TGF-β: A Fibrotic Factor in Wound Scarring and a Potential Target for Anti-Scarring Gene Therapy

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Abstract: Hypertrophic scar and keloid are common and difficult to treat diseases in plastic surgery. Results of wound healing research over the past decades have demonstrated that transforming growth factor-β (TGF-β) plays an essential role in cutaneous scar formation. In contrast, fetal wounds, which heal without scarring, contain a lower level of TGF-β than adult wounds. How to translate the discovery of basic scientific research into the clinical treatment of wound scarring has become an important issue to both clinicians and basic researchers. The development of gene therapy techniques offers the potential to genetically modify adult wound healing to a healing process similar to fetal wounds, and thus reduces wound scarring. This article intends to review the roles of TGF-β in the formation of wound scarring, the possible strategies of antagonizing wound TGF-β, and our preliminary results of scar gene therapy, which show that wound scarring can be significantly reduced by targeting wound TGF-β.

Keywords: Wound scarring, TGF-β, gene therapy, adenovirus, gene transfer

INTRODUCTION

Cutaneous wound healing is a complicated process, which involves many different aspects including growth factors, extracellular matrices and various cell types [Clark, 1996]. This healing process in adult human being or adult animals of higher vertebrate is usually overactive and can cause scar formation.

Scarring is an unpleasant lesion to patients physically and psychologically. Sometimes, it also causes functional disability in severe burn patients. Keloid is an example of aberrant scarring in human being, which usually extends beyond the boundary of an original wound and is difficult to treat clinically [Tuan and Nichter 1998]. Fortunately, the scarless wound healing process in fetal wound repair may provide insights into the potential conversion of adult wound healing into a healing process similar to that of fetal scar-free wound. The development of gene therapy techniques offers a powerful tool to genetically modify the healing process of adult wounds to a repair process similar to fetal wounds.

Because of the close relationship between sustained expression of transforming growth factor-β (TGF-β) and the formation of keloid and hypertrophic scar [Wang *et al.*, 2000; Schmid *et al.*, 1998; Lee *et al.*, 1999; Chin *et al.*, 2001; Chodon *et al.*, 2000; Wang *et al.*, 1999b; Polo *et al.*, 1999; Smith *et al.*, 1999b; Bettinger *et al.*, 1996; Younai *et al.*, 1994; Peltonen *et al.*, 1991] as well as transient, lower level expression of TGF-β in fetal scarless wound healing [Whitby and Ferguson, 1991; Martin *et al.*, 1993; Krummel *et al.*, 1988; Houghton *et al.*, 1995], this article intends to review the roles of TGF-β in scar formation, the strategies of antagonizing wound TGF-β and our preliminary results of scar gene therapy by targeting wound TGF-β.

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TGF-β LIGANDS AND THEIR RECEPTORS

The term TGF-β generally represents a family of different TGF-β isoforms, each of which is highly homologous in their amino acid sequences, although they may have different functions in wound healing [Roberts and Sporn 1996; Shah *et al.*, 2000]. Mammalian cells express three TGF-β isoforms, designated as TGF-β1, -β2 and -β3. TGF-β1 is the most abundant isoform in all tissues, and is the only isoform in human platelets. TGF-β1 is also the major isoform in wound fluid [Roberts and Sporn 1996], while the other two isoforms participate in wound healing process as well [Polo *et al.*, 1999; Smith *et al.*, 1999b; Shah *et al.*, 1995].

Wound TGF-β is released from the degranulating platelets and is secreted by all major cell types participating in wound repair, including lymphocytes, macrophages, endothelial cells, epithelial cells and fibroblasts. TGF-β produced by most of the cells is in latent forms and is functionally inactive. It associates either with the latency-associated protein (LAP) to form a small latent complex or with both LAP and the latent TGF-β-binding protein (LTBP) to form a large latent complex. The latent TGF-β is activated by its dissociation with the latent proteins. Mannose-6-phosphate, plasmin, transglutaminase, and acidification are involved in the activation [Roberts and Sporn, 1996]. The major roles of TGF-β in wound healing include chemotaxis (for monocytes, lymphocytes, neutrophils and fibroblasts), angiogenesis, production and

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remodeling of extracellular matrix, and mitogen for dermal fibroblasts [Roberts and Sporn, 1996].

TGF-β binds all three types of receptors. However, it signals through TGF-β receptors I and II, the serine/threonine kinase receptors, and their down-stream signaling molecules Smad [Massague and Watton 2000]. The type III receptor, a membrane anchored proteoglycan (betaglycan), contributes to the signaling by increasing the binding affinity between the ligands and their signaling receptors. Once bound to the ligands, the receptor II associates with the receptor I to form a heterodimer or a tetramer and then transphosphorylates receptor I. The phosphorylated receptor I further activates Smad molecules to transduce the signaling into nucleus. Among the Smads, Smad2, 3 and 4 mediate the signaling, whereas Smad6 and 7 (inhibitor Smads) block the signaling by competitive inhibition of the functions of Smad4 and Smad2,3 respectively [Massague and Watton, 2000].

TGF-β AND FETAL SCARLESS WOUND HEALING

Unlike adult wounds repaired by wound scarring, fetuses heal their wounds by tissue regeneration without scar formation. The comparative studies have revealed the distinct differences in the healing processes between fetal and adult wounds [Longaker and Adzick 1991]. These differences include the intrinsic function of fetal and adult dermal fibroblasts [Chen *et al.,* 1989; Lorenz *et al.,* 1995], the extracellular matrix components [Whitby and Ferguson, 1991; Longaker *et al.,* 1989], the inflammatory response to tissue injury [Cowin *et al.,* 1998; Robinson and Goss 1981] and the cytokine profiles [Hsu *et al.,* 2001; Liechty *et al.,* 1998; Haynes *et al.,* 1994], etc.

A lower level of TGF-β expression in fetal wounds clearly plays an essential role in the scarless wound healing. In 1991, Whitby and Ferguson found that TGF-β1 and TGF-β2 could be detected with immunolocalization in neonatal and adult wounds, but not in fetal wounds [Whitby and Ferguson, 1991]. Martin *et al.* also demonstrated that both gene expression and protein production of TGF-β1 were rapid and transient in fetal wounds. In adult wounds, however, its expression and production appeared to be slower but sustained longer [Martin *et al.,* 1993]. Furthermore, Krummel [Krummel *et al.,* 1988] and Houghton [Houghton *et al.,* 1995] found that fetal scarless wound healing could be converted into a scar-forming healing process if exogenous TGF-β was added into the wounds, indicating that the absence of TGF-β in fetal wounds is important for scarless wound healing.

Other studies have provided the possible mechanisms of lower TGF-β expression levels in fetal than in adult wounds. The absence of platelets in the wounds of early stage fetuses may result in the lack of an initial dose of TGF-β that is usually released in adult wounds by degranulating platelets [Shaw, 2000]. Additionally, a weaker inflammatory response to dermal wounding in fetuses may also lead to the lower levels due to the less infiltration of TGF-β producing cells, monocytes and macrophages [Cowin *et al.,* 1998]. Furthermore, more TGF-β modulator fibromodulin that inhibits TGF-β activation is presented in fetal than in adult wounds, causing a lower level of bioactive TGF-β [Soo *et al.,* 2000]. Moreover, the gene expression level of TGF-β receptor II in the dermal fibroblasts of rat fetuses is much higher in the late gestational stage (scar forming) than in the early stage (scar free) [Hsu *et al.,* 2001], providing another way of modulating TGF-β function in fetal wounds. All these data indicate that the diminished TGF-β effect plays an important role in fetal scarless wound healing. Therefore, reducing TGF-β production or attenuating its effect in adult wounds might be helpful to the inhibition of wound scarring.

TGF-β AND WOUND SCARRING

In contrast to fetal wounds, enhanced expression of TGF-β and its receptors has been observed in hypertrophic scar and keloids. Wang *et al.* demonstrated that the hypertrophic scar tissue expressed five times as much TGF-β1 mRNA as normal skin tissue expressed. The *in vitro* cultured fibroblasts derived from the hypertrophic scar also expressed TGF-β1 mRNA in a level significantly higher than that of the normal fibroblasts [Wang *et al.,* 2000]. Schmid *et al.* found that more TGF-β receptors I and II were expressed in the granulation tissues than in the normal skin at both mRNA and protein levels. However, the expression levels decreased gradually in the normal healing excisional wounds during the granulation tissue remodeling. By contrast, the receptor expression in the hypertrophic scar maintained at high levels up to 20 months post-injury, suggesting that a persistent autocrine loop of TGF-β may exist and contribute to hypertrophic scar formation [Schmid *et al.,* 1998]. Similarly, Lee *et al.* have shown in an *in vitro* study that keloid fibroblasts produced more TGF-β1 and TGF-β2 protein, but not TGF-β3, than normal fibroblasts [Lee *et al.,* 1999]. Chin *et al.* further demonstrated an increased protein expression of TGF-β receptors I and II in the cultured keloid fibroblasts as compared with the normal dermal fibroblasts [Chin *et al.,* 2001]. These results suggest that overexpressed TGF-β ligands and their receptors are associated with scar and keloid formation.

CELLULAR AND MOLECULAR MECHANISMS OF TGF-β IN SCAR FORMATION

Although the mechanism of scar formation involves many different aspects and is too complicated to be reviewed here, a number of studies have shown that TGF-β plays a key role in scar formation via different mechanisms such as inflammation, matrix production, matrix remodeling, and the regulation of cell proliferation and apoptosis, etc [Roberts and Sporn, 1996].

During wounding, the initial dose of TGF-β appears to be an important factor to trigger the later cascade of TGF-β overproduction and scar formation. When a wound is created, TGF-β released from the platelets can recruit monocytes and other cells into the wound, and these cells further produce more TGF-β in the wound. Additionally, the initial dose of TGF-β in the early wound induces fibroblasts, the key player of scar formation, to produce their own TGF-β in the later stage wound via autocrine regulation, leading to the overproduction of wound TGF-β and eventually to scar formation [Roberts and Sporn, 1996] [Singer and Clark, 1999].

TGF-β is responsible for the most distinctive pathogenic processes of wound scarring, fibroblast proliferation and collagen production. *In vitro* studies have shown that TGF-β1 promoted cell proliferation and collagen production of both keloid and normal dermal fibroblast [Bettinger *et al.*, 1996; Younai *et al.*, 1994], and the collagen production stimulated by TGF-β was mediated by Smad3 and Smad4 [Chen *et al.*, 2000]. *In vivo,* overexpression of TGF-β 1 gene has been observed at the peripheral area of keloid tissues, where highly proliferated cells reside. In addition, TGF-β 1 gene expression is also co-localized to the area where collagen gene is actively expressed, suggesting that TGF-β 1 produced by keloid fibroblasts also promotes their proliferation and collagen production in the local environment of keloid tissues [Peltonen *et al.*, 1991].

Deposition of excessive collagen in hypertrophic scar or in keloids is also caused by inhibited proteolytic degradation of extracellular matrix (ECM) in wounds. Plasminogen activator (PA) and matrix metalloproteinases (MMP) are the two major ECM degrading enzymes, which are negatively regulated by "plasminogen activator inhibitor 1 (PAI-1) and tissue inhibitor of metalloproteinase-1" (TIMP-1), respectively. Increased expression of TGF-β in wounds not only enhances collagen production, but may also inhibit collagen degradation by up-regulating PAI-1 [Cao *et al.*, 1995] and TIMP-1 [Yang *et al.*, 2002] expression and by down-regulating MMP expression [Eickelberg *et al.*, 1999]. In an *in vitro* fibroplasia assay, Tuan *et al.* found that PAI-1 expression was strikingly higher in keloid fibroblasts than in normal dermal fibroblasts. In contrast, urokinase plasminogen activator (uPA) level was much lower in keloid fibroblasts, which led to the inhibited degradation of a fibrin gel when compared with the fibrin gel that contained normal fibroblasts [Tuan *et al.*, 1996]. A similar result has also been observed by Higgins *et al.* [Higgins *et al.*, 1999], suggesting that TGF-β is involved in the altered ECM degradation process in keloids.

Wound tension is also known to promote scar formation [McCarthy *et al.*, 1990]. In an *in vitro* assay, Peled *et al.* have shown that mechanical strain could up-regulate the gene expression of TGF-β1 and its receptors as well as type I procollagen in normal dermal fibroblasts, indicating that enhanced expression of TGF-β1 and its receptors by cellular strain may contribute to the tension-induced scarring [Peled *et al.*, 2000].

Regulation of cell apoptosis during wound healing is important in scar establishment and the development of pathological scarring. Several studies have revealed the reduced apoptosis in keloid fibroblasts [Luo *et al.*, 2001; Chipev *et al.*, 2000; Chodon *et al.*, 2000]. Mutation or down-regulated expression of apoptosis related genes has also been observed [Sayah *et al.*, 1999; Saed *et al.*, 1998]. Interestingly, TGF-β seems to contribute to reduced apoptosis observed in keloid fibroblasts, because high apoptosis rate of normal fibroblasts can be reduced by adding TGF-β to the culture media [Chipev *et al.*, 2000] [Chodon *et al.*, 2000]. Likewise, apoptosis rate of keloid fibroblasts can be increased by adding TGF-β neutralizing antibody [Chodon *et al.*, 2000].

The mechanism by which keloid fibroblasts regulate their TGF-β expression remains incompletely understood. The higher expression levels of TGF-β1, TGF-β2 [Lee *et al.*, 1999] and TGF-β receptors I and II [Chin *et al.*, 2001] in keloid fibroblasts than in normal dermal fibroblasts suggest that a TGF-β autocrine loop may play a role. Recently, we have demonstrated that the TGF-β1 autocrine regulation existed in keloid fibroblasts (Fig. **1**) [Liu *et al.*, 2002].

TGF-β autocrine regulation is usually self-limited in normal wound healing process. This self-restriction is

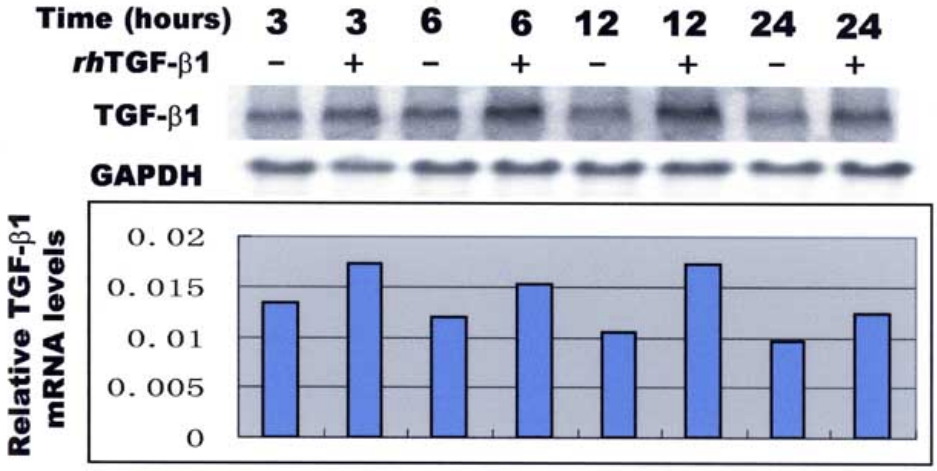

Fig. (1). rhTGF-β1 up-regulates gene expression of TGF-β1 in keloid fibroblasts. Keloid cells were either not treated or treated with recombinant TGF-β1(*rh*TGF-β1, 5ng/ml) for 3, 6, 12 and 24 hours respectively. Non-treated and treated cells were harvested at various time points to isolate total RNA. A Northern Blot assay was performed to analyze the gene expression of TGF-β1. The relative mRNA expression level was determined by dividing the expression level of TGF-β1 with GAPDH expression level.

possibly mediated by the regulated expression of Smads. The study by Mori *et al.* has shown that in normal dermal fibroblasts, Smad3 gene expression was down-regulated by exogenous TGF-β1, while Smad7, an antagonistic signaling molecule, was up-regulated by TGF-β1 [Mori *et al.*, 2000]. Thus, an elevated expression level of TGF-β1 in normal cells may elicit a negative feedback that limits the autoinduction process. Although a comparative study of TGF-β1 autocrine regulation between keloid and normal fibroblasts has not yet been performed, the sustained high expression levels of TGF-β ligands and their receptors in keloid cells imply that this autoinduction process may not be self-restricted, which might promote the unlimited growth of keloids clinically. In addition, the abnormal TGF-β signaling in keloid fibroblasts is likely to contribute to the abnormal TGF-β autocrine loop. This assumption is supported by the fact that Smad3 is over-phosphorylated in keloid fibroblasts as compared with normal fibroblasts [Chin *et al.*, 2001]. This abnormal signaling pathway may also explain why keloid fibroblasts exhibit an altered response to TGF-β treatment, such as increased cell proliferation and collagen production [Bettinger *et al.*, 1996] [Younai *et al.*, 1994]. Babu *et al.* also demonstrated that in the absence of serum, the TGF-β1-induced increase in fibronectin biosynthesis occurred more rapidly in keloid fibroblasts than in normal fibroblasts, indicating that keloid overproduction of extracellular matrix components might be due to an inherent modification of the TGF-β regulatory program [Babu *et al.*, 1992].

STRATEGIES OF BLOCKING TGF-β BIOLOGICAL ROLES IN WOUNDS

Wound scarring remains difficult to cure. Conventionally, scarring is considered as a natural result of wound healing and is inevitable. The study by Longaker *et al.* in a sheep model, however, demonstrated that the cleft lip defect of early gestational fetal sheep could be repaired without any scar formation [Longaker *et al.*, 1992]. This interesting phenomenon indicates that scar is not the necessary result of healing mammalian wounds, and adult wounds may be ideally repaired by tissue regeneration rather by fibrous tissue if their healing processes can be modified to mimic the mechanism of fetal scarless wound healing. A pioneer study by Shah *et al.* demonstrated for the first time that the scar formation in adult rodent wounds could be inhibited by neutralizing wound TGF-β with anti-TGF-β antibodies [Shah *et al.*, 1992]. This study provides direct evidence that manipulation of TGF-β effects in adult wounds is a good strategy of reducing wound scarring. Importantly, local manipulation of wound TGF-β can avoid the adverse effect of systemic manipulation of TGF-β, which may cause a lethal effect on experimented animals [Koch *et al.*, 2000].

Manipulation of wound TGF-β at early stage is essential to the reduction of scarring, because the initial dose of TGF-β can trigger the cascade of TGF-β overproduction in wounds via its chemotaxis and autoinduction, which stimulates fibroblast proliferation and collagen production, inhibits matrix degradation and eventually leads to scar formation [Shah *et al.*, 2000].

Antagonizing TGF-β effects in wounds can be conducted at either protein or gene level. Neutralizing wound TGF-β with an antibody proves to be a simple and effective method. Shah *et al.* injected a polyclonal neutralizing antibody to TGF-β1,2 into full-thickness cutaneous wounds of adult rodents just prior to wounding or within 24 hours of wounding and repeated daily for two days post-wounding. This approach has successfully reduced the wound scarring without reduction in the wound tensile strength. The study has also shown that the treated wounds had reduced inflammatory reaction and decreased angiogenic response. Furthermore, the treatment could improve the architecture of the neodermis, which closely resembled the normal dermis [Shah *et al.*, 1992; Shah *et al.*, 1994]. Interestingly, the treated wounds produced less TGF-β1 than the control wounds as evaluated with immunohistochemistry, suggesting that the early neutralization of wound TGF-β can inhibit TGF-β1 autoinduction and reduce inflammatory and angiogenic response [Shah *et al.*, 1994]. Shah *et al.* later demonstrated that adding TGF-β3 in the wounds was helpful to the reduction of wound scarring, indicating that different TGF-β isoforms might have different roles in wound scarring [Shah *et al.*, 1995].

The second way of manipulating wound TGF-β effects at protein level is the use of TGF-β antagonists. Several different molecules are found to be able to bind TGF-β ligands and block their biological effects. Decorin is a small chondroitin/dermatan sulfate proteoglycan [Fransson *et al.*, 2000]. The core protein fragment (Leu155-Val260) of decorin binds TGF-β [Schonherr *et al.*, 1998] and inhibits TGF-β mediated biological effects [Zhao *et al.*, 1999; Abdel-Wahab *et al.*, 2002]. Border *et al.* injected decorin molecules intravenously and successfully inhibited the fibrotic process of glomerulonephritis, in which TGF-β also plays an important role in the pathogenesis [Border *et al.*, 1992]. Biglycan is another small proteoglycan that is able to bind TGF-β. Nevertheless, it may regulate TGF-β's effect *in vivo* differentially from decorin [Kolb *et al.*, 2001]. Soluble forms of TGF-β receptors I and III (betaglycan) have been found in different cell types as a result of natural process of mRNA splicing [Choi 1999; Andres *et al.*, 1989]. Because the binding regions for TGF-β ligands are located at the extracellular domains, the soluble receptors remain able to bind their ligands but fail to signal intracellularly, and thus can serve as TGF-β antagonists. Since TGF-β receptor II initiates the ligand binding and the intracellular signaling, most studies used recombinant soluble TGF-β receptor II as an antagonist to neutralize various TGF-β effects [Lammerts *et al.*, 2002; Tsang *et al.*, 1995; Zheng *et al.*, 2000; Wang *et al.*, 1999a]. The recombinant soluble receptor II is usually fused to the Fc region of a human immunoglobulin [Ruocco *et al.*, 1999; Smith *et al.*, 1999a] to maintain its solubility.

The third approach is to block TGF-β activation. Natural TGF-β is secreted as a biologically latent form, which can be activated by transient acidification, proteolysis, and chaotropic agents [Brown *et al.*, 1990]. Since TGF-β can be activated via the binding of mannose-6-phosphate (M6P) residues on LAP to the M6P receptors on cell surface [Harpel *et al.*, 1992], excessive M6P injected into wounds can compete with LAP and inhibit the activation of wound

TGF-β, and thus reduce scarring [McCallion and Ferguson 1996]. An antibody to LTBP or to transglutaminase and transglutaminase inhibitor may also inhibit the activation of latent TGF-β [Kojima *et al.,* 1993].

Manipulation of wound TGF-β effects can also be conducted at gene level. The strategy of antisense oligonucleotide was first tried to reduce wound scarring. Choi *et al.* injected antisense TGF-β1 oligonucleotides into adult mouse wounds and demonstrated that the treated wounds had much less scarring than the control wounds where sense TGF-β1 oligonucleotides were injected [Choi *et al.,* 1996]. However, this approach is not likely to neutralize the initial amount of wound TGF-β released by the degranulating platelets. Another approach can be the introduction of therapeutic genes into wounds to antagonize TGF-β' effects. As described above, several natural molecules like decorin and soluble TGF-β receptors can serve as the antagonists of wound TGF-β. Therefore, a gene expression vector carrying the cDNA that encodes one of these molecules can be transfected into the cells of a wound to express an antagonist protein and thus inhibits TGF-β's effects. The additional approach is to block the TGF-β signaling pathways of wound cells, which renders them unable to response to wound TGF-β. Yamamoto *et al.* applied a strategy of blocking TGF-β signaling at the receptor level to the deletion of TGF-β mediated diverse effects [Yamamoto *et al.,* 1996]. To achieve this goal, the cDNA coding region of the kinase domain of TGF-β receptor II was deleted to form a truncated receptor II. When overexpressed, the truncated receptor II can compete with the wild-type receptor II for the ligand binding, and thus blocks the signaling by its dominant negative effect. As a result, the overexpressed mutated receptor II in endothelial cells and mink lung epithelial cells completely deleted TGF-β's effect on inhibiting ectodermal cell proliferation. It also abrogated the effect on enhancing collagen production in smooth muscle cells [Yamamoto *et al.,* 1996]. Recently, the same approach has been applied to our keloid research. The result showed that blocking TGF-β signaling at the receptor level could significantly down-regulate endogenous TGF-β1 gene expression (Fig. **2**) [Liu *et al.,* 2002]. In addition, keloid cells proliferated much more slowly when their TGF-β signaling was blocked, suggesting that this approach may disrupt TGF-β1 autoinduction in keloid fibroblasts and inhibit TGF-β's effect on keloid cell proliferation.

TGF-β signaling can also be disrupted more specifically at Smad signaling molecules. It has been found that collagen production in dermal fibroblasts stimulated by TGF-β is mainly mediated by Smad3 [Chen *et al.,* 2000], while fibronectin synthesis is mediated by a c-Jun N-terminal kinase-dependent, Smad4-independent pathway [Hocevar *et al.,* 1999]. The advantage of targeting a specific Smad may diminish TGF-β mediated undesired effects like collagen overproduction, whereas maintain the beneficial effect, such as fibronectin mediated wound epithelialization [Garat *et al.,* 1996].

To explore the role of Smad proteins in wound healing, Ashcroft *et al.* used Smad3 knock-out mice for the wound healing study. In an incisional wound model, they found that the absence of Smad3 molecule in the wounds caused fast re-epithelialization. In addition, significantly reduced local infiltration of monocytes was observed in the wounds

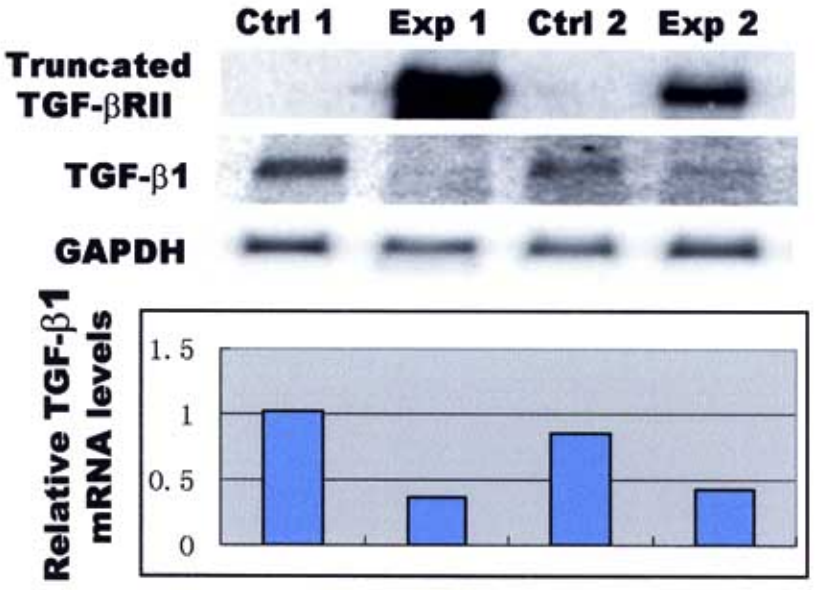

Fig. (2). Overexpressed truncated TGF-β receptor II down-regulates TGF-β1 gene expression in keloid fibroblasts. Keloid cells were treated either with β-galactosidase adenovirus (Ctrl) or truncated TGF-βRII adenovirus (Exp). Cells were harvested at day 5 post-viral infection for RNA extraction. A Northern Blot assay was performed to analyze the gene expression of TGF-β1. The relative mRNA expression level was determined by dividing the expression level of TGF-β1 with GAPDH expression level.

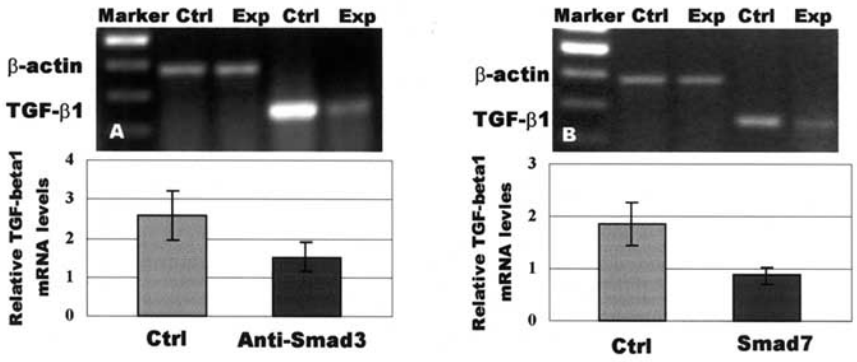

Fig. (3) Transfection of antisense Smad3 cDNA (A) or sense Smad7 cDNA (B) down-regulates endogenous TGF-β1 gene expression in NIH3T3 fibroblasts. NIH3T3 cells were transfected with either antisense Smad3 or sense Smad7 cDNA as the experimental cells (Exp) or transfected with empty pcDNA3 vector DNA as control cells (ctrl). RT-PCR assays was used for semi-quantitative analysis of relative TGF-β1 gene expression and the experiment was performed three times. The relative mRNA expression level was determined by dividing the expression level of TGF-β1 with β-actin expression level. Paired t-test indicates a significant difference in relative TGF-β1 expression level between two groups ($p<0.05$).

due to the blunted chemotactic response of these cells to TGF-β. As a result, much less matrix was produced and deposited in the wounds, which led to a narrow wound area. This result provides direct evidence for the first time that Smad3 is involved in wound repair via modulation of keratinocyte and monocyte function *in vivo* [Ashcroft *et al.*, 1999]. The same group also showed that ionizing radiation caused less TGF-β production and less infiltration of inflammatory cell in the skin of Smad3 null mice than in the skin of control mice, suggesting that the inhibition of Smad3 function might decrease tissue damage and reduce fibrosis after exposure to ionizing irradiation [Flanders *et al.*, 2002].

Recently, we have transfected the antisense Smad3 cDNA into NIH3T3 cells and found that the overexpressed antisense mRNA could down-regulate endogenous TGF-β1 gene expression (Fig. **3a**). Additionally, the proliferation of transfected cells was also significantly inhibited when compared with the control cells that were transfected with an empty vector DNA (Fig. **4a**). Since Smad7 serves as a negative regulator of TGF-β signaling, we have also transfected the full-length sense Smad7 cDNA into NIH3T3 cells. The results demonstrated that the overexpressed Smad7 functioned similarly to antisense SMAD3 in down-regulating TGF-β1 gene expression (Fig. **3b**) when compared with the control cells transfected with an empty vector DNA. Likewise, the cell proliferation of Smad7 transfected cells were significantly inhibited as compared to the control cells (Fig. **4b**). These data indicate that blocking TGF-β signaling at Smad level can modify cell behaviors. As described earlier, evidences suggest that the aberrant growth of keloid is closely related to TGF-β overproduction [Lee *et al.*, 1999; Bettinger *et al.*, 1996; Younai *et al.*, 1994] and its abnormal signaling [Chin *et al.*, 2001], therefore we are using the above strategy to genetically modify the TGF-β signaling in keloid fibroblasts and hope to find out an effective way to change the aberrant behaviors of keloid cells.

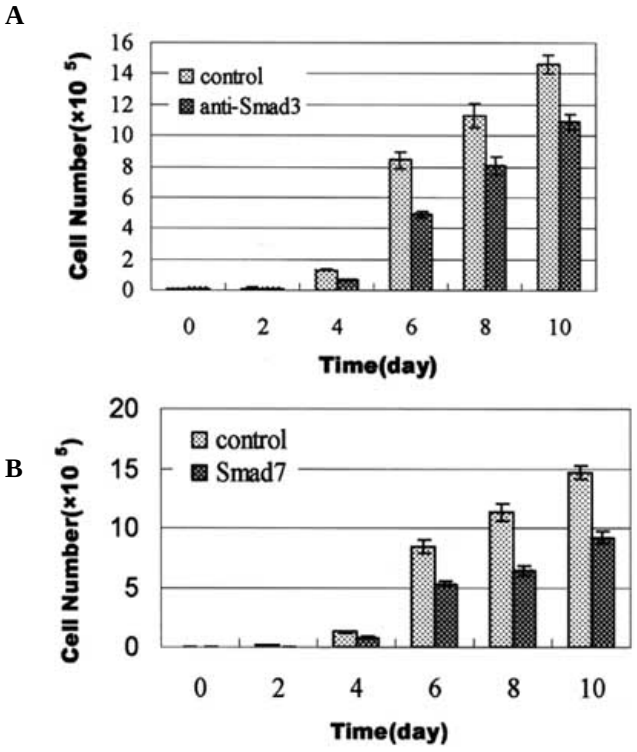

Fig. (4). Transfection of antisense Smad3 cDNA (A) or sense Smad7 cDNA (B) inhibits NIH3T3 cell proliferation. Transfected cells were seeded on 6-well culture plates with a density of $5x10^3$/well. Cells from triplicate wells were harvested at the time points of days 2, 4, 6, 8 and 10 post-seeding for cell counting. The experiment was performed twice and paired t-test demonstrated a significant difference of cell numbers at each time point between control and antisense Smad3 transfected cells ($p<0.05$), and between control and sense Smad7 transfected cells ($p<0.05$).

GENE THERAPY VECTORS AND ANIMAL MODEL

Gene transfer vectors include both viral and non-viral gene delivery vehicles. Currently, the available viral vectors are retrovirus, herpes simplex virus, adeno-associated virus and adenovirus, etc. Generally, viral vectors have much higher efficiency of gene transfer than non-viral vectors.

The most commonly used recombinant retroviral vector is derived from Moloney murine leukemia virus [Mulligan, 1993]. The recombinant virus is constructed by replacing the viral genes of *gag*, *pol* and *env* with a therapeutic gene and therefore, is replication-deficient. For wound healing application, retrovirus is more often used to transduce skin cells *in vitro*, such as the transfer of human growth hormone gene into keratinocytes to express a biologically active hormone [Morgan *et al.*, 1987]. The genes of other growth factors [Ghahary *et al.*, 1998; Eming *et al.*, 1998] have also been transfected into keratinocytes and the transduced cells were used to construct artificial skin for *in vivo* transplantation [Supp *et al.*, 2000]. However, the conventional retrovirus only infects rapidly dividing cells, and therefore may not be able to acquire the gene transfer efficiency that is high enough to achieve a therapeutic effect of anti-scarring. A recently developed retroviral vector system based on human immunodeficiency virus (lentiviral vector) is able to transduce both dividing and non-dividing cells [Naldini *et al.*, 1996], and thus may provide a more powerful retroviral vector for wound gene transfer.

As mentioned above, the initial dose of TGF-β released by the platelets in early stage wound is important for scar formation and scar gene therapy should focus on targeting TGF-β in early wound stage. Thus, a transient intervention of the functions of TGF-β ligands and their receptors is more preferable than to permanent knock-out of the functions. From this point, retrovirus is not an optimal vector for scar gene therapy. Besides, insertional gene mutation of transduced cells caused by retrovirus may also become a major concern for its application in scar gene therapy. However, retroviral vector such as lentivirus may potentially be used for controlling keloid growth since it is a long-term disease. Keloids usually grow unlimitedly, invade and expand continually into the surrounding normal tissue. Using retrovirus to introduce a therapeutic gene, such as Smad7 or truncated TGF-β receptor II, into keloid tissue may control keloid growth and its invasion and expansion. As other anti-tumor gene therapies mediated by retroviral vectors in clinical trials, this approach may still be beneficial for keloid (benign and uncontrolled tumor) treatment even the potential risk of insertional mutagenesis exits.

Herpes simplex virus and adeno-associated virus (AAV) are also the commonly used vectors for gene therapy. However, their application in wound healing is very limited [Deodato *et al.*, 2002]. This is possibly due to the fact that the former targets more specifically to neuron [Ozuer *et al.*, 2002] and the latter may cause DNA integration [Linden *et al.*, 1996]. Thus, AAV is possibly more suitable for long-term expression of transfected genes.

In contrast, adenovirus is the most frequently used viral vector in wound healing research because of its high gene transfer efficiency and its ability to target a wide spectrum of cell types, either dividing or non-dividing cells. Jaakkola *et al.* demonstrated that adenovirus could mediate gene expression in the cells located at epidermal, dermal and subcutaneous areas and maintained the gene expression up to 10 days, indicating that adenovirus can serve as an ideal gene transfer vector for wound gene therapy [Jaakkola *et al.*, 2000].

Recombinant adenovirus is replication-deficient due to the deletion of its E1 and E3 genes. The major concern of using adenovirus is its immunogenecity and the unpredictable adverse effects derived from host immune response. Although the E1 and E3 genes are deleted in recombinant adenovirus, the remaining viral genes can produce other viral proteins and trigger a strong inflammatory response when adenoviruses are administered *in vivo*. This immune reaction will eliminate injected viruses and is considered responsible for *in vivo* transient gene expression mediated by adenoviral vector [Yang *et al.*, 1996]. Nevertheless, adenovirus remains to be a favorite viral vector for wound gene therapy because a transient, rather than long-term gene expression is preferred. Liechty *et al.* found that adenovirus could mediate LacZ gene expression in adult rabbit cutaneous wound and the expression lasted up to two weeks [Liechty *et al.*, 1999a]. Although *in vivo* administration of adenovirus (LacZ) could slightly impair the healing of an ischemic wound when compared with the vehicle control wound, adenovirus-mediated VEGF expression in an ischemic wound could override this undesired effect and promote wound healing much better than the control vehicle [Liechty *et al.*, 1999b]. In a human skin SCID mouse model, Sylvester *et al.* found that injection of adenovirus into human skin did not interfere normal would healing process, although it caused acute inflammation [Sylvester *et al.*, 2000].

However, adenovirus induced inflammation is not preferable for the study of scar gene therapy because it will recruit more TGF-β producing cells into wounds and increase wound scarring. To conquer this undesired effect, we have developed a newborn rat incisional wound model by taking the advantage that a relatively immature immune system of newborn rats may not elicit a strong immune response to the injected adenoviruses. In addition, the newborn rats may heal their incisional wounds faster than adult rats, and thereby close the wounds before the injected adenoviruses lose their gene expression [Liu *et al.*, 2000]. Our study showed that in unwounded skin of 10-day-old newborn rats, injected adenoviruses mediated their gene expression as early as day 1 post-injection and reached a peak level at day 3 post-injection. The gene expression lasted up to day 7 post-intradermal injection or up to day 9 post-subcutaneous injection, while the incisional wound healed at day 7 post-wounding. Interestingly, the injected adenoviruses did not elicit a strong immune response because no obvious infiltrated inflammatory cells were observed at the virus-injected dermis, even at day 3 post-injection when the exogenous gene expression reached a peak level. Grossly, no obvious side effect was observed in the injected newborn rat skin [Liu *et al.*, 2000]. In contrast, skin edema was formed in the injected skins of adult rats.

In vivo administration of adenoviruses more than once usually induces a severe immune response and thus prevents

the repeated use of adenoviral vector [Ilan *et al.*, 1998]. This phenomenon was also observed in our model system when the adenoviruses were injected at the second time, 7 days after the first injection, suggesting that new versions of adenoviruses with less immunogenecity might be the optimal tools for cutaneous gene therapy. To overcome the shortcomings of first-generation adenovirus, new adenoviral vectors have been developed to reduce their immunogenecity and cytotoxicity and to prolong their transgene expression by deleting most of the viral genes, and thus are called "gutless" adenoviruses [Morsy *et al.*, 1998]. When prepared, the recombinant vectors are supplied *in trans* with structural proteins required for viral packaging and rescue, and therefore are also called helper-dependent adenovirus [Ehrhardt and Kay 2002]. Due to the less immunogenecity, the "gutless" adenovirus may be more suitable for scar gene therapy study in adult animals.

Gene transfer using non-viral vector such as plasmid DNA can avoid the undesired pathogenic effects, such as induced inflammation and insertional mutagenesis, derived from the viral vectors. The muscle tissue was found to be the best tissue target for naked DNA injection [Wolff *et al.*, 1990]. Gene expression in skin tissue following direct plasmid DNA injection has been observed in different animals [Hengge *et al.*, 1995]. Although the gene transfer efficiency is much lower than that of viral vectors, the study by Dupre *et al.* demonstrated that the expression level of a transgene in skin mediated by naked DNA injection was higher enough to elicit a significant biological response, such as DNA vaccination [Dupre *et al.*, 2001]. Meuli *et al.* have shown that the surgically wounded mouse skin could be transfected by local injection of plasmid DNA and the gene expression could be maintained up to 2 weeks post-injection. Additionally, when injected locally into the wound, the plasmid containing the gene encoding G-CSF (granulocyte colony stimulating factor) could produce a high level and activity of the cytokine [Meuli *et al.*, 2001], indicating that intradermal injection of plasmid DNA might be useful for anti-scarring gene therapy.

In order to enhance the transferred gene expression mediated by naked plasmid DNA, physical methods or chemical compounds have also been applied to the increase of gene transfection rate. Microseeding or puncture-mediated gene transfer [Eriksson *et al.*, 1998; Ciernik *et al.*, 1996] and gene gun [Nanney *et al.*, 2000; Eming *et al.*, 1999] have been developed for delivering naked plasmid DNA into skin. Compared with DNA direct injection and gene gun mediated gene transfer, microseeding can achieve a much higher gene expression level [Eriksson *et al.*, 1998]. Chemical compounds like cationic liposome have also been used to improve the gene transfer efficiency when mixed with plasmid DNA [Sun *et al.*, 1997; Taub *et al.*, 1998]. The major concern of using liposome is its cytotoxicity. Recently developed polymer-based gene delivery system can enhance the transfection efficiency of plasmid DNA with low or no cytotoxicity [Putnam *et al.*, 2001]. Collectively, the use of helper-dependent adenovirus or non-viral vectors may avoid the inflammatory reaction induced by first generation adenovirus and can be applied to the study of anti-scarring gene therapy in adult animal models.

EXPERIMENTAL STUDY OF SCAR GENE THERAPY

Compared with protein therapy, gene therapy is less expensive in cost and more effective in treatment. In addition, gene therapy is able to provide an appropriate dose of growth factors or recombinant proteins in wounds, and thus has a great potential for clinical application [Yao and Eriksson 2000]. Once the gene expression vector is constructed, either the viral vector or the plasmid DNA is easy to produce and purify in a large scale. The most important advantage of using gene expression vectors is their ability to maintain a relatively long period of gene expression in wounds and therefore, repeated injections within a short time period, which might interfere with the normal process of wound healing, will not be necessary.

Cutaneous scar gene therapy remains to be an unexplored area in the field of gene therapy and has only few reports [Choi *et al.*, 1996; Liu *et al.*, 2001]. We have recently applied the strategy of blocking TGF-β signaling [Yamamoto *et al.*, 1996] to the scar gene therapy using the newborn rat incisional wound model and the recombinant adenovirus carrying a gene of truncated TGF-β receptor II (Ad-tTGF-βRII). In ten-day-old Sprague-Dawley rats, 1×10^9 plaque forming units (pfu) of Ad-tTGF-βRII were injected intradermally into the right side of dorsal skin as the experimental group, and the same dose of recombinant adenoviruses carrying a LacZ gene or the same volume of phosphate buffered saline was injected at the left side skin of the same rats as the control group. A full-thickness incisional wound deep to and through the panniculus muscle was created at the injected area of both sides at day 2 post-injection, because the previous study showed that the expression of transferred gene mediated by adenovirus approached to a peak level 2 days after injection [Liu *et al.*, 2000]. The wounds were allowed to heal by themselves.

The result of the experiment showed that the wounds transfected with truncated TGF-βRII gene had less infiltration of inflammatory cells than control wounds when observed at day 3 post-wounding. In addition, re-epithelialization completed in the experimental wounds but not in the control wounds at day 3. Furthermore, the gene-transfected wounds contained less TGF-β1 than the control wounds when examined with immunohistochemistry at day 3. At day 14 post-wounding, the experimental wound healed with much less scarring than its control wound in each of the rats (n=11) (Fig. **5a** and **5b**). Quantitative analysis of wound scarring using Image Pro-Plus software (Media Cybernetics) showed that an average of 49 percent reduction of wound scar area was achieved in the experimental wounds as compared with the control wounds, a statistically significant difference ($p<0.01$) between the experimental and the control wounds (Fig. **5c**).

To determine the mechanism, we have also performed an *in vitro* assay. When infected with Ad-tTGF-βRII, the dermal fibroblasts could overexpress the truncated TGF-β receptor II gene, which dramatically down-regulated TGF-β1 gene expression. How overexpressed truncated receptor II regulates the gene and the protein expression of the other two TGF-β isoforms is unknown. It is, however, highly possible that the autoinduction of the other two isoforms

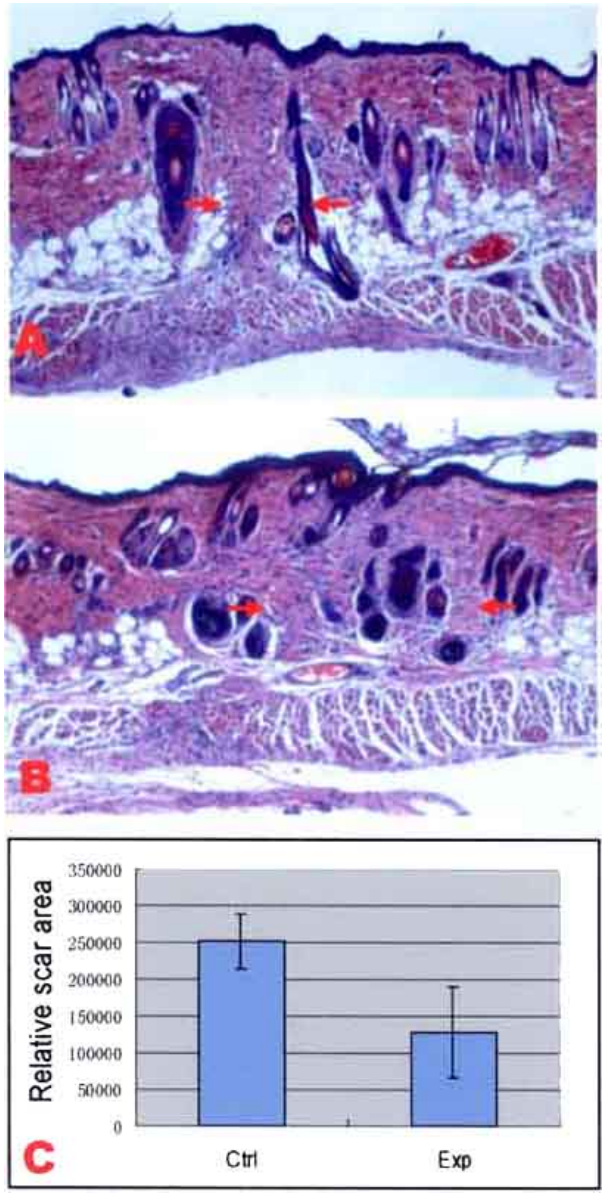

Fig. (5). Adenovirus-mediated overexpression of truncated TGF-β receptor II inhibits wound scarring in newborn rats. Truncated TGF-βRII adenoviruses (1×10^9 pfu) and β-gal adenoviruses (1×10^9 pfu) were intradermally injected into the right and the left side of 10-day-old SD rat dorsal skin respectively. A 0.5cm long full-thickness incisional wound deep to panniculus muscle was created at the injected sites two days post-injection. Wound tissues were harvested at day 14 post-wounding for histological examination. Quantitative analysis shows a 49 percent decrease of wound scar area in the experimental wound as compared to the control wound area, and the difference is statistically significant ($p<0.01$). (A) Experimental wound; (B) Control wound. (H&E staining, original magnification ×100); (C) Quantification of scar area.

will be blocked as well because all three isoforms bind the same TGF-β receptors. Thus, blocking multiple isoforms' effect may contribute to the reduction of wound scarring. Based on the *in vitro* and *in vivo* data, we hypothesize the possible mechanism of this anti-scaring gene therapy approach. When a wound is created, the initial dose of TGF-βs released by the platelets immediately bind the overexpressed truncated type II receptors and become unavailable to other cells. Thus, their chemotactic effect is blocked, which leads to the reduced infiltration of TGF-β producing cells, such as macrophage and monocytes, and the significant reduction of wound TGF-βs. On the other hand, the bound TGF-βs also lose their ability to induce proliferation, collagen production and TGF-β autoinduction of transduced wound cells because of the blocked signaling. All these effects eventually lead to the reduced scaring.

However, there are several factors that might affect the scar reduction level in this approach. Firstly, the overexpressed truncated TGF-β receptor II remains cell membrane bound, therefore, part of the wound cells which are not transduced remain able to respond to TGF-β's effect on cell proliferation, collagen production and TGF-β autoinduction. Secondly, the overexpressed truncated receptor II may block the functions of all three TGF-β isoforms, and thus also blocks the possible anti-scarring effect of TGF-β3. Thirdly, the time of gene expression mediated by conventional adenovirus is still relatively short even in this newborn rat model, possibly due to the immunogenic reactivity. The use of new generation adenoviral vectors may prolong the gene expression time and further enhance the anti-scarring effect.

Although this study was performed in the newborn rat model, the strategy of anti-scarring via blocking TGF-β signaling developed by this study can be applied to the study in adult animal when "gutless" adenovirus or plasmid DNA is used as a gene transfer vehicle. More importantly, anti-scarring gene therapy studies in an animal model that closely mimic human hypertrophic scar [Aksoy *et al.*, 2002] or keloid may provide more valuable information for future clinical application.

SUMMARY

Wound scarring remains difficult to treat clinically and is considered inevitable conventionally. However, fetal wound healing studies demonstrate that scar formation is not the inevitable result of healing mammalian wounds if adult wound healing can be modified to a healing process resembling fetal wounds. Difference in the amount of wound TGF-β is one of the most distinctive features between fetal and adult wounds, and thus wound TGF-β may be a key target for wound gene therapy in order to reduce scarring. Gene transfer of TGF-β antagonists and the blockers of TGF-β signaling may serve as good strategies for scarless healing. Furthermore, the use of helper-dependent adenovirus and physically or chemically assisted plasmid DNA transfer may further enhance the cutaneous gene transfer efficiency and improve the anti-scarring effect of gene therapy. Finally, further effort should be made to bridge the gap between the animal study and the clinical application.

MATERIAL AND METHODS

Cell Culture

Human normal dermal fibroblasts, human keloid fibroblasts and NIH 3T3 cells were cultured in Dulbecco's modified Eagles's medium (DMEM; Gibco-BRL, Grand Island, N.Y.), 10% fetal bovine serum (FBS), 1% penicillin-streptomycin at 37°C in a humidified CO_2 incubator with 5% CO_2. When reaching confluence, cells were subcultured at a ratio of 1:3 with the treatment of 0.05% trypsin-ethylenediamine tetraacetic acid (EDTA; Gibco-BRL). Human fibroblasts of low passages were used in this study.

Amplification and Purification of Recombinant Adenoviruses

Recombinant adenoviruses of truncated TGF-β receptor II (Ad-tTGF-βRII) and β-galactosidase adenovirus (Ad-β-gal) were kindly provided by Dr. H. Ueno of Kyushu University, Japan [Yamamoto *et al.*, 1996]. Adenovirus were amplified in 293 cells and purified by cesium chloride gradient centrifugation as previously described [Liu *et al.*, 2000].

TGF-β 1 Autocrine Regulation

When cells reached 90% confluence, culture medium was replaced with serum-free DMEM medium containing human recombinant TGF-β 1 (*rh*TGF-β 1, 5ng/ml, R&D, Minneapolis, MN). Cells without *rh*TGF-β 1 treatment served as controls. Both treated and non-treated cells were harvested at the time points of 3h, 6h, 12h and 24 h post-*rh*TGF-β 1 treatment, respectively. Total RNA was extracted with Trizol (Gibco-BRL) as previously described [Hsu *et al.*, 2001].

Blocking TGF-β Signaling

When cultured fibroblasts reached 90% confluence, culture medium was removed and serum-free DMEM medium containing Ad-tTGF-βRII (100 pfu/cell) was added and incubated for 1 hour at 37°C to completely infect cells. Virus containing medium was then removed and DMEM+10%FBS was added and incubated for three days to overexpress the transfected gene. After changing medium, cells were incubated for another two days and then collected for RNA extraction. In control group, Ad-β-gal (100 pfu/cell) was added and the rest of the procedure was similar to that of the experimental cells.

Probe Preparation

TGF-β 1 was a full-length human cDNA from a plasmid (ATCC#59954). TGF-β receptor II extracellular probe was a 500 base pair fragment from a plasmid kindly provided by Dr. R.G. Wells [Wells *et al.*, 1997]. Each probe (100 ng) was labeled with 50 μCi of α-[P^{32}]-deoxycytidine triphosphate as previously described [Hsu *et al.*, 2001].

Northern Blot Analysis

Northern blot analysis was performed as previously described [Hsu *et al.*, 2001]. Gene expression was quantified by densitometry using Image Quant image analysis software (Molecular Devices). The relative mRNA expression level was determined by dividing the expression level of interested gene with GAPDH expression level.

Animals

Ten-day-old postnatal Sprague-Dawley (SD) rats were used in this study. The experimental protocols of all studies were approved by the Animal Experiment Committee of Shanghai Second Medical University.

In Vivo Gene Therapy

For gene therapy study, 1×10^9 pfu of Ad-tTGF-βRII were intradermally injected at the right side dorsal skin of the newborn SD rats as the experimental group (n=15). At the left side of the same rat, either 1×10^9 pfu of Ad-β-gal (n=11) or equal volume of phosphate buffered saline (n=4) was injected as the control group. At day 2 post-injection, a 5mm-long full thickness incisional wound deep to and including the panniculus muscle was made on the injected skins and the wound was left unrepaired as previously described [Liu *et al.*, 2000]. Animals were sacrificed at days 3 (n=2), 7(n=2) and 14(n=11) to harvest wound tissues for histological examination and immunohistochemistry.

Scar Area Quantification and Statistical Analysis

After being stained with H&E, tissue sections of day 14 wounds were observed under the microscope (Nikkon, Japan) at a fixed magnification (×40), and the images were digitally recorded into the computer by Image-Pro Plus system (Media Cybernetics, Silver Spring, MD). The relative area of wound scar below the epidermis and above the panniculus muscle was measured and automatically recorded by the Image-Pro Plus software (Media Cybernetics). Paired t-test was used to analyze the difference in scar area between the experimental wounds and the control wounds. A *p*-value less than 0.05 was considered as statistically significant.

Plasmid Construction and Gene Transfection

Human Smad3 cDNA was kindly provided by Dr. Y. Zhang [Zhang *et al.*, 1996], which shares 70 percent homology with murine counterpart. Briefly, full-length human Smad3 cDNA was excised from the original plasmid and then cloned into pcDNA3 vector (Invitrogen) in an antisense orientation. The plasmid of full-length human sense Smad7 cDNA (80 percent homology with murine counterpart) in pcDNA3 vector was kindly provided by Dr. S.J. Chen [Chen *et al.*, 1999]. For transfection, NIH3T3 cells were grown in 6 well-plates and cells were transfected with either antisense Smad3 plasmid or sense Smad7 plasmid by Fugene 6 (Roche), respectively as the experimental groups. In the control groups, empty pcDNA3 vector DNA was transfected into NIH3T3 cells. All transfected cells were selected by G418 (300μg/ml) to obtain stable transfected cells. The mRNA expression of transfected cDNA was confirmed by RT-PCR using designed primers that amply human Smad mRNAs.

NIH3T3 Cell Proliferation Assay

After selection with G418, Smad transfected cells and vector transfected control cells were subjected to a

proliferation assay. Briefly, both experimental and control cells were seeded on 6-well culture plates (BD Falcon) with a density of $5x10^3$/well. Cells from triplicate wells were harvested at the time points of days 2, 4, 6, 8 and 10 post-seeding. Cells from each well were counted twice to get an average cell number. The triplicate numbers were averaged to get mean and standard deviation. The experiment was performed twice. A paired t-test was used for statistical analysis and a p-value less than 0.05 was considered as statistically significant.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed to detect mRNA expression of transfected Smad cDNAs and to determine their effects on regulating TGF-β1 gene expression of transfected NIH3T3 cells. Total RNA was extracted from control and experimental cells and subjected to RT and PCR reactions using a RT-PCR kit (Takara, Japan) and a T3 thermocycler (Biometra). Briefly, 2μg of RNA was used for reverse transcription by incubating the samples at 30°C for 10min, 42°C for 60min, 99°C for 5min and 5°C for 5min. Aliquots of the resulting cDNAs were then subjected to PCR amplification in the presence of specific primers, and the reactions were performed at 95°C for 2min, in a thermal cycle of 95°C for 1min, 60°C for 30s and 72°C for 1.5min for total 28 times, then stopped at 72°C for 10min. For Smad7, the annealing temperature was designed as 62°C. The sequences of the primers are listed as the followings. Smad3 primers (upstream:5'-ctaacttccccgcaggcatcgag-3'; down-stream: 5'-gagggcagcgaactcctggttgttg-3') detected a 563bp fragment of expressed antisense human Smad3 mRNA; Smad7 primers (upstream: 5'-caactgcagactgtccagatg-3'; downstream: 5'-ctgctgcataaactcgtggtc-3') detected a 503bp fragment of expressed human Smad7 mRNA. TGF-β1 primers (upstream: 5'-gaagtggatccacgagcccaag-3'; down-stream: 5'-gctgcacttgcaggagcgcac-3') detected a 247bp fragment of TGF-β1 mRNA expression. β-actin primers (upstream: 5'-ctgggacgacatggagaag-3'; downstream: 5'-ctcagctgtggtggtgaag-3') detected a 382bp fragment. To quantitatively analyze the effects of Smad transfection on TGF-β1 gene expression, the PCR bands in the gels were digitally recorded and quantified by Gel-Pro-Imager (Media Cybernetics, Silver Spring, MD). The relative mRNA expression level was determined by dividing the expression level of interested gene with β-actin expression level. Experiments were performed three times and the difference of relative TGF-β1 mRNA levels between the experimental and control groups was statistically analyzed with a paired t-test and a p-value less than 0.05 was considered statistically significant.

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