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LETTER TO THE EDITOR

Modulation of transforming growth factor-β1 production by vector-based RNAi in hypertrophic scar fibroblasts: A therapeutic potential strategy for hypertrophic scar

KEYWORDS
Hypertrophic scar; Transforming growth factor-β1; Gene therapy

Hypertrophic scars can cause significant aesthetic and functional symptoms and to date no optimal treatment has been established. A number of studies have implicated transforming growth factor-β1 (TGFβ1) plays as essential role in wound healing and hypertrophic scarring [1—4]. RNAi is a post-transcriptional gene-silencing mechanism that can be initiated by double-stranded RNA (dsRNA) homologous in sequence to the target gene. A vector-based approach can achieve suppression of gene expression in mammalian cells [5]. This study was focused on the gene-silencing and anti-scarring effects of TGFβ1 siRNAs in the cultured fibroblasts isolated from surgically excised hypertrophic scar, indicating the potential for this inhibitory TGFβ1 to blunt excessive scarring in patients.

Eight patients (five men and three women with age range of 13—42 years) who received no previous treatment for burn hypertrophic scar before surgical excision were enrolled in each experiment of this study with their written informed consent. The hypertrophic scar fibroblasts were cultured by the explant method previously described [6]. When fibroblasts outgrowth had become well established, the skin explants were dislodged prior to subculture of the monolayer. The cells were grown to confluence and only low passage cultures (passages 4—6) were analyzed for further experiments.

For specific gene inhibition, we used a shRNA vector expression plasmid, named TGFβ1 pSUPER (which was generously provided by Dr. G. Eisele, Department of Immunology, University of Tubingen, Tubingen, Germany) [7], and introduced into the passages 4—6 of hypertrophic scar fibroblasts using liposome GenePORTER 2 (Gene Therapy System Inc., San Diego, CA) (50 nM/l). In parallel wells, to act as controls, scar fibroblasts were transfected with transfection reagent alone (Mock) and nonspecific pSUPER (50 nM/l) (a present from Dr. G. Eisele too) according to the GenePORTER2 manufacturer's instructions.

Post 24 h transfection, cells were replaced fresh growth media. Twenty-four to forty-eight hours after transient transfection, total RNA from the hypertrophic scar fibroblasts was prepared using Trizol® Reagent (Invitrogen) according to the manufacturer's instructions. cDNA synthesis was conducted according to the RNA PCR kit protocol (TAKARA). The primers were used as follows: TGFβ1 (forward primer, CAGCAACAATTCCTGGCGATA; reverse primer, AAGGCGAAAGCCCTCAATTT; 136 bp expected), Smad2 (forward primer, GTCTTGCCTCCAGTCTTATT; reverse primer, GAGAGCCTGTGTCCATACTT; 126 bp expected), Smad3 (forward primer, AGGAATTTGCTGCCCTCCTAG; reverse primer, GCCTTTGACGAAGCTCATGC; 169 bp expected), Col1A2 (forward primer, TGAGAGAGGGGTTGTTGGAC; reverse primer, AGGTTCACCCTTCACACCTG; 142 bp expected), and GAPDH (forward primer, GCACCGTCAAGGCTGAGAAC; reverse primer, TGGTGAAGACGCCAGTGGA; 138 bp expected). Real-time PCR was performed using the Chromo 4™ Four-Color Real-Time System (Bio-Rad Laboratories Inc., CA, USA) and was carried out with SYBR Premix Ex Taq (Perfect Real Time) kit (TAKARA) in a 25 μl volume of the PCR reaction solution. PCR products were measured by the threshold cycles (C_T), at which specific fluorescence became detectable. The C_T was used for kinetic analysis and was proportional to the initial number of target copies in the sample. A melting curve analysis was performed to assess the specificity of the amplified PCR products.

To detect expression of TGFβ1 protein in culture supernatants, 24—48 h after transient transfection, the extracellular medium was collected and centrifuged with removal of cell sediment for determination of secreted TGFβ1 protein. The concentration of TGFβ1 in culture supernatant was measured using

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a commercial TGFβ1 enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (Jingmei, China).

The results of this research showed that the expression of TGFβ1 mRNA and protein were significantly decreased after 24 h transfection, while the TGFβ1 expression in fibroblasts transfected nonspecific pSUPER and transfection reagent alone was basically no difference ($P > 0.05$) (Fig. 1A and B). And after 48 h transfection, the expression of TGFβ1 mRNA and protein further decreased (Fig. 1A and B). The possible mechanism might be that shRNA was

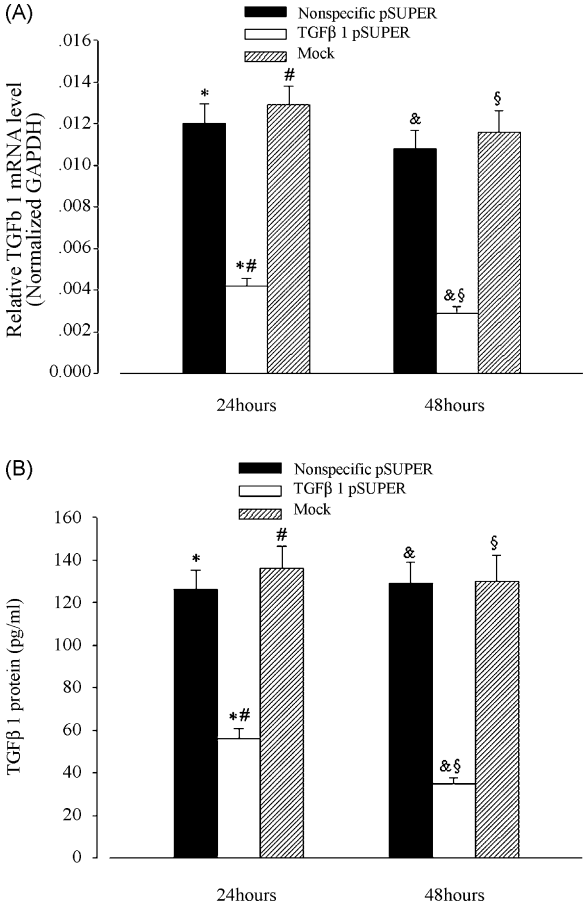

Fig. 1 Effectiveness of transforming growth factor TGFβ1 pSUPER mediated gene silencing. Nonspecific pSUPER or TGFβ1 pSUPER (50 nM/l) were introduced into hypertrophic scar fibroblasts, respectively, at subconfluence using GenePORTER. In parallel wells, to act as negative control, fibroblast cells were transfected with transfection reagent alone (Mock). Twenty-four and forty-eight hours after transfection, RNA was extracted. TGFβ1 mRNA levels were determined by Real Time RT-PCR (A). Media was replaced at 24 h after transfection, and conditioned media was collected at 24 and 48 h for measurement of TGFβ1 using ELISA (B). Statistical comparisons between groups were evaluated using ANOVA. Results are mean ± S.E.M. A value of $P < 0.05$ was accepted as significant (*$P < 0.001$, &$P < 0.001$, #$P < 0.001$ and §$P < 0.001$).

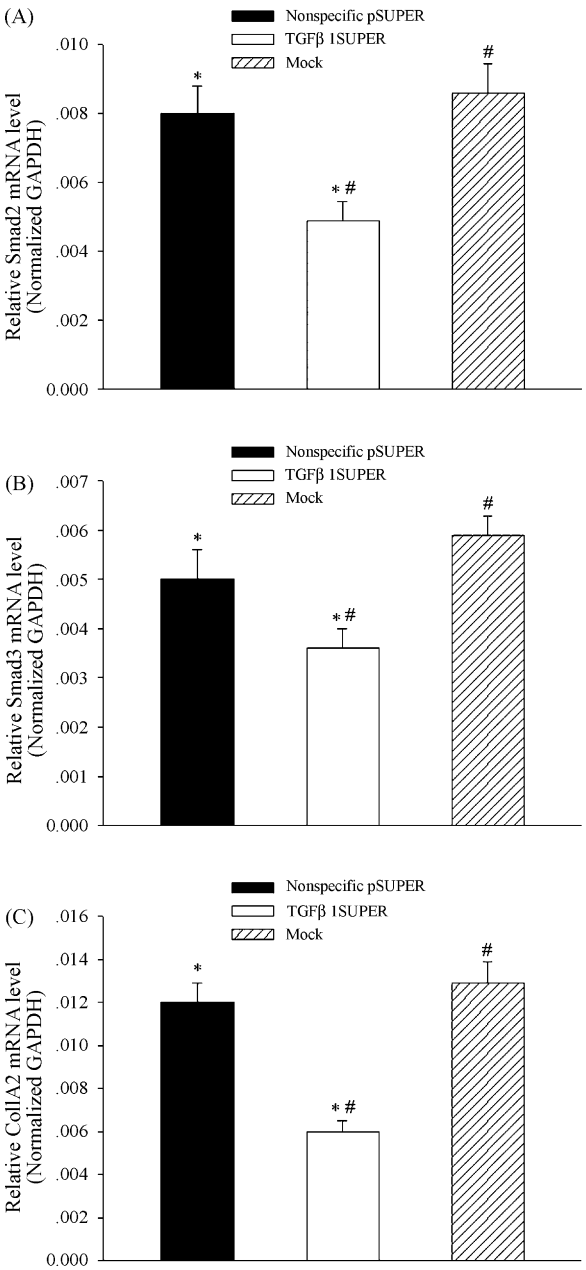

Fig. 2 Suppressed Smad2, Smad3 and COL1A2 silenced hypertrophic scar fibroblasts in the TGFβ1 silenced cells. Nonspecific pSUPER or TGFβ1 pSUPER (50 nM/l) were introduced into hypertrophic scar fibroblast cells, respectively, at subconfluence using GenePORTER. In parallel wells, to act as negative control, fibroblast cells were transfected with transfection reagent alone (Mock). Twenty-four hours after transfection, cells were replaced with fibroblast growth media for another 24 h, and then RNA was extracted to verify that the Smad2 (A), Smad3 (B) and COL1A2 (C) gene was effectively silenced by Real Time RT-PCR. Statistical comparisons between groups were evaluated using ANOVA. Results are mean ± S.E.M. A value of $P < 0.05$ was accepted as significant (*$P < 0.05$, #$P < 0.05$).

formed after the cells were transfected with TGFβ1 pSUPER plasmid vectors and then processed into siRNA, which specifically bound to TGFβ1 mRNA and degraded it, thus resulting in gene silencing or suppression of expression. However, the down regulation of TGFβ1 expression was not achieved a complete of gene function, which might be associated with GenePORTER transfection efficiency in hypertrophic scar fibroblasts. The other mechanism of this might be related to the death of a part of fibroblasts for the toxicity of GenePORTER. How to improve the transfection efficiency in hypertrophic scar fibroblasts by GenePORTER has become the objective of our further research. Moreover, the TGFβ/Smad signaling downstream genes such as Smad2, Smad3, COL1A2 were highly inhibited too (Fig. 2A—C), which was agreement with the finding that disrupting TGFβ signaling by knocking out or neutralizing these genes [8—10] can also be disrupted more specifically. These data raise the possibility that the use of local vector-based TGFβ1 shRNA could decrease the tendency for hypertrophic scar. However, before clinical application can be considered, a number of problems need to be solved. One major problem is the selection of effective and highly efficient target sites, and how to identify the best target using a simple method. Another problem is how to efficiently and specifically deliver shRNA-expression vectors to target cells. Advances in RNAi techniques may thus provide a promising strategy for gene therapeutic applications to prevent hypertrophic scar.

In conclusion, on the basis of our *in vitro* evidence we can state that modulation of TGFβ1 production by vector-based RNAi in hypertrophic scar fibroblasts may be a therapeutic potential strategy for hypertrophic scar. We are presently striving to establish an effective method (such as: lentiviral or adeno-associated virus vector) that can stably express shRNA in order to investigate the possibility of long-term analysis of gene function and provide foundation for further animal and clinical experiments.

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