ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2001 Oct; 22 (10): 876 - 880
http://www.chinaphar.com

Effects of recombinant human basic fibroblast growth factor on restenosis after arterial endothelial injury in rats

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KEY WORDS percutaneous coronary transluminal angioplasty; basic fibroblast growth factor; vascular smooth muscle

ABSTRACT

AIM: To study the preventive effect of recombinant human basic fibroblast growth factor (rh-bFGF) on restenosis after percutaneous transluminal coronary angioplasty (PTCA). **METHODS**: Sixty male Wistar rats were randomly divided into sham operation group, intima injured group, and intima injured plus rh-bFGF treated group. Rat carotid arteries were injured using a balloon catheter except sham operation group. The rats of rh-bFGF treated group were injected im rh-bFGF 10 $kU \cdot kg^{-1} \cdot d^{-1}$ after intima was injured. Ten rats in each group were killed on d 7 and d 14 after injury, respectively. [^{3}H] Thymidine incorporation assay and pathological examination were carried out to each vessel. **RESULTS**: (1) Seven days after injury, the average intimal thickness in sham operation group, intima injured group, and rh-bFGF treated group was (7 ± 1), (32 ± 11), and (17 ± 3) μm; average intimal area was (384 ± 145), (1530 ± 817), and (586 ± 185) μm^2; the numbers of smooth muscle cells in neointima per transect were 0 ± 0, 146 ± 18, and 105 ± 26; the ratio of the collagen area to intimal area plus medial area were 0.29 ± 0.09, 0.7 ± 0.3, and 0.30 ± 0.14; [^{3}H]thymidine incorporation were (17 ± 6), (62 ± 23), (20 ± 8) kBq/g tissue, respectively. (2) Fourteen days after injury, the average intimal thickness of sham operation group, intima injured group, and rh-bFGF treated group was (8 ± 1), (41 ± 9), and (20 ± 3) μm; average intimal area was (391 ± 134), (1761 ± 337), and (731 ± 124) μm^2; the numbers of smooth muscle cells in neointima per transect were 0 ± 0, 145 ± 9, and 102 ± 6; the ratio of collagen area to intimal area plus medial area were 0.28 ± 0.14, 0.59 ± 0.21, and 0.38 ± 0.03; [^{3}H] thymidine incorporation was (15 ± 4), (57 ± 11), and (22 ± 6) kBq/g tissue, respectively. **CONCLUSION**: Large dosage of rh-bFGF inhibits neointimal hyperplasia and reduces restenosis after balloon injury.

INTRODUCTION

Percutaneous transluminal coronary angioplasty (PTCA) damaged arterial endothelial cells and caused absence of endothelial cells in the area of injury. Platelets adhere to the injured vessel wall, where they aggregate and release mitogenic growth factors such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), transform growth factor-β, and basic fibroblast growth factor (bFGF), *etc*, which stimulate vessel smooth muscle cells (SMC) to proliferate and migrate from the media to the intima and to replicate continually there. This leads to the neointima formation and restenosis[1]. Some articles reported that bFGF stimulated SMC to proliferate *in vitro*[2,3], however, bFGF also stimulated endothelial cell proliferation and promoted endothelial cells to repair rapidly, which protected the injured area from thrombosis by inducing synthesis of plasminogen activator and reducing the production of plasminogen inhibitors[4]. Restenosis was mainly caused by SMC proliferation after endothelial injury. Therefore, bFGF possesses dual effects on restenosis formation. Up to date, there are few reports about the role of exogenous bFGF on restenosis *in vivo* at pharmacological dosage. For its clinical application, it is valuable to explore whether large dosage of exogenous bFGF affects artery SMC proliferation and restenosis. In this study, we applied the model of rat artery balloon catheter injury to observe the effect of large dosage of recombinant human bFGF (rh-bFGF) on SMC proliferation after endothelial cells injury, and discussed

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Received 2000-10-08 Accepted 2001-05-31

the value of application of rh-bFGF in clinical treatment after PTCA.

MATERIALS AND METHODS

Reagents Recombinant human bFGF (1 mg = 1 × 10^6 U) was offered by Torita Bio-Pharma Co Ltd (Zhuhai, China), stored at −20 ℃, and diluted with normal saline just before using. [^{3}H]Thymidine (specific activity, 18.5 MBq/mol) was obtained from Beijing Institute of Nuclear Research, China. Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco Laboratories. 2,5-Diphenyl oxazole and 1,4-bis-[5-phenyl oxazoyl-2]-benzene were purchased from Sigma Company. The other reagents were of analytical purity.

Rats Sixty male Wistar rats, weighing 300 g ± 50 g, were obtained from the Experimental Animal Center of Gansu Academy of Medical Sciences (Clean grade, Certificate № 14-010). They were randomly divided into sham operation group, intima injured group, and intima injured plus rh-bFGF treated group (called rh-bFGF treated group). There were 20 rats in each group.

Experimental instruments The FJ2101 liquid scintillation counter was the product of the 262th Factory (Xi-an, China). The Cmias8 computer image analysis system was the product of Beijing University of Aeronautics and Astronautics. 2F balloon catheter was obtained from Peking University.

Surgical procedures The rats were anesthesized with sodium pentobarbital ip 30 mg/kg. A median incision about 2-cm long into the neck skin was made, and the left common carotid artery and the left external carotid artery were isolated carefully. A 2F balloon catheter was inserted into left common carotid artery through the left external carotid artery and advanced close to the aortic arch, and inflated with 0.1 mL normal saline to a diameter of about 2 mm. The balloon catheter was pulled and pushed thrice in the left common carotid artery. After removal of the catheter, the left external carotid artery was ligated and the neck wound was closed. rh-bFGF was injected im 10 kU·kg^{-1}·d^{-1} for 7 d or 14 d. The rats in intima injured group were injected im normal saline 1 mL·kg^{-1}·d^{-1}. The same treatments were given to the sham operation group except no insertion of 2F balloon catheter. The rats were killed on d 7 and d 14 after denudation, respectively. The left common carotid artery about 2-cm long was retrieved and cut into two segments. One was used for pathological examination, and the other was used for [^{3}H]thymidine incorporation assay by culture *in vitro*.

Pathological examination The left common carotid artery was flushed with normal saline after rat was killed, and fixed at 4 ℃ with 4 % paraformaldehyde dissolved in phosphate buffered solution (PBS, 0.1 mol/L, pH 7.2), and then put into 30 % buffered sucrose overnight at 4 ℃. On the next day, the left common carotid artery was removed and embedded into paraffin. Artery sections 4-μm thick (six sections in each artery) were prepared and stained with hematoxylin and eosin, smooth muscle cell special stain, and Masson's stain. The average thickness and area of intima and media, SMC numbers, and collagen area in neointima and media of artery per transect was measured by pathological image analysis system.

***In vitro* proliferation assay** The vessel about 5-mm long was cut into vascular loops with 1-mm length, and the vascular loops were incubated in DMEM containing 10 % fetal bovine serum with 55 kBq [^{3}H]thymidine in a 5 % CO_2 incubator at 37 ℃ for 24 h. The vascular loops were taken out and flushed with PBS (0.1 mol/L, pH 7.2, 4 ℃) thrice, and the vascular tissue was weighed. After the vascular loops were digested for 2 h at 80 ℃ in 0.25 mL 88 % methyl acid, the digested liquid was completely transferred into scintillation cuvette and scintillation liquid 7 mL and 95 % ethanol 3 mL was added. The radioactivity was measured through a liquid scintillation counter.

Statistical analysis Data were expressed as the $\bar{x} \pm s$. Statistically significant differences between two groups were calculated by t test.

RESULTS

Effects of rh-bFGF on neointimal and medial thickness of rat common carotid artery Compared with the sham operation group, the average intimal and medial thickness in intima injured group increased markedly on d 7 and d 14 after balloon injury ($P < 0.01$), indicating that the animal model was successfully prepared (Tab 1). The average intimal thickness in rh-bFGF treated group was attenuated greatly as compared with intima injured group ($P < 0.01$). But the difference of average medial thickness between rh-bFGF treated group and the intima injured group was not significant ($P > 0.05$). These data indicate that rh-bFGF inhibited neointimal hyperplasia of rat common carotid artery after balloon injury, but did not affect the

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2001 Oct; 22 (10)
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proliferation of medial SMC.

Tab 1. Effects of rh-bFGF on neointimal and medial thickness of rat common carotid artery. $n = 10$ rats. $\bar{x} \pm s$. $^aP > 0.05$, $^cP < 0.01$ *vs* intima injured group.

Groups	Neointimal and medial thickness/μm		
	Intimal	Intimal + medial	Medial
d 7			
Sham operation	7 ± 1[c]	17 ± 2[c]	10 ± 2[c]
Intima injured	32 ± 11	74 ± 21	42 ± 26
rh-bFGF treated	17 ± 3[c]	57 ± 14[c]	40 ± 14[a]
d 14			
Sham operation	8 ± 1[c]	17 ± 1[c]	9 ± 4[c]
Intima injured	41 ± 9	70 ± 12	29 ± 8
rh-bFGF treated	20 ± 3[c]	45 ± 4[c]	25 ± 5[a]

Effects of rh-bFGF on neointimal area, medial area, and the ratio of collagen area to neointimal area plus medial area of rat common carotid artery On d 7 and d 14 after injury, the average neointimal area, medial area, and the ratio of collagen area to neointimal area plus medial area in intima injured group increased greatly as compared with sham operation group ($P < 0.01$). rh-bFGF 10 kU/kg im showed a suppressive effect on the neointima formation and collagen synthesis compared with intima injured group ($P < 0.01$), whereas the effect on average medial area was not significant ($P > 0.05$). These data showed that the increase of neointimal area of rat common carotid artery after balloon injury was inhibited by rh-bFGF, but the proliferation of medial SMC was not. Recombination human bFGF also inhibited collagen synthesis in this experimental model (Tab 2).

Effects of rh-bFGF on SMC numbers in neointima and media of rat common carotid artery SMC were not found in the intima of rat common carotid artery in sham operation group (Tab 3), but in the intima injured group, there were extraordinarily excessive SMC on d 7 and d 14 after balloon injury ($P < 0.01$). The SMC numbers in neointima in rh-bFGF treated group were reduced compared with that in intima injured group ($P < 0.01$). But the average medial SMC numbers showed no significant difference between rh-bFGF treated group and intima injured group ($P > 0.05$). These data implied that rh-bFGF inhibited medial SMC to migrate to intima and replicate there continually, but rh-bFGF did not affect the proliferation on medial SMC.

Tab 3. Effects of rh-bFGF on SMC numbers in neointima and media of rat common carotid artery. $n = 10$ rats. $\bar{x} \pm s$. $^aP > 0.05$, $^cP < 0.01$ *vs* intima injured group.

Groups	SMC numbers		
	Neointima	Media	Intima + media
d 7			
Sham operation	0 ± 0[c]	154 ± 15[c]	154 ± 15[c]
Intima injured	146 ± 18	303 ± 17	449 ± 35
rh-bFGF treated	105 ± 26[c]	313 ± 49[a]	418 ± 75[c]
d 14			
Sham operation	0 ± 0[c]	160 ± 15[c]	160 ± 15[c]
Intima injured	145 ± 9	193 ± 18	338 ± 27
rh-bFGF treated	102 ± 6[c]	201 ± 11[a]	303 ± 17[c]

Effects of rh-bFGF on [^{3}H]thymidine incorporation of rat common carotid artery [^{3}H]Thymidine incorporation of intima injured group was increased as compared with sham operation group on d 7 and d 14 after balloon injury ($P < 0.01$), suggesting that

Tab 2. Effects of rh-bFGF on neointimal area, medial area, and the ratio of collagen area to neointimal area plus medial area of rat common carotid artery. $n = 10$ rats. $\bar{x} \pm s$. $^aP > 0.05$, $^cP < 0.01$ *vs* intima injured group.

Groups	Neointimal and medial area/μm^2			Collagen content in intima plus media area	
	Intimal area	Medial area	Intimal + medial area	Collagen area/μm^2	Collagen ratio
d 7					
Sham operation	384 ± 145[c]	3855 ± 1224[c]	4240 ± 1301[c]	1230 ± 253	0.29 ± 0.09[c]
Intima injured	1530 ± 817	7557 ± 875	9087 ± 1104	6088 ± 1019	0.67 ± 0.32
rh-bFGF treated	586 ± 185[c]	7447 ± 942[a]	8032 ± 1032[c]	2410 ± 354	0.30 ± 0.14[c]
d 14					
Sham operation	391 ± 134[c]	3741 ± 1195[c]	4232 ± 1297[c]	1185 ± 275	0.28 ± 0.14[c]
Intima injured	1761 ± 337	5770 ± 607	7531 ± 270	4443 ± 983	0.59 ± 0.21
rh-bFGF treated	731 ± 124[c]	5727 ± 1238[a]	6458 ± 1114[c]	2454 ± 427	0.38 ± 0.03[c]

the DNA synthesis was increased after balloon injury. The [^{3}H] thymidine incorporation in rh-bFGF treated group was decreased greatly as compared with intima injured group ($P < 0.01$), indicating that rh-bFGF inhibited the DNA synthesis in SMC of rat common carotid artery (Tab 4).

Tab 4. Effects of rh-bFGF on [^{3}H] thymidine incorporation of rat common carotid artery (kBq/g tissue). $n = 10$ rats. $\bar{x} \pm s$. $^cP < 0.01$ *vs* intima injured group.

Groups	[^{3}H] Thymidine	
	d 7	d 14
Sham operation	17 ± 6^c	15 ± 4^c
Intima injured	62 ± 23	57 ± 11
rh-bFGF treated	20 ± 8^c	22 ± 6^c

Effects of rh-bFGF on SMC layers of rat common carotid artery wall The SMC layers were arranged alternately with elastic membrane. The proliferation of SMC companied with the proliferation of elastic membrane. There are about 2 – 3 layers of SMC in common carotid artery wall of sham operation group. However, there were about 4 layers of SMC in intima injured group (Tab 5). The difference was statistically significant ($P < 0.01$). The SMC layers were all 3 layers in rh-bFGF treated group both on d 7 and d 14 after injury. When it was compared with the intima injured group, the difference was significant ($P < 0.01$), indicating that rh-bFGF inhibited the hyperplasia of rat common carotid artery wall after balloon injury.

Tab 5. Effects of rh-bFGF on SMC layers of rat common carotid artery wall. $n = 10$ rats. $\bar{x} \pm s$. $^cP < 0.01$ *vs* intima injured group.

Groups	SMC layers	
	d 7	d 14
Sham operation	2.5 ± 0.5^c	2.4 ± 0.6^c
Intima injured	4.1 ± 0.9	3.8 ± 0.2
rh-bFGF treated	3.0 ± 0.0^c	3.0 ± 0.0^c

DISCUSSION

At present time, there are two opinions on the roles of bFGF in SMC proliferation. One takes bFGF for the potent mitogen of SMC. It stimulates SMC to proliferate and leads to restenosis[2,3]. Cultured SMC treated with bFGF resulted in phosphorylation of p42 mitogen-activated protein kinase (p42MAPK), as well as the phosphorylation and increased expression of c-*myc*. bFGF also increased protein kinase C (PKC) activity in SMC in a time-dependent manner. The cultured rat aortic SMC treated with angiotensin Ⅱ (Ang Ⅱ) increases fibroblast growth factor-2 (FGF-2) but not FGF-1 mRNA levels[5]. FGF-2-induced extracellular signal-regulated kinases (ERK) activation mediates the endothelial response to wounding and induces the proliferation of SMC[4]. The other opinion suggested that bFGF was not necessary to SMC proliferation. Immunocytochemical staining showed the presence of bFGF in the uninjured arterial wall, and that bFGF was decreased after balloon injury. Western and Northern blot analyses likewise showed that the amount of bFGF protein and mRNA decreased after injury. A neutralizing antibody to bFGF was administered 4 and 5 d after injury and was found to have no effect on intimal smooth muscle cell proliferation. Systemic injection of a neutralizing antibody against bFGF prior to balloon catheterization markedly decreased the induced SMC proliferation by 80 %. The intimal lesion that developed within 8 d after injury, however, was not significantly reduced[6,7].

On the basis of above data, we can see that bFGF stimulates cultured SMC to proliferate in early stage after injury *in vitro*, however, the role of bFGF may be more complicated *in vivo* than *in vitro*, especially in late stage after injury. In our study, we administered large dosage of rh-bFGF to rats after balloon catheter injury. Compared with intima injured group, the [^{3}H] thymidine incorporation was reduced markdely on d 7 and d 14 after injury, indicating that DNA synthesis in SMC decreased. At the same time, rh-bFGF also reduced the neointimal thickness and its area. However, the thickness and area of media were not reduced at two time points as compared with intima injured group. This indicated that rh-bFGF inhibited the migration and replication but not the proliferation of SMC. Recombinant human bFGF also decreased the content of collagen in neointima and media, suggesting that rh-bFGF inhibited the extracellular matrix deposit. Possibly, it is related to the following factors: as a wounding repair factor, bFGF promoted the proliferation of vascular endothelial cells, which protected the injured area from thrombosis by inducing synthesis of plasminogen activator and reducing the production of plasminogen inhibitors[4]. Therefore, bFGF has anti-coagulative activity. rh-bFGF possesses role of anti-

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2001 Oct; 22 (10)
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damage caused by free radicals[8]. Large dosage of rh-bFGF possibly inhibits the release of others polypeptide growth factors, such as PDGF, EGF, and Ang Ⅱ, which promote proliferation. Our study indicated that rh-bFGF did not inhibit medial SMC proliferation, but did inhibit SMC migrating to intima and replicating there, consequently, inhibited neointimal hyperplasia. Thus, application of large dosage of rh-bFGF does not promote restenosis, on the contrary, it can inhibit the generation and development of restenosis.

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重组人碱性成纤维细胞生长因子对大鼠动脉内皮损伤后再狭窄的影响

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关键词 经皮冠状动脉经腔血管成形术；碱性成纤维细胞生长因子；血管平滑肌

目的：探讨重组人碱性成纤维细胞生长因子（rh-bFGF）对经皮冠状动脉腔内成形术后再狭窄的预防效果. **方法**：用2F球囊导管造成大鼠左颈总动脉内皮损伤. 治疗组每天 im rh-bFGF 10 kU/kg. 分别于术后7天和14天，每组各处死大鼠10只，取左颈总动脉进行[^{3}H]胸腺嘧啶掺入测定和病理形态学检查. **结果**：在第7天和第14天时，与模型对照组相比，rh-bFGF治疗组颈总动脉平均新生内膜厚度明显变薄；平均中膜面积缩小；平滑肌细胞和弹力板层数减少；胶原含量及[^{3}H]胸腺嘧啶掺入量也比模型对照组明显降低. **结论**：适当应用rh-bFGF有抑制气囊损伤后动脉新生内膜增厚，降低再狭窄发生的作用.

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