Experimental Studies

A Study to Determine if Basic Fibroblast Growth Factor (bFGF) Reduces Myocardial Infarct Size in Acute Coronary Arterial Occlusion

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SUMMARY

We investigated the angiogenic and myocardial salvage effects of bFGF. Twelve beagles with ligated left anterior descending coronary arteries were divided into two groups: a FGF group administered bFGF intravenously, and a Control group, after CAG immediately post-ligation. One week post-ligation, CAG was repeated. The heart was sliced along the short axis. For each section, the fluorescein Na staining deficit area (DA) and ratio of DA to total area (DAR), TTC staining of the infarct area (IA) and ratio of IA to total area (IAR), and Masson trichrome staining of the fibrosed area (MA) and ratio of MA to total area (MAR), were calculated. The increase in the number of collateral vessels, seen on CAG from post-ligation to 1 week later, was significantly greater in the FGF group. No significant differences in IAR or MAR were seen between the groups. However, DAR and DA/IA were significantly less in the FGF group. In conclusion, bFGF had no effect on infarct size, but stimulated the growth of collateral vessels and improved coronary blood flow in IA. (Jpn Heart J 1999; 40: 165–178)

Key words: Basic fibroblast growth factor (bFGF), Angiogenesis, Myocardial infarction, Coronary collateral circulation

It is generally accepted that well-developed preexistent coronary collaterals are beneficial to the promotion of the healing process of acute myocardial infarction (AMI). Patients with well-developed collaterals in the early period of AMI and successful thrombolysis showed an improvement in ejection fraction (EF) from the acute phase to the chronic phase, but in patients with no or less well-developed collaterals and successful thrombolysis, EF was similar in patients with unsuccessful thrombolysis. Hirai et al. reported that the collateral perfusion

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existing at the onset of AMI exerted a beneficial effect on the prevention of left ventricular aneurysm formation.

One of the prototypes of fibroblast growth factors (FGF), basic FGF (bFGF), with an affinity for heparin is classified into low (18 kD) and high (22, 25.5 and 24 kD) molecular weight types and possesses angiogenic effects. Proliferation, migration and the formation of capillary-like tubular structures of vascular endothelial cells are stimulated by bFGF, as is the stimulation of secretion of plasminogen activator and collagenase by endothelial cells, and it is a vascular smooth muscle mitogen.

In our laboratory, we have examined the efficacy of bFGF in acute peripheral circulatory failure. We ligated the femoral artery in adult beagles, and conducted femoral arteriography 1 week later. The development of collateral vessels was markedly increased in the bFGF group compared to the Control group, and in particular many neovascular plexi were seen. In addition, these vessels formed an effective collateral circulation, significantly increasing the circulation distal to the obstruction.

Yanagisawa et al.\textsuperscript{5} reported that when bFGF was administered immediately following coronary artery occlusion in an experimental myocardial infarction model, there was increased neoangiogenesis, the infarct size was reduced, and cardiac function improved. In general, it has been considered that the golden time for reperfusion treatments in acute myocardial infarction is 6 hours. However, it has been pointed out that it takes from several hours to several days after the administration of an angiogenic agent for neoangiogenesis to commence,\textsuperscript{4-6} and the role of angiogenic agents immediately following coronary artery occlusion has not been fully elucidated. In the present study, we administered low molecular weight bFGF immediately following coronary artery occlusion, and investigated its angiogenic and myocardial salvage effects in myocardial ischemia.

\textbf{METHODS}

All experiments were performed in accordance with the Guidelines of the Committee on Animals of Tokyo Medical University, and with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 85–23, Revised 1985).

Twelve adult female beagles (weight 10.0–11.5 kg) were given an intramuscular injection of ketamine hydrochloride (20 mg/kg), anesthetized with pentobarbital sodium (25 mg/kg, i.v.), intubated and positive pressure ventilation maintained with a respirator (Shinano Model SN-480–3). Respiratory rate and tidal volume were controlled, keeping arterial blood gas pH and pCO\textsubscript{2} within the normal range. An 8Fr sheath was then inserted into the right femoral vein and a
5Fr sheath into the femoral artery; these were fixed in place, and the electrocardiograms and blood pressure were monitored. Pulmonary capillary wedge pressure (PCWP) and cardiac output (CO) were measured using a Swan-Ganz catheter, and left coronary arteriography was performed using a 4Fr Judkins catheter in the right anterior oblique position. Thoracotomy was then performed via the left fifth intercostal space, and the heart exposed through a vertical incision in the pericardium. The left anterior descending artery was separated just below the first diagonal branch. This site was ligated with a 2.0 silk suture, and occlusion confirmed with CAG in the same position as before.

The dogs were randomly assigned to either an FGF group (n = 6) given bFGF or a Control group (n = 6) given physiological saline. The FGF group was given bFGF (Kaken Pharmaceutical Co., Tokyo, Japan) 20 μg dissolved in 10 ml physiological saline, whereas the Control group was given the same volume of physiological saline alone, administered intravenously. All procedures were conducted aseptically. At no stage in the experiment was heparin used.

After closure of the thoracotomy, an intravenous injection of CMZ (1 g) dissolved in 10 ml of physiological saline was administered and the animals kept in their normal environment. On day 3 post-occlusion, the FGF group was given bFGF (20 μg) dissolved in 10 ml physiological saline, and the Control group was again given physiological saline alone, administered intravenously. On day 7 post-occlusion, once again under general anesthesia, hemodynamic parameters were assessed via a Swan-Ganz catheter, CAG was conducted, and thoracotomy performed. At this stage 2% fluorescein Na (1 ml/kg) was administered intravenously, followed 30 seconds later by potassium chloride (3 g/20 ml) also intravenously, and the heart was quickly removed. The removed heart was sliced into 8 mm sections starting from the apex along the short axis, and the sections photographed in a dark room under 20 W ultraviolet illumination to obtain fluorescein images. Photographs were taken using Ektachrome Dyna Ex 100 film, with a green filter, at F11 for a 2 minute exposure. Triphenyl tetrazolium chloride (TTC) 1000 mg was dissolved in 100 ml of 0.2 M, pH 7.8 Tris buffer solution in a constant temperature bath at 37°C. The myocardial sections were stained by placing them in this solution for 30 minutes, and the infarct area determined (TTC staining). After TTC staining each section was photographed using Ektachrome Dyna Ex 100 film under room lighting. After fixing in formalin each section was sliced thinly, stained with Masson trichrome, and directly photographed.

We investigated changes in haemodynamic parameters by measuring BP, CO, and PCWP before, immediately post-occlusion and 1 week post-occlusion.

In assessing the CAG results, we counted all the distinguishable vessels that branched towards the infarct area from the left circumflex artery, and all the
collateral vessels to the anterior descending artery distal to the occlusion, either intracoronary or intercoronary anastomoses. For both groups, we derived the score, Δ number of collaterals = (number of vessels 1 week later) - (number of vessels immediately post-occlusion), and compared the results. Next, we scored the collateral development for both groups according to Rentrop’s classification, deriving the Δ Rentrop score = (Rentrop score 1 week later) - (Rentrop score immediately post-occlusion), and compared the results. We then derived the ratio of LAD distal to the occlusion to the total left coronary artery length from the CAG measurements, and compared the area at risk for the two groups.

We then scanned the photographs of each section stained with fluorescein Na, TTC and Mason trichrome, using a Coolscan scanner (Nikon Corp), a Power Macintosh 7600/120 and Photoshop 4.0J (Adobe). Scanning was conducted on the three sections from the apex up, which included an infarcted area, and then the stained and non-stained areas were measured for each stain using the NIH image. The left ventricular lumenal diameter and, from the TTC stained sections, the ratio between the wall thickness of the infarcted area and the adjacent non-infarcted area (infarcted area wall thickness/non-infarcted area wall thickness) were also calculated. The mean for the three sections was derived and the results for the two groups compared.

All measurements are expressed as mean ± SE. Mann-Whitney U-test and Wilcoxon signed rank test were used to assess the significance of differences between the groups, with p values less than 0.05 considered significant.

RESULTS

A transient but significant drop in BP was seen immediately post-ligation in both the FGF and Control groups, but no significant difference was seen between the two groups before or immediately post-ligation, or 7 days later. No significant difference was seen in the rate of rise in BP from immediately post-ligation to 7 days later between the two groups (Control; before, 184.5 ± 7.995 mmHg vs immediately post-ligation, 162.67 ± 7.017 mmHg vs 7 days later, 175 ± 6.633 mmHg, respectively; not significant) (FGF; before, 169.67 ± 6.23 mmHg vs immediately post-ligation, 162.67 ± 5.31 mmHg vs 7 days later, 164.83 ± 12.46 mmHg, respectively; not significant) (Figure 1). A transient but significant rise in PCWP was seen immediately post-ligation in both the FGF and Control groups, but no significant difference was seen between the two groups before or immediately post-ligation, or 7 days later. No significant difference was seen in the rate of fall in PCWP from immediately post-ligation to 7 days later between the two groups (Control; before, 8.83 ± 1.42 vs immediately post-ligation, 11.17 ± 1.72 vs 7 days later, 9.5 ± 1.23, respectively; not significant) (FGF; before, 7.17 ± 1.78 vs
immediately post-ligation, 10 ± 1.57 vs 7 days later, 8.33 ± 2.11, respectively; not significant) (Figure 1). Again, a transient but significant fall in CO was seen immediately post-ligation in both the FGF and Control groups, but no significant difference was seen between the two groups before or immediately post-ligation, or 7 days later, and no significant difference was seen in the rate of rise in CO from immediately post-ligation to 7 days later between the two groups (Control; before, 1.6 ± 0.37 vs immediately post-ligation, 1.01 ± 0.2 vs 7 days later, 1.31 ± 0.28, respectively; not significant) (FGF; before, 1.87 ± 0.14 vs immediately post-ligation, 1.35 ± 0.13 vs 7 days later, 1.46 ± 0.17, respectively; not significant) (Figure 1). No significant difference was seen between the two groups in the ratio between the length of LAD distal to the ligation and the sum of the lengths of the left coronary artery main trunk, LAD and circumflex arteries, as
Figure 2. A: Ratio of length of LAD distal to ligation. B: Increase in collateral vessel numbers from immediately post-ligation to 1 week later. C: Increase in Rentrop score from immediately post-ligation to 1 week later.

measured on CAG (Control, 37.58 ± 1.62% vs FGF, 36.17 ± 3.08%, respectively; not significant) (Figure 2). Thus, we concluded that there was no significant difference in the size of the risk area between the two groups. Development of collateral circulation was poor even at 1 week post-ligation as seen on the CAG for the Control dogs, with Rentrop scores of 1 for one animal, 2 in four animals, and 3 in one animal. However, in the dogs administered FGF, the development of collateral circulation consisting mainly of a number of newly grown vessels toward the infarct area from the left circumflex artery, as well as intracoronary anastomoses from the diagonal branch to the distal LAD, and intercoronary anastomoses through the apical region, were seen at 1 week post-ligation (Figure 3). A Rentrop score of 3 was given to 5 out of 6 animals, with the LAD clearly visible distally. In the FGF group, Δ number of collaterals was significantly greater (Control, 6.16 ± 0.54 vs FGF, 10.83 ± 1.19, p < 0.05) (Figure 2), and Δ Rentrop score was also significantly greater than those of the Control group. (Control, 1.17 ± 0.17 vs FGF, 2.33 ± 0.33, p < 0.05) (Figure 2).

Under ultraviolet light, in fluorescein-stained sections of myocardium, perfused areas exhibited yellow-green fluorescence, whereas non-perfused areas did not fluoresce and appeared black. With TTC staining, normal myocardium appeared dark red, whereas infarcted areas appeared white or grey due to the loss of dehydrogenase activity. Under Masson trichrome staining, normal myocardium appeared red, whereas fibrosed areas were stained purple (Figure 4).
No significant difference was seen between the two groups in the infarct area ratio (IAR), derived from the total area of each section and the area not stained by TTC (IA), (Control, 34.68 ± 4.08% vs FGF, 33.35 ± 5.37%). No significant difference was seen between the two groups in the fibroed area ratio (MAR), derived similarly from the total area of each section and the area stained by Masson trichrome (Control, 49.13 ± 4.27% vs FGF, 40.24 ± 5.05%). However, in a comparison of the fluorescein-stained sections, the ratio (DAR) of non-perfused, or non-fluorescing, area (DA) to total area was significantly less in the FGF group than in the Control group (Control, 30.14 ± 3.29% vs FGF, 17.04 ± 3.38%, p < 0.05). Similarly, the DA/IA ratio was significantly less in the FGF group than in the Control group (Control, 88.78 ± 2.591% vs FGF, 47.48 ± 4.274%, p < 0.01) (Figure 3). No significant difference was seen between
Figure 4. C: Control group. F: FGF group. (1) Fluorescein fluorescent stain section photographs. Perfused areas exhibit yellow-green fluorescence, whereas unperfused areas appear black. (2) TTC stain section photographs. Normal myocardium is dark red, whereas the infarcted area appears white or grey. (3) Masson trichrome stain section photographs. Normal myocardium is red, whereas fibroed tissue appears purple.

the two groups for left ventricular luminal diameter (Control, 1.71 ± 0.153 cm vs FGF, 1.59 ± 0.22 cm) (Figure 6). Again, no significant difference was seen between the two groups in the ratio of wall thickness of infarcted and non-infarcted areas (Control, 97.9 ± 1.2% vs FGF, 98.4 ± 5.67%). (Figure 6).

DISCUSSION

In this study, we administered bFGF to beagle dogs immediately following acute coronary artery occlusion, and observed significant neoangiogenesis, with a significant decrease in the area of non-perfusion within the infarct area as shown by fluorescein uptake. However, no decrease in the size of the TTC staining infarct area was seen, and no significant reduction in myocardial fibrosis, as shown by Masson trichrome staining, was seen following administration of bFGF.

In the canine acute myocardial infarction model, at 4 hours after coronary artery occlusion over 75% of the infarct area tissue has undergone irreversible damage⁸, and after roughly 40 minutes following coronary artery occlusion, the
necrosis spreads from the endocardium towards the epicardium, and after about 24 hours it is said to extend through the full thickness of the heart muscle.\textsuperscript{10}

The development of collateral circulation in response to coronary artery occlusion is thought to consist of (1) collateral vessels, already present pre-occlusion, that enlarge passively in response to the post-occlusion pressure gradient, and (2) newly grown vessels actively produced post-occlusion. Pre-existing collateral vessels are small and thin-walled, with a diameter of 40–100 \textmu m in dogs,\textsuperscript{11} and 20–200 \textmu m in humans.\textsuperscript{12} In response to coronary artery occlusion they become enlarged and overextended, and are termed overstretch arterioles.\textsuperscript{15} In a model of acute occlusion of the left circumflex artery in dogs, immediately post-occlusion blood flow increased towards the centre of the infarct area in pre-existing collateral vessels at a rate of approximately 9 ml/min per 100 g of tissue,\textsuperscript{14,16} and myocardial blood flow in the ischemic area immediately post-occlusion has been shown to be increased 120\% 5 seconds later.\textsuperscript{15} However, this blood flow to the ischemic myocardium immediately following the coronary artery occlusion period increases up to 3 hours post-occlusion, but after this time no further increase can be seen.\textsuperscript{10} This is thought to be due to the limited number of pre-existing collateral vessels.
In active neoangiogenesis, endothelial DNA synthesis commences rapidly in response to FGF following coronary artery occlusion. However, in experiments with endothelial cell cultures, the sprouting of new vessels requires 2 or 3 days after commencement of stimulation with bFGF. \(^{(5)}\) In vivo, as the time required for DNA transcription before the first endothelial cell division is relatively long, it is said to require 22 hours from the commencement of the ischemic stimulus. \(^{(6)}\)

In the present study, 1 week post-ligation blood flow to the distal LAD improved to a Rentrop score grade of 3 in 5 out of 6 dogs on CAG, and the non-perfused area within the infarcted area as expressed by the DA/IA ratio was significantly reduced. This improvement in blood flow is thought to be due to enlargement of pre-existing collateral vessels in response to a pressure gradient, and also to the growth of new vessels. However, as pre-existing collateral vessels are thought to be no different between the two groups, the significant improvement in blood flow in the FGF group may be considered to be due to neoangiogenesis as well as the maturation of pre-existing vessels.

Padua et al. \(^{(9)}\) reported that bFGF is cardioprotective in myocardial ischemia reperfusion injury, employing an isolated rat heart model. In addition, bFGF has been shown to inhibit apoptosis of endothelial cells, vascular smooth
muscle cells, and some other cells. Tanaka et al. have reported that hypoxia induces apoptosis in cultured rat cardiomyocytes. In this way, it is possible that, independent of its angiogenic effect, bFGF protects damaged cells and directly affects cell viability. However, in our observation period of 7 days, no statistical difference was found between the two groups in infarct size as demonstrated by TTC staining or in the extent of myocardial fibrosis, and no improvement in hemodynamic parameters was observed. From these observations, we concluded that the angiogenic and cardioprotective effects of bFGF are insufficient in the early stages after coronary artery occlusion, allowing completion of the infarct, and thus no decrease in infarct size or improvement in hemodynamic parameters.

At present, treatments such as thrombolytic therapies, percutaneous coronary angioplasty (PTCA), coronary arterial stents and bypasses are widely used in acute myocardial infarction, all of which aim to relieve ischemia by re-establishing blood flow. Bonaduce et al. performed reperfusion therapies in 24 myocardial infarction patients more than 4 hours post-onset, and reported the prevention of expansion and remodeling. Weisman et al. performed PTCA on myocardial infarction patients 6–48 hours post-onset, and reported a reduction in expansion and fatal arrhythmias, giving an improved prognosis.

In our observation period of 7 days, no significant difference was observed between the two groups in wall thickness of the infarcted area or left ventricular luminal diameter. However, at 1 week following coronary artery occlusion, a definite improvement in blood flow was observed, improving in the LAD distal to the occlusion to a Rentrop score of 3 in 5 out of 6 animals. Scheinowitz et al. administered bFGF immediately following myocardial infarction and after reperfusion in an experiment using rats, and reported prevention of left ventricular dilatation, suggesting the possibility that this treatment may improve the prognosis in myocardial infarction. This suggests that in cases where direct stenosis-relieving procedures such as PTCA cannot be performed, or have been tried and failed, or where thrombolytic therapies are contraindicated, treatment with bFGF may be useful. Furthermore, there is a need to confirm its efficacy as an adjunct to reperfusion therapies.

**Limitations of the present study:** Researchers are trying a number of different dosages and methods of administration of bFGF. Among the methods of administration being attempted are direct injection into the myocardium, via a coronary artery, and a method of applying bFGF via impregnated gauze applied to the area adjacent to the coronary artery. In this study, we used the intravenous route of administration, readily used in cases where the above-mentioned interventional therapies cannot be performed, or in conditions such as exertional angina with coronary artery stenosis. Yanagisawa-Miwa et al. reported a reduc-
tion in infarct size with administration of bFGF into the coronary artery immediately following coronary arterial occlusion. More recently, Scheumacher et al.\textsuperscript{31} have reported the clinical finding of significant development of collateral vessels following intracardiac administration of bFGF during bypass surgery. We feel there is a need to confirm the efficacy of the non-intravenous routes of administration. With regard to dosage, there have been reports of exacerbation of ischemia,\textsuperscript{32} and of hypertrophy in myocardial cell cultures,\textsuperscript{33} with large doses of bFGF. In our study using an acute peripheral circulatory failure model, we observed a significant neangiogenic effect, but none of the above adverse effects. In this study, we again used the simplest, intravenous, route of administration at the same dosages. However, Lazarous et al.\textsuperscript{31} administered a massive dose of 1.74 mg/day for a period of 4 weeks to dogs with ligated coronary arteries. An important issue for future study is the dose-responsiveness of the angiogenic effect of bFGF. As mentioned earlier, even when large doses of bFGF are administered in the early stages of myocardial infarction, there is little chance of significant neangiogenesis occurring inside the golden time for myocardial salvage.

In the study, infarct size reduction had no effect, but it is necessary when comparing infarct sizes to adjust the risk area. No significant difference was seen between the two groups in the ratio between the length of LAD distal to the ligation and the sum of the lengths of left coronary artery main trunk LAD and circumflex arteries, as measured on CAG.\textsuperscript{35} Furthermore, no significant difference was seen between the two groups in hemodynamic parameters immediately following coronary artery ligation, from which we assume that there was also no significant difference in risk areas. However, due to individual variation it is difficult to standardize risk areas with complete certainty.

**Conclusion:** Administration of bFGF for the treatment of acute coronary artery occlusion induced significant development of collateral circulation, and improved blood flow to the infarcted area was observed. No effect was observed on infarct size, but a better prognosis can be expected due to the improved blood flow. From the angiogenic effect seen in the ischemic area, its use may also be considered in the treatment of angina pectoris and ischemic heart disease.

**References**


