Toward surgical angiogenesis using slow-released basic fibroblast growth factor

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Abstract

Objective: Therapeutic angiogenesis using basic fibroblast growth factor (bFGF) in coronary artery disease has been documented in a number of papers. However, the effectiveness is discrepant among documents. In this study, we evaluated the distribution of bFGF in the rat heart by different administration methods, and investigated the efficacy of slow-released administration of bFGF using biodegradable hydrogel microspheres (bFGF microspheres) in a pig infarction model toward an enhanced coronary bypass surgery.

Methods: Heart failure due to myocardial infarction was induced in rats and pigs. In the rat study, free form of bFGF (central venous injection, intracoronary injection, and intramyocardial administration) and bFGF microspheres (intramyocardial administration) were given 4 weeks later. The remaining radioactivity of bFGF in the hearts was estimated 1, 24, and 72 h later. On the other hand, the pigs were randomized into two groups 4 weeks after myocardial infarction. While the control group (n = 8) had gelatin hydrogel microspheres with saline, the FGF group (n = 8) received bFGF microspheres in the left ventricular (LV) wall.

Results: In the rat study, after intramyocardial administration of bFGF microspheres, more bFGF remained in the rat heart 72 h later compared with the other methods (P < 0.001). In the pig study, 4 weeks after the treatment, the FGF group had smaller LV diastolic diameter (48.7 ± 5.3 vs. 56.7 ± 5.2 mm, P < 0.01) than the control group. LV end-systolic elastance was higher in the FGF group (2.96 ± 1.2 vs. 1.06 ± 0.3 mmHg/ml, P < 0.01). In microscopic examinations, many neovessels were found in and around the scar tissue, and the vascular density in the FGF group was significantly higher (61.5 ± 18.3 vs. 153.0 ± 29.0/mm², P < 0.01). In addition, the infarcted LV walls were less expanded and more thickened in the FGF group.

Conclusions: Biodegradable hydrogel microspheres with bFGF improved LV function and inhibited LV remodeling by angiogenesis in pigs with chronic myocardial infarction. bFGF microspheres into ischemic myocardium may revascularize small ungraftable vessels and may potentially increase distal run-off when applied in coronary bypass surgery.

Keywords: Angiogenesis; Basic fibroblast growth factor; Myocardial infarction

1. Introduction

The induction of coronary collateral growth represents a novel and potentially important therapeutic approach for patients who are not candidates for standard revascularization such as coronary angioplasty (PTCA) or bypass surgery (CABG). Basic fibroblast growth factor (bFGF) is one of the powerful angiogenic growth factors that have been evaluated as agents to promote angiogenesis. In spite of a lot of studies about its effectiveness for ischemic heart disease, the results were discrepant among the reports due to various methods and/or routes for bFGF administration. Some have reported that bFGF administration had no or insufficient effects on ischemic hearts [1–3]. Others have documented that it increased regional myocardial blood flow, reduced infarction size, and improved ventricular function [4–11]. In general, the biological effects of growth factors in its free form are very limited because its half-time in vivo is too short. Hence, several growth factors including vascular endothelial growth factor have been applied by means of gene transfection [12]. We previously documented that polymer hydrogels are a preferable matrix candidate for...
release of growth factors because of their biosafety and high inertness toward protein drugs [13]. Biodegradable gelatin microspheres incorporating bFGF (bFGF microspheres) have been developed using acidic gelatin hydrogels, enabling bFGF to be released at the site of action over a period of time that is required for sufficient effectiveness [14,15].

In the present study, we evaluated the bFGF distribution in the heart by the different administration methods and investigated the efficacy of bFGF microspheres in pigs with myocardial infarction.

2. Materials and methods

2.1. Animal information

Fifty-five male rats weighing 220–280 g and 18 male pigs weighing 19–22 kg were used in this study. All animals received humane care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources, National Research Council and published by the National Academy Press.

2.2. Preparation of bFGF incorporating gelatin hydrogels

Human recombinant bFGF was supplied by Kaken Pharmaceutical Co., Tokyo, Japan. A gelatin sample was isolated from the bovine bone (Nitta Gelatin Co., Osaka, Japan). The bFGF-microspheres were prepared as described previously [15]. Briefly, gelatin microspheres were prepared through glutaraldehyde cross-linking of gelatin. Then the microspheres were washed with acetone (4°C) and recovered by centrifugation. Basic FGF was radioiodinated according to the method and immersed in the microspheres for 1 h before use [16].

2.3. In vivo distribution of bFGF

The left anterior descending arteries (LAD) of 55 rats were ligated to create myocardial infarction. Four weeks later, four administration regimens were conducted for 48 survived rats with myocardial infarction: (1) free bFGF by central venous bolus injection (group I, n = 12); (2) free bFGF by intracoronary injection, injection via LV apex during ascending aorta clamping (group II, n = 12); (3) free bFGF by intramyocardial administration into the infarct and peri-infarct area (group III, n = 12); (4) bFGF microspheres by intramyocardial administration (group IV, n = 12). All the bFGF was labeled by 125I. Blood samples and the hearts were obtained at different time intervals: 1, 24 and 72 h. Each ventricular wall was divided into the infarct area including peri-infarct zone and the non-infarct area. The remaining radioactivity of bFGF in each sample was measured on a gamma counter (ARC-301B, Aloka Co. Ltd., Tokyo, Japan).

2.4. Chronic myocardial infarction model in pigs and study group

A flexible 2.6-F catheter (Radifocus SP Catheter, Terumo) was introduced through the left common carotid artery and placed in the distal LAD between the first and second diagonal branches under fluoroscopy guidance under general anesthesia. Homogenized gelatin sponges (Spongell, Yamanouchi, Tokyo) were slowly injected through the SP catheter into the distal LAD under electrocardiogram (ECG) monitoring. After completion of embolization, coronary angiography showed that the middle portion of LAD was occluded with the ST-segment in ECG elevated dramatically. Four weeks after the LAD embolization, left ventricular (LV) dysfunction and myocardial infarction were evaluated by echocardiography. Then, 16 pigs were randomized into two groups; eight pigs received an intramyocardial injection of gelatin hydrogel microspheres with saline (Control group), eight received an injection of bFGF microspheres (FGF group).

2.5. bFGF microspheres administration

Median sternotomy was performed under general anesthesia. The LAD was ligated to secure the infarction. In each group, saline or bFGF microspheres were injected with a fine syringe at five points, one at the ligation site and the others around the peri-infarct area of the LV free wall. In the FGF group, 0.2 ml of saline including 40 μg bFGF microspheres was injected at each point, totally 200 μg.

2.6. Assessment of left ventricular function

2.6.1. Echocardiography

Echocardiographic measurements were performed with a 7-MHz ultrasound transducer (connected with SSA-260A Ultrasound system, Toshiba Medical Inc., Tokyo, Japan) at the time of before and 4 weeks after the embolization and 4 weeks after the treatment. The following parameters were derived from the M-mode tracing: LV end-diastolic dimension (EDD; mm), LV end-systolic dimension (ESD; mm), LV ejection fraction (EF; %). Infarction size was estimated by the percentage of the akinetic region divided by the LV endocardial circumference at end-diastolic phase. The images were analyzed off-line by a single observer blinded to the profile of the animals. All parameters were measured by the American Society for Echocardiology leading-edge method from at least three consecutive cardiac cycles.

2.6.2. LV pressure–volume study

LV pressure–volume study was performed to have more precise assessment of global LV function before and 4 weeks...
after the treatment. A thermodilution catheter (7 F, CritiCath, Thermodilution Catheter, Omeda, Singapore) was placed in the pulmonary artery via percutaneous femoral approach. A median partial sternotomy was made and the heart was exposed. A ten-electrode conductance catheter (Millar, Houston, TX) and a micromanometer-tipped catheter (Millar) were inserted into the LV for pressure and volume recording and connected to a signal-conditioner processor (Leycom Sigma-5, CardioDynamics BV, Zoetermeer, The Netherlands). The parallel conductance was evaluated by the injection of hypertonic saline solution into the pulmonary artery. Pressure–volume relations were obtained at steady state and during transient inferior vena cava occlusion.

### 2.7. Coronary angiography

Coronary angiography was performed before and 4 weeks after treatment under a general anesthesia. A 5-F Judkins catheter was introduced through the left common carotid artery and placed in the LAD under fluoroscopy.

### 2.8. Histological study

After all physiological measurements, the pigs were killed. The hearts were fixed in 10% buffered formalin and five peri-infarct portions treated by saline or bFGF were cut out. The transmural sections were stained with hematoxylin–eosin and with anti-factor VIII antibody (Dako) to detect vessels more precisely. The vessels per unit area (1 mm²) in the peri-infarct zone were counted in five randomly chosen fields per slide of each portion by two blinded pathologists. The average number of the vessels in one portion was used for assessment of vascular density.

### 2.9. Plasma brain natriuretic peptide (BNP) levels

In all pigs, 5 ml of blood was sampled at three time points: before the LAD embolization, before and 4 weeks after the treatment. Plasma levels of BNP were measured with enzyme immunoassay using a high sensitivity kit (BNP-32 porcine EIA Kit, Peninsula Co., CA).

### 2.10. Data analysis

All values were calculated as the mean ± SD. The pairwise comparisons of individual groups in the rat study were conducted by means of one-way analysis of variance followed by Scheffe’s test. Continuous variables between the two groups were compared by paired Student’s t-test at before and 4 weeks after the treatment in the pig study. Statistical analyses were performed with Statview for Windows version 5.0 (SAS Institute Inc., Cary, NC). Values of \( P < 0.05 \) were considered statistically significant.

### 3. Results

#### 3.1. In vivo distribution of bFGF

In group I, there was little bFGF remaining in the heart only 1 h after central venous injections of bFGF. Group II had similar results to those of group I. In group III, the bFGF in the heart remained more than that in the groups I and II. In group III, remaining bFGF levels decreased to about the one tenth of the administrated bFGF 72 h after its administration. An initial drop was found in group IV, but there were no significant changes in remaining bFGF for 72 h. No remaining bFGF in the blood was detected in all groups at any sampling points (Table 1).

#### 3.2. Animal model characteristics

Eighteen animals underwent coronary embolization, of which two died due to irreversible ventricular fibrillation soon after the embolization. Therefore, the data from the 16 pigs that survived the first operation were analyzed. Body weights at the baseline did not differ between the control and FGF groups (31.8 ± 2.2 vs. 34.0 ± 3.9 kg). The body weights at 4 weeks after myocardial infarction in the control group were significantly lower than the FGF group (38.0 ± 1.9 vs. 42.4 ± 3.4 kg, \( P < 0.01 \)).

#### Table 1

Distribution of bFGF in the heart

<table>
<thead>
<tr>
<th>Group</th>
<th>1 h (n = 4, at each time point)</th>
<th>24 h (n = 4, at each time point)</th>
<th>72 h (n = 4, at each time point)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MI (%)</td>
<td>No-MI (%)</td>
<td>MI (%)</td>
</tr>
<tr>
<td>I (n = 12)</td>
<td>&lt;0.1*†</td>
<td>&lt;0.1</td>
<td>&lt;0.1*†</td>
</tr>
<tr>
<td>II (n = 12)</td>
<td>1.4 ± 0.9*†</td>
<td>2.0 ± 1.1</td>
<td>0.2 ± 0.03*†</td>
</tr>
<tr>
<td>III (n = 12)</td>
<td>16.2 ± 6.9*</td>
<td>1.3 ± 1.1</td>
<td>19.9 ± 4.4*</td>
</tr>
<tr>
<td>IV (n = 12)</td>
<td>35.7 ± 1.0</td>
<td>0.7 ± 0.1</td>
<td>27.3 ± 9.8</td>
</tr>
</tbody>
</table>

Data are shown as mean ± SD. MI, the myocardial infarction area including the peri-infarct zone; No-MI, the myocardium except the MI area. Group I received free bFGF by central venous bolus injection, group II received free bFGF by intracoronary injection, group III received free bFGF by intramyocardial administration, and group IV received bFGF microspheres by intramyocardial administration. \(* P < 0.0001 \) vs. group IV; † \( P < 0.001 \) vs. group III.
4 weeks after treatment. In the FGF group, LV dilation was inhibited and LVEDD at 4 weeks showed significantly smaller levels compared with the control group (control vs. FGF: 56.7 ± 5.2 vs. 48.7 ± 5.3 mm, \( P < 0.01 \)), and similar results were recognized in LVESD (41.3 ± 3.8 vs. 34.4 ± 5.4 mm, \( P = 0.011 \)). There were significant differences in EF between the groups (50.8 ± 2.6 vs. 57.6 ± 7.2%, \( P < 0.05 \)). Infarction size did not reveal any significant differences between the two groups at each time point. However, the changes in the infarction size between baseline and post-treatment in the control group were positive, while those in the FGF group were negative (+0.96 ± 1.3 vs. −2.46 ± 3.5%, \( P < 0.05 \) (Fig. 1D,E).

### 3.3. LV function

#### 3.3.1. Echocardiography

There were no differences in data between the two groups at baseline (Fig. 1A–C). LVEDD and LVESD in the control group increased and were larger than baseline levels at 4 weeks after treatment. In the FGF group, LV relaxation (\( \tau \)) and end-systolic elastance (\( E_{es} \)) as a marker of global systolic and diastolic function, respectively. We found significantly lower \( \tau \) in the FGF group than the control group at 4 weeks after treatment. \( E_{es} \) in the FGF group progressively increased, whereas that

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control group (n = 8)</th>
<th>FGF group (n = 8)</th>
<th>( P )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR (bpm)</td>
<td>82.5 ± 13.2</td>
<td>81.5 ± 15.6</td>
<td>0.89</td>
</tr>
<tr>
<td>CO (l/min)</td>
<td>3.7 ± 1.1</td>
<td>3.9 ± 1.9</td>
<td>0.81</td>
</tr>
<tr>
<td>LVEDV (ml)</td>
<td>116.0 ± 35.1</td>
<td>108.9 ± 22.0</td>
<td>0.63</td>
</tr>
<tr>
<td>LVESV (ml)</td>
<td>68.1 ± 20.2</td>
<td>63.4 ± 11.2</td>
<td>0.59</td>
</tr>
<tr>
<td>LVEDP (mmHg)</td>
<td>14.1 ± 6.0</td>
<td>12.8 ± 5.5</td>
<td>0.64</td>
</tr>
<tr>
<td>+dP/dt (mmHg/s)</td>
<td>783.8 ± 73.6</td>
<td>835.3 ± 79.9</td>
<td>0.20</td>
</tr>
<tr>
<td>−dP/dt (mmHg/s)</td>
<td>850.5 ± 92.9</td>
<td>851.0 ± 121.0</td>
<td>0.99</td>
</tr>
<tr>
<td>SV (( \tau ))</td>
<td>37.2 ± 3.6</td>
<td>39.3 ± 11.1</td>
<td>0.61</td>
</tr>
<tr>
<td>( E_{es} ) (mmHg/l)</td>
<td>1.30 ± 0.24</td>
<td>1.69 ± 0.59</td>
<td>0.11</td>
</tr>
</tbody>
</table>

#### 3.3.2. LV pressure–volume study

Hemodynamic data are shown in Table 2. There were no differences in all parameters between the two groups at baseline. The parameters except heart rate improved in the FGF group at 4 weeks after the treatment. We calculated the time of data between the two groups at each time point. However, the changes in the infarction size between baseline and post-treatment in the control group were positive, while those in the FGF group were negative (+0.96 ± 1.3 vs. −2.46 ± 3.5%, \( P < 0.05 \) (Fig. 1D,E).

### Table 2

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</tbody>
</table>

Data are shown as mean ± SD. HR, heart rate; CO, cardiac output; LVEDV, left ventricular end-diastolic volume; LVESV, left ventricular end-systolic volume; LVEDP, left ventricular end diastolic pressure; +dP/dt, plus time-derivative; −dP/dt, minus time-derivative; \( \tau \), the time constant of isovolumetric relaxation; \( E_{es} \), end-systolic elastance.
in the control group decreased during the 4 weeks (Fig. 1F,G).

3.4. Coronary angiography

The LAD was confirmed to be occluded at the middle portion after the embolization, although all animals barely had the patency of the LAD and no collateral arteries, in the coronary angiography performed just before the treatment 4 weeks later. Four weeks after the treatment, the LAD in the control group was completely occluded. On the other hand, the FGF group showed the forward flow to the distal end through collateral arteries in all cases (Fig. 2).

3.5. Hormonal study

The average levels of BNP in all pigs were 540 ± 190 pg/ml before myocardial infarction was created. There were no differences in BNP values between the two groups before treatment (1194 ± 250 vs. 1415 ± 399 pg/ml, P = 0.21). Four weeks after the treatment, BNP levels in the FGF group were significantly lower than those in the control group (1481 ± 290 vs. 1160 ± 298 pg/ml, P < 0.05) (Fig. 3).

3.6. Histological study

LV volume in the control group was significantly larger, as shown in Fig. 4. Microscopic examination showed the following findings. There were much more neovessels in the peri-infarct area in the FGF group 4 week after bFGF treatment compared with the control group (Fig. 5). In addition, vascular density in the FGF group was significantly higher. In particular, the large-size vessels, of more than 50 μm diameter, were significantly more induced in the FGF group (Fig. 5).

4. Discussion

A number of angiogenic growth factors have been evaluated experimentally as agents to promote coronary collateral development for patients with ischemic heart disease [7,8,12]. bFGF, one of the most powerful angiogenic factors, induces the migration and proliferation of endothelial and smooth muscle cells, the formation of vascular tubes and networks [17,18]. Some in vivo investigations demonstrated that bFGF administration increased collateral vessels, enhanced cardiac function and reduced infarction size [4–11]. In other papers, however, bFGF administration following the development of myocardial infarction exerted no additional effects on collateral growth [1–3]. We speculate that the discrepancies may have come from differences in study models, study subjects (human, pig, dog, etc.), delivery routes (intravenous, intracoronary, intramyocardial, etc.), administration doses and frequencies, and forms of bFGF administration (gene, protein form, etc.).

In this first part of our study, we found that there was little bFGF in the heart 1 h after central venous or intracoronary injections of bFGF. On the contrary, intramyocardial injections of bFGF kept more bFGF in the heart than central venous or intracoronary injection. The results were compatible to previously reported data about the pharmacodynamics of bFGF after its administration from different routes [19]. In addition, our data showed that there was 15-fold more remaining bFGF in the heart 72 h after the intramyocardial administration of bFGF microspheres compared with intramyocardial injection of free bFGF in solution. We concluded that the action of bFGF was exhibited more efficiently by intramyocardial administration of bFGF microspheres.

The biological half-time of bFGF is too short and was reported as less than 50 min [10]. In addition, the initial response of endothelial cells begins almost 1 day after stimulation of bFGF [20]. In fact, several researchers reported that a single intracoronary infusion of bFGF was not effective [11]. Lazarous and associates found rich collateral arteries after repeated bFGF administration via the left atrium for 5–9 weeks, although there were some complications, such as hypotension, anemia and thrombocytopenia, due to high plasma levels of bFGF [10]. These results imply that enough concentrations of bFGF should be maintained in the local tissue for a certain period of time in order to obtain its sufficient effectiveness. In this context,
the slow-release system with gelatin microspheres may be very beneficial, because it enables bFGF to locally exert its activities for a long period and induce angiogenesis.

There were a few studies about therapeutic angiogenesis using bFGF that precisely estimated cardiac function. Therefore, in the second study, we estimated LV function by cardiac echocardiography and catheterization after the treatment. Moreover, we measured plasma levels of BNP, which has been demonstrated to well reflect global LV performance. In this study, both systolic and diastolic LV function in the FGF group were significantly improved compared with the control group. Likewise, BNP levels in the FGF group were significantly lower. In addition, it should be noted that our results demonstrated more dramatic improvement of LV function compared with other reports.

We speculate the reasons for the promising data of the control-released bFGF as follows. First, as mentioned before, control-released bFGF administration enabled bFGF to work over a long period in situ, which induced rich neovascularization and good collateral formation, and increased regional blood flow around bFGF-injected sites: in the peri-infarct area and in the distal perfusion area of the ligated LAD. In this study, the distal portion of the LAD was opacified through these collateral arteries in coronary angiography. Moreover, histological study showed a lot of neovessels induced in the peri-infarct zone. Although we did not evaluate the regional blood flow, Yamamoto et al. have documented significant improvement of regional blood flow using the same formula as ours [9]. Similar results have been reported experimentally and clinically in some earlier studies using heparin-alginate beads including bFGF [6,8]. In addition, sustained bFGF activities may have worked as a potential anti-apoptotic or cardioprotective factor in the peri-infarct area [21–24].

Second, cardiac remodeling secondary to myocardial infarction may have been inhibited by injecting bFGF in the peri-infarct area rather than in healthy or infarct myocardium. Myocardial stress is not homogeneous in the remodeling heart. Miwa and associates demonstrated oxidative stress in the peri-infarct area was significantly higher than those in the non-infarct area at the chronic phase after myocardial infarction in a rat model [25]. This finding suggests that the peri-infarct zone is very important in the process of cardiac remodeling in the chronic myocardial infarction heart at the compensation phase. In this study, bFGF was selectively administered into the peri-infarct area for a long time. We consider that this may be related to the good results in the present study and may be critically important for treatment.

The therapeutic angiogenesis using bFGF showed very promising results for ischemic heart disease. Although administration of this agent alone was very effective, we consider that the method should work more effectively when applied in combination with conventional treatments such as percutaneous transluminal coronary angioplasty and/or coronary artery bypass grafting (CABG). At present, patients with poor distal run-off of the coronary arteries are untreatable by those conventional treatments. However, these patients may benefit from the method using bFGF microspheres during CABG. During operation, bFGF microspheres can be precisely administered to the intra-myocardium where we expect neovascularization. By using our method, we may be able to achieve a good run-off in target vessels and complete revascularization even for patients with poor coronary vasculature.

In conclusion, this study demonstrated that bFGF microspheres sustained the concentration of myocardial bFGF for a long time compared with free bFGF in solution. In addition, it improved LV function and inhibited LV

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![Fig. 4. Photograph of the heart section at the level of the papillary muscles 4 weeks after the treatment. Arrows indicate the anterior parts.](image)

![Fig. 5. (A) Neovascularization in the peri-infarct area of the left ventricular free wall in the control and the FGF groups 4 weeks after the treatment. Scales indicate 100 μm (hematoxylin–eosin; original magnification ×100). (B) Capillary density in the peri-infarct area in each treatment group is expressed as the number of capillary vessels per field. Data are shown as mean ± SEM. *P < 0.01 vs. Control.](image)
remodeling in pigs with chronic myocardial infarction. This method for ischemic myocardium may revascularize small vessels and increase the distal run-off of the target arteries. When it is applied during CABG operation, it may give better outcomes.

4.1. Study limitations

There are several limitations to the present study. First, the animals used in our study had no risk factors for ischemic heart disease, such as hypertension, atherosclerosis and diabetes mellitus. The beneficial efficacy of bFGF microspheres administration may be impaired by such unfavorable conditions. Second, we used just one administration regimen. Therefore, we need more detailed information to obtain the optimal efficacy (e.g. doses, timing).

Acknowledgements

The authors thank Dr Goditha Premarathene, Dr Xue Li and Mr Hashino (Taisho Biomed Instruments Co. Ltd.) for the preparation of the experiments.

References


Appendix A. Conference discussion

Dr G. Lutter (Freiburg, Germany): This underlines results we have obtained already in Freiburg showing that FGF is a good inductor of angi- and arteriogenesis. Therefore, my question will be: Did you also analyze arteriogenesis in your histologic stainings (arteriole-counting) to show whether FGF does induce more arterio- than angiogenesis? Can you comment on this?

Dr Sakakibara: After bFGF treatment we were able to find more vessels compared with VEGF or with HGF treatments. Actually, in the findings on coronary angiography, we found forward flow through the collateral arteries in all cases. I don’t know the mechanism exactly. However, I think that there are two important things. One is that FGF was maintained for a long time locally, and the other is that we had the bFGF administered to the peri-infarct area. The peri-infarct area is very important in remodeling hearts. So I guess that bFGF worked there for a long time as a more powerful angiogenic, anti-apoptotic and cardioprotective factor.