Effects of Intramyocardial Administration of Slow-Release Basic Fibroblast Growth Factor on Angiogenesis and Ventricular Remodeling in a Rat Infarct Model

Zhan-Qiang Shao, MD; Kentaro Takaji, MD; Yukihiro Katayama, MD; Ryuji Kunitomo, MD; Hisashi Sakaguchi, MD; Zhong-Fang Lai, MD*; Michio Kawasuji, MD

Background  Basic fibroblast growth factor (bFGF) stimulates neoangiogenesis. Incorporation into bioavailable gelatin hydrogels provides the sustained release of bFGF. The effects of intramyocardial injections of slow-release bFGF on neoangiogenesis in a rat model of infarction were investigated.

Methods and Results  Myocardial infarction was induced in rats using coronary artery ligation. A total of 124 rats received an intramyocardial injection of 20 μg of bFGF, the same amount of bFGF incorporated into gelatin hydrogel (bFGF+gel), gelatin hydrogel (gel) or saline. Ventricular function was evaluated by echocardiography 2 or 4 weeks later. Morphometric and histological analyses were used to evaluate infarct size, vascular density and myocardial apoptosis. Capillary density in the infarct border zone was higher in the bFGF and bFGF+gel groups than in the saline and gel groups at 4 weeks (p<0.001). Arteriolar density was higher in the bFGF+gel group than in the other 3 groups (p<0.05). The bFGF and bFGF+gel groups contained fewer apoptotic cardiomyocytes in the border zone than the saline and gel groups (p<0.01). The bFGF+gel group had thicker (p<0.05) and less expanded infarcts (p<0.01) compared with the saline group at 4 weeks.

Conclusions  Incorporation of bFGF in gelatin hydrogels enhanced the effects of bFGF on arteriogenesis, ventricular remodeling and cardiac function. (Circ J 2006; 70: 471–477)

Key Words:  Angiogenesis; Apoptosis; Ischemic heart disease; Myocardial remodeling

Both coronary artery bypass grafting and percutaneous coronary intervention ameliorate angina pectoris, prevent myocardial infarction and improve the long-term survival of patients with atherosclerotic coronary artery disease. However, the nature of coronary arteries significantly impacts the quality of life. Standard therapies for myocardial revascularization are often limited because of diffuse lesions or small-caliber vessels. Angiogenic therapy to induce myocardial neovascularization is not dependent on vessel caliber and provides an alternative treatment alone or in combination with standard revascularization. Basic fibroblast growth factor (bFGF) is a potent angiogenic protein that induces endothelial and smooth muscle cell proliferation in vivo and elicits angiogenesis that includes the migration and proliferation of endothelial cells, vascular tube formation and linkage to the extant vascular network. Intracoronary injections of bFGF reduce infarct size in a canine model of myocardial infarction and improve myocardial function in chronically ischemic porcine hearts. We have previously shown that the intramyocardial administration of bFGF increased the number of capillaries and arterioles in the peri-infarct region, increased regional myocardial blood flow and consequently improved ventricular function in a canine infarction model. However, angiogenesis induced by growth factors has not been always successful. Despite high binding affinity for acidic polysaccharides such as heparin and heparan sulfate in the extracellular matrix, bFGF has a short biological half-life in tissues. The sustained release of bFGF might help to enhance its angiogenic activity in vivo. Gelatin is a nontoxic, biodegradable, natural polymer with low antigenicity and gelatin hydrogel is considered to be a preferable matrix for the sustained release of protein drugs, such as growth factors. However, the effects of intramyocardial injections of slow-release bFGF on vascular growth, cardiomyocyte apoptosis and cardiac function have not been investigated in detail. The present study evaluates the ability of gelatin hydrogels to enhance the benefits of bFGF on neoangiogenesis, cardiomyocyte apoptosis, myocardial fibrosis, ventricular remodeling and cardiac function in a rat infarction model.

Methods

Preparation of bFGF

Human recombinant bFGF and gelatin hydrogels were provided by Kaken Pharmaceutical, Tokyo, Japan. Gelatin with an isoelectric point of 4.9 was prepared using alkaline treatment of bovine collagen with Ca(OH)₂. To obtain gelatin hydrogels that incorporated bFGF, 0.7 ml of saline containing 350 μg of bFGF was impregnated into freeze-dried hydrogels. The release of biologically active bFGF is sustained as a result of hydrogel degradation in vivo. Empty gelatin hydrogels free of bFGF were similarly prepared.
Myocardial Infarction Model

Adult male Sprague–Dawley rats (n=124) weighing 275–320g received humane care in compliance with the Rules of the Animal Experimentation Committee, Kumamoto University, Graduate School of Medical Sciences and the Guidelines for Animal Experimentation (1987), published by the Japanese Association for Laboratory Animal Sciences. The study protocol was approved by the Animal Experimentation Committee, Kumamoto University, Graduate School of Medical Sciences and the guidelines of the Animal Experimentation Committee, Kumamoto University. Ventilated animals were anesthetized using ether inhalation and a subsequent intraperitoneal injection of pentobarbital. Left thoracotomy through the fourth intercostal space was performed under sterile conditions. The left anterior descending coronary artery was ligated approximately 2–3 mm distal from its origin with a 6-0 polypropylene suture. Thirty minutes later, surviving animals were assigned to 4 groups. The myocardium of the bFGF group (n=35) was injected at 6 points of the border zone surrounding the infarct with 20 μg of bFGF in 57 μl of saline using a micro-needle and a microsyringe. The bFGF + gel group (n=35) received 20 μg of bFGF incorporated into 57 μl of gelatin hydrogels. The gel group (n=14) received 57 μl of gelatin hydrogels in the same manner. The saline group (n=40) received the same volume of saline. After reaching hemodynamic stability, the chest was closed and the animals were allowed to recover.

Cardiac Function Study

Cardiac function was measured 2 or 4 weeks after coronary ligation using a commercially available echocardiography system (SONOS 4500, Philips, Eindhoven, Netherlands). Left ventricular pressure and dp/dt were measured and data were recorded on a thermal arraycorder (WR8500-UM-101; Graphtec, Yokohama, Japan).

Morphometric Study

After completing the physiological study, the hearts were arrested at diastole by infusing 15% KCl through the left carotid artery, excised and weighed. Specimens were coded and fixed in 10% buffered formalin. Four short-axis, 3-mm-thick slices were prepared from the ventricle, embedded in paraffin and then cut into 5-μm-sections for subsequent morphometric and histopathological analyses. The sections were then stained with Sirius red (Polysciences, Netherlands).
Angiogenesis With Slow-Release bFGF

Warrington, PA, USA) and evaluated using a colored-image analysis system. Images of the samples were acquired using a microscope (SZH10, Olympus, Tokyo, Japan) with a video camera at 7× magnification (KY-F55MD, Olympus, Tokyo, Japan) (Fig 1). Ventricular morphology was evaluated using image analysis software (Mac SCOPEC, Mitani Corporation, Fukui, Japan). The bottom of the third slice from the apex of the heart, which represented the mid-papillary level of the left ventricle, was morphometrically analyzed. Infarct size was determined as the percentage of epicardial and endocardial circumference occupied by the infarct on all heart slices and calculated as follows: Infarct size (%) = (Infarct endocardial length/Total endocardial length)/2 + (Infarct epicardial length/Total epicardial length)/2. The infarcted wall thickness was determined as the ratio of the thickness of the infarcted wall to that of the noninfarcted septal wall. The left ventricular cavity area and total left ventricular area were measured and the expansion index was calculated as follows: Expansion index = (Left ventricular cavity area/Total left ventricular area)×(Non-infarcted septum thickness/Infarct wall thickness).

Collagen-positive areas were calculated as Sirius red-stained collagen using the same image analysis software. Collagen density is expressed as the percentage of the collagen area compared to the total myocardial tissue area.

Histological Study

To detect angiogenic activity, endothelial cells were stained with anti-von Willebrand factor antibody (Zymed, South San Francisco, CA, USA) and smooth muscle cells were stained with an antibody to α smooth muscle actin (Dako, Glostrup, Denmark). Capillaries were identified as having a diameter <20 μm, a thin layer of endothelial cells and no smooth muscle cells. Arterioles were similarly identified as being ≥20 μm, <100 μm and having a thin layer of smooth muscle cells. Ten fields in the border zone at 400× magnification were selected, and the number of blood vessels are presented as the mean per unit area (0.2 mm²).
Cardiomyocyte apoptosis was determined using terminal deoxynucleotide transferase-mediated dUTP nick-end labeling (TUNEL) assays using an Apop Tag® Plus In Situ Apoptosis Detection Kit (Chemicon, Temecula, CA, USA). The number of TUNEL-positive cardiomyocyte nuclei and the total number of cardiomyocyte nuclei in the same area of 8 fields were counted in the border zone at 400× magnification. The number of apoptotic cardiomyocytes is expressed as the proportion of the TUNEL-positive cardiomyocyte nuclei to the total number of cardiomyocyte nuclei. Myocardial fibrosis was evaluated by staining with Masson’s trichrome and then the ratio of fibrotic to left ventricular areas was calculated.

Statistical Analysis
All values are expressed as means ± SD. Data were statistically evaluated using the analysis of variance (StatView 5.0, SAS Institute, Berkeley, CA, USA). Dichotomous variables were compared with Fisher’s exact test. Differences were considered statistically significant at p<0.05.

Results
Of the 124 rats that underwent coronary artery ligation, 103 (83.1%) survived for 2 or 4 weeks. All 29 (82.9%) rats in the bFGF group, 30 (85.7%) in the bFGF + gel group, 12 (85.7%) in the gel group and 32 (80%) in the saline group that survived had transmural infarctions. Gelatin hydrogels were found in the myocardium of 11 (64.7%) of 17 hearts in the bFGF + gel group and in 3 (50%) of 6 hearts in the gel group at 2 weeks, but not in that of 19 hearts in the 2 groups at 4 weeks.

Cardiac Function
Heart rate, left ventricular systolic pressure and end-diastolic pressure or dp/dt did not significantly differ between the 4 groups (Table 1). Echocardiographic data showed that left ventricular end-diastolic and end-systolic dimensions significantly increased at 4 weeks compared with those at 2 weeks in each group. Significantly more fractional shortening was evident at 2 weeks in the bFGF + gel group than in the saline group. Left ventricular end-systolic and end-diastolic dimensions, as well as fractional shortening did not significantly differ among the 4 groups at 4 weeks.

Morphological Analysis
Infarct size (%) was significantly smaller in the bFGF + gel group (p<0.05) at 2 weeks than in both the saline and gel groups, but did not differ at 4 weeks between the 4 groups (Fig 2A). The ratio of infarcted to non-infarcted wall thickness was significantly higher in both the bFGF (p<0.05) and bFGF + gel (p<0.001) groups at 2 weeks than in the saline group, and significantly higher in the bFGF + gel (p<0.05) group than in the saline group at 4 weeks (Fig 2B). The left ventricular expansion index was significantly lower in both bFGF (p<0.01) and bFGF + gel (p<0.01) groups compared with the saline group at 2 and 4 weeks (Fig 2C).

Histological Analysis
Capillary density in the infarct border zone did not differ among the 4 groups at 2 weeks but was significantly higher in the bFGF (27.5±7.6/0.2 mm², p<0.001) and bFGF + gel (28.1±5.9/0.2 mm², p<0.001) groups compared with the saline (19.5±4.0/0.2 mm²) and gel (15.1±5.5/0.2 mm²)
Fig. 4. Effect of bFGF and bFGF+gel on arteriogenesis. (A) Heart tissues in infarct border zone at 4 weeks. Arterioles (20\(\mu\)m≤ diameter <100\(\mu\)m) were identified in saline, bFGF, gel and bFGF+gel groups as having a thin layer of smooth muscle cells stained with anti-\(\alpha\)-smooth muscle actin antibody. Scale bar, 100\(\mu\)m; 400× magnification. (B) Arteriolar density expressed as vessels per visual field (0.2 mm\(^2\)). Arteriolar density is higher in the bFGF+gel group at 2 and 4 weeks. bFGF, basic fibroblast growth factor; Gel, gelatin hydrogel; bFGF+gel, basic fibroblast growth factor+gelatin hydrogel.

Fig. 5. Effect of bFGF and bFGF+gel on cardiomyocyte apoptosis. (A) Heart tissues in infarct border zone at 4 weeks. Cardiomyocyte apoptosis in saline, bFGF, gel and bFGF+gel groups evaluated as terminal deoxynucleotide transference-mediated dUTP nick-end labeling (TUNEL)-positive cardiomyocytes (arrows). Scale bar, 100\(\mu\)m; 400× magnification. (B) Ratio (%) of TUNEL-positive cardiomyocytes in infarct border zone is lower in the bFGF and bFGF+gel groups at 2 and 4 weeks. bFGF, basic fibroblast growth factor; Gel, gelatin hydrogel; bFGF+gel, basic fibroblast growth factor+gelatin hydrogel.
groups at 4 weeks (Fig 3). Compared with that at 2 weeks, capillary density significantly dropped in the saline and gel groups at 4 weeks (p<0.05), but did not significantly change in the bFGF and bFGF + gel groups. Arteriolar density was significantly higher in the bFGF + gel group than the saline, bFGF and gel groups at both 2 and 4 weeks (Fig 4). TUNEL-positive cardiomyocytes were detected in the infarct, border zone and non-infarcted tissues in all 4 groups at both 2 and 4 weeks. The ratio of TUNEL-positive cardiomyocytes in the border zone was significantly lower in the bFGF and bFGF + gel groups (p<0.01) compared with the saline and gel groups at 2 and 4 weeks (Fig 5). The ratio of fibrotic to left ventricular areas (data not shown) and collagen density (%) did not differ among the 4 groups at 2 and 4 weeks.

Discussion

The major findings of the present study are as follows: in the rat heart, the intramyocardial administration of gelatin hydrogels incorporating bFGF prolonged the duration of bFGF activity at least at 4 weeks after injection, stimulated neoangiogenesis and decreased cardiomyocyte apoptosis in the infarct border zone. Slow-release bFGF significantly reduced thinning of the infarction wall, attenuated left ventricular remodeling and consequently improved cardiac function.

Our previous study found that a single administration of free bFGF protein in a canine infarction model increased capillary and arteriolar density in the infarct border zone, increased regional myocardial blood flow and consequently improved ventricular function. However, the results of clinical studies using bFGF are inconsistent, probably due to differences in study design, route of administration and method of delivery. Biologically active, free bFGF is easily inactivated and degraded. Thus, delivery of a DNA encoding bFGF using adenoviral vectors or slow-release bFGF protein has been advocated to achieve sustained release. Slow-release bFGF protein has the advantages of simplicity, consistent delivery and safety over DNA encoding bFGF protein. Sellke et al have described the safety and clinical effectiveness of therapeutic angiogenesis with bFGF delivered by heparin-aginate slow-release devices. Gelatin hydrogel is a preferable matrix candidate for the sustained release of growth factors. Tabata et al demonstrated that a slow-release drug delivery system using gelatin hydrogel can increase the half-life of bFGF in vivo from days to weeks. We injected bFGF directly into the myocardium because myocardial distribution and retention might be superior to those of other modes of delivery. The intramyocardial administration of bFGF can avoid high levels of circulating angiogenic activity that might stimulate plaque angiogenesis or the growth of concealed neoplasms.

Iwakura et al reported that an intramyocardial injection of a gelatin hydrogel incorporating bFGF into the border zone of scar tissue 4 weeks after coronary artery ligation induced significant angiogenesis and improved left ventricular function in a rat infarct model. However, we observed that both scar tissue formation and left ventricular wall thinning were established 4 weeks after infarction and that ventricular function did not seem to recover in the saline control group. In addition, it is technically difficult and even dangerous to inject gelatin hydrogel into the thin ventricular wall of the infarcted rat heart. The acute infarction model of the present study does not represent the chronic myocardial ischemia that is widespread in the clinical setting. However, this model enabled evaluation of the effects of angiogenic growth factor under proangiogenic conditions during the period of healing after myocardial infarction. The present study showed that the increased capillary density at 2 weeks after myocardial infarction declined at 4 weeks in the saline and gel groups. Ren and coworkers found significant capillary growth after 7–14 days, which decreased after 4 weeks in a canine model of myocardial ischemia and reperfusion. Ischemia and hypoxia following myocardial infarction induces intense inflammatory reactions that promote angiogenesis. Monocyte-derived macrophages and mast cells produce the cytokines and growth factors necessary for fibroblast proliferation and neovascularization, leading to effective repair and scar formation. The early phase of angiogenesis stimulated by bFGF was masked by post-infarction inflammatory reactions, which essentially disappeared by 4 weeks. In contrast to the saline and gel groups, the increased capillary density persisted for 4 weeks in the groups treated with bFGF. This suggests that angiogenesis stimulated by exogenous bFGF is more durable than the transient angiogenesis induced by inflammation. In addition, the arterial system develops as smooth muscle cells differentiate around endothelial cells during vascular myogenesis and stabilizes vessels during arteriogenesis. Our data also showed significantly increased arteriolar density in the bFGF + gel group, suggesting that slow-release bFGF promotes smooth muscle proliferation and accelerates arteriogenesis.

Alterations in ventricular structures involving both infarcted and noninfarcted areas comprise ventricular remodeling that includes myocardial hypertrophy, cardiac fibroblast proliferation, fibrosis and cell death. Cardiomyocyte apoptosis is an important factor leading to cardiac remodeling and heart failure according to several experimental and clinical studies. Palojoki et al reported that the ratio of apoptotic cardiomyocytes in a rat infarction model tended to be higher in the border zone adjacent to the infarct. The present study demonstrated that the administration of both sustained release and free bFGF reduced cardiomyocyte apoptosis in the infarct border zone. Iwai-Kanai et al have suggested that bFGF is a protective factor against myocardial cell apoptosis mediated by inducible nitric oxide synthase.

After myocardial infarction, various growth factors such as transforming growth factor β, insulin-like growth factor, platelet-derived growth factor, bFGF, angiotensin II and endothelin play important roles in the regulation of collagen production, angiogenesis, and ventricular remodeling. They might all stimulate an increase in the production of collagen by cardiac fibroblasts. Exogenous bFGF stimulates the proliferation of cardiac fibroblasts and therefore might increase collagen production. However, the present study showed that the intramyocardial administration of bFGF did not increase collagen deposition (cardiac fibrosis) after myocardial infarction and that rats treated with slow-release bFGF had thicker and less expanded infarcts compared with those of the saline and gel groups. These results suggest that slow-release bFGF attenuates ventricular remodeling through enhanced neoangiogenesis, which subsequently protects the ischemic myocardium and decreases myocardial apoptosis in the border area adjacent to the infarct. Slow-release bFGF effectively improved cardiac function at the early post-infarction period. However,
its effect on cardiac function was decreased at 4 weeks, in association with progressive left ventricular dilatation. Vessel growth and maturation are critical for scar formation and cardiac repair.9 Despite increased capillary density and attenuated ventricular remodeling, cardiac function in the bFGF group did not differ from that in the saline and gel groups. The results of the present study suggest that arteriogenesis, which was significantly induced by slow-release bFGF, plays an important role in improvement of cardiac function.

Five rats in the bFGF + gel group and 2 in the gel group died during the operative procedure in the present study. We considered that 5 of these deaths resulted from the intramyocardial injection, which induced thrombosis in the anterior cardiac vein, probably with subsequent systemic embolization. We injected gelatin hydrogel containing 20 μg of bFGF, which was considerably less than 100 μg of bFGF incorporated into gelatin hydrogel in another rat infarct model.10 The potential increase in risk associated with higher-dose bFGF therapy will require further investigation.

In conclusion, the intramyocardial administration of slow-release bFGF incorporated into a biodegradable gelatin hydrogel can enhance bFGF activity in a rat infarction model. Slow-release bFGF significantly increased the number of capillaries and arterioles, reduced cardiomyocyte apoptosis in the infarct border zone, attenuated ventricular remodeling after myocardial infarction and improved cardiac function.

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