Intradermal Injection of Transforming Growth Factor-β1 Gene Enhances Wound Healing in Genetically Diabetic Mice

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Purpose. To evaluate the biologic effect of direct cutaneous TGF-β1 gene delivery on impaired wound healing models using genetically diabetic mice.

Methods. Diabetic mice (C57BKS.Cg-m +/+ Leprdb female mice) with 1 cm × 1 cm excisional wounds were intradermally injected with 60 μg of plasmid DNA encoding TGF-β1 gene. The wound closure was measured up to 14 days postwounding. At days 7 and 14 postwounding, sections of skin were taken for hematoxylin and eosin and Masson's trichome staining to examine the morphology and collagen deposition. The cell proliferation and TGF-β1 gene expression were studied using immunohistochemical stainings for 5-bromo-2-deoxyuridine and for TGF-β1.

Results. A higher cell proliferation rate and a denser and more organized new extracellular matrix were observed in the treated wound site. Complete wound closure was detected as early as 7 days for TGF-β1–treated group in comparison with 11–14 days for the untreated, control plasmid DNA- and PBS-treated groups.

Conclusion. A single intradermal injection of TGF-β1 plasmid DNA was sufficient to enhance wound healing. This approach represents a new strategy that may be applied to the treatment of excisional wounds in human diabetic patients.

KEY WORDS: gene transfer; TGF-β1; diabetics; wound healing.

INTRODUCTION

Wound healing is severely impaired in diabetic patients. These patients show prolonged inflammation and impaired neovascularization at the site of the wound. Different healing steps (e.g., inflammation, granulation tissue formation, angiogenesis, epithelialization, and remodeling) are controlled in large part by growth factor proteins released at the site of the wound. The topical application of recombinant growth factor proteins at the site of the wound has been shown to enhance wound healing in animal models (1–4). However, their efficacy is limited by their short half-lives which in turn necessitate high initial doses and daily applications. A gene therapy approach is an attractive alternative to treat wound healing because growth factor proteins can be continuously synthesized and released at the site of the wound for a prolonged period of time, thereby alleviating the necessity for repeated applications.

Among nonviral gene transfer technologies, particle-mediated gene delivery has been used successfully to treat wounds in healthy rats (5,6). Sun *et al.* (7) investigated the effect of cationic liposome/DNA complexes, or lipoplex, on wound healing in genetically diabetic mice. The wound-healing process in these mice is markedly delayed similarly to the human condition. This healing impairment is characterized by delayed cellular infiltration and granulation tissue formation. The authors topically applied DNA encoding for fibroblast growth factor complexed with lipoplexes to excisional wounds once daily for three consecutive days after wounding. Their results showed that the closure of the wounds was significantly accelerated by transfection with the fibroblast growth factor plasmid. Recently, Meuli *et al.* (8) reported efficient gene expression in incisional wounds following intradermal injection of plasmid DNA.

Furthermore, cross reactivity of TGF-β1 between human and mouse has been reported (9). Human TGF-β1 significantly regulates murine macrophage proliferation. Therefore, we have used a mouse model to evaluate the biologic response of the human TGF-β1 gene. In this study, we have assessed the biologic efficacy of a single direct cutaneous injection of DNA encoding for human recombinant TGF-β1 in the wound margins of a full-thickness wound in genetically diabetic mice.

MATERIALS AND METHODS

Animals

C57BKS.Cg-m +/+ Leprdb female mice, 8 weeks old, were used as the model for genetically diabetic mice (Jackson Laboratories, Bar Harbor, Maine). The diabetes mutation is a result of a spontaneous point mutation in the leptin receptor gene, *Lepr.* These mice become obese around 3 to 4 weeks of age. Elevation of plasma insulin begins at 10 to 14 days and of blood sugar at 4 to 8 weeks based on the information provided by the vender. All animal protocols were approved by the IACUC committee of the University of Pittsburgh.

Plasmids

Plasmids encoding the enhanced green fluorescence protein (EGFP) cDNA driven by the immediate early promoter of CMV (pCMV-EGFP) was amplified in the DH5α strain of *Escherichia coli*. Human TGF-β1 cDNA in pcDNA3.1/GS (Invitrogen Corporation, Carlsbad, CA) was amplified in TOP10 competent cells (Invitrogen Corporation, Carlsbad, CA). All plasmids were isolated by alkaline lysis and purified by ion-exchange column chromatography (Qiagen Inc. Valencia, CA).

Wounding Protocol and Treatment

Full-thickness skin wounds 1 cm × 1cm (through the panniculus carnosus) were made on the backs of diabetic mice under isoflurane inhalation anesthesia. Immediately after the wounding, 60 μg of TGF-β1 plasmid DNA dissolved in 50 μl of PBS was injected into the dermis in the left skin margin of the wound. The opposite margin received 50 μl of PBS alone. As control, one group of wounded mice received a single intradermal injection of 60 μg of pcDNA3.1/GS plasmid DNA without the TGF-β1 gene (empty plasmid) and a single

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ABBREVIATIONS: TGF-β1, transforming growth factor β1; EGFP, enhanced green fluorescence protein; PBS, phosphate-buffered saline.

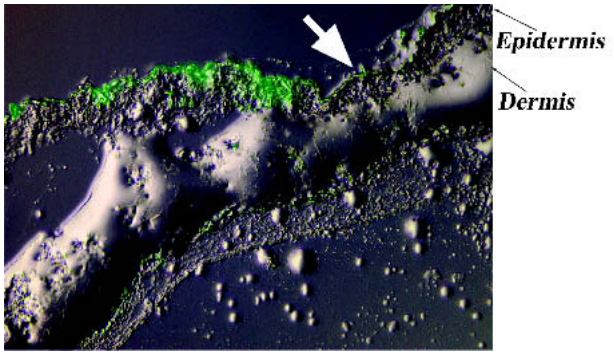

Fig. 1. EGFP gene expression located in the epidermis and the dermis at the edge of the wounded skin *(arrow)*. Magnification 200×.

intradermal injection of PBS solution. Other controls were a group of untreated mice and a group of mice treated with two intradermal injections of PBS solution on both the left and right wound margins. There were four mice in each group. After wounding and treatment, the wounds were left undressed, and the mice were kept individually in separate cages.

Wound Closure Analysis

Each 2 days, for a total of 14 days, the wound area was measured with a caliper and calculated as the percentage of wound area covered with new granulation tissue: [(day 0 area – day *N* area)/ (day 0 area)] × 100. Data are represented as mean ± SD and were compared among different groups using the Student *t* test.

Histology

At days 7 and 14, wounds were harvested and embedded in OCT medium and frozen in isopentane (Sigma, St Louis, MO) at –80°C. Frozen wounds were equilibrated at –20°C in a cryostat before sectioning. Sections 10 μm thick and perpendicular to the wound surface were obtained. Hematoxylin and eosin staining was performed for histology. The degree of collagen formation was evaluated by Masson's trichrome staining (10).

Detection of the Enhanced Green Fluorescence Protein (EGFP)

Mice were intradermally injected with 60 μg of EGFP plasmid immediately after wounding. Twenty-four hours postwounding, the skin was frozen and cryosectioned as described above. EGFP expression in the sample was observed with a fluorescence microscope (Nikon TE 300). The images were converted to gray scale.

Immunohistochemical Staining for TGF-β1

Immunostaining was performed using chicken polyclonal anti-TGF-β antibody (R&D Systems). Endogenous peroxidases were blocked by using a freshly prepared solution of 3% hydrogen peroxide in methanol for 10 min at room temperature. After rinsing in PBS, sections were blocked using normal rabbit serum. Incubation with polyclonal anti-TGF-β antibody was carried out for 2 h at 37°C in a humidified chamber at a dilution of 1/50. After washing in PBS, sections were incubated 1 h at room temperature with a biotinylated secondary antibody against the chicken IgY raised in rabbit (Jackson ImmunoResearch laboratories, West Grove, PA) at a dilution 1/250. This was followed by a 30-min incubation with the ImmunoPure ABC peroxidase reagent (Pierce, Rockford, IL) and subsequent detection with ImmunoPure Metal Enhanced DAB substrate (Pierce, Rockford, IL). The reaction was stopped after 10 min, and the sections were stained with hematoxylin.

Cell Proliferation

5-Bromo-2-deoxyuridine (BrdU) is an analog of thymidine that is incorporated into the cellular DNA at the S phase of the cell cycle. Cell proliferation levels in the granulation tissue were examined at days 4 and 7 after wounding. BrdU (Sigma, St Louis, MO) was injected intraperitoneally 3 h before sacrifice at a dose of 50 mg/kg. To detect proliferating

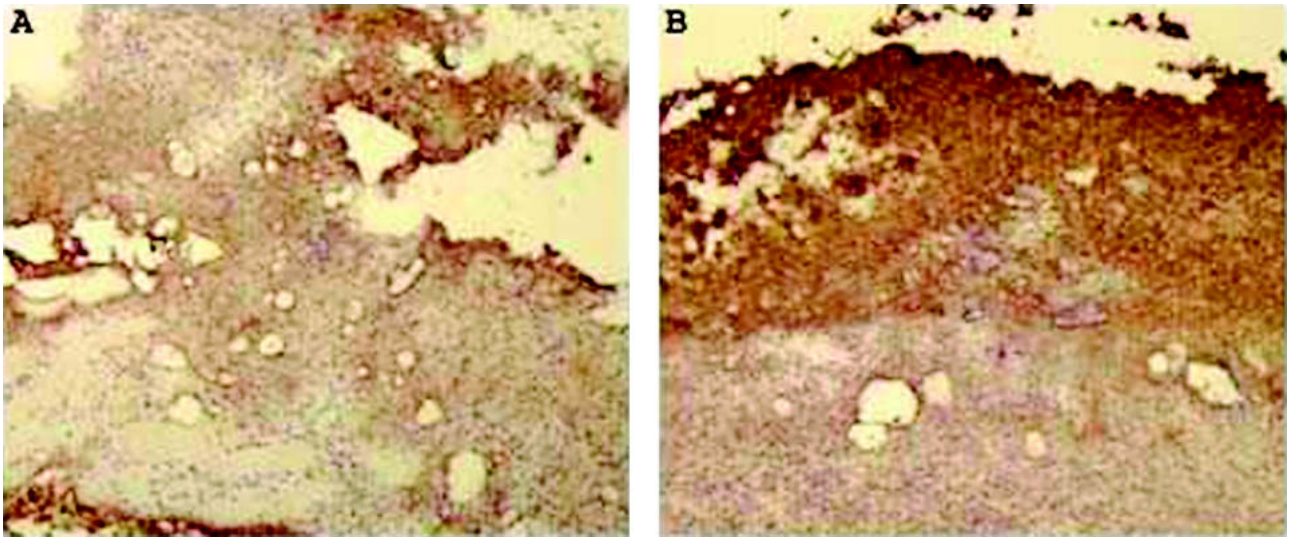

Fig. 2. Expression of TGF-β1 protein in the wound bed 7 days postwounding in the skin treated by intradermal injection of PBS (A) or by TGF-β1 plasmid DNA (B).

cells, immunostaining was performed using a monoclonal anti-BrdU antibody (Sigma, St Louis, MO). Endogenous peroxidases were blocked using a freshly prepared solution of 3% hydrogen peroxide in methanol for 10 min at room temperature. After washing in PBS, sections were immersed in 2 N HCl for 30 min at 37°C to denature DNA. Then, 0.1% trypsin solution was applied for 30 min at 37°C. After rinsing in PBS, sections were blocked using normal rabbit serum. Incubation with monoclonal anti-BrdU antibody was carried out for 2 h at 37°C in a humidified chamber at a dilution of 1/250. After washing in PBS, sections were incubated for 1 h at room temperature with a biotinylated secondary antibody against mouse IgG raised in rabbit (Jackson ImmunoResearch Laboratories, West Grove, PA) at a dilution of 1/250. This was followed by a 30-min incubation with the ImmunoPure ABC peroxidase reagent (Pierce, Rockford, IL) and subsequent detection with ImmunoPure Metal Enhanced DAB substrate (Pierce, Rockford, IL). The reaction was stopped after 10 min of incubation, and the sections were stained with hematoxylin. Sections were taken from each of three animals.

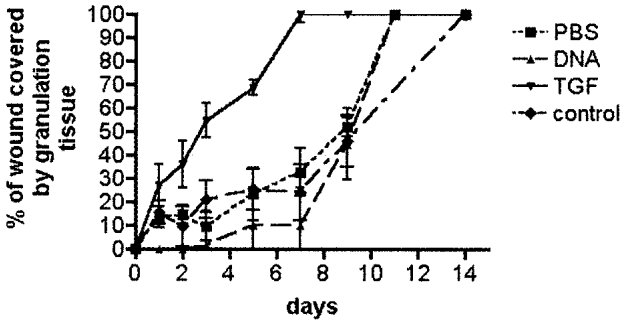

Fig. 3. Evaluation of the wound closure by formation of new granulation tissue as a function of time. Control (untreated group), PBS (50 μl of PBS solution was injected on both sides of the wound), DNA (60 μg of plasmid DNA without the gene encoding for TGF-β1 dissolved in 50 μl PBS was injected at one side of the wound, and PBS solution on the other side), TGF (60 μg of plasmid DNA with the gene encoding for TGF-β1 dissolved in 50 μl PBS was injected at one side of the wound, and PBS solution on the other side). Four mice were treated per group.

RESULTS

Efficiency of Gene Transfer at the Wound Site

First, the efficacy of gene transfer was evaluated following the intradermal injection of 60 μg of EGFP plasmid immediately after wounding. Figure 1 shows gene expression 24 h postwounding. Green fluorescence could be seen predominantly in the dermis and the epidermis of the skin next to the wound. This verifies that efficient gene expression can be obtained in the diabetic mouse skin by intradermal injection of naked DNA dissolved in PBS, similar to what we have reported in the normal mouse skin (11). Based on this result, PBS was used to dissolve the plasmid DNA in the entire experiment. A dose of 60 μg plasmid DNA was determined to give significant levels of gene expression predominantly located in the dermis and the epidermis. Intradermal injection of smaller amounts of the gene resulted in much lower gene expression (data not shown).

As a result of cross reaction between murine and human TGF-β1 proteins, the intensity of gene expression at day 4 was comparable between the treated and untreated wounds. However, Fig. 2 shows that, 7 days postwounding, TGF-β1 proteins were still expressed in the wound bed in animals treated with intradermal injection of TGF-β1 gene. The expression was significantly higher than in the one treated with PBS. The TGF-β1 expression was more prominent on the top of the granulation tissue where reepithelialization occurred.

Therapeutic Effect of TGF-β1 Gene following Intradermal Injection

Concomitant with higher and more sustained production of TGF-β1 proteins, the wound closure analysis indicated a rapid wound closing with new granulation tissue for mice treated with TGF-β1 plasmid DNA (Fig. 3). Delayed wound closure of approximately 4 or 7 days was observed for empty plasmid DNA, PBS, or untreated, after which rapid closure was noticed. This result indicates that the injection of TGF-β1 plasmid DNA was effective in enhancing the healing rate of the wound, primarily promoting the early phase of the healing. That delayed wound closure in the early phase was observed for empty plasmid DNA compared to the untreated may be caused by nonspecific inflammatory cytokine production induced by the unmethylated CpG motifs in the DNA (12).

Evaluation of Extracellular Matrix Deposition

The process of wound healing involves four major steps: inflammation, formation of new granulation tissue, reepithelialization, and tissue remodeling (13). During inflammation, the formation of a blood clot at the site of the wound gives rise to a provisional matrix through which macrophages and neutrophils can migrate. These cells release proinflammatory cytokines such as TGF-α, TGF-β, and IL-1, which are responsible for the initiation and formation of new granulation tissue. Three or four days after injury, dermal fibroblasts from the wound edges begin to proliferate and migrate into the provisional matrix. During the first week after wounding, the fibroblasts stimulated by TGF-β1 will be responsible for the synthesis of a rich collagen matrix, which will replace the provisional matrix (14). The formation of a healthy granulation tissue is very important because it will provide a good medium for new blood vessel synthesis as well as epidermal cell migration and proliferation for reepithelialization (15).

The deposition of collagen at the wound site was evaluated by histology. Collagen can be identified as a blue color on skin sections stained with Masson's trichrome staining solution. A clear difference in blue intensity was observed on the skin wound bed 7 days postwounding between PBS and TGF-β1 gene-treated wounds (Fig. 4). The wounds treated with PBS contained less collagen than the wounds treated with TGF-β1 gene. A denser and more organized granulation tissue could also be seen on wounds treated with TGF-β1 gene, suggesting better healing for these wounds. The appearance of organized collagen fibers as bundles also suggests increased tensile strength of the new skin tissue. Concomitant with more collagen and granulation tissue formation, a higher cellular density was also recorded in these wounds. Figure 5 shows skin sections 14 days postwounding, the time when

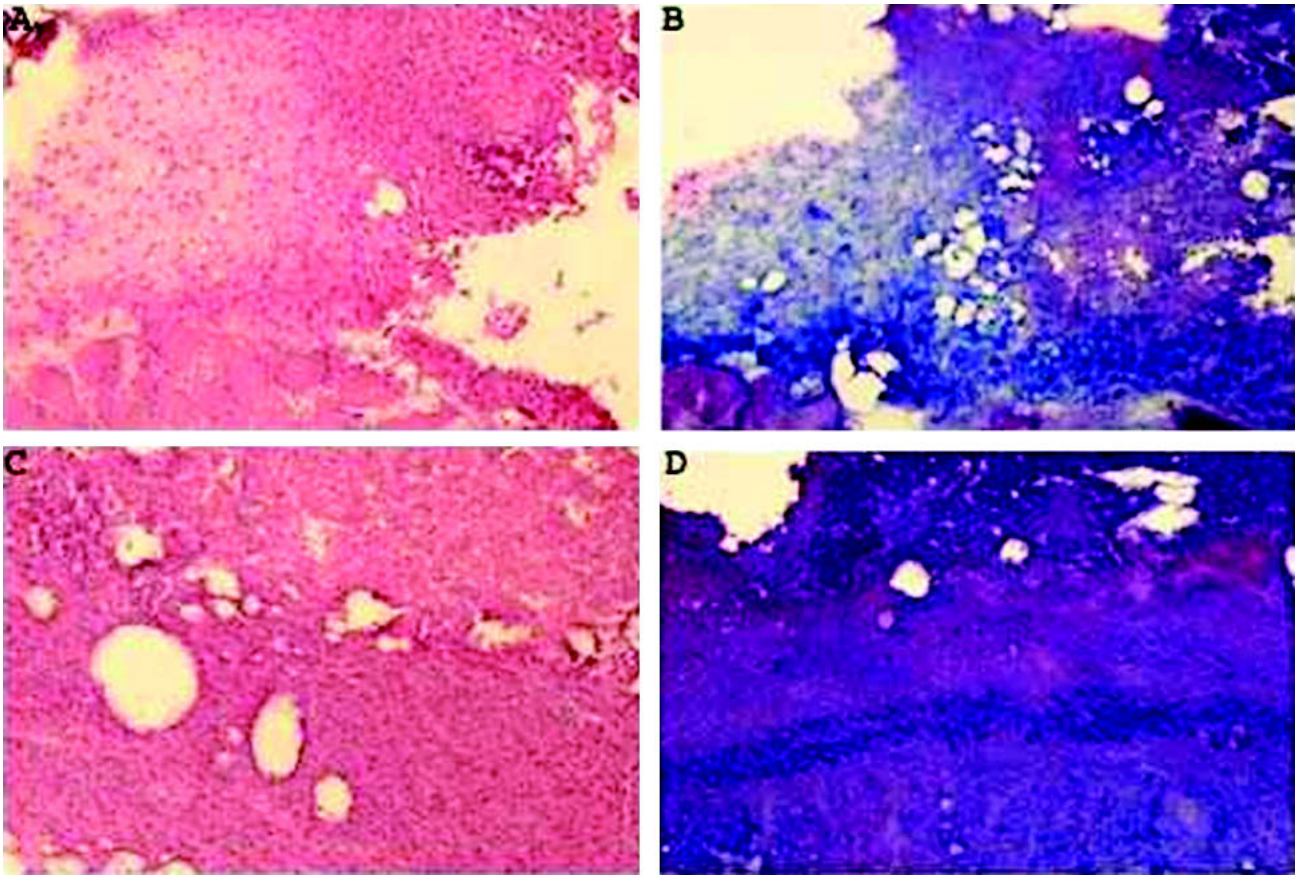

Fig. 4. Morphology and extracellular matrix deposition of skin wound treated with TGF-β1 gene. Hematoxylin and eosin staining of the wound bed from a skin treated by intradermal injection of PBS (A) or TGF-β1 gene (C) 7 days after wounding. Masson's trichrome staining of the wound bed from skin treated by intradermal injection of PBS (B) or TGF-β1 gene (D) 7 days after wounding. Blue and dark purple colors in (B) and (D) indicates collagen staining.

complete wound closure occurred in all groups. It is evident that a denser and more organized extracellular matrix was found in the wound treated by TGF-β1 gene than in the untreated wounds or the wounds treated with PBS.

Cell Proliferation

Cell proliferation was evaluated by histology by immunohistochemical staining for BrdU. Figure 6 shows that at day 7, TGF-β1–treated wounds displayed higher levels of stained cells than untreated wounds. The cells were distributed homogeneously in the wound bed. Furthermore, the appearance of BrdU-positive stained cells was already visible at day 4 after wounding. However, the cells were mostly located on the wound edges, with practically no cells detected in the wound bed (data not shown). This result suggests that the local production of TGF-β1, resulting from the direct DNA intradermal injection, induced cell proliferation as early as day 4, and the effect lasted until day 7 postwounding. As a control, nonwounded mice were injected with BrdU, and their skins were harvested and sectioned. No positively stained cells were noticed in these sections (data not shown).

DISCUSSION

In chronic human diabetic wounds, the healing repair is prevented by several abnormalities including prolonged inflammation, impaired neovascularization, decreased synthesis of collagen, and defective macrophage functions (16). These characteristics can also be seen in animal models such as the genetically diabetic mouse C57BKS Cg.m +/+ Leprdb. Significant delays in the rate of wound closure, cellular infiltration, and formation of granulation tissue have been described (3,4). Frank *et al.* (17) reported that the level of TGF-β1 is significantly reduced in genetically diabetic mice compared to nondiabetics. Addition of recombinant TGF-β1 protein to the site of the wound has shown promising effects in the repair process (1,2,18). In this study, we evaluated the effect of a single intradermal injection of TGF-β1 gene at the site of the wound on the repair process in genetically diabetic mice.

Meuli *et al.* (8) reported that direct cutaneous injection of DNA alone into wounded skin from normal mice was efficient enough to transfect the skin with gene expression levels that were still detectable after 8 weeks. In our study, intradermal injection of EGFP plasmid DNA into the edges of wounded skin from genetically diabetic mice was examined for gene expression 24 h after injury. The injection of TGF-β1 plasmid DNA at a dose of 60 μg resulted in more rapid healing compared with untreated and PBS-treated wounds. This enhanced healing resulted most likely from a robust and sustained production of TGF β1 protein at the site of the wound by the transgene. TGF-β1 has several biologic effects, among which it is a potent chemoattractant for monocytes, macro-

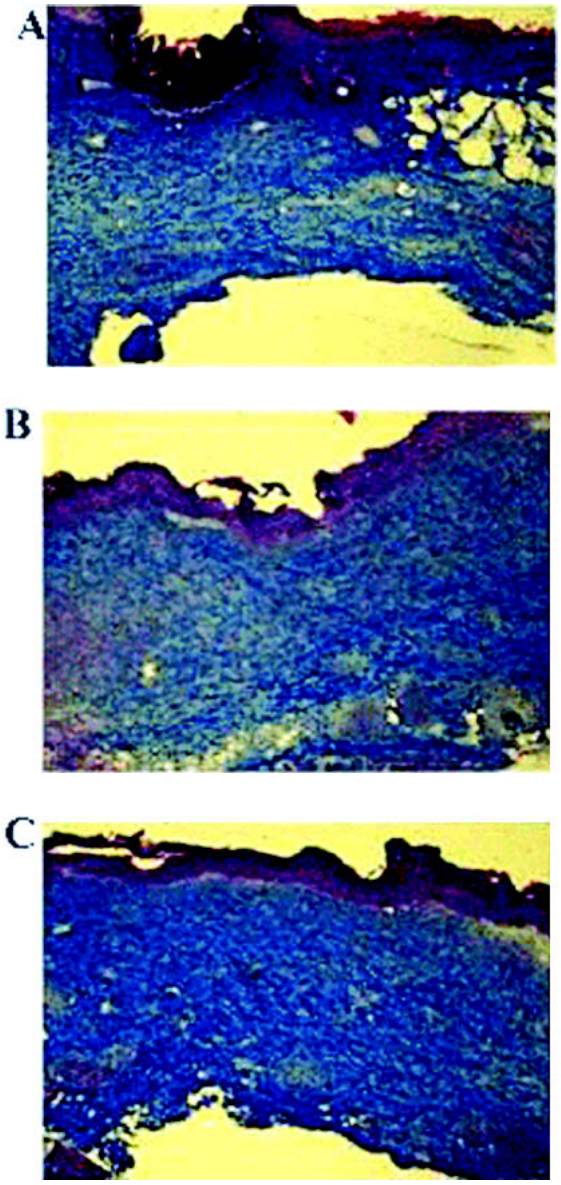

Fig. 5. Masson's trichrome staining of skin 14 days after wounding: (A) untreated skin, (B) skin treated by intradermal injection of PBS, (C) skin treated by intradermal injection of TGF-β1 plasmid DNA.

phages, lymphocytes, and neutrophils during the inflammatory phase (14). All these cells need to be present very quickly in the provisional matrix because they will act as reservoirs for the release of cytokines necessary for the initiation and propagation of granulation tissue formation. Darby *et al.* (19) observed that cellular infiltration in wounds from genetically diabetic mice was delayed compared to normal, nondiabetic mice. In our study the presence of an increased amount of TGF-β1 proteins in the wound margins, as a result of transgene expression, could provide a compensatory source of cytokines that would initiate more efficient and more rapid proliferation and migration of cells from the surrounding dermis into the wound bed. Indeed, we have observed a higher cell proliferation rate in the granulation tissue and in the wound edges of TGF-β1–treated wounds compared to PBS-treated wounds as early as day 4. Frank *et al.* (17) have described the kinetics of TGF-β1 expression in wounds from genetically diabetic mice and reported that TGF-β1 mRNA levels 24 h after wounding were up-regulated up to ninefold during next 4 days and then decreased. Seven days after wounding, our results showed that TGF-β1 expression could still be detected in the granulation tissue from TGF-β1–treated wounds in comparison with lower detectable levels in PBS-treated wounds. The increased expression of TGF-β1 in the granulation tissue could be explained by higher cellular proliferation and infiltration and by the autoinductive effect of TGF-β1 on its own expression (14). We have also noticed that the effect of TGF-β1 became visible as early as day 4. Another role of TGF-β1 is to be an important regulator of the formation of the new extracellular matrix by acting on the fibroblasts that have begun to migrate into the wound bed to increase collagen deposition (17). We have observed a complete wound covering by new granulation tissue at 7 days in the TGF-β1–treated group in comparison with 11 to 14 days for the untreated, control plasmid DNA–treated, and PBS-treated groups. The TGF-β1–treated wounds were characterized by a more organized and denser collagen matrix, suggesting better healing of improved quality.

In conclusion, all our results confirm that a single local injection of TGF-β1 gene had beneficial effects on wound healing in genetically diabetic mice. The biologic effect of TGF-β1 was more pronounced in the early stage of the heal-

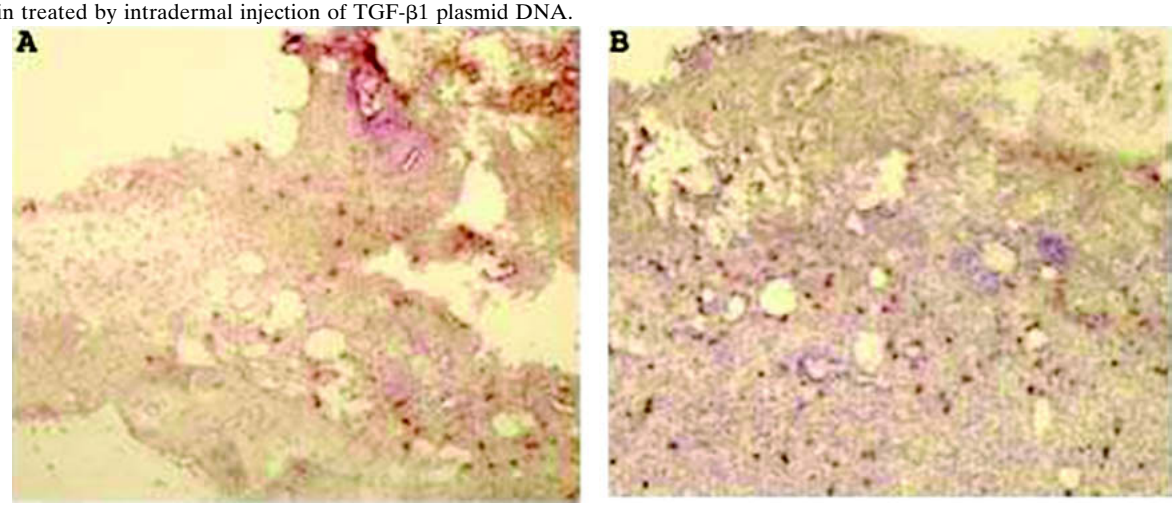

Fig. 6. Cell proliferation at the wound site. Anti-BrdU staining of skin 7 days postwounding. Skin was treated with intradermal injection of PBS (A) or TGF-β1 plasmid DNA (B). Dark brownish stain indicates BrdU-positive cells.

ing, probably as a result of increased cellular proliferation and migration of dermal cells into the provisional matrix. Consequently, the release of TGF-β1 in the wound bed activated collagen synthesis by fibroblasts. The gene transfer technique is simple and could be applied to human skin to enhance the formation of granulation tissue in diabetic patients, thereby limiting the occurrence of infection in prolonged untreated wounds. The combination of several genes expressing different cytokines could also be studied because Brown *et al.* (4) reported that the addition of PDGF and IGF-II had synergistic effects on wound healing in genetically diabetic mice. These experiments will be performed in the future.

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