Therapeutic Use of Short Hairpin RNA in Acute Liver Failure

Yoshiaki Mizuguchi[1,2], Shigeki Yokomuro[1], Takuya Mishima[2], Yasuo Arima[1],
Tetusuya Shimizu[1], Yutaka Kawahigashi[1,2], Toshihiro Takizawa[2] and Takashi Tajiri[1]

[1]Department of Surgery for Organ Function and Biological Regulation, Nippon Medical School
[2]Department of Molecular Anatomy and Functional Morphology, Nippon Medical School

Introduction

Disruption of transforming growth factor-beta (TGF-β) signaling underlies many human disorders. In the liver, TGF-β plays an essential role in hepatocyte apoptosis, growth inhibition, and the progression of fibrogenesis. The pathophysiology underlying acute liver failure (ALF) involves over-efficient apoptosis and inhibition of hepatocyte regeneration associated with TGF-β signaling. Hence, TGF-β receptor II (TGFβRII) is an attractive target for therapy and the analysis of pathophysiology in many human disorders. To approach this problem, we investigated the effects of a TGFβRII short hairpin RNA (shRNA)-coding plasmid (shTGFβRII) using hepatocyte injury in a mouse model of liver injury.

Materials and Methods

Constructing shTGFβRIIs

To construct vectors that express mouse and human shTGFβRIIs, we used the p*Silencer* (Ambion) plasmid.

Mice, Culture Cells, and shRNA Coding Plasmid Treatment

The Ethics Review Committee for Animal Experimentation of Nippon Medical School approved our experimental protocols. Male BALB/c mice, aged 6 to 7 weeks and weighing 20 to 25 g, were purchased. Mouse BNL CL.2 cells were cultured with Dulbecco's minimum essesntial medium (Gibco, Tokyo, Japan) supplemented with 10% fetal calf serum. Plasmids encoding shRNA were delivered into mice using a modified hydrodynamic injection method, in which 100 μg of plasmid dissolved in 2.5 m*l* phosphate-buffered saline was injected into mice via a tail vein over 6 to 8 seconds. Cell transfection was performed with Lipofectamine 2000 (Invitrogen, Tokyo, Japan) according to the manufacturer's instructions. Cells were transfected three times at intervals of 16 to 18 hours.

Animal Models

For the *in vivo* study, we used a Fas agonist (Jo-2 antibody, BD PharMingen, CA, USA). Apoptosis induced by a Fas agonist is an excellent system for studying ALF. Recent reports have indicated that TGF-β-induced apoptosis in hepatocytes uses pathways similar to those activated by Fas ligation and that Fas ligation rapidly
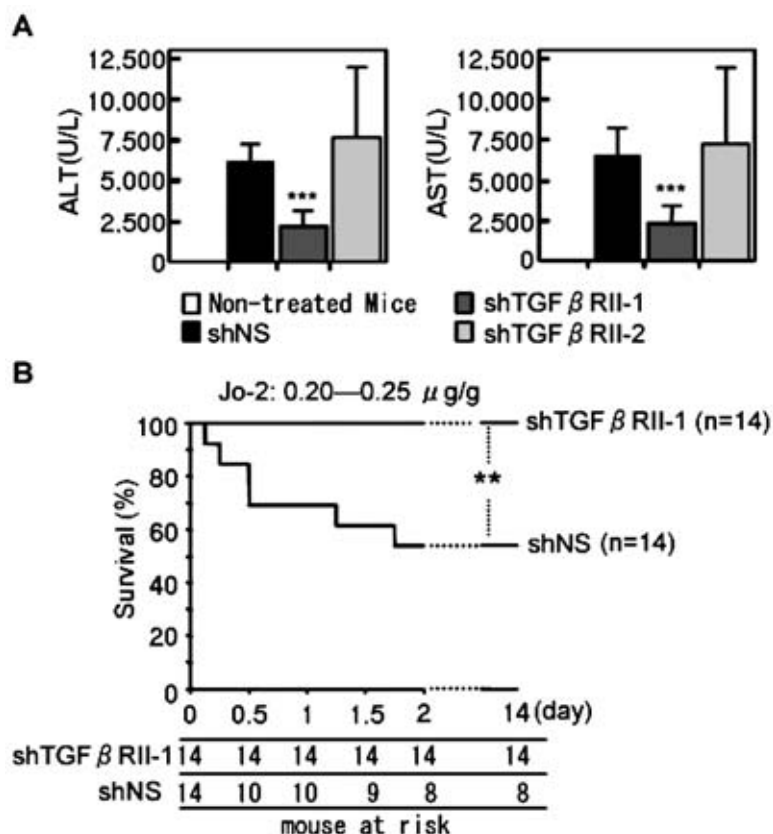
Fig. 1　Modulating TGF-β signaling in mice with ALF. (A) Therapeutic effects of silencing on the release of serum AST and ALT. Mice were treated IV with a sublethal dose of Jo-2 antibody (0.10~0.15 μg/g) after shNS, shTGFβRII-1, or shTGFβRII-2 injection. Values are expressed as means±SD. n=6. *, $P<0.05$;**, $P<0.01$ compared with shNS (black bar). The bar for the untreated mice is not visible because the values were within the normal limits (AST, 20~48 U/L; ALT, 10~40 U/L). (B) Mice were treated IV with a 50% lethal dose of Jo-2 antibody (0.20~0.25 μg/g) and were followed up for 14 days. The Kaplan-Meier method was used to calculate survival, and the log-rank test was used to compare survival between groups. n=14 per group. **, $P<0.01$.

induces TGF-β. For the acute hepatitis model, mice were received intravenous (IV) injections of a sublethal dose of Jo-2 monoclonal antibody (0.10~0.15 μg/g, IV) dissolved in pyrogen-free phosphate-buffered saline (containing Ca^{++} and Mg^{++}). After 8 hour, serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured using a standard clinical automatic analyzer. For the survival study, we used an ALF model in which mice received injections of a 50% lethal dose of Jo-2 antibody (0.20~0.25 μg/g, IV) and were followed up for 14 days. The Kaplan-Meier method was used to calcuate survival, and the log-rank test was used to compare survival between the groups.

Modulating TGF-β Signaling in Mice with ALF

In our study, hydrodynamic injection of pEGFP-C1 (100 μg) with shNS (100 μg) induced massive e- g- f- p-(EGFP) expression in the liver. Furthermore, we extracted the plasmid encoding transfected shRNA and identified clones with the shRNA insert using sequence analysis (data not shown).

ALF results in high mortality. We performed experiments in BALB/c mice with Jo-2 antibody-induced acute

liver injury.

In mice with acute hepatitis (Jo-2; 0.10～0.15 μg/g, 8 h), shTGFβRII-1 and shTGFβRII-2 significantly decreased *TGFBR2* mRNA by 75% and 60%, respectively, compared with non-specific shRNA coding plasmid (shNS) ($P<$ 0.01 and $P<0.05$, respectively).

To confirm the physiological relevance of TGFβRII silencing on the clinical condition, we tested its effect using clinical variables. As shown in **Figure 1A**, 8 hours after Jo-2 injection (0.10～0.15 μg/g), shTGFβRII-1 pretreatment strongly prevented the release of aminotransferases from damaged hepatocytes compared with shNS (AST, 2,220 ± 832 vs. 6,192 ± 934, $P<0.001$; ALT, 2,363 ± 1,014 vs. 6,538 ± 1,680, $P<0.001$). Furthermore, in a survival study of mice with ALF, about 43% (6 of 14) of the mice pretreated with shNS died within 48 hours of injection. In contrast, all mice pretreated with shTGFβRII-1 were alive 14 days after injection ($P<0.01$) **(Fig. 1B)**.

Discussion

Our findings demonstrate that RNA interference (RNAi) can be used to silence TGFβRII involved in the cause or pathway of many human disorders with a plasmid vector encoding shRNA.

One of the main problems with applying RNAi technologies to human diseases is the delivery system. In this study, we used a hydrodynamic injection method, but further optimization is needed to achieve clinical acceptance. Nevertheless, our findings show that the use of shRNA targeting TGFβRII has great potential as an analytical tool for TGFβRII in TGF-β signaling and in gene-specific therapies for human disorders.

———————————————————