American Journal of Pathology, Vol. 136, No. 6, June 1990

PDGF and FGF Stimulate Wound Healing in the Genetically Diabetic Mouse

David G. Greenhalgh,* Katherine H. Sprugel,‡ Mark J. Murray,‡ and Russell Rosst

From the Department of Surgery and the University of Washington Burn Center, the Department of Pathology,† University of Washington, and ZymoGenetics, Inc.,‡ Seattle, Washington*

To examine the effects of recombinant growth factors* in vivo, *impaired wound healing was studied in genetically diabetic C57BL/KsJ-db/db mice. Large full-thickness skin wounds made on the backs of these mice exhibited significant delays in the entry of inflammatory cells into the wound, the formation of granulation tissue, and in wound closure when compared to their nondiabetic littermates. Recombinant human platelet-derived growth factor (rPDGF-BB, 1 or 10 μg), recombinant human basic fibroblast growth factor (rbFGF, 1 μg), or combinations of both were applied topically to the wounds for 5 to 14 days after wounding. Diabetic mouse wounds treated with rPDGF-BB or rbFGF had many more fibroblasts and capillaries in the wound bed at 10 and 21 days than did wounds treated with the vehicle alone. The animals treated with growth factors also had significantly greater wound closure at 21 days than those treated with the vehicle. Combinations of rPDGF-BB and rbFGF improved all parameters of healing but not to a greater extent than either growth factor alone. The effectiveness of rPDGF-BB and rbFGF suggest that recombinant growth factors may be useful in the treatment of patients with deficient wound repair. (Am J Pathol 1990, 136:1235–1246)

While normal, healthy people rarely have problems with healing, many medical and surgical complications can be attributed to deficiencies in wound repair. Open wounds have lost the barrier that protects tissues from bacterial invasion and allows for the escape of vital fluids. Without expeditious healing, infections become more frequent. Most wound complications, such as dehiscence, anastomotic breakdown, or skin graft loss, are associated with some form of host impairment such as malnutrition, infection, diabetes, or treatment with steroids, chemotherapy, or radiation.[1-3]

Recently, attempts have been made to improve healing using growth factors. Many *in vitro* experiments suggest that growth factors can act as chemoattractants[4-9] and mitogens[10-14] for the cells involved in wound repair. Growth factors can stimulate angiogenesis,[14-15] extracellular matrix production and degradation,[16-22] and cytokine release.[23,24] Initial studies of wound repair processes in animals used partially purified mixtures of growth factors with some success.[25,26] Relatively few wound healing studies have been performed in humans with growth factors. Knighton[27] has shown that application of an autologous platelet releasate containing a mixture of growth factors improved the healing of chronic leg ulcers. Improved healing in such ulcers has also been reported after application of bovine platelet extracts.[28] The availability of larger quantities of purified growth factors through biotechnology has enabled new studies in animals and the initiation of studies in human subjects. Recombinant epidermal growth factor (EGF) stimulates an improvement in the healing rate of human skin donor sites.[29] EGF[30] or transforming growth factor alpha (TGF-a)[31] also improve the healing of partial-thickness wounds in pigs. In subcutaneous wound chambers, growth factors have been found to increase cellular invasion and fibroplasia.[32-36] Attempts to improve healing in animals with growth factors in full-thickness wounds have resulted in observations varying from slight improvement[37-40] to no effect.[41,42] Several investigators have improved isolated aspects of the normal healing process but the beneficial effects do not appear to change the overall time required for complete wound repair. One explanation for the modest effects seen after the application of growth factors to wounds in normal animals may be that the healing process already proceeds at a near-optimal rate. Consequently, a more useful approach may be to use growth factors in wounds that demonstrate a clinically relevant healing impairment such as that which occurs in diabetes. Recent reports suggest that growth factors can reverse some of the deficits in impaired healing models.[34,35,42]

Accepted for publication January 5, 1990.

Address reprint requests to Katherine H. Sprugel, PhD, ZymoGenetics, Inc., 4225 Roosevelt Way NE, Seattle, WA 98105.

In this report, we present a model of impaired wound healing in homozygous, genetically diabetic (db/db) mice. These animals develop obesity, insulin resistance and severe hyperglycemia that resembles human adult onset diabetes. Wound healing in these diabetic mice is markedly delayed when compared with their heterozygous, nondiabetic littermates. The healing impairment is characterized by delayed cellular infiltration and granulation tissue formation. In such wounds, topical application of recombinant human platelet-derived growth factor (rPDGF-BB) and basic fibroblast growth factor (rbFGF) profoundly stimulate the repair process.

Methods

Animals

Male and female C57BL/KsJ-db/db mice were obtained from Jackson Laboratories (Bar Harbor, ME). The animals were 8 to 12 weeks old at the start of all experiments. During the experiments the animals were housed one per cage and maintained in a central animal care facility with a 12-hour light/dark cycle. Water and standard rodent laboratory chow were supplied *ad libitum*. The animal care facilities were maintained by professionals who followed federal guidelines, and all procedures were approved by the University of Washington and ZymoGenetics Animal Care Committees.

The C57BL/KsJ-db/db mice were chosen because they exhibit a series of characteristics similar to those of human adult onset diabetes. The metabolic abnormalities result from a single autosomal recessive mutation on chromosome 4 (db+).[43] Only the homozygous (db+/db+) animals develop diabetes while the heterozygous (db+/+m) littermates show no signs of diabetes or obesity. The heterozygotes were used as controls for comparison with the diabetic homozygotes.

Serum glucose values were obtained in several of the experiments after anesthetization and before the animals were killed. Blood was obtained from the retro-orbital plexus using heparinized capillary tubes. Serum was analyzed for glucose content using a Beckman Glucose Autoanalyzer (Beckman Instruments, Towson, MD).

Insulin levels were obtained for some of the mice in heparinized blood samples. The serum was collected, frozen at −20° C, and later analyzed for insulin levels by radioimmunoassay.[44]

Wounding

The animals were anesthetized with a mixture of ketamine (110 mg/kg, Vetalar®, Parke Davis, Morris Plains, NJ) and xylazine (7 mg/kg, Rompun®, Miles Laboratories, Shawnee, KA). The hair on the back was clipped, and the skin washed with povidone-iodine solution and wiped with sterile water. A template was used to mark a 1.5 × 1.5 cm square on the midback and a full-thickness wound corresponding to the template was made by excising the skin and panniculus carnosus. Tincture Benzoin Compound (Paddock Laboratories, Minneapolis, MN) was applied outside the perimeter of the wound and the semipermeable polyurethane dressing OpSite® (Smith and Nephew, Massillon, OH) was placed over the wound and sealed at the edges by the benzoin. The growth factor mixture was applied by injecting it through the OpSite with a 27-gauge needle and allowing it to spread over the wound bed. The animals were given 1 ml of subcutaneous Ringer's solution at completion of the surgical procedure.

Growth Factors

Human recombinant PDGF-BB was produced in a yeast expression system similar to that previously described.[45] This system secretes the rPDGF-BB into the yeast growth medium from which it is purified by ion exchange chromatography. The rPDGF-BB used in these studies is more than 95% pure and was quantitated by amino acid analysis. The full-length 155 amino acid form of human rbFGF was produced by cytoplasmic expression in yeast. The rbFGF was purified from the yeast cytoplasm by acid extraction followed by heparin-sepharose chromatography[46] to more than 95% purity and quantitated by amino acid analysis. The biologic activity of each factor was measured as the ability to stimulate ^{3}H-thymidine incorporation by Swiss 3T3 cells in a mitogenesis assay.[47] Values were assigned by comparison to standards with known activity.

The BB homodimer of rPDGF-BB was used in doses of 1 and 10 μg/wound/day. The doses used for rbFGF were 0.4 and 1 μg/day. These doses were chosen based on the responses described in previous reports[36,37,39] and preliminary dose-response studies. The growth factors were mixed in a vehicle of 5% polyethylene glycol (PEG) (Carbowax PEG 8000, USP grade, Union Carbide, Danbury, CT) in phosphate-buffered saline, lyophilized, and stored at −80° C until the day of use. Mitogenic activity of the preparations was measured after lyophilization. Typically, approximately 20% of the activity was lost during the processing. The doses referred to in the text are the prelyophilization values. The vials were prepared and coded by laboratory personnel who were not involved with the histologic evaluation of the wounds. Each animal was randomly assigned to a coded and blinded treatment regimen. For each treatment, 0.1 cc of the reconstituted treatment mixture (growth factor or vehicle) was injected through the OpSite and over the wound bed. For most of

the experiments the growth factor was applied daily for 5 days starting immediately after wounding. In a few experiments, the treatments were given daily for 10 or 14 days. Endotoxin levels were determined in each preparation of PEG, rPDGF-BB, and rbFGF and were always less than 10 pg/ml (QCL 1000™ Quantitative Chromogenic Limulus Amebocyte Lysate test, Whittaker Bioproducts, Walkersville, MD).

Wound Analysis

The animals were weighed and the wounds were checked 3 to 5 times each week. Wounds were considered closed if moist granulation tissue was no longer apparent and the wound appeared covered with epithelium. Histologic analysis confirmed the presence of complete re-epithelialization under these circumstances.

The edge of the wound was traced onto a glass microscope slide and the wound area was determined by planimetry using ImageMeasure® (Microscience, Inc., Federal Way, WA) or Optimas® (Bioscan, Edmonds, WA). The trace taken immediately after wounding was used as the reference or original area and all further areas were recorded as the percentage of the original area. When the animals were killed, the extent of re-epithelialization was determined by inspection as the border between the moist central, open wound and the dry surroundings. In the rare cases in which a scab was present at the end of the experiment, the edge of the scab was considered the edge of the re-epithelialization (a conservative interpretation). Wound closure is reported as the percentage closed and calculated as:

$$\% \text{ Closed} = [(\text{Area on Day 0} - \text{Open Area on Final Day})/\text{Area on Day 0}] \times 100$$

After the final tracing, the entire wound, including a margin of approximately 5 mm of unwounded skin, was excised down to the fascia and removed. The wound was divided in half and placed in methanol Carnoy's solution for histologic analysis. Each histologic specimen was embedded in paraffin so that the mid-portion of the wound was cut in 5-μ sections. The slides were stained with hematoxylin and eosin or Masson's trichrome for the analyses. Each slide was given a histologic score ranging from 1 to 12, with 1 corresponding to no healing and 12 corresponding to a completely re-epithelialized wound (Table 1). The scoring was based on the degree of cellular invasion, granulation tissue formation, vascularity, and re-epithelialization. The histologic score was assigned separately by two of the investigators and averaged for analysis. Typically the scores of the evaluators were within 1 to 2 units of one another. The wounds were also ranked on the basis of histology from the least healed to the most healed for statistical comparisons of the different groups. The code describing each animal's treatment was broken after the scoring and ranking were completed by all observers.

Table 1. *Scoring of Histology Sections*

Score	Criteria
1–3	None to minimal cell accumulation. No granulation tissue or epithelial travel.
4–6	Thin, immature granulation that is dominated by inflammatory cells but has few fibroblasts, capillaries or collagen deposition. Minimal epithelial migration.
7–9	Moderately thick granulation tissue, can range from being dominated by inflammatory cells to more fibroblasts and collagen deposition. Extensive neovascularization. Epithelium can range from minimal to moderate migration.
10–12	Thick, vascular granulation tissue dominated by fibroblasts and extensive collagen deposition. Epithelium partially to completely covering the wound.

Data Analysis

Values are expressed as the mean ± standard error of the mean. The Student's *t*-test was used for comparisons of biochemical data between the control and diabetic animals. Statistical analysis of the histology rankings was performed using the Kruskal-Wallis test,[48] with individual comparisons performed by the Dunn's procedure ($P < 0.05$)[49] The data comparing the percentages of wound closure were compared by analysis of variance (ANOVA) and individual comparisons were performed by Tukey's procedure ($P < 0.05$).[50] The analyses were performed using the StatView II® statistical software package (Abacus Concepts, Inc., Berkeley, CA).

Results

Impaired Wound Healing in the Diabetic Mouse

The C57BL/KsJ-db/db (db/db) mice demonstrated diabetic characteristics documented in previous reports.[43,51–53] The diabetic mice were obese, weighing 40 to 50 g, in contrast to their nondiabetic littermates that weighed 25 to 32 g. The diabetics were markedly hyperglycemic with average glucose levels of 927 ± 35 mg/dl (n = 52) compared with 205 ± 8 mg/dl (n = 27) for the nondiabetic animals ($P < 0.0001$ by Student's *t*-test). The hyperglycemia produced classic signs of diabetes including polydipsia, polyuria, and glycosuria. The diabetics had approximately 2.5 times the insulin levels of their nondiabetic littermates (diabetic, 48.5 ± 11.0 *vs.* nondiabetic, 19.7

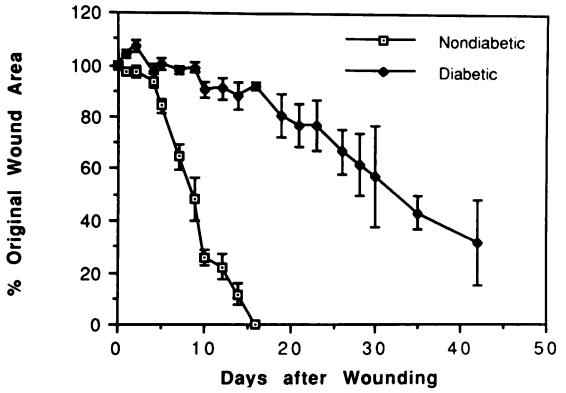

Figure 1. *Time course of wound closure in nondiabetic and diabetic mice. Wound areas were measured immediately after wounding and at various times during the healing process. Values are presented as percentage of original area (mean ± SE, n = 5 to 19), calculated as described in Methods.*

± 3.1 μunits/ml, with n = 15 for both groups, $P < 0.005$ by Student's *t*-test).

Full-thickness wounds were created in the backs of the anesthetized animals and the exposed area was immediately covered with the semipermeable, transparent dressing OpSite. Both the diabetic and nondiabetic animals tolerated the wounding procedure without problems. Neither of the groups had significant changes in food consumption or weight loss after wounding. The OpSite remained in place until the wound healed. Wounds were evaluated by gross inspection and the edge of the open wound was traced through the OpSite to determine wound areas at several time points. On the day of analysis, animals were killed, a final wound area tracing was performed after removal of the OpSite, and the midportion of the wound was preserved for histologic evaluation.

Initial experiments compared the differences in wound healing between the diabetic and nondiabetic animals. The wounds were inspected and their areas measured for 6 weeks. Histologic sections were obtained from randomly chosen animals from both groups at various time points after wounding. The wounds of the nondiabetic animals closed completely within 10 to 16 days, whereas 4 to 6 weeks was required for closure to occur in the diabetic animals. After a 2- to 3-day lag, the nondiabetic wounds rapidly contracted to 10% to 20% of their original area within 10 days (Figure 1). The remaining 10% to 20% of the original area healed by re-epithelialization over new granulation tissue. Wounds created in the diabetic animals did not show any gross evidence of healing for the first 2 to 3 weeks. At as late as 21 days they often remained unchanged from their appearance immediately after wounding. Gradually the wounds developed erythema and granulation tissue. The diabetic wounds eventually closed but rarely before 4 weeks, and one animal examined had a chronic wound at 90 days. The areas of the wounds measured at various times after injury reflect the significant delay in diabetic wound closure when compared to those of the nondiabetic animals (Figure 1).

Histologic evaluation of wounds at various days after wounding showed a close correlation between the gross and microscopic appearances of the wounds. At day 5, wounds in the nondiabetic controls had developed abundant granulation tissue and had partially re-epithelialized. By 10 days, the wounds in the nondiabetic animals contained granulation tissue rich in fibroblasts, collagen, and capillaries. In many cases, new epithelium had completely covered the wound (Figure 2a). In contrast, minimal cellular infiltrates and granulation tissue developed in wounds of the diabetic mice 10 days after wounding (Figure 2b). This lack of cellular ingrowth and granulation tissue formation in the diabetic wounds paralleled the gross appearance of minimal healing and was observed as late as 3 weeks after wounding. Three to four weeks after skin excision, the diabetic wounds started to be invaded by inflammatory cells, fibroblasts, and capillaries. The wounds eventually developed thick, cellular and vascular granulation tissue. For both the nondiabetic and diabetic wounds, re-epithelialization appeared to follow the development of this new granulation tissue. Once healed, wounds in the diabetic mice (Figure 2c, 35 days after wounding) had a normal gross and microscopic appearance.

Wound contraction contributed less to the healing of full-thickness skin excisions in the diabetic mice than in their nondiabetic littermates. This was evident qualitatively on inspection of the wounds as they healed. In the nondiabetic mice, the skin rapidly contracted to cover the defect and only a small region healed by development of granulation tissue and re-epithelialization. In contrast, wounds in the diabetic animals exhibited minimal early contraction and were filled primarily by granulation tissue formation and re-epithelialization. Some contraction was observed in the later stages of healing in the diabetic mice. While difficult to quantify precisely, an approximation of the extent of contraction was achieved by comparing the area bordered by the original edge of the wound at 21 days to the area of the wound at day 0. Roughly 90% of the wound closure in the nondiabetic mice could be attributed to contraction, while only about 40% of the wound closure in the healed diabetic mice was due to contraction.

Effects of rPDGF-BB and rbFGF on Wound Healing in the Diabetic Mouse

Wounds treated for the first 5 days after surgery with rPDGF-BB (1 or 10 μg/day) or the vehicle alone were analyzed at 10 days. Grossly, diabetic animals treated with either dose of rPDGF-BB developed thicker and more erythematous wounds than the vehicle control animals. Histologic scoring was based on the degree of cellular infil-

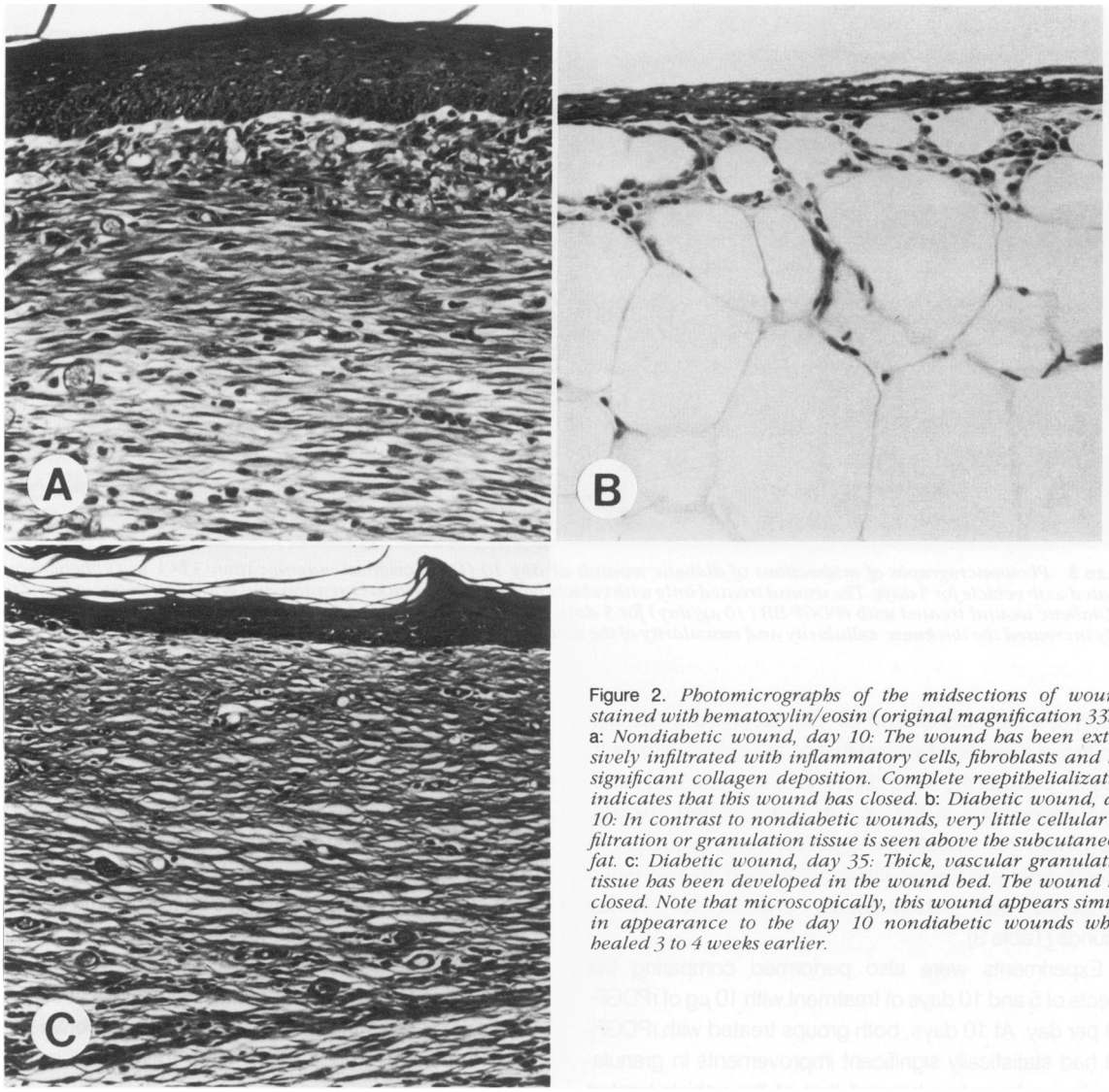

Figure 2. *Photomicrographs of the midsections of wounds stained with hematoxylin/eosin (original magnification 33×).* a: *Nondiabetic wound, day 10: The wound has been extensively infiltrated with inflammatory cells, fibroblasts and has significant collagen deposition. Complete reepithelialization indicates that this wound has closed.* b: *Diabetic wound, day 10: In contrast to nondiabetic wounds, very little cellular infiltration or granulation tissue is seen above the subcutaneous fat.* c: *Diabetic wound, day 35: Thick, vascular granulation tissue has been developed in the wound bed. The wound has closed. Note that microscopically, this wound appears similar in appearance to the day 10 nondiabetic wounds which healed 3 to 4 weeks earlier.*

tration, granulation tissue formation, and re-epithelialization (Table 1). Wounds treated with rPDGF-BB had higher average histologic scores (Table 2), which was consistent with a statistically significant improvement in histologic ranking compared to those in which the vehicle was the sole treatment. Diabetic wounds treated with vehicle alone had minimal cellularity at 10 days (Figure 3a). Those wounds treated with rPDGF-BB developed thick granulation tissue composed of many macrophages, fibroblasts, and new capillaries (Figure 3b). Although rPDGF-BB application improved the granulation tissue in diabetic wounds at 10 days, no statistically significant improvements could be demonstrated in the degree of wound closure at 10 days (Table 2).

Table 2. *The Effects of 5 Days of rPDGF-BB Treatment on Diabetic Wounds Evaluated at 10 Days*

Treatment*	Histologic score†	% Wound closure‡	N
Vehicle	4.3 ± 0.4	35.8 ± 2.9	20
1 μg rPDGF-BB	6.7 ± 0.6§	46.3 ± 5.6	13
10 μg rPDGF-BB	7.9 ± 0.3§	39.0 ± 3.3	8

* Full-thickness skin wounds on diabetic mice were treated with vehicle or rPDGF-BB (1 or 10 μg/day) for 5 days.

† Histology scores were assigned to each coded specimen by two investigators using the scale in Table 1. Values are expressed as mean ± SEM.

‡ Values are expressed as mean ± SEM. There were no significant differences between groups.

§ $P < 0.05$ when compared to vehicle treatment by ranking the histologic specimens and determining statistical significance using the Kruskal–Wallis test with individual comparisons performed using Dunn's procedure.

Topical application of rPDGF-BB (10 μg/day), rbFGF (1 μg/day), or a combination of the two growth factors (10 μg/day rPDGF-BB and 1 μg/day rbFGF) for 5 days produced more erythema and thicker-appearing granulation tissue at 10 days than did vehicle alone. Histologic evaluation confirmed these observations. There was a greater degree of cellular infiltration and capillary ingrowth in the growth factor-treated wounds (Table 3). The combi-

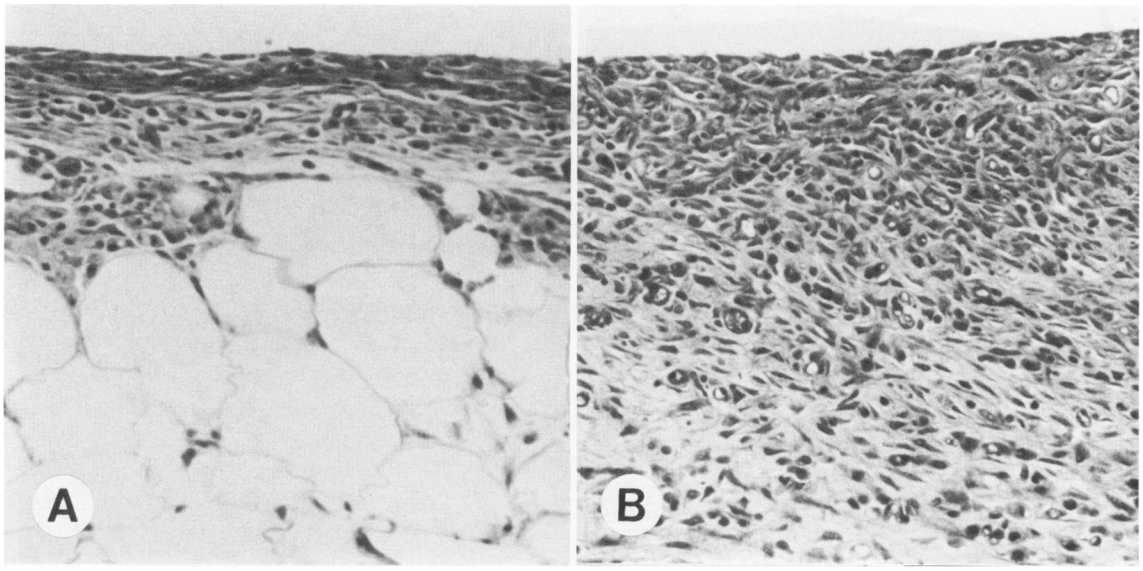

Figure 3. *Photomicrographs of midsections of diabetic wounds at day 10 (H&E, original magnification 33×).* a: *Diabetic wound treated with vehicle for 5 days: The wound treated only with vehicle has minimal cellular invasion and granulation tissue formation.* b: *Diabetic wound treated with rPDGF-BB (10 μg/day) for 5 days: In contrast to vehicle treatment, application of rPDGF-BB markedly increased the thickness, cellularity and vascularity of the new granulation tissue.*

nation of rPDGF-BB and rbFGF did not improve granulation tissue formation beyond that of the individual growth factors. Despite the clear differences in gross and histologic appearance in the growth factor-treated animals, there were no statistically significant improvements in wound closure in the growth factor-treated animals when compared with vehicle-treated animals for 10-day wounds (Table 3).

Experiments were also performed comparing the effects of 5 and 10 days of treatment with 10 μg of rPDGF-BB per day. At 10 days, both groups treated with rPDGF-BB had statistically significant improvements in granulation tissue formation beyond that of the vehicle-treated wounds (Table 4). Animals treated with rPDGF-BB for 10 days exhibited a trend toward a decrease in their wound areas when compared to vehicle-treated mice, but this difference lacked statistical significance (Table 4).

By gross observation, many of the wounds developed a distinct red color at 4 to 5 days. After breaking the code we found that only those animals treated with growth factors developed this erythema. To try to correlate the gross appearance of the wounds with histologic findings, wounds were treated with vehicle or rPDGF-BB (10 μg/day) for up to 5 days and animals werer killed at 3, 5, or 7 days. At 3 days there were no differences between the two treatment groups. By 5 days there were more in-

Table 3. *The Effects of 5 Days of Treatment with rPDGF-BB and/or rbFGF on Diabetic Wounds Evaluated at 10 Days*

Treatment	Histologic score†	% Wound closure‡	N
Vehicle	2.7 ± 0.3	27.3 ± 3.1	5
10 μg rPDGF-BB	6.2 ± 1.0	41.5 ± 4.8	6
1 μg rbFGF	7.4 ± 0.5§	48.5 ± 6.5	5
rPDGF-BB/rbFGF	7.8 ± 0.7§	48.3 ± 7.0	6

* Full-thickness skin wounds on diabetic mice were treated with vehicle, rPDGF-BB (10 μg/day), rbFGF (1 μg/day), or a combination of both for 5 days.

† Histology scores were assigned to each coded specimen by two investigators using the scale in Table 1. Values are expressed as mean ± SEM.

‡ Values are expressed as mean ± SEM. There were no significant differences between groups.

§ $P < 0.05$ when compared to vehicle treatment by ranking the histologic specimens and determining statistical significance using the Kruskal–Wallis test with individual comparisons performed using Dunn's procedure.

Table 4. *The Effects of 5 or 10 Days of Treatment with rPDGF-BB (10 μg/day) on Diabetic Wounds Evaluated at 10 Days*

Treatment*	Histologic score†	% Wound closure‡	N
Vehicle: 5 days	3.5 ± 0.3	24.4 ± 4.2	6
Vehicle: 10 days	4.5 ± 0.5	24.9 ± 4.9	4
rPDGF-BB: 5 days	7.3 ± 0.5§	34.6 ± 3.4	10
rPDGF-BB: 10 days	9.1 ± 0.1§‖	51.9 ± 2.7	5

* Full-thickness skin wounds on diabetic mice were treated with vehicle or rPDGF-BB (10 μg/day) for 5 or 10 days.

† Histology scores were assigned to each coded specimen by two investigators using the scale in Table 1. Values are expressed as mean ± SEM.

‡ Values are expressed as mean ± SEM. There were no significant differences between groups.

§ $P < 0.05$ when compared to vehicle treatment for 5 days by ranking the histologic specimens and determining statistical significance using the Kruskal–Wallis test with individual comparisons performed using Dunn's procedure.

‖ $P < 0.05$ when compared to vehicle treatment for 10 days by ranking the histologic specimens and determining statistical significance using the Kruskal–Wallis test with individual comparisons performed using Dunn's procedure.

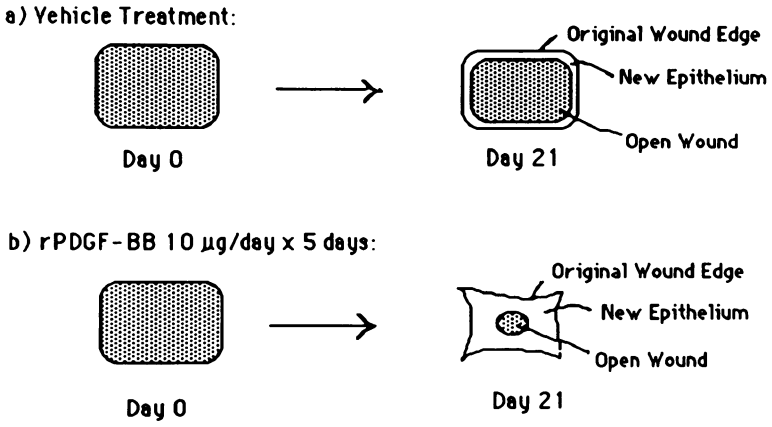

Figure 4. *Schematic representations of 21 day diabetic wounds show the marked difference in the extent of wound closure after treatment with growth factors.* a: *A schematic of a 21-day wound treated with vehicle. A large portion of the wound remains open.* b: *Treatment with rPDGF-BB (10 µg/day) for 5 days. There is significant re-epithelialization.*

flammatory cells present in the wounds treated with rPDGF-BB than in those treated with the vehicle alone. The rPDGF-BB–treated wounds appeared reddened and more granular than did those treated with vehicle. Marked differences were seen by 7 days. At this time, a thick, vascular granulation tissue was observed in the animals treated with rPDGF-BB, whereas vehicle-treated wounds showed little evidence of healing, resembling the wound bed immediately after wounding. The erythema observed grossly correlated with the histologic finding of early capillary infiltration into the wound. The vehicle-treated wounds had little evidence of inflammation or capillary ingrowth in the first week.

The studies of the first 10 days of healing demonstrated that rPDGF-BB and rbFGF can accelerate the rate of granulation tissue formation, but the growth factors had no significant effect on wound closure at 10 days. To determine whether the improvement in granulation tissue formation would lead to an increase in wound closure, studies were extended to 21 days. Diabetic animals were treated with rPDGF-BB (10 µg/day), rbFGF (1 µg/day), or a combination of the two for 5 days. On gross inspection, wounds treated with any of the growth factor regimens appeared to have closed more than those receiving only the vehicle. A schematic of wounds treated with rPDGF-BB and the vehicle illustrate the changes in wound areas from the day of wounding to day 21 (Figure 4a and b). Wound area measurements (Table 5) confirmed that the 21-day wounds had a significantly greater wound closure after treatment with any of the growth factor regimens when compared to application of vehicle alone ($P < 0.05$ by ANOVA, multiple comparisons by Tukey's procedure). By 21 days, application of either growth factor resulted in 80% to 90% reduction in open wound area, whereas vehicle-treated wounds had only a 50% reduction. The amount of wound closure that could be attributed to contraction did not change with growth factor treatment. Wounds treated with either growth factor alone or the combination had significantly improved histologic rankings at 21 days when compared with those wounds treated with the vehicle alone (Table 5). As in the 10-day study, the improved histologic scores correlated with much thicker and more cellular granulation tissue formation (Figure 5). In addition, histologic analysis at 21 days confirmed that growth factor-treated wounds had greater epithelial coverage of the new granulation tissue. Combining the two growth factors failed to improve any of the healing parameters beyond that of the individual growth factors.

To further improve the healing response to rPDGF-BB, we tested the effects of treating the wounds for different periods of time. Wounds treated with 10 µg rPDGF-BB/day for 10 days had a statistically significant improvement in histologic appearance and percentage of wound closure at 21 days relative to vehicle-treated animals (Table 6). However, the apparent improvement seen after treatment with 1 µg rPDGF-BB/day for 10 days did not reach statistical significance. In separate experiments, diabetic wounds were treated with 10 µg rPDGF-BB/day for 5, 10, or 14 days after wounding. Treatment with rPDGF-BB for any of the time periods resulted in significant improve-

Table 5. *The Effects of 5 Days of Treatment with rPDGF-BB and/or rbFGF on Diabetic Wounds Evaluated at 21 days*

Treatment*	Histologic score†	% Wound closure‡	N
Vehicle	5.1 ± 0.5	48.0 ± 5.1	17
10 µg rPDGF-BB	9.7 ± 0.7§	90.6 ± 5.5‖	9
1 µg rbFGF	8.2 ± 0.4§	79.5 ± 3.7‖	16
rPDGF-BB/rbFGF	8.0 ± 0.7§	78.7 ± 5.7‖	8

* Full-thickness skin wounds on diabetic mice were treated with vehicle, rPDGF-BB (10 µg/day), rbFGF (1 µg/day), or a combination of both for 5 days.

† Histology scores were assigned to each coded specimen by two investigators using the scale in Table 1. Values are expressed as mean ± SEM.

‡ Values are expressed as mean ± SEM.

§ $P < 0.05$ when compared to vehicle treatment by ranking the histologic specimens and determining statistical significance using the Kruskal–Wallis test with individual comparisons performed using Dunn's procedure.

‖ $P < 0.05$ when compared to vehicle treatment by ANOVA and individual comparisons by Tukey's procedure.

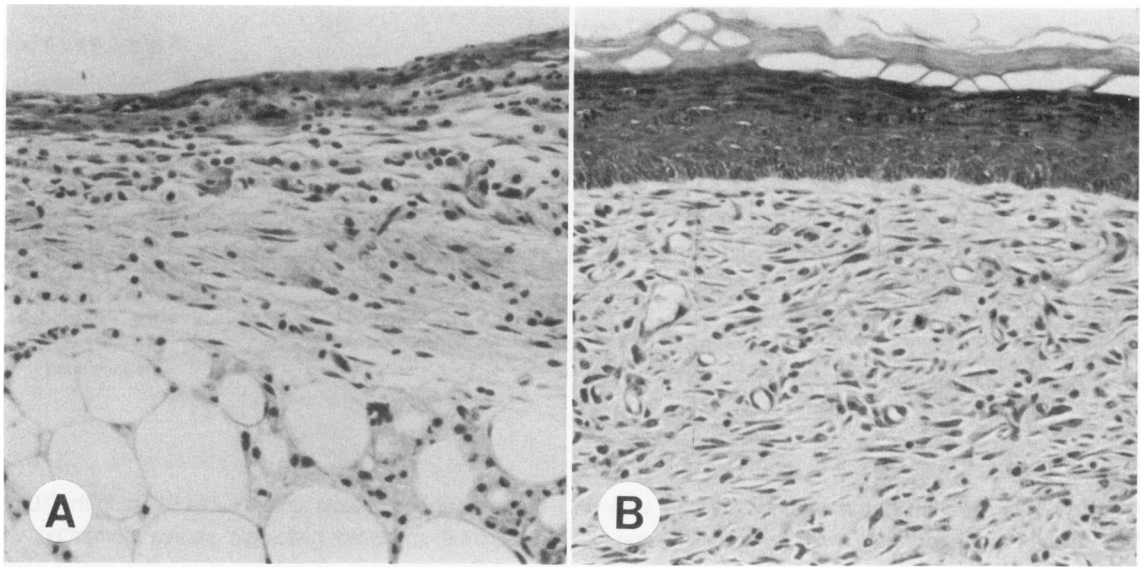

Figure 5. *Photomicrographs of midsections of diabetic wounds at day 21 (H&E, original magnification 33×).* **a:** *Diabetic wounds treated with vehicle for 5 days: Treatment with vehicle alone resulted in wounds with only moderate granulation tissue accumulation.* **b:** *Diabetic wounds treated with rPDGF-BB (10 μg/day) for 5 days: Thicker, more cellular and vascular granulation tissue is seen after treatment with rPDGF-BB. Complete re-epithelialization has occurred, closing the wound.*

ments in wound closure in 21-day diabetic wounds when compared to the appropriate vehicle controls (Figure 6). Interestingly, there was no difference in the response to rPDGF-BB, regardless of the duration of treatment. This suggests that treatment with a short course of rPDGF-BB is as effective in reversing the healing impairment as is treatment for a longer duration. The stimulatory effects of rPDGF-BB would thus appear to act in the early stages of the healing process.

Effects of rPDGF-BB and rbFGF on Wound Healing in Nondiabetic Animals

In contrast to the diabetic animals, application of different doses of rPDGF-BB (1 or 10 μg/day) and/or rbFGF (0.4 or 1.0 μg/day) for 5 consecutive days did not produce any appreciable improvement in wound closure or histologic appearance in their nondiabetic littermates when evaluated at 10 days (data not shown). Similar rates of closure were observed in animals treated with any combination of the growth factors or the vehicle alone. Their wounds closed within 10 to 14 days regardless of whether growth factors were applied.

Table 6. *The Effects of 10 Days of rPDGF-BB Treatment on Diabetic Wounds Evaluated at 21 Days*

Treatment*	Histologic score†	% Wound closure‡	N
Vehicle	4.7 ± 1.1	47.7 ± 12.7	5
1 μg rPDGF-BB	9.6 ± 0.5	76.9 ± 8.5	6
10 μg rPDGF-BB	10.5 ± 0.8§	88.2 ± 5.4‖	7

* Full-thickness skin wounds on diabetic mice were treated with vehicle or rPDGF-BB (1 or 10 μg/day) for 10 days.

† Histology scores were assigned to each coded specimen by two investigators using the scale in Table 1. Values are expressed as mean ± SEM.

‡ Values are expressed as mean ± SEM.

§ $P < 0.05$ when compared to vehicle treatment by ranking the histologic specimens and determining statistical significance using the Kruskal–Wallis test with individual comparisons performed using Dunn's procedure.

‖ $P < 0.05$ when compared to vehicle treatment by ANOVA and individual comparisons by Tukey's procedure.

Discussion

Wound Healing in the db/db Mouse

The full-thickness wound in the C57BL/KsJ-db/db diabetic mouse results in a clinically relevant and reproducible model of impaired wound healing. The db/db mice provide an excellent analogy to human diabetes. They are obese and develop hyperglycemia that is resistant to insulin. Complications seen in human diabetes such as peripheral neuropathy, microvascular lesions, basement membrane thickening, glomerular filtration abnormalities, immune complex deposition, and immunodeficiency have all been observed in the db/db mice.[52] These metabolic similarities, together with our observations that wounds in these mice exhibit a marked delay in cellular infiltration, granulation tissue formation, and time required for wound closure suggest that healing in this animal may be relevant to the healing impairment seen in human diabetes.

Much of the previously published work on impairment of wound healing in diabetic animals was performed using

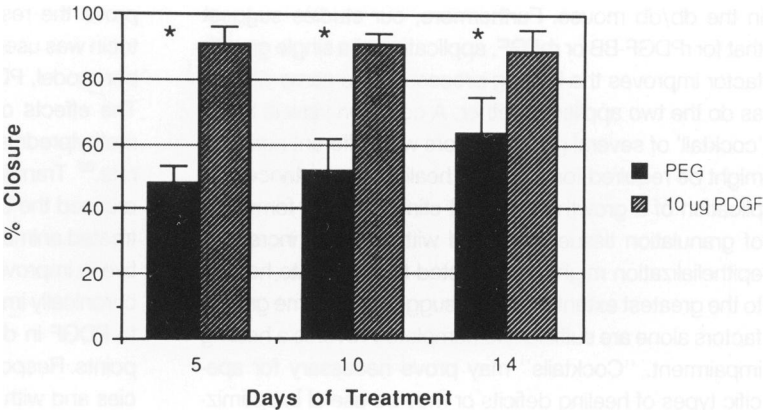

Figure 6. *Effect of duration of treatment on wound closure: Treatment with rPDGF-BB (10 μg/day) for 5, 10, or 14 days after wounding significantly improved the extent of wound closure in 21-day diabetic wounds when compared with the corresponding treatment with vehicle alone (Bars represent mean ± SE and * indicates* $P < 0.05$ *by ANOVA and multiple comparisons by the Tukey's procedure,* $n = 6–16$*). There were no differences amongst the wounds treated with different time courses of the PEG vehicle or between any of the rPDGF-BB regimens.*

the cytotoxic drugs alloxan or streptozotocin to induce the diabetic state. These treatments result in decreases in collagen synthesis in subcutaneous chambers and in tensile strength in incisions.[54–57] A confounding factor in streptozotocin-induced diabetes is that the drug is toxic not only to pancreatic beta cells but also to the liver, renal tubules, and exocrine pancreas.[58] In addition, alloxan and streptozotocin alter macrophage phagocytosis and depress T-cell function.[59] We chose not to use a cell toxin to induce diabetes because of these potential confounding side effects.

The healing impairment in the C57BL/KsJ-db/db diabetic mice is at least partially due to the marked delay in cellular infiltration into the wound space. Wounds in these animals are almost devoid of cells for several days after injury. Once the cells responsible for initiating the healing response arrive in the wound, an apparently normal granulation tissue forms. The reason for the delayed cellular infiltration in these wounds is not yet known. One possibility is that the cells involved in wound repair may not function normally in the db/db mice. The obesity of the mice also may be a factor. The increased adipose tissue could restrict the ability of the skin to contract or form a diffusion barrier that might affect recruitment of cells to the wound and increase the distance cells must migrate to reach the wound.

Goodson and Hunt have examined wound healing in a different strain of genetically diabetic mice. They made full-thickness wounds in the backs of C57BL/6J-ob/ob (ob/ob) obese mice and demonstrated a delay in the closure rate.[60,61] Experiments with diet restriction and insulin treatment suggested that part of the wound healing impairment (as measured by decreased collegen accumulation) was a complication of obesity rather than glucose regulation.[61] The differences between the ob/ob and db/db mice for studies of impaired wound healing remain to be determined because impairments occur despite some significant differences in the two strains of mice. The mutations underlying the defects in the two strains of mice are on different chromosomes.[43,52] The patterns of hyperglycemia and hyperinsulinemia vary with age and are different in each strain. In general, the degree of hyperglycemia is much greater in the db/db mice (serum glucose values averaging 900 mg/dl) than in the ob/ob mice (serum glucose average 200 mg/dl[61]). Comparisons between these strains of mice in future wound healing studies may provide useful information about the mechanisms by which growth factors act to stimulate the tissue repair process.

PDGF and FGF Stimulate Healing in db/db Mice

Both of the growth factors used in the present study have been shown to stimulate several *in vitro* activities related to healing. Platelet-derived growth factor is both a chemoattractant[4,5] and a mitogen[10] for fibroblasts and smooth muscle cells. Platelet-derived growth factor can also increase the synthesis of collagen as well as collagenase and proteoglycans,[20,22] potentially affecting the balance of extracellular matrix production and degradation. Basic FGF can attract and stimulate the growth of fibroblasts and endothelial cells,[8,9,11,12] is an angiogenic factor,[14] and increases the synthesis of collagenase.[21] In our studies, treatment with human recombinant PDGF-BB, bFGF, or a combination of both increased the rate of cellular infiltration and capillary ingrowth into the wounds of db/db mice. These *in vivo* effects presumably result from direct or indirect chemotactic and mitogenic effects of the growth factors. As a result, a thicker and more cellular granulation tissue was formed than in the vehicle-treated animals. The granulation tissue induced in the treated animals created an adequate bed for rapid re-epithelialization in the full-thickness wounds. While the role of wound contraction in wound closure in the diabetic mice is difficult to assess precisely, the differences observed after treatment with growth factors were predominantly the result of differences in granulation tissue and re-epithelialization. No obvious differences in wound contraction were observed.

Our results demonstrate that treatment with a single growth factor, ie, PDGF, can provoke more rapid healing

in the db/db mouse. Furthermore, our studies suggest that for rPDGF-BB or rbFGF, application of a single growth factor improves the healing process to the same degree as do the two applied together. A common view is that a 'cocktail' of several growth factors with different activities might be required for complete healing. For instance, application of a growth factor that stimulates the formation of granulation tissue combined with one that increases epithelialization might be expected to accelerate healing to the greatest extent. Our data suggest that some growth factors alone are sufficient to completely reverse a healing impairment. "Cocktails" may prove necessary for specific types of healing deficits or may be useful in optimizing healing responses, but in the db/db mouse a beneficial response can clearly be achieved with a single factor. Similar *in vivo* responses to growth factors that have a different spectrum of *in vitro* activities might be explained if each factor were to initiate or affect a common pathway leading to healing. Further understanding of the mechanism of healing impairment in the db/db mouse and how growth factors reverse this defect may aid in understanding clinical problems of healing in human diabetes.

Application of rPDGF-BB or bFGF for a relatively brief period (5 days) had the same effect as did application for 10 or 14 days. Apparently chemotactic and mitogenic stimulation by PDGF at the early stages of healing triggers the repair process, allowing a return to a more normal rate. Extending the growth factor treatment beyond this early time provides no additional improvement of healing. As a corollary, this finding suggests that in the db/db mice the healing deficit is related to the early, cellular phase of wound repair. Once the cells arrive in the wound, healing may proceed without additional significant delay.

While rbFGF appeared to be more effective at a lower dose than rPDGF-BB in these experiments, we are cautious about interpreting this as a difference in potency between the factors. Rigorous dose-response studies have not been performed with these growth factors in this model. The doses used were based on reports from other laboratories[37,38,40,41] and our own preliminary dose-response studies. In addition, the pharmacokinetics of the factors in the wound space are not known. Differential binding to extracellular matrix components or different susceptibility to degradation could affect the concentration of active growth factor available to stimulate a response. PDGF and bFGF, with different molecular weights, isoelectric points, and binding proteins probably have different pharmacokinetics in the wounds.

Other investigators have examined the effects of growth factors in different impaired wound healing models. In two animal models, conflicting results have been reported when looking at changes in cellularity and collagen accumulation in subcutaneous chambers. When the chemotherapeutic agent doxorubicin was used to impair granulation tissue accumulation, PDGF did not improve the response, but TGF-β did.[34] When streptozotocin was used to impair healing in a subcutaneous chamber model, PDGF restored responses to normal levels.[35] The effects of TGF-β and PDGF have been studied in methylprednisolone-impaired healing of linear incisions in rats.[62] Transforming growth factor β but not PDGF increased the breaking strength of incisions in the steroid-treated animals. In our studies, rPDGF-BB was very effective in improving healing in genetically diabetic mice with chronically impaired wounds. The variability of responses to PDGF in different studies highlights several important points. Responses to a given growth factor vary with species and with the underlying cause of the healing impairment. A single factor may not be effective in improving all types of impaired healing. Animals with impaired healing induced by drugs may be more prone to variation in the degree of impairment between individuals than animals in which the impairment has a genetic basis. There may be advantages to comparing different factors in the same model in the future.

In vitro studies have provided insight into the actions of growth factors under relatively simple and controlled conditions, suggesting the possible usefulness of such factors in tissue repair. In a wound, the interactions among the various cell types, the extracellular matrix, and the many cytokines present are complex and still incompletely understood, so the growth factors may not behave as predicted from *in vitro* studies. In addition, healthy organisms heal wounds so rapidly that changes in healing can be hard to measure. Models of impaired wound healing present opportunities to see pronounced growth factor effects and offer the potential to explore the mechanisms by which the factors act.

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Acknowledgments

The authors thank their colleagues in the yeast expression, protein chemistry, and bioassay groups at ZymoGenetics for production and characterization of the recombinant growth factors used herein, Paul Goodwin and Megan Lantry for their able technical assistance, and Margo Rogers for preparing the manuscript. Thanks also go to the laboratory of Daniel Porte Jr., M.D. for performing the insulin radioimmunoassays. Finally, the authors appreciate the helpful discussions with Drs. David Heimbach and Joe Leibovich. DGG was supported in part by the Northwest Burn Foundation.