CELL/ GENE THERAPY FOR DIABETIC WOUND HEALING

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Submitted to the Graduate Faculty of

School of Engineering in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2005

UNIVERSITY OF PITTSBURGH

SCHOOL OF ENGINEERING

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Wounds in diabetes are difficult to heal. Current standard strategy employs series of medical treatments to clean and remove the infected tissue, and keep moisture with adequate blood supply. However, the standard treatments may not be sufficient enough. According to ADA, there are an increasing number of amputation cases in diabetes. In this thesis, recent development of therapies in wound healing is reviewed and results of using a TGF-β1 plasmid DNA or stem cells in genetically diabetic mouse model are reported.

In previous study, we have found that the diabetic wound healing has been improved by intradermally injecting TGF-β1 plasmid DNA. This finding supports the feasibility of using naked DNA as a therapeutic approach for treating diabetic wounds. Since naked DNA approach yields low efficiency of gene transfer, we seek strategies that can enhance the gene expression. Hydrogel as well as electroporation which involves an application of electric pulses has been shown to enhance gene transfection. On the other hand, electrical stimulation (ES) which involves the application of a different condition of electric pulses from electroporation or hydrogel wound dressing has been shown to improve wound healing. In this thesis project, we develop a more effective strategy to improve diabetic wound healing by combining the available wound therapy and gene therapy.

However, application of exogenous single cytokine gene may not be sufficient for severe wound problems. Owing to the self renewal and multipotent characteristics of stem cells, stem cells may have the potential to differentiate into some of the essential cells in wound healing such as

macrophages, keratinocytes and fibroblasts. We develop a strategy to topically apply three different types of stem cells individually with the thermosensitive hydrogel in an attempt to improve wound repair.

Three new strategies in this thesis project are reported. (1) Intradermal injection of TGFβ-1 plasmid DNA followed by electroporation or (2) Topical application of TGFβ-1 plasmid DNA with themosensitive hydrogel made of PEG-PLGA-PEG triblock copolymer. (3) Topically application of the thermosensitive hydrogel with three different types of stem cells: muscle derived stem cell, mesenchymal stem cells or hematopoietic stem cells.

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PREFACE

I would like to thank my advisor, Dr. Leaf Huang for his support, guidiance and patience. His advice on science and my career is invaluable. Furthermore, I would like to thank my committee members, Dr. Johnny Huard, Dr. Kacey Marra, Dr. Patricia Hebda for their advice on my projects and my thesis writing. Their suggestions on my writing and my projects are greatly helpful. I also greatly appreciate with the help from Dr. Simon Watkins's laboratory for the immunofluorescence study. I would like to thank everyone in the Center for Pharmocogeneics, particularly the people who cheer me up when I am upset and share experience with me.

Finally, I want to thank my family and my fiancé Alan, who gives me tremendous support and care in the toughest and my most frustrating moment.

1.0 INTRODUCTION

Wound healing impairment is a complication of diabete mellitus, which affects dominately the people in the developed countries. There are two common types of diabetes: Type I (10%) and Type II (90%). According to American Diabetes Association (ADA), 18,000,000 people were diagnosed with diabetes in the United States in 2002 and 15% (approximately 2,400,000) will develop ulcers and risk of amputation (Pecoraro et al 1990). There has been an increasing number of amputations among diabetic patients. According to a recent statistics report by ADA in 2001, 84,000 more cases of amputation among diabetics are performed each year, or approximately 2-fold increase each year. Furthermore, the morbidity and mortality associated with diabetic foot ulcers is substantial. 39-68% of patients who have an amputation will die within 5 years.

The incidence of amputation in diabetic patients is attributed to single or multiple causes. There are seven individual potential causes: ischemia, infection, neuropathy, poor wound healing, minor trauma, cutaneous ulceration and gangrene. 72% of the amputations occur with a combination of potential causes, from minor trauma, cutaneous ulceration and wound healing failure. Among all the individual causes, poor wound healing is one of the main causes that contribute to the amputation (81% of the amputation cases) (Pecoraro et al 1990).

1.1 BIOLOGY OF WOUND HEALING

1.1.1 Normal wound healing

Normal (acute) wound healing involves a cascade of sequential biological processes (Enoch and Price 2005). Cellular and biochemical activities including phagocytosis, cell migration, cell proliferation, collagen synthesis and cytokine production are well coordinated in normal wound healing. Those activities occur in 4 major overlapping steps: inflammation, re-epithelialization, granulation tissue formation and tissue remodeling (Singer et al. 1999, Martin 1997).

1.1.1.1 Inflammation

Tissue injury can cause leakage of blood from damaged blood vessels. The formation of a blood clot reestablishes hemostasis and provides a provisional matrix through which cells can migrate during the repair process. The clot acts as a reservoir of cytokines and growth factors. Vasoactive mediators and chemotactic factors generated at the site of the wound attract neutrophils to cleanse the bacteria and foreign particles. In response to specific chemoattractants such as TGF-β produced by platelets, monocytes also migrate to the wound site and later become activated macrophages stimulated by the binding to the extracellular matrix (Singer and Clark 1999, Dipietro et al 2001). Macrophage seems to play a vital role in regulating inflammation and initiation of wound repair (Riches 1996). The binding of macrophage to extracellular matrix induces the production of many important cytokines that are necessary for tissue repair such as transforming growth factor (TGF)-β, interleukin-1 (IL-1), TGF-α, platelet derived growth factor (PDGF). For example, TGF-β-1 and β-2 are related to induce keratinocyte migration while PDGF is related to induce fibroblast proliferation (Singer and Clark 1999). Moreover, macrophages are also important in the phagocytosis of any remaining damaged cell debris

(Brown 1995). After 2-3 days, the number of neutrophils begins to decline but macrophages continue to accumulate at the wound site.

1.1.1.2 Re-epithelialization

Reepithelialization is the process to regenerate epithelium. Within hours, keratinocytes at the edges of the wound begin to migrate to cover the wound (Singer and Clark 1999). The provisional matrix formed at the wounds helps keratinocyte migration. Furthermore, growth factors such as TGF-β1 enhance the motility of keratinocytes through the wound by stimulating their expression of integrins. Concomitantly, the expression of tissue-type plasminogen activator (tPA) and urokinase type plasminogen activator (uPA) is also upregulated in the keratinocytes. These activators are necessary to transform plasminogen into plasmin, an enzyme that lyses the fibrin clot thereby facilitating keratinocyte migration across the wound. Matrix metalloproteinase family such as MMP9 can also facilitate the epithelial regeneration probably by digesting the provisional matrix (Mohan et al 2002). One or two days after injury, keratinocytes at the wound edge begin to proliferate behind the previous migrating ones (Clark 1996). The growth factors regulating the proliferation and motility of keratinocytes are part of the epidermal growth factor (EGF) family comprising EGF, TGF-α and heparin binding-epidermal growth factor (HB-EGF). Their major sources of production include platelets, macrophages and keratinocytes. In addition, keratinocyte growth factor (KGF) is produced by fibroblasts and has also been characterized as having mitogenic effects on keratinocytes (Singer and Clark 1999).

1.1.1.3 Formation of granulation tissue

Approximately 3 or 4 days after injury, dermal fibroblasts begin to migrate into the provisional matrix where they proliferate and contribute to new extracellular matrix production (Clark 1993). As previously mentioned, the degradation of the provisional extracellular matrix is required to permit fibroblasts and endothelial cells to move into the wound space. In order to do so, an active proteolytic system of enzymes including plasminogen activator and various members of the matrix metalloproteinase family (MMPs) such as, MMP2 and MMP 9 (Arumugam et al 1999) play an important role. After injury, the fibroblasts stimulated perhaps by TGF-β1 will be responsible for the synthesis of a rich collagen matrix, which will replace the provisional matrix and give the wound tensile strength. During the formation of the granulation tissue, angiogenesis occurs to form new blood vessels/ capillaries to support the formation of granulation tissue. Macrophages provide a good source of cytokines such as acidic fibroblast growth factor (aFGF), basic fibroblast growth factor (bFGF), TGF-β and vascular endothelial growth factor (VEGF) which can stimulate wound angiogenesis (Wiseman et al 1988, Bates and Jones 2003, Tonessen et al 2000). When most of the provisional extracellular matrix is replaced by collagen in the granulation tissue, the formation of new blood vessels stop and those new blood vessels will be degenerated (Tonesen et al 2000).

1.1.1.4 Tissue remodeling

Tissue remodeling involves the continuous synthesis, degradation and reorganization of collagen. During this process, random deposited collagen in the granulation tissue remodels to a more organized structure. Type III collagen is the main component but is replaced with type I collagen as the wound matures. This remodeling process contributes to developing the tensile strength

(Clark and Singer 1999). MMPs are apparently essential in collagen degradation (Clark and Singer 1999) in addition to the degradation of the provisional extracellular matrix. For example MMP-2 and MMP-9 in the gelatinase subgroup of the MMPs, have been known to denature collagens (Birkedal-Hansen 1995) such as collagen type I (Corbel et al 2000).

1.2 PATHOLOGIC WOUND HEALING CONDITIONS IN DIABETES

The relationship between pathophysiologic and impaired wound healing in diabetes is not completely understood. However, alterations of the cellular and biochemical activities have been implicated in the failure of wound healing in diabetes. As a result, the entire wound healing process is disrupted: abnormal cellular infiltration, defective cytokine production and impaired tissue remodeling have all been found in diabetic wounds (Brown et al 1997).

1.2.1 Abnormal cellular activity

As discussed in the previous session, both macrophages and fibroblasts are essential cells in the normal wound healing process. Macrophages and fibroblasts both demonstrated defective migration or proliferation in diabetic wounds (Lerman et al. 2003, Wetzler et al 2000). It was also shown that the infiltration of macrophages and neutrophils was prolonged in diabetes (Wetzler et al 2000). Furthermore, in diabetes, the macrophages produce in a reduced level of cytokines (Zykova et al 2000).

1.2.2 Defective cytokine production

The dysfunction of macrophages and fibroblasts in cytokine production or extracellular matrix regulation contributes to the impairment of wound healing. Macrophages in diabetics

demonstrated a decreased release of many potent chemokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-1beta (IL-1β). Both fibroblasts and macrophages demonstrated a decreased release of vascular endothelial growth factor (VEGF), a potent angiogenic factor (Zykova et al 2000). It has been suggested that type II diabetic patients have defective signaling of IL-1β and IFN in macrophages and neutrophils.

1.2.3 Impaired tissue remodeling

When the wound matures, collagen should replace the provisional matrix thereby promoting tensile strength (Veves et al 2002) and wound contraction (Veves et al 2002). In the diabetic condition, collagen deposition is reduced due to decreased synthesis and accelerated degradation (Bowersox 1986, Seibold et al. 1985). On the contrary, provisional matrix components such as chondroitin sulfate and fibronectin excessively accumulate and extendedly exist in the wound bed (Loots el al. 1998). Diabetic fibroblasts have been shown to produce reduced levels of collagen (Seibold et al. 1985). Excessive MMP-9 (Lerman et al 2003) and MMP 2 (Wall et al 2003) was also found in diabetic wounds with increased concentrations of 14-fold and 6 fold respectively compared with the concentrations in the non-diabetic wounds (Lobmann et al 2002).

1.3 RECENT DEVELOPMENT OF WOUND THERAPIES

Foot ulcer is a common problem resulting from diabetes (Boulton 2004). Foot ulcer can be only a loss of epidermis or can extend to dermis and deeper layers such as bone and muscle (Boulton 2004). Standard management can include a series of treatments: debridement, sufficient application of dressings, frequent dressing changes, and efforts to reduce the pressure points on a patient's feet. The purpose of the series treatments is to clean or remove the infected tissue as

well as to keep a moist wound environment with adequate blood supply. If the necrotic or infected tissue is thick, patients may need deeper debridement by surgery, which creates more pain and a larger open wound. Commonly for diabetic patients, multiple debridements may be necessary. The effectiveness of this complicated procedure varies. As mentioned before, there has been an increasing number of death associated-amputation cases which are mainly caused by the wound healing failure. Furthermore, the cost of treating poorly healing wounds is 1.5 billions dollars in US per year. These facts urge researchers to develop new, more effective wound treatments for diabetic patients.

New technologies for diabetic patients who do not respond to conventional methods employ living cell constructs and growth factor proteins. However, the efficacy in clinical applications is not uniform. Furthermore, these technologies require frequent application and they mostly show promise in wound healing when they are used with standard wound care techniques. The technologies include living cell constructs, Graftskin (Apligraf; Organogenesis, Inc) (Curren and Plosker 2002) and Dermagraft (Dermagraft; Advanced Tissue Sciences) (Marston et al 2003)as well as a protein construct becaplermin (Regranex; Ortho-McNeil) (Smiell et al 1999). Apligraf is made from bovine collagen and living fibroblasts and keratinocytes derived from human infant foreskin. In a controlled clinical trial, 47 % of the non-healing patients treated with Apligraf along with standard therapy showed improved wound closure, compared with 19% of the patients treated with standard therapy (Curran and Plosker 2002). Dermagraft is a cryopreserved human fibroblasts-derived dermal substitute. Dermagraft is made from extracellular matrix, a bioabsorbable scaffold and human fibroblasts derived from neonatal foreskin tissue. Dermagraft demonstrates less wound closure improvement in clinical trial than Apligraf. In a different controlled clinical trial, 30% Dermagraft treated patients

showed complete wound closure; while 19% of the conventional therapy treated patients showed complete wound closure (Marston et al 2003). The therapeutic effect of the two living cell constructs varies more than that of the growth factor therapy; the clinical outcome of these constructs show 15- 20 % increase in improving diabetic wound healing compared to treating with saline soaked gauze, a control conventional therapy approved by the FDA (Hansbrough et al 1997, Philips 1993, Veves et al 2001). Regranex^{TM}, the first bioengineered product approved by the FDA for treating diabetic ulcers, is a topical gel consisting of platelet-derived growth factor (PDGF). PDGF is the only growth factor protein commercialized to date and approved for use in diabetic ulcers. Again, the clinical outcome shows that the effectiveness is not uniform. Although one clinical study found that approximately 57.5% of the patients treated with Regranex^{TM} had resulted in complete healing compared with 36% of placebo-treated patients (Smiell et al 1999), another study showed only 28% of the treated patients with complete wound closure (Niezgoda et al 2005). This disappointing clinical experience may limit the ability of other growth factor proteins to reach the marketplace.

1.4 TRANSFORMING GROWTH FACTOR-BETA (TGF-β) in WOUND HEALING

Although the clinical outcome of growth factor therapy has not yet yielded a totally satisfactory result in the PDGF study described above, growth factor approach is still believed to be important in treating nonhealing wounds. In chronic wounds such as those occurring in the lower extremities of individuals with diabetes, there is a significant deficiency of growth factors, proteins that mediate the growth and proliferation of cells or growth factor receptors. Beer et al (1997) demonstrated a reduced expression PDGF A and B and of PDGF A-type receptor in wounded and unwounded diabetic skin. Insulin-like growth factor (IGF)-I was found to be

reduced by 42 % in wound fluid and by 48% in serum in diabetes-related impairment (Bitar and Labbad, 1996). Decrease of transforming growth factor-beta (TGF-β) expression is predominant in diabetic wound. A 55% reduction of TGF-β expression in diabetic wound fluid has also been shown in the study of Bitar and Labbad (1996).

TGF-β family plays an important role and is multifunctional in wound healing (Martin et al 1993, Frank et al 1996). All three isoforms TGF-β1, 2 and 3 are found in wounds (Frank et al 1996). However, studies have found that only TGF-β3 (Schmid et al 1993) and TGF-β1 (Kane et al 1991) expresses constuitively in human intact skin. Exogenous application of TGF-β1, 2 and 3 has been shown to enhance wound closure, reepithelialization and angiogenesis (Grose and Werner 2004, Hebda 1988). In addition to the enhanced wound repair, exogenous TGF-β3 has been shown to correlate to reduce scar formation (Ferguson and O'Kane 1999). Interestingly, another study showed that exogenous TGF-β3 increased the granulation tissue formation but not reduce the scarring within the dose which can enhance wound repair (Wu et al 1997). Furthermore, TGF-β family is known as potent modulator of the synthesis of various growth factors (Strutz et al 2001, Abboud 1993, Miyazono et al 2001). While TGF-β induces the synthesis of PDGF (Abboud 1993. Janat et al 1992), fibroblast growth factor (FGF) (Strutz et al 2001) and IGF-I (Simmons et al 2002), TGF-β inhibits the synthesis of epidermal growth factor (EGF) (Miyazono et al 2001).

The lack of TGF-β1 may suggest the tendency of disrupted wound healing such as in the case of the chronic wounds. TGF-β1 was not found in chronic wounds but in acute wounds (Schmid et al 1993, suggesting that the use of exogenous TGF-β1 for treating chronic wound treatment may be attractive. TGF-β1 is a potent cytokine and is produced in macrophages and

platelets in response to wound healing and has been shown to have multiple functions in wound healing. On the other hand, TGF-β1 is known as a chemokine for different cells such as macrophages, myofibroblasts, keratinocytes and fibroblasts, which are substantial cells in inflammation phase, wound contraction, reepithelialization and tissue remodeling, respectively.

In a study from Lanning's group (Lanning et al 2000), TGF-β1 has been shown to induce myofibroblast production, resulting in a significantly reduced wound size in a non-contractile fetal rabbit model. Sidhu et al (1999) demonstrated that TGF-β1 increased migration of myofibroblasts, fibroblasts and macrophages and enhanced collagen content as well as reepithelialization (Sidhu 1999). TGF-β1 is known to be an inhibitor of keratinocytes proliferation *in vitro* (Yang et al 1996, Hashimoto 2000, Haber et al 2003). Interestingly, TGF-β1 perhaps can induce keratinocyte proliferation *in vivo (*Fowlis et al 1996). Fowlis et al showed that an overexpression of TGF-β1 may promote the epidermal cell growth in TGF-β1 transgenic mouse model (Fowlis et al 1996). In addition, TGF-β1 induces keratinocyte migration by up-regulating the synthesis of laminin 5, which has a dual function in keratinocyte adhesion or migration (Decline et al 2003). Since an enhanced formation of capillary by TGF-β1 was found *in vitro* (Sakuda et al 1992), TGF-β1 is possibly a potent chemokine for endothelial cells. In summary, TGF-β1 is beneficial to wound healing by enhancing reepithelialization, wound contraction, collagen deposition and neovascularization. For these reasons, TGF-β1 was employed in this thesis project.

1.5 CUTANEOUS DELIVERY OF NAKED DNA IN THE WOUND ENVIRONMENT

To date, only PDGF-BB is approved by FDA for treating diabetic ulcers, yielding only a limited improvement of healing in patients. The disappointing clinical outcome is perhaps due to the inherent difficulties in delivering the growth factors which are high molecular weight and liable molecules in sufficient quantities and duration of activity. This may be due to the proteins' short half life, degradation by wound proteases and difficulty to maintain the level of active protein in the wound above the therapeutic threshold. All of the limitations support the discovery of a better alternative to protein therapeutics. Growth factor gene therapeutics has therefore attracted attention as a cost-effective alternative to protein therapeutics. It is speculated that a gene therapy approach will prolong the availability of therapeutic proteins, yielding improved healing responses. Overexpression of a therapeutic gene is not necessary after wound healing is complete. Thus, only temporary gene expression is required for wound repair in wound healing, which is not as the case in gene therapy for the inherited diseases. Therefore, gene therapy for wound healing may be more easily achieved.

The choice of gene transfer strategies in wound healing depends on whether the modification of gene expression is temporary or permanent. Long-term genetic modification can be achieved by most of the viral vectors such as retrovirus, adeno-associated virus and the HIV-based lentivirus. On the other hand, the majority of the nonviral vectors and some of the viral vectors such as adenovirus and herpes simplex virus can achieve temporary genetic modification. In wound healing, as mentioned, a permanent genetic modification is not necessary. Thus, most of the viral vector strategies may not be suitable to deliver therapeutic genes in wounds. Furthermore, using viral vectors in transient genetic modification may not be preferred. Since viral transfection strategies are based on the natural ability of viruses to infect cells, the

transfection efficiency could be associated with increased antigenicity owing to the viral sequence for replication. One example is the adenovirus. The strong immunogenicity of viral vectors revealed from clinical experiences presents a risk to compromise the wound repair, particularly in the nonhealing wounds where inflammation is disrupted. In order to reduce the immunogenicity, researchers have developed a new generation of helper dependent viral vectors, in which all the viral coding sequences have been removed. However, more animal and clinical studies are necessary to test whether or not the immunogenicity is successfully reduced.

Nonviral gene transfer strategies evade some of the drawbacks associated with the viral gene transfer strategies. In particular, there is no concern of viral integration and replication. It is therefore believed to have lower cytotoxicity and antigenicity compared with viral gene transfer strategies. The efficiency varies widely from 5%- 20% and up to 90% depending on the target cells and tissues. Examples of nonviral gene transfer techniques include: the simplest approach which is direct injection of plasmid DNA, physical methods of hydrodynamic pressure, electroporation, particle bombardment or microseeding and chemically methods using liposome polymers and proteins.

The success of a gene delivery method depends on whether a gene is able to transport to the cells of the target tissue for gene expression. Skin is one of the attractive targeted tissue for therapeutic delivery (Spirito et al 2001, Pfutzner and Vogel 2000). Skin is the most accessible organ and it is therefore believed that a relatively simple and minimally invasive method, such as local injection and topical application, is feasible. However, due to the complexity of skin structure, gene transfer strategy in skin is more limited than expected. In fact, diffusion of macromolecules was shown to be increasingly hindered by high level of collagen type I (Pluen et al 2001), one of the major extracellular matrix proteins in skin, in particular, during the process

of wound healing. Thus, transport of large-sized molecules such as plasmid DNA without accompanying of vectors could be favorable. Indeed, it has been shown that delivery of naked DNA is relatively efficient compared with delivery of DNA in liposome or in the form of lipoplex (lipid /DNA complex) (Sawamura et al 1997, Udvardi et al 1999, Meuli et al 2001). Following the diffusion in the extracellular environment, internalization of DNA is a crucial process for allowing the transgene to be expressed in the cells. Both keratinocytes and fibroblasts, which are essential cells in wound repair, are able to internalize naked DNA (Udvardi et al 1999, Kamiya et al 2002). Therefore, the delivery of exogenous naked DNA to skin appears to be feasible.

1.6 METHODS FOR DELIVERING NAKED DNA TO SKIN

The simplest approach to non-viral vectors is direct injection of naked DNA. The promise of direct injection of naked DNA was found in Wolff's study (Wolff et al 1990) on the expression of a reporter gene following direct intramuscular injection of DNA. Their study revealed that the reporter gene delivered with the naked DNA could lead to a long-term expression of the gene in the muscle. Several experiments have since been conducted to introduce naked DNA into skin by direct injection (Hengge et al 1995; Ciernik et al 1996; Eriksson et al 1998). Injection of plasmids encoding the β-galactosidase (Lac Z) reporter gene into the superficial dermis of porcine skin resulted in a visible expression of the encoded protein. The expression of the reporter gene lasted for three days, whereas the expression of the encoded protein was visualized for up to three weeks (Hengge et al 1995).

Without the protection of vectors, DNA tends to be degraded easily and the efficiency of gene transfer can be low. Although long-lasting gene expression is not necessary, enhanced gene

expression efficiency is important because sufficient gene expression is required to overcome the therapeutic threshold. Various physical approaches have been developed in an attempt to increase the gene transfer efficiency in the target tissues.

Hydrodynamic pressure by rapidly injecting large volume of DNA solutions through the tail vein (so called hydrodynamics-based intravenous injection) induced gene expression in the kidney, spleen and heart, but primarily in the liver (Liu et al 1999). Hydrodynamic pressure is commonly used in systemic administration of therapeutics while only local administration of therapeutics is necessary in the case of wound healing.

Several other physical approaches have been developed to increase gene transfer efficiency. Jet injection of a low volume of DNA solution in pressurized air was employed as the driving force for efficient gene transfer in tumor (Walther et al 2002). Electroporation is one of the most common techniques used in laboratory. Electroporation involves the uses of brief electric pulses to induce the formation of transient pores in the membrane of the host cell (Neumann et al 1999). Such pores appear to act as passageways through which the naked DNA can enter the host cell (Neumann et al 1999, Nishikawa et al 2001). Gene gun is a particle mediated approach to enhance gene delivery. Gene gun uses particle bombardment to shoot DNA-coated microscopic pellets through the cell membrane (Nishikawa et al 2001). Compared to the performance of electroporation in gene delivery, the application of gene gun has been limited to superficial tissues due to the short penetration depth (<0.5mm in murine muscle) into tissue (Zelenin et al 1997). Furthermore, the high pressure may cause mechanical damage of tissue. Until recently, a significant improvement in tissue penetration has been achieved using a new design of gene gun by Dileo et al (2003). The gene gun which delivers DNA-coated gold

beads at a high pressure allows the transgene accessing to subcutaneous tissues, such as muscle or tumors, and consequently achieving longer-term gene expression (Dileo et al 2003).

1.6.1 Electrical-mediated gene delivery

Electroporation has demonstrated success in preclinical trials of gene therapy (Bjordal et al 2003, Evans et al 2001). Enhancement of gene transfer using electroporation is generally about 100-1,000 fold greater than the delivery of naked DNA alone. Optimization of electrical parameters such as voltage, duration of each pulse and number of pulses, for *in vivo* electroporation is important to gene delivery. Voltage that is optimal in one tissue may not be optimal in another. For example, an electric voltage of 200V in a tumor was used to enhance 100 fold gene expression whereas 100V was necessary in muscle (Cichon et al 2002). In addition to the voltage, the duration and number of pulses are also necessary for optimization because intense application of electric pulses causes local inflammation and tissue damage. Electroporation has been reported to increase gene transfer in liver and muscle (Aihara and Miyazaki 1998, Suzuki et al 1998). Due to the complexity of the skin, a harsh condition of electroporation is required to accomplish effective gene transfer. Most studies used an electric field strength that ranged from 700-2000V/cm (Titomirov et al 1991, Heller et al 2001). However, typical applied transdermal voltage is 50-150V (Vanbever et al 1996). Higher voltage may cause local heating within the skin and could lead to tissue damage. Indeed, high current and voltage applied to the skin can cause tissue damage and electrolysis (Pliquett 1999). Electroporation has an attractive potential in wound healing. Investigators have shown that electrical stimulation (ES) can accelerate wound closure of chronic ulcers (Houghton et al 2003, Gardner et al 1999) and diabetic foot ulcers (Baker et al 1997). In addition, many applications of ES for wound-related treatments have been

long used in clinics with an excellent safety record (Crevenna et al 2001). In Chapter 2, we will report the feasibility of a proper electric condition that can combine electrical and gene therapies to treat diabetic wound healing without damaging the tissue.

1.6.2 Topical gene delivery using biodegradable polymer

A topical delivery method is ideally suited for wound healing because it is minimally invasive and capable to treat for a large surface area. In addition, topical delivery is easy to apply and can be done in an outpatient setting. Biodegradable polymers have been used as topical drug carriers with encouraging results (Puolakkainen et al 1995, Sawada et al 1990). Polymers can protect the drug or gene from degradation. Biodegradable polymers themselves will be degraded in a relatively brief period and surgical removal is not necessary. Hydrogels composed of hydrophobic and hydrophilic block copolymers are especially suitable as the potential use of hydrogels are broad due to their flexible release kinetics, which are mediated by adjusting the hydrophobic portion or hydrophilic portion of the block copolymer. Some drugs or proteins such as TGF-β1 require transient administration to accelerate wound healing since persistent TGF-β1 administration causes excessive extracellular matrix component accumulation resulting in skin fibrosis or scar formation (Ito et al 2001). In contrast, antitumor drugs such as doxorubicin require prolonged and sustained release. However, most of biodegradable polymers are not convenient for formulating with drugs since many of the polymers are solid at room temperature. Thus, a thermosensitve hydrogel made of a triblock copolymer poly [ethylene glycol-b-(D, L-lactic acid-co-glycol acid)-b-ethylene glycol] (PEG-PLGA-PEG), which is liquid at room temperature but forms a viscous gel at the body temperature due to its gel-sol transition, is advantageous. In addition to the potential for drug delivery, some polymers such as

glucosaminoglycan, have demonstrated to be a suitable wound dressing. However, no one has combined the characteristics of wound dressing and gene carrier in a delivery system, to the best of our knowledge. In Chapter 3, we will report the feasibility of the thermosensitive hydrogel, PEG-PLGA-PEG, to combine the wound dressing and gene carrier in diabetic wound healing.

1.7 STEM CELL-MEDIATED THERAPY

Wound healing involves regeneration of macrophages, keratinocytes and fibroblasts for the recovery. Since stem cells are capable of self-renewal and multipotency/pluripotency, it is therefore hypothesized that stem cells can improve the wound healing.

1.7.1 Characteristic of stem cells

Stem cells have the capacity to self-renew and display long-term proliferation capacity. Stem cells can generate different types of cells. There are two types of stem cells: those from embryonic/fetal origin and those from adult origin. Embryonic stem cells are long known to be pluripotent (Gerecht-Nir and Itskovitz-Eldor 2004, Tiedemann et al 2001). However, major concerns of embryonic stem cells including embryonic stem cell-associated tumor formation (Chambers and Smith 2004, Andrews 2002) and immune rejection upon differentiation (Drukker et al 2002). There is also an ethical concern in using embryonic stem cells for therapeutic applications. Today, researchers have begun investigating adult stem cells because their multipotent characteristics have been recently identified. For example, it has been found that hematopoietic stem cells can differentiate to neurons, blood cells and macrophages. It has been found that muscle-derived stem cells can differentiate into adipogenic, osteogenic, chrondrogenic

and hematopoietic lineages (in vitro or in vivo) (Shen et al 2004, Sinanan et al 2004, Cao et al 2003).

1.7.2 Muscle-derived stem cells (MDSC)

Cell-based therapy has been proposed as a direct way for regenerating muscle. Satellite cells isolated from skeletal muscle may have potential in treating Duchenne muscular dystrophy (Bachrach et al 2004, Morgen and Patridge 2003). The disrupted muscle regeneration in Duchenne muscular dystrophy is perhaps correlated to the altered proliferation (Maier and Bornemann 1999) or differentiation (Schuierer et al 2005) of satellite cells. Satellite cells lie between the muscle fiber and the basement membrane and are typically quiescent (Morgen and Patridge 2003, Menasche 2003). However, it is reported that satellite cells can proliferate and fuse with the myotube in vitro (Bischroff 1975). In response to injury, the satellite cells can proliferate and differentiate to regenerate muscle (Morgen and Patridge 2003). In addition to the ability to regenerate myogenic lineage (Bachrach et al 2004), satellite cells can commit to adipogenic and osteogenic lineages (Asakura et al 2001, Wada et al 2002), indicating that satillete cells are multipotent. Recently, preplate technique was used to isolate a population of cells from skeletal muscle of postnatal animals and was found apparently different from the satillete cells (Qu et al 2002). The cells has been called LTP cell and the phenotype of LTP cells preserved for more than 200 passages (Deasy et al 2005). Furthermore, the LTP can differentiate into various lineages from muscle to neurons, osteogenic cells, endothelial cells, and hematopoietic cells. Another group also found that MDSC could have the hematopoietic potential supporting the mulitpotency of MDSC (Howell et al 2002). Transplantation of MDSC has been shown to restore partially the dystrophin expression (Gussoni et al 1999) and prevent

early cell death after skeletal muscle transplantation. Qu et al (2002) also found that the MDSC were almost negative for the expression of MHC-1 (0.5%), which plays an essential role in the immune rejection following transplantation.

MDSC may have a higher proliferation rate suggesting that MDSC transplantation may be an attractive approach to regenerate muscle (Qu-Peterson et al 2002). MDSC displays several characteristics of bone marrow derived stem cells, including the antigen Sca-1, a marker which presents in subpopulations of hematopoietic cells with stem cell-like characteristics (Gussoni et al. 1999). MDSCs may have a potential to differentiate to skin cells. It has been demonstrated that MDSCs can differentiate to myofibroblasts in the muscle injury site where TGF-β1 is overexpressed (Li et al 2002).

To summarize, the studies on MDSC have been shown as a promising approach for regenerating muscle and possibly other tissues including skin.

1.7.3 Bone marrow-derived stem cell (BMDSC)

There are three kinds of stem cells in the bone marrow cavity, hematopoietic stem cell (HSC), mesenchymal stromal cell (MSC) and endothelial progenitor cells (EPC). Stem cells are less than 0.1 % of all nucleated cells in bone marrow. All bone marrow stem cells are derived from the mesoderm and are all round in morphology. While HSC and MSC shares common morphological characteristics, HSC and MSC is different in adherence properties. For example, HSC are non-adherent whereas MSC are adherent (Turksen 2004). The isolation of MSC is based on their adherence properties whereas HSC can be isolated using CD34 (Engelhardt et al 2002). Recently, HSCs were also found in CD34- fraction (Engelhardt et al 2002, Turksen 2004). In contrast to MDSC, bone marrow- derived stem cells have been studied since the 60s

(Cudkowicz et al 1964, Lewis et al 1964). Bone marrow–derived stem cells were studied extensively because they are relatively easy to harvest from patients. With the advanced techniques and instruments, the harvest of MDSCs is relatively non-invasive and can be achieved by using a simple syringe.

1.7.4 Stem cell therapy in wound healing

Keratinocytes, fibroblasts and macrophages all have an important role in wound healing. While the majority of the mature cells have a limited self renewal capability, stem cells are able to be self-renewed and pluripotent/ multipotent. Stem cells would be a valuable cell source for regenerating tissues in wound healing. Stem cells have been used in different wound healing studies. Several types of stem cells such as BMDSCs, HSCs or MDSCs have been shown to enhance the healing in bone, cartilage, muscle or skin after injury (Evangelos et al 2003, Peng et al 2003, Stocum 2001). BMDSCs are able to be recruited and incorporated into a cutaneous wound site (Badiavas et al 2003). In their study, they injected bone marrow derived stem cells intravenously and found the stem cells incorporating into the hair bulge region at the wound site and differentiating into epithelial cells.

1.8 ORGANIZATION OF DISSERTATION

At the beginning of this thesis project, we found that the diabetic wound healing has been improved by a simple approach which is a direct injection of naked DNA. Chesnoy et al (2002) have shown that intradermal injection of TGF-β1 plasmid DNA significantly enhances the diabetic wound repair. This finding supports the feasibility of using naked DNA as a therapeutic approach for treating diabetic wounds. Since naked DNA approach yields low efficiency, we

seek strategies that can enhance the therapeutic level of gene expression and comtribute to improvements in wound healing. One of the primary goals in this thesis project is to develop a more effective strategy to improve diabetic wound healing compared with the conventional methods for treating wounds such as electrical stimulation (ES) and hydrogel wound dressing. Additionally, we use muscle derived stem cells in an attempt to improve wound repair as the stem cells may have the potential to differentiate into essential cells in the wound such as macrophages, keratinocytes and fibroblasts. The following are the three specific aims in this thesis project:

1) To test a positively synergistic effect on wound healing using electroporation for delivering TGF-β1 gene. Based on the findings that electroporation can enhance gene transfer (Aihara and Miyazaki 1998, Suzuki et al 1998) and electric therapy can accelerate wound recovery in diabetic patients (Baker et al 1997), it is hypothesized that using electroporation to deliver the TGF-β1 gene at the condition where both electroporation and electric therapy will synergistically accelerate the wound healing. Two different types of electrodes i.e. caliper and syringe electrodes will be tested to deliver TGF-β1 gene to the wound. In Chapter 2, we will discuss this topic in detail.

2) To test whether wound repair synergistically accelerates using thermosensitive hydrogel as a carrier of TGF-β1 gene.

There is growing evidence that wet and moist environments result in improved wound healing of full and partial thickness wounds (Svensjo et al 2000, Vogt et al 1995, Chen et al 1992). A hydrogel dressing is an available treatment for providing a moist environment in the wound (Vogt et al 2001, Eisenbud et al 2003). In addition, hydrogels have been shown to be effective drug delivery systems, for growth factors (Iwakura A et al 2003, Hatano et al 2003) and DNA

(Li et al 2003) in animal models (Amant et al 1999, Vogt et al 2001, Reimer et al 2000) and in clinical trials. Based on all these facts, it is hypothesized that using thermosensitive hydrogel, PEG-PLGA-PEG as a wound dressing and as a vehicle to deliver TGF-β1 gene improves synergistically wound repair. In Chapter 3, we will describe the feasibility of the thermosensitive hydrogel as a gene delivery vehicle and wound dressing in the diabetic wounds.

3) To test whether using thermosensitive hydrogel to assist the delivery of muscle derived stem cells accelerate wound repair.

Previously we have found that the thermosensitive hydrogel promotes cell proliferation. Together with the finding of muscle derived stem cells differentiating into myofibroblasts (Li et al 2002), which are one of important cells in wound contraction, it is hypothesized that the muscle derived stem cells possibly accelerate the wound healing and differentiate to essential cells in wound healing such as keratinocytes, fibroblasts or macrophages. In Chapter 4, we will discuss the approach using muscle derived stem cells in diabetic wound healing in detail.

2.0 WOUND CLOSURE STUDY AND HISTOLOGIC EVALUATION OF THE SYNERGISTIC EFFECT OF ELECTROPORATIC THERAPY AND DELIVERY OF TGF-β1 ON DIABETIC WOUND HEALING

(Lee PY, Chesnoy S, Huang L. Electroporatic delivery of TGF-beta1 gene works synergistically with electric therapy to enhance diabetic wound healing in db/db mice. J Invest Dermatol. 123(4):791-8, 2004.)

2.1 ABSTRACT

Electrical stimulation is a therapeutic treatment for wound healing. Electroporation, a type of electrical stimulation, is a well-established method for gene delivery. Since both processes involve electrical treatment at the wound site, we hypothesize that proper conditions can be found with which both electrical and gene therapies can be additively applied to treat diabetic wound healing. For the studies of TGF-β1 local expression and therapeutic effects, full thickness excisional wound model on db/db mouse was used. We measured TGF-β1 cytokine levels using ELISA at 24 h postwounding and examined wounds histologically using H&E, picrosirius, anti BrdU and anti factor VIII-related antigen staining. Furthermore, wound closure was evaluated by wound area measurements at each day for a total of 14 days. We found that syringe electrodes were more effective than the conventional caliper electrodes. Furthermore, diabetic skin was more sensitive to the electroporative damage than the normal skin. The optimal condition for diabetic skin was 6 pulses of 100V/cm with the duration of 20ms. Under such conditions, the healing rate of electrically treated wounds was significantly accelerated. Furthermore, when the TGF-β1 gene was delivered by electric pulses, the healing rate was further enhanced. Five to

seven days postapplication of intradermal injection of plasmid TGF-β1 followed by electroporation, the wound bed showed an increased reepithelialization rate, collagen synthesis and angiogenesis. Our data indicate that the electric effect and gene effect were synergistic in the genetically diabetic model.

2.2 INTRODUCTION

For decades, investigators have attempted to treat problems related to wound impairment by electrical stimulation (ES). Most of the ES applications are safe and effective. For example, ES has long been used in clinics for wound-related pain control (Bjordal et al 2003, Evans et al 2001) and neuromuscular rehabilitation with an excellent clinical safety record (Crevenna et al 2001). Bjordal et al reported that electrical nerve stimulations reduced analgestic consumption in postoperative patients in an average of 35.5%. Chronic heart failure patients were not observed with heart abnormalities during administration of ES on the thigh muscle (Crevenna et al 2001), suggesting that ES can be highly safe to apply in patient. Furthermore, electric pulses can accelerate the healing of diabetic ulcer (Baker et al 1997). In other human studies, ES has been shown to accelerate healing of various types of chronic wounds (Gardner et al 1999).

Electroporation is a type of electrical treatment which can enhance cell permeability to allow penetration of macromolecules such as DNA (Banga and Prausnitz 1998). It has been reported to increase gene transfer in liver and muscle (Aihara and Miyazaki 1998, Suzuki et al 1998). Delivery of chemotherapeutic agents for cancer using electroporation has progressed to Phase II clinical trials (Jaroszeski et al 1999, Heller et al 1999). Due to the complexity of the skin, the harsh condition of electroporation, ranged from electric field strength of 400-2,000V/cm, was primarily used to accomplish effective gene transfer (Titomirov et al 1991,

Heller et al 2001). Typical transdermal voltage for drug delivery is 50-150V (Vanbever et al 1996). High current and voltage applied to the skin can cause tissue damage and electrolysis (Pliquett 1999).

Administration of exogenous growth factors has been successfully used to accelerate the pathologic wound healing in animal models. Topical application of PDGF shortens the prolonged inflammatory phase in genetically diabetic mice (Beer et al 1997). A single dose of TGF- β in a collagen vehicle can restore the diabetes-related decrease in tensile strength of collagen (Bitar and Labbad 1996). Broadley et al (1989) also reported that injection of TGF-β induced accumulation of granulation tissue, and collagen production and maturation. However, one disadvantage of the application of the growth factor is the high expense. Another problem is the need to have continuous or repeated delivery of the growth factor, which becomes inactivated in the wound. Instead of using growth factors, an alternative approach is to administer a gene that encodes the growth factor (Yao and Eriksson 1989). The contribution of growth factor gene therapy to problematic wound healing appears as successful as the growth factor itself, although no direct comparison has been reported. Subcutaneous injection of interleukin-6 plasmid to mice restores abnormal wound healing (Gallucci et al 2001). In a dermal ulcer model, topical application of PDGF embedded in collagen sponges promotes reepithelialization, wound closure and new granulation tissue formation (Tyrone et al 2000). In our previous study, intradermal injection of TGF-β1 gene accelerated wound closure (Chesnoy et al 2003). TGF-β1 is a multifunctional growth factor. TGF-β1 is a chemokine for fibroblasts, and enhances wound contraction rate, extracellular matrix production in *vivo* (Lanning et al 2000, Mustoe et al 1991) and the formation of capillaries *in vitro* (Sakuda et al 1992). TGF-β1 influences tissue repair by activation of Smad signaling (Dijke et al 2003). TGF-β1 signals through heteromeric complexes

of type II and type I transmembrane Ser/Thr kinase receptors which then initiate phosphorylation cascades involving receptor-regulated Smads, a co-Smad, and inhibitory Smads.

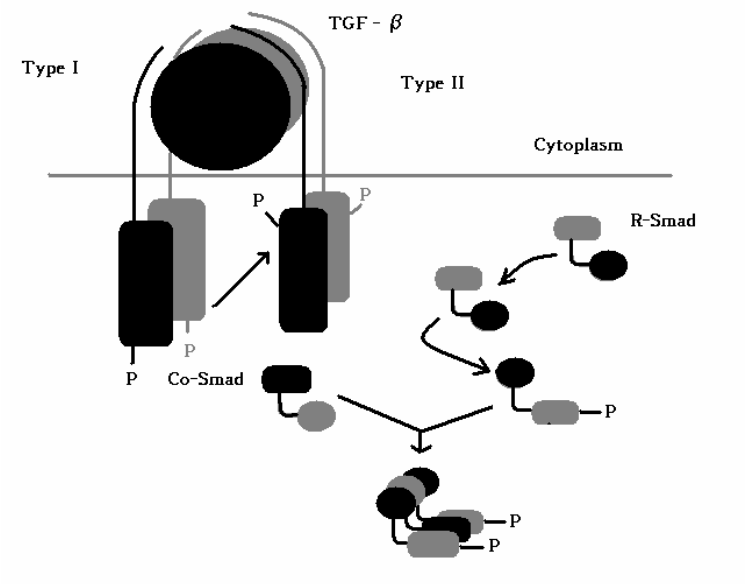


Figure 2.1 The signaling of TGFβ. TGF-β1 signals through heteromeric complexes of type II and type I transmembrane Ser/Thr kinase receptors which then initiate phosphorylation cascades involving receptor-regulated Smads, a co-Smad, and inhibitory Smads.

We hypothesized that proper conditions could be found in which both electrical and gene therapies could be additively applied to treat diabetic wound healing without damaging the tissue. The first step of our study was the optimization of parameters such as applied voltage, pulse duration, number of pulses and type of electrodes. We then examined if the condition optimal for TGF-β1 gene transfer at the wound site could also benefit healing due to its electric effect.

2.3 METHODS

2.3.1 Animal Model

Female mice (C57BL/6 or C57BKS.Cg-m +/+ Lepr^{db} (type II diabetes)) in 7-9 weeks old, were obtained from Jackson Laboratories (Bar Harbor, Maine). C57BKS.Cg-m +/+ Lepr^{db} mice have been used as a model for wound healing in diabetics, especially for studies involving cytokines and growth factors (Greenhalgh et al 1990, Okumura et al 1996). Mice homozygous for the diabetes spontaneous mutation (*Lepr^{db}*) become identifiably obese around 3 to 4 weeks of age. Elevations of plasma insulin begin at 10 to 14 days and elevation of blood sugar at 4 to 8 weeks. All mice were housed in the animal facility at the University of Pittsburgh. All animal protocols were approved by IACUC of the University of Pittsburgh.

2.3.2 Preparation of Plasmids

Human TGF-β1 cDNA in 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). Plasmid pNGVL-luc, which encodes luciferase as a reporter protein (National Gene Vector laboratory) was obtained similarly.

2.3.3 Wounding Protocol and Treatment

A total of 36 C57BL/6 and 136 C57BKS.Cg-m +/+ Lepr^{db} mice were anesthetized by inhalation of Isoflurane and randomly divided into different control and treatment groups. Forty micrograms of plasmid pNGVL-luc (dissolved in 50μL 1.5X PBS) intradermally injected into the skin of C57BKS.Cg-m +/+ Leprdb or C57BL/6, followed by electroporation using a caliper (BTX Gentronics, Inc. San Diego, CA) or a syringe (Liu and Huang 2002) electrodes for optimization of the electric conditions. Intradermal injection of plasmid pNGVL-luc was also conducted for comparison. Two 7 x 7mm full thickness excisional wounds were created in parallel on the back of each mouse after the mice were anesthetized. Human recombinant plasmid TGF-β1, (30μg dissolved in 50μL PBS), was intradermally injected to the lateral sides of a wound followed by electroporation using a syringe electrode. Control mice received no treatment, electroporation only, intradermal injection of empty plasmid with or without electroporation, or intradermal injection of plasmid TGF-β1 without electroporation.

2.3.4 Measurement of luciferase reporter gene expression

Six mice (n=12 wounds) in each group were sacrificed and skin biopsies were collected and homogenized. Luciferase gene expression (activity) was measured using a luminometer. The activity was presented as relative unit per mg soluble tissue protein (RLU/mg protein).

2.3.5 Local expression of TGF-β1 using immunoassay

At 24 h post-application, three mice (n= 6 wounds) in each group were sacrificed and wound biopsies were harvested and homogenized with protease inhibitor (Roche Diagnostics). TGF-β1

protein concentration was measured with human TGF-β1 ELISA kit (R&D Systems) following the supplier's protocol.

2.3.6 Wound closure analysis

Six mice in each group were examined. Area of wounds was measured using a caliper at each day, for a total of 14 days, and evaluated as percentage of wound closure using the equation:

% wound closure = 100 x (wound area at day 0 – wound area at day n) /wound area at day 0

2.3.7 Histology

Three mice from each group were sacrificed and skin biopsies were harvested at days 3, 5 and 7 postwounding. The harvested tissue was formalin-fixed and embedded in paraffin. Sections of 4μm thickness were prepared using a microtome, then deparaffinized, hydrated and stained with Hematoxylin and Eosin for observing the morphology and picrosirius red staining using a 0.1% picrosirius red solution (Sweat et al 1964).

2.3.8 Cell proliferation using anti 5-bromo-2-deoxyuridine (BrdU) immunohistochemistry

At day 3 postwounding, BrdU (Sigma, St Louis, MO) labeling was performed by intraperitioneal injection at a dose of 50 mg/kg at 3 h prior to sacrifice. Paraffin sections were taken from specimens at the wound site. The sections were deparaffinized, hydrated, pretreated with 2N HCl and trypsin (Sigma). 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 were peroxidase-labeled with Vetastain Elite ABC Kit (Vector Laboratories, Burlingame, CA). The immunoprecipitate was visualized by 3,3'-diaminobenzidine

tetrahydrochloride (DAB) chromogen and Gill 1X hematoxylin (Fisher Scientific) counterstain. Sections were observed under a microscope (Nikon, Japan), and positively stained cells were counted in 3 representative fields at 200X magnification.

2.3.9 Angiogenesis using anti-factor VIII related antigen immunohistochemistry

Paraffin sections were taken from specimens at the wound site at day 7 post-wounding. Factor VIII related antigen immunochemical staining was performed with incubation of rabbit polyclonal antisera for Factor-VIII antigen. All other steps were performed as the same as mentioned in the method for anti-BrdU staining. Sections were observed at 200X magnification.

2.3.10 Statistical analysis

Data were expressed as means ± standard deviation (SD) and analyzed by two-tailed Student's t-test using the PRISM software program (GraphPad Software, San Diego, CA, USA). The alpha value (Type I error) adjustment was done by using Bonferroni correction in case of multiple comparisons.

2.4 RESULTS

2.4.1 Optimization of electroporative condition

In vivo electroporative gene delivery is commonly accomplished using two kinds of electrodes: calipers and syringe electrodes, a special type of needle electrode. The design of the syringe electrode has been previously described (Liu and Huang 2002). To compare caliper and syringe electrodes in the effectiveness on gene delivery, we intradermally injected 40μg of luciferase

reporter gene to C57BL/6 or C57BKS.cgmLepr^{db} mice followed by electroporation using caliper or syringe electrodes. We found that the syringe electrodes were more effective in gene transfer compared with the caliper electrodes. In Figure 2.1A, the luciferase gene expression in the syringe-electroporated skin was 10 fold higher compared with the caliper- electroporated when the same applied voltage and duration (100V in 20ms) were used. Therefore, in our further experiments, we decided to use the syringe electrodes.

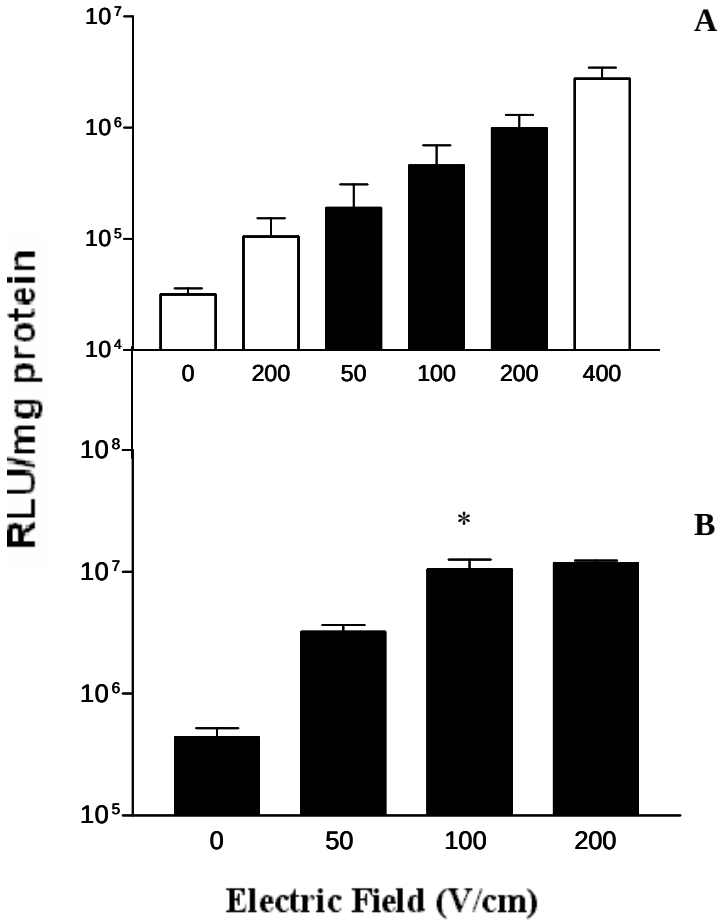


Figure 2.2 Luciferase expression in the normal (A) or diabetic (B) skin 24 h postapplication of electroporation using either caliper (white bars) or syringe electrodes (black bars). * $p<0.001$, comparing data of 100V using syringe electrode with 50V or 0V in (B). n = 6.

To optimize the electroporative condition for the syringe electrodes, we tested different applied voltage and found that 100V in the duration of 20ms with 6 pulses were the optimal conditions for gene transfer in the skin tissue (Fig. 2.1B). Higher voltage did not induce a corresponding increase in the gene expression. Furthermore, when we compared the luciferase gene expression of two skin types (diabetic and normal), the diabetic skin demonstrated a 10-fold higher expression compared with the normal skin at these conditions. Bubbles, a sign of electrolysis, were observed surrounding the electrodes in the diabetic skin when 200V/cm field strength was applied. This observation did not appear in the normal skin, suggesting that the diabetic skin is more sensitive to electroporation in mouse model.

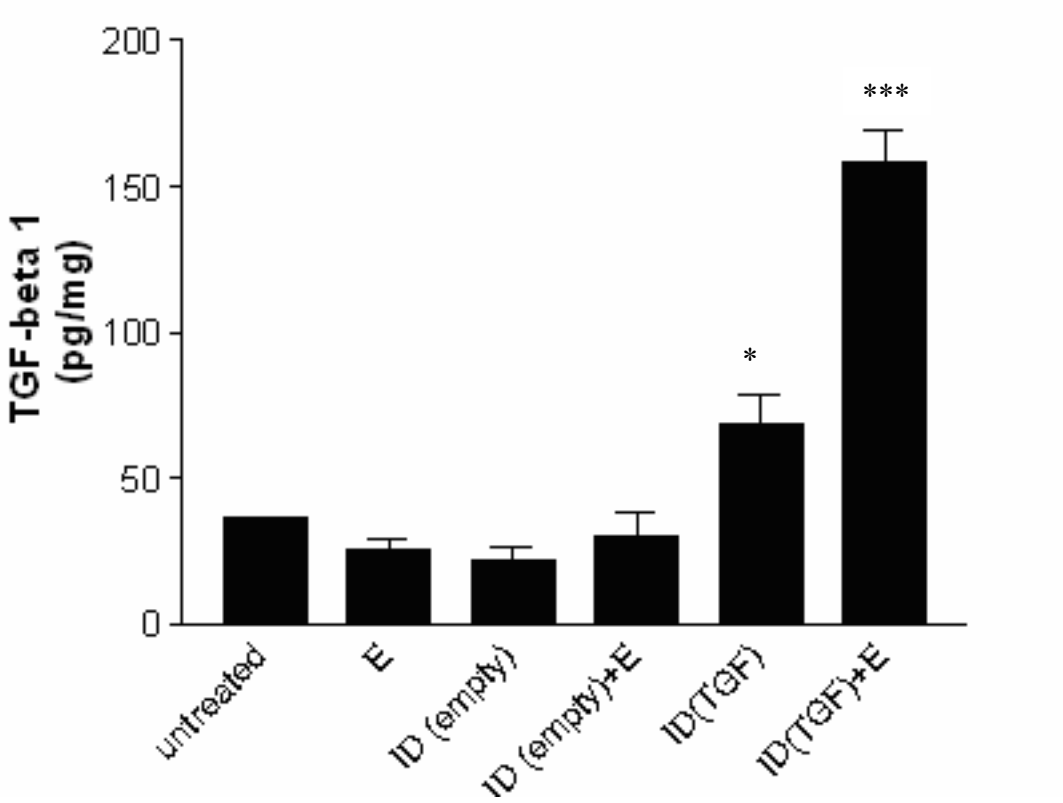


Figure 2.3 TGF-β1 cytokine level 24 h after the following treatments: electroporation only [E], intradermal injection of empty plasmid [ID(TGF)] or plasmid with TGF-β1 gene [ID(empty)], electroporation following intradermal injection of plasmid with [ID(TGF)+E] or without TGF-β1 gene [ID(empty)+E]. ***$p<0.001$, comparing treatment of TGF-β1 gene by electric pulses with all other treatments. *$p<0.05$, comparing treatment of TGF-β1 gene by intradermal injection with E, [ID(empty)+E], [ID(empty)], or [untreated] . n = 6.

2.4.2 Local expression of TGF-β1 at the wound bed

Next, we transfected the diabetic skin tissue with our therapeutic gene, TGF-β1, using the optimized conditions. To measure the skin transfection by the plasmid, we used an ELISA kit, which was coated with TGF-β1 receptor to detect human TGF-β1. As shown in Figure 2.2, intradermal injection of plasmid TGF-β1 gave a significantly ($p<0.05$) higher expression of TGF-β1 compared with the untreated, electroporation alone or intradermal injection of the empty plasmid. Intradermal injection of plasmid TGF-β1 followed by electroporation produced a two-fold higher cytokine level compared with the intradermal injection of plasmid TGF-β1 alone, suggesting that electroporation enhanced the gene transfer as previously reported (Heller et al 2000). On the other hand, the untreated tissue produced low level of TGF-β1 cytokine. Furthermore, wound treated with intradermal injection of empty plasmid or electroporation alone also produced TGF-β1 cytokine as low as the untreated. These low levels of activity might arise from the cross reactivity of the antibody with endogenous murine TGF-β1.

2.4.3 Wound healing parameters

Wound healing is a multiple step process. In order to investigate the progress of wound healing, wound healing phases such as reepithalization, wound closure, collagen synthesis and angiogenesis were examined in the diabetic skin.

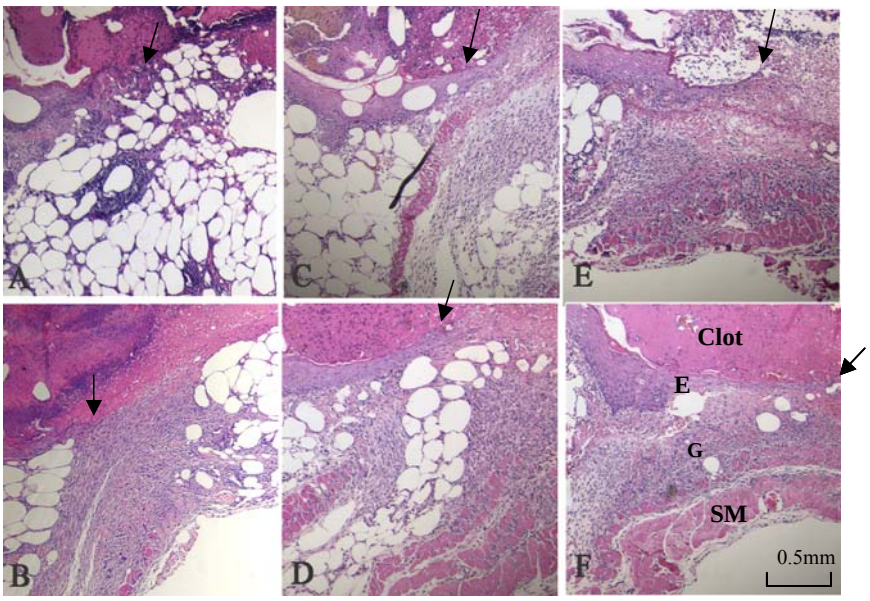


Figure 2.4 Wound morphology shown by H&E staining at day 5 postwounding following the treatment without electroporation (A, C & E) or with electroporation (B, D & F). No plasmid (A & B), empty plasmid alone (C & D) or with TGF-β1 gene (E & F). The leading end of epithelial tongue is indicated by black arrows. Epithelial tissue, granulation tissue or smooth muscle are indicated respectively by E, G or SM. Magnification X 100.

2.4.3.1 Reepithelialization

Reepithelialization is a process involving keratinocyte migration followed by keratinocyte proliferation. At day 5 postwounding, H& E staining showed that the leading end of epithelium, (i.e. the epithelial tongue schematically shown in Fig. 2.4A), of all wounds migrated toward the center (from left to right in Fig. 2.3). Epithelial tongue (indicated by arrow in Fig. 2.3) moved the fastest in the wound treated with plasmid TGF-β1 injection followed by electroporation. The electric treatment appears to induce the fibroblast migration. As can be seen in Figure 2.3, cell density in the granulation tissue (schematically shown in Fig. 2.4A) in the wound treated with electroporation alone (Fig. 2.3B) was higher than the untreated (Fig. 2.3A). The majority of the observed cells in the granulation tissue were spindle-like, similar to the shape of fibroblasts. Anti-BrdU immunohistochemical staining further showed that epithelial cells were the most

actively proliferated in the electro-TGF-β1 gene treated wound. Cell proliferation was also significantly induced by simple plasmid TGF-β1 injection. On the contrary, other treatments produced comparable level of cell proliferation as the untreated.

When we investigated the epidermal cell proliferation at the edge of the wound, the proliferation rate (Fig 2.4B) at region 2 (Fig 2.4A) was higher than at region 1, which is the leading edge of the epithelial tongue. The difference tends to be significant ($p < 0.05$) in the actively proliferated wound bed which was received the plasmid TGF-β1. In both locations, the

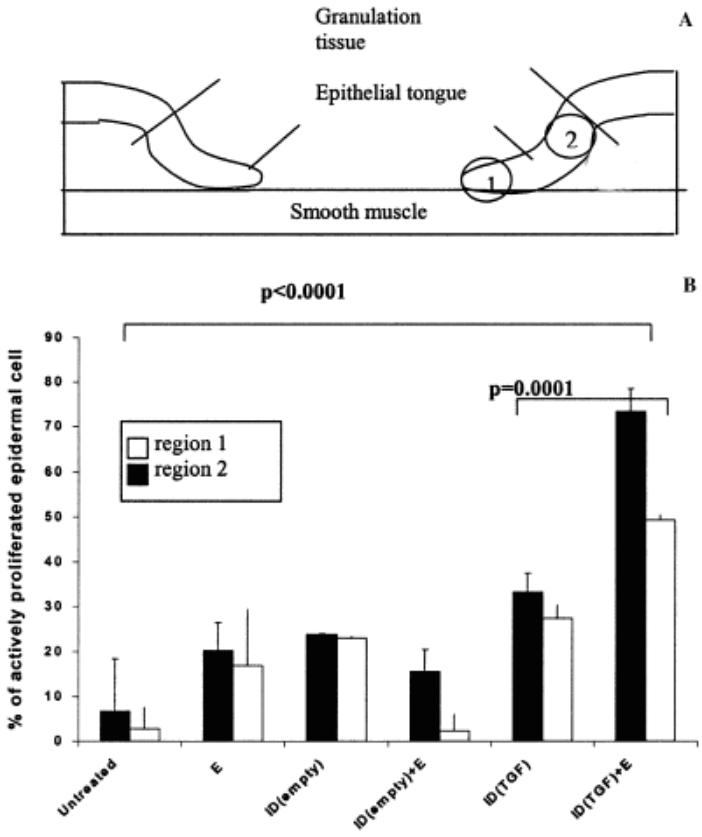


Figure 2.5 Immunostaining for Brdu-positive keratinocytes at the wound edge of epithelium at day 3 postwounding following different treatments. (A) Schematic drawing of a wound indicating the location of cell counts taken (Regions 1 and 2). (B) BrdU-positive cell count at region 1 (□) or at region 2(■) following different treatments. (n = 3). Region 1 indicates the area of migrating cells. Region 2 indicates the area of proliferating cells.

wound treated with intradermal injection of TGF-β1 gene followed by electroporation induced higher proliferation than other treatments. Intradermal injection of plasmid TGF-β1 also induced proliferation significantly ($p<0.05$) in both positions compared with the untreated, electroporation, intradermal injection of the empty plasmid with or without electroporation.

2.4.3.2 Wound Closure

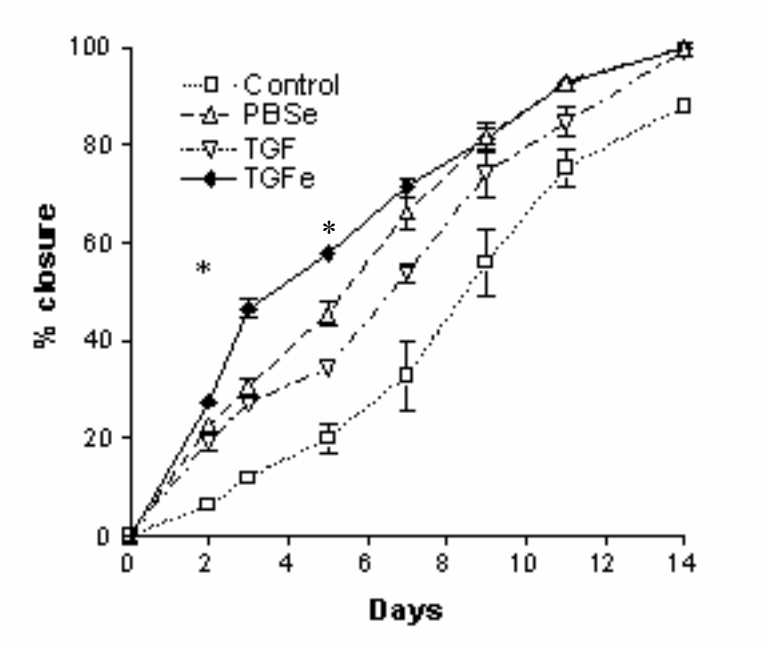


Figure 2.6 Wound closure at day 5 postwounding of the untreated (Control) following the applications of electroporation alone (PBSe), intradermal injection of TGF-β1 with (TGFe) or without electroporation (TGF). n=12. * $p<0.05$, comparing the treatment of TGF-β1 gene with or without electric pulses.

Wound contraction is the process to minimize open area by pulling the neighboring tissue towards the wound center. Myofibroblasts differentiated from fibroblasts generate the contractile force (Feugate et al 2002, Jester et al 1999). It occurs faster than reepithelialization because no cell proliferation is involved. We measured the wound contraction rate by percentage wound

closure until wound was completely closed at day 14 (Fig. 2.5). When compared with the untreated group, electroporation alone or intradermal injection of plasmid TGF-β1 with or without electroporation induced wound contraction during the 14 days. Wound closure rate was significantly ($p<0.05$) accelerated at early stage (day 2- 5) in the electro-TGF-β1 gene treated wound bed compared with TGF-β1 gene treated wound bed. This suggests that the additive effect of electroporation combined with TGF-β1 gene treatment on wound closure occurred only in the early phase of wound healing.

2.4.3.3 Collagen synthesis

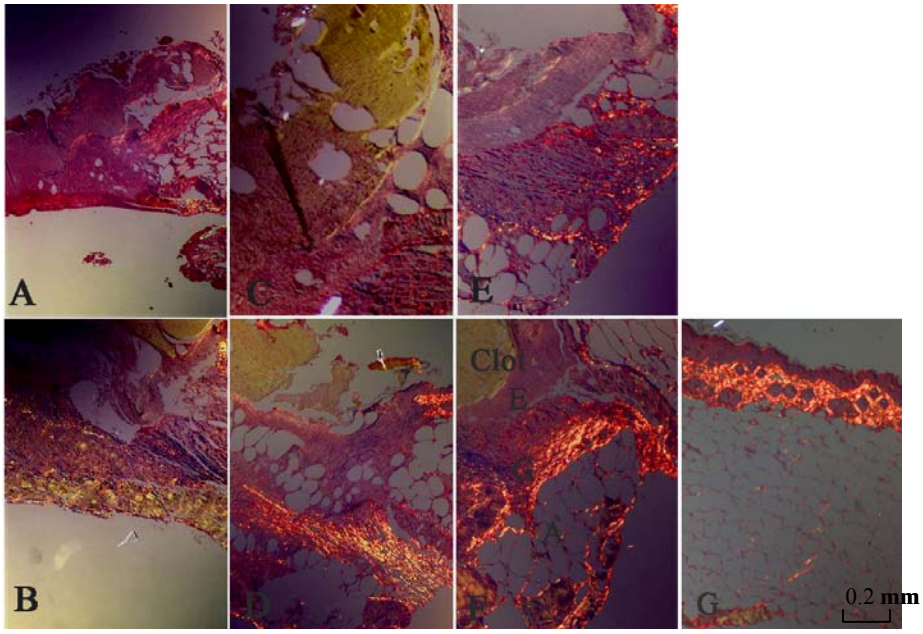


Figure 2.7 Collagen formation shown by picrosirius staining and polarized microscopy at day 5 postwounding following the treatment of electroporation (B, D&F) or without electroporation (A,C&E). No injection of plasmid (A&B), injection of empty plasmid (C&D) of plasmid with TGF-β1 gene (E&F). Unwounded skin (G). Collagen is indicated as glowing yellowish orange. Epidermal tissue, granulation tissue, adipose tissue and smooth muscle are marked as E, G, A and SM, respectively. Magnification 200X.

At day 5 after treating with intradermal injection of plasmid TGF-β1 with or without electroporation, a high intensity of collagen was found in the newly formed granulation tissue at the epithelial tongue, with the former greater than the latter (Fig. 2.6). Picrosirius/polarized light microscopy reveals regions of organized collagen which increases as the wound matures. The collagen organization in the electric-gene treated wound appeared the most mature, as it resembled the unwounded skin at day 5 (Fig. 2.6 F&G). In addition, we observed that the collagen organization in the smooth muscle layer (white arrow, Fig. 2.6D) is more dispersed from the granulation tissue.

2.4.3.4 Angiogenesis

Angiogenesis is a process for new capillary growth and one of its components is endothelial cell migration (Veves et al 2002). At day 7, sections of harvested wound bed were stained with anti-factor VIII related antigen to identify endothelial cells in the newly synthesized granulation tissue in the wound. A higher density of endothelial cells was found in the electro-TGF-β1 gene treated wound than all other treatments. In the wound bed treated with simple injection of TGF-β1 gene, stained endothelial cells could be observed in one of the three representative fields (black arrow, Fig 2.7E). On the other hand, with electroporation, more intensely stained endothelial cells were observed in all three fields (Fig. 2.7F). The number of stained endothelial cells found in different treatment groups is shown in Fig. 2.7G. Only electro TGF-β1 gene therapy appeared to enhance endothelial cell migration significantly ($p<0.05$), suggesting an enhanced angiogenesis.

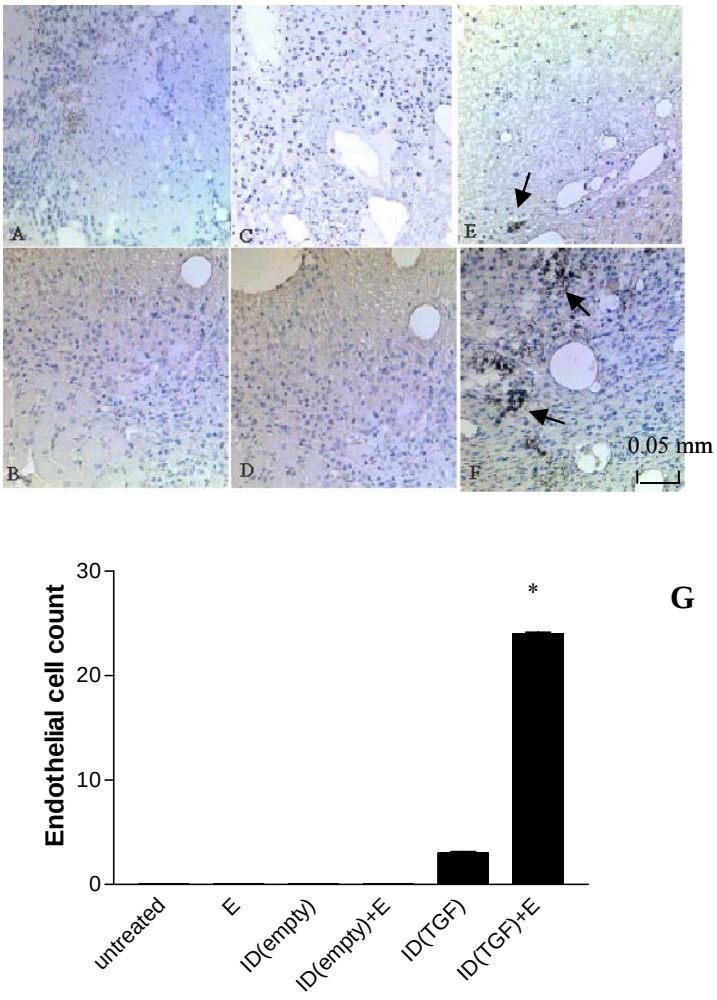


Figure 2.8 Angiogenesis in the granulation tissue at the center of the wound bed (region 3) at day 7 postwounding following the treatment without (A, C&E) or with electroporation. (B, D&F). No injection of plasmid (A&B), injection of empty plasmid (C&D) or plasmid with TGF-β1 gene (E&F). Endothelial cells are indicated by black arrow. Magnification 400X. (G) Endothelial cell count in 3 representative fields. *$p<0.05$, comparing the treatment of TGF-β1 gene by electric pulses with all other treatments.

2.5 DISCUSSION

This chapter is to report the potential of the combination of gene and electric therapies to treat diabetic wound healing. For this purpose, we assessed various conditions in which both TGF-β1 gene transfer and electrical treatment would occur. We applied electric pulses immediately after injection of the luciferase reporter gene. We found that 6 pulses of 100V/cm in 20 ms duration

with the syringe electrodes was the most effective condition and accomplished transfection level of approximately 10^7 RLU/mg protein. Compared with the condition used by most of other investigators, 700V/cm – 1,200V/cm, our condition used < 700V/cm to reach a comparable transfection level (Heller et al 2001). This is important since the diabetic skin as shown in Figure 2.1 is more sensitive to electroporation than the normal skin in this mouse model. The use of a milder condition was necessary to prevent tissue damage.

Next we determined if our optimal condition could result in the therapeutic effects of TGF-β1 gene transfer and electrical therapy. We investigated several well-recognized wound healing parameters such as reepithelialization, wound closure, collagen deposition and angiogenesis with our diabetic excisional wound model. We used the same syringe for the injection of TGF-β1 gene and the application of 6 pulses of 100V/cm in 20 ms duration without withdrawing the needle. In this experimental setting, we determined that electric pulses alone could induce cell migration (Fig 2.3) and wound closure (Fig 2.5). Simple injection of TGF-β1 plasmid could induce reepithelialization, wound closure, collagen deposition and angiogenesis. As we expected, the application of electric pulses along with the delivery of TGF-β1 plasmid further enhanced all wound healing parameters. An increase of reepithelialization rate, wound contraction, collagen synthesis and angiogenesis was found in the wound 5-7 day post-application of intradermal injection of TGF-β1 followed by electroporation. Not surprisingly, the improvements in reepithelialization rate, collagen synthesis and angiogenesis were greater than the additive effects of gene and electric treatments. There was a clear synergism between the two treatments.

TGF-β1 is a multifunctional cytokine and the response is complicated in wound healing. It is a potent chemokine for fibroblasts. TGF-β1 enhances granulation tissue and collagen

formation. The effect of TGF-β1 on reepithelialization is complicated. TGF-β1 is a potent inhibitor of keratinocyte proliferation in *vitro* (Garlick et al 1996, Garlick and Taichman 1994). One possible reason is that TGF-β1 pathway is a negative feedback mechanism for epidermal growth factor-induced proliferation of human keratinocyte (Yamasaki et al 2003). Interestingly, in a steroid-impaired rabbit wound model, application of TGF-β1 enhanced reepithelialization (Beck et al 1991). Another study also shows that TGF-β1 probably can induce keratinocyte proliferation *in vivo* (Fowlis et al 1996). Fowlis et al showed that overexpression of TGF-β1 promoted the epidermal cell growth in TGF-β1 transgenic model (Fowlis et al 1996), suggesting the complicated biological activity of TGF-β1. In addition to keratinocyte proliferation, keratinocyte migration is important in reepithelialization. TGF-β1 can induce keratinocyte migration by up-regulating the synthesis of laminin 5, which has a dual function in keratinocyte adhesion and migration (Decline et al 2003). Taken together, TGF-β1 is beneficial to wound healing. Indeed, the therapeutic effect of plasmid TGF-β1 has been fully documented by our group (Chesnoy et al 2003). Our data is consistent with the previous findings.

Electrical stimulation has been known as a cell migration promoter. Studies showed that electrical field stimulates macrophage, corneal epithelial cells and fibroblast migration (Cho et al 2000, Wang et al 2003, Brown and Loew 1994). Our results also showed that electroporation induces cell migration (Fig 2.3B). The induction is associated with an alteration of cell movement including cell crawling and possibly cell rolling without changing the cell morphology (Cho et al 2000). The mechanism of electric field –induced migration is not yet clearly elucidated, but integrin-dependent signaling may be involved in electric field-induced macrophage migration (Cho et al 2000). Furthermore, the electric stimulation also enhances the activation of ERK1/2, a signaling molecule in the MAP kinase pathway (Wang et al 2003).

In our study, we found that the actively migrating keratinocytes at the leading end (region 1, Fig.2.4A) do not proliferate. Instead, most of the proliferation occurred in the vicinity of leading end (region 2, Fig. 2.4A). A previous study reported that reepithelialization is temporally and spatially coordinated: keratinocyte migrates into the wound followed by transiently burst proliferation at wound margin, and the actively migrating keratinocytes do not proliferate (Garlick and Taichman 1994). Thus, our data are consistent with previous findings.

In conclusion, we have developed an innovative strategy for therapeutic treatment of diabetes-induced wound impairment with a combination of electric and gene therapies which may have a significant implication for clinical applications.

3.0 THERMOSENTIVE HYDROGEL AS A TGF-β1 GENE DELIVERY VEHICLE ENHANCES DIABETIC WOUND HEALING

(Lee PY, Li Z, Huang L. Thermosensitive hydrogel as a Tgf-beta1 gene delivery vehicle enhances diabetic wound healing. Pharm Res. 20(12):1995-2000, 2003.)

3.1 ABSTRACT

This chapter is to report the feasibility to accelerate diabetic wound healing with TGF-β1 gene delivery system using a thermosensitive hydrogel made of a triblock copolymer, PEG-PLGA-PEG. Two 7x7mm full thickness excisional wounds were created in parallel at the back of each genetically diabetic mouse. The hydrogel containing TGF-β1 gene 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. We found that the thermosensitive hydrogel alone is slightly beneficial for reepithelialization at early stages of healing (day 1- 5), but significantly accelerated reepithelialization, increased cell proliferation and organized collagen were observed in the wound bed treated with thermosensitive hydrogel containing TGF-β1 gene. The accelerated reepithelialization was accompanied with enhanced collagen synthesis and more organized extracellular matrix deposition. Humatrix® alone or

mixed with TGF-β1 gene, had little effect. In conclusion, thermosensitive hydrogel composed of PEG-PLGA-PEG triblock copolymer provides excellent wound dressing activity and delivers TGF-β1 gene to promote wound healing in a diabetic mouse model.

3.2 INTRODUCTION

Of the growth factors, TGF-β family members play a central role in tissue repair. The biological activities of TGF-β1 in the wound healing process have been previously reported. Lanning et al. (2000) showed TGF-β1 induces myofibroblast production, resulting in a significantly reduced wound in a non-contractile fetal rabbit model. Sidhu et al. (1999) 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 (Jude et al 2002) and PDGF (Beer et al 1997), to overcome impaired wound healing in diabetes (Greenhalgh et al 1990). 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 (Chesnoy et al 2003). 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 (Wang et al 2002) and canine osteotomy model (Bonadio et al 1999) 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 (Border et al 1994).

In this study, we have tested the ability of a thermosensitive hydrogel to deliver naked DNA to the wound surface. We have previously characterized the hydrogel made of a triblock copolymer, PEG-PLGA-PEG, for naked DNA delivery at the wound site (Li et al 2003). Upon water evaporation at the skin temperature, the liquid copolymer solution formed an adhesive film in situ. Adherent interaction prevents wound desiccation as well as reduces the risk 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.

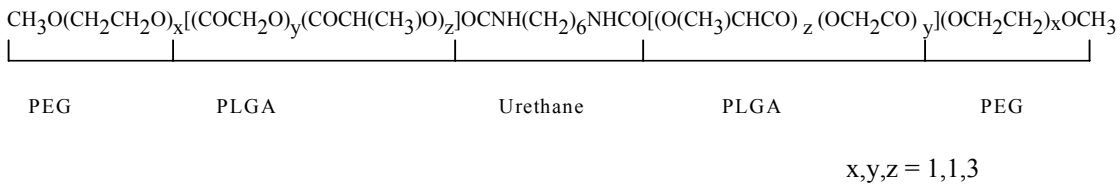
3.3 MATERIALS AND METHODS

3.3.1 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.

3.3.2 Synthesis of in situ hydrogel solution

We have synthesized a triblock co-polymer, poly [ethylene glycol-b-(D, L-lactic acid-co-glycol acid)-b-ethylene glycol] (PEG-PLGA-PEG), according to published procedure (Jeong et al 2000, Jeong et al 1999) with Mw = 29,659, Mn = 15,732 and polydispersity = 1.877. Briefly, the triblock copolymer was prepared by ring opening polymerization of D, L-lactide (LA) and glycolide (GA) onto monomethoxy poly (ethylene glycol) (mPEG, Mw = 750 Da), followed by coupling of the resulting diblock copolymer (mPEG 750-PLGA) using hexamethylene diisocyanate (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 chloroform ($CDCl_3$) 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.



3.3.3 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).

3.3.4 Wounding protocol and treatment

The mice were anesthetized by inhalation of isoflurane. Two 7x7mm 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 200μg (dissolved in 20μL PBS), was mixed with 50μL of PEG-PLGA-PEG (30% w/v). TGF-β1 plasmid was also mixed with Humatrix® (Care-Tech® Laboratories, St Louis, MO) prior to the application of treatment. Humatrix® is a commercial wound dressing primarily consists of chondroitin sulfate.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 70μL of one of the polymer wound dressings alone.

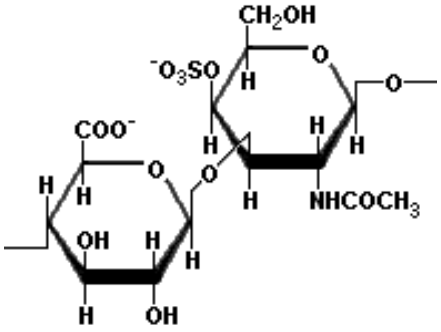


Figure 3.1 Structure of chondroitin sulfate

3.3.5 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:

% wound closure = 100 x (wound area at day 0 – wound area at day N) /wound area at day 0

3.3.6 Histology

Skin biopsies were harvested at day 5. The harvested tissue was formalin-fixed, dehydrated, and embedded in paraffin. Sections of 4mm thickness were then deparaffinized, dehydrated and observed either the morphology with H&E staining following or collagen with picrosirius red staining (Sweat et al 1964).

3.3.7 Cell proliferation using anti 5-bromo-2-deoxyuridine (BrdU) immunohistochemistry

At day 5 postwounding, 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 37C° and incubated with 0.01% trypsin 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 7 representative fields with 400X magnification.

3.3.8 Statistical analysis

All statistics are performed in PRISM software for Student t test.

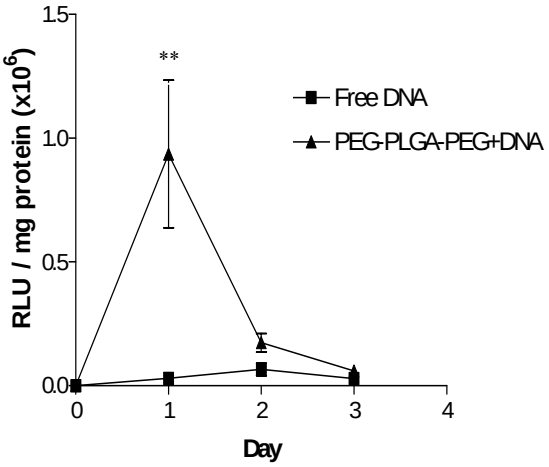


Figure 3.2: Time lapse of gene expression in mice, which received free luciferase cDNA (no vehicle), or luciferase cDNA+PEG-PLGA-PEG (vehicle). n=4. **p< 0.02.

3.4 RESULTS

3.4.1 Expression of gene released from the hydrogel made of PEG-PLGA-PEG

A 30% aqueous PEG-PLGA-PEG solution formed a hydrogel 45 min after its application to the wound due to evaporation of water at the wound site. Therefore, the film of PEG-PLGA-PEG covered the entire area of the wound. With the modified triblock copolymer as a vehicle, we proceeded to evaluate the efficiency of gene expression, which can be seen in Figure 3.1. At day 1, the PEG-PLGA-PEG vehicle significantly enhanced the gene expression compared to no vehicle (0.1M sodium phosphate buffer). Maximum gene expression was seen at day 1 and then

decreased on the following days. This demonstrates that the hydrogel formed by the modified polymer is a fast release vehicle, which is ideal for TGF-β1 gene therapy, as TGF-β1 is a cytokine that exerts its activity in the early phase of wound healing.

3.4.2 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 stages (day 1- day 5), we observed significantly accelerated reepithelialization when the plasmid TGF-β1 was delivered by the triblock copolymer hydrogel. Furthermore, the wound dressing effect of the hydrogel was slightly beneficial for reepithelialization in early

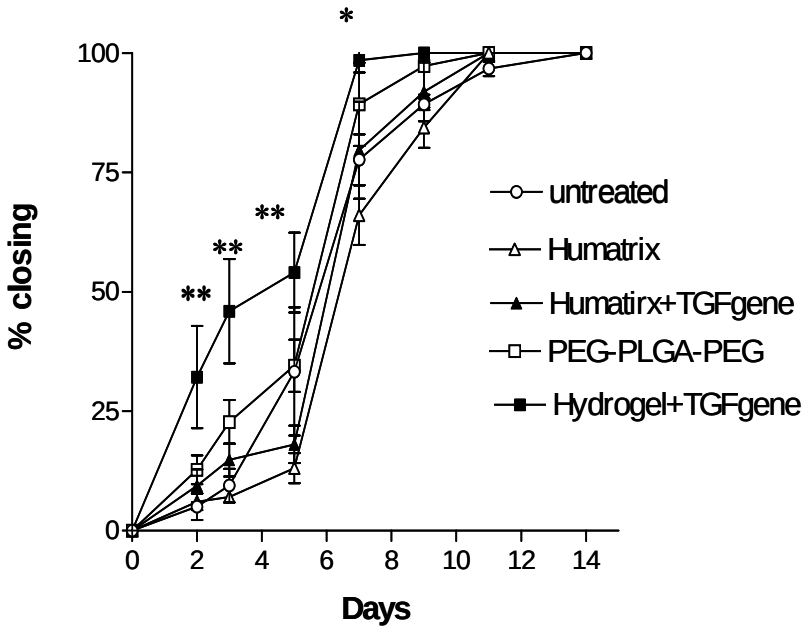


Figure 3.3: Wound closure presented as % closing of the wounds in untreated mice or mice treated with Humatrix, PEG-PLGA-PEG, 200μg TGF-β1 gene with Humatrix or PEG-PLGA-PEG. Large wounds (7x7 mm) were used. n=5. **p<0.01, *p<0.05.

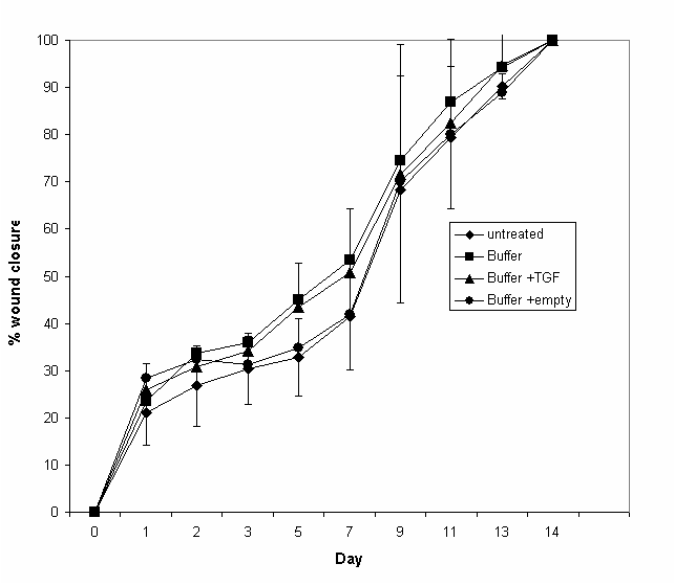


Figure 3.4 Wound closure presented as % closing of the wound in the untreated mice or mice treated with sodium phosphate buffer (Buffer) or 200μg of empty plasmid with (Buffer + TGF) or without TGF-β1gene in sodium phosphate buffer (Buffer + empty). Large wounds (two 7x7 mm) were used. n=5.

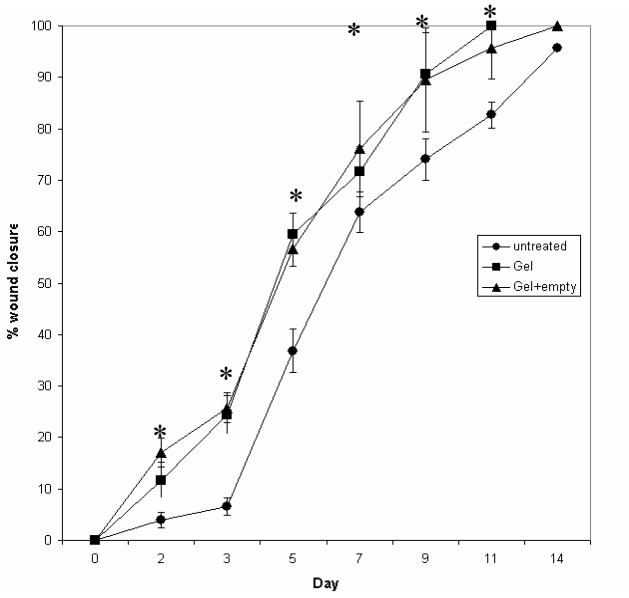


Figure 3.5 Wound closure presented as % closing of the wound in the untreated mice or mice trated with PEG-PLGA-PEG dissolved in sodium phosphate buffer (gel) or 200μg of empty plasmid mixed with PEG-PLGA-PEG which is dissolved in sodium phosphate buffer (gel+empty). Large wounds (two 7x7 mm) were used. n=5. *$p<0.05$ when untreated compared with gel or gel+empty.

healing stages. 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. Wound treated either with plasmid TGF-β1 or empty plasmid in buffer did not demonstrate significantly accelerated wound closure. Since the activity of gene has been shown independent of plasmid or oligonucleotide form (Hofman et al 2001), the increased closure rate in wound treated with plasmid TGF-β1 in hydrogel in comparison with in saline suggests that hydrogel is a suitable 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**.**

Figure 3.2 demonstrates the formation of an adhesive film at 1 h after the treatment in wounds treated with triblock copolymer hydrogel (B&D in upper panels). In wounds 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 (Figure 3). 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.

3.4.3 Histological examination

H & E staining demonstrated a visibly accelerated migration of epithelium (shown with black arrow in Figure 3.3) 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 Figure 3.3) was observed in the wound bed with the treatment of the synthetic hydrogel containing plasmid TGF-β1 than other treatment.

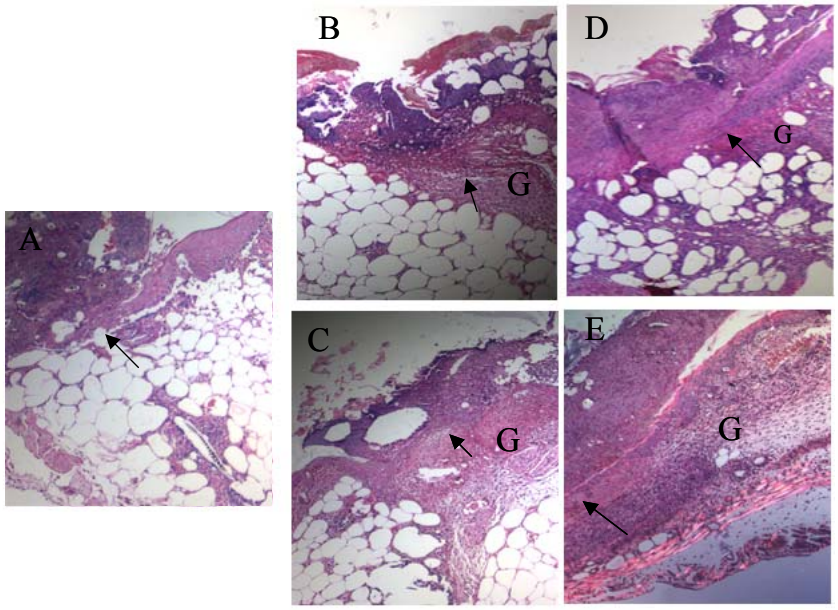


Figure 3.6: Hydrogel mediated TGF-β1 gene therapy on the morphology of wounded skin at day 5 postwounding. H&E staining of the wound bed from untreated skin (A), skin treated with wound dressing alone, either Humatrix® (B) or the synthetic hydrogel (D), skin treated with TGF-β1 gene in either Huamtrix® (C) or the synthetic hydrogel (E). Black arrows indicate the end of epithelial tongue. Granulation tissue was indicated by G. Magnification (100X).

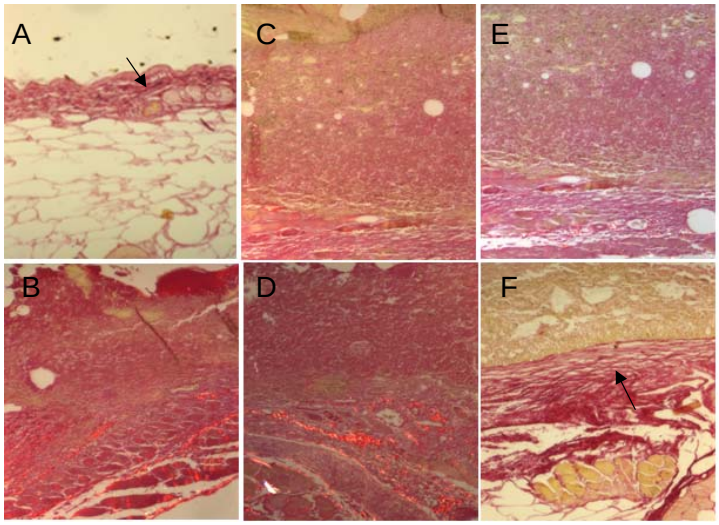


Figure 3.7: Collagen deposition at day 5 postwounding shown by picrosirius red. Skin section were collected from unwounded animal (A), wounded mice which are untreated (B), or treated with PEG-PLGA-PEG alone (C) or with TGF-β1 gene (D). Reddish orange color indicates collagen. Black arrow indicates well-aligned, basket weaved pattern, collagen. 40X magnification.

In Figure 3.4, 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 Figure 3.4).

Active fibroblast proliferation undergoes during wound healing to synthesize extracellular matrix and wound contraction. Because wound closure at day 5 showed the greatest difference between both hydrogel formulations, we expected hydrogel formulated with TGF-β1 gene increased fibroblast proliferation at that time. Actively proliferating fibroblasts in the granulation tissue were identified with anti-BrdU antibody. In the center of 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 ($p<0.0001$) (Figure 3.5), 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.

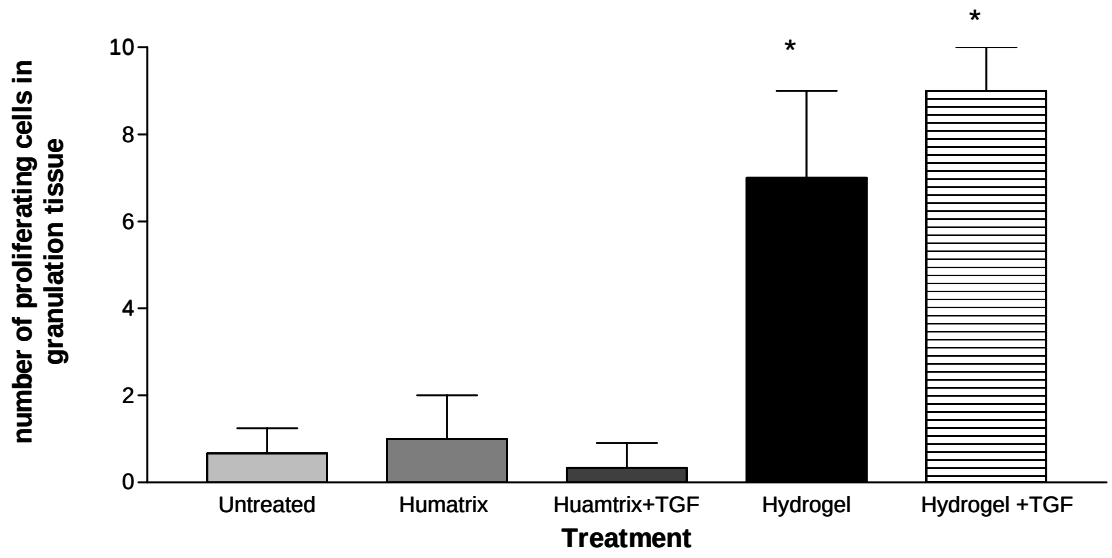


Figure 3.8: Cell proliferation at the wound bed measured by immunostaining of BrdU positive cells at day 5. The group treated with copolymer hydrogel, with or without TGF-β1 gene, is significantly different from all other groups. * $p<0.01$. n =7

3.5 DISCUSSION

Hydrogels promote wound healing by moist retention to maintain homeostatic environment. However, hydrogels may be inconvenient in application. Most of the commercial hydrogels, such as Humatrix® need several re-applications daily with an overlying occlusive wound dressing. Moreover, occlusive self-adhesive membrane requires some degree of expertise and

causes pain during changes. The thermosensitve hydrogel alleviates the necessity of repeated and complicated applications. Furthermore, the potency of the hydrogel as a wound-healing promoter appears not to be inhibited by drying. The liquid copolymer left uncovered and formed a hydrogel in situ at 45 minutes 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 the hydrogel is necessary because hydrogel becomes less useful when homeostatic environments recover with the coverage of clots and skin cells. In our study, the wound after a 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 than Humatrix.

The DNA release from the hydrogel (PEG-PLGA-PEG triblock copolymer) is driven by diffusion and biodegradation as previously described (Li et al 2003). The copolymer-hydrogel slowly releases the entrapped DNA with a half-life of approximately 5 days at 37°C (Li et al 2003). 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 (Figure. 3.1). 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 a previous report, enhanced biodegradation was observed with localized pH change, which usually occurs during acute inflammation and infection (Zaikov 1985).

TGF-β1 is used in this experiment because TGF-β1 is a chemokine for fibroblasts. When plasmid TGF-β1 formulated with the copolymer, accelerated reepithelialization, increased fibroblast proliferation, and organized and mature collagen fibers were observed at the early stages of the healing process. One biological effect of TGF-β1 is to enhance reepithelialization.

During wound healing, fibroblasts stimulated by the TGF-β1 signaling migrate to the injured tissue and synthesize collagen (Frank et al 1996). Tensile strength increases as collagen matures. The granulation tissue bed, mainly comprised of collagen and proliferating fibroblasts, serves as a foundation for keratinocyte migration resulting in an enhanced reepithelialization.

Skin fibrosis, characterized by disorganized collagen formation (Ito et al 2001) and epidermal architecture (Christner et al 2000) is the possible adverse effect reported from cutaneous delivery of TGF-β1. From previous studies, the pathologic fibrosis and scar occurs mostly due to persistent presence of TGF-β1 caused by sustained release or reapplication in vivo or in vitro (Lanning et al 1996, Bettinger et al 1996). Therefore, transient expression with single dose using our copolymer as a vehicle appears to be advantageous in wound healing. Indeed, 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 (Theoret et al 2002), and the robust therapeutic effects were observed at the early stages of wound healing, our copolymer hydrogel delivery system seems to mimic the temporal sequence of the endogenous TGF-β1. While 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®. The further formulation of the thermosensitive hydrogel with a growth factor gene might be highly applicable in treating problematic wound healing.

4.0 THERMOSENSITIVE HYDROGEL ASSISTS MUSCLE DERIVED STEM CELLS TO ACCELERATE DIABETIC WOUND HEALING

4.1 ABSTRACT

Muscle derived stem cells (MDSC) have been shown to enhance muscle regeneration and bone healing. PEG-PLGA-PEG is a biodegradable and biocompatible triblock copolymer which forms a thermosensitive hydrogel and promotes cell proliferation in the cutaneous wound of the db/db diabetic mice. We applied the MDSCs with the PEG-PLGA-PEG in db/db mice and performed the wound closure study until the wounds were completely closed. Furthermore, the histological studies were performed at days 5, 9, 14 post-wounding including immunofluorescence to observe the localization and differentiation pattern of the β-gal engineered MDSCs, and picrosirius red staining to observe collagen. From the results, MDSCs accelerated the wound healing only when the PEG-PLGA-PEG was applied on MDSCs. Furthermore, the hydrogel could enhance the engraftment for the MDSCs; 30 % of the transplanted MDSCs were found at day 9 and 15% remained when the wound was closed at day 20. From the double immunofluorescent study, we found that some MDSCs differentiated into fibroblasts and endothelial cells. In conclusion, we demonstrated the potential of MDSCs in improving diabetic wound healing and the differentiation pattern of MDSCs at the diabetic cutaneous wound environment.

4.2 INTRODUCTION

The development of stem cells as a new method for tissue regeneration has become attractive in the past few years. Due to ethical issues and the tumor induction potential associated with the embryonic stem cells, there is an increasing recognition of the importance of multipotent post-natal stem cells in tissue regeneration. Several types of stem cells have been used to regenerate a variety of tissues including bone, skeletal muscle, cardiac muscle and skin (Peng and Huard 2004, Arinzeh et al 2005, Kocher et al 2001, Orlic et al 2001, Orlic et al 2001) and improve the function of the new tissue (Penn et al 2004). Hematopoietic stem cells (HSCs) can differentiate into neurons, blood cells and macrophages (Shen et al 2004, Sinanan et al 2004) while postnatal muscle derived stem cells (MDSCs) can differentiate into adipogenic, osteogenic, chrondrogenic and hematopoietic lineages (Cao et al 2003).

Recently, adult stem cells have been shown to enhance wound repair. Bone marrow derived stem cells, HSCs or MDSCs have been documented to accelerate the healing in bones, cartilage, muscle or skin after injury (Badiavas et al 2003, Badiavas and Falanga 2003, Peng et al 2003, Stocum 2001). Bone marrow derived stem cells (BMDS) are recruited and incorporated into cutaneous wound sites (Badiavas et al 2003). In the study of Badiavas et al (2003), the researchers injected bone marrow derived stem cells intravenously and found the cells incorporating into the hair bulge region at the wound site and differentiating into epithelial cells. However, there was little incorporation of BMDS at the wound site in the genetically diabetic db/db mouse model (Stepanovic et al 2003). If BMDS is used in the cutaneous wound healing, the efficiency of skin regeneration might be hindered by low efficiency of the cell engraftment to the wound site. On the other hand, the MDSCs might be suitable for the cutaneous wound

healing, as they can remain in the engraftment site for a prolonged period and differentiate into myofibroblasts (Li et al 2004), essential cells for wound contraction (Darby et al 1990).

PEG-PLGA-PEG is a biocompatible and biodegradable triblock copolymer that forms a thermosensitive hydrogel under the appropriate conditions (Jeong et al 2000, Jeong et al 2000, Li et al 2003). The polymer solution is in a liquid state at room temperature and becomes a viscous gel and then eventually a thin film when applied to the cutaneous wounds (Lee et al 2003). The triblock copolymer displays a relatively low cytotoxicity compared with poly-L-lysine (Li et al 2003). PEG-PLGA-PEG has been shown the potential to sustain drug release in bladder (Tyagi et al 2004). Recently, PEG-PLGA-PEG has been shown to enhance reepithelialization and assist cell proliferation (Lee et al 2003) in wound healing.

In this study, we report the findings of using the thermosensitive hydrogel with three different stem cells, HSCs, MSCs or MDSCs in diabetic wounds. A db/db mouse model, which is a relevant model for the impaired wound healing in diabetes, have been used in this study (Tsuboi et al 1992). The first step of our study was to test the wound closure activity of the three stem cells with the application of the hydrogel. We then examined engraftment activity and differentiation pattern at the diabetic wound bed.

4.3 RESULTS

4.3.1 Wound closure effect by MSC, HSC and MDSC

We tested the MSC, HSC and MDSC for the capability of enhancing wound healing in the db/db mouse. As previously reported, HSC and MSC can promote wound healing (Badiavas et al 2003,

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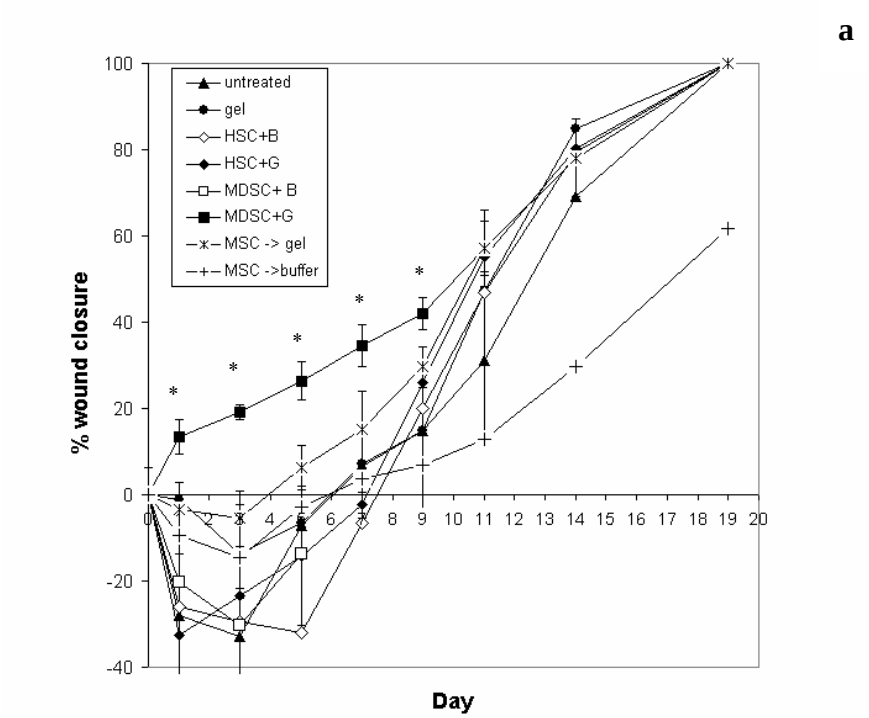
untreated
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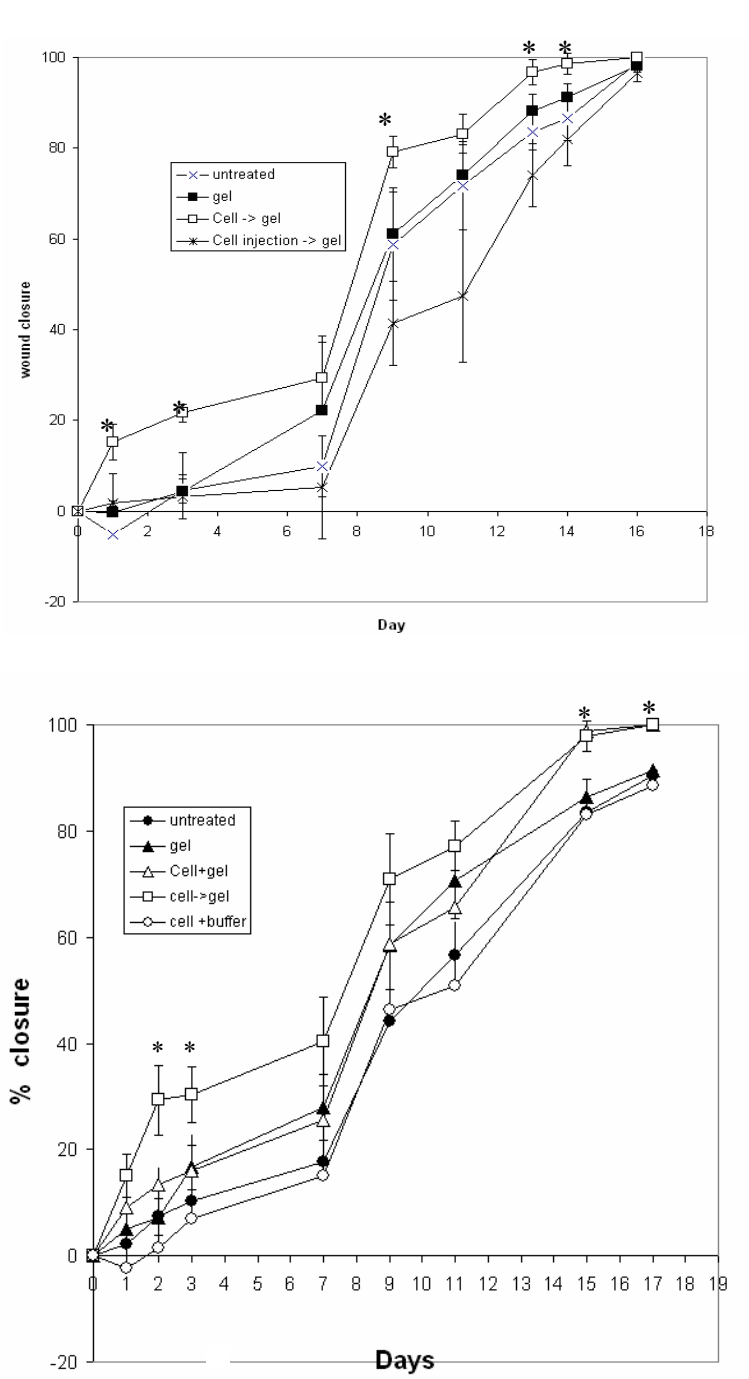

Figure 4.1 (a) Wound closure effect of MDSCs, HSC or MSC mediated by PEG-PLGA-PEG hydrogel copolymer. Wounds were untreated, treated with PEG-PLGA-PEG or with MDSC, MSC or HSC overlaid by sodium phosphate buffer alone (MDSC+buffer, MSC + buffer or HSC+buffer, respectively) or with PEG-PLGA-PEG hydrogel (MDSC+ gel, MSC+gel or HSC+gel, respectively) (b) Wound closure effect by two different application protocols, topical application or injection using MDSCs. Wounds were left untreated (untreated), treated topically with PEG-PLGA-PEG hydrogel alone or with the PEG-PLGA-PEG applied on the MDSCs (cell + gel) or intradermal injection of MDSCs at wound edges followed by the topical application of PEG-PLGA-PEG (cell injection -> gel). (c) Wound closure effect of the mixing and overlaying protocols using MDSC and the PEG-PLGA-PEG hydrogel. Wounds were untreated, treated with PEG-PLGA-PEG (gel), MDSC mixed with the sodium phosphate buffer (cell+ buffer) without or with the hydrogel (cell + gel) and MDSC overlaid by the hydrogel (cell ->gel).n=4. * $p<0.05$.

Badiavas and Falanga 2003, Stocum 2001). We evaluated the wound closure rate after the application of various stem cells followed by overlaying with the sodium phosphate buffer with or without PEG-PLGA-PEG. As shown in Fig. 4.1, the wound closure rate of all stem cell-buffer treated wounds was not significantly increased compared with the untreated. With the overlaid PEG-PLGA-PEG hydrogel, the wound closure of MDSC treated wounds was greatly enhanced while the wound closure of other stem cell treated wounds was not significantly enhanced compared with the untreated (Fig.4.1a). To see if the protocol we used was optimized for the application of MDSC, we tested several methods in our wounding model including injection of MDSC with the topical application of the hydrogel on the wound (Fig. 4.1b), MDSC mixed with the hydrogel polymer (Fig 4.1c) or the topical application of MDSC followed by overlaying with the hydrogel copolymer (Fig. 4.1b). We found that the latter (hydrogel overlaying method) was more favorable for wound healing application in our model.

The morphology of the wound showed that the migration of epithelial tongue was achieved faster in the wound treated with MDSC overlaid by the hydrogel than the wound left untreated (arrows, Fig. 4.2), suggesting that the former promoted reepithelialization.

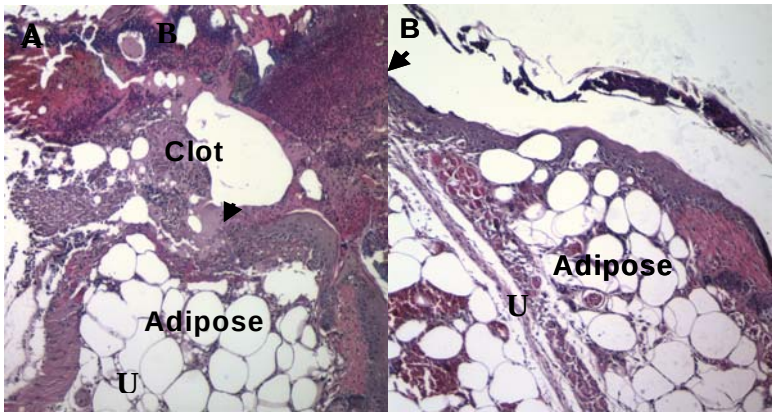

Figure 4.2 The morphology at day 5 postwounding using H& E staining in the wound left untreated (A) and the wound treated with MDSC with the coverage of PEG-PLGA-PEG (B). Arrows indicated the leading end of the epithelial tongue. U indicated the unwounded portion. The epithelium migrated from the unwounded to the wounded tissue toward the left.

4.3.2 The engraftment of MDSC mediated by thermosensitive hydrogel, PEG-PLGA-PEG

To observe if the hydrogel plays a role in enhancing the engraftment of MDSC, we evaluated the percentage of the MDSC remained at the wound bed when the cells were overlaid by the copolymer hydrogel or by a clinically used hydrogel, Humatrix^{R}. Since MDSCs were stably transduced with a β-gal gene, we measured the β-gal activity of the harvested wound tissue. During the wound healing, we found that the β-gal activity in the wound bed treated with PEG-PLGA-PEG and MDSCs was higher than all other groups (Fig. 4.3).

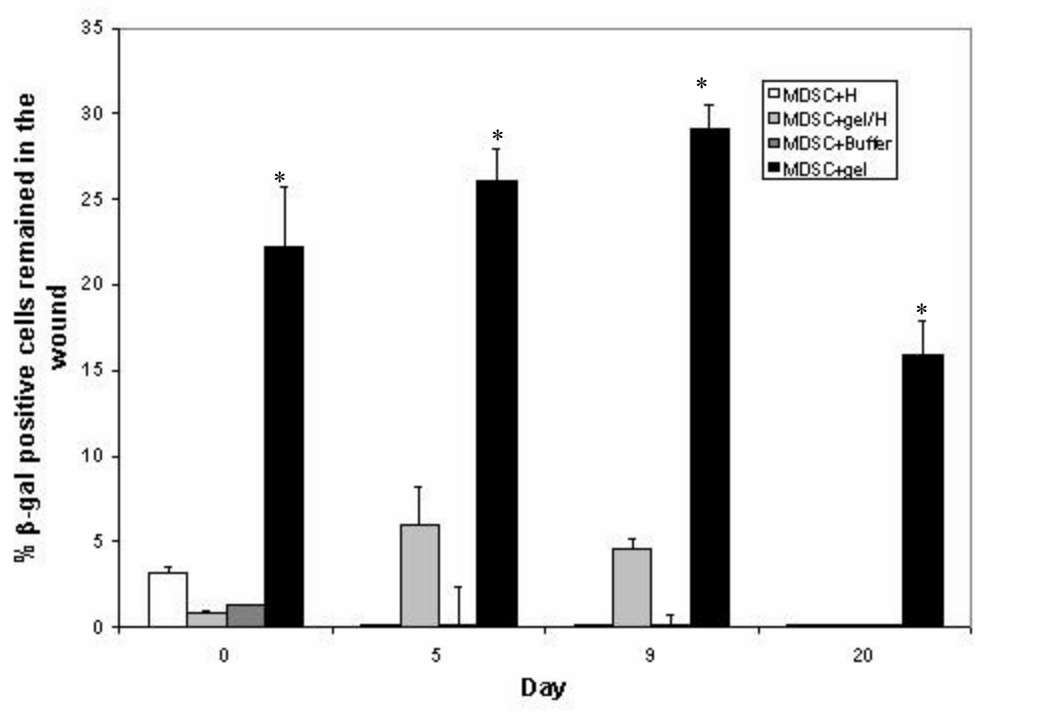

Figure 4.3 The percentage of MDSC remained at the wound bed evaluated by β-gal assay. The wound was treated with the MDSC overlaid by Humatrix (MDSC+ H), 1:1 mixture of Humatrix with the PEG-PLGA-PEG hydrogel (MDSC+ gel/H) or sodium phosphate buffer alone (MDSC+Buffer) or with the PEG-PLGA-PEG hydrogel (MDSC+gel). β-gal assay was evaluated at day 0, 5, 9 or 20 (complete reepithelialization) postwouding. Data was presented after normalization of the background from the untreated wound. n=5. * $p<0.05$.

Up to 30% of the original β-gal activity was detected in the wound at day 9. When the wound was completely closed (at day 20 postwounding), 15 % of the β-gal expressing MDSCs still remained while no detectable level of β-gal activity was found in the wound bed covered by the mixture of Humatrix and hydrogel polymer (1:1 ratio) or Humatrix alone (Fig. 4.3), suggesting that the copolymer hydrogel facilitated the engraftment of the MDSCs. Furthermore, 22% of the original β-gal activity at day 3 postwounding increased to 30% at day 9, suggesting that the copolymer hydrogel might facilitate the proliferation of the MDSCs.

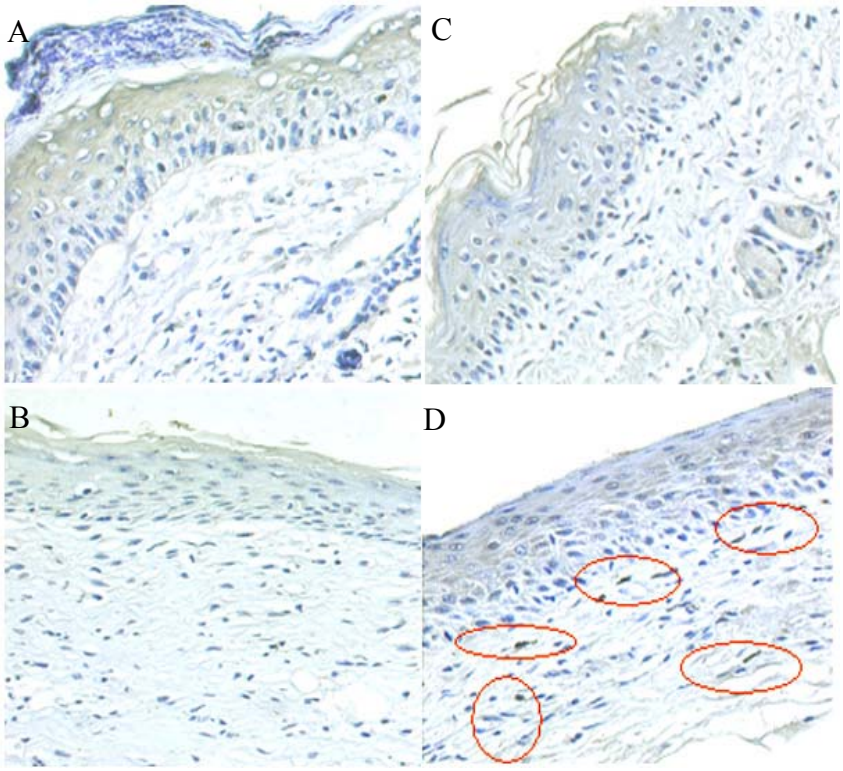

Figure 4.4 Anti β-gal immunostaining of skin sections at 14 days postwounding. (A) Untreated. Wound treated with PEG-PLGA-PEG hydrogel (B), MDSC overlaid with Na phosphate buffer alone (C) or with PEG-PLGA-PEG hydrogel (D). Blue indicates nuclei counterstained by hematoxylin. Grey indicates positively stained β-gal cells (red circles). 200x field.

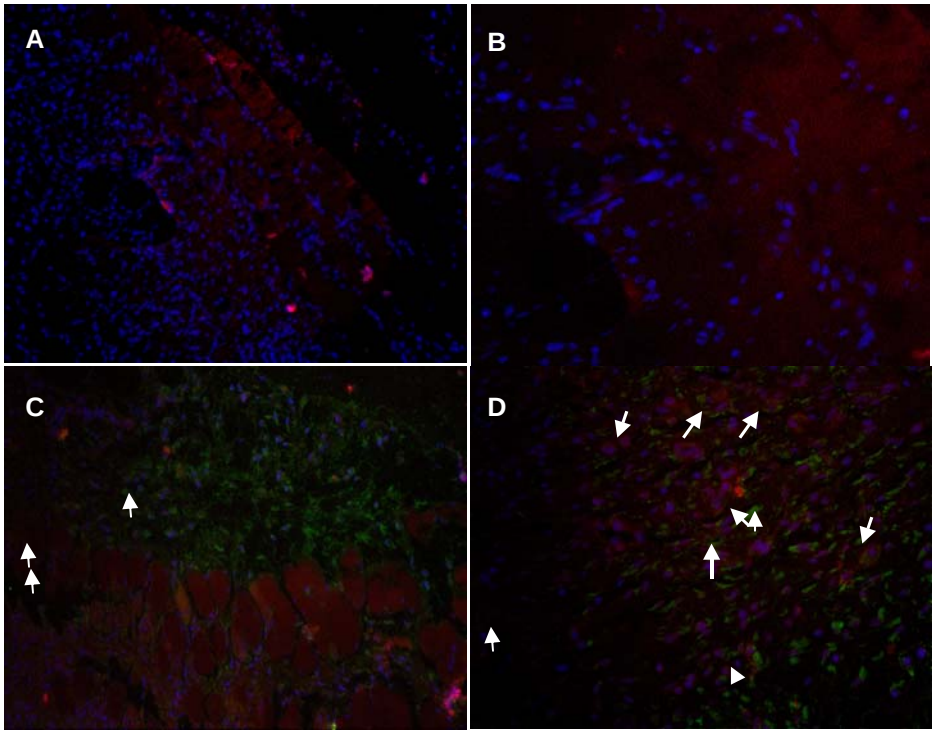

Figure 4.5 Double immunofluorescence of anti-β-gal (green) and anti-smooth muscle actin (red) in skin wounds at day 9 postwounding. (A&B) untreated. (C&D) skin was treated with MDSC overlaid with PEG-PLGA-PEG hydrogel. Magnification in 20x field (A&C) or in 40x field in the dermal layer (B&D). Nuclei were counterstained with Hoechst and appeared in blue. Arrows indicate the cell stained positively with both anti-β-gal and anti alpha smooth muscle actin while arrowheads indicate the cell stained positively with anti-β-gal.

4.3.3 The differentiation of MDSC at the wound bed

To localize the engrafted MDSC in the wound bed, we have used immunofluorescence with anti-β-gal antibody (Fig. 4.4). At day 14 postwounding, no visible β-gal positive cells appeared in the untreated wound (A), wounds treated with hydrogel alone (B), or MDSC in Na phosphate buffer (C). But the β-gal positive cells were abundantly found in wounds treated with MDSC in hydrogel (D); the cells exclusively localized in the dermis layer of the wound bed (Fig. 4.4D). From the morphology and the dermis location of β-gal positive cells, we predicted some of the

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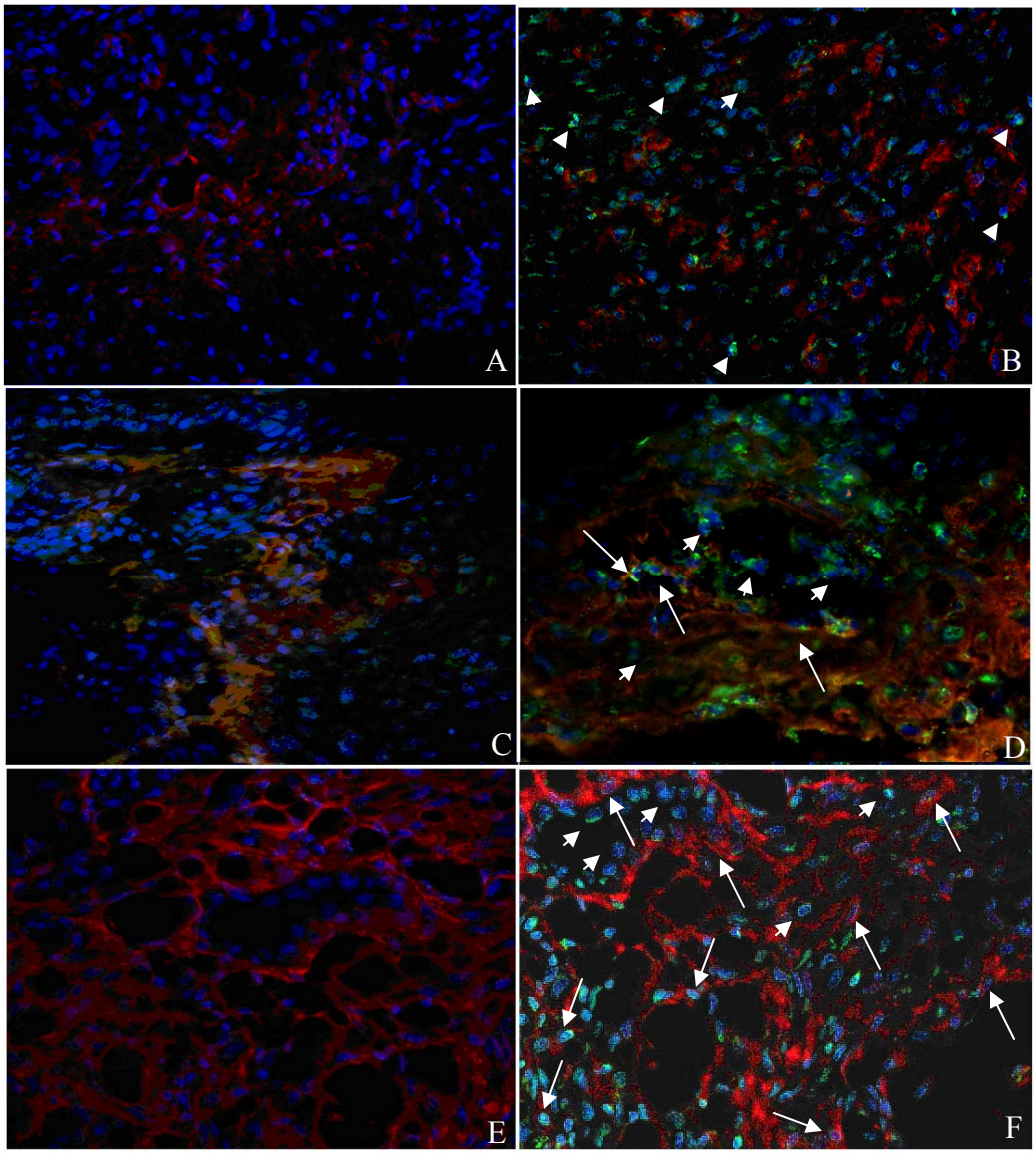
A
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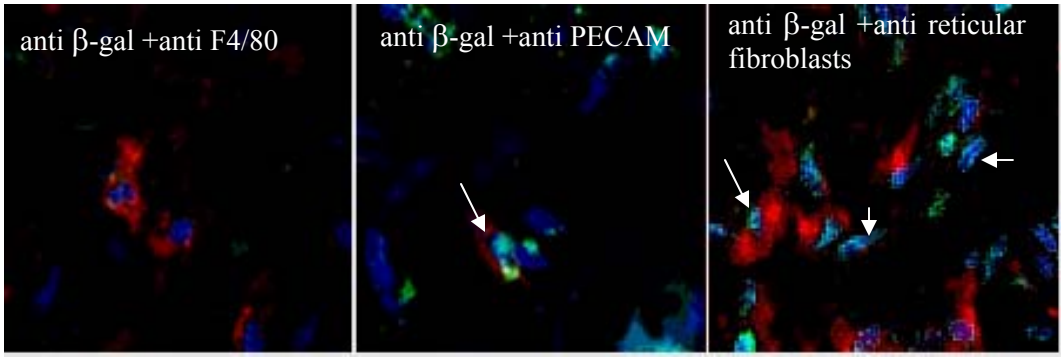
II


anti β-gal +anti F4/80
anti β-gal +anti PECAM
anti β-gal +anti reticular fibroblasts

III

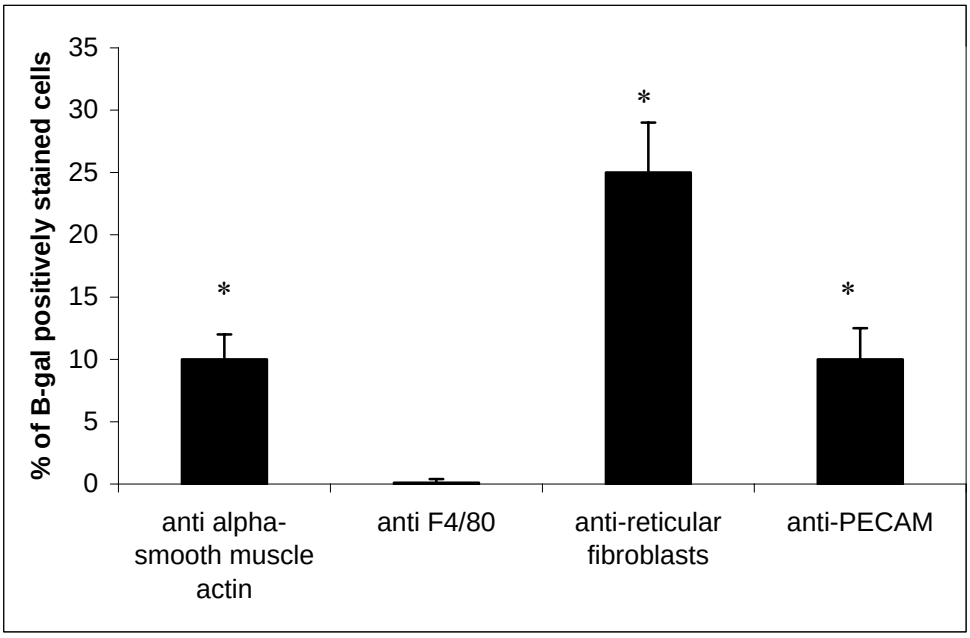


Figure 4.6 Double immunofluorescence of anti-β-gal (green) and anti-F4/80 (A&B), anti-PECAM (C&D) or anti-reticular fibroblasts (E&F) respectively (red) was performed on sections of skin wound at day 9 postwounding. (A, C &E) untreated. (B, D&F) wounded skin was treated with MDSC overlaid by PEG-PLGA-PEG hydrogel. Magnification in 400x field in the dermal layer (B&D). Nuclei were counterstained with Hoechst and appeared in blue. Arrows indicate the cell stained positively with both anti-β-gal and antibody for cell markers while arrowheads indicate the cell stained only positively with anti-β-gal. Panel II demonstrates a zoom in of Panels IB, D &F (from left to right, respectively). Nuclei were counterstained with Hoechst and appeared in blue. Arrows indicate the cell stained positively with both anti-β-gal and antibody for cell markers while arrowheads indicate the cell stained only positively with anti-β-gal. Panel III α- smooth muscle actin (data from Fig 4.5), F4/80, reticular fibroblasts or PECAM-positive cell count that is β-gal positive in (n=5) representative fields. * $p<0.05$.

stem cells might have differentiated into fibroblasts (Fig. 4.4 D). To confirm this, we performed the double immunofluorescence for β-gal either with α-smooth muscle actin or reticular fibroblast (Fig. 4.5, Fig. 4.6 I F). We found that there were many β-gal positive cells (green) in the dermis of the wound bed at day 9 postwounding. The results also showed that some β-gal positive cells were indeed α-smooth muscle actin (approximately 10%, Fig 4.6 III) or reticular fibroblast (approximately 25%, Fig 4.6 III) positive (arrows in Fig. 4.5B, arrow in Fig 4.6 I F),

confirming the hypothesis that MDSC had differentiated into fibroblasts. Furthermore, we also wanted to identify if the stem cells differentiated into other cell types such as macrophages or endothelial cells which are essential cells appeared in the dermal layer in wound healing. Thus, we performed double immunofluorescence for β-gal either with F4/80 or PECAM. The result showed that some β-gal positive stem cells (approximately 10%, Fig 4.6 III) were PECAM positive (arrows in Fig. 4.6 I D) while no β-gal positive cells were noticeably F4/80 positive (arrowheads in Fig 4.6 I B). The data suggest that some of the stem cells may have differentiated into endothelial cells but not macrophages. In each set of the double immunofluorescence study, there were many β-gal positive cells that were not α- smooth muscle actin, reticular fibroblast, F4/80 or PECAM positive (Fig. 4.5 arrowheads, Fig 4.6 arrowheads), suggesting the presence of the quiescent, undifferentiated stem cells in the dermis layer of the healed wound.

4.3.4 Collagen deposition at the wound site treated with MDSC

Collagen in granulation tissue could be produced by dermal fibroblasts (Gabbiani et al 1979, Kayne 1981). If more fibroblasts were present in the dermis, it would be possible to observe thicker and more mature collagen deposition in the dermis layer at the wound bed. To evaluate the collagen deposition, we performed the picrosirius red staining on the sectioned skin wounds. Compared to the untreated wound or the wound treated with MSC, more dispersed glowing orange staining appeared in the granulation tissue in MDSC-hydrogel treated wound bed (Fig. 4.7), indicating thicker and more mature granulation tissue collagen formation (Berry et al 1998) in the MDSC-hydrogel treated wound bed. The amount of collagen in the wound bed was further quantified by using Metamorph program to analyze the microphotograph in Figure 4.7. The

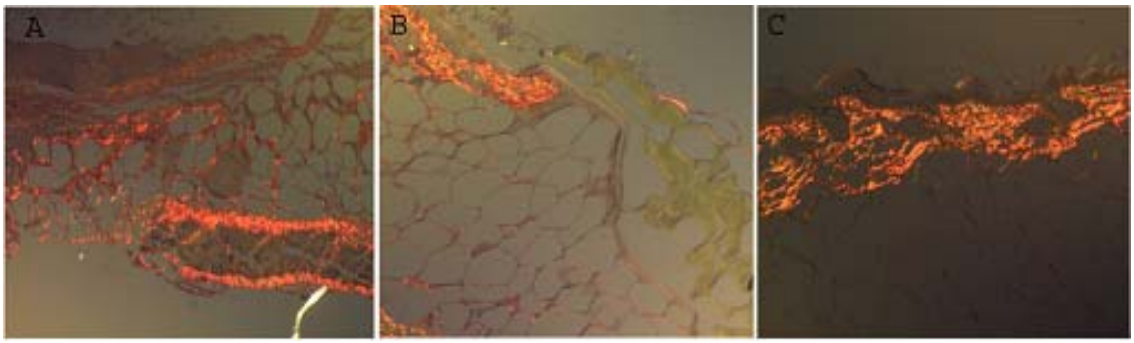


Figure 4.7 Collagen deposition at the wound site mediated by MSC or MDSC. (A) Untreated wound, or wound treated with MSC (B) or MDSC (C) followed by covering of the copolymer hydrogel. Glowing orange indicates collagen Magnification 200x. G indicates the granulation tissue.

result indeed indicates that MDSC promoted collagen deposition, probably due to increased dermal fibroblasts from the differentiation of MDSC.

4.4 DISCUSSION

We have tested several different types of stem cells in combination with the thermosensitive hydrogel composed of the polymer, PEG-PLGA-PEG, for treating impaired wound healing in a diabetic mouse model. Compared to HSC and MSCs, which have been previously documented to regenerate tissue successfully (Satoh et al 2004, Wang et al 2004), MDSC may have a higher potential in accelerating the wound closure. This study is the first to demonstrate that MDSC can enhance cutaneous wound healing. Furthermore, MDSC only significantly accelerated wound closure when MDSCs were covered with the thermosensitive hydrogel made of copolymer, PEG-PLGA-PEG. We predicted that the improved wound closure by MDSC and exclusively with the hydrogel might be because the hydrogel enhanced the MDSC engraftment. Indeed Fig 4.3 showed the enhancement of the engraftment of the MDSC promoted by the hydrogel. When

we compared the hydrogel with sodium phosphate buffer, pure Humatrix or Humatrix-PEG-PLGA-PEG mixture, greater amounts of MDSC remained in the wound when the cells were treated with the PEG-PLGA-PEG hydrogel. Throughout the wound healing until the wound was closed, 15% or more of MDSC remained in the wound bed when we used the hydrogel. The amount of MDSC was undetectable at the time of complete wound closure when we used the sodium phosphate buffer, pure Humatrix or Humatrix-PEG-PLGA-PEG mixture. Many reports showed that failure of cell transplantation was associated with the fact that the cells were not able to survive in the recipients (Murry et al 2002). While our system showed a 30% of MDSC remained at day 9 postapplication, a myocardial muscle repair study showed a 10 % of embryonic stem cells remained at day 4 and a drop to 1% at day 10 at the transplanted site (Murry et al 2002). Using fibroblast grafting in skin wound model, no cells remained at day 7 postwounding (Price et al 2004). The increased engraftment of MDSC may be due to the presence of large component of PEG in the copolymer hydrogel. PEG has been associated with the capability to reduce immune cell attack and has been shown to be favorable for cell survival (Lee et al 2004, Lacasse et al 1998) (already checked. No spelling problem).

PEG-PLGA-PEG is apparently a good scaffold for MDSC compared to Humatrix and the mixture of Humatrix with PEG-PLGA-PEG. We observed that there was an increase of MDSC at the wound site (22% of the implanted cells remained at day 0 and 30% at day 9) during the wound healing process when the PEG-PLGA-PEG was used at the time the wound was closed (Fig 4.3). This would suggest that the PEG-PLGA-PEG might have the capability to promote the cell proliferation of MDSC. This is consistent with the finding of the ability of the polymer to promote proliferation of skin cells (Lee et al 2003).

Another novel finding is about the differentiation potential of the MDSC to the cells which are essential for cutaneous wound healing. Our result suggests that some of the MDSC can differentiate into fibroblasts and relatively smaller population of endothelial cells during wound healing (Fig. 4.5). There are three possibilities that may cause the differentiation of MDSCs into fibroblasts and endothelial cells: (1) The thermosensitive hydrogel induces the differentiation of the MDSCs. (2) The wound environment containing growth factors and the neighboring skin cells promote the differentiation of MDSC to fibroblasts. (3) The MDSC from the wild type animals correct the deficiency of cytokines (Bitar and Labbad 1996) such as FGFs, which commonly occurs in the diabetic wound. The increased level of these cytokines may induce the differentiation into fibroblasts or even endothelial cells.

Fibroblasts are highly populated in the dermis layer during the healing process. One of the functions of fibroblasts is to produce collagen (Kayne 1981, Wiencke et al 1968), which gives tensile strength to the wound and promotes cell migration (Scott et al 1985, Hou et al 2000). In our results, we found that collagen deposition in the wound treated with MDSC and PEG-PLGA-PEG was thicker at day 5 post-wounding compared to the untreated wound or the wound treated with MSC. The observation of thicker collagen deposition supports that MDSC are highly possible to differentiate into fibroblasts. More importantly, the results suggest that the differentiated fibroblasts are highly possibly mature as collagen is mostly produced by the mature fibroblasts. This observation can be significant in treating diabetic wound healing in the db/db mice as it is well known that collagen synthesis and growth factor production is impaired in the db/db model (Brown et al 1997, Bitar and Labbad 1996).

Since MDSC can be readily transduced with viral vectors (Cao et al 2003, Li et al 2002), gene therapy using MDSC is an attractive approach. Gene therapy with growth factor genes is

potentially attractive for skin wound healing as shown in our previous findings (Chesnoy et al 2002, Lee et al 2003, Lee et al 2004). Due to the self-renewal property of the MDSC and the differentiation capacity to skin cells, MDSC may be a good source to produce the growth factor proteins. The stem cell/gene therapy might further improve the wound healing by treating multiple defective wound healing processes. This approach offers the advantage of the release of a growth factor protein at the wound site and alleviates the necessity for repeated application of a protein. The increased engraftment of stem cells in our system may result in further applications not limited to wound healing. Many disease states might be treatable with the protein products generated by the engrafted stem cells.

4.5 METHODS

4.5.1 Animals

Female mice of C57BL/6 or C57BKS.Cg-m +/+ Leprdb (a model for type II diabetes) in 7-9 weeks old were obtained from Jackson Laboratories. (Bar Harbor, Maine). Mice homozygous for the diabetes spontaneous mutation (*Lard*) become identifiably obese around 3 to 4 weeks of age. Elevations of plasma insulin begin at 10 to 14 days and elevation of blood sugar at 4 to 8 weeks. Twenty-six weeks old genetically diabetic mice (db/db) (Jackson laboratory, 40g) were used in the study. All mice were housed in the animal facility at the University of Pittsburgh.

4.5.2 Wounding protocol and treatments

All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at University of Pittsburgh. Two 7x7mm excisional wounds were created on the back of

anesthetized mice using a pair of scissors. The wound were treated with the 10^5 of MDSC cells in 0.1 M sodium phosphate buffer, 32% PEG-PLGA-PEG triblock copolymer solution in 0.1M sodium phosphate buffer, Humatrix or a 1:1 mixture of the Humatrix with the PEG-PLGA-PEG copolymer solution.

4.5.3 Stem cell preparation

4.5.3.1 Muscle derived stem cell

The muscle derived stem cells were given by Dr. Huard's lab and was prepared by preplating technique as described (Qu et al 2002). Briefly, harvested muscle from the forelimb and the hindlimbs was minced using a razor. Cells were enzymatically dissociated with 0.2% collagenase XI and subsequently the muscle cell extract was preplated on the collagen coated flasks. According to the time needed for the cells to adhere, the cells were isolated. Preplate 1(PP1) represents a population of cells that adhere in the first hour after isolation. PP2 represents in the next 2h. PP3 represents in the next 18 h. The cells obtained at 24 hours interval was represented by PP4-6. Clone FN12A5.6E of pp6 after passage 11 was used in our experiment and had been used in other applications (Lee et al 2001). β-gal has been shown to localize in the nucleus of MDSC using the monoclonal anti-β-gal antibody (Sigma Aldrich, St Louis, MO) (Lee et al 2000).

4.5.3.2 Mesenchymal stromal cells (MSC) from bone marrow

The mouse MSCs were obtained from the Tulane University. They prepared the cells as previously described (Peister et al 2003). Briefly, MSCs were obtained from femurs and tibiae of C57BL/6 mice. The femurs and tibiae were harvested and immersed into complete isolation

medium (CIM consists of IMDM supplemented with 9% Fetal bovine serum, 9% Horse Serum, 100U/mL penicillin, 100mg/mL streptomycin and 12mM L-glutamine) before exposing the marrow by clipping the ends of each tibia and femur. The marrow was collected by centrifugation followed by resuspension in CIM and filtrating through a 70μm nylon filter. Since MSCs are adherent, the adherent cells are collected and cultured.

4.5.3.3 Hematopoietic stem cell (HSC)

HSCs from C57Bl/6 female mice were obtained from Dr. Tao Cheng's laboratory. They prepared the HSCs as described (Stier et al 2002). Briefly, bone marrow was obtained from the mice and the procedure for exposing the marrow was similar as described for the preparation of MSCs. The isolation of HSC was performed by immunoprecipitating with Sca1 microbeads. Cells with a selection for Sca1+ were collected.

4.5.4 Triblock copolymer PEG-PLGA-PEG

Triblock copolymer, PEG-PLGA-PEG was synthesized according to published procedure (Jeong et al 1999, Jeong et al 2000). The triblock co-polymer, was characterized with Mw = 29,659, Mn = 15,732 and polydispersity = 1.877 (Li et al 2003). An aqueous solution (32%, w/v) of this polymer flows freely at room temperature, but forms an adhesive hydrogel film at the wound site in approximately 30 min.

4.5.5 β-gal assay

Biopsies of the entire wounded tissue were harvested followed by homogenization, 4 freeze-thaw cycles and centrifugation. The supernatant of cell lysate from centrifugation was collected for the

evaluation of the β-gal activity using a β-gal assay kit (Invitirogen, Carlsbad, CA) according to the vender's protocol.

4.5.6 Histology

The harvested tissue of entire wound site was formalin-fixed and embedded in paraffin. Sections of 4μm thickness was prepared using a microtome, then was deparaffinized, hydrated and the staining was performed either with Hematoxylin and Eosin for observing the morphology, picrosirius red for collagen staining using 0.1% picrosirius red solution as previously described (Lee et al 2004). Collagen was analyzed from Figure 4.7 microphotograph with the Metamorph software provided by the CBI at the University of Pittsburgh.

4.5.7 Double immunostaining for β-gal and different cell markers

The double staining was performed by an incubation of the deparaffinized sections or frozen sections with a mixture containing a mouse biotinylated monoclonal anti-β-gal antibody diluted to 1:100 in PBS (Sigma Aldrich, St Louis, MO) and a cy3-conjugated anti-alpha smooth actin muscle or antibody for other cell markers, rat anti F4/80, rat anti PECAM or rabbit anti reticular fibroblasts diluted to 1:1000 in 0.5% BSA (Sigma Aldrich, St Louis, MO) for overnight at 4°C. Sections were then incubated with streptavidin conjugated Alexa 488 at the concentration of 1:500 in PBS (Molecular Probes). For staining for F4/80, PECAM or reticular fibroblasts, sections were incubated with secondary antibody, either Alexa 596 conjugated anti rat or Alexa 555 anti rabbit respectively, at the dilution of 1:500 in 0.5% BSA for 1h. Sections were then observed under a fluorescent microscope (Nikon, Japan) at 200X and 400X magnification.

Double positive cells were counted in 5 representative fields and presented as percentage of β-gal-positive cells.

4.5.8 Statistical analysis

Data were expressed as means ± standard deviation (SD) and analyzed by two-tailed Student's t-test using the PRISM software program (GraphPad Software, San Diego, CA, USA).

5.0 CONCLUSION AND FUTURE DIRECTION

5.1 GENERAL DISUSSION

The conventional clinical approach for treating diabetic wound healing largely employs a series of palliative treatments involving removal of infected tissue and moisture retention. Unlike the conventional therapy such as saline soaked gauze, new technology is bioactive instead of palliative. Furthermore, those new technologies such as growth factors, bioengineered skin are designed in an attempt to correct the problematic wound environment instead of treating symptoms. Growth factor therapy, the first FDA approved therapeutic approach for treating diabetic ulcer, is a direct way to correct the decreased level of growth factors in diabetic wounds. However, the challenge is to maintain the level of therapeutic proteins at sufficient time and amount. Thus, patients need to receive multiple injections until the wound heals. Gene therapy is an attractive option to protein therapy as the introduction of a gene rather than its product is potentially more efficient to correct the growth factor levels and less expensive. Although there are limited gene delivery methods available to sustain a long-term gene expression, transient gene expression is relatively desirable in treating wounds as the expression of the introduced gene is not necessary after complete healing. Furthermore, skin is an attractive target for gene delivery due to the easy accessibility. Thus, it is highly feasible to choose the least invasive but the most effective way from a wide variety of modalities to introduce certain genes into the wounds. In particular, topically application or simple injection can be feasible. To date, most of the work related to growth factor gene therapy has been conducted in animals; however, certain

approaches may be feasible in humans. For example, introduction of naked plasmid DNA yielded promising results in a clinical trial (Isner et al 1998). The data indicates the importance of naked plasmid DNA approach as an alternative to treat non-healing wounds.

Bioengineered living cell constructs such as Dermagraft and Apligraft is another way to correct the abnormal environment. Clinical result shows those living cell constructs improve the wound healing in some patients. However, unlike growth factor therapy, living cell constructs are not as widely accepted by clinicians (Falanga et al 2004). This may be due to the fact that the cells in the construct will die before the wounds heal (Philips et al 2002). Although the mechanism is not fully understood, it is possible that the cells may release the growth factors and serve to protect the wound, which are applying the same concepts as wound dressing and growth factor therapy. Stem cells may be a good alternative because of their self-renewal ability. Furthermore, the multipotent characteristic of stem cells potentially allows the regeneration of epidermal, dermal and vascular components in skin.

In this thesis project, several strategies were taken to improve wound healing in diabetes. All of the approaches were developed to use a physical treatment to benefit biological therapy (either nonviral gene therapy or cell therapy) with a protocol of single administration. For gene therapy approaches, we used a simple and safe technique, naked DNA. To improve the gene expression, we used electroporation as the first approach (Chapter 2) and a thermosensitive hydrogel composed of PEG-PLGA-PEG as the second approach (Chapter 3). The third approach used stem cells with the PEG-PLGA-PEG thermosensitive hydrogel (Chapter 4).

5.2 DELIVERY OF TGF-β1 BY ELECTROPORATION

A prior study from our lab reports a promising treatment approach by simple injection of TGF-β1 naked plasmid DNA (Chesnoy et al 2003). As mentioned, TGF has multiple functions in wound healing. Intradermal injection of TGF-β1 followed by applying electric pulses at the optimal condition yields an increased gene expression of TGF-β1 and synergistic improvement in diabetic wound healing compared with saline treated wounds. Multiple processes of wound healing including angiogenesis, re-epithelialization and wound closure can be observed a synergistic improvement in recovery. We also found that diabetic skin was more sensitive to the electric condition. Using 100V/cm, the gene expression was 10 fold higher compared with the normal skin. Applying 200V/cm or higher to the diabetic skin, we observed electrolysis which did not occur the normal skin. The corresponding higher sensitivity might be due to the difference in the diabetic cell structure or the difference in chemical components in the diabetic wound environment. Further studies will be needed to determine the difference of cellular or biochemical components in diabetic wound environment using cells harvested from the diabetic skin. Those further studies may give more insight on the biology and the biochemistry in diabetic wound environment.

Not only may the derivation occur between the diabetic and normal wound environment, but the mechanism of action about electric pulse may vary in different cell types even in the same wound environment. Based on our result that the therapeutic effect of electric pulses on wound closure was larger than angiogenesis and reepithelialization, the action of electric pulses may vary from keratinocytes, fibroblasts to endothelial cells. Further studies need to be conducted to determine the difference of the action in different cell types using different types harvested from the skin.

5.3 DELIVERY OF TGF-β1 BY THERMOSENSITIVE HYDROGEL

Previous study has shown the promising approach of using thermosensitive hydrogel made of PEG-PLGA-PEG to enhance naked DNA delivery (Li et al 2003). Given that hydrogel wound dressings can enhance wound healing in humans, it is logical to select the hydrogel for improving diabetic wound healing. The treatment of the wound with the hydrogel mode of the copolymer, and TGF-β1 plasmid synergistically enhanced scab rejection, reepithelialization, collagen synthesis and wound closure in the early phase of wound healing. However, we used a high loading of DNA (200μg) for topical application using PEG-PLGA-PEG. In further studies, we suggest to lower the loading amount of DNA by enhancing the delivery efficiency of DNA, i.e. maximize the amount of DNA uptake by the cell. For example, to increase the hydrophobic of polymer surfaces to make more cells accessible to DNA. More cells attached to the polymer may allow more cells to be transfected by DNA. Since hydrogel wound dressings allow the topical application, a patient-friendly administration mode, this simple and effective strategy is attractive as it could be feasible in an outpatient setting.

5.4 STEM CELL DELIVERY USING THE THERMOSENSITIVE HYDROGEL

From this study, we report that MDSC (muscle-derived stem cells) uniquely improved the wound closure with the thermosensitve hydrogel, PEG-PLGA-PEG, which functions to promote the survival of stem cells. Furthermore, we find that it is highly possible that some of the MDSCs differentiate to fibroblasts in the wound environment.

Cell development and cell differentiation are influenced by stimuli in tissue microenvironment (niche) including neighboring cells, extracellular matrix and growth factors.

For example, mesenchymal stem cells (MSC) will differentiate to airway epithelial cells (AEC) when coculturing with AEC (Wang et al 2005). Growth factors could be another influencing factor to drive stem cell differentiation (Chang et al 2003). The diabetic wound environment is known to have a decreased expression of multiple growth factors such as IGF, TGF-β, FGF EGF TNF-α IL-1β and VEGF (Bitar et al 1996, Zykova et al 2000). It is possible that such an altered wound environment drives the differentiation of MDSC into fibroblasts. Another possibility is that that the diabetic wound environment consists of some growth factors or extracellular matrix that is uncommon in the normal wound environment to induce the differentiation of MDSC to skin cells. Further studies will be necessary to identify the novel component(s) or the change of the biochemical and cellular conditions in diabetic wound environment. So far, MDSC promotes skin repair as well as bone and muscle regeneration (Qu et al 2002, Li et al 2004). However, the control of the stem cell differentiation and the underlying mechanism has not yet to be fully elucidated. Thus, the proposed study may contribute to the tissue engineering by creating an effective and regulated regenerative therapy for skin ulcers through the understandings of the biology of the stem cell.

Since MDSC can be readily transduced with viral vectors (Cao et al 2003, Li et al 2002), gene therapy using MDSC is also an attractive approach. By transducing the stem cells with growth factor; or cytokine genes in particular leptin gene which play an important role in wound healing, the stem cell/gene therapy may further improve the wound healing by treating multiple defective wound healing processes. As shown in our previous findings, gene therapy for growth factors is very attractive for skin wound healing (Chesnoy et al 2002, Lee et al 2003, and Lee et al 2004). Due to the self-renewal property of the MDSC and the differentiation capacity to the cells which are essential for wound healing, MDSC may be a good source to produce the growth

factor proteins. This approach offers the advantage of the release of a growth factor protein at the wound site and alleviates the necessity for repeated application of a protein. The prolonged presence of stem cells in our system may also allow the application not limited to wound healing. Many disease states might be treatable with the protein products generated by the engrafted stem cells.

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