Thermosensitive Hydrogel as a Tgf-β1 Gene Delivery Vehicle Enhances Diabetic Wound Healing

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Purpose. To accelerate diabetic wound healing with TGF-β1 gene delivery system using a thermosensitive hydrogel made of a triblock copolymer, PEG-PLGA-PEG.

Methods. Two 7 × 7 mm full thickness excisional wounds were created in parallel at the back of each genetically diabetic mouse. The hydrogel containing plasmid TGF-β1 was administered to the wound and formed an adhesive film *in situ*. Controls were either untreated or treated with the hydrogel without DNA. We used a commercial wound dressing, Humatrix®, either with or without DNA, to compare the therapeutic effect with the thermosensitive hydrogel.

Results. We found that thermosensitive hydrogel alone is slightly beneficial for reepithealization at early stage of healing (day 1–5), but significantly accelerated repithelializaion, increased cell proliferation, and organized collagen were observed in the wound bed treated with thermosensitive hydrogel containing plasmid TGF-β1. The accelerated reepithelialization was accompanied with enhanced collagen synthesis and more organized extracellular matrix deposition. Humatrix® alone or with plasmid TGF-β1, had little effect.

Conclusions. Thermosensitive hydrogel made of PEG-PLGA-PEG triblock copolymer provides excellent wound dressing activity and delivers plasmid TGF-β1 to promote wound healing in a diabetic mouse model.

KEY WORDS: gene therapy; TGF-β1; diabetic wound healing; triblock copolymer; thermosensitive hydrogel.

INTRODUCTION

Of the growth factors, TGF-β family members play a central role in tissue repair. The biologic activities of TGF-β1 in the wound healing process have been previously reported. Lanning *et al.* (1) showed TGF-β1 induces myofibroblast production, resulting in a significantly reduced wound in a noncontractile fetal rabbit model. Sidhu *et al.* (2) demonstrated TGF-β1 locally improved neovascularization, increased migration of myofibroblasts, fibroblasts, and macrophages and produced higher collagen content, resulting in an accelerated reepithelialization.

Exogenously administered growth factors can compensate for decreased expression of endogenous growth factors such as TGF-β1 (3) and PDGF (4), to overcome impaired wound healing in diabetes (5). We have recently shown that plasmid TGF-β1 delivered by intradermal injection of naked DNA effectively accelerated wound healing in a genetically diabetic mouse model (6). Accelerated collagen deposition and cell proliferation were observed in the plasmid TGF-β1 treated wound.

Use of biodegradable polymer implants to deliver naked DNA to muscle (7) and canine osteotomy model (8) results in a sustained transgene expression. However, persistent overproduction of growth factors may cause adverse effect. For example, transient TGF-β1 administration accelerates wound healing, yet persistent TGF-β1 administration causes excessive extracellular matrix component accumulation resulting in skin fibrosis (9).

In this study, we test the ability of a thermosensitive hydrogel to deliver naked DNA to the wound surface. We previously characterized the hydrogel made of a triblock copolymer, PEG-PLGA-PEG, for naked DNA delivery at the wound site (10). On water evaporation at the skin temperature, the liquid copolymer solution formed an adhesive film *in situ* at the wound site. Adherent interaction prevents wound desiccation and reduces the chance of bacterial infection. In addition to the wound dressing effect, the hydrogel serves as a DNA-release carrier. Here, we report the findings of using the triblock copolymer as a TGF-β1 gene delivery hydrogel for diabetic wound healing.

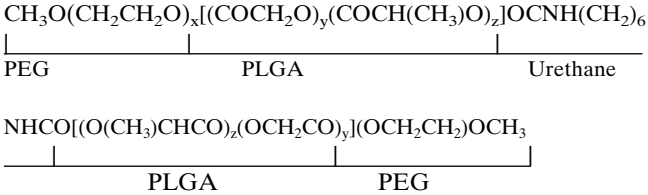
MATERIALS AND METHODS

Animal

C57BKS.Cg-m +/+ $Lepr^{db}$ female mice, 9 weeks old, were used as a model for genetically diabetic mice (Jackson Laboratories, Bar Harbor, Maine). Mice homozygous for the diabetes spontaneous mutation (*$Lepr^{db}$*) become identifiably obese around 3 to 4 weeks of age. Elevation of plasma insulin begins at 10 to 14 days and elevation of blood sugar at 4 to 8 weeks. The mice were housed in the animal facility at the University of Pittsburgh. All animal protocols were approved by IACUC of the University of Pittsburgh.

Synthesis of *in Situ* Hydrogel Solution

We have synthesized a triblock co-polymer, PEG-PLGA-PEG,



according to published procedure (11,12) with Mw = 29,659, Mn = 15,732, and polydispersity = 1.877. Briefly, the triblock copolymer was prepared by ring opening polymerization of DLLA and GA onto mPEG-750, followed by coupling of the resulting diblock copolymer (mPEG 750-PLGA) using HMDI. The resulting triblock copolymer was dried in a pressurized oven. The structure and composition of resulting products were confirmed by ^{1}H nuclear magnetic resonance (NMR) spectra recorded at 30°C with a Burker DPX-300 spectrometer using CDCl as a solvent. An aqueous solution (30%, w/v) of the triblock copolymer flows freely at room temperature, but form an adhesive hydrogel film at the wound site.

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ABBREVIATIONS: TGF-β1, transforming growth factor β_1; H&E, Hematoxylin and Eosin

Plasmids

Human TGF-β1 cDNA in pcDNA3.1/GS or empty plasmid, pcDNA3.1/GS, (Invitrogen Corporation, Carlsbad, CA) was amplified in TOP10 competent cells (Invitrogen Corporation, Carlsbad, CA). The plasmid DNA was isolated by alkaline lysis and purified by ion exchange column chromatography (Qiagen Inc. Valencia, CA).

Wounding Protocol and Treatment

The mice were anesthetized by inhalation of isoflurane. Two 7 × 7mm full thickness wounds were created in parallel on the back of each mouse. Human recombinant TGF-β1 plasmid or the empty plasmid, in an optimized dose 200 μg (dissolved in 20 μL PBS), was mixed with 50 μL of PEG-PLGA-PEG (30% w/v). TGF-β1 plasmid was also mixed with Humatrix® (Care-Tech® Laboratories, St Louis, MO) right before the application of treatment. The mixture was spread evenly with a sterile pipette tip on the wound and left uncovered. Control mice (n = 6) received either no treatment, or a 70 μL of one of the polymer wound dressings alone.

Wound Closure Analysis

Area of the wound was measured using a caliper at each day, in a total of 14 days, and evaluated as percentage of wound closure using the equation:

$$\% \text{ wound closure} = 100 \times (\text{wound area at day } 0 - \text{wound area at day N})/\text{wound area at day } 0$$

Histology

Skin biopsies were harvested at day 5. The harvested tissue was formalin-fixed, dehydrated, and embedded in paraffin. Sections of 4-mm thickness were then deparaffinized, dehydrated, and observed either the morphology with H&E staining following Degroat's protocol or collagen with picrosirius red staining (13).

Cell Proliferation Using Anti 5-Bromo-2-Deoxyuridine (BrdU) Immunohistochemistry

At day 5 postwoundng, BrdU (Sigma, St Louis, MO) labeling was performed by intraperitoneal injection at a dose of 50 mg/kg at 3 h prior to sacrifice. Paraffin sections were taken from specimens at the wound site. Sections were deparaffinized, hydrated, pretreated with 2N HCl for 20 min at 37°C and incubated with 0.01% trysin at 37°C for 3 min. BrdU immunochemical staining was performed by incubation of a rat monoclonal anti-BrdU antibody (Accurate Chemical & Scientific Corp, Westbury, NY) for 18 h at 37°C. Sections were then incubated with biotinylated mouse adsorbed rabbit anti-rat IgG and peroxidase-labeled with Vetastain Elite ABC Kit (Vector Laboratories, Burlingame, CA). The immunoprecipitate was visualized by 3,3′-diaminobenzidine tetrahydrochloride chromogen and Gill 1X hematoxylin (Fisher Scientific, Pittsburgh, PA) counterstain. Positively stained cells were counted in seven representative fields with ×400 magnification.

Statistical Analysis

All statistics are performed in PRISM software for Student's *t* test.

RESULTS

Wound Closure

We examined whether plasmid TGF-β1 can elicit therapeutic effect. First, we examined the wound closure until the wound completely reepithelialized (at day 14 post-wounding). In the early healing stage (day 1–5), we observed significantly accelerated reepithelialization when the plasmid TGF-β1 was carried by the triblock copolymer hydrogel. Furthermore, the wound dressing effect of the hydrogel was slightly beneficial for reepithelialization in early healing stage. Wound bed treated with hydrogel containing plasmid TGF-β1 produced 56% wound closure at day 5 while only 30% wound closure was found in animals treated with the hydrogel alone and 12% with no treatment. The closure rate of the wound treated with the empty plasmid in hydrogel was not different from the hydrogel alone (data not shown). Wound treated either with plasmid TGF-β1 or empty plasmid in saline did not demonstrate significantly accelerated wound closure (data not shown). Although the activity of gene has been shown independent of plasmid or oligonucleotide form (14), the increased closure rate in wound treated with plasmid TGF-β1 in hydrogel in comparison with in saline suggests that hydrogel is a good gene vehicle.

On the contrary, both Humatrix®(a commercial wound dressing primarily consists of chondroitin sulfate) containing plasmid TGF-β1 and Humatrix® alone did not produce significantly beneficial effect on reepithelialization in the early stage. Reepithelialization was complete at day 9 in the wound bed treated with the hydrogel containing the plasmid TGF-β1, at day 11 either with Humatrix® containing plasmid TGF-β1 or both wound dressings alone and at day 14 without any treatment (Fig 1).

Figure 2 demonstrates the formation of an adhesive film at 1 h after the treatment in wounds treated with tiblock copolymer hydrogel (B&D in upper panels). In wounds

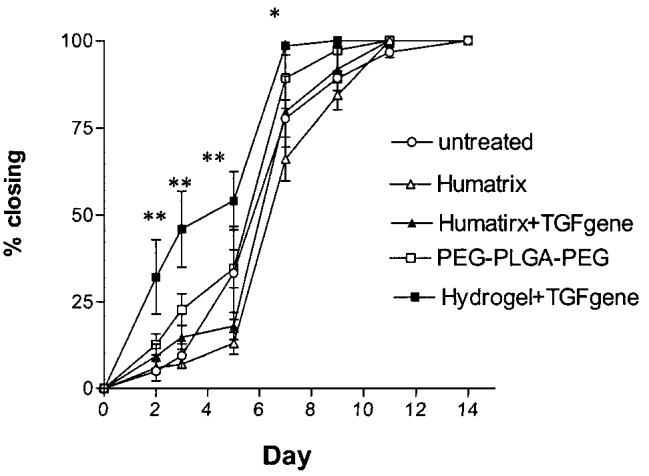

Fig. 1. Kinetics of reepithelialization by wound closure following the application of different wound dressings as a function of time. Humatrix (Humatrix® hydrogel alone), PEG-PLGA-PEG (PEG-PLGA-PEG hydrogel alone), Humatrix + TGFgene (TGF-β1 plasmid DNA in Humatrix®), PEG-PLGA-PEG +TGFgene (TGF-β1 plasmid DNA in PEG-PLGA-PEG). $^{**}p < 0.01$, $^{*}p < 0.05$ represents the comparison of wound closure for the treatment of hydrogel containing TGF-β1 gene with other treatments, n = 6.

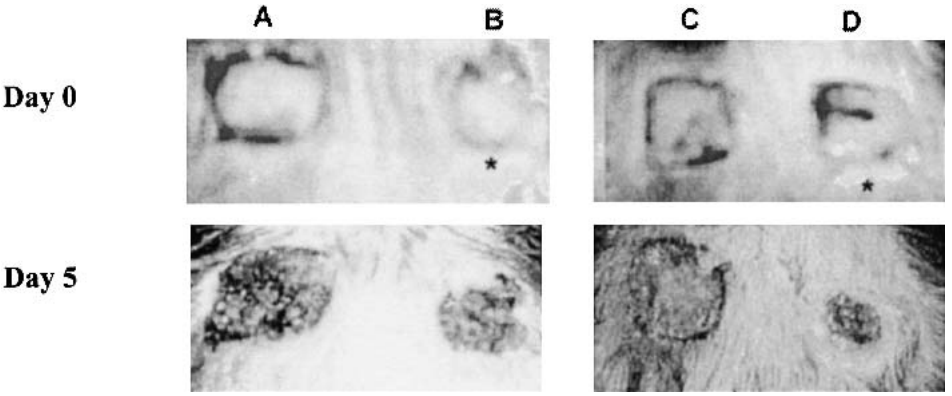

Fig. 2. Gross morphology of wounds at 1 h after the treatment (upper panels) and at day 5 (bottom panels). Skin treated with wound dressing alone either with Humatrix® (A), or PEG-PLGA-PEG (B). Skin treated with TGF-β1 gene either in Humatrix® (C) or PEG-PLGA-PEG (D). Asterisk (*) indicates wound with formation of adhesive film *in situ.*

treated with Humatrix®, only an opaque viscous liquid covered the wound (A&C in upper panel). Gross morphology at day 5 shows that reepithelialization in wounds treated with plasmid TGF-β1 in hydrogel was the fastest among any other treatments, such as hydrogel alone or Humatrix® with or without plasmid TGF-β1 (Fig. 2). Moreover, scab rejection was found at day 5 in the wounds treated with the hydrogel but not in those treated with Humatrix®, indicating that the hydrogel is capable of retaining moisture.

Histologic Examination

H & E staining demonstrated a visibly accelerated migration of epithelium (shown with black arrow in Fig. 3) in the wound bed treated with synthetic hydrogel containing plasmid TGF-β1. Under the same region of the wound bed, the migration of epithelial tongue was significantly slower in wounded tissue treated with either Humatrix® containing the gene, or controls, which was either one of the wound dressings alone or untreated. Furthermore, visibly thicker granulation tissue (marked with "G" in Fig. 3) was observed in the wound bed with the treatment of the synthetic hydrogel containing plasmid TGF-β1 than other treatments.

In Fig. 4, basket-weave collagen organization was solely found in the wound bed treated with the synthetic hydrogel containing plasmid TGF-β1. This collagen pattern resembled the unwounded dermis (shown with black arrow in Fig. 4).

Active fibroblast proliferation occurs during wound healing to synthesize extracellular matrix and wound contraction. Because wound closure at day 5 showed a great difference between both hydrogel formulations, we expected hydrogel formulated with TGF-β1 gene increased fibroblast proliferation at that time. Actively proliferating fibroblasts in the

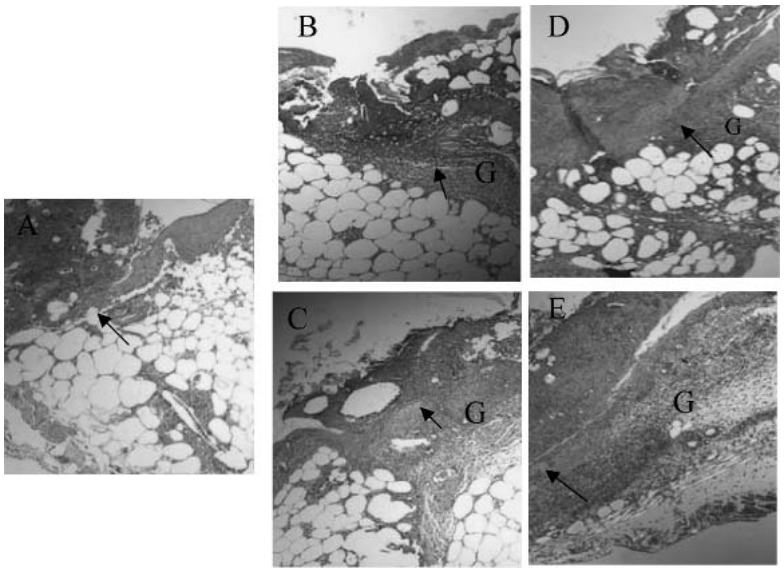

Fig. 3. Effect of TGF-β1 gene therapy on the morphology of wounded skin at day 5 postwounding. H&E staining of the wound bed from untreated skin (A), skin treated with wound dressing alone, either Humatrix® (B), or the synthetic hydrogel (D), skin treated with TGF-β1 gene in either Huamtrix® (C) or the synthetic hydrogel (E). Black arrows indicate the end of epithelial tongue. Granulation tissue was indicated by G. Magnification (×100).

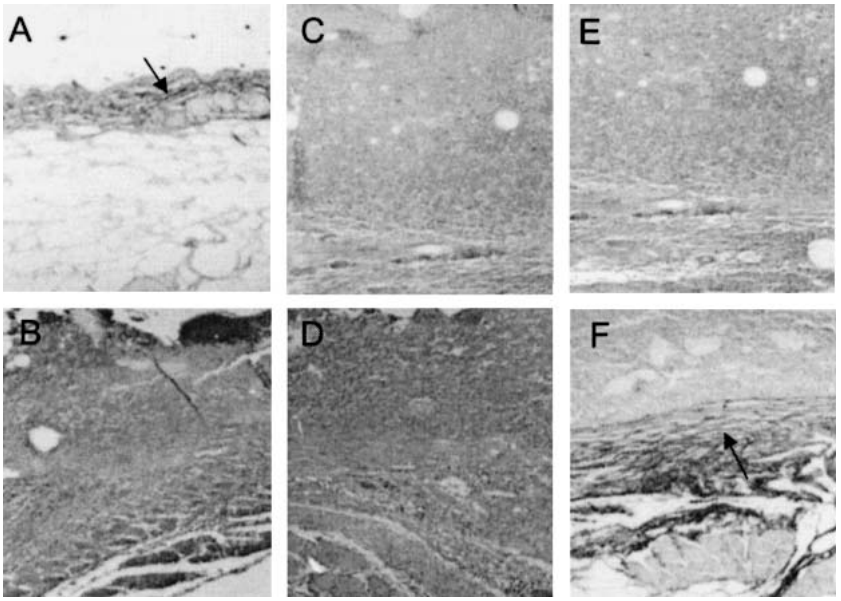

Fig. 4. Effect of TGF-β1 gene therapy on the collagen deposition of the wounded skin at day 5 postwounding. Picrosirius red staining of the wound bed from unwounded skin (A), untreated wounded skin (B), skin treated with wound dressing alone, either Humatrix® (C) or the synthetic hydrogel (E), skin treated with TGF-β1 gene in either Huamtrix® (D) or the synthetic hydrogel (F). Reddish orange color indicates collagen fibers. Basket-weave collagen pattern was shown by black arrows. Magnification (×200).

granulation tissue were identified with anti-BrdU antibody. In the center of the wound bed at day 5, there were few stained cells in the granulation tissue of the animals with no treatment or treated with Humatrix® with or without plasmid TGF-β1. However, the number of actively proliferated cells was noticeably higher in the wound bed treated with the synthetic hydrogel alone or with plasmid TGF-β1 ($p < 0.0001$) (Fig. 5), with the latter higher than the former ($p < 0.05$). However, the enhancement of fibroblast proliferation between both hydrogel formulations is not as high as in wound closure, suggesting that fibroblast proliferation is not the sole factor for reepithelialization.

DISCUSSION

Hydrogel promotes wound healing by moisture retention to maintain homeostatic environment. However, it is incon-

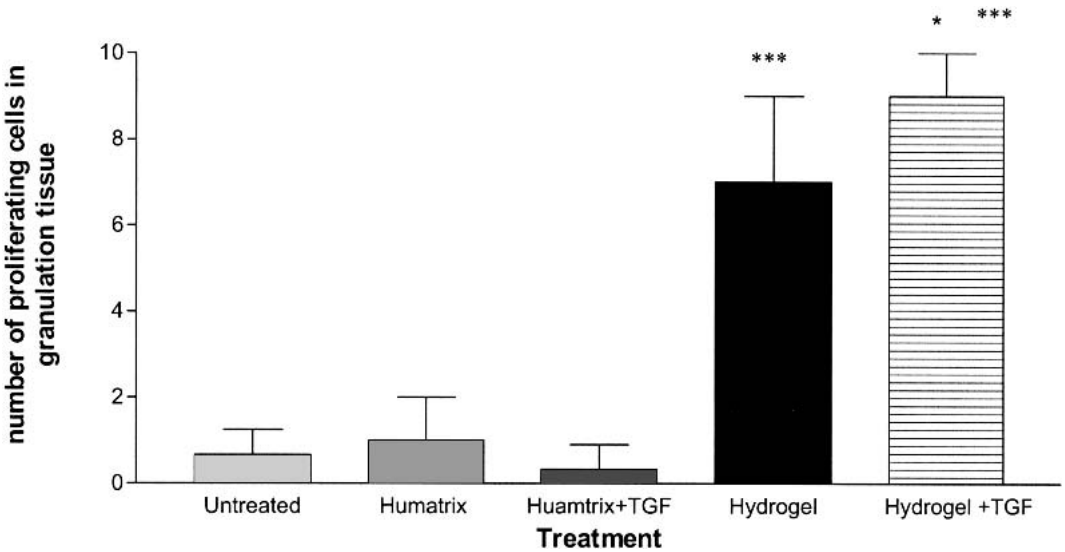

Fig. 5. Fibroblast proliferation at the wound site at day 5 measured by antiBrdU immunohistochemical staining. Humatrix (Humatrix® hydrogel alone), PEG-PLGA-PEG (PEG-PLGA-PEG hydrogel alone), Humatrix+ TGF-gene (TGF-β1 plasmid DNA in Humatrix®), PEG-PLGA-PEG+TGFgene (TGF-β1 plasmid DNA in PEG-PLGA-PEG). Positively stained cell from each treatment group were counted in 7 representative ×400 magnification fields. * $p < 0.05$ in comparison of hydrogel treatments with or without TGF-β1 gene; and ***$p < 0.0001$ in comparison with these two treatments with all other groups.

venient in application. Most of the commercial hydrogel, such as Humatrix® need several re-applications a day with an overlying occlusive wound dressing. Besides, occlusive self-adhesive membrane requires some degree of expertise and causes pain during changes. The thermosensitve hydrogel avoids the necessity of repeated and complicated application. Furthermore, its potency as a wound-healing promoter does not appear to be inhibited by drying. The liquid copolymer left uncovered and formed a hydrogel *in situ* 45 min after topical application, followed by formation of an adhesive film. The film was intact until 3 days after application to prevent wound fluid evaporation, and thereafter biodegraded. The biodegradation of hydrogel is necessary because hydrogel becomes useless when homeostatic environment recover with the coverage of clots and skin cells. In our study, the wound after single treatment with the hydrogel compared with Humatrix® was visibly smaller even at day 5 post-application, suggesting that the copolymer film is a better wound-healing promoter.

The DNA release from hydrogel (PEG-PLGA-PEG triblock copolymer) is driven by diffusion and biodegradation as previously described (10). The copolymer- hydrogel slowly releases the entrapped DNA with a half-life of approximately 5 days at 37°C (10). When the DNA was amalgamated with triblock copolymer and delivered to the wound, an early and transient gene expression occurs and peaks at day 1 (10). We believe some other mechanisms induce the rapid release of DNA to skin cells. One mechanism may be initiated by the inflammation condition in the wound site. In previous report, enhanced biodegradation was observed with localized pH change, which usually occurs during acute inflammation and infection (15).

TGF-β1 is used in this experiment because it is a chemokine for fibroblasts. When plasmid TGF-β1 formulated with the copolymer, accelerated reepithelialization, increased fibroblast proliferation, organized and mature collagen fibers were observed at the early stage of the healing process. One biologic effect of TGF-β1 is to enhance repeithelialization. During wound healing, fibroblasts stimulated by the TGF-β1 signaling migrate to the injured tissue and synthesize collagen (16). Tensile strength increases, as collagen matures. The granulation tissue bed, mainly made of collagen and proliferating fibroblasts, serves as a foundation for keratinocyte migration resulting in an enhanced reepithelialization.

Skin fibrosis, characterized by disorganized collagen formation (17) and epidermal architecture (18) is the possible adverse effects reported from cutaneous delivery of TGF-β1. From previous studies, the pathologic fibrosis and scar occurs mostly because of persistent presence of TGF-β1 caused by sustained release or reapplication *in vivo* or *in vitro* (19,20). Therefore, transient expression with single dose using our copolymer as a vehicle seems to be advantageous in wound healing. None of these pathologic conditions were observed in our studies. Instead, TGF-β1 expression resulting in robust therapeutic effect was observed with our new DNA delivery system at the early stages of wound healing.

Since endogenous TGF-β1 expression peaks in the early stages of normal wound healing process (21), and the robust therapeutic effects were observed at the early stage of wound healing, our copolymer hydrogel delivery system seems to mimic the temporal sequence of the endogenous TGF-β1. Although the mechanism of the transient expression is not clear, our copolymer system seems to be ideally suitable for delivering TGF-β1 gene for promotion of wound healing.

Diabetes mellitus is one of the major contributors to chronic wound healing problems. When diabetic patients develop an ulcer, they become at high risk for major complications, including infection and amputation. These patients show prolonged inflammation, impaired neovascularization, and defective collagen formation. It is reported that deficiency of endogenous growth factors is the underlying mechanism. Therefore, our new DNA delivery method might be advantageous for wound healing in diabetic patients in future.

In conclusion, the thermosensitive triblock copolymer, PEG-PLGA-PEG is a wound-healing promoter, which is clearly superior to the commercially available wound dressing, Humatrix®. Its further formulation with a growth factor gene might be highly applicable in treating problematic wound healing.

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REFERENCES

1. Lanning DA, Diegelmann RF, Yager DR, Wallace ML, Bagwell CE, and Haynes JH. Myofibroblast induction with transforming growth factor-beta1 and -beta3 in cutaneous fetal excisional wounds. *J. Pediatr. Surg.* **35**(2):183–187; discussion 187–188, (2000).
2. G. S. Sidhu, H. M. Mani, J. P. Gaddipati, A. K. Singh, P. Seth, K. K. Banaudha, G. K. Patnaik, and R. K. Maheshwari. Curcumin enhances wound healing in stretozocin induced diabetic rats and genetically diabetic mice. *Wound Rep. Reg.* **7**:362–374 (1999).
3. E. B. Jude, R. Blakytny, J. Bulmer, A. J. Boulton, and M. W. Ferguson. Transforming growth factor-beta 1, 2, 3 and receptor type I and II in diabetic foot ulcers. *Diabet. Med.* **19**:440–447 (2002).
4. H. D. Beer, M. T. Longaker, and S. Werner. Reduced expression of PDGF and PDGF receptors during impaired wound healing. *J. Invest. Dermatol.* **109**:132–138 (1997).
5. D. G. Greenhalgh, K. H. Sprudel, M. J. Murray, and R. Ross. PDGF and FGF stimulate wound healing in the genetically diabetic mouse. *Am. J. Pathol.* **136**:1235–1246 (1990).
6. S. Chesnoy, P. Y. Lee, and L. Huang. Intradermal injection of transforming growth factor-β1 gene enhances wound healing in genetically diabetic mice. *Pharm. Res.* **20**:345–350 (2003).
7. J. Wang, P. C. Zhang, H. Q. Mao, and K. W. Leong. Enhanced gene expression in mouse muscle by sustained release of plasmid DNA using PPE-EA as a carrier. *Gene Ther.* **9**:1254–1261 (2002).
8. J. Bonadio, E. Smiley, P. Patil, and S. Goldstein. Localized, direct plasmid gene delivery in vivo: prolonged therapy results in reproducible tissue regeneration. *Nat. Med.* **5**:753–759 (1999).
9. W. A. Border and N. A. Noble. TGF β in tissue fibrosis. *N. Engl. J. Med.* **10**:1286–1292 (1994).
10. Z. Li, W. Ning, J. Wang, A. Choi, P. Y. Lee, P. Tyagi, and L. Huang. Control gene delivery system based on thermosensitive biodegradable hydrogel. *Pharm. Res.* **20**(6):884–888 (2003).
11. B. Jeong, Y. H. Bae, and S. W. Kim. In situ gelation of PEG-PLGA-PEG triblock copolymer aqueous solutions and degradation thereof. *J. Biomed. Mater. Res.* **50**:171–177 (2000).
12. J. Byeongmoon, H. You, and W. Sung. Biodegradable thermosenstive micelles of PEG-PLGA-PEG triblock copolymers. *Colloids and Surfaces B: Biointerfaces* **16**:185–193 (1999).
13. F. Sweat, H. Puchtler, and S. Rosenthal. Sirius red F3BA as stain for connective tissue. *Archs. Pathology* **78**:69–72 (1964).
14. C. R. Hofman, J. P. Dileo, Z. Li, S. Li, and L. Huang. Efficient in

vivo gene transfer by PCR amplified fragment with reduced inflammatory activity. *Gene Ther.* **8**(1):71–74 (2001).
15. G. E. Zaikov. Quantitative aspects of polymer degradation on the living body. *JMS-Rev. Macromol. Chem.Phys.* **C25**(4):551–597 (1985).
16. S. Frank, M. Madlener, and S. Werner. Transforming growth factors β1, β2, and β3 and their receptors are differentially regulated during normal and impaired wound healing. *J. Biol. Chem.* **271**:10188–10193 (1996).
17. Y. Ito, P. Sarkar, Q. Mi, N. Wu, P. Bringas Jr., Y. Liu, S. Reddy, R. Maxson, C. Deng, and Y. Chai. Overexpression of Smad2 reveals its concerted action with Smad4 in regulating TGF-beta-mediated epidermal homeostasis. *Dev. Biol.* **236**:181–194 (2001).
18. P. J. Christner, C. M. Artlett, R. F. Conway, and S. A. Jimenez. Increased numbers of microchimeric cells of fetal origin are associated with dermal fibrosis in mice following injection of vinyl chloride. *Arthritis Rheum.* **43**:2598–2605 (2000).
19. D. A. Lanning, B. C. Nwomeh, S. J. Montante, D. R. Yager, R. F. Diegelmann, and J. H. Haynes. TGF-beta1 alters the healing of cutaneous fetal excisional wounds. *J. Pediatr. Surg.* **34**(5):695–700 (1999).
20. D. A. Bettinger, D. R. Yager, R. F. Diegelmann, and I. K. Cohen. The effect of TGF-beta on keloid fibroblast proliferation and collagen synthesis. *Plast. Reconstr. Surg.* **98**(5):827–833 (1996).
21. C. L. Theoret, S. M. Barber, T. N. Moyana, and J. R. Gordon. Preliminary observations on expression of transforming growth factors beta1 and beta3 in equine full-thickness skin wounds healing normally or with exuberant granulation tissue. *Vet. Surg.* **31**: 266–273 (2002).