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Transdermal siRNA-TGFβ1-337 patch for hypertrophic scar treatment

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ABSTRACT

Hypertrophic scarring (HSc) is a fibroproliferative disorder of the dermis characterized by erythematous, swollen, and pruritic lesions of healing skin. An increased understanding of the role of TGFβ1 in the development of HSc provides the potential for treating HSc by down-regulating TGFβ1 expression. siRNAs that effectively interfered with TGFβ1 expression were screened. It was concluded that the siRNA-TGFβ1-337 was able to effectively down-regulate TGFβ1 expression in HSc fibroblasts. The effects of siRNA-TGFβ1-337 on cell proliferation, cell cycle, and apoptosis of HSc fibroblasts were investigated. It was shown that it inhibited cell proliferation, arrested cells in the G1 stage of the cell cycle, and induced apoptosis of HSc fibroblasts. The transdermal patch of siRNA-TGFβ1-337 was a combination of siRNA-TGFβ1-337 and a pressure-sensitive adhesive hydrogel. The treatment effects of the transdermal patch were assessed in an animal model established by transplanting human HSc to nude mice. Decreased expression of TGFβ1 was observed with treatment with the transdermal siRNA-TGFβ1-337 patch. Consequently, the treatment resulted in type I collagen down-regulation and regularly arranged scar fibroblasts being significantly reduced and undergoing apoptosis; the scar size was decreased significantly. Thus, our findings indicate that a transdermal siRNA-TGFβ1-337 patch is a potential treatment for hypertrophic scars.

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1. Introduction

A hypertrophic scar occurs when there is an overgrowth of fibrous tissue at the site of an injury (Tredget et al., 1997). It is often characterized by swollen, erythematous, hard tissue, usually with abnormal sensations, including pain and tenderness (Rudolph, 1987; Ehrlich and Kelley, 1992). HSc is a common fibroproliferative disorder of the dermis due to excessive production, deposition, and contraction of extracellular matrix. The development of HSc is a complex interplay between fibroblasts and cytokines, and it is well established that cytokines are important regulators in wound healing and scar formation. Transforming growth factor beta (TGF-β) has been suggested to account for much of the altered phenotype of fibroblasts in HSc following thermal injury (Bellemare et al., 2005).

TGF-β is the currently known closest and most representative cytokine that promotes fibrosis and scarring in numerous tissues (Tredget et al., 1998). The TGF-β family includes three isoforms, TGFβ1, TGF-β2, and TGF-β3 (Basler et al., 1993). These isoforms have 60–80% homology and are believed to activate the same intracellular signaling pathways and appear to have similar biological functions *in vitro* (Roberts and Sporn, 1992). They play wide-ranging and diverse roles in differentiation, development, and homeostasis. They inhibit proliferation in most cell types and induce apoptosis in epithelial cells, but stimulate mesenchymal cells to proliferate and produce extracellular matrix and induce a fibrotic response in various tissues *in vivo* (Massague, 1998; McCartney-Francis et al., 1998). TGF-β acts as an antiproliferative factor in normal epithelial cells and at early stages of oncogenesis, and it has been found in higher concentrations in platelets and lymphocytes, mononuclear cells, macrophages, and fibroblasts, and it played effects through autocrine, paracrine, and endocrine signaling. However, there are differences in some biological activities. TGFβ1 has been implicated in certain fibrotic disorders such as pulmonary fibrosis, glomerulonephritis, and cirrhosis of the liver (Border et al., 1990; Broekelmann et al., 1991; Castilla et al., 1991). TGF-β2 has been implicated in proliferative vitreoretinopathy (Connor et al., 1989). Whereas the scarless wound healing seen in fetal wounds was due to the increased levels of TGF-β3 (Chen et al., 2005).

Abbreviations: HSc, hypertrophic scar; TGFβ1, transforming growth factor, beta 1; TGF-β2, transforming growth factor, beta 2; TGF-β3, transforming growth factor, beta 3; siRNA, small interfering RNA; G1, G1 phase of the cell cycle; LAP, latency-associated peptide; IHC, immunohistochemistry; qPCR, quantitative PCR; WB, Western blotting; NF, normal fibroblast; FB, hypertrophic scar fibroblast; RIPA, radio immunoprecipitation assay; HRP, horseradish peroxidase; ECL, enhanced luminol-based chemiluminescent; WST-8, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt; PI, propidium iodide; PVP, polyvinylpyrrolidone; PVA, polyvinyl alcohol; PET, polyethylene glycol terephthalate film; DEPC, diethylpyrocarbonate; DAPI, 4′,6-diamidino-2-phenylindole; H&E, Hematoxylin and eosin stain; DAB, 3,3′-diaminobenzidine; SD-208, 2-[(5-Chloro-2-fluorophenyl)pteridin-4-yl]pyridin-4-yl-amine; Akt, protein kinase B; ERK, extracellular regulated protein kinases.

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It was shown that HSc tissue and fibroblasts produce more TGFβ1 than normal skin tissue and fibroblasts, and that the size of a scar is proportional to the amount of TGFβ1 (Ghahary et al., 1995; Sullivan et al., 1995; Yang et al., 2002; Wang et al., 2007). Upon cutaneous injury, TGFβ1 is induced rapidly in neutrophils, macrophages, and fibroblasts (Wahl et al., 1987; Kane et al., 1991). It has been found that TGFβ1 could delay apoptosis of keloid cultures, and plays a key role throughout the repair process (Chipev et al., 2000). Also, throughout the repair process, overexpression of the TGF-β ancillary receptor endoglin can promote proliferation of fibrotic mesenchymal cells by suppressing Smad3-dependent responses in cell culture (Rodriguez-Pena et al., 2001; Diez-Marques et al., 2002; Leask et al., 2002). Activation of TGF-β receptors collectively activates extracellular matrix synthesis and fibroblast growth and differentiation into myofibroblasts (Shi and Massague, 2003; Daniels et al., 2004). TGFβ1-treated wound fibroblasts demonstrated active collagen fibrillogenesis and accretion of subfibrils at the ultrastructural level. This suggested a key role for TGFβ1 in HSc formation (Tredget et al., 2006) and that it may be an attractive target for the therapy of HSc (Pierce et al., 1991). Blocking TGF-β through a number of TGF-β inhibitors, such as decorin, biglycan, and LAP, may block fibrotic TGF-β signaling, but it does not affect the TGF-β immune response (Zhang et al., 2003). It has been shown that exogenous addition of TGFβ1 antibodies in HSc inhibits fibrosis in animal models (Shah et al., 1995; McCormick et al., 1999).

Much research has focused on the pro-fibrotic growth factor TGFβ1, which is key to reducing the activity of TGFβ1 and preventing scar proliferation in the early stage of trauma. However, there are some unsolved problems for the use of TGFβ1, such as its short half life, complex mechanism, and low content after systemic medication. In this study, we focus on the combination of transdermal patch and siRNA targeting TGFβ1.

A transdermal patch is a medicated adhesive patch that is placed on the skin to deliver a specific dose of medication through the skin. It has unique advantages beyond systemic administration, and can extend the functional time, enhance therapeutic efficacy, is suitable for self-administration, and minimizes undesirable side effects. As soon as RNA interference (RNAi) was found to work in mammalian cells, research quickly focused on harnessing this powerful and specific mechanism of gene silencing for human therapy. Phase I clinical studies have already begun to test the therapeutic potential of small RNA drugs that silence disease-related genes by RNAi. Small interfering RNA (siRNA), sometimes known as silencing RNA, is a class of double-stranded RNA molecules, 20–25 nucleotides in length. siRNA plays many roles, but it is most notable in the RNA interference (RNAi) pathway, where it interferes with the expression of specific genes with a complementary nucleotide sequence.

2. Materials and methods

2.1. Cell cultures

Hypertrophic scar tissues were obtained through Southern Hospital of Guangzhou, P.R. China. Patients who had suffered thermal or wound injuries and underwent plastic surgery to correct excess scar tissue were subjects for this study. All had developed hyperemic, raised, thickened, pruritic, and non-compliant scars, confined to the site of injury. After obtaining informed consent and using procedures previously approved by the Southern Hospital Institutional Review Board, samples of the scars and nearby unaffected skin were obtained by full-thickness punch biopsy. Fibroblasts were established from these tissues as described previously (Nakano and Scott, 1986). Fibroblasts were cultured in DMEM medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (GIBCO, Australia) and grown at 37 °C in an atmosphere of 5% CO_2. The following experiments were conducted on the fibroblasts at passage 4 or 5.

2.2. TGFβ1 siRNA

siRNAs (siRNA-TGFβ1-337, siRNA-TGFβ1-418, siRNA-TGFβ1-499, siRNA-TGFβ1-828) with the sequences listed in Table 1, targeting TGFβ1, were synthesized (GenePharma, China). Each siRNA duplex was transfected into hypertrophic scar fibroblast using Entranster-R (Engreen, China) following the manufacturer's protocol. siRNA-TGFβ1-NC, siRNA-NC-FAM (labeling with Carboxyfluorescein) and siRNA-GAPDH are respectively served as negative control, transfecting control and siRNA positive control targeting GAPDH gene (Table 1).

2.3. qPCR

Total RNA was prepared using the Trizol reagent (Invitrogen, U.S.A.) according to the manufacturer's instructions. Reverse transcription was performed on total RNA using a PrimeScript RT reagent kit with gDNA Eraser (Takara, China). qPCR was performed using an ABI7500 Real-Time PCR system and a SYBR Premix Ex Taq Kit (Takara, China); β-actin was used as an internal control, and each sample was normalized to its β-actin content. All experiments were performed in duplicate and repeated twice. Primers for quantitative PCR are shown in Table 2.

2.4. Western blotting

For the extraction of total protein, cells were lysed in RIPA lysis buffer and quantitated using a protein assay (Bio-Rad, U.S.A.). Then,

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Table 1
siRNA sequences.

siRNA	Sequence	Target gene	Modification
siRNA-TGFβ1-337	5′-GGUGGAAACCCACAACGAATT-3′	TGFβ1	Methylation
	5′-UUCGUUGUGGGUUUCCACCAT-3′		Methylation
siRNA-TGFβ1-418	5′-CCGAGAAGCGGUACCUGAATT-3′	TGFβ1	Methylation
	5′-UUCAGGUACCGUUCUCGGAG-3′		Methylation
siRNA-TGFβ1-499	5′-CGUGGAGCUGUACCAGAAATT-3′	TGFβ1	Methylation
	5′-UUUCUGGUACAGCUCCACGTG-3′		Methylation
siRNA-TGFβ1-828	5′-GCCGAGCCCUGGACACCAATT-3′	TGFβ1	Methylation
	5′-UUGGUGUCCAGGGCUCGGCGG-3′		Methylation
siRNA-GAPDH	5′-GUAUGACAACAGCCUCAAGTT-3′	GAPDH	Methylation
	5′-CUUGAGGCUGUUGUCAUACTT-3′		Methylation
siRNA-TGFβ1-NC	5′-UUCUCCGAACGUGUCACGUTT-3′	Negative control	Methylation
	5′-ACGUGACACGUUCGGAGAATT-3′		Methylation
siRNA-NC-FAM	5′-UUCUCCGAACGUGUCACGUTT-3′	Negative control with FAM modification	5′FAM and methylation
	5′-ACGUGACACGUUCGGAGAATT-3′		5′FAM and methylation

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