis. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a molecule that may contribute to the apoptotic removal of activated HSC through binding to its dedicated receptors. In the present study, we investigated the potential application of recombinant receptor-specific TRAIL proteins in the efficient elimination of activated HSCs. Our finding revealed differential contribution of TRAIL receptors among HSCs populations with activated hepatic stellate cells expresses more TRAIL receptors DR5. In vitro treatment of activated HSCs with DRS-specific or wild-type TRAIL variants induced a significant reduction in viability and extracellular matrix production, whereas no significant decrease in viability was associated with the treatment of cells by DR4-specific TRAIL. Our analysis indicate the successful application of the DRS receptor-specific TRAIL variant in the targeted elimination of activated HSCs via interference with collagen production and simultaneous induction of apoptosis via activation of the caspase pathway. DRS receptor-specific TRAIL may thus represent a new therapeutic compound for the treatment of liver fibrosis.

Introduction

Following chronic injury, the liver develops to a pathologic state regarded as fibrosis. The key factor in the liver fibrosis process is a cell type called the hepatic stellate cell (HSC). Quiescent HSCs are dedicated to retinoid storage, yet through an activation process due to injury, they proliferate and transform to a myofibroblastic phenotype. In this form, activated HSCs start secretion of extracellular matrix proteins mainly collagen I and III that accumulate over time and impair the functional structure of the liver [1]. During activation, HSC become more susceptible to apoptotic cell death by apoptotic factors, including tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL). TNFα, FasL and TRAIL are among the most studied factors in the TNF family members that employed by a number of immune cells, specially natural killer cells (NK) and cytotoxic lymphocyte (CTL), to induce controlled apoptosis in tumor cells or infected cells [2]. Whereas TRAIL, CD95L and TNFα may have a proliferative effect on HSCs in certain conditions [3–5] and could cause massive hemorrhagic necrosis of various tissues, including the liver [6]. Thus, TRAIL is the most promising apoptotic ligand to eliminate activated HSCs. There are two death receptors DR4 (TRAIL-R1) and DR5 (TRAIL-R2) that are responsible for the induction of apoptosis upon binding to TRAIL, yet TRAIL also can bind to the anti-apoptotic decoy receptors DcR1 (TRAIL-R3), DcR2 (TRAIL-R4) and osteoprotegerin (OPG). The application of TRAIL agonists has been suggested as a potential strategy to eliminate activated HSCs [7,8]. On the other hand, the ubiquitous expression of TRAIL receptors and the complex role of apoptosis in inducing inflammation make it difficult to explore TRAIL ligands for the treatment of liver fibrosis [9,10]. TRAIL receptor-specific agonistic molecules have been introduced both in the form of TRAIL variants or monoclonal antibodies against specific TRAIL receptors [11,12]. The dynamics of wild-type (wt) and mutant TRAIL interactions with TRAIL death receptors have been well characterized [13]. Receptor-specific agonists may reduce the decay receptor-mediated antagonism [14]; hence, by using receptor-specific TRAIL variants, the therapeutic dose is expected to be lower [13,15]. The application of receptor-specific agonists for DR5 drastically reduced hepatotoxicity [16] that is associated with wild-type human TRAIL [11,12,16,17]. DR5-specific TRAIL lower toxicity could be attributed to negligible amount of DR5 on the surface of healthy hepatic cells in comparison to DR4 [16,17]. In this study, we investigate the potential application of two receptor-specific TRAIL variants for targeted elimination of activated HSCs.

Methods

Cell lines and culture

The LX-2 immortalized human HSC line was kindly provided by Prof. Scott Friedman (Mount Sinai Hospital, New York) and was cultured in Dulbecco’s minimum essential medium (DMEM) containing 10% fetal bovine serum, (100 U/mL) penicillin, 100 g/mL streptomycin, (50 g/mL) gentamicin and (100 nmol/L) insulin. The HepG2 human hepatoma cell line (ATCC#HB-8065) and HEK-293
human embryonic kidney cell line (ATCC#CRL-1573) were cultured in DMEM containing 10% fetal bovine serum. Cells were cultured in a humidified atmosphere containing 5% CO₂ at 37 °C.

**Primary hepatic cells and animals**

Specified pathogen-free male Wistar rats were purchased from Harlan (Zeist, the Netherlands). They were housed under standard laboratory conditions and had free access to standard laboratory chow and water. Each experiment was performed following the guidelines of the local committee for care and use of laboratory animals. For isolation and culture of rat hepatocytes and HSCs, primary rat hepatocytes [18] and HSCs [19] were isolated and cultured as described previously. Hepatocyte viability and purity were always more than 90% as judged by Trypan blue exclusion. Cells were cultured in a humidified atmosphere containing 5% CO₂ at 37 °C.

**Antibodies and TRAIL variants**

The following antibodies (Ab) were used: anti-TRAIL monoclonal Ab anti-Human CD253 (TRAIL) (eBioscience Affymetrics Co); mouse anti-human EGFR IgG Ab; mouse anti-human α-smooth muscle actin (α-SMA) IgG Ab; goat anti-human collagen I IgG Ab; anti-β actin mouse IgG (Sigma-Aldrich, St. Louis, MO). Receptor-specific TRAIL proteins DHER and 4C7 were produced and purified from prokaryotic expression systems as previously described [13,15]. For the apoptosis assay, a Annexin V-FITC (IQ products, IQP-193F) and mouse IgG2b-FITC (IQ products, IQP-193F) was used. DR4-specific, DR5-specific and wild-type TRAIL antibodies were used as previously described [13].

**In vitro evaluation of receptor-specific TRAILs for inducing apoptosis in HSCs**

To activate the LX-2 cells or primary rat HSCs, cells (1000 cells/well) were seeded and incubated in 96-well, flat-bottomed uncoated plastic plates in DMEM, Glutamax (Invitrogen) media containing 10% FBS for up to 7 days as previously described [7]. HSCs were exposed for 48 h to various concentrations of WT TRAIL or receptor-specific TRAIL variants DHER or 4C7, diluted in DMEM medium. Primary hepatocytes were seeded at a concentration of (8000 cells/well) in 96-well, flat-bottomed uncoated plastic plates overnight before treatment with receptor-specific TRAILs for 48 h. The effect of different proteins and cell viability were assessed 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 manufacturer's protocol (Sigma) in triplicate. Cell only controls received DMEM 1% FBS.

**Immunohistochemistry and western blotting**

For SDS-PAGE and western blotting, LX-2 cells were cultured to 50% confluency. At the indicated time points, TRAIL proteins were added at concentrations ranging from 10 to (100 ng/ml) in DMEM. For control cells, DMEM was added. After 48 h, the supernatants were removed and the cells were washed with PBS. The cell pellets were separated on 12.5% SDS-polyacrylamide gels as previously described [8]. For western blot analysis with Abs against collagen I, α-SMA and β-actin, gels were blotted on blotting membrane as previously described [8], followed by incubation for 2 h at room temperature with either an Ab against human collagen I (1:1000), α-SMA (1:1000) or β-actin (1:5000) in PBS with 1% BSA and 0.05% Tween-20. After washing, the membrane was incubated for one hour with the second Ab (horseradish peroxidase (HRP) conjugated rabbit anti-mouse IgG; Dako, Denmark) for β-actin and α-SMA or mouse anti-goat IgG-HRP (Dako, Denmark) in the case of collagen I, all diluted 1:1000 in the buffer mentioned earlier. The membrane was then washed four times for 15 min in the washing solution as above. The blot was developed using an AEC staining solution system (Sigma) according to the manufacturer's instructions. For immunohistochemistry of cultured activated LX-2 cells, first LX-2 cells were cultured on uncoated plastic in 96-well plates with 1% FCS in DMEM for 7 days and incubated with different concentrations of TRAIL. After 24 h, the medium was removed and the cells were washed three times with PBS, fixed with absolute methanol (Sigma) at −20 °C for 20 min, air-dried for 30 min and re-hydrated with PBS for 5 min. Next, the cells were incubated with either anticollagen I (1:75), anti-α-SMA (1:600) in PBS or 1:10,000 Hoechst 33342. Finally, the cells were washed with PBS 3 times and incubated with the second Ab (rabbit anti-mouse IgG-HRP; Dako, Denmark) for α-SMA and (mouse anti-goat IgG-HRP; Dako, Denmark) for collagen I all diluted 1:100 in PBS. The integrated density of intracellular insoluble collagen or α-SMA was then calculated through analysis of five separate pictures from triplicate experiment for each treatment. All acquisition parameters were kept constant for samples and respective controls. Images were imported into Image J 1.48 software. For each wavelength, back ground fluorescence has been subtracted from the selected region of interest. The integrated cell density was then calculated and normalized for the total number of cells for all pictures.

**Profiling TRAIL receptor expression on HSCs**

Flow cytometer analysis of TRAIL receptors was performed as follows. 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 14,000 rpm for 5 min at 4 °C, the supernatant was discarded, and the cells were separately suspended in 100 μL of rabbit anti-DR4 (10 μg/mL), rabbit anti-DR5 (10 μg/mL), rabbit anti-DcR1 (10 μg/mL) or rabbit anti-DcR2 (10 μg/mL) antibodies for TRAIL DR5 and incubated for 45 min at 4 °C. The cells were then washed twice with PBS and were incubated for an additional 45 min with 1:100 dilution anti-rabbit PE-conjugated secondary antibody under cold and dark conditions. After two final washings, cells were suspended in 300 μL PBS (containing 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 flow cytometry analysis (Calibur 1).

For the analysis of TRAIL receptor distribution in activated HSC side populations (SP), LX-2 cells cultured on plastic plates for 7 days in DMEM containing 10% FBS were detached by trypsinization for 3 min. The number of viable cells was estimated by Trypan blue staining. Cells were collected by centrifugation and washed with 5 mL of PBS. To detect the SP, LX-2 cells (1×10⁵) were incubated with Hoechst 33342 (5 mg/mL) in DMEM/10% FBS for 90 min at 37 °C with vortexing every 15 min. Cells were then stained with specific TRAIL receptor antibodies as explained above. As previously described [20] to discriminate between SP and non-SP cells, cells were incubated with the Hoechst dye in the presence or absence of verapamil (50 mM). At the end of the incubation period, 1 mg/mL propidium iodide (Pi; Sigma) was added to
identify dead cells before analysis by flow cytometry for Blue 450 nm (blue Hoechst) and 675 (red Hoechst) laser using the BD LSR II Flow Cytometer [20]. Gated SP cells were analyzed for expression of different TRAIL receptors. All FACS data analyzed with FlowJo VX software.

**RNA isolation and real-time RT-PCR analysis**

Total RNA from cultured LX-2 cells was isolated using the SV total RNA isolation system (Promega Z3100) (all tests carried out in triplicate). The amount of RNA was measured by NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and analyzed qualitatively by gel electrophoresis. Subsequently, synthesis of first-strand cDNA from total RNA was performed with Revers Transcription system (Promega A3500) in a volume of (20 μL) containing (250 ng) of oligo dT (Promega, Madison, WI). The obtained cDNA was diluted with Millipore water (Millipore Corporation, Billerica, MA) to a concentration of (10 ng/μL), and (1 μL) was applied for each PCR. The α-SMA primer was ordered as Assays-by-Design (Applied Biosystems, Foster City, CA) (4331348/ assay name ACT-R-ACT2). GAPDH was used as a housekeeping gene (Applied Biosystems, Foster City, CA). DR5- and DR4-specific receptors primers were used as previously described [7]. The PCR was carried out in TaqMan PCR Master Mix (Applied Biosystems, Foster City, CA) with a final concentration of (200 mM) GAPDH primers and (250 mM) for the primers of the other genes studied. The amplification reaction was performed in an ABI PRISM 7900HT sequence detector (Applied Biosystems) with the following cycling conditions: 2 min at 50 °C, 10 min 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 was performed in triplicate, and the averages of the obtained threshold cycle (Ct) values were processed for further calculations according to 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. The average value of ΔCt obtained from day 0 culture LX-2 was subtracted from the average of the ΔCt value of each sample, yielding the ΔΔCt value. Finally, the gene expression level was calculated as $2^{-ΔΔCt}$, giving the final value that is normalized to the housekeeping gene and relative to the control sample values of the studied α-SMA gene.

**Total soluble collagen assay**

The Sircol assay (Biocolor, UK) was used for measuring soluble collagen in culture supernatants. One ml of Sircol dye reagent and (150 μL) of sample were mixed and incubated for 30 min at room temperature. Collagen–dye complexes were centrifuged for 10 min at 14,000 rpm, washed with 1 mL of ethanol and dissolved in 1 mL of (0.5 M) NaOH. The absorbance was measured at 540 nm. The assay was performed in triplicate, and the mean of data was calculated for each sample. For caspase and apoptosis assay, caspase 3/7 activities were assayed using the Apo-ONE™ Homogeneous Caspase-3/7 Assay kit (Promega). Briefly, 1 × 10⁴ HSCs treated with (100 ng/mL) of the different TRAIL variants or DMEM medium (control). 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 sealer. After incubation for 2 h, fluorescence of each well was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. The measurements are reported in relative fluorescence units (RFU).

For the evaluation of apoptosis, LX-2 cells were treated with TRAIL variants as described earlier and harvested at the indicated time points. Briefly, cells were washed with calcium buffer ((0.15 M) NaCl, (11.7 mM) HEPES and (23 mM) CaCl₂) buffer and resuspended in 1/10 dilution of antiannexin V in 60 μL of calcium buffer. Cells were incubated for 30 min at 4 °C, washed in calcium buffer and resuspended in calcium buffer supplemented with 0.5 μg/ml propidium iodate. Finally, fluorescence-activated cell sorting (FACS) was performed using the BD LSR II Flow Cytometer. Gated cells were analyzed for apoptotic signaling of annexin V. All FACS data were analyzed with FlowJo VX software.

**Statistical analysis**

All experiments were performed in triplicate and averages were used to calculate for significance between different treatments. The significance of the difference in TRAIL receptors expression was analyzed by the Student’s t-test on three separate experiments. p values <.05 were considered to be significant. Comparisons between different primary cells and or cell lines treated with TRAIL variants were calculated by using a nonparametric one-way analysis of variance (ANOVA) with Sidak’s multiple comparison tests with a single pooled variance (*p <.05) using GraphPad Prism version 5.0 for Windows (GraphPad Software, La Jolla, CA).

**Results**

**TRAIL receptors expression increases during progressive activation of LX-2 cells in cell culture**

LX-2 cells were activated by culturing the cells for 9 days on polyethylene plastic plates. This model of progressive activation in culture has been established for LX-2 cells for the in vitro study of liver fibrosis [7,8]. Activation of LX-2 cells confirmed by the phenotypic transition and increase in α-SMA production. LX-2 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 fivefold in mRNA and protein expression of α-SMA was associated with progressive activation (Figure 1(A)). This model was further used to evaluate the effect of different TRAIL variants on activated HSCs. We assessed the expression of the different TRAIL receptors using both real-time PCR and FACS analysis. Real-time analysis showed that there was an increased DR4 and DR5 receptor mRNA expression in accordance with the observed activation profile for α-SMA expression in activated HSCs. The increase in mRNA expression was 100-times more for the DR4 receptor compared to the DR5 receptor (Figure 1(B)). Flow cytometry analysis showed that there was a 100% and 10% shift in mean fluorescent intensity of DR4 and DR5, respectively. However, the level of DR5 expression in comparison to DR4 was 10-fold higher, which could indicate that DR5 may play a more important role in TRAIL-mediated apoptosis of HSCs (Figure 1(C)). The expression of both DcR1 and DcR2 seem to be absent on LX2 cells and only detectable at low levels in activated LX2 cells.

**DR5-specific TRAIL decreases extra cellular matrix (ECM) production by activated HSC**

One of the characteristic of activated HSCs is the increased expression of fibrotic markers such as αSMA and collagen I. It has been previously shown that TRAIL and TRAIL derivatives could reduce the expression of ECM produced by activated HSCs. This has been clearly demonstrated in previous studies. In short, TRAIL-induced heat-shock factor 1 (HSF1) phosphorylation and
inactivation, consequently leading to the suppression of heat-shock protein 47 (Hsp47)-dependent collagen production in activated human HSCs. Hsp47 is associated with chaperonin folding of pre-collagen structure into mature soluble collagen form that will be secreted out of cells. Thus, inhibiting the production of Hsp47 through HSF1 would result in sedimentation of insoluble collagen in the cells [8,21]. To evaluate the potential effect of receptor-specific TRAIL on collagen I expression, LX-2 cells were cultured on a plastic surface for 7 days and different amounts of receptor-specific or wt TRAIL were added to cell cultures. After 48 h, cells were analyzed for collagen production using the Sircol assay and western blotting. Both DR5 and wt TRAIL caused a substantial decrease (80%) in collagen production, even at very low dose (10 ng/mL), whereas DR4-specific TRAIL was only capable of a similar effect at a high dose (Figure 2(A)). It has been reported that the expression of collagen correlates closely with the expression of collagen-specific molecular chaperone Hsp47 in activated HSCs. Therefore, we next investigated the expression of HSP47 in LX-2 cells treated with increasing amounts of TRAIL variants. The results suggest that a decrease in collagen production was associated with down regulation of HSP47 in TRAIL-treated cells. Furthermore, DR5 receptor-specific TRAIL showed a greater capability in reducing HSP47 expression at lower concentration (Figure 2(B,C)). However, the reduction of collagen was significantly higher in DR5 receptor-specific TRAIL compared to the DR4 receptor-specific TRAIL. Finally, staining of TRAIL treated LX-2 cells with anticollagen I and anti-α-SMA revealed increase in intracellular insoluble collagen is associated with decrease in α-SMA. Moreover, the aggregation of insoluble collagen and reduction in the expression of α-SMA were more pronounced in cells treated with DR5-specific TRAIL, thus indicating with DR5 specific TRAIL seems to interfere more efficiently with collagen folding mechanism (Figure 3).

Different receptor-specific TRAILs have different potential to eliminate activated HSCs

To evaluate the potential cytotoxic effect of receptor-specific TRAIL, different amounts from each receptor-specific or wt TRAIL
protein were added to activated or quiescent LX-2 HSCs and the viability of cells was determined 72 h later by the MTS assay (Figure 4). Activation of LX-2 cells confirmed by the phenotypic transition and increase in α-SMA production showed a substantial decrease in viability by increasing doses of TRAIL. A comparison between the different TRAIL variants showed that DR5-specific TRAIL was more potent in reducing viability (60%), whereas treatment with DR4-specific TRAIL did not affect the viability of activated HSC, only in the presence of high concentrations of TRAIL. Treatment of quiescent LX-2 with different types of TRAIL, on the other hand, did not significantly affect the viability even at the highest concentrations (1 μg/ml). Finally, to examine the specificity of the different TRAIL variants to HSCs, the hepatic parenchymal cancer cell line HepG2 was treated with increasing concentrations of DR4, DR5 and wt TRAIL. Whereas no detectable difference in viability of HepG2 cells was observed after application of wt TRAIL,

![Figure 2](image-url). Interfering in LX2 cells collagen production by receptor-specific TRAIL variants. Representative sircol assay for collagen (A) and protein production (B) in LX2 cells treated with sublethal dose of different receptor specific variants and wt TRAIL; (C) HSPA47 mRNA expression and (D) protein production in LX2 cells treated with two different concentration of TRAIL proteins.

![Graph A](image-url)

- **Control media**
- **DR4 specific TRAIL**
- **WT TRAIL**
- **DR5 specific TRAIL**

![Graph B](image-url)

- **Control media**
- **DR4 specific TRAIL**
- **WT TRAIL**
- **DR5 specific TRAIL**

![Graph C](image-url)

- **Control media**
- **DR4 specific TRAIL**
- **WT TRAIL**
- **DR5 specific TRAIL**
a decrease in viability was observed with the DR4 or the DR5 specific TRAIL proteins (Figure 5(C)). These findings confirm the selectivity of the wt TRAIL protein toward activated HSC. The inhibition of the viability in primary activated rat HSC was lower than in LX-2 cells and was only found at high doses of DR5-specific TRAIL (1 μg/mL), which showed a 20% reduction in viability (Figure 5(E)). No significant reduction in viability was observed after treatment with the different TRAIL variants of quiescent rat HSCs or rat hepatocytes. We next evaluated the contribution of the different TRAIL receptor variants with respect to induce apoptosis using the caspase 3/7 activity assay. The measurement of caspase activity in HSCs treated with TRAIL showed a higher caspase activity for DR5- and wt-treated cells versus DR4-treated LX-2 cells. Data obtained were normalized to the untreated cell line (Figure 5(A)). To further
investigate the apoptosis-inducing activities of the different TRAIL variants, LX-2 cells were incubated with PBS or the TRAIL variants proteins for 48 h. Consistent with the cell viability results, only WT and DR5-specific TRAIL-induced robust apoptosis of LX-2 cells, whereas DR4-specific TRAIL was less effective as measured by Annexin V FACS analysis (Figure 5(B)).

Discussion

Persistent liver injury lead to enhanced ECM production and accumulation, a condition referred to as liver fibrosis. HSCs have a central role in this process, it has been shown that activated HSCs significantly amplify the hepatic response to liver injury, hence depleting fibrotic livers from activated HSCs may ameliorate the fibrotic condition [22]. TRAIL-induced apoptosis was successfully used to eliminate activated HSC [7]. In order to increase the specificity and to be able to lower the effective dose of TRAIL, we evaluated the different properties of receptor-specific TRAILs as a selective treatment for the elimination of activated HSCs to improve the remedy from liver fibrosis. To uncover the dynamics of TRAIL receptor expression during time-dependent HSC activation, the expression pattern of different TRAIL receptors on the surface of quiescent and activated HSCs was evaluated. Our findings cast light on the differential contribution of TRAIL receptors in HSCs. An increase in the level of TRAIL-DR5 and DR4 receptors was observed both in mRNA and protein expression upon activation of HSCs. Despite the increase in TRAIL-DR4 receptor expression during activation, the net expression remained significantly lower than that of the TRAIL-DR5 receptor. An increase in the expression of TRAIL receptors was concomitant with an increase in expression of TRAIL decoy receptors, especially DcR2. As part of our study, we demonstrated that the DR5 receptor was the single most dominant receptor on HSCs in comparison with the HSC side population, whereas decoy receptor DcR2, was predominantly expressed on the surface of HSC side population. This finding is particularly important since it has been proven that HSCs may directly undergo mesenchymal-to-epithelial transition to transdifferentiate into liver progenitors [23,24] or indirectly augment regeneration of hepatic cells by its produced cytokines [25,26]. The exact mechanisms by which HSCs may help mediate liver regeneration and the relative importance of different subtypes of HSCs remain to be determined. However, crucial regenerative role of the SP in multiple organs imply the possibility for
this population to have similar role in liver regeneration. Thus, next to the higher expression of TRAIL receptors on activated HSCs the selectivity of TRAIL variants could be partially explained by the higher expression of the decoy receptors in SP HSCs than in rest of population. Our findings therefore, show the protective role of TRAIL decoy receptor 2 in HSCs SP.

To further evaluate the application of different receptor-specific TRAIL agonists in eliminating activated HSCs, we explored the functional capacity of receptor-specific TRAIL variants [13,15,26] in inducing apoptosis and reducing ECM production in HSCs. ECM-producing cells, such as HSC, are highly responsive to a series of factors, such as EGF, PDGF and TGF-β, that are released from damaged liver parenchyma during the fibrotic process. Affected HSCs start producing excessive amount of ECM. The production of ECM, especially collagen I, could in return accelerate HSC proliferation and fibrosis [27,28]. Addressing this phenomenon, we evaluated the capability of different TRAIL variants in blocking ECM production by HSCs. All TRAIL variants suppressed the production of soluble collagen by activated HSCs with DR5 receptor-specific and wt TRAIL being the most potent molecules. This observation was accompanied by downregulation of the pro-collagen specific molecular chaperone Hsp47 and aggregation of insoluble collagen in TRAIL-treated cells. These findings cast light on the supporting mechanism of receptor-specific TRAIL mediated collagen regulation through interfering with the natural folding and secretion process of pre-collagen in activated HSCs. A direct relation has already been established between fibrogenesis and the number of α-SMA positive cells [29]. Since the increase in accumulation of insoluble collagens in our study was associated with a reduced number of α-SMA positive cells, it may be speculated that the decreased expression of ECM proteins is caused by the reduction in HSC activation and proliferation. The exact mechanism for this phenomenon yet to be defined, but it has been shown that TRAIL could inhibit cellular growth in the absence of cell death induction due to cyclin dependent cell cycle arrest in S-G2/M phase [30–32]. Hence, inhibition of HSC activation and proliferation by TRAIL could directly reduce secretion of ECM including collagen I. An alternative mechanism for reduced activation of TRAIL-affected HSCs could be attributed to role of pericellular collagen on HSCs proliferation. There is accumulating evidence that HSC biology is tightly regulated by their peripheral ECM environment. It has been shown collagen I, the major component of fibrosis, enhances the proliferation of HSC in vitro via different mechanisms, that is, interacting with type-2 discoidin domain receptor [33,34]. Thus, TRAIL property for in vitro eliminating ECM accumulation through interfering with pro-collagen folding and secretion could eliminate HSCs proliferation and promote reversion of the fibrotic phenotype of activated HSCs [35].

Our findings revealed a substantial decrease in LX-2 HSC viability achieved through exposure to DR5-specific and wt TRAIL, whereas DR4-specific TRAIL showed only a marginal effect on LX-2 cell viability. This finding is in concordance with findings regarding the functional role of different TRAIL variants in inducing apoptosis as measured by increase in caspase 3/7 and Annexin V [7]. Selectivity of TRAIL variants is an important issue since apoptosis in itself could have a dual function in ameliorating or deteriorating fibrosis progression. Here, we showed that the effects of receptor specific TRAIL variants are limited to the HSCs rather than the

Figure 5. Comparison of caspase 3/7–8 enzyme activity and Annexin V in activated HSCs treated with different TRAIL variants. An increase in the amount of caspase-3/7–8 activity in LX2 cells (A); treated with TRAIL variants is concurrent with increase in apoptosis marker Annexin V in FACS analysis with a significant difference between DR5- and DR4-specific apoptosis induction (**p < .05) (B).
hepatocytes. The observed sensitivity of HepG2 cells towards receptor-specific TRAIL could be partially explained through its over expression of pro-apoptotic TRAIL receptors and DcR2 in hepatocellular carcinoma cell line [36]. Since fibrosis is considered rather a long term and persistent problem, a durable and efficient approach need to be pursued. Our current strategy to dissect the functional properties of different TRAIL variants is particularly beneficial since TRAIL shows a very short half-life (6 h) in vivo and thus needs high dosages and frequencies of administration for clinical benefit. We speculate that an advantage of treatment with receptor-specific TRAIL variants is the specific targeting of activated HSCs and lowering required dosage for having the same effect. Moreover, given that TRAIL variants carry both separate functions for simultaneously inducing apoptosis through TRAIL receptors and reducing ECM production via blocking relevant signaling pathways in HSCs, it appears to be an ideal combination for eliminating fibrotic process. Hence, a loss of viability in TRAIL-treated activated HSCs could be followed by a lower proliferation and reversion to quiescent phenotype of remaining activated HSC. This is particularly important for sustaining the effect of TRAIL. Furthermore, TRAIL variants may be applied as genetic therapy [37] resulting in highly effective long-term in vivo elimination of activated HSCs and reduction of ECM production. In this regard, our study is the first to show a significant antifibrotic effect of different receptor-specific TRAIL variants, which warrants further in vivo studies (Figure 6).

TRAIL is a molecule that have been long known for its properties in cancer therapy. Due to its natural role in mediating resolution of liver fibrosis TRAIL has been recently addressed as a potential attractive candidate for treatment of liver fibrosis. Despite the extensive work carried out to elucidate the molecular mechanism of TRAIL and its dedicated receptors in cancer cells, less is known about the role of TRAIL receptors in elimination of hepatic stellate cells. In this study, we investigated receptor specific TRAIL or wild-type TRAIL for their role in the elimination of activated HSCs as the major player in liver fibrosis process. Our results indicate more than one receptor is responsible for recognition and signal transduction of TRAIL into activated HSCs. DRR5 receptors were demonstrated to be the most frequent receptor on the surface of activated hepatic stellate cells. Consequently, we demonstrate DRR5-specific TRAIL is highly favorable to eliminate this type of cell. Decrease in viability due to exposure with different TRAIL variants associate with an increase in caspase activity and apoptosis induction in activated hepatic stellate cells. Also, the application of sub toxic dosage of different TRAIL variants result in pronounced reduction in extra cellular matrix production correlate with their inhibitory effect on production of pro-collagen specific folding molecule chaperonin HSP-47. The overall finding from this study shed light on the involvement of different TRAIL receptors in eliminating activated HSCs that could pave the way for its future utility of receptor specific TRAIL-based antifibrosis therapy.

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Disclosure statement
The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.
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