Effects of Basic Fibroblast Growth Factor on Irradiated Porcine Skin Flaps

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Objective: To determine the vascular and collagen effects of supplemental basic fibroblast growth factor (bFGF) in irradiated porcine skin flaps.

Intervention: Animals were subjected to 2 fractions of 650 cGy orthovoltage radiation. Following this, the skin flaps were administered bFGF intracuticularly for 6 days before and after surgery. The animals were sacrificed 3 weeks after the start of bFGF administration. Tissues were analyzed for vascularity, collagen content, wound-breaking strength, and histopathological analysis.

Results: The bFGF-treated flaps showed a 62% increase in vascularity compared with controls (10.4%±2.4% vs 6.43%±2.27%; P<.05). The bFGF flaps had a significantly lower collagen concentration compared with control flaps when measured by hydroxyproline content (0.0619±0.0211 nm/µg vs 0.0784±0.0150 nm/µg). Wound-breaking strength was not significantly different, although the bFGF flaps had a trend toward lower breaking strength. Histologically, the bFGF-treated flaps showed increased cellularity, fibroblasts, and extracellular mucopolysaccharides compared with controls.

Conclusions: This study provides evidence that supplemental bFGF can increase vascularity to skin flaps in previously irradiated porcine skin tissue. Histologically, radiation did not prevent the angiogenic effect of bFGF.


Surgery and radiation therapy are combined frequently in treating patients with head and neck cancer. Unfortunately, wound healing following surgery is significantly impaired in previously irradiated soft tissue. Under these circumstances, surgical wounds are more susceptible to poor healing because of decreased vascularity and abnormal fibrosis in these tissues. Decreased vascularity in irradiated tissues is attributable to a lower number of blood vessels and to obliteratorative endarteritis.1, 2 The progressive loss of vascularity proceeds in an almost linear fashion for the rest of the patient's life.3 In addition, a steep oxygen gradient in irradiated tissues is lacking, which is necessary for macrophage chemotaxis and later release of angiogenic growth factors.4 This occurrence leads to delayed healing in response to injury. Poor wound healing can lead to significant clinical problems in the head and neck that include oral cutaneous fistulas, pharyngocutaneous fistulas, salivary fistulas, and open wounds. Even more severe complications are an exposed carotid artery and carotid artery rupture. Prevention of these sequelae is of paramount importance to decrease patient morbidity and mortality.

Increasing the blood supply to irradiated tissues is desirable to improve wound healing. Use of medications has been tried to increase blood flow to skin flaps by vasodilation.6 However, these effects may be only transient. A more advantageous approach would be to increase the number of blood vessels in the tissues. Recently, angiogenic growth factors have shown potential to enhance both vascularity and collagen production in tissues by influencing endothelial cells, fibroblasts, and macrophages.5 By enhancing cellular proliferation and mobilization in these wounds, healing could be improved. Potential beneficial effects of angiogenic growth factors have stimulated much interest in skin flap healing and survival.6, 7

Basic fibroblast growth factor (bFGF) is one of the most potent angiogenic growth factors known. It is a 146-amino acid protein with a molecular weight of approximately 18 000. It is produced by macrophages and endothelial cells. It is found in tissues such as brain, pituitary, retina, corpus luteum, kidney, placenta, adrenals, and prostate. The main target cells of bFGF are fibroblasts and endothelial cells that are responsible for collagen production and neovascularization, respectively.8 Basic fibroblast growth factor has been shown to increase...
MATERIALS AND METHODS

Sixteen white Yorkshire/Hampshire pigs (9-10 kg) were used in the study. The animal use protocol was approved by the Institutional Animal Care and Use Committee of the Minneapolis Medical Research Foundation in Minneapolis, Minn. Each animal had 4 ventrally based arterial skin flaps, 2 on each side measuring 4×7 cm (28 cm²) separated by 4 cm. The blood supply to these flaps was based on the superficial epigastric artery. On each side, one flap was randomly chosen to be treated with bFGF with the other one serving as the control. A reciprocal setup was used on the opposite side. Each pig was used as its own control to eliminate metabolic and nutritional factors between animals, which can affect flap survival and healing.

A radiation port measuring 9×14 cm was marked on each side of the pig, giving a 1-cm border around the flaps (Figure 1). The ports were irradiated with an orthovoltage x-ray machine (Phillips Medical Co, Shelton, Conn). The irradiation parameters were 225 kilovolt (peak) (kVp) 15 mA, filtration with both 3.5-mm copper and built-in 1 mm of aluminum. The skin-to-source distance was 60 cm and the dose rate was 102.3 Gy/min. A 1.5-cm bolus was placed on each port prior to irradiation to reduce visceral organ exposure. Each pig was irradiated in 2 fractions during a 2-week period. An initial dose of 650 Gy was given to each side of the pig. The radiation dose was confirmed by standard thermoluminescent dosimetry. After receiving this initial radiation fraction, the pigs were again irradiated 1 week later under the same conditions with 650 Gy. Six weeks after the initial radiation treatment, the pigs were prepared for bFGF administration and surgery.

A radiation dose of 650 Gy in 2 fractions was used to induce vascular and collagen soft tissue changes. A previous study has shown that a single irradiation dose greater than 1000 Gy decreases endothelial cell density and reduces vascularity in porcine skin after 24 days. Another study showed that a dose of 1863 Gy significantly impairs the survival of porcine skin flaps 5 to 6 weeks after irradiation by altering the blood supply. In the rabbit ear model, irradiation doses greater than 1000 Gy decreased the number of capillaries to less than 20% of that in controls. In this laboratory as a pilot study, 1000 Gy was administered in a fraction to each side of the animal. However, radiation enteritis ensued and a catabolic state became evident among the pigs. To circumvent these complications, the radiation dose was reduced. A dose of 650 Gy in 2 separate fractions is biologically approximated to 900 Gy in a single fraction. Using this radiation protocol preoperatively, no pigs developed signs of radiation enteritis clinically or at autopsy.

Six weeks after radiation exposure, the pigs were prepared for growth factor treatment and skin flap elevation. Basic fibroblast growth factor was administered 6 days before flap construction in a serial intracuticular fashion to the planned flap sites. The growth factor was injected every other day. This made it possible for each planned treated flap to be primed with bFGF before flap elevation. On one side of the animal, the planned treated flap site was chosen at random, with the reciprocal setup on the other side. Each planned flap site was marked with a 1-cm² grid. From this grid marking, each square centimeter of the planned treated flap site was injected intracuticularly with 0.10 mL of bFGF at a concentration of 100 µg/mL. This provided 10 µg of bFGF to each square centimeter of treated tissue. The amount of bFGF chosen (10 µg/cm²) in this study is greater than the levels of bFGF needed to induce an angiogenic response in previous skin flap studies. The planned control flap sites received equal volumes of phosphate-buffered saline solution in a similar fashion. Each flap site received 28 injections over the 28 cm² flap area. During these flap injection procedures, the pigs were sedated with xylazine hydrochloride (15 mg/kg) and ketamine hydrochloride (30 mg/kg) by intramuscular injections.

Before flap construction the pigs were intubated and maintained under general anesthesia with 2% halothane. The ventrally based arterial skin flaps were elevated in a plane below the panniculus carnosus muscle layer. The arterial supply is by the direct cutaneous arteries from the superficial epigastric artery. The flaps were then returned to their original position and stapled in place. A soft elastic dressing (Co-Flex, Andover, Salisbury, Mass) was circumferentially wrapped around the midportion of the pig to prevent the animal from mechanically disrupting the flaps during the healing process. After flap construction, the injections were continued every other day (as before surgery) for the next 6 days, resulting in 7 total injection cycles. Thus, the skin flaps were surgically elevated at the midpoint of the injection sequence. The pigs were sacrificed on postoperative day 14, 8 days after the last injection cycle. Tissue samples were obtained at this time for angiographic analysis, collagen concentration via hydroxypoline analysis, wound-breaking strength measurements, and histological analysis (Figure 2).

At the time of sacrifice, each pig had one set of treated and control flaps randomly selected for the angiographic

RESULTS

Sixteen pigs were involved in the study. To eliminate metabolic and nutritional variability between pigs, treated and control flaps were compared within the same animal. In the vascularization measurements, 13 pigs had angiograms, for a total of 30 flaps (one set of angiograms was not obtained because of technical factors in performing the angiograms). Of 15 treated flaps, 13 treated flaps showed a higher percentage area of vascularization by digital image analysis compared with their respective controls. The treated flaps had a mean (±SD) area of vascularization of 10.4%±2.49% while the controls showed a mean (±SD) of 6.43%±2.20%. Using the...
analysis while the other set was used for histological analysis, collagen content analysis, and wound-breaking strength measurements. To give an accurate assessment of the wound-breaking strength, fresh tissue samples without fixatives were sectioned and measured from one set of flaps. In addition, full-thickness tissue samples from the flaps were taken for analysis of hydroxyproline analysis to measure collagen content.

Neoangiogenesis of the treated and control porcine flaps was evaluated by digitalizing the skin flap angiograms. Angiographic preparation was performed at the time of animal sacrifice. The pigs were sedated and anesthetized as described previously for skin flap elevation. After a thoracotomy the left ventricle was cannulated with a standard intravenous line secured to the myocardium with a silk suture. Heparin at 1 mg/kg was infused intravenously. With the pigs under general anesthesia, they were sacrificed with an intravenous administration of 60 meq of potassium chloride. Thirty percent barium infusion was administered through the intravenous line in the left ventricle. After several minutes, an incision was made in the right atrium and the animal was exsanguinated for 30 minutes. Next, 30% barium in 10% formalin solution was infused for a total volume of 1000 mL. At this point, the flaps were removed and fixed in 10% formalin solution for at least 24 hours. This allowed for preservation of the tissue for imaging the flap vasculature.

The angiograms for each pig were imaged by mammography techniques to maximize the fine detail of the capillary vessels. The mammography exposure settings were at 27 kVp, magnification ×1.7 using 20×25-cm film. A computerized digitizer (ICU Digitizer, Kodax, Rochester, NY) was used to store the image in a computer, 2048×3260 pixels. Two separate regions measuring 2×4 cm were randomly chosen on each flap. These areas were then analyzed on the computer to calculate the percentage of vascular area. These regions were filtered to remove low-frequency variations in density then thresholded to form binary image-segmenting vascular areas from the background. The vascular area of the treated flaps was compared with that of the control flaps on the same pig. New collagen formation was measured by 2 modalities, the hydroxyproline assay and wound-breaking strength measurements. Collagen quantification of the flap samples was obtained by determining hydroxyproline concentration. One set of flaps from each animal was used for hydroxyproline analysis. A full-thickness core biopsy specimen was obtained from each of the treated and control flaps. These tissue specimens were removed from the midline of the flap, 1 cm from the distal edge. Since hydroxyproline is the basic subunit of collagen, assessment of its concentration indirectly reflects the amount of collagen present in the skin flaps. The amount of tissue sampled was measured on a standard balance (Mettler BasBal No. BB240, Mettler-Toledo Inc, Hightstown, NJ). These samples were measured fresh, allowing determination of the wet mass of the biopsy specimens. The tissues were then hydrolyzed and processed from the method proposed by Moore and Stein. The resultant material was run on an amino acid analyzer (6300 Amino Acid Analyzer, Beckman Instruments Inc, Edina, Minn) using a software program (Beckman System Gold Data System, version 8.1) to determine hydroxyproline content. The hydroxyproline concentration was determined by dividing the amount of hydroxyproline present by the known mass of the sample. These sample measurements were compared with each other from the same animal.

Wound-breaking strength measurements were made from the same flaps used for the hydroxyproline analysis. A total of 9 pigs (18 flaps) were analyzed. These measurements were made on fresh tissues less than 8 hours old. This quantity gives an indirect measurement of relative collagen formation and cross-linking. Three strips of skin were removed from one treated flap and from one control flap using a standardized industrial cutter at the time of animal sacrifice. Thus, each pig had 3 control and 3 treated samples of skin. These tissues were maintained in saline solution surrounded by ice until the measurements were performed. The tissues were mounted on an industrial tensiometer (Instron Model No. 1130, Instron Corp, Canton, Mass, courtesy of Medtronics, Minneapolis, Minn). The tension on the skin samples was increased until separation of the tissues occurred at a constant crosshead speed of 0.51 cm/min. The maximal force required to disrupt the incision line was defined as the wound-breaking strength. Three measurements were performed with 3 incisions on each flap. These measurements of the treated and the control flap samples were compared statistically within each animal.

Histopathological analysis was also carried out on tissue samples obtained from the porcine flaps at the time of sacrifice. Each skin flap was sectioned transversely at the proximal, middle, and distal thirds. The tissues were fixed in a 10% neutral buffered formalin solution and embedded in paraffin. Sections were stained with hematoxylin-eosin. The tissue sections were qualitatively analyzed for radiation-induced changes and bFGF effects. The treated and control flaps from the same animal were compared against each other in a blinded fashion.

Nine animals (18 flaps) were used for wound-breaking strength analysis. With these measurements, a large variability was evident within the same flap among the 3 incisional samples for each animal. Averaging these 3 measurements proved impractical because of this large variability. Therefore, it was decided to take the highest one of these measurements from each flap when the control and treated values in each animal were compared. The bFGF-treated incisions had a mean (±SD) value of 3.180±1.297-lb wound strength while the control incisions had a mean (±SD) of 4.714±1.927-lb wound strength. There was a trend toward lower wound strength in the treated group compared with the control group in the same animal. However, this difference was not significant (paired Student t test, P=.07) (Figure 5).
By gross inspection, the bFGF-treated flaps showed increased flap thickness in the dermal, subcutaneous, and muscular layers. Histological analysis demonstrated that radiation-induced changes in the treated and control flaps. These changes were most evident in the vascular structures in the flaps. Histologically, the vascular findings resulting from radiation included mucoid degeneration of the vessel wall; balloon degeneration of the endothelial cells; hyperchromasia of endothelial cell nuclei; vessel lumen narrowing; and smooth muscle damage in the vessel wall. These alterations varied in intensity but were consistently present (Figure 6). In the bFGF-treated flaps, there was cellular proliferation in the wall of dermal vessels as well as an increased number of smooth muscle cells in arrectores pili muscles (Figure 7).

Among the treated flaps, the epidermal layer was thickened and displayed acanthosis; the dermal layer had increased cellular density. The collagen matrix appeared somewhat less dense in the treated flaps because of an increase in extracellular substance. The bFGF-treated flaps showed increased numbers of fibroblasts and extracellular mucopolysaccharides in the dermis. This resulted in a greater distance between collagen fibers, thereby decreasing the collagen density in the dermis (Figure 8).

The subcutaneous tissues in the treated flaps demonstrated similar findings, but to a lesser degree than those described in the dermis. The septa in the panniculus carnosus of treated flaps were thicker and hypercellular due to fibroblastic proliferation.

The tissues deep to the panniculus carnosus muscle also showed similar histological changes in the treated
flaps. In addition, prominent inflammatory infiltrates consisting of lymphocytes and variable numbers of neutrophils and eosinophils were evident. By gross inspection, this layer of tissue deep to the panniculus carnosus muscle appeared thicker among treated flaps compared with the controls. The gross and histological findings in each flap were similar at the proximal, middle, and distal flap sites.

**COMMENT**

In this study, supplemental bFGF, when given by serial intracuticular injections, increased skin vascularity in previously irradiated porcine dermal tissues. Among the treated flaps, the growth factor continued to increase neovascularization over controls even in the presence of previous radiation. Thus, the vascular effect of bFGF on endothelial cells to stimulate angiogenesis was not arrested by irradiation. This is an important finding since one of the main deleterious effects of radiation to surgical wounds is a decreased blood supply. Whether this increased vascularization from bFGF in porcine flaps is clinically relevant remains undetermined.

The bFGF-treated flaps showed a lower hydroxyproline concentration compared with control flaps, corresponding to a lower collagen content. This finding was also evident from the histological findings of increased extracellular mucopolysaccharides present in the dermis of the bFGF-treated flaps. An increased amount of extracellular matrix and fluid between the collagen fibers would lower the collagen concentration. Fibroblasts and the FGFs are not only involved in producing collagen but are also involved in the production of acid mucopolysaccharides. These findings are consistent with some studies showing that bFGF-treated wounds result in less collagen accumulation. Additionally, the treated flaps were grossly more edematous than the controls. This factor may have played a role in hydroxyproline analysis since the tissue samples were weighed fresh as wet weight. Thus, the latter measurement would be larger secondary to tissue edema. Correspondingly, the hydroxyproline concentration in the treated flap samples would then be lower.

Wound-breaking strength, defined as the force necessary to disrupt the incision, is influenced by collagen production and cross-linking. Wound-breaking strength was lower in the bFGF-treated flaps compared with that in controls. Several possibilities may contribute to this finding. One possible explanation is that bFGF flaps were consistently thicker and more edematous, putting additional stress on the flap incision. Another possible explanation is that a lower concentration of collagen, which was evident in the treated flaps, resulted in a weaker flap incision in treated flaps. A previous study in the rat model also showed a decreased wound-breaking strength after application of bFGF. In addition, other studies have shown that bFGF stimu-
lates collagenase production from granulation tissue–derived fibroblasts that would decrease wound-breaking strength. The inflammatory process present could also have played a role. Collagenases are released by macrophages and neutrophils during this process to assist in their movements. Thus, as the treated flaps demonstrated higher numbers of inflammatory cells, there may have been a correspondingly higher amount of collagenases in these flaps. Recently, bFGF has been shown to down-regulate type I collagen gene expression in keloid fibroblasts and normal fibroblasts. In addition, the time course of the collagen measurements within the flap from bFGF may also be an important variable. This is because in other porcine skin models, bFGF demonstrated increased collagen deposition only within a 3- to 7-day interval after wounding.

The histological effects of bFGF were not prevented by irradiation. While both treated and control flaps showed alterations consistent with radiation therapy, the treated flaps showed bFGF-mediated changes. Whether these effects from bFGF are clinically significant remains to be evaluated. The fact that the changes observed in histological sections from the proximal, middle, and distal thirds of each flap were not different indicates that the protocol of bFGF administration results in uniform distribution and similar effects throughout the treated flaps.

Several limitations of this study must be stated. The radiation dose given to the porcine skin is not completely analogous to that given preoperatively to humans. In addition, the time course of testing the skin 5 to 6 weeks after irradiation does not allow enough time for the late effects of radiation to fully manifest. However, in this study, attempts were made to reveal the short-term changes of vasculature and collagen by bFGF in the setting of previous irradiation. The porcine skin flap model was chosen since pig skin is closely analogous to human skin in its vascular anatomy.

In conclusion, this study demonstrates the potential of bFGF to increase vascularization in irradiated porcine skin flaps. Could it be that in irradiated soft tissue wounds, native bFGFs are more depleted or inactive? By answering such questions and by improving our understanding of the effects of bFGF in irradiated soft tissue, it may be possible to better manage these difficult-to-heal wounds. Further studies are needed to explore the role of bFGF in irradiated soft tissues.

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