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Arabpour, Mohammad

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Chapter 6

General discussion and future perspectives

Mohammad Arabpour

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

Liver fibrosis is the major problem in patients with chronic liver disease. Activation of hepatic stellate cells (HSCs) and deposition of extra cellular matrix is the major underlying mechanism of liver fibrosis, which imposes an especially hard economic and health burden on health care systems due to the cost of liver replacement therapy, the only viable option in the end stage of the disease. Therefore, the pursuit of a workable alternative therapy to reverse the fibrotic process is desired. We investigated the application of TRAIL derivatives and variants as a drug therapy to target activated HSCs. Activation of HSCs is known to be an important factor in the development of liver fibrosis. HSCs activation also occurs concurrently with the over-expression of death-inducing receptors such as Tumor Necrosis Factor Related Apoptosis-Inducing Ligand (TRAIL) receptors, including DR4 and DR5, thus rendering HSCs susceptible to the apoptotic effects of TRAIL agonists. The application of TRAIL agonists has previously been described as a potential strategy to eliminate activated HSC [1]. TRAIL agonists have been used successfully in targeting different tumors in clinical settings, proving its safety in humans. However, in order to ensure both specific targeting and efficient elimination of HSC in the fibrotic liver, further modification of TRAIL is required. It has been demonstrated that a series of human growth factors play a role in HSC proliferation in the fibrotic liver because their corresponding receptors are highly expressed in activated HSCs. Particularly, it has been shown that epidermal growth factor and platelet derived growth factor receptors expression is up-regulated on the surface of activated HSCs [2][3]. Based on this finding, we reasoned that the coupling of a single chain antibody or peptide specific to these receptors would enhance the efficiency of

TRAIL both in specific targeting and in higher loading capacity. The fusion protein produced by the coupling would be specifically targeted to and would eliminate activated HSC in the fibrotic liver. In addition, by targeting TRAIL molecules specifically to HSCs, we expected to keep them efficiently away from the hepatocytes.

In **Chapter 2** of this thesis we described the application of anti EGFR scFv–TRAIL fusion protein in eliminating activated HSCs [4]. TRAIL receptors' over expression on activated HSCs make it an ideal target for TRAIL agonists. We and others have shown that TRAIL can preferentially induce apoptosis in activated HSCs. In this chapter we present results that demonstrate the targeted elimination of activated HSCs via EGFR and simultaneous activation of the caspase pathway. Interestingly, enhanced apoptosis of activated HSCs was reduced by separately eliminating the EGFR signaling pathway through monoclonal Ab. This later finding, however, highlights the importance of coupling death signaling and survival inhibition for enhanced killing in form of an adenoviral derived fusion protein.

The application of single chain antibody for targeted delivery of TRAIL was a success, as described in **Chapter 2**. However, antibodies possess a high molecular weight, limited tissue penetration and species-specific recognition that might be disadvantageous for targeting applications in *in vivo* models and also in clinical applications. Alternatively, we imbedded GE11 and pPB peptides that recognize the PDGF receptor and EGF receptor, respectively in **Chapter 3** [5–7]. Theses peptides were embedded in form of fusion proteins with TRAIL. Whereas anti EGFR scFv-

TRAIL receptor specific peptide- TRAIL fusion proteins did not further enhance TRAIL efficiency in eliminating activated HSCs, we show that receptor expression level, proportion of surface turnover or speed of receptor internalization did not impact the efficiency of TRAIL fusion proteins in eliminating activated HSCs. In contrast, TRAIL load that was targeted to HSCs correlated with the efficiency of TRAIL fusion constructs to induce caspase dependent apoptosis in activated HSCs.

The experiments described in **Chapter 4** focus on the characterization of receptor specific TRAIL or wild type TRAIL for their role in the elimination of activated HSCs. The results presented in Chapter 4 provide evidence that more than one receptor system is involved in the recognition and signal transduction of TRAIL into activated HSCs. DR5 receptors were demonstrated to be the most frequent receptor on the surface of activated HSCs receptors. Consequently, we conclude that the selectivity of DR5 specific TRAIL is highly favorable to eliminate this type of cell. We show a substantial decrease in LX2 cell viability achieved through exposure to DR5-specific and wt TRAIL, whereas DR4-specific TRAIL was shown to have only a marginal effect on cell viability. The decrease in viability due to exposure with different TRAIL variants is concurrent with an increase in caspase 3/7 and Annexin V in HSCs. These findings suggest that the different TRAIL variants protein reduced viability in activated HSCs via caspase-associated apoptotic pathways. This finding concurs with the functional role of TRAIL in induction of death through the extrinsic caspase pathway and caspase-8 dependent activation [8].

TRAIL in non-lethal concentrations was shown to reduce production of extra cellular matrix by interfering with collagen specific HSP47 folding mechanism [9]. Our *in vitro* studies demonstrate that all TRAIL variants are capable of decreasing the expression of pro-fibrotic gene expression, such as collagen I and α -SMA production in HSCs. However, DR5 specific and wt TRAIL were proven to be the most effective proteins. We show that a reduction in collagen I and α -SMA production due to treatment with receptor specific or wt TRAILs were well correlated with a decrease in HSP 47. Together, the evidence presented argues for the successful application of the DR5 receptor-specific TRAIL variant in the targeted elimination of activated HSCs via interference with the collagen production and simultaneous induction of apoptosis via activation of the caspase pathway.

In **Chapter 5**, a combination of TRAIL variants and inhibitors for histone acetyltransferase or deacetylase (HDAC) or (HAT) was used to evaluate the potential application of different epigenetic modifications on TRAIL induced apoptosis in carcinoma cell line. Our findings indicate that simultaneous application of HDAC inhibitor SAHA could render carcinoma cell lines substantially more susceptible to different TRAIL variants. Our findings also indicate that receptor specificity of TRAIL variants did not significantly contribute to their killing effect in presence of SAHA. However, the effects of combining HAT and HDAC inhibitors in augmenting TRAIL killing effect has not yet been investigated. Our findings indicate that HAT inhibitor C646 even at low concentrations increases TRAIL-induced cytotoxicity in multiple carcinoma cell lines. In conclusion, current evidence indicates that in

conjunction with HAT inhibitors TRAIL constitutes a potent apoptotic stimulus in human carcinoma cells.

In summary, in this thesis we demonstrate that TRAIL exerts two functions in the therapy for liver fibrosis. First, a targeted form of TRAIL can be used as an inducer of caspase dependent apoptosis in activated HSCs. Second, TRAIL variants may modulate different processes such extra cellular matrix production and proliferation in activated HSCs. Thus, the dual functionality of TRAIL as a drug therapy may be successfully exploited and constitutes a new approach in the treatment of liver fibrosis.

Future Perspectives

Currently, various strategies are being investigated for their efficacy in resolving liver fibrosis, yet an ultimate solution for use in humans remains elusive. Low specificity or high toxicity of experimental drugs towards HSCs in the liver often undermines the anti-fibrotic effects *in vivo* compared with results obtained with these same compounds *in vitro*.

TRAIL application for Liver fibrosis treatment has proven its potential in therapy via *in vitro* and *in vivo* preclinical studies. However, due to the short half-life of TRAIL *in vivo*, practical use of TRAIL for chronic diseases such as liver fibrosis requires sophisticated manipulation of TRAIL in order to ensure maximum targeted uptake and persistent treatment for a long-term therapy. In this study we therefore addressed these issues by employing TRAIL in the form of a fused protein in an Ad vector for targeted elimination of activated HSCs. While the

application of adenovector ensures the long term expression of fused TRAIL protein in the liver, fusing TRAIL with targeting moieties for activated HSCs increases the chance of selective uptake by targeted cells. We also explored the efficacy of different TRAIL types in the elimination of extra cellular matrix production where different receptor specific TRAILs showed varying potential for HSC elimination and inhibition of collagen secretion mechanism by activated HSCs. These approaches open the possibility to selectively modulate the action of HSCs involved in the fibrotic process. We believe progress in vector technology and monitoring techniques could promote and enhance the idea of safely using TRAIL in treating liver fibrosis. Replacement of viral vectors by less toxic and less complex non-viral vectors that offer large carrying capacities could increase the chance of bringing the TRAIL products for treating liver fibrosis to clinic in future. Also, hopefully ever growing knowledge on molecular pathways could pave the way for application of alternative safer molecules like monoclonal antibodies for more specific targeting in eliminating activated HSCs. However, future studies using TRAIL for treatment of liver fibrosis will have to demonstrate their practical applicability in a clinical setting.

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