Mechanism of Acceleration of Wound Healing by Basic Fibroblast Growth Factor in Genetically Diabetic Mice

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To elucidate the role of basic fibroblast growth factor (bFGF) in the wound healing process, we investigated the ability of the factor to modulate an inflammatory reaction at the wound site and to influence endothelial cells and fibroblasts *in vitro*. A single, topical application of bFGF to a full-thickness wound of genetically diabetic mice caused an increase in the volume of wound exudate in a dose-dependent manner. bFGF induced the infiltration of a large number of leukocytes in the wound exudate. Transforming growth factor- β (TGF- β) positive cells, such as macrophages, monocytes and fibroblasts, appeared in the granulation tissue in bFGF-treated diabetic mice. These phenomena were comparable to those in normal animals, suggesting that the treatment with bFGF restored the inflammatory response in wound healing of diabetic mice. The effects of bFGF on cell proliferation, migration and angiogenesis were histologically recognized as shown in enhanced granulation tissue formation and neovascularization. It is suggested that bFGF promotes the recruitment of inflammatory cells into the wound site to induce a cascade reaction of growth factors including TGF- β in a wound healing process, and so would accelerate wound healing.

Key words basic fibroblast growth factor; wound healing; inflammatory cell; transforming growth factor- β

Wound healing is initiated by an inflammatory phase followed by the deposition of a conditional matrix, collagen synthesis and remodeling. It is well accepted that various growth factors and cytokines are involved in the wound healing process in an orderly progression manner.^{1–3)} Among them, basic fibroblast growth factor (bFGF) is considered to participate in an early stage of the wound healing process.⁴⁾ bFGF promoted the migration, proliferation, proteolytic enzyme production and capillary growth of endothelial cells.⁵⁾ The most important role of bFGF is presumed to be the formation of capillaries. Genetically diabetic and steroid-treated animals are known to be impaired wound healing models.^{6,7)} In these animals, inflammatory reactions induced after wounding are largely suppressed. bFGF shortened the period of wound healing in diabetic mice to the level in normal mice.^{7–9)}

Transforming growth factor- β (TGF- β) is also an important factor in the wound healing process.⁴⁾ In a normal healing process, TGF- β is thought to be involved in the migration of inflammatory cells such as macrophages, monocytes and neutrophils in early inflammatory reactions post wounding.^{10–12)} TGF- β stimulates fibroblasts to promote collagen synthesis and plays a role in remodeling in later stages of wound healing.⁴⁾ Some papers have shown evidence that bFGF and TGF- β modulate the activities of each other *in vitro*,^{13,14)} suggesting that various growth factors may exert their biological activities through intricate interactions among themselves at an appropriate time during wound healing.

Since even a single application of bFGF promoted wound healing in diabetic mice,⁹⁾ it seemed that application at an early stage of the wound healing process was important in accelerating the process by bFGF. In the present paper, we investigated both the infiltration of inflammatory cells and their expression of TGF- β due to treatment with bFGF in a full-thickness wound of diabetic mice, as well as the mechanisms of action of bFGF in the

wound healing process.

MATERIALS AND METHODS

Reagents Recombinant human bFGF (rh-bFGF) was obtained from Scios Nova, Inc. (Mountain View, CA). Histofine SAB-PO(R) and DAB (3,3'-diaminobenzidine tetrahydrochloride) substrate kits (Nichirei Corp., Tokyo) were used for immunohistology. Human TGF- β 1 and human platelet-derived growth factor (PDGF) were obtained from R & D System, Inc. (Minneapolis, MN). Human epidermal growth factor (EGF) was purchased from Wakunaga Pharmaceutical Co., Ltd. (Osaka). Rabbit anti-TGF- β polyclonal antibody (specific for TGF- β 1 and β 2) was purchased from King Brewing Co., Ltd. (Kakogawa).

Animals Genetically diabetic female mice (C57BL/KsJ-db/db) and their heterozygous (m+/db) littermates were purchased from Jackson Laboratories (Bar Harbor, ME) at the age of 5–8 weeks. We used animals which indicated normoglycemia (100–200 mg/dl) and hyperglycemia (300–above 500 mg/dl) in normal and diabetic mice, respectively. The animals were 7–10 weeks old at the start of all experiments and were housed in individual cages during the experiments.

Wounding The animals were shaved on the back with an electric shaver under anesthetization with ether on the day before wound surgery. The skin was sterilized with 70% ethanol. A full-thickness wound was made by excising both the skin and panniculus carnosus corresponding to a 1.6 cm diameter template. Twenty μ l of saline or a solution of rh-bFGF (1 mg/ml) was applied to the wound bed (*ca.* 2 cm²), and a transparent occlusive dressing (Bioclusive; Johnson & Johnson, Arlington, TX) was placed over the wound. The dressing was replaced every other day. On days 3, 5 and 10, four animals from each set were sacrificed by euthanasia to excise the skin, including the wound site, for histology. Exudate from the

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wound site was accurately collected with a micropipette to measure the volume on day 4 and to measure the number of leukocytes using the counting chamber of Bürker Türk.

Immunostaining The excised skins were fixed in 10% buffered formalin, followed by paraffin embedding procedures. Three μm sections of the tissues were mounted and dried on poly-L-lysine-coated slide glass (Muto Pure Chemicals Co., Ltd., Tokyo). After being washed with xylene, the sections were dried and dipped in 100% ethanol, and were then treated with methanol including 3% hydrogen peroxide for 15 min to block endogenous peroxidase activity. The sections were washed with flowing water and equilibrated with 0.01 M phosphate buffered saline (PBS, pH 7.2) for immunostaining using a Histofine SAB-PO(R) kit. The sections were blocked for 10 min by 1–2 drops of blocking reagent before a 1-h treatment with anti-TGF- β antibodies (1/150 dilution with 0.01 M PBS) at 37 °C, and were then washed 3 times with 0.05% Tween 20/PBS (T-PBS) and PBS, respectively, and were reacted with the second antibody (biotinylated anti-rabbit IgG) for 10 min at room temperature. After the same washing procedure with T-PBS and PBS, the peroxidase-labeled streptavidin reagent was dropped on the sections and was incubated at room temperature for 5 min. A solution of DAB was applied after the third washing procedure with T-PBS and PBS, followed by washing with 50 mM Tris-HCl, pH 7.0. The sections were washed with water after the 3-min reaction. The nuclei were counterstained with methyl green. Another section of the same tissue was stained with hematoxylin/eosin.

Cell Cultures Baby hamster kidney (BHK-21) cells and bovine adrenal cortex capillary endothelial (ACE) cells were obtained from Scios Nova, Inc. BHK-21 cells were plated at 2×10^5 cells per 100 mm dish (Falcon Labware, Becton-Dickinson Co., Lincoln Park, NJ) in Dulbecco's Modification of Eagle's Medium (DMEM)/F-12 (DME/F-12) (Sigma Chemicals Co., St. Louis, MO) with 10% fetal calf serum (FCS) (Flow Laboratories, ICN Biomedicals, Inc., Costa Mesa, CA) and were used at subconfluence. All media were supplemented with 50 units/ml penicillin and 50 $\mu\text{g}/\text{ml}$ streptomycin. ACE cells were plated at 2.5×10^5 cells per T-75 flask (Falcon) in DMEM supplemented with 1 ng/ml rh-bFGF and 10% calf serum (CS) (Hyclone Laboratories, Inc., Logan, UT). The culture medium was exchanged into the medium supplemented 10% CS without rh-bFGF at confluence, and the cells were used for the experiments after incubation for 2 or 3 d.

Proliferation Assays BHK-21 cells were plated at 1×10^3 cells per well on 96-well plates (Falcon) in DME/F-12 containing 2 mg/ml bovine serum albumin (BSA) (Sigma), 1 $\mu\text{g}/\text{ml}$ transferrin (Sigma) and 5 $\mu\text{g}/\text{ml}$ insulin (Sigma), and were incubated for 72 h at 37 °C in 95% air and 5% CO_2 . ACE cells were plated at 2.5×10^3 cells per well on 96-well plates in DMEM supplemented with 10% CS and were incubated for 48 h at 37 °C in 93% air and 7% CO_2 . rh-bFGF was added at the initiation of the incubation in a range of 0.14–100 ng/ml as final concentrations. The cell proliferation was evaluated using a colorimetric assay^{15,16} based on the tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bro-

mide (MTT) (Chemicon, Inc., Temecula, CA).

Labelling of rh-bFGF rh-bFGF was labelled with Na^{125}I (17.4 Ci/mg) (Dupont/NEN, N. Billerica, MA) using chloramine T (Nacalai Tesque, Kyoto) according to the method described elsewhere.^{17,18} Labelled rh-bFGF was purified by a chromatography of heparin Sepharose (Pharmacia Biotech, Uppsala). The specific activity of the ^{125}I -rh-bFGF obtained was 999 cpm/fmol.

Binding Assays Binding assays were performed according to the method of Moscatelli.¹⁹ Confluent cultures of BHK-21 and ACE cells in 24-well plates were incubated for 3 h at 37 °C in serum-free DME/F-12 with 0.15% gelatin (Sigma) before incubation for 20 min in the presence of 50 μM suramin (Miles Inc., FBA Pharmaceuticals, West Haven, CT). Cells were then washed twice with cold PBS, and 300 μl of cold DME/F-12 containing 25 mM HEPES, pH 7.5, 0.15% gelatin and the desired concentration of ^{125}I -rh-bFGF were added to each well. The cells were incubated ice-cold for 3 h. At the end of the incubation, cells were washed 3 times with cold PBS and once with 1 ml of 2 M NaCl in 20 mM HEPES and were then solubilized twice for 10 min with 0.25 ml of 0.5% Triton X-100 in 0.1 M sodium phosphate, pH 8.1. The radioactivities in the incubation medium (including PBS wash), 2 M NaCl wash and Triton X-100 extract were determined in a gamma scintillation counter G5500B (Beckman Instruments, Inc., Fullerton, CA). All experiments were run in triplicate. In all the experiments, representative wells were trypsinized to count the cells.

Chemotaxis Assay The migration of ACE cells was determined using a membrane filter (Nuclepore, Pleasanton, CA) of 5 μm pore size in a 48-well microchemotaxis chamber (Neuroprobe, Bethesda, MD). The lower wells were filled with serial dilutions of rh-bFGF, TGF- β , PDGF or EGF in serum-free DMEM supplemented with 0.2% BSA. Fifty μl of ACE cell suspensions (1×10^4 cells/well) were added to the upper wells. Incubation was carried out for 3 h at 37 °C in 95% air and 5% CO_2 . Migrated cells at the lower surface of the membrane were fixed with ethanol, and were stained with hematoxylin. The total migrated cell number was counted with a microscope (Olympus, Tokyo).

Plasminogen Activator Assay ACE cells were suspended in DMEM containing 0.5% CS and were seeded in 6-well plates (Falcon) at 1.5×10^5 cells/2.5 ml per well. rh-bFGF in a range of 0–30 ng/ml as a final concentration was added 3 h after seeding. After a 48-h incubation, the cells were washed with cold PBS, and plasminogen activator (PA) was extracted with 1 ml of cold 50 mM Tris-HCl, pH 8.5, containing 0.05% Triton X-100. The protein concentration of the cell extracts was measured using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA) using BSA as a standard. PA activity was measured using a Chromolize urokinase-type PA (uPA) assay kit (Biopool AB, Umeå) in which samples were incubated with glu-plasminogen, a plasmin chromogenic substrate (D-But-CHT-Lys-pNA) and human pro-uPA. Measured uPA was expressed as ng/mg extracted protein.

Capillary Tube Formation Assay Forty-eight well plates (Sumitomo Bakelite Co., Ltd., Tokyo) were coated with 150 μl of type I collagen (Koken, Tokyo) gel per well,

and ACE cells were plated overnight at 7.5×10^3 cells/0.5 ml per well in DMEM supplemented with 10% CS. Cultures were sucked the medium and were covered with 100 μ l of type I collagen gel. After an hour, 0.25 ml of fresh medium and 1 μ l of rh-bFGF in a range of 0–5 μ g/ml (final concentrations of 0–10 ng/ml) were added on the gel in each well. The incubation was carried out for 45 h at 37°C in 93% air and 7% CO₂. After fixation with 5% buffered formalin, the length of tube in an area of 6.1 $\times$ 6.1 mm was observed ($\times 40$ objective, $\times 0.55$ video camera magnification) with a computer-assisted image analyzer SP-1000 (Olympus).

RESULTS

Promotion of Exudation by rh-bFGF in Full-Thickness Wound Healing The delayed healing in diabetic mice may be partly due to diminished inflammatory reactions at the wound site during an early stage after wounding. Figure 1 shows the amount of exudate and the number of infiltrated leukocytes, mainly neutrophils, into the exudate at 4 d after the application of rh-bFGF or saline as a control to a full-thickness wound in diabetic mice. A volume of exudate at the wound site was immeasurable or very little in most of the mice treated with saline. On the other hand, the application of rh-bFGF induced significant increases in both the volume of exudate and the number of leukocytes. It is likely that bFGF enhanced the inflammatory reactions in diabetic mice. Twenty μ g of rh-bFGF per wound site was enough to induce a maximal response for wound healing and to infiltrate leukocytes into the exudate. The high dose (200 μ g/wound)

of rh-bFGF, however, reduced the increase in the number of leukocytes. It seemed that bFGF initiated wound healing through mobilizing leukocytes to support early inflammatory reactions after wounding.

Expression of TGF- β in a Full-Thickness Wound When rh-bFGF (20 μ g/wound) was applied once to the surface of a full-thickness wound in diabetic mice on day 0, granulation tissue formation was initiated within 3 d in association with the proliferation and migration of fibroblasts and endothelial cells (Fig. 2). Immunostaining with anti-TGF- β antibody on day 3 revealed that both the epidermis near the wound edge and the sebaceous glands were intensively positive in healthy mice treated with saline (healthy group, Fig. 2A) and in diabetic mice treated with rh-bFGF (rh-bFGF-treated group, Fig. 2E), but weak in diabetic mice treated with saline (diabetic control group, Fig. 2C). In addition, the rh-bFGF-treated group (Fig. 2F) showed the infiltration of as many macrophages and lymphocytes, which were TGF- β -positive, to the wound site as the healthy group (Fig. 2B) did. In the diabetic control group (Fig. 2D), both granulation tissue formation and infiltration of leukocytes to the wound site were delayed in comparison with either the healthy group or the rh-bFGF-treated group. On day 5, the healthy group and the rh-bFGF-treated group showed further increases in the thickness of granulation tissue and the number of TGF- β -positive cells, and the diabetic control showed similar histogenesis to that of the healthy group on day 3 (data not shown). It seemed that rh-bFGF normalized early inflammatory reactions post-wounding in diabetic mice.

Effects of rh-bFGF on the Proliferation of Fibroblast and Endothelial Cells It is very probable that bFGF causes the migration and proliferation of fibroblasts and endothelial cells *in vivo*.⁹⁾ The promotion of granulation tissue formation by rh-bFGF treatment was presumed to occur based on the thickening of granulation tissue (Fig. 2E). It was also observed that the thickness of the loose connective tissue around the wound site was temporally increased by rh-bFGF treatment and then returned to normal by day 10 (data not shown). Figure 3 shows loose connective tissues of subcutaneous fat around the wound edge on day 3 post-wounding. rh-bFGF would increase the thickness of the tissue wall due to the proliferation of fibroblasts and neovascularization. It was histologically observed that the fibroblasts lying between subcutaneous fat cells proliferated following treatment with rh-bFGF. Furthermore, more capillary blood vessels were observed in the tissue of the rh-bFGF-treated group (Fig. 3C) compared with that of the control group (Fig. 3B). These observations are presumably the result of the activities elicited by rh-bFGF *in vitro* as follows. rh-bFGF is a potent mitogen of fibroblasts and endothelial cells as shown in Fig. 4. The proliferation of BHK-21 and ACE cells was promoted by rh-bFGF in a dose-dependent manner. Maximal responses were obtained at around 10 ng/ml for BHK-21 cells and around 3 ng/ml for ACE cells, respectively. The mitogenic activity of rh-bFGF is the result of specific binding for the receptor. The Scatchard analyses²⁰⁾ of receptor binding assays revealed the presence of two kinds of binding sites in both

Fig. 1. Increase in Exudation by rh-bFGF Treatment in the Full-Thickness Wound

The volume of exudate for 24 h from day 3 to day 4 after application of an indicated dose of rh-bFGF or saline to the full-thickness wound in diabetic mice was measured by a micropipette (Upper). The number of leukocytes in the wound fluid was counted using a counting chamber (Lower). Data from each mice are plotted. Means (+) and error bars (S.D.) are illustrated from 7–9 samples. A similar experiment was performed twice. The data were statistically evaluated using the Kruskal-Wallis test (* p < 0.05, ** p < 0.01).

Fig. 2. Immunohistochemical Localization of TGF- β at the Wound Edge on Day 3

The full-thickness wound sections were immunoreacted with anti-TGF- β antibody and the nuclei were counterstained with methyl green as described in Materials and Methods. Photos show representative samples of the wound skin sections from 4 animals in each group, respectively. The healthy mice (photos A, B) and the diabetic control mice (C, D) were treated with saline on day 0. The animals treated with rh-bFGF (E, F) received 20 μ g of rh-bFGF to the wound site on day 0. Magnifications of A, C and E are $\times 40$. B, D and F show strong magnification ($\times 400$) of the portion of granulation tissue under the excisional site (ES), respectively. TGF- β positive cells were stained brown. Representative macrophages, fibroblasts and lymphocytes were shown as m, f and l, respectively.

cell types, and K_d s for the high affinity binding receptors were 17.2 pM for BHK-21 cells and 21.4 pM for ACE cells (Fig. 5). These values are in a good agreement with a previous report¹⁹⁾ which used bFGF purified from human placenta. K_d s for low affinity binding receptors in BHK-21 cells and ACE cells were 110.6 pM and 5200 pM, respectively. The proliferation of fibroblasts would lead to granulation tissue formation involved in the production of a collagen matrix for capillary endothelial cells to establish neovascularization.

Angiogenesis and the Production of Plasminogen Activator by rh-bFGF Rapid neovascularization by rh-bFGF might be responsible for promoting the recruit-

ment of macrophages and lymphocytes which express other growth factors such as TGF- β . The *in vitro* activities of rh-bFGF for ACE cells could explain its promotion of neovascularization *in vivo*. Figure 6 shows the activity of rh-bFGF for ACE cell migration using a microchemotaxis chamber. rh-bFGF above 1 ng/ml promoted the migration of ACE cells. TGF- β , PDGF or EGF were without this effect. uPA, which is produced by endothelial cells, is an important protease which degrades and enables cells to invade the tissue extracellular matrix during the formation of new capillaries. In addition, the enzyme is known to activate a latent form of TGF- β .²¹⁾ rh-bFGF increased the production of uPA in ACE cells

Fig. 3. Cell Proliferation and Neovascularization in Loose Connective Tissues of Subcutaneous Fat around the Wound Edge 3d after Wounding

Wound sections were stained with hematoxylin/eosin; (A) normal mouse, (B) diabetic mouse applied saline, (C) diabetic mouse applied rh-bFGF (20 µg/site), magnification: $\times 400$. Arrowheads indicate capillary blood vessels which are located in the tissue wall of subcutaneous fat cells (F).

(Fig. 7). An increase in the protein content shows the promotion of cell proliferation. Figure 8 shows capillary tube formation by ACE cells in a three-dimensional collagen matrix. The total capillary length in sight (area: 6.1×6.1 mm) increased in a concentration-dependent manner with rh-bFGF. Three ng/ml of rh-bFGF elicited the maximal response. It can be easily conjectured that the potentiation of cell migration, cell proliferation, uPA

Fig. 4. Effects of rh-bFGF on the Proliferation of BHK-21 Cells and ACE Cells

BHK-21 cells (A) were incubated for 72 h at 37 °C on 96-well plates in serum-free DME/F-12 medium to which was added various concentrations (0.14–100 ng/ml) of rh-bFGF. ACE cells (B) were incubated for 48 h on 96-well plates in DMEM supplemented 10% CS and various concentrations (0.14–100 ng/ml) of rh-bFGF. The cell proliferation was evaluated using an MTT assay kit. Each data indicates the mean and S.D. from six runs.

Fig. 5. Binding Assays of ^{125} I-rh-bFGF to BHK-21 Cells and ACE Cells

BHK-21 cells (A) and ACE cells (B) were incubated ice-cold with various concentrations of ^{125} I-rh-bFGF for 3 h. The binding assays were performed as described in Materials and Methods. Non-specific bindings ($\blacktriangle$) were determined in the presence of 3 µg/ml (for BHK-21 cells) or 6 µg/ml (for ACE cells) of cold rh-bFGF, and were subtracted from the total binding ($\circ$) to determine specific binding ($\bullet$). The cell-associated radioactivities were analyzed by the method of Scatchard.²⁰⁾

synthesis and capillary tube formation, which rh-bFGF exerts at the wound site, contributes to the acceleration of granulation tissue formation.

Fig. 6. Migration of ACE Cells by rh-bFGF, TGF- β , PDGF and EGF

Cell migration was assayed using a microchemotaxis chamber. Lower wells were filled with serum-free DMEM supplemented with various concentrations of rh-bFGF (○), TGF- β (△), PDGF (▽), or EGF (+). ACE cells were incubated for 3 h on the membrane in upper well filled with serum-free DMEM. Migrated cells were counted using a microscope. The data show the mean $\pm$ S.D. from 3 experiments which were performed by 3 runs (Dunnett's one way analysis, * $p < 0.05$, ** $p < 0.01$).

Fig. 7. Production of Plasminogen Activator by rh-bFGF in Endothelial Cells

The assays were carried out in DMEM supplemented with 0.5% CS. PA was extracted with cold 50 mM Tris-HCl (pH 8.5) containing 0.05% Triton X-100 after a 48-h incubation of endothelial cells with rh-bFGF. Plasminogen activator produced (●) was expressed as ng per mg of extracted protein (○). The data (mean $\pm$ S.D.) were plotted as the results from 3 runs.

DISCUSSION

In a full-thickness wound model of diabetic mice, the topical application of rh-bFGF enhanced the recruitment of inflammatory cells and accelerated granulation tissue formation (Fig. 2E). Werner *et al.*^{22,23} have shown that the expression of bFGF mRNA after injury is impaired in diabetic mice. Since a cascade reaction of growth factors is important for the normal wound healing process, any disturbance in this orderly expression would result in impairment of the healing. The present findings on immunohistology suggest an attenuation of TGF- β expression in an early stage of wound repair in diabetic mice (Fig. 2C). bFGF has been reported to stimulate the expression of TGF- β in osteoblastic cells²⁴ and in endosteal cells.²⁵ A supplement of rh-bFGF to the wound site in diabetic mice may allow a normal rate of healing by rapidly mobilizing TGF- β -positive inflammatory cells.

TGF- β is a strong inhibitor of the proliferation of endothelial cells *in vitro*,²⁶ but it induces the formation of capillaries *in vivo*.²⁷ Fafeur *et al.*¹⁴ demonstrated that

Fig. 8. Capillary Tube Formation of ACE Cells in a Three-Dimensional Collagen Matrix

ACE cells were plated on 48-well plates coated with type I collagen and were covered with the same collagen gel after overnight incubation in DMEM supplemented with 10% CS. The total tube length in the same area of sight was measured. The data represent the average $\pm$ S.D. of 4 or 5 runs.

bFGF reduced the inhibitory effect of TGF- β on cell proliferation through a decrease in the number of TGF- β receptors on endothelial cells. Furthermore, it is hypothesized that the ability of bFGF to promote angiogenesis *in vivo* would be potentiated by the co-stimulation of angiogenesis-related endothelial cell functions and counteraction of the inhibitory signal of TGF- β on endothelial cells. It is likely that the application of rh-bFGF to the wound site alters the expression of TGF- β and the exertion of its activity. Recently, it was reported that bFGF had a potent synergistic effect with TGF- β 1 or vascular endothelial growth factor on the induction of angiogenesis *in vitro*.²⁸⁻³⁰ rh-bFGF recruited a number of TGF- β -positive cells into granulation tissue, suggesting an association with TGF- β in the acceleration of the wound healing process (Fig. 2).

Macrophages, which expressed TGF- β and would play a pivotal role in wound healing, increased at the wound site in the rh-bFGF-treated group (Fig. 2F) compared with the diabetic control group (Fig. 2D). Leukocytes in the wound exudate were mainly neutrophils, but their infiltration might not be essential to the healing process.³¹ Leibovitch and Ross³² demonstrated that the depletion of circulation blood monocytes and tissue macrophages resulted in a marked delay in fibroblast proliferation and wound fibrosis. Rappolee *et al.*³³ showed that infiltrating wound macrophages produced not only TGF- β but also other growth factors, including interleukin-1 α , PDGF, and insulin-like growth factor-1. These growth factors may induce a further increase in connective tissue volume.

bFGF is not chemotactic for neutrophils and monocytes, but TGF- β is a potent chemoattractant for macrophages.¹⁰ There should be another way to achieve a rapid increase in macrophage numbers at the site of inflammation. Newman *et al.*³⁴ demonstrated that the

phagocytosis of effete neutrophils by incoming macrophages was one of the mechanisms in the final stage of an inflammatory reaction. Stewart *et al.*³⁵⁾ reported that peripheral blood monocytes were converted to macrophages. bFGF may be intricately involved with inflammatory reactions during the early wound healing process.

An increase in the volume of wound exudate by rh-bFGF might be a result of progression in neovascularization, because the rh-bFGF-treated mice showed swollen loose connective tissue containing many capillaries (Fig. 3C). rh-bFGF stimulated the migration of ACE cells (Fig. 6) and promoted capillary tube formation (Fig. 8). Pepper *et al.*²⁸⁾ reported that the tube formation of bovine lymphatic endothelial cells was also promoted by bFGF. rh-bFGF may urge inflammatory cells to exudate into the wound site by generating capillaries in both the blood vessel and lymphatic systems. In clinical use, it is predicted that an application of rh-bFGF to the wound site may temporarily induce the promotion of exudation.

It is highly possible that the ability of rh-bFGF to potentiate cell migration, proliferation, uPA production, and tube formation is involved in the acceleration of wound healing in a combined fashion. Three to ten ng/ml of rh-bFGF gave maximal responses in each activity studied in the present paper. The results of our binding assays for the high affinity binding sites on cultured cells were in good agreement with those reported by Moscatelli¹⁹⁾ using human placental bFGF; K_d s were 18.4 pM for BHK cells and 20.0 pM for bovine capillary endothelial cells. However, the K_d for BHK cells shown by Neufeld and Gospodarowicz³⁶⁾ using bovine pituitary bFGF was 270 pM. The reason for this discrepancy is unclear, and may be due to differences in the origin species of bFGF. In addition, the K_d calculated for the low affinity binding disagreed with that reported by Moscatelli.¹⁹⁾ Since the low affinity binding sites are heparin or heparan sulfate, the difference may reflect the presence of a clonal variation or differences in culture conditions.

rh-bFGF stimulated the production of uPA on ACE cells (Fig. 7). TGF- β mobilized by inflammatory cells may be released in a latent form and may be retained on the extracellular matrix.³⁷⁾ uPA produced by ACE cells after stimulation by rh-bFGF could generate mature TGF- β from the latent form through converting plasminogen into plasmin.²¹⁾ The increase in mature TGF- β would suppress the actions of bFGF, followed by progression to remodeling stage.

Recently, Hackshaw and Shi³⁸⁾ reported that bFGF induced an increase in the intracellular calcium of peritoneal macrophages. However, further study is remained to elucidate whether bFGF directly promotes the production of TGF- β in the macrophages.

In conclusion, our data suggested that the primary action of rh-bFGF in wound healing may be the recruitment of inflammatory cells such as neutrophils, monocytes and macrophages into the wound site. Exogenous rh-bFGF would thus play a role in the acceleration of wound healing by inducing various growth factors including TGF- β , through the mobilization of inflammatory cells. In the course of wound healing, the

growth factors would modulate each other to advance the healing process, would sometimes compete or cooperate with the others, and might sometimes inhibit overreactions. These interactions might lead to the remodeling stage to complete the healing.

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