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Basic Fibroblast Growth Factor Caused No Change in Collateral Flow or Infarct Size of Acutely-Infarcted Myocardium in Rats

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INAGAKI, M., KIMURA, A., MIYATAKA, M. and ISHIKAWA, K. *Basic Fibroblast Growth Factor Caused No Change in Collateral Flow or Infarct Size of Acutely-Infarcted Myocardium in Rats*. Tohoku J. Exp. Med., 2000, **191** (2), 101-111 —— The extent to which local administration of basic fibroblast growth factor (bFGF) increased regional myocardial blood flow (Qm) to acutely-infarcted areas of the heart, thereby mediating myocardial salvage, was examined in this study. Myocardial infarction was induced in two groups of rats by ligation of the left coronary artery. The bFGF group ($n=16$) received 100 μg bFGF in physiological saline by intramyocardial injection into the infarcted area, while the control group ($n=7$) received only saline. The rats were then maintained for four weeks. Among the controls, Qm decreased in the infarcted areas to 6.5±6.7% of that in the noninfarcted areas immediately after coronary ligation, then increased to 11.5±8.6% during the four-week maintenance period. In the bFGF group, Qm immediately decreased to 17.5±14.7% following ligation and remained stable thereafter (18.3±9.1%). There were no significant differences between the bFGF and control groups with respect to Qm, the number of viable myocardial cells or the extent of myocardial fibrosis. In this study we failed to show any significant effect of bFGF on coronary angiogenesis in acutely-ischemic myocardium in rats. Application of bFGF using different dosage, different routes of administration, measuring new capillaries morphologically will be needed to confirm the present negative results. ———— basic fibroblast growth factor; myocardial infarction; collateral circulation; angiogenic therapy © 2000 Tohoku University Medical Press

Angiogenic therapy for ischemic heart disease has been the focus of much research during the past several years (Gospodarowicz 1974; Yanagisawa-Miwa et al. 1992; Engler 1996). Yanagisawa-Miwa et al. (1992) documented the angiogenic effect of basic fibroblast growth factor (bFGF) on myocardial infarction in dogs. They first induced infarction by embolizing the canine left anterior

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descending coronary artery; then they administered 2 bolus injections of 10 μg bFGF into the left circumflex artery. Left ventricular ejection fraction and infarct size were measured one week later, and significant preservation of the left ventricular ejection fraction, limitation of the infarct size and, importantly, an increase in new blood vessels were observed. Since then, the angiogenic effect of bFGF has been replicated in both dogs and pigs by several other investigators, including ourselves (Battler et al. 1993; Lazarous et al. 1995; Uchida et al. 1995; Miyataka et al. 1998).

In our study of acutely-infarcted rabbits, bFGF caused an increase in regional myocardial blood flow (Qm) and myocardial salvage in the border zone, but not in the infarcted area (Hasegawa et al. 1999). This suggests the effect of bFGF on the acutely-infarcted myocardium may vary depending upon the species or the preexisting number of native collaterals. Our preliminary study in rats using 10 μg bFGF intravenously failed to increase Qm at the infarcted area (Inagaki et al. 1996). However, the dosage of bFGF might be too small to induce any angiogenic effect. We hypothesized that increment of dosage may cause angiogenic effect in rats to limit infarct size. The purpose of this study was to find out any effects of large dose (100 μg) of bFGF on the acutely-infarcted myocardium in rats.

Methods

Animal preparation

Sprague-Dawley rats (14.8 $\pm$ 2.0 weeks of age, mean $\pm$ s.d.; 436 $\pm$ 45 g in weight) were anesthetized by inhalation of diethylether and subsequent intraperitoneal injection of 30 mg/kg pentobarbital and then ventilated by positive pressure. The hearts were exposed, and the left coronary artery was ligated with 5-0 silk thread approximately halfway between a point directly beneath the left atrium and the apex. In the bFGF group ($n = 16$), 100 μg of human recombinant bFGF (Kaken Pharmaceutical Co., Tokyo) dissolved in 0.04 ml of physiological saline were injected into the left ventricular wall in the infarcted area immediately after coronary artery ligation. The control group ($n = 7$) received 0.04 ml of physiological saline intramyocardially exactly as in the bFGF group. The thorax was then closed, the rats were allowed to recover, and they were maintained for an additional four weeks.

Nonradioactive colored microspheres for measuring Qm

To measure Qm, 200 000 nonradioactive, colored microspheres (E-Z Trac Co., Los Angeles, CA, USA [Hale et al. 1988]) were injected three times during the experiment; a different color was used for each injection. The microspheres were suspended in 0.2 ml of 13.3% glucose solution, which had the same specific gravity as the microspheres, and were injected into the left atrium over 10 seconds. The first injection was administered just prior to the coronary ligation; the second was

made about 1 minutes after the ligation and injection of bFGF; and the third injection was made when the chest was reopened after the four-week maintenance period.

Heart specimen

After the final microspheres injection, the rats were killed by intravenous administration of an overdose of pentobarbital, and the heart was removed. The distances between the apex and the base of the resected heart (a) and between the apex and the ligation site in the coronary artery (b) were measured, and the ligated position was defined as b/a (%). The resected hearts were then frozen on dry ice and cut on a microtome from the base to the apex into 1 mm thick, short-axis, serial slices. Each slice was macroscopically divided into the three regions: the infarcted region (MI, where myocardial thinning was complete); the noninfarcted region (where the myocardium was of normal thickness); and the border zone (BZ, situated between the infarcted and normal regions). The respective areas of the three regions were measured, and the sizes of the MI (Fletcher et al. 1981) and BZ regions, expressed as the ratio of the slices to the total area of the left ventricle (Total), were calculated according to the following expressions:

Infarct size (%) $= \Sigma \mathrm{MI}/\mathrm{Total} \times 100\%$,
Border zone (%) $= \Sigma \mathrm{BZ}/\mathrm{Total} \times 100\%$.

Flow into the three regions was assessed in slices by microsphere counting (Hale et al. 1988; Ishikawa et al. 1994).

Myocardium score and the extent of fibrosis

The degree of necrosis in the infarcted area was assessed from the myocardium score and the extent of fibrosis, which were measured as described previously (Miyataka et al. 1998; Hasegawa et al. 1999). One slice from the center of the infarcted region was fixed in 10% formalin for approximately one month, stained with Azan Mallory and photographed under a light microscope at a magnification of 200×. Each slice was divided into central, right-hand and left-hand portions, and the infarcted areas of each portion was further subdivided into the epicardial area, the mid area and the endocardial area. Each of the 3 areas was photographed 5 times yielding a total of 15 pictures. BZ on the right and left, adjacent to the infarcted area, and the noninfarcted region were also subdivided into epicardial, mid and endocardial areas, and 2 pictures each were taken (12 and 6 pictures, respectively). A 10×10 grid was then placed over each photograph and the amount of viable myocardium, defined as myocardium still retaining striations, was assessed in each area using the point counting method (Baandrup and Olsen 1981; Miyataka et al. 1998; Hasegawa et al. 1999). The myocardium score was defined as the number of viable myocardial cells situated at the 100 intersecting

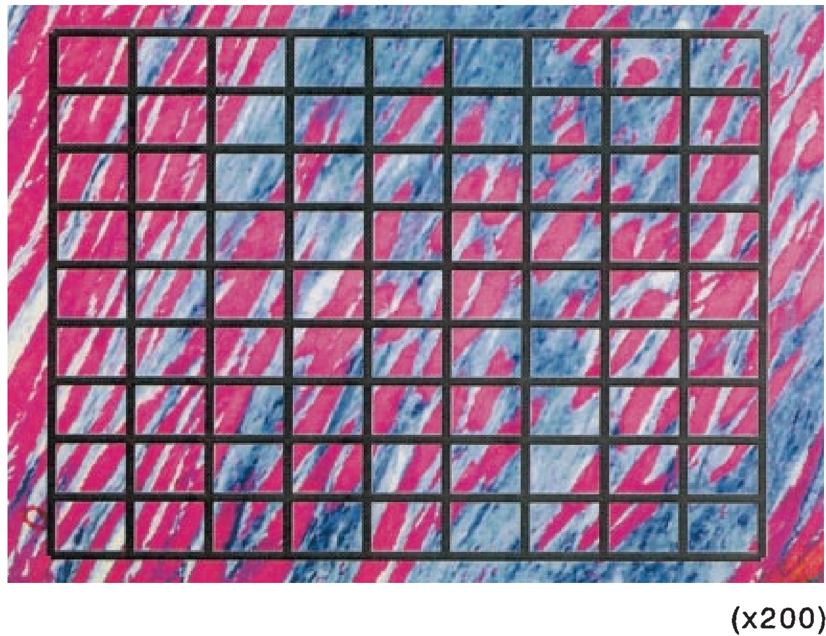

Fig. 1. Myocardium score calculated by point counting method (Azan Mallory staining) (Baandrup and Olsen 1981; Miyataka et al. 1998; Hasegawa et al. 1999). The score was defined by the number of viable (striated) cells situated at the 100 points of intersection in the grid.

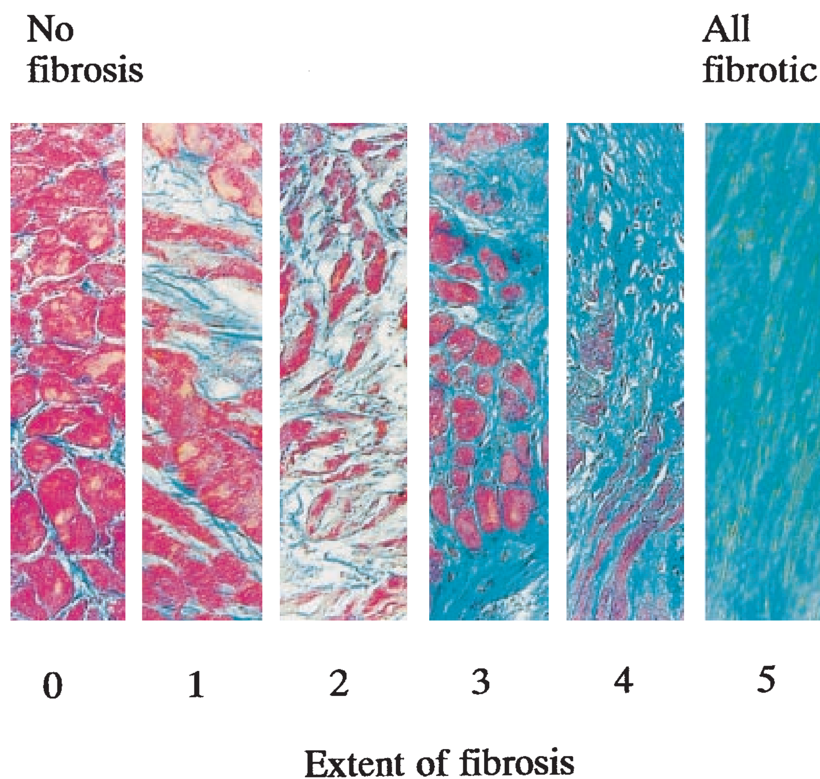

Fig. 2. Extent of fibrosis (Azan Mallory staining) (Miyataka et al. 1998; Hasegawa et al. 1999). Panels 0-5 are indicative of the 6 degrees of fibrosis assessed.

points of the grid (Fig. 1). After Azan Mallory staining, the extent of myocardial fibrosis was likewise visually graded on a scale of 0 to +5, where 0 represented no myocardial fibrosis and +5 represented complete fibrosis (Fig. 2) (Miyataka et al. 1998; Hasegawa et al. 1999). Three investigators independently judged the extent of fibrosis, and the mean value was accepted as the score for the specimen.

Calculation of Qm

The method for measuring Qm was previously described in detail (Ishikawa et al. 1994). Briefly, the collected myocardial slices containing the microspheres were weighed and then dissolved in boiling 2 N NaOH for 30 minutes while stirring. The resultant solution was centrifuged to recover the microspheres. The numbers of microspheres retrieved from 1 g of myocardium were counted using a Fuchs-Rosenthal hemocytometer under a microscope. Absolute Qm values (ml/min/g) before and after coronary ligation could not be determined because some of the microspheres were lost during the four-week, postinfarction, maintenance period (Murdock and Cobb 1980). Consequently, Qm in the infarcted areas were normalized to values in noninfarcted areas, and the resultant ratios (% noninfarcted) were defined as indices of the changes in Qm according to the following expression (Lazarous et al. 1995; Inagaki et al. 1996; Miyataka et al. 1998; Hasegawa et al. 1999):

$$\text{Qm (\% noninfarcted)} = (Q/Qc)_{\text{infarcted}}/(Q/Qc)_{\text{noninfarcted}} \times 100\%;$$

where Qc = Qm prior to myocardial injection and coronary ligation, and Q = Qm at each time point.

Statistical analysis

Values are expressed as the mean ± standard deviation (S.D.). Comparisons between means of the two groups were made using Student's *t*-test for unpaired samples.

Results

The chests were closed after coronary ligation in 24 rats in the control group. Among them, 17 died during the four-week maintenance period, leaving 7 available for final measurement (mortality 71%). Among 57 in bFGF group, 41 died during the four-week maintenance period, 16 rats available for final measurement (mortality 72%). There was no significant difference in the mortality between the 2 groups. High mortality during convalescence in both groups may be attributable to the bleeding following left atrial injection of colored microsphere. The two groups did not differ in body weight, heart weight or ligated position, and there were no significant differences in the sizes of either the infarct regions or the border zones (Table 1).

Table 2 shows Qm values obtained immediately after coronary ligation and

TABLE 1. *Body weight, heart weight and infarct size in the 2 groups*

Age at acute phase (week)	Body weight		Heart weight				Number of slices	Infarct and border		Ligated position b/a (%)
	Acute phase (g)	4 weeks later (chronic phase) (g)	Total heart weight (g)	Noninfarcted zone (g)	Infarcted zone (g)	Border zone (g)		Infarct size (%)	Border zone (%)	
Control										
12.3 ±1.3	382 ±36	433 ±25	1.502 (0.27) ±0.165 (±0.03)	0.509 ±0.089	0.062 ±0.026	0.216 ±0.070	15.0 ±1.0	19.9 ±6.3	25.7 ±7.6	52.8 ± 7.7
bFGF										
15.9 ±1.0	459 ±24	472 ±20	1.447 (0.25) ±0.180 (±0.03)	0.671 ±0.131	0.090 ±0.035	0.202 ±0.101	16.0 ±2.1	17.9 ±7.8	20.4 ±7.7	50.8 ±14.6

Mean ± S.D. A total heart weight as % of body weight at chronic phase.

TABLE 2. *Regional myocardial blood flow (Qm) as measured by % of noninfarcted*

	Infarcted zone		Border zone	
	Acute phase	4 weeks later (chronic phase)	Acute phase	4 weeks later (chronic phase)
	(% of noninfarcted)		(% of noninfarcted)	
Control	6.5 ± 6.7	11.5 ±8.6	63.4 ±10.1	64.1 ±14.6
bFGF	17.5 ±14.7	18.3 ±9.1	77.9 ±19.2	81.0 ±24.3

Mean±S.D.

TABLE 3. *The amount of viable myocardium (myocardium score), extent of fibrosis at the infarcted and border zone areas*

	Myocardium score (%)		Extent of fibrosis (0-5)	
	Infarcted zone	Border zone	Infarcted zone	Border zone
Control	15.8 ±6.2	54.0 ±14.2	4.2 ±0.3	2.0 ±0.7
bFGF	12.8 ±5.5	50.5 ±18.2	4.4 ±0.2	2.2 ±0.9

Mean±S.D.

four weeks after creating the infarct. Among the controls, Qm in the infarcted region was reduced to 6.5±6.7% of the noninfarcted region immediately after coronary ligation ; after four weeks, Qm had increased slightly to 11.5±8.6%. In the bFGF group, coronary ligation decreased Qm to 17.5±14.7% where it remained stable over the four-week maintenance period. There were no significant differences in Qm between the bFGF and control groups at any time point. Qm in the BZ considerably decreased immediately after coronary ligation, but again there were no significant differences between the bFGF and control groups during the acute phase or after four weeks.

The number of viable myocardial cells (myocardium score) and the extent of myocardial fibrosis (extent of fibrosis) were then histologically compared in bFGF and control hearts (Table 3). Although the myocardium score decreased markedly in the infarcted regions of both groups, there was no significant difference between the bFGF and control hearts; similarly, there was no significant difference in the extent of fibrosis. With respect to the BZs, again there were no significant differences in the myocardium scores or the extent of fibrosis.

DISCUSSION

This present study showed that bFGF failed to increase myocardial blood flow or salvage myocardium in acutely-infarcted rat hearts, a finding that is contrast to previous studies carried out in dogs (Yanagisawa-Miwa et al. 1992;

Lazarous et al. 1995; Uchida et al. 1995; Shou et al. 1997; Miyataka et al. 1998), pigs (Battler et al. 1993; Harada et al. 1994; Lopez et al. 1995) and rabbits (Hasegawa et al. 1999).

The capacity of bFGF to limit infarct size was initially observed by Yanagisawa-Miwa et al. (1992) following intracoronary injection of bFGF in dogs. A similar effect was subsequently found by the same group after intrapericardial injection (Uchida et al. 1995). Consistent with that finding, we also observed that injection of 300 μg of bFGF directly into the myocardium elicited a notable increase in Qm and a reduction of infarct size (Miyataka et al. 1998). On the other hand, injection of bFGF 30 μg into the contralateral coronary artery did not increase Qm or promote myocardial salvage (Miyataka et al. 1996), suggesting that the effect of bFGF may be dose dependent. Indeed, Lopez et al. (1995) reported that the effect of bFGF was proportional to dose; i.e., the effect of 100 μg bFGF was superior to that of 10 μg in pigs. Previous study in rats using bFGF 10 μg continuous intravenous injection for 24 hours failed to show any angiogenic effect (Inagaki et al. 1996). Further studies in our laboratory using bFGF 10 μg intramyocardial injection failed to show any effect on Qm or infarct size in rats (Ishikawa 1998). Local intramyocardial injection of 100 μg in rats in this study was an extraordinarily large dose compared to 300 μg in dogs (Miyataka et al. 1998) or 10 μg or 100 μg in pigs (Lopez et al. 1995). This suggests that the present negative result is not due to the insufficient dose. bFGF in slow-release (Sellke et al. 1994), intrapericardial injection (Uchida et al. 1995) or injection in the marginal zone (Harada et al. 1994) may exert different effects. We need to clarify how much of bFGF and in what way bFGF should be administered to be effective. Also, it is necessary to find out whether or not capillaries or arterioles caused any increase in the bFGF group to conclude angiogenic effect of bFGF in rats.

Battler et al. (1993) injected Affigel beads infiltrated with bovine pituitary gland fluids into the infarct-related coronary arteries of pigs with acute myocardial infarction. Two weeks later, a marked increase in the number of capillaries was observed histologically. In addition, Sellke et al. (1994) induced a fourfold increase in arteriolar density in the infarcted areas of pig hearts by injecting bFGF incorporated into calcium alginate microcapsules around the coronary arteries while subjecting the arteries to gradual ligation with an ameroid constrictor. Similar angiogenesis and myocardial salvage has been demonstrated in pigs by other investigators (Harada et al. 1994; Lopez et al. 1995) using ameroid constrictors (Battler et al. 1993; Harada et al. 1994; Sellke et al. 1994; Lopez et al. 1995) to gradually occlude the infarct-related coronary arteries over a period of approximately one week (Lazarous et al. 1995; Shou et al. 1997). The gradual occlusion of the arteries makes the results obtained in those studies difficult to compare with the present study where the affected artery was rapidly ligated. This is especially true for pigs, rabbits or rats (Maxwell et al. 1987) whose hearts

contain few native collaterals and suggests that the effects of bFGF might vary depending on how rapidly and how completely myocardial ischemia develops. In other words, bFGF may have a chance to exert angiogenic effects in rats if the coronary artery was gradually occluded.

We previously reported that in rabbits, bFGF increases Qm significantly in the noninfarcted myocardium, increases Qm slightly in the border zone and salvages myocardium in this area, but does not increase Qm or salvage the infarcted region (Hasegawa et al. 1999). Compared with dogs (15.9%), native collaterals are scarce in rabbits (2.0%), as they are in pigs (0.6%) and rats (6.1%) (Maxwell et al. 1987). The angiogenic actions of bFGF may thus be determined in part by the number of native collaterals. In addition, our data suggest that experimental results obtained with human recombinant bFGF may vary in different species depending upon its relative homology with the primary structure of the native bFGF. For example, Barrios et al. (1995) reported bFGF from bovine pituitary gland induced neovascularization in normal rat hearts, whereas human recombinant bFGF in this study did not. However, there is a high sequence homology between FGFs of different species, this is particularly evident with bFGF of human, bovine and rat (Baird and Böhlen 1991). Accordingly, application of human recombinant bFGF may not be the reason of the present negative results. As a matter of fact, several studies showed angiogenic effect of human recombinant bFGF in the ischemic rat limbs (Yang and Feng 2000) but, this has not been proved in the ischemic rat heart.

The present investigation suggests that the angiogenic effect of bFGF may be affected by the number of native collaterals, the rapidity with which ischemia develops and/or the primary structure of the bFGF isoform used. Clinical application of bFGF to myocardial infarction was started in USA (Shou et al. 1997), and there is a report (Schumacher et al. 1998) of intramyocardial injection of FGF-1 during coronary artery bypass surgery in Germany. A phase I clinical study of direct myocardial gene transfer of vascular endothelial growth factor for patients with symptomatic myocardial ischemia has been reported by Isner's group (Losordo et al. 1998). In summary, our present experiment serves a warning that the efficacy of bFGF in patients with ischemic heart disease may vary depending on the pathophysiological status of each individual myocardium. Also, the present negative results need to be varified using morphological analysis measuring development of new capllaries, using bFGF of different dosage, different route of administration as well as application of bFGF of rat itself.

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