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Changes of Expressions of VEGF, bFGF, and Angiogenesis, and Effect of Benazepril, bFGF on Angiogenesis in Acute Myocardial Infarction Model of the Rabbits

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Objective To explore the changes of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and angiogenesis, and the effects of bFGF, angiotensin converting enzyme inhibiter(ACEI) benazepril on the angiogenesis in acute myocardial infarction (AMI) model of rabbits, and to provide a probable evidence for the treatment of AMI. **Methods** AMI model was established by ligating anterior descending branch of coronary artery of Japan-Sino hybridization white rabbits. The postoperative rabbits were randomly divided into 6 groups and each group was treated with different drugs. Groups 1 and 2 were treated with normal saline (NS) for 28 and 14 days (d), group 3 and 4 with bFGF for 28 and 14 d, groups 5 with benazepril for 14 d, and group 6 with benazepril and bFGF for 14 d respectively. The rabbits were killed on the 14th or 28th d and their hearts were excised, sectioned and stained with HE, Masson trichrome to observe VEGF, bFGF and CD_{34} under a microscope, which were quantified with a computer-assisted morphometry. **Results** Compared with group 1, the granulation tissue of infarction zone (IZ) in group 2 freshened up, and the capillary density (CD) in IZ was increased (P=0.002). The CD in the IZ as well as VEGF and bFGF in groups 3 and 4 were increased respectively (P=0.011-0.037). In group 5 the changes of VEGF and bFGF were not found in the IZ and the border zone (BZ) while CD was significantly increased (35.4% and 25.6%, P=0.036 and 0.037). Compared with group 2, the CD in the IZ and BZ of group 6 was significantly increased (63.4% and 44.3% P=0.007 and 0.007), meanwhile VEGF and bFGF were increased. Compared with group 5, only VEGF was increased. **Conclusion** Intravenous bFGF may increase VEGF and bFGF significantly, thus promoting the angiogenesis in the IZ and BZ in cardiac infarction as VEGF and bFGF are the potent angiogenic growth factors. Benazepril may promote angiogenesis in the IZ and BZ in cardiac infarction, but its mechanism is irrelative to the expression of VEGF and bFGF. The combination of benazepril and bFGF may promote, to some extent, the expression of VEGF and bFGF, but their effect on angiogenesis has not been found.

Key words: Myocardial infarction; Angiogenesis; Basic fibroblast growth factor; Vascular endothelial growth factor; Benazepril

INTRODUCTION

Other than traditional drug treatment, interventional therapy and surgical intervention which may improve blood perfusion in ischemic myocardium, the concept of molecular

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bypass or therapeutic angiogenesis has been adopted in the treatment of coronary disease recently[1], in which growth factors (proteins or genes) are used to enhance angiogenesis in the ischemic area of myocardium. The proliferation of capillary in the border of myocardial infarction is typical angiogenesis[2]. A great number of growth factors and corresponding inhibitors participate in the process of angiogenesis, among which VEGF and bFGF are considered as two critical factors. VEGF and bFGF are both found increasing in ischemic myocardium and its border in animal models of acute or chronic myocardial ischemia and in patients' specimens[3-5]. In acute or chronic animal models of myocardial ischemia, intervention of VEGF or bFGF promotes angiogenesis, reduces infarction area and ameliorates cardiac function[6-8].

Because of wide use of ACEI in cardiovascular disease and influence of angiotensin system on angiogenesis and growth factors, scholars have shown great interests in the relationship between ACEIs and angiogenesis in myocardial infarction recently. But generally speaking, the studies about their effect on angiogenesis and growth factors involved are relatively few and controversial. We established an AMI model of rabbits, observed the changes of VEGF, bFGF and angiogenesis, and studied the effects of bFGF, ACEI and benazepril on the changes of bFGF, VEGF and angiogenesis of the heart in order to provide a probable evidence for the therapy of AMI.

MATERIALS AND METHODS

Animals and Grouping

Forty-eight male Japan-Sino hybridization white rabbits weighting 2.0-2.5 kg (Certificate No. zd00-002), were provided by Animal Center of Zhejiang Academy of Medical Sciences. They were randomly divided into six groups and each group was treated with different drugs. Group 1: administered normal saline (NS), 8 mL iv qd×28 days; group 2 (control group) : NS, 8 mL iv qd×14 days; group 3: bFGF, 10 μg/kg iv qd×28 days; group 4: bFGF, 10 μg/kg iv qd×14 days; group 5: benazepril, 10 mg/kg/d ip.×14 days; group 6: benazepril, 10 mg/kg/d ip.×14 days and bFGF, 10 μg/kg iv qd×14 days. The initiation time of drug administration was 1 hour after ligation.

Drugs and Reagents

Benazepril powder was presented by Beijing Novartis Pharmaceutical Company Ltd, China, and bFGF injections were gifted by EssexBio Group, China. Masson trichrome stain kit was purchased from Fujian Sanqiang Biological and Chemical Engineering Company Ltd, China. Streptavidin/peroxidase stain kit, rat anti-human primitive hematopoietic CD34 monoclonal antibody, rat anti-human VEGF polyclonal antibody, rabbit anti-human bFGF polyclonal antibody and DAB stain kit were purchased from Beijing Zhongshan Bio-Tech Company Ltd. 10% formaldehyde, dimethylbenzene, ethanol of different concentrations, hematoxylin and eosin stain liquid were got from pathologic department of our hospital.

Establishment of AMI Model

Rabbits were anesthetized with intravenous 0.3% pentobarbital sodium (1 mL/kg). Under sterile conditions, left thoracotomy was performed through the third or fourth intercostal space (where heart beat was the strongest). The pericardium was opened and the signal of inferior margin of left auricular appendage and great cardiac vein, the anterior descending branch of coronary artery were transfixed twice. After 15 minutes' observation,

when the anterior wall of the heart turned purple and elevated ST segment was detected on the bed side monitor, electrocardiocustodium, ischemia of myocardium was confirmed and then thoracotomy was closed. Antibiotics (penicillin) were injected intramuscularly for three days. After operation the rabbits were fed carefully.

Specimens

Rabbits were killed by 10% potchloride 5 mL intravenously on the 14th or 28th day, and the hearts were excised and fixed in formaldehyde.

Tissue Analysis

After fixation for at least 2 days, the hearts were cut into 2 mm slices from apex to base and embedded with paraffin. The basal side of each equatorial slice was sliced up and the sections were stained with HE and Masson trichrome. The slices were also stained by the method of SP immunohistochemistry with VEGF, bFGF and CD34. The processes of the staining were similar, but the dilutions of each antibody and incubation time were different. Rabbit anti-VEGF (which reacts with rabbit VEGF) was diluted at 1:100, and incubated at room temperature for 3 h, rabbit anti-bFGF (which reacts with rabbit bFGF) was diluted at 1:250, and incubated at room temperature for 2 h, rat anti-CD34 (which reacts with rabbit CD34) was diluted at 1:100, and incubated at room temperature for 3 h. Immunohistochemical stain was used as negative control (stained with non-specific antibodies)

Computer-assisted Morphometry

VEGF, bFGF and CD34 immunohistochemically stained slices were observed through an omnipotent microscope with 10 (ocular lens) ×20 (object lens) by a pathologist without knowledge of the treatment, and photographed by a color video camera. The software of HPIAS-1000 high clarity colorful image and word report system were used to observe the pathologic changes in the infarction zone (IZ), the border zone of infarction (BZ) and the zone beyond infarction (FZ). Ten visual fields were randomly selected, and analysis on the positive signal of selected fields was made. In the IZ, we calculated the ratio of the positively stained area and took the mean as the average rate of positive expression, while in the BZ and FZ, we calculated the ratio of positive myocardial cells, and took the mean as the ratio of positive cells. As to the CD34 immunohistochemically stained slices, the number of capillaries in one unit area was calculated.

Data Analysis

Data were reported as $\bar{x}\pm s$. Statistical analysis was made with SPSS/10.0 software. Independent *t* or *t'* test and one-way analysis of variance were used for analysis. A *P* value <0.05 was considered statistically significant.

RESULTS

Basic Conditions

All the rabbits in groups 1 to 4 were alive, and one rabbit in group 5 and two rabbits in group 6 crocked up day by day and died at last, which might be due to the injury of lavage. The survival rate was 93.8%. No difference between weight and heart rate of each group was found.

Result of Masson Trichrome Staining

The infartion zone stained with bright green corresponded with that of HE stain and was more clear suggesting the model of myocardial infarction was well established. It could be used to judge the zone of infarction, the border zone of infarction and the zone of non-infarction.

Results of Immunohistochemistry

In the infarction zone, the positive region of VEGF and bFGF immunoreactivity was in the extracellular matrix, while in the border zone of infarction and the zone beyond infarction, VEGF and bFGF immunoreactivity was in cytoplasm of myocardium, endothelium. The immunoreactivity was different in each group. Additionally, CD34 immunoreactivity was only found in cytoplasm of endothelial cells lining capillaries.

Changes of Myocardial Tissue After AMI

On the sections of the 2nd or 3rd layer of the rabbit's heart of HE staining, the myocardium in the anterior wall was replaced by granulation tissue, fibrous tissue and fatty tissue from endocardium to epicardium, while in the posterior lateral wall and interventricular septum, the cardiac muscle fibers arranged regularly and tightly, and cross striations were clear and orderly. Compared with group 1, granulation tissue of the IZ in group 2 freshened up and was composed of more fibroblast cells and more abundant vessels. In the center of infarction there were unabsorbed myocardial cells, infiltration of inflammatory cells, such as macrophages and neutrophils. Compared with group 1, capillary density (CD) in the IZ (P=0.002), VEGF in the BZ and FZ of group 2 were significantly increased (P=0.030 and 0.003) (Table 1).

Effect of bFGF on VEGF, bFGF and Angiogenesis After AMI

In group 3, the expression of VEGF was increased in the IZ and BZ (79.5%, 78.5%, P=0.011, 0.037), the CD was increased in the IZ (65.1%, P=0.008) compared with group 1. In group 4, and the expression of VEGF and bFGF in the IZ was increased compared with group 2 (VEGF: 53.1%, P=0.029; bFGF: 56.7%, P=0.006), and the CD in the BZ was increased by 30.4% (P=0.003). Compared with group 3, the CD in the IZ of group 4 was increased (P=0.004) (Table 2).

Effect of Benazepril on VEGF, bFGF and Angiogenesis After AMI

No changes of VEGF and bFGF were found in the IZ and BZ in group 5, while the CD in the IZ and BZ was significantly increased (35.4% and 25.6% respectively, P=0.036 and 0.037) (Table 3).

Effect of Combination of Benazepril and bFGF on VEGF, bFGF and Angiogenesis After AMI

Compared with group 2, the CD in the IZ and BZ of group 6 was significantly increased (63.4% and 44.3% respectively, P=0.007 and 0.007), meanwhile the amount of VEGF in the IZ, bFGF in the IZ and BZ were incre ased (VEGF: 56.1%, P=0.039; bFGF: 65.9% and 36.9%, P=0.007, 0.004). When compared with group 5, VEGF in the BZ was significantly increased (65.7%, P=0.004), while no change of CD was found (Table 4).

TABLE 1

Comparison of VEGF, bFGF, and CD in Groups 1 and 2

Group	*n*	IZ			BZ			FZ		
		VEGF	bFGF	CD	VEGF	bFGF	CD	VEGF	bFGF	CD
Group 1	8	20.23±9.12	11.16±4.63	7.11±2.17	11.54±8.51	25.07±7.69	27.49±6.95	3.77±3.05	8.76±4.25	24.91±9.49
Group 2	8	29.31±12.90	15.16±5.11	12.03±2.81[a]	19.85±4.76[a]	30.67±10.09	28.04±6.16	9.92±3.79[a]	8.59±2.83	26.35±7.40

Note. t test , [a] vs group 1, $P<0.05$.

TABLE 2

Comparison of VEGF, bFGF, and CD in Groups 1, 2, 3, and 4

Group	*n*	IZ			BZ			FZ		
		EGF	bFGF	CD	VEGF	bFGF	CD	VEGF	bFGF	CD
Group 1	8	0.23±9.12	11.16±4.63	7.11±2.17	11.54±8.51	25.07±7.69	27.49±6.95	3.77±3.05	8.76±4.25	24.91±9.49
Group 2	8	9.31±12.90	15.16±5.11	12.03±2.81[a]	19.85±4.76[a]	30.67±10.09	28.04±6.16	9.92±3.79[a]	8.59±2.83	26.35±7.40
Group 3	8	36.35±6.43[a]	8.12±5.03	11.74±3.56[a]	20.60±7.71[a]	32.26±10.03	32.49±9.34	5.16±3.81	9.03±4.21	28.21±5.80
Group 4	8	44.87 ± 11.51[b]	23.75±8.74[b]	19.02±5.59[b,c]	26.47±6.10	42.71±11.34	36.57±6.99[b]	7.05±4.46	10.24±4.94	28.23±6.41

Note. One-way ANOVA, [a]vs group 1, $P<0.05$, [b]vs group 2, $P<0.05$, [c]vs group 3, $P<0.05$.

TABLE 3

Comparison of VEGF, bFGF, and CD Between Groups 2 and 5

Group	*n*	IZ			BZ			FZ		
		VEGF	bFGF	CD	VEGF	bFGF	CD	VEGF	bFGF	CD
Group 2	8	29.31±12.90	15.16±5.11	12.03±2.81	19.85±4.76	30.67±10.09	28.04±6.16	9.92±3.79	8.59±2.83	26.35±7.40
Group 5	7	31.55±11.45	19.33±4.30	16.29±4.20a	18.94±5.26	31.08±6.24	35.22±5.75[a]	6.86±2.61	8.37±4.75	28.56±5.14

Note. t test, [a]vs group 2, $P<0.05$.

TABLE 4

Comparison of VEGF, bFGF, and CD Between Groups 2, 5, and 6

Group	*n*	IZ			BZ			FZ		
		EGF	bFGF	CD	VEGF	bFGF	CD	GF	FGF	CD
Group 2	8	29.31±12.90	15.16±5.11	12.03±2.81	19.85±4.76	30.67±10.09	28.04±6.16	9.92±3.79	8.59±2.83	26.35±7.40
Group 5	7	31.55±11.45	19.33±4.30	16.29±4.20[a]	18.94±5.26	31.08±6.24	35.22±5.75[a]	6.86±2.61	8.37±4.75	28.56±5.14
Group 6	6	45.75±11.47[a]	25.15±7.17[a]	19.66±5.65[a]	31.38±8.47[b]	44.15±12.84[a]	40.50±8.96[a]	8.09±3.67[a]	11.39±2.89	24.74±5.61

Note. One-way ANOVA, [a]vs group 2, $P<0.05$, [b]vs group 5, $P<0.05$. The unit of VEGF, bFGF expression :%. The unit of capillary density: no./unit area.

DISCUSSION

Changes of Myocardial Tissue After Infarction

In the condition of ischemia or hypoxia, the expression of bFGF, VEGF and their receptors in myocardium were increased[3,12]. Ventricular-biopsy specimens from 37 patients undergoing coronary bypass surgery were collected by Lee *et al.*, and VEGF mRNA and protein were detected in myocardial specimens in the early period of ischemia and infarction (within 48 h)[12]. After two hours' ligation of the left anterior descending coronary artery of dogs, bFGF in myocardium increased and reached its peak in 1-2 weeks and disappeared in 8 weeks[4]. After the combination with its receptor, VEGF exhibited important effects on the whole process of angiogenesis, including the increase of vascular permeability, degration of extracellular matrix, endothelial cell proliferation and migration, formation of cords and lumens, showing an unsubstitutional role in angiogenesis[14]. bFGF could induce proliferation of many types of cells including endothelial cells, smooth muscle cells and fibroblasts, and promote the expression of VEGF[13,16].

In our experiment, the expression of VEGF in the IZ, BZ and FZ in hearts of rabbits killed 4 weeks after infarction was significantly lower than that in hearts of those killed 2 weeks after infarction. Accordingly, the capillary density of infarction area of the former was lower than that of the latter. Though there was no significant difference of bFGF in the IZ or BZ between groups 1 and 2, it showed a tendency to decrease. In HE staining, compared with group 2, fibroblast cells in IZ of group 1 became more mature, turning into fibrocytes and the number of capillary was less, while there was no significant difference of bFGF and CD in the FZ between the two groups. The results suggested that the concentration of VEGF and bFGF in the IZ and BZ increased compensatively and transitorily because of stimulation of ischemia and hypoxia after myocardial infarction. But when infarction turned from granulation tissue into scar gradually, VEGF and bFGF in these tissues decreased gradually, so did the capillary network. As a result, the local concentration of VEGF and bFGF increased transitorily after myocardial infarction, which might not be able to meet the need of creating sufficient angiogenesis as far as time was concerned. This deduction might explain the reason why there were no effective newly born vessels or lateral circulation though VEGF and bFGF significantly increased in myocardium after infarction. Lee *et al.* reported that in the early period after infarction, VEGF was expressed in ischemic myocardium and its border, and increased with the prolongation of ischemia. The present result was not all identical with Lee's finding, which might be interpreted by the fact that the observation time after infarction was not the same[3,12].

Effect of bFGF on Angiogenesis After AMI

Since the beginning of 1990s, the growth factors have been adopted in a variety of acute or chronic animal myocardial ischemic models. With the development of biological agents, growth factors and their genes have been used in clinical experiments[17,19]. When they were administered in local myocardium or through coronary artery etc., they could ameliorate clinical symptoms, increase local perfusion and reduce infarction areas[20,21]. Since intravenous injection was convenient and repeatable, the rabbits were given bFGF 10 μg/kg intravenously for 2 or 4 weeks. The results showed that bFGF administered at 10 μg/kg/d for two or four weeks aroused a significant increase in the expression of VEGF and the capillary density in the infarction zone or its border. The results suggest that giving bFGF through vein may increase VEGF and bFGF significantly and enhance the angiogenesis in the infarction zone

and the border as VEGF and bFGF are potent angiogenic growth factor.

In the experiment, when comparing the group with two-week administration of bFGF to the group with four-week administration, we found that though the dosage was the same, the capillary density in the infarction zone of the former was higher than that of the latter. The difference between them might be due to the fact that compensatory angiogenesis of myocardium in 2 weeks after infarction was more active than that in 4 weeks as showed in our experiment. This suggests that when the area of infarction changes from granulation tissue to scar, the angiogenic action of bFGF becomes weak. So angiogenic growth factor should be applied early after infarction and it may produce more significant effect.

Sasame *et al.* proved recently that when bFGF 20 μg was given for intravenously three days to canines after anterior wall myocardial infarction, on the 7th day after operation, capillary density of infarction area in bFGF treated group increased significantly compared with the control group[22]. Our result was also consistent with Sasame's.

Effect of Benazepril and bFGF on Angiogenesis After AMI

ACEI was used in myocardial infarction mainly to ameliorate myocardium reconstitution. There exsits controversy about the effect of AECI on angiogenesis after AMI. In our experiment bFGF and VEGF did not increase in the IZ and BZ in the benazapril treated group while the capillary density increased significantly (35.4% and 25.6%, respectively). Some experiments showed that angitensin II (Ang II) could increase the expression of VEGF protein or gene of human vascular smooth muscle cells, rat heart endothelial cells and bovine retinal endothelial cells, etc.[23-25]. But in our experiment bFGF and VEGF did not increase in the IZ and BZ in the benazapril treated group when compared with the control group, indicating that the inherent inhibitory effect of ACEI on Ang II does not inhibit the expression of bFGF and VEGF of infarcted cardiac muscle cells. Now we know that under the condition of ischemia or hypoxia, expression of bFGF, VEGF in myocardium increases, which plays an important role in the process of angiogenesis[3,4], thus our result may be explained by the inference that hypoxia *in vivo* may be independent of AngII and is an important factor that may enhance the expression of growth factors (however, the expression is limited).

Silvestre *et al.* examined neovascularization induced by ACEI perindopril in B_2 receptor-deficient (B_2 -/-) mice in a model of surgically induced hindlimb ischemia (compared with wild-type mice). The mice were administered perindopril, 3 mg/kg/d for 28 days. Vessel density and capillary number in the ischemic leg of the wild-type mice were raised by 1.8- and 1.4-fold respectively, and meanwhile the leg perfusion ratio was raised by 1.5-fold and it was associated with a 1.7-fold increase in tissue eNOS (endothelial nitric oxide synthase) protein level but not with the change in VEGF protein level. Conversely, ACEI did not have such an effect on ischemic hindlimb of (B_2 -/-) mice[26]. When the stroke-prone spontaneously hypertensive rats were given ramipril and bradykinin B_2-receptor antagonist Icatibant at the same time, ramipril increased capillary density significantly but the effect was blocked by Icatibant[27]. This also suggests that the effect of ACEI on angiogenesis is related to ACEI-induced potentiation of endogenous bradykinin. Morbidelli *et al.* proved that bradykinin could promote growth of endothelial cells by B_1 receptor[28]. It is known that Ang II could stimulate the NADH oxidase present in vascular wall, increase the generation of superoxide anions capable of degradating NO[29], thus ACEI can increase the bioavailability of NO by inhibiting the conversion of Ang II. Therefore ACEI can promote the accumulation of NO in coronary microvessels. The upregulation of VEGF could increase the secretion of NO of endothelium, which is essential to the VEGF-induced

angiogenesis and has a close relation to angiogenesis[30]. The present experiment revealed that benazapril did not induce angiogenesis by directly stimulating the expression of growth factors. From the above findings we may reasonably infer that the effect of ACEI on the promotion of angiogenesis is related rather to bradykinin B_1 and B_2 receptor signal transduction, eNOS upregulation and accumulation of NO in coronary system, than to growth factors.

The experiment showed that compared with the control group, the capillary density in the IZ and BZ of group 6 (intervention by a combination of benazepril and bFGF) increased significantly, meanwhile the amount of VEGF and bFGF increased in the IZ and BZ. When compared with group 5 (intervention by benazepril only), the amount of VEGF of group 6 increased while no increase of capillary density was found. The results show that the combination of benazepril and bFGF may promote the expression of VEGF and bFGF to some extent, but their effect on angiogenesis has not been found. When benazepril was singly administered the capillary density in the IZ and BZ increased while no change of bFGF and VEGF was found, when benazepril was administered in combination with bFGF, the expression of growth factors increased. In this sense, the combination seems much more beneficial.

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