Basic Fibroblast Growth Factor Stimulation of Epidermal Wound Healing in Pigs

Patricia A. Hebda, Corine K. Klingbeil, Judith A. Abraham, and John C. Fiddes
Department of Dermatology (PAH), University of Pittsburgh, School of Medicine, Pittsburgh, PA, and California Biotechnology, Inc. (CKK, JAA, JCF), Mountain View, CA, U.S.A.

Basic fibroblast growth factor (bFGF) has recently been shown to be a mitogen for keratinocytes. This observation has now been extended in a porcine model of epidermal wound healing. A single application of recombinant human bFGF given at the time of injury to healthy animals accelerated the rate of epithelialization by 20%; multiple applications gave no greater effect than the single application. Histologic analysis of biopsies of these partial-thickness wounds taken during bFGF-mediated healing supported the assessment of an enhanced rate of epithelialization and an earlier onset of dermal healing. Because no histologic abnormalities were observed, bFGF induced an acceleration of what appears to be the normal healing process. *J Invest Dermatol 95:626–631, 1990*

Basic FGF is a polypeptide growth factor characterized by a high affinity for the glycosaminoglycan heparin (for recent reviews see Refs. [1–3]). Basic FGF is a potent mitogenic and chemotactic agent in vitro for many cells of mesodermal origin [4]. These include endothelial cells and fibroblasts, two cell types whose proliferation is key to the dermal healing response of repair and remodeling. As predicted from these in vitro activities, it has been shown that exogenously administered bFGF can enhance the deposition of connective tissue in dermal wound healing models such as polyvinyl alcohol sponges implanted subcutaneously in rats [5–7]. In these experiments, a single dose of bFGF was found to be as effective a stimulator of the deposition of connective tissue as multiple doses, suggesting that bFGF is able to trigger a sequence of cellular events, and that its continued presence is not needed to complete the healing process.

Recently, it has been reported that bFGF is also a potent mitogen for human keratinocytes [8,9], an ectodermal cell type that is the predominant cell in the basal layer of the epidermis. This observation raises the possibility that bFGF may be capable of accelerating epidermal as well dermal healing. To test this possibility, we applied recombinant human bFGF to partial-thickness excisional wounds generated in porcine skin by an electrodermatome. This type of wound has been used previously as a model for human epidermal healing [10,11]. Porcine skin is similar to human skin with respect to the relative thicknesses of the epidermis and dermis, the relative sparsity of hair, and the presence of a subcutaneous adipose layer. Epithelialization of this wound type depends on the migration and proliferation of keratinocytes from the wound margins and from epidermal appendages such as the hair follicles located throughout the wound. This model has been used previously to show that topical application of the keratinocyte mitogen epidermal growth factor (EGF) can enhance epidermal healing; however, multiple doses of EGF over several days are required to achieve a stimulator effect [12,13]. The ability of this animal model to predict the effects of a growth factor on human wound healing was established in a recent report of the stimulatory effects of EGF on epithelialization of graft donor sites [14]. The results of this clinical trial were in close agreement with the preclinical evaluation in the porcine wound model [12,13].

In the experiments described below, recombinant human bFGF was evaluated using both single and multiple applications, and at several concentrations, in order to determine an effective schedule and dosage for epidermal healing. Daily biopsies of representative wounds were taken for histologic assessment of the course of epidermal healing as well as repair and remodeling of the papillary dermis [15].

MATERIALS AND METHODS

Basic FGF A cDNA encoding the 155-amino acid form of human bFGF [16] was expressed under the control of the *trp* promoter in *Escherichia coli* [17]. The resulting recombinant protein was purified using heparin-Sepharose affinity chromatography and shown to be active as a mitogen for capillary endothelial cells. Preparations of recombinant bFGF were shown by the Limulus amebocyte lysate assay (Pyrotell kit from Associates of Cape Code Inc., Woods Hole, MA) to contain low levels of endotoxin (<0.06 endotoxin units/application). Sterile test solutions were prepared in pyrogen-free phosphate-buffered saline (PBS) and were kept at 4°C until immediately before use.

Experimental Design and Model Young white female Yorkshire pigs (20–30 lbs, approximately 2–3 months old) were housed in individual pens in an American Association for the Accreditation of Laboratory Animal Care (AAALAC)-accredited animal facility with controlled temperature (19–21°C) and light (12 h/12 h light/dark). The pigs were fed a basal diet ad libitum. The hair was clipped with standard animal clippers and the skin on the back and sides was prepared for wounding by washing with a mild soap and water and rinsing. Other antiseptics were not used because of the potential effect on the healing process. Before surgery, the pigs were

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Reprint requests to: Dr. Patricia A. Hebda, Department of Dermatology, University of Pittsburgh, School of Medicine, Pittsburgh, PA, 15261.

Abbreviations:
- AAALAC: American Association for the Accreditation of Laboratory Animal Care
- bFGF: basic fibroblast growth factor
- BS-I: *Bandeirea simplifolia* agglutinin-I
- EGF: epidermal growth factor
- HT_{50}: time at which 50% of the wounds are healed
- PBS: phosphate-buffered saline

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Animal Number:

1	2	3	4	5
C V1 / T1 V2 / T2	V2 T2 / C V1 / T1	V1 T1 / V2 T2 / C	T2 C / V1 T1 / V2	T1 V2 / T2 C / V1

Figure 1. Experimental design. The complete block design shown was used to evaluate bFGF in PBS at two different application schedules *(T1, T2)*, as well as the PBS vehicle for each application schedule *(V1, V2)* and the control (*C*, untreated or air-exposed). The details of these treatment groups are given in Fig 2*a,b*. The matrices represent the backs of the five experimental animals divided into five treatment areas with the head of each animal at the top of the matrix. Each treatment area contained 30–35 individual wounds separated by at least 1 cm of intact skin. The design allows for uniform distribution of the five treatment groups on the five experimental animals.

anesthetized with ketamine (300 mg intramuscularly) and halothane (3%, open mask) administered with nitrous oxide and oxygen (40:60, 5 l/min). A total of five animals was used in the first experiment, four animals in the second. On each animal, 150–175 small rectangular wounds, approximately 1 cm², and 0.3-mm deep, were made in the paravertebral and thoracic areas with a Castroviejo electrodermatome (Storz Instruments, St. Louis, MO). This standard wound has been shown to achieve the complete removal of epidermis and the most superficial dermis, leaving the epidermal appendages intact. [10,18].

The wounds were divided into treatment groups as shown in the experimental design for the first experiment (Fig 1). The position of each treatment group was rotated on the animals to control for any anatomical variation in the wound-healing response, while accommodating the inclusion of all the treatment groups in every animal. In this porcine wound-healing model, the variation in response is greater between individuals than between anatomical regions [11]. In practice, we have found the latter to be negligible.

Wound Healing Within 1 h of wounding, all traces of clotted blood and wound fluid were removed from the wounds with a damp swab and the wounds were blotted dry with clean paper towels. Each wound was either treated with 10 μl of the bFGF solution, the vehicle, or left untreated according to the design in Fig 1. Treatments were left undisturbed for 30 min until the small volume was absorbed into the tissue of the wound beds. The wounds were left uncovered, anesthesia was discontinued, and the animals were returned to their pens. Occlusive dressings were not used to avoid masking a stimulatory effect of bFGF, with the positive effect resulting from occlusion [18].

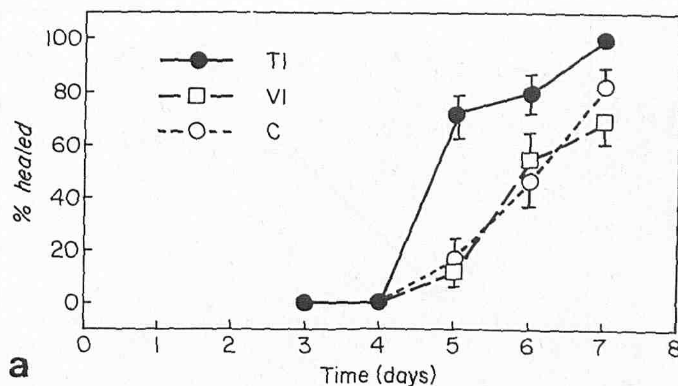
Table I. Basic FGF Stimulation of Epidermal Wound Healing

Treatment Groups	HT_{50}[a]	Relative Rate of Healing[b]	Net Effect of Treatment[c]
T1 10 μg FGF/PBS Day 0	4.9	+18%	+21%
V1 PBS	6.2	− 3%	
T2 10 μg FGF/PBS Day 0, 1, 2	5.0	+17%	+10%
V2 PBS Day 0, 1, 2	5.6	+ 7%	
C Air-exposed Control	6.0		

[a] n = 20.

[b] Relative rate of healing (RRH) = $\frac{HT_{50}\,(\text{control}) - HT_{50}\,(\text{treatment})}{HT_{50}\,(\text{control})} \times 100\%$.

[c] Net effect of treatment = RRH (treatment) − RRH (vehicle).



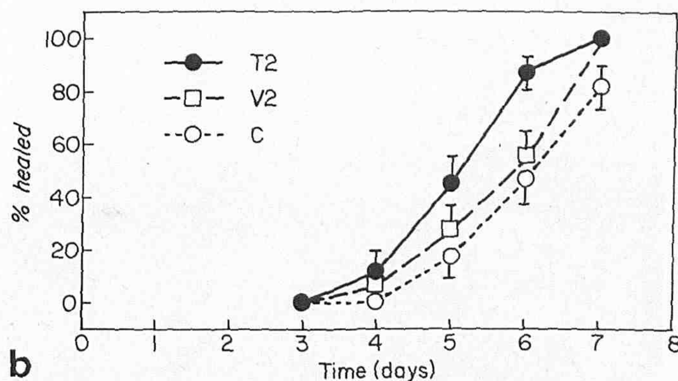


Figure 2. Stimulation of the rate of epidermal wound healing by recombinant bFGF. *(a)*, treatment with a single dose of bFGF. The percentage of fully healed epidermal specimens (±standard error, n = 20–25) is shown on days 3, 4, 5, 6, and 7 post-wounding, for *T1* (10 μg bFGF in 10 μl PBS on day 0), *V1* (10 μl PBS vehicle on day 0), and *C* (air-exposed control). The bFGF-treated group showed a greater percentage of wounds healed on days 5, 6, and 7 compared to vehicle-treated or untreated controls ($p < 0.05$). *(b)*, treatment with multiple doses of bFGF. The percentage of fully healed epidermal specimens (±SE, n = 20–25) is shown on days 3, 4, 5, 6, and 7 post-wounding for *T2* (10 μg bFGF in 10 μl PBS on days 0, 1, and 2), *V2* (10 μl PBS vehicle on days 0, 1, and 2), and *C* (air-exposed control). The bFGF-treated group showed greater healing on days 5, 6, and 7 compared with untreated controls and on day 6 compared with vehicle-treated controls ($p < 0.05$).

Macroscopic evaluation of the rate of epidermal healing over the next seven days was performed as described previously [10,11]. Briefly, 4–5 wounds per day were excised from each treatment group in each of the five pigs, resulting in a total of 20–25 wounds/d/group collected on days 3 through 7. The samples excised were of sufficient size (22 × 33 mm) and depth (0.5–0.8 mm) to include the entire wound bed of the original partial-thickness injury and some surrounding normal tissue. Following excision, the samples were incubated in 0.5 M sodium bromide at 37°C for 24 h to separate the epidermis from the dermis at the basement membrane [19]. The epidermal specimens were evaluated for the presence or absence of a defect, and were mounted on cardboard for preservation. By this means of analysis, only wounds that were completely healed, with no visible defect in the epidermis, were scored as healed. The samples taken from all five animals were pooled to obtain a value of percent wounds healed of the total wounds evaluated (n = 20–25).

Data Analysis The data are shown graphically as the percent of wounds healed vs days after wounding. When graphed in this form, data from each treatment group are depicted as a smooth sigmoidal

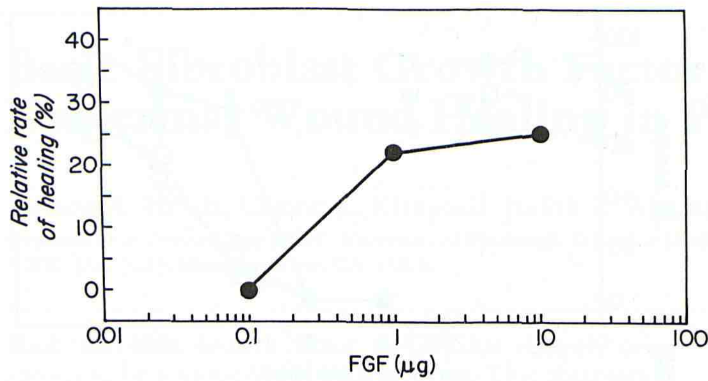
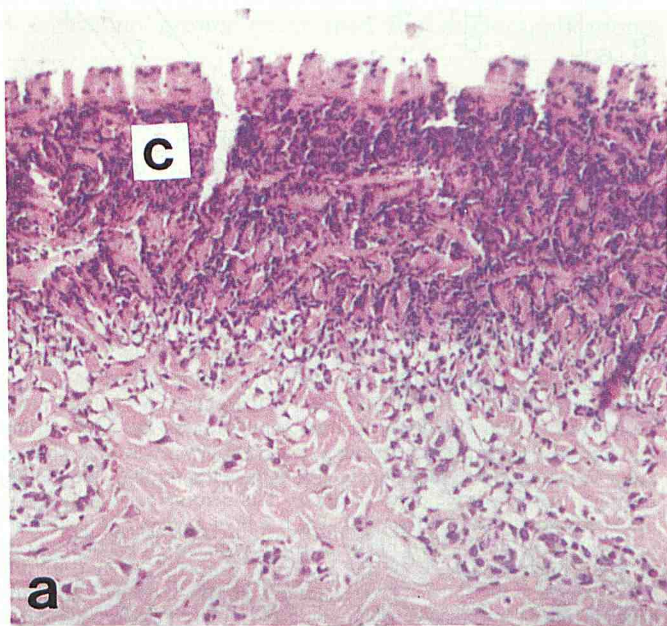
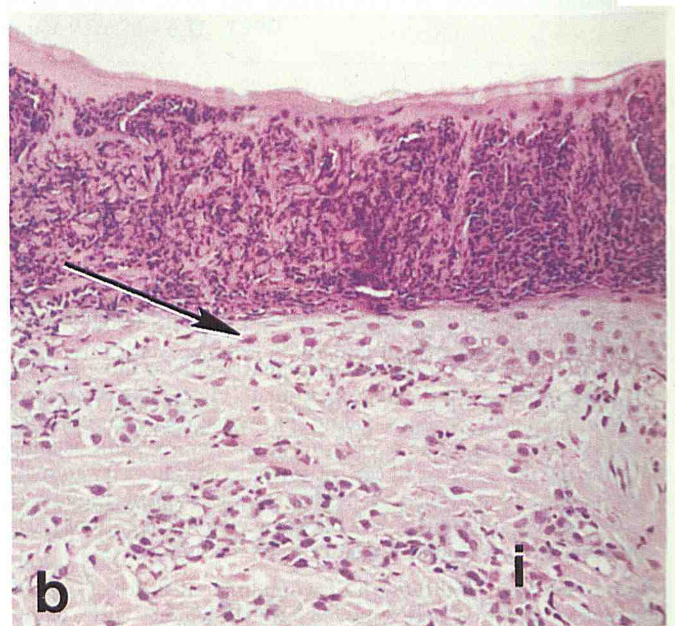


Figure 3. Dose-response study of bFGF-stimulated epidermal healing. The pigs were treated as described in Fig 1, but the objective in this experiment was to determine the effect on epidermal wound healing of different bFGF doses: 10 μg, 1.0 μg, and 0.1 μg. Results are plotted as the relative rate of healing (defined in Table I) vs the amount of bFGF administered once, on day 0. Treatment with bFGF produced greater healing compared with controls on days 5, 6, and 7 at doses of 10 μg and 1.0 μg per wound ($p < 0.05$), but not at 0.1 μg per wound.





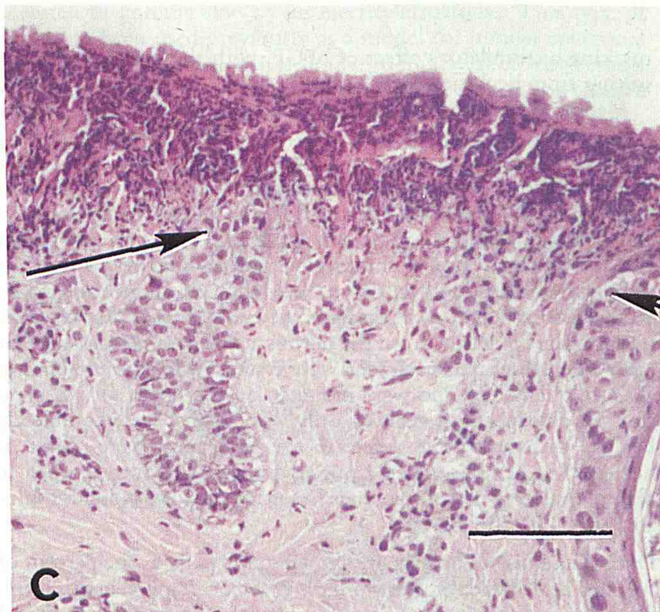


Figure 4. Wound histology. Representative partial-thickness wounds from each of the control and treatment groups were biopsied, fixed in formalin, and stained with hematoxylin and eosin. Examples from one such series of day 2 wounds are shown: *(a)*, air-exposed control, *(b)*, bFGF-treated, and *(c)*, PBS vehicle-treated control. The dried exudate or crust covering all the wounds at this stage is indicated by *"c"* in *panel a*. Migrating epithelium in the bFGF-treated wound is indicated by an *arrow* *(panel b)*. In contrast, epidermal appendages that lack the migrating morphology are indicated by *arrows* in the vehicle-treated control *(panel c)*. The bFGF-treated wounds characteristically showed a more extensive cellular infiltrate (labeled "i" in *panel b*) than did wounds in either of the control groups. Magnification × 40. *Bar,* 100 μm.

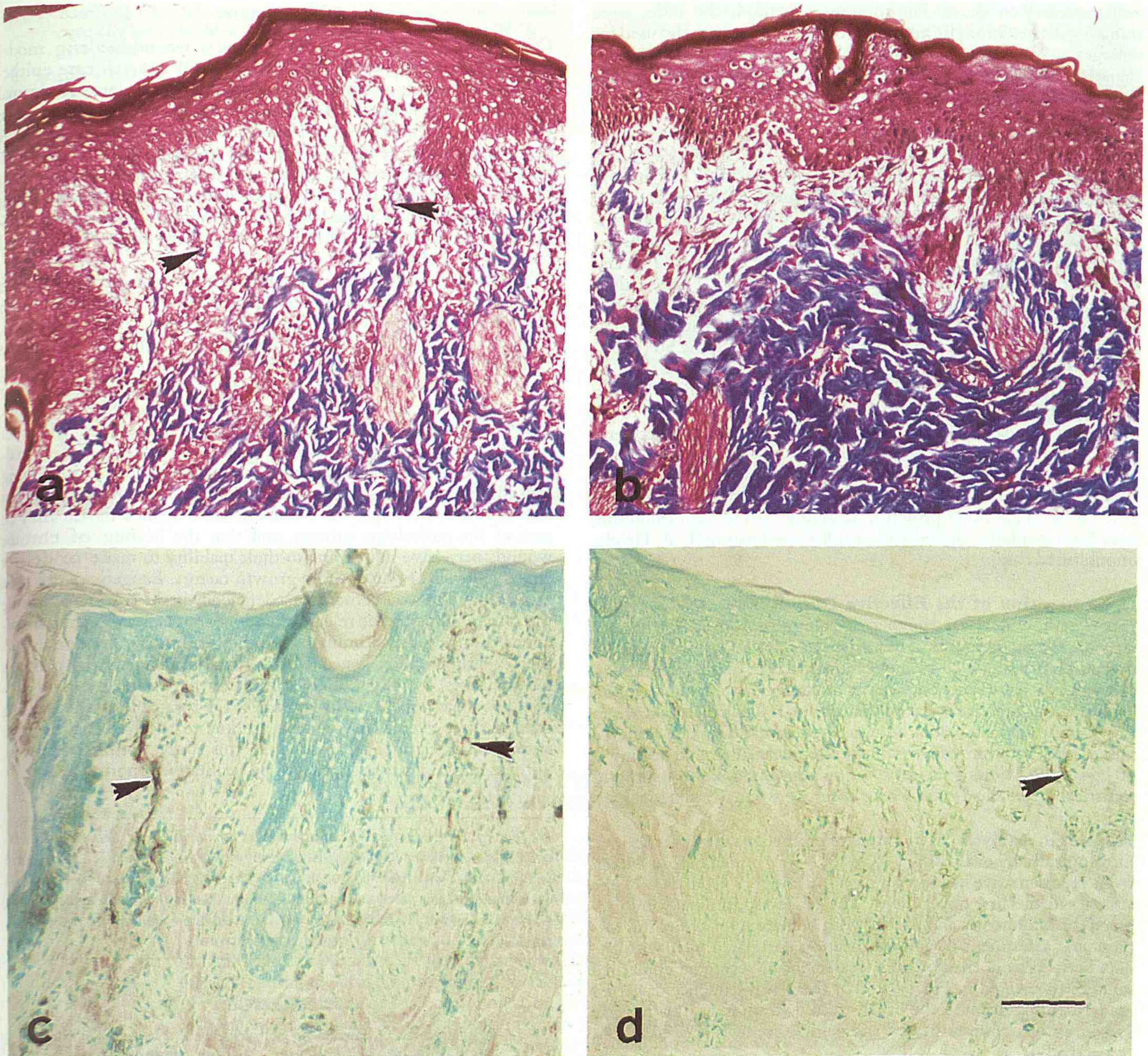


Figure 5. Dermal wound healing. Biopsies taken on day 6 from either *(a)* bFGF-treated or *(b)* vehicle-treated control wounds were stained with Masson's trichrome [22] to detect collagen fibers. A greater deposition and organization of new collagen fibers (indicated in panel *A* by *arrows*) was present in the bFGF-treated wounds than in the control wounds. Similarly, biopsies taken on a day 6 from *(c)* bFGF-treated wounds or *(d)* vehicle-treated control wounds were stained to identify endothelial cells using the *Bandeirea simplicifolia* agglutinin-I (BS-I) followed by visualization of the bound lectin with an avidin-biotin-peroxidase complex [23]. In the bFGF-treated wounds, there was more extensive angiogenesis than in the control wounds as indicated by the darkly-stained endothelial cells *(arrows, panel c)*. Magnification × 40. *Bar,* 100 μm.

curve progressing from 0% to 100%. The value of interest is the time point when 50% of the wounds have healed, HT_{50}. However, as the HT_{50} values can only be estimated from such a graph, it is necessary to express the data in a linear format, from which the HT_{50} values can be derived precisely. The data were converted to a linear format using step-wise logistic regression with the aid of a computer software package for statistical analysis [20]. The three variables were: natural logarithm of time (ln time), number of samples healed, and total number of samples. The computer program determined the slope of the line and the y intercept. The HT_{50} value for each group was then derived by solving the equation

$$HT_{50} = \ln^{-1}\frac{(-\text{y intercept})}{\text{slope}}.$$

The data were also analyzed for differences between groups on each day after wounding using the Z test (a chi-square type of analysis that is appropriate when the sample size is less than 40) [21]. This test was used to obtain the p values reported in Fig 2*a,b*.

Histology Daily biopsies (4-mm trephine) were taken from two animals in each experiment. These were fixed in 10% formalin and embedded in paraffin; 4–6-μm transverse serial sections were cut

and mounted on slides. For routine evaluation, the slides were stained with hematoxylin and eosin. Special stains were also used for collagen—Masson's trichrome [22]—and endothelial cells—*Bandeirea simplifolia* agglutinin-I (BS-I)—followed by avidin-biotin-peroxidase staining [23].

RESULTS

Evaluation of Single Vs Multiple Applications The first experiment (Fig 1) compared single (day 0) versus multiple (days 0, 1, and 2) doses of bFGF administered at 10 μg per wound. The combined data from the epidermal evaluation of the five animals are shown graphically in Fig 2*a* for the single dose on day 0 *(T1)*, and Fig 2*b* for the multiple doses on days 0, 1, and 2 *(T2)*. These healing curves indicate that both single and multiple applications of bFGF in saline accelerated epidermal wound healing. The data were analyzed by logistic regression to derive the Healing Time 50 (HT_{50}) values shown in Table I. A single application of 10 μg per wound on day 0 was most effective, producing a 21% enhancement of healing when compared with the vehicle. Multiple applications of 10 μg bFGF given on days 0, 1, and 2 induced essentially the same acceleration of healing as the single application when compared to the air-exposed control. With multiple applications, there was a slight beneficial effect from the vehicle. The observed stimulation is specific for bFGF, as other proteins, including bovine serum albumin, have been similarly applied with no effect on healing (P. A. Hebda, unpublished data).

Determination of the Effective Dose Range In the second experiment, which was designed to examine dose-response characteristics, various amounts of bFGF (0.1, 1.0, or 10 μg) were applied to wounds in a single 10-μl application on day 0. In this experiment, the wounds were distributed over at total of four animals in an experimental design similar to that in Fig 1. The results shown in Fig 3 indicate that, when administered in a phosphate-buffered saline (PBS) solution, bFGF stimulated epidermal wound healing at 10 μg and 1 μg wound but not at 0.1 μg per wound. Within limits of statistical error, the 1- and 10-μg doses were equally effective.

Histologic Analysis The extent of epithelialization was verified histologically by evaluating a series of 4-mm punch biopsies taken from representative wound on days 0 through 7 from two of the animals in the first experiment. The biopsy specimens were prepared for microscopy and stained with hematoxylin and eosin. Microscopic assessment of epithelialization correlated well with the data derived from macroscopic assessment of separated epidermal samples (presented in Fig 1), in that there was evidence of earlier epidermal migration in the bFGF-treated wounds, as illustrated in Fig 4*b*, compared to untreated and vehicle controls (Fig 4*a* and 4*c*, respectively).

In addition, the histologic analyses indicated that bFGF also accelerated wound healing events in the papillary dermis. The biopsies were, therefore, evaluated for evidence of connective tissue formation and angiogenesis. Following fixation, the specimens were stained either with Masson's trichrome to detect newly-formed collagen fibers (Fig 5*a,b*), or with the lectin BS-I to detect endothelial cells (Fig 5*c,d*). The Masson's trichrome-stained sections provided evidence that bFGF-treated wounds had an earlier deposition of new collagen in the superficial dermis. New collagen fibers were detectable by histology in bFGF-treated wounds on day 6, but not in vehicle-treated control wounds from the same day (Fig 5*a,b*). Control wounds from day 7 exhibited new collagen fiber formation (data not included) and closely resembled bFGF-treated wounds from day 6 in this feature. Thus, bFGF treatment brought about an earlier maturation of acute dermal wound healing by about one day. The BS-I lectin binding study indicated that neovascularization was enhanced by bFGF treatment in wound biopsies taken on day 6 (Fig 5*c,d*). The bFGF-treated wounds consistently exhibited more advanced growth of new vessels. This effect on angiogenesis agrees with studies in other wound models [5-7].

DISCUSSION

Our results in a porcine partial-thickness wound-healing model demonstrate quantitatively the ability of bFGF to accelerate epithelialization; there is also qualitative evidence for accelerated dermal repair and angiogenesis. These findings suggest that bFGF may be effective in the treatment of partial-thickness human wounds. A single application of bFGF on the day of injury was sufficient to induce a 20% acceleration in the rate of epidermal healing. No further acceleration was noted with multiple applications. This level of stimulation is considered to be substantial because the maximum stimulation achieved by other topical agents in this wound model is about 30% [11].

The ability of bFGF to accelerate healing in a single application may be advantageous for clinical treatment over therapies with growth factors such as EGF that require repeated treatment [14]. Because the bFGF-accelerated healing was achieved in acute wounds in healthy animals that normally exhibit a rapid healing response, it is conceivable that the relative acceleration of healing may be even greater in subjects that show an impaired healing response. Impaired healing can result from a number of different problems such as diabetes, obesity, diminished circulation, and malnutrition, or result from various pharmacologic agents including steroids and immuno-suppressants. It has been speculated that the lack of one or more growth factors in the wounded tissue may be part of the pathologic process, and that the healing of chronic wounds may have stalled due to their inability to make or deliver effective levels of one or more growth factors. Exogenous growth factors may thus compensate for a deficiency in the tissue and prime the system inducing the release of endogenous factors so that subsequent endogenously stimulated wound healing can proceed. Further experimentation is needed to obtain an understanding of the mechanism(s) by which bFGF stimulates the wound healing process, but it has now been shown that bFGF is capable of facilitating at least three events in vivo that are key to the healing process: angiogenesis, dermal repair, and, in this study, epithelialization, with an end result that appears to be qualitatively normal.

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CONFERENCE ON THE PREDICTION OF PERCUTANEOUS PENETRATION

Following the highly successful conference held in April 1990, the Organizing Committee is pleased to announce the second Conference on the Prediction of Percutaneous Penetration. The purpose of the Conference is to provide the latest information on techniques for the prediction of chemical penetration through the skin. It will be of particular relevance to scientists in the pharmaceutical and agrochemical industries, toxicologists, universities, and government (e.g., MAFF, HSE, EPA, FDA). Carefully selected, internationally respected speakers will address topics relating to their own expertise. The ability to predict absorption will be a central theme. In addition, there will be free communication (oral and poster) sessions and targeted workshops. Attendance is expected to be international, but will be limited to 300.

Date and Venue: 10–12 April 1991, The University of Southampton, United Kingdom.

Conference Topics: dermal metabolism, in vivo/in vitro studies, human volunteer studies, non-invasive techniques, penetration enhancement, ASAR/modeling, and regulatory affairs/risk assessment.

Organizing Committee: The principal organizers are Dr. R.C. Scott (tel: 0625 514508), Professor J. Hadgraft (tel: 0222 874180), Professor R.H. Guy (tel: USA 415 476 4830), and Dr. H.E. Bodde (tel: Netherlands 71 274350), from whom fuller program information can be obtained.