Gelatin Sheet Incorporating Basic Fibroblast Growth Factor Enhances Healing of Devascularized Sternum in Diabetic Rats

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Background—Poor healing of the sternum often limits the use of bilateral internal thoracic arteries (BITAs) after coronary bypass surgery in diabetic patients. We have reported that a gelatin sheet that incorporates basic fibroblast growth factor (bFGF) accelerates sternal healing after BITA removal in normal rats. This study evaluated the effects of the above method for sternal healing in diabetic animals.

Methods and Results—Diabetic Wistar rats with blood glucose levels >400 mg/dL and body-weight loss >20 g were established by a single intravenous injection of streptozotocin (55 mg/kg). After median sternotomy and BITA removal, 16 diabetic rats received either a gelatin sheet that incorporated bFGF (100 μg/sheet) on the posterior table of the sternum (FGF group, n=9) or no gelatin sheet (control, n=7). Peristernal blood flow, as measured by a noncontact laser Doppler 4 weeks after surgery in the FGF group, recovered to the preoperative level (106±10% versus 82±9%, P<0.01), and marked angiogenesis was also observed around the sternum in the FGF group (30.5±3.2 versus 15.8±2.7 vessels/unit area, P<0.01). Deep sternal wound complications developed in 5 control rats but only in 1 rat in the FGF group (P<0.05). In the FGF group, histological examination showed improved sternal healing (excellent in 6 rats and slow/poor healing in 3). Bone mineral content as assessed by dual-energy x-ray absorptometry was greater in the FGF group (75.9±18.1 versus 48.9±10.7 mg, P<0.05). Bone mineral density of the sternum was similar between the 2 groups.

Conclusions—A gelatin sheet that incorporates bFGF may offset sternal ischemia and accelerate sternal bone regeneration and healing, even in diabetic patients. (Circulation. 2001;104[Suppl I]:I-325-I-329.)

Key Words: angiogenesis ■ growth substances ■ diabetes mellitus

Recently, some reports have demonstrated that the use of bilateral internal thoracic arteries (BITAs) for CABG improves long-term survival compared with use of a single internal thoracic artery (ITA).1,2 However, it is widely recognized that harvesting BITAs raises the potential for an increase in sternal wound complications after median sternotomy, because BITAs provide the major blood supply to the sternum. Sternal wound complications prolong the hospital stay, increase healthcare costs considerably, and may delay return to work or social activities. Although deep sternal wound infection is rarely encountered, occurring in 0.7% to 2.3% of cases,3,7 the potential for serious morbidity and mortality is well known. Previous studies7,8 confirmed that BITA grafting in patients with diabetes is a risk factor for sternal wound complications. Therefore, despite their benefits, the use of BITA grafts is limited for diabetic patients, whose coronary arteries are more complicated than those of nondiabetics.

Basic fibroblast growth factor (bFGF) is one of the potent mitogens regulating proteins that induce the proliferation of a variety of cells, including epithelial and mesenchymal cells, and promote the growth and regeneration of organs and tissues in vivo.9 Because bFGF in free form did not have biological activities sufficient to induce the expected results, we developed a biodegradable hydrogel composed of acidic gelatin to enable bFGF to be released at the site of action for a sufficient time period.10 We have already reported that the topical use of a gelatin sheet that incorporates bFGF on the sternum after removal of the BITA accelerates sternal healing in rats by accelerating normal bone regeneration and remodeling of the sternum.11,12 The present study evaluated the effectiveness of the gelatin sheet with bFGF on sternal healing in rats in which diabetes was induced by administration of streptozotocin.
Methods

Preparation of bFGF-Incorporating Gelatin Hydrogel Sheets

Gelatin with an isoelectric point of 4.9 was isolated from bovine bone collagen by an alkaline process with Ca(OH)\textsubscript{2} (Nitta Gelatin Co). Human recombinant bFGF with an isoelectric point of 9.6 was supplied by Kaken Pharmaceutical Co (Tokyo, Japan). Gelatin hydrogel sheets were made by the same process described previously.\textsuperscript{19} Sheets were freeze-dried, followed by impregnation with an aqueous solution containing 100 \mu g of bFGF, to obtain gelatin hydrogels that incorporated bFGF. The prepared hydrogel sheets were rectangular (1 × 10 mm) and 0.7 mm thick. All experimental processes were conducted under sterile conditions.

Animal Experiments

Wistar male rats weighing between 300 and 400 g were used for diabetic models. Diabetic rats were created by a single intravenous injection of 55 mg/kg streptozotocin (Wako Chemicals) in 0.1 mol/L citrate buffer (pH 4.9). Diabetes mellitus was defined by both an increase in blood glucose levels >400 mg/dL and a loss of body weight >20 g 1 week after the injection.

Sixteen diabetic rats were orally intubated after anesthesia with 99.5% ether and ventilated on a small volume-cycled animal ventilator (dove ventilator model 683, Harvard Apparatus). Anesthesia was maintained during surgery with 1% to 2% isoflurane. After a midline skin incision was made with the animal in a supine position, the bilateral major pectoral muscles were divided from the junction of the sternum, and intercostal muscles on both sides of the sternum were exposed. A median sternotomy was carefully performed with a rotating saw (D-7200, Aesculap), leaving part of the narrow sternum on both sides. Bleeding from the bone marrow was stopped with bone wax. BITAs were ligated with 6-0 polypropylene sutures near the takeoff and at the distal bifurcation, and the BITAs and their beds were destroyed with an electrical coagulator. The rats were randomly divided into 2 groups: rats in the FGF group (n=9) had the BITA removed, and a gelatin hydrogel sheet that incorporated bFGF was placed on the posterior surface of the sternum before the sternum was closed; rats in the control group (n=7) had the BITA removed and the sternum closed without use of the sheet. When the sheets incorporating bFGF (100 \mu g/sheet) were placed in the animals, the destroyed ITA beds were also completely covered by the sheet from the inside of the chest wall, and the implant was stabilized with 6-0 polypropylene sutures on all sides to avoid movement behind the sternum. After positive end-expiratory pressure was applied to fully inflate the lung, the sternum was closed parasternally with 4 interrupted braided polyester sutures. The muscle layer and skin were then carefully sutured with 4-0 nylon monofilaments. Streptomycin (50 mg per rat) was given intramuscularly just after skin closure. All animals were killed by intravenous administration of a lethal dose of sodium pentobarbital 4 weeks after surgery. The sternum was excised and fixed in 10 wt% EDTA solution in PBS solution for 4 days to assess the extent of bone regeneration. All of the animal experiments were performed according to the institutional guidelines on animal experimentation at Kyoto University.

Measurement of Peristernal Blood Flow

Peristernal blood flow (mL · min\textsuperscript{-1} · 100 g\textsuperscript{-1}) at the capillary blood perfusion level was measured with a noncontact laser flowmeter (ALF21N, Advance) before median sternotomy, after closure of the sternum, and 4 weeks after surgery. A beam of laser light was directed through an optic fiber to a measuring probe with a diameter of 3.0 mm. The probe was placed over the intercostal muscles near the sternum, separated by 10 mm in a straight line, so that the area of measurement was ~5 mm in diameter and 1 mm deep. The He-Ne light was then switched to a diode laser (2 mW, 780 nm) to measure blood flow, which was calculated based on Doppler shift. Three readings for each measurement were recorded after a stable baseline had been obtained, and the 3 values were averaged.

Histological Assessment of Angiogenesis

Arterioles (>25 and <100 \mu m in external diameter) and capillaries (<25 \mu m in external diameter) were counted in preparations stained with hematoxylin and eosin. Five fields were chosen randomly from the connective tissue around the sternum. Two pathologists blinded to treatment counted the number of vessels per unit of area (200 × 200 \mu m\textsuperscript{2}) using a grid method,\textsuperscript{13} whereby the density of arterioles in each 5 × 5-mm field was assessed by determining the mean number of vessels in 5 randomly chosen unit areas (200 × 200 \mu m) using a section ocular micrometer (Olympus) at ×400 magnification. The total number of vessels in 25-U areas (5 fields with 5-U areas per field) was counted and averaged. To maintain randomness, an optic lens containing a protractor and micrometer was used for selection of the 5 portions.

Histological Assessment of Sternal Bone Formation

Bone regeneration in the sternum was assessed by soft x-ray analysis and histological examination. Soft (high-contrast) x-ray pictures of the sternum were taken at 46 kV, 2 mA, for 45 seconds with an x-ray apparatus (type CMB; Koizumi X-Senkoshis). Photographs of formalin-fixed bone specimens from different experimental groups were taken with the same type of x-ray film.

Bone specimens were demineralized in 10 wt% EDTA solution at 4°C for 3 days, embedded in paraffin, and sectioned at 10 \mu m thickness. The sections were obtained at the third, fourth, and fifth intercostal spaces of the sternum and stained with hematoxylin-eosin 2 and 4 weeks after surgery. The histological sections were analyzed with a microscope equipped with a video camera, which was connected to an image-analysis system (SP-1000, Olympus). The area of new bone in each section prepared from the sternum was measured at ×2 magnification.

Qualitative and Quantitative Analysis of Regenerated Sternal Formation

Bone regeneration of the sternum was assessed by dual-energy x-ray absorptiometry and histological examinations. The bone mineral density and bone mineral content of each sternum was measured with dual-energy x-ray absorptiometry utilizing a bone mineral analyzer (Dichroma Scan 600, Aloka Co) 4 weeks after surgery. The instrument was calibrated with a phantom of known mineral content. Each scan was performed at a speed of 20 mm/s, and the scanning length was 1 mm.

Statistical Analysis

Experimental results are expressed as mean ± SD. Statistical analysis comparing 2 groups was performed with unpaired 2-tailed Student’s t test for means or \chi\textsuperscript{2} test for categorical variables. Results of statistical analyses were regarded as significant when the P value was <0.05.

Results

Assessment of Angiogenesis Around the Sternal

Figure 1 shows peristernal blood flow just after removal of the BITA and 4 weeks after surgery in each group. These flows just after BITA removal had shown similar decreases in both groups (FGF group 57 ± 7% versus control group 55 ± 4% of preoperative level). Four weeks after surgery, peristernal blood flow in the FGF group recovered to the preoperative level (106 ± 10% versus 82 ± 9% in control group, P<0.01).

Histological sections of tissue around the sternum also showed an increase in vascular number in the FGF group. There were more capillaries and arterioles (10 to 50 \mu m in diameter) around the sternum in the FGF group than in the control group (Figure 2). Figure 3 shows the number of arterioles and capillaries per unit area around the sternum 4
weeks after surgery in both groups. In the FGF group, a larger number of vessels were seen in the connective tissue around the sternum, and the number of arterioles and capillaries per unit area around the sternum increased to a greater extent in the FGF group than in the control group (FGF group 30.5±3.2 versus control group 15.8±2.7 vessels/unit area, \( P<0.01 \)).

**Histological Assessment of Bone Formation**

Four weeks after surgery, deep sternal wound complications (DSWCs) developed in 5 control rats but only in 1 rat in the FGF group (71.4% versus 11.1%, \( P<0.05 \)). DSWC was diagnosed by a soft x-ray photograph of the sternum. Obvious dehiscence of the separated original sternum was observed only in rats with DSWCs (Figure 4C). All rats with sternal dehiscence had developed macroscopic abscesses in the anterior mediastinum. Moreover, the control group without DSWCs did not demonstrate sternal regeneration (Figure 4B). In contrast, all but 1 rat in the FGF group had no sternal dehiscence and had almost complete bone regeneration (Figure 4A).

Figure 5 shows histological sternum sections 4 weeks after surgery by different procedures. Slight enchondral ossification around the original sternum was observed in the control group (Figure 5B). Conversely, FGF rats had almost completely healed sternums filled with regenerated bone tissue and bone marrow (Figure 5A), except for 2 rats with slow sternal healing and 1 rat with poor sternal healing due to DSWC.

**Qualitative and Quantitative Analysis of Regenerated Sternal Formation**

Bone mineral content assessed by dual-energy x-ray absorptometry was significantly higher in the FGF group, as illustrated in Figure 6 (75.9±18.1 versus 48.9±10.7 mg, \( P<0.05 \)). However, the bone mineral density of the regenerated sternum was quite similar between the 2 groups (50.6±15.6 versus 45.9±5.0 mg/mm²), which suggests normal bone quality in all groups.

**Discussion**

Diabetic patients have been demonstrated to be at higher risk of complications and to have a higher long-term mortality rate after CABG than nondiabetic patients. Specifically, diabetes mellitus might cause DSWC after CABG. Cosgrove et al described diabetes mellitus as a risk factor for wound complications after CABG on multiple logistic regression test. However, the population of diabetic patients who undergo CABG has a tendency to increase, and these patients usually have diffuse coronary artery lesions that involve distal vessels. ITAs have been preferred for CABG in diabetic patients because the ITA graft maintains excellent late graft patency, whereas saphenous vein grafts may not remain patent. Recently, some reports have demonstrated that the use of BITA grafts improves long-term survival and postoperative ischemic events after CABG compared with the use of a single ITA graft. Unfortunately, the use of BITA grafts usually has been avoided in diabetic patients because of the higher prevalence of DSWCs.

Recently, harvesting of BITAs in a skeletonized fashion has been recommended to prevent a decrease in sternal blood flow postoperatively. Cohen et al showed that although a pedicled ITA graft to the left anterior descending artery reduced blood flow to the left side of the sternum by assessment with single photon emission computed tomography, a skeletonized ITA did not result in such a reduction. Jesus and Acland classified the collaterals into 6 types in a human anatomy study. If the collaterals connect to the side wall of the ITA (ie, no vertical common channel), even a careful skeletonization method can destroy the collaterals from the lateral chest wall to the sternum. Thus, skeletonization may be useful for some but not all patients who need or would potentially benefit from extended use of BITA for coronary revascularization. Our previous study showed that our method improved sternal healing after BITA removal compared with that in the control group with no ITA removal;
this suggests that the method enhances sternal healing even if the sternal blood supply is well maintained.\textsuperscript{11,12} Calafiore et al\textsuperscript{19} analyzed 1146 patients undergoing isolated myocardial revascularization using pedicled or skeletonized BITA grafts and found that sternal wound-healing problems were present in 1.7\% of cases despite the use of skeletonized BITA conduits. Moreover, the incidence of these complications in diabetic patients was also higher than that in nondiabetic patients in their large series. Conversely, our novel trial using bFGF for sternal healing showed that the use of BITA grafts can increase blood supply to the devascularized sternum and enhance bone regeneration even in diabetic rats. With regard to sternal blood supply, peristernal blood flow in the FGF group increased significantly 4 weeks after surgery compared with that in the control group. Our histological study of blood vessels around the sternum showed an increase in vascular number, which suggests that the increased peristernal blood flow in the FGF group was associated with the angiogenic effect of bFGF. Unfortunately, a DSWC developed in 1 rat in the FGF group despite improvement of the sternal ischemia. Intensive diabetic therapy with improved blood glucose control has been shown to prevent impairment of the ability of the white blood cells to phagocytose and effectively kill bacteria.\textsuperscript{20,21} Our diabetic rats did not receive any treatment during the perioperative period, and their elevated blood glucose level remained $>400$ mg/dL postoperatively. We believe that our method can prevent DSWC more effectively when used in combination with perioperative glucose control.

It has been reported that bFGF is not only a potent angiogenic mitogen but also an effector that can stimulate bone formation.\textsuperscript{22} This stimulation occurs by callus formation, as a result of the mitogenic effect on periosteal cells, and by osteoclastic callus resorption. Our results also revealed

\begin{figure}
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\includegraphics[width=\textwidth]{figure4.png}
\caption{Radiograph shows sternal bone regeneration 4 weeks after surgical treatment in diabetic rats. A, Almost complete sternal regeneration was seen in FGF group. B, Slow/poor sternal regeneration was shown in control group. C, Obvious sternal dehiscence due to DSWC was observed in control group (arrow).}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure5.png}
\caption{Histological cross sections of hematoxylin and eosin–stained sternum were obtained 4 weeks after surgical treatment. A, Regenerated sternal bone with bone marrow completely surrounded original sternum in FGF group. B, Regenerated sternal bone with bone marrow was observed partially in control group. NB indicates new bone with bone marrow; OS, original sternum.}
\end{figure}
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that bFGF enhanced normal sternal regeneration by quantitative and qualitative analyses with dual-energy x-ray absorptiometry. However, detailed histological assessment of the regenerated sternum showed slow healing in 2 rats in the FGF group. It is well known that bone fractures in diabetic patients heal poorly. A variety of mechanisms have been proposed to explain the inhibition of the repair process.23,24 Kawaguchi et al25 demonstrated that all of the healing steps were markedly inhibited in the diabetic rat fracture model. In addition, their immunohistochemical studies revealed that the appearance of bFGF at the fracture site was markedly impaired in diabetic rats and could be restored by insulin treatment. Control of the blood glucose level in diabetic patients may be important for bone regeneration.

There were some limitations in this experimental study. First, our models in which BITAs were destroyed by an electrical coagulator may not be analogous to a patient with BITA harvest. The second limitation was that this model has a narrow and thin sternum with anatomic features that differ from those of humans. Additional investigations with large animal models are needed before studies in humans can be performed.

In conclusion, the use of a gelatin hydrogel sheet that incorporates bFGF offsets sternal ischemia and facilitates healing despite BITA removal even in diabetic rat models, probably because of the angiogenic and osteogenic effects of bFGF. Moreover, acceleration of normal sternal healing by bFGF decreased the incidence of DSWCs. This therapeutic approach would potentially facilitate the sternal healing by bFGF decreased the incidence of DSWCs. Moreover, acceleration of normal diabetic rat models, probably because of the angiogenic and healing despite BITA removal after sternotomy even in performed.

References


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