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Improving Wound Healing with Topical Gene Therapy

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Abbreviations and Acronyms

HIF-1α = hypoxia-inducible factor alpha
mRNA = messenger RNA
p53 = tumor protein 53
RNA = ribonucleic acid
SDF1 = stromal cell-derived factor 1
siRNA = small interfering ribonucleic acid
SMAD3 = mothers against decapentaplegic homolog 3
TGF-β = transforming growth factor beta
VEGF = vascular endothelial growth factor

Background: Impaired wound healing remains a major clinical problem with many etiologies. Altering gene expression to enhance healing is an innovative therapeutic approach. In recent years, we have developed a means to topically silence genes at the post-transcriptional level to locally alter wounds and improve the healing process.
The Problem: Many types of chronic wounds have been associated with alterations in the expression of genes that mediate healing. Targeting the expression of these genes in a way that can improve healing while limiting systemic side effects has been very challenging.
Basic/Clinical Science Advances: Our laboratory's recent work has focused on the use of topically applied small interfering ribonucleic acid (siRNA) to inhibit messenger RNA expression of certain mediators involved in healing in two different types of cutaneous injury—radiation-induced cutaneous injury and the diabetic excisional wound. By successfully inhibiting specific gene mediators with topical siRNA, we reversed downstream signaling pathways, which led to expedited wound healing in diabetic wounds and restoration to a more normal phenotype in radiation-induced skin injuries.
Clinical Care Relevance: The signaling pathways and gene mediators that we targeted and inhibited in murine models are present in humans. Applying parallel treatment strategies in humans may provide novel means of treating these burdensome and costly conditions.
Conclusion: Our novel method for local gene silencing is effective in treating various types of cutaneous murine wounds. Topical gene silencing with siRNA obviates the side effects of systemic medication and has the potential to be effective in healing or preventing a wide array of cutaneous human conditions.

BACKGROUND

Aberrations in the mechanisms of wound repair result in a wide spectrum of impaired healing. Grossly, pathologies can range from impaired wound healing as seen in persistent diabetic wounds, to a scarring phenotype as seen in radiation-induced skin fibrosis. Impaired healing conditions have been associated with distinct aberrations in messenger RNA (mRNA) and protein expression profiles. Therapies that could potentially target wound healing pathways at the cellular level are attractive.

Since the discovery of RNA interference in 1998,[1] and its subsequent application in human cell lines in 2001,[2] RNA interference through the use of small interfering ribonucleic acid (siRNA) has offered a new

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approach to manipulating gene expression. Briefly, siRNA is a double-stranded RNA homolog of a gene of interest that can be synthesized, modified, and administered to cells both in culture and *in vivo*. Once in the cytoplasm, siRNA interacts with RNA-induced silencing complexes, unwinds into single stranded RNA, and then degrades its endogenous mRNA target. This process "knocks down" the quantity of synthesized protein in the cytoplasm, which then alters any cellular signaling that involves the target gene. The specificity of siRNA therapy offers intriguing intervention modalities and we have focused its use in preventing radiation-induced skin fibrosis and in enhancing diabetic wound closure.

TARGET ARTICLES

1. Lee J, Tutela J, Zoumalan R, Thanik V, Nguyen P, Varjabedian L, *et al.*: Inhibition of Smad3 expression in radiation-induced fibrosis using a novel method for topical transcutaneous gene therapy. Arch Otolaryngol Head Neck Surgery 2010; **136:** 714.

2. Nguyen P, Tutela J, Thanik V, Knobel D, Allen R, Chang C, *et al.*: Improved diabetic wound healing through topical silencing of p53 is associated with augmented vasculogenic mediators. Wound Repair Regen 2010; **18:** 553.

CLINICAL PROBLEM ADDRESSED

Pathologic wound states are associated with a lower quality of life and are a common cause of morbidity and mortality in patients. Chronic diabetic wounds are often complicated by infection and sepsis and contribute significantly to healthcare cost. Also, radiation-induced fibrosis, a common adverse effect of cancer therapy, complicates subsequent surgical intervention, leads to strictures, and heals poorly. Impairments in healing in the aforementioned cutaneous injuries are currently understood, at least in part, on a cellular pathway level.[3–6] Thus, we focused our efforts on mitigating two different pathologic phenotypes by altering mRNA expression with siRNA specifically targeted to the genetic dysregulations, which characterize these pathways.

RELEVANT BASIC SCIENCE CONTEXT

Early damage in irradiated skin consists of erythema, desquamation, and ulceration, whereas more long-term effects include skin atrophy, decreased elasticity, fibrosis, microvascular damage, and impaired wound healing.[7] Transforming growth factor β (*TGF-β*) is a central regulator for the inflammation and fibrosis that results following radiation.[3,4] Recent studies have demonstrated that the fibrosis is largely mediated by an intercellular mediator, mothers against decapentaplegic homolog 3 (*SMAD3*), which is downstream to activation of the *TGF-β* receptor.[8] Once activated by the *TGF-β* receptor complex, *SMAD3* is transported into the nucleus, where it acts as a transcription factor for genes coding for multiple types of collagen and extracellular matrix proteins.[9] Recent studies revealed that Smad3 knockout mice are protected from radiation-induced cutaneous injury.[10] In our investigation, we attempted to achieve similar protective effects by knocking down *SMAD3* locally with topical siRNA to *SMAD3*. In addition, by limiting therapy to the epidermis and dermis, we attempted to obviate the untoward side effects of systemic gene knockdown.

In our second line of investigation, we aimed to improve wound closure in diabetic wounds. Recent advances in the understanding of the pathogenesis of diabetic wound healing correlated hyperglycemia, poor wound healing, and higher levels of apoptosis.[11] Hyperglycemia is associated with a diminished hypoxic response, which is critical in inducing and attracting the appropriate inflammatory mediators to a wound.[5] This hypoxic response is normally mediated by hypoxia-inducible factor alpha (*HIF-1α*), a transcription factor that plays a significant role in wound healing; *HIF-1α* is dramatically reduced in diabetic wound.[5] Studies have also revealed a role for the tumor suppressor gene *p53* in promoting the proteasomal degradation of *HIF-1α*.[12] Tumor protein 53 (p53) is responsible for both cell arrest and apoptosis.[6] As *p53* (and apoptosis) is increased during diabetic wound healing,[13] we postulated that locally silencing *p53* expression in the diabetic wound would inhibit apoptosis and stimulate the *HIF1-α* dependent pathways, ultimately leading to improved wound healing.

EXPERIMENTAL MODEL OR MATERIAL: ADVANTAGES AND LIMITATIONS

Murine skin is composed of epidermal and dermal architecture similar to human skin.[14] Further, the mechanisms of wound healing and scarring in mouse skin are also very similar to those in human skin.

One limitation to rodent wound models is that due to the laxity of their skin and the mobility of the underlying panniculus carnosus, their primary mechanism of wound healing is by epithelial con-

tracture healing (by primary intention). In contrast, large wounds in humans heal via re-epithelialization and granulation tissue (by secondary intention).[15] We circumvented this criticism by using our previously established splinted wound model[16] composed of a stent enforced with sutures around a given wound, thus preventing wound contracture and allowing healing by secondary intention.

With regard to siRNA, we have developed a topical agarose matrix-based siRNA delivery system that reproducibly delivers siRNA into the epidermis and through the dermis,[17] not penetrating beyond the dermis. One advantage of this is the fact that gene suppression occurs locally, thus obviating systemic delivery and undesirable side effects. Weekly application was necessary for a sustained effect as the molecules are active for 5–7 days.[17] Administering topical siRNA to an open wound has potential clinical applicability for human use. We have extended this technology for use on intact skin by disrupting the stratum corneum with a detergent, which then allows siRNA penetration. This current strategy is limited by the fact that the detergent used is irritating and painful to skin; nanoparticle-based delivery systems are being devised to circumvent this limitation. Ultimately, these technologies will require additional refinements in larger animal models, particularly the porcine model, before the consideration of human use.

DISCUSSION OF FINDINGS AND RELEVANT LITERATURE

Using a published model of skin-only radiation injury, topical *SMAD3* siRNA treatment before irradiation demonstrated near-complete inhibition of Smad3 in the epidermis and dermis by week 1 with an effect limited to the area of application on the skin. The area of *SMAD3* knockdown was limited to the treatment area. After weekly application of siRNA, *SMAD3* inhibition persisted and remained limited to the dermis. Figure 1 illustrates a schematic of topical siRNA application. After 4 weeks, quantitative measurements of epidermal thickness on histology revealed treated skin to be markedly thinner compared with controls. Histology also revealed siRNA-treated skin to have dermal collagen architecture similar to that of nonirradiated skin, whereas irradiated control specimens displayed dense collagen architecture. Moreover, a comparison of tissue elasticity quantified by tensiometry determined *SMAD3* siRNA-treated skin to be significantly more elastic than nonsense siRNA-treated controls, approaching the elasticity of normal, nonirradiated skin.

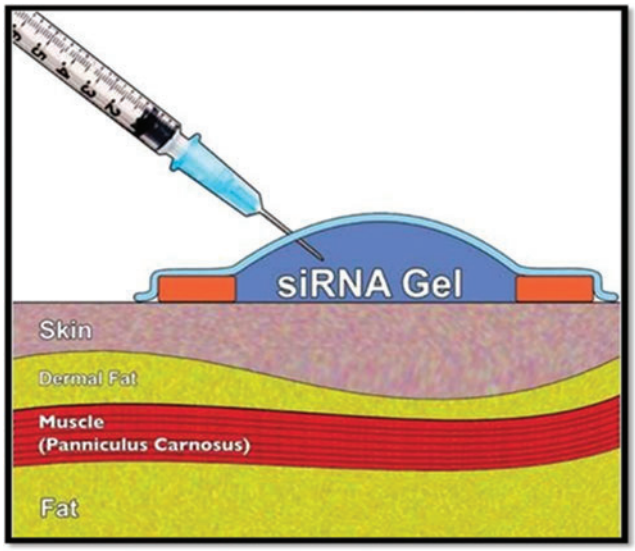

FIG. 1. A schematic of topical small interfering ribonucleic acid (siRNA) application on intact skin. A complex of siRNA with agarose is applied over a designated treatment area of skin. siRNA penetrates through the epidermis and has intracellular effects in the epidermis and dermis. Color images available online at www.liebertpub.com/wound

By inhibiting *SMAD3* expression in irradiated skin, downstream pathways responsible for collagen and extracellular matrix protein synthesis were attenuated and skin fibrosis following radiation was minimized.

In our *p53*-suppressed diabetic wound investigation, wounds received a complex of siRNA to *p53* in agarose on postwound days 1 and 8. Complete wound closure occurred in 18 days, and robust granulation tissue was already present at 10 days. In comparison, untreated diabetic wounds healed in 28 days (wild-type nondiabetic wounds healed in 14 days).

The level of *p53* mRNA was shown to be suppressed three-fourths in *p53* siRNA-treated wounds at 10 days postwounding. Immunohistochemistry showed near-complete knockdown of *p53* protein expression as well (Fig. 2). *p53* inhibition was accompanied with an increase in mRNA and protein expression of multiple vasculogenic cytokines at postwounding day 10. Specifically, there was a near 2-fold increase in *HIF1-α* and stromal cell-derived factor 1 (*SDF-1*) mRNA, and a 1.5-fold increase in vascular endothelial growth factor (*VEGF*) mRNA in p53 siRNA-treated wounds compared with control wounds. Parallel increases in *HIF1-α*, *SDF-1*, and *VEGF* protein expression were noted. Finally, *p53* siRNA-treated wounds demonstrated increased vascularity (CD31 staining). Illustrating the transient effect of siRNA, *p53* mRNA levels in healed wounds returned to low levels equal to controls at day 30. Moreover, rewounded formerly *p53*

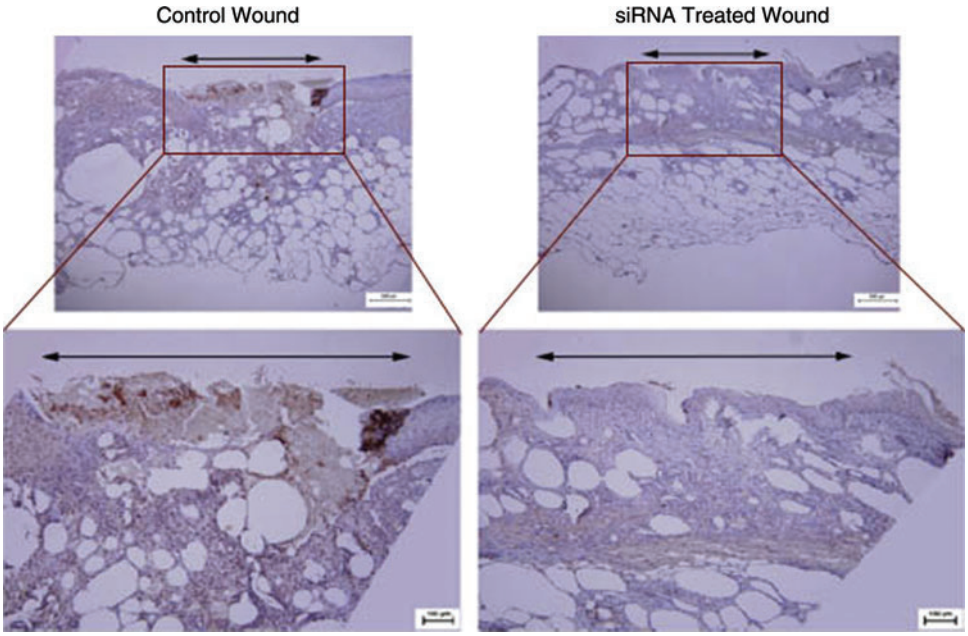

FIG. 2. Immunohistochemical staining for *p53*. Reduced expression of *p53* (brown staining) is demonstrated within the *p53*-silenced wound beds compared with control wound beds at postwounding day 10. Color images available online at www.liebertpub.com/wound

siRNA-treated wounds and nonsense siRNA-treated (control) wounds demonstrated similar levels of *p53* mRNA in their healing wounds at all healing time points.

By suppressing *p53* expression in a diabetic wound, expression of *HIF1-α*, *SDF-1*, and other important mediators in the hypoxic response increased and was associated with a more normal wound healing interval and phenotype.

Better understanding of normal and pathologic wound healing pathways will provide even more selective targets for siRNA therapy. Additionally, new approaches to siRNA delivery will improve local siRNA knockdown and broaden the therapeutic scope of siRNA to more systemic pathologies.

INNOVATION

In both investigation arms, a central cellular mediator was successfully suppressed and phenotypic pathologies in wound healing were reversed to a more normal phenotype. Our findings shed light on the potential diversity of clinical applicability of this therapeutic modality.

TAKE-HOME MESSAGE

Basic science advances

Wound healing pathologies are grossly and mechanistically diverse. Many cutaneous conditions have been previously associated with specific cellular aberrations. Thus, cutaneous diseases or poor wound healing conditions offer an excellent opportunity for the modification of gene expression through topically applied siRNA. We showed that targeting a single upstream modulator can be an efficient and effective way to alter the expression of a target gene and the expression of the downstream products in associated cellular pathways. With the proper target, these changes can restore intracellular and extracellular environments to those that more closely resemble the normal healing state. Further, these modifications are transient, localized to the treated area, and avoid off-target effects.

Clinical science advances

Strategic modulation of wound healing pathways using transcutaneous siRNA improves healing of cutaneous pathologies. More thorough understanding of the pathways involved in wound healing will improve our outcomes through more specific targeting of downstream modulators. Further, siRNA therapy involving multiple targets may prove to be more effective than the current single-target approach.

Relevance to clinical care

Improving wound healing remains paramount to improving patient quality of life and limiting morbidity and mortality. Local manipulation of molecular targets using topically applied siRNA can yield favorable wound healing outcomes in murine models. Work is required to assure minimal patient risk in future clinical trials that employ parallel treatment strategies.

Locally administered siRNA could potentially be used to downregulate any gene product of a known molecular target. This innovative technology can be applied to most skin pathology for which known genes are responsible, including contact hypersensitivity, autoimmune alopecia, psoriasis, scleroderma, and skin cancer ranging from basal cell carcinoma to squamous cell carcinoma to melanoma. For example, one candidate for local gene knockout with siRNA is an integrin-linked kinase, which regulates angiogenesis in melanoma[18]; in theory, downregulating the kinase would slow disease progression.

CAUTION, CRITICAL REMARKS, AND RECOMMENDATIONS

Although our findings supporting the use of transcutaneous siRNA for the modulation of pathologic wounds are promising, a better understanding of the cellular components of the biologic pathways of normal wound healing and various states of pathologic wound healing is necessary to better target siRNA therapy. Certainly, silencing a tumor suppressor gene such as *p53* even topically has theoretical risks and more selective downstream effectors of diabetic wound healing may represent more clinically relevant targets.

Finally, our transcutaneous siRNA model for intact skin (in the irradiated murine model) required a pretreatment regimen to disrupt the stratum corneum to allow siRNA penetration. Although effective, this process can be cumbersome and labor intensive. Less-invasive delivery systems need to be developed before this technology becomes more clinically applicable. Current efforts to circumscribe this problem include nanoparticle-based delivery systems.

FUTURE DEVELOPMENT OF INTEREST

Truly individualized medicine will begin to evolve as technologies improve. Therapies that can safely and effectively alter gene expression will play an important role in individualized medicine. One might imagine that in the future a patient with a specific condition may be able to have their genetic data analyzed, the etiology of their pathology determined, and a specific siRNA target molecule synthesized to abrogate their disease process.

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