Acidic Fibroblast Growth Factor: Evaluation of Topical Formulations in a Diabetic Mouse Wound Healing Model

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The efficacy of topical formulations of acidic fibroblast growth factor (aFGF) in healing of full-thickness wounds has been studied in a diabetic db+/db+ mouse model. The effect of several formulation variables, dose, and application frequency was examined. It was found that wound healing in diabetic animals treated with aFGF or placebo was slower than in their nondiabetic littermates. The availability of aFGF from the viscous vehicle employed in this study (1% hydroxyethyl cellulose) was demonstrated *in vitro* using diffusion cells. The viscous formulation of aFGF was equally effective in wound healing as a nonviscous formulation in phosphate-buffered saline. A formulation containing heparin (necessary for full biological and conformational stability of aFGF) at a mass ratio of 3:1 to aFGF was more efficacious than formulations with lower heparin: aFGF ratios. Wounds treated with three doses of 3.0 μg/cm^2 aFGF healed faster than those treated with a single dose of 3.0 μg/cm^2 aFGF. Three applications of 3.0 or 0.6 μg/cm^2 aFGF were equally effective in accelerating wound healing.

KEY WORDS: acidic fibroblast growth factor; wound healing; diabetic mouse; topical formulations.

INTRODUCTION

Acidic fibroblast growth factor (aFGF),[6] a 15.9-kDa protein is one of the growth factors currently under intense investigation for their potential in wound healing (1–5). Common properties of these proteins are stimulation of mitogenesis, angiogenesis, cell proliferation, and differentiation. Several papers reported acceleration of healing by epidermal growth factor (6,7), basic FGF (8–12), platelet-derived growth factor (PDGF) (13), and transforming growth factor-beta (12,14,15). The effectiveness of aFGF in the healing of full-thickness dermal wounds has also been demonstrated in the normal mouse and rat (16).

The purpose of this study was to evaluate *in vivo* the influence of formulation components on growth factor efficacy by utilizing an animal model of impaired healing, i.e., the db+/db+ diabetic mouse. Several recent publications reported delayed healing in these animals, which may be relevant to impaired healing observed in humans with diabetes mellitus (8,10,11,13,17). The starting point for these investigations was a topical formulation of aFGF developed previously based on *in vitro* data (18) and a nonviscous composition evaluated recently *in vivo* (16). This formulation consisted of a solution of aFGF in 1% hydroxyethyl cellulose (HEC; a viscosity enhancing agent) in phosphate-buffered saline (PBS), containing heparin:aFGF at a mass ratio of 3:1. An additional objective of this study was to compare different application regimens for treatment of wounds with aFGF.

MATERIALS AND METHODS

Genetically diabetic female C57BL/KsJ db+/db+ mice and their nondiabetic heterozygous littermates (db+/+m) were received from Jackson Laboratories (Bar Harbor, ME). The mice ranged in age from 6 to 8 weeks upon arrival and were 8 to 12 weeks old at the beginning of the study. Animals were housed a minimum of 2 weeks prior to use, and fasted serum glucose levels were determined a week before initiation of the study. Blood was withdrawn from the retroorbital plexus via heparinized capillary tubes. Serum was analyzed for glucose content using a Beckman Glucose Autoanalyzer (Beckman Instruments, Towson, MD). Treatment groups (6–11 animals) were balanced according to their blood glucose levels. Animals whose glucose levels were lower than 200 mg/dL were not considered diabetic and not included in the study. Mice were housed individually in microisolator cages (Lab Products, Maywood, NJ); positions of cages in laminar airflow Carworth units (Becton, Dickinson & Co., New York) were randomized. All manipulations were performed under aseptic conditions. All animals were cared for and used in accordance with rules and guidance of the Merck Research Laboratories Institutional Animal Care and Use Committee and the *Guide for the Care and Use of Laboratory Animals* [DHHS Publication No. (NIH) 85-23, revised 1985].

Growth Factor Formulation

Human recombinant aFGF (>99% purity) was isolated (Merck Research Laboratories, West Point, PA) from transformed *Escherichia coli* cells (19,20) with the final isolation steps described elsewhere in detail (21,22). Formulations of aFGF were assayed for protein mass by size-exclusion HPLC (17). Biological activity of aFGF was assessed by its ability to stimulate DNA synthesis in the Balb/C 3T3 fibroblast cell line (23). Except where specified, aFGF was formulated in 1% HEC (52,000H, Union Carbide, Danbury, CT) dissolved in PBS (Merck Research Laboratory, West Point, PA), pH 7.2, and contained bovine heparin (MW ~16 kDa, Hepar Industries, Inc., Franklin, OH):aFGF at a mass ratio

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[6] *Abbreviations used:* aFGF, acidic fibroblast growth factor; db+/db+, diabetic mice; db+/+m, nondiabetic mice; HEC, hydroxyethyl cellulose; HT90, time to 90% wound reduction; PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor.

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of 3:1. Sterile formulations stored at 4°C have maintained stability for over 1 year (18).

Wounding and Wound Care

The animals were anesthetized by i.m. injection of a diluted mixture of ketamine (Ketaset, 140 mg/kg; Aveco Co., Fort Dodge, IA) and xylazine (Anased, 20 mg/kg; Lloyd Laboratories, Shenendoah, IA). The hair on the back was clipped and the skin washed with 70% ethanol solution and dried with sterile gauze. The wound area was marked in the middorsal area with a glass culture tube (13-mm diameter) dipped in Betadine solution (Purdue Frederick Co., Norwalk, CT). A 2-cm^2 full-thickness wound was created by blunt excision with scissors. Following application of the dose (12 $\mu L/cm^2$), wounds were protected with Bioclusive dressing (Johnson & Johnson Products Inc., New Brunswick, NJ). Wound perimeters were traced in triplicate onto sterile microscope slides on the day of wounding and every 3–4 days postwounding prior to replacement of dressing. Dressings were changed on each observation day, and wounds were also photographed to record the progress in healing. Wounds were visually examined and considered healed if moist granulation tissue was no longer visible and the wound was covered by a continuous layer of epithelium. Healed animals were fasted overnight and blood withdrawn the following day for glucose determination. The animals were then sacrificed and wound areas, with surrounding skin, were excised and fixed in formalin solution for histological evaluation. The studies were terminated on either day 23, day 24, or day 31 postwounding depending on the protocol employed. Any nonhealed animals were sacrificed (1 day after the last measuring day) and skin samples prepared as described previously.

Measurement of Wound Area and Contraction

Wound areas were measured from microscope slide tracings in triplicate by an image analysis system designed specifically for our use. Contraction of wounds during the course of the study was measured for each animal on every observation day from enlarged photographs enlarged by ~10 fold. Contraction was estimated by measuring the differences between the initial wound area (on day 0) and the area of the wound site on a given day postwounding (healed plus unhealed area), both normalized for the initial wound size.

Tissue Preservation, Preparation for Histological Evaluation, and Histologic Evaluation

At the time the animals were sacrificed, the wound sites and surrounding normal skin down to subcutis were removed, placed on cardboard, covered with an empty teabag (UPI Southern Tea Co., Lansdale, PA) that was stapled in place, and immersed in 10% neutral buffered formalin. Standard 6-μm hematoxylin-and-eosin-stained sections of the center of each wound were prepared for histologic evaluation. In addition, an avidin–biotin–peroxidase complex immunoperoxidase procedure using *Bandeirea simplicifolia* isolectin B-4 binding as a marker of vascular endothelium was performed on 5-μm sections of each wound. This was a modification of a previously published technique by Alroy *et al.* (26) using the more specific B-4 isolectin of BS-1 lectin (16).

A "blinded" comparative microscopic evaluation was performed on all histologic preparations. Individual sites were compared for such factors as approximate dermal and epidermal volume, degree of cellular infiltrate, density of new vessel growth, and degree of wound coverage by new epidermis.

In Vitro Release of aFGF from a Viscous Vehicle

Release of aFGF from viscous formulation composed of 0.2 mg aFGF, 0.6 mg heparin in 200 μL of 1% HEC was measured employing diffusion cells (24) thermostated at 32°C. A viscous solution was evenly spread on a polycarbonate membrane with a 0.4-μm pore diameter (Nucleopore, Pleasanton, CA). Samples (1 mL) were withdrawn from the 13-mL receptor phase (PBS containing 0.6 mg/mL heparin) at 1, 3, 5, 7, and 24 hr and volume replaced after each sample. Permeation of aFGF was quantitated in the receptor fluid by size-exclusion chromatography/HPLC and permeation of HEC was determined by a colorimetric assay (25).

Statistical Analyses

Average aFGF- and placebo-treated wound areas at fixed follow-up times were compared using *t* tests. Time to 90% reduction of wound area (HT90) was analyzed by log-rank tests (27) since animals which did not achieve a 90% reduction in wound size were censored. HT75 and HT50 values were calculated but found to be less discriminating in assessing differences among parameters tested. All *P* values were two-sided.

RESULTS AND DISCUSSION

First, the utility of diabetic mice to examine the efficacy of aFGF *in vivo* was evaluated. The rates of healing of wounds treated with a single dose of 3.0 $\mu g/cm^2$ aFGF containing 9.0 $\mu g/cm^2$ heparin in 1% HEC or matching placebo (9.0 $\mu g/cm^2$ heparin in 1% HEC) in db+/db+ mice and nondiabetic db+/+m mice were compared. In Fig. 1, the decreases in mean wound areas (normalized for their initial size) as a function of time are illustrated. Results indicate that wounds in nondiabetic db+/+m animals treated with either aFGF or placebo were healing much faster than in corresponding db+/db+ animals. Diabetic mice treated with aFGF formulation were the only group with wounds not expanding on day 3 postwounding. A similar effect was previously reported by Kleinberg *et al.* (10) in db+/db+ mice treated with bFGF and was attributed to stimulation of myofibroblasts (that contribute to contraction of wounds) by bFGF. The enlargement of placebo-treated db+/db+ wounds compared to nonexpanding aFGF-treated wounds was marginally statistically significant ($P = 0.053$). For the db+/+m mice, differences in area of aFGF- versus placebo-treated wounds were not statistically significant at any time. In db+/db+ animals, these differences were significant starting from day 16 postwounding ($P < 0.01$). Assessment of complete closure of wounds revealed that at the conclusion of the study (day 23), 66% of the aFGF-treated db+/

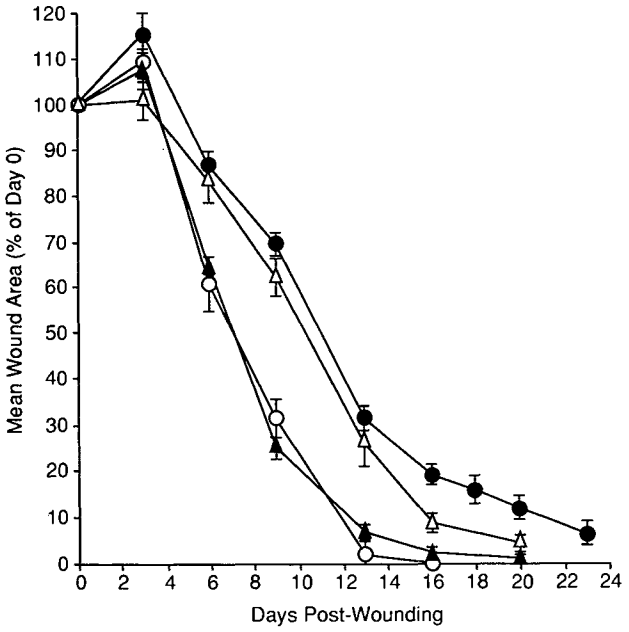

Fig. 1. Time course of wound healing in diabetic (db+/db+) and nondiabetic (db+/+m) mice treated with a single dose of 3.0 μg/cm² aFGF in 1% HEC containing 9.0 μg/cm² heparin or placebo (9.0 μg/cm² heparin in 1% HEC). Values are presented as a percentage of the original wound area (mean ± SE). (●) db+/db+ treated with placebo (n = 8); (△) db+/db+ treated with aFGF (n = 9); (○) db+/+m treated with placebo (n = 6); (▲) db+/+m treated with aFGF (n = 6).

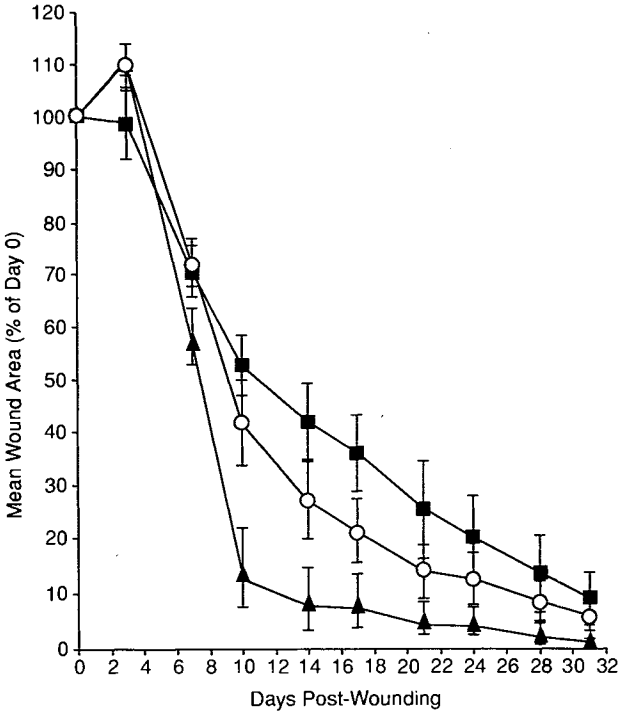

Fig. 2. Time course of wound healing in diabetic (db+/db+) mice treated with a single dose of 3.0 μg/cm² aFGF containing 9.0 μg/cm² heparin in 1% HEC (▲; n = 9), 3.0 μg/cm² aFGF containing 3.0 μg/cm² heparin in 1% HEC (○; n = 9), or 3.0 μg/cm² aFGF containing 1.0 μg/cm² heparin in 1% HEC (■; n = 8). Values are presented as a percent of the original wound area (mean ± SE).

db+ wounds were closed, while only 11% of the placebo-treated group were healed.

Progress in healing was additionally analyzed by time to 90% wound reduction (HT90). In diabetic mice the median HT90 for aFGF-treated animals was 15.4 days and was statistically different (P = 0.01) from that of placebo-treated animals, 20.7 days. The difference in healing was not significant in db+/+m mice: the median HT90 was 12.9 days for aFGF-treated and 12.5 days for placebo-treated mice.

Since aFGF requires heparin to maintain full biological activity and conformational stability (23,28), the optimal heparin concentration in the formulation was determined. It was shown recently (18) that due to the multivalent nature of aFGF–heparin complexes, almost-complete protection against heat-induced aggregation was achieved at mass ratios of heparin:aFGF as low as 1:3 (at 55°C); the complete protection at 55°C was achieved at a 3:1 mass ratio. The effects of a single dose of 3.0 μg/cm² aFGF formulated in 1% HEC containing heparin:aFGF at mass ratios of 3:1, 1:1, or 1:3 (i.e., 9.0, 3.0, and 1.0 μg/cm² heparin) on the rate of healing in db+/db+ mice are compared in Fig. 2, where for clarity the healing progress for corresponding placebo groups was not included. It is apparent that the rate of healing increases with the increase in heparin concentrations in aFGF formulations. It should be pointed out that the differences between the mean wound areas for aFGF- and those for placebo-treated wounds were significant starting from day 7 post-wounding for the highest heparin concentrations and were not significant over the course of the study for two lower heparin concentrations. Median HT90's were 9.8, 22.9, and 23.4 days for the aFGF formulations containing heparin and for the corresponding placebos were 13.4, 19.6, and 23.8 days (from the highest to the lowest heparin ratio, respectively). The formulation containing 3:1 heparin:aFGF gave significantly faster healing rates than the two others (P = 0.008 for 3:1 vs 1:1 and P = 0.001 for 3:1 vs 1:3) and was therefore used in subsequent experiments. The observed trend of a decrease in HT90's with an increase in heparin concentrations in placebo formulations (not significant) may reflect a stimulation effect of heparin on wound healing. Comparison of the numbers of totally healed wounds was in good agreement with the above findings: treatment of wounds with the formulation containing the highest ratio of heparin resulted in 56% of the wounds being healed at the end of the study, whereas only 33 and 17% of wounds treated with 1:1 and 1:3 ratios, respectively, were completely closed. Higher ratios of heparin were not investigated since an exploratory study at a 6:1 mass ratio of heparin to aFGF revealed excessive bleeding observed shortly (0.5–1 hr) post-application of either aFGF or placebo formulations.

The rate of healing of wounds treated with a single or multiple dose of 3.0 μg/cm² aFGF containing 9.0 μg/cm² heparin in 1% HEC was also investigated. It was found (data not shown) that three applications of that formulation were statistically more effective than a single dose and the differences between treatments were significant from day 7 post-wounding until the conclusion of the study at day 24. The difference in median HT90 was 1.5 days (P = 0.015). The differences between the mean size of aFGF- and that of pla-

cebo-treated wounds were statistically significant ($P < 0.05$) starting from day 10 postwounding for both treatments. Since treatment with three applications was found to be more efficacious, this dosing regimen was used in the subsequent experiments. It should be pointed out that in this and the following experiments, the initial wound expansion (occurring between day 0 and day 3) was not observed; gentle stretching of the skin surrounding freshly excised wounds eliminated this phenomenon.

The effect of aFGF concentration on healing was examined by comparing the rate of healing of wounds treated with three applications of 3.0 or 0.6 μg/cm^2 aFGF formulated in 1% HEC and containing heparin:aFGF at a ratio of 3:1. The rate of healing was similar for both aFGF concentrations over the course of the study (data not shown). Median HT90's for the higher and lower concentrations of aFGF were not statistically significantly different (14.0 and 13.3 days, respectively). Both aFGF-containing formulations were statistically more effective than their corresponding placebos starting from days 10 and 7 (for the higher and lower aFGF concentrations, respectively).

Figure 3 illustrates the effect of vehicle in which aFGF (3.0 μg/cm^2) was administered in either PBS (nonviscous vehicle) or 1% HEC (viscous vehicle). Progress of healing was nearly identical for both vehicles: the median HT90 was 12.5 days in PBS and 12.7 days in 1% HEC ($P > 0.1$), although data were less scattered in the viscous vehicle. The unhealed wounds were observed at the end of the study in animals treated with either PBS alone or heparin in PBS. The viscosity enhancing agent, HEC, was incorporated into the aFGF formulation to prevent or minimize runoff of the applied dose from the wound site. All formulations containing HEC were well tolerated by animals and no adverse reaction in wound sites was observed. The polymer appears to have a slightly positive effect on healing progress.

The release of aFGF from 1% HEC was demonstrated by measuring *in vitro* permeation of aFGF from 1% HEC containing heparin:aFGF at a 3:1 ratio. Results are shown in Fig. 4, where permeation of HEC (7–8% at 24 hr) is also illustrated. It was found that 97.5 ± 3.7% of aFGF applied to the cell was released from the formulation, indicating a lack of interaction between aFGF and HEC. The much slower rate of permeation of HEC indicated that aFGF present in the receiver side of the diffusion cell was not bound to the polymer.

Histologic evaluation was performed on wounds treated with 3.0 μg/cm^2 aFGF in 1% HEC/9.0 μg/cm^2 heparin versus those treated with a 1% HEC/heparin placebo formulation. There was a good correlation between the gross and the microscopic assessment of total closure of wounds. It was found that the new dermis present in aFGF-treated wounds was generally slightly thicker and more densely cellular than that present in placebo-treated wounds (Fig. 5). Since few of the wounds treated with the placebo formulation were healed, the amount of epidermis present was sparse in this group. Even though aFGF-treated sites had comparatively greater amounts of new connective tissue (dermis) deposition, the amount of tissue present was never excessive and the new skin organization (from epidermis to subcutis) was always normal. There was occasionally mixed inflammatory cellular infiltration in the new dermis and in the subcutis in close proximity to the wounds of aFGF- and placebo-treated animals. Immunoperoxidase staining of endothelial cells using a lectin binding technique also revealed a comparatively larger amount of new vessel formation in the new dermis of aFGF-treated wounds vs placebo-treated wounds (Fig. 6).

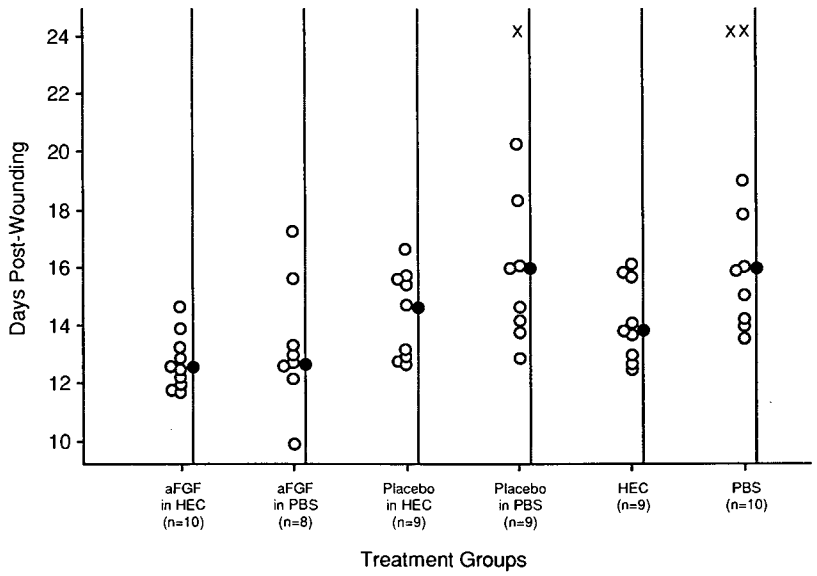

Fig. 3. Estimated HT90 for diabetic (db+/db+) mice treated with three doses (applied on day of wounding and days 3 and 7 postwounding) of 3.0 μg/cm^2 aFGF containing 9.0 μg/cm^2 heparin in either 1% HEC or PBS or corresponding placebos (9.0 μg/cm^2 heparin in 1% HEC or PBS) or vehicles (1% HEC or PBS). HT90 represents numbers of days necessary for 90% reduction of wound area. (○) One animal; (●) group median; (X) wound did not heal 90% by the end of the experiment.

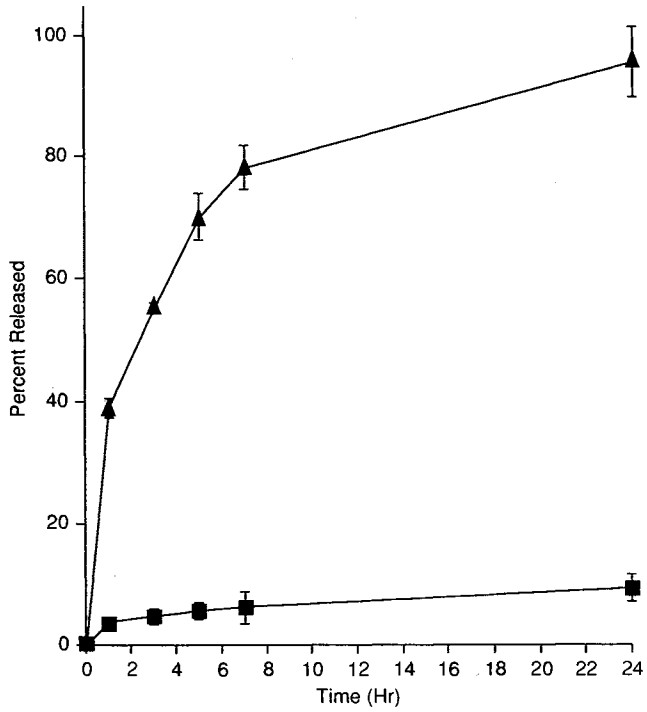

Fig. 4. *In vitro* release of aFGF from 1% HEC in the presence of heparin: 200 μL of solution containing 0.2 mg aFGF and 0.6 mg heparin in 1% HEC was applied to the diffusion cell. $n = 4$; mean ± SE. (▲) Cumulative percentage of aFGF in the receptor side; (■) cumulative percentage of HEC in the receptor side.

Wound contraction was evaluated for all formulations and treatment regimens investigated in diabetic mice. It was estimated that contraction contributed greatly to healing: circa 70% of the decrease in wound area by the end of the healing process was due to contraction. This number is higher than the 40% estimated (based on evaluation at one point time) previously by Greenhalgh *et al.* (13) in the same animal model following treatment with basic FGF and platelet-derived growth factor. Generally, however, no significant effect of treatment on contraction was observed in our studies (data not shown).

The effect of the initial glucose level was also investigated. Blood glucose was found to be surprisingly different between animals from the same shipment (from 160 to 1300 mg/dL). Despite significant differences in blood glucose levels, no relationship between the initial (or final) blood glucose level and the rate of healing was observed (data not shown).

Based on the literature and data reported in this paper, diabetic mice appear to be a sensitive animal model for wound healing studies. Similarly to previously reported data employing the same animal model for evaluating the effects of bFGF (8,10,11,13) and PDGF (13), this report confirmed the ability of aFGF to accelerate the healing process in a healing-impaired animal model. As recently reported by Mellin *et al.* (16), aFGF induces marked stimulation of angiogenesis, granulation tissue formation, and growth of new epithelium in healthy rodents. Acceleration of healing of 6-mm-diameter full-thickness wounds (measured by the ratio of the mean wound area of aFGF-treated wounds to that of placebo-treated wounds) following two applications daily of 0.25 μg bovine aFGF for 10 days (in PBS, containing 2 μg each of heparin and mouse serum albumin) was significant for 2 to 6 days postwounding in the mouse, reaching 1.8-"fold improvement" on day 4 postwounding. The 10 day treatment in rats with 1.0 μg (two 0.5-μg doses daily) of human aFGF in PBS containing a total of 3.0 μg heparin resulted in a maximal 1.8-fold improvement on day 8 postinjury. Stimulation of healing in db+/db+ animals by different growth factors has been demonstrated in several publications. Klingbeil *et al.* (11) established a threshold of 1 μg/cm^2

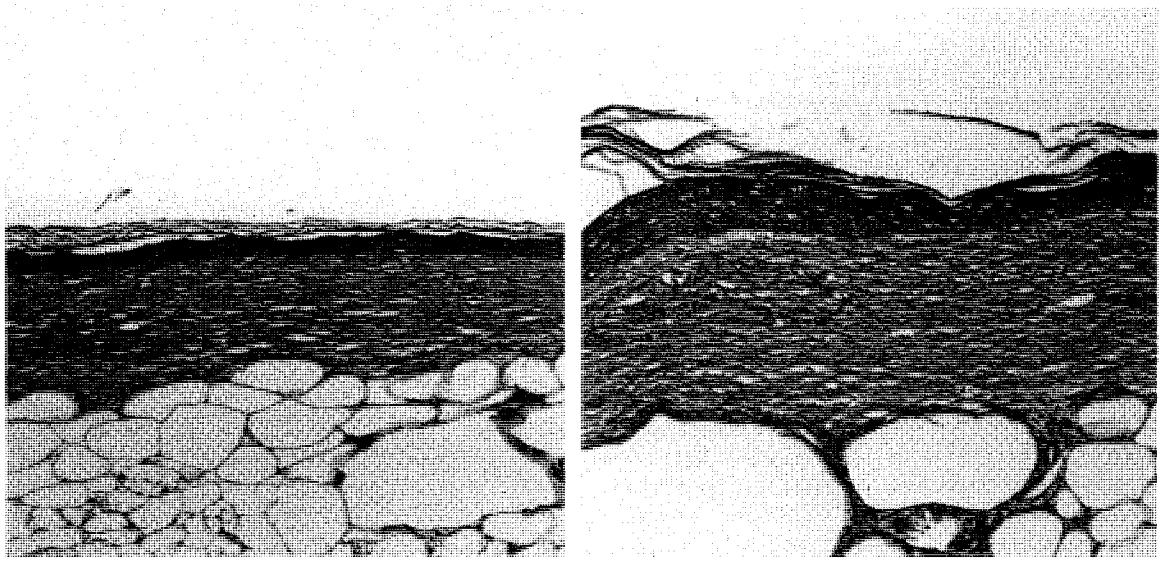

Fig. 5. Skin: Placebo-treated (left) vs aFGF-treated (right) wound sites (hematoxylin-and-eosin stained). Although the new dermis was generally thicker and more densely cellular in aFGF-treated wounds, the organization and proportions of the epidermal and dermal components were consistently within normal limits. 108.5×

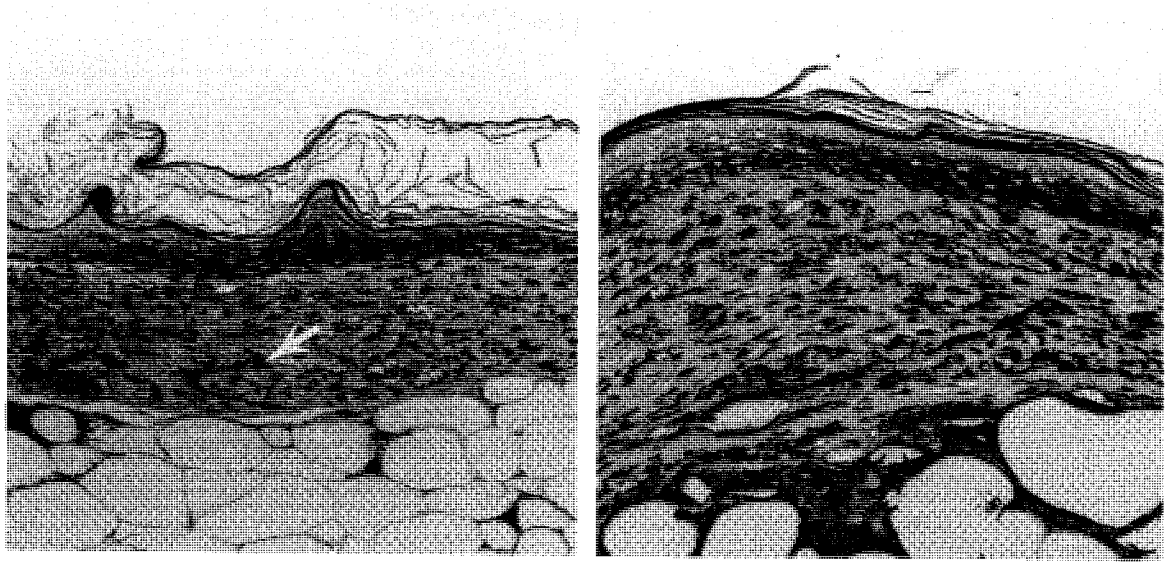

Fig. 6. Skin: Placebo-treated (left) vs aFGF-treated (right) wound sites (avidin–biotin–peroxidase complex immunoperoxidase stain using BS-1 lectin as a marker for vascular endothelium). New endothelial cells in the dermis are darkly stained (arrows). There was generally a greater density of new vessels in the dermis of aFGF-treated wounds compared to that of placebo-treated wounds. 108.5×

for effectiveness of a single dose of bFGF under the protocol which we followed in our studies. The bFGF was formulated in isotonic sodium acetate and contained no heparin. A minimum effective dose of 0.5 μg/0.28 cm^2 (6-mm-diameter biopsy) bFGF formulated in 1.5% carboxymethyl cellulose in sterilized PBS containing 0.5% mouse serum was reported by Tsuboi and Rifkin (8). Greenhalgh and coauthors (13) reported acceleration of healing by 1 μg/2.25 cm^2 (1.5 × 1.5-cm wound) bFGF applied daily for 5 or 14 days and also by 10 μg/2.25 cm^2 PDGF under the same protocol. Growth factors were formulated in 5% polyethylene glycol 8000 in PBS in this study The combination of both factors provided no additional improvement in healing. Generally, however, caution must be exercised in attempting to compare the healing potency of particular growth factors. The differences in formulation and/or protocols may likely influence results and make comparisons between them difficult.

The viscous formulations containing HEC employed in this report were well tolerated by all animals used in this study and adverse affects were not observed by either visual observations or histological examining (compared to untreated surrounding skin; data not shown).

It should be noted, however, that the response to the same treatment, i.e., three applications of a dose of 3.0 μg/cm^2 aFGF (used as a control in all experiments), was variable in experiments reported herein. A considerable effort was undertaken to identify the reason for these discrepancies, and no significant relationship between initial and final blood glucose level, animal weight, and age was found. Additional studies would be required to distinguish whether this is an intrinsic problem of the model or whether it is related to our protocol. Nevertheless, the difference in time to 90% would healing comparing aFGF with placebo from 2.1 to 7.8 days among these experiments was always statistically significant ($P < 0.05$).

CONCLUSIONS

The diabetic mouse model was shown to be a suitable model for measuring the relative efficacy of various formulations of aFGF. The formulation containing heparin:aFGF at a 3:1 ratio was the most efficacious *in vivo*. Although a full range of aFGF dose responses was not investigated, doses of 0.6 and 3.0 μg/cm^2 were shown equipotent. Three applications made over a 7-day period appear to stimulate healing to a greater extent than a single dose. The viscosity enhancing agent, HEC, did not interfere with the healing rate of the active formulation and, in placebo studies, slightly promoted healing compared to PBS alone.

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