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Original Research

Basic fibroblast growth factor up-regulates the expression of vascular endothelial growth factor in primary cultured rat astrocytes

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KEY WORDS: basic fibroblast growth factor; astrocytes; endothelial growth factors; cultured cells; genetic transcription; polymerase chain reaction; message RNA; immunohistochemistry

ABSTRACT

AIM: To examine the effect of recombinant human basic fibroblast growth factor (bFGF) on the expression of vascular endothelial growth factor (VEGF) in primary cultured rat astrocytes. **METHODS**: Semiquantification PCR (SQ-PCR) and immunocytochemistry were used to investigate the effect of bFGF on VEGF mRNA level and protein level, respectively. **RESULTS**: Treatment with bFGF dose-dependently increased the VEGF mRNA level in astrocytes. The up-regulation of VEGF mRNA induced by bFGF (10 μg/L) was detected as short as 3-h treatment. The increase of VEGF mRNA level reached the maximum after 24-h treatment with bFGF. The immunocytochemical staining showed that the VEGF protein level in astrocytes also increased after the cells were incubated with bFGF. **CONCLUSION**: bFGF induced a marked time- and concentration-dependent increase in VEGF expression in primary cultured astrocytes, suggesting that the effect of bFGF on angiogenesis in brain may act partly by up-regulating VEGF expression in astrocytes.

INTRODUCTION

Basic fibroblast growth factor (bFGF) is a polypeptide with neurotrophic effects, which can protect neuronal cells against ischemic insults *in vivo* and the effect may partly result from its capability to promote cerebral angiogenesis in ischemic brain[1-6]. bFGF is an endothelial mitogen *in vitro* thus it can have "direct" effect on endothelial cells *in vivo*[7,8]. In the present study, we intended to study if bFGF could also have "indirect" angiogenesis-promoting effect by modulating the expression of the direct angiogenic growth factor, vascular endothelial growth factor (VEGF), in cells other than endothelial cells.

MATERIALS AND METHODS

Cell culture Astrocyte cultures were prepared from 1-day-old rat pups[9] given birth by one pregnant Wistar ♀ rat (obtained from Shanghai Experimental Animal Center, Chinese Academy of Sciences, Grade II, Certificate № 005). The cells were seeded into 162-cm^2 tissue culture flasks (Costar) and allowed to grow for 3 d at 37 ℃ and 5 % CO_2 in Dulbecco's modified Eagle's medium (DMEM)/F12 medium (1∶1, Gibco) with 10 % heat-inactivated fetal bovine serum and benzylpenicillin 100 $kU \cdot L^{-1}$, streptomycin 100 mg $\cdot L^{-1}$. Media were changed every 2-3 d until 10-11 d, at which time the flasks were shaken at 200 cycles/min in a rotary shaker overnight to remove contaminating cells, mainly microglia. The cultures generated by this procedure were consistently >95 % astrocytes. Astrocytes were then detached from the flasks into cell suspension using 0.25 % trypsin (Gibco) and then subcultured on poly-*L*-lysine (Sigma)-coated 6-well plates (Costar). The subcultured cell density was 1×10^6 cells/well. After 24 h, cultures were ready to be treated with bFGF (provided by Torita Bio-Pharma Co Ltd, Zhuhai, China) or vehicle.

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RNA extraction and reverse transcription After desired incubation period, cells were collected. Total RNA was isolated from the cells with TRIzol (Gibcol/BRL) according to the manufacturer's guidelines. For cDNA synthesis, reverse transcription mixture 20 μL contained total RNA templet 3 μL (1 μg), each of dNTPs 2 mmol·L^{-1} 10 μL (Forward, China), Oligo $(dT)_{15}$ primer 1 μL (1.6 μg) (Sangon, Canada), ribonucleade inhibitor 1 μL (20 U) (Promega, USA), M-MuLV reverse transcriptase 1 μL (20 U) (MBI, Lithuania) and 5 × reaction buffer 4 μL (Tris-HCl 250 mmol·L^{-1}, KCl 250 mmol·L^{-1}, $MgCl_2$ 20 mmol·L^{-1}, DTT 50 mmol·L^{-1}). The mixture was incubated at 37 ℃ for 90 min and then the reverse transcriptase was inactivated by heating to 95 ℃ for 10 min.

PCR procedures The forward primer for VEGF was 5′-CAAGGATCCATGAACTTTCTGCTGTCTTGGG-3′, and the reverse primer was 5′-TCTAAGCTTATCACCGCCTCGGCTTGTCACATCT-3′. To ensure that reverse transcriptase efficiencies were comparable between test groups, β-actin cDNA was also amplified. The forward primer for β-actin was 5′-TTGTAACCAACTGGGACGATA-3′ and the reverse primer was 5′-GATCTTGATCTTCATGGTGCT-3′. VEGF and β-actin PCR were carried out in the same procedure. One μL each of reverse transcription solution was added to PCR mixture containing 10 × buffer 5 μL, dNTPs 2 mmol·L^{-1} 2 μL, each of the primers 1 μL (10 pmol) and Taq DNA polymerase 0.75 μL (3.5 U) (Sangon, Canada) in a total volume of 50 μL. PCR was performed on a thermal cycler PTC-150 (MJ, USA) with the following thermocycle parameters: a 5-min initial denaturation at 98 ℃ followed by 25 – 35 cycles of 45-s denaturation at 94 ℃, 45-s annealing at 60 ℃, 90-s extension at 72 ℃, and finally a 10-min extension at 72 ℃. VEGF and β-actin PCR products 5 μL were separated by silver stained 8 % PAGE. The density of each band was measured by densitometer. The ratio of $A_{VEGF}/A_{\beta\text{-actin}}$ was used to express the level of VEGF mRNA level in astrocytes.

Immunocytochemistry The streptavidin-biotin-peroxidase complex (SABC) method was used to detect VEGF protein expression in the cells. The cells were fixed in 4 % paraformaldehyde at 4 ℃ for 20 min and then were microwaved in PBS containing 0.1 % Triton X-100 at 95 – 98 ℃ for 10 min. After preincubation with 1 % H_2O_2-methanol and normal serum, a rabbit anti-VEGF antibody (Santa Cruz, USA) were applied at 4 ℃ in a moist chamber overnight. Secondary biotinylated-antibody was added and then incubated at 37 ℃ for 1.5 h. The streptavidin biotinylated peroxidase complex was subsequently added at 37 ℃ for another 1 h. The peroxidase activity was made visible with 0.003 % diaminobenzidine (DAB, Sigma, USA) and 0.001 % H_2O_2 in PBS.

Statistics Data were expressed as $x \pm s$ and assessed by t test.

RESULTS

VEGF mRNA level after bFGF stimulation Low level of VEGF mRNA was detected in control astrocytes. When astrocytes were incubated with bFGF (1 – 100 μg·L^{-1}), VEGF mRNA level was increased dose-dependently (Fig 1, Tab 1) and time-dependently (Fig 2). The inductive effect of bFGF on VEGF mRNA seemed to be very rapid since the rise in VEGF mRNA level was clearly evident within 3 h of incubation with bFGF and reached the maximum by 24-h incubation.

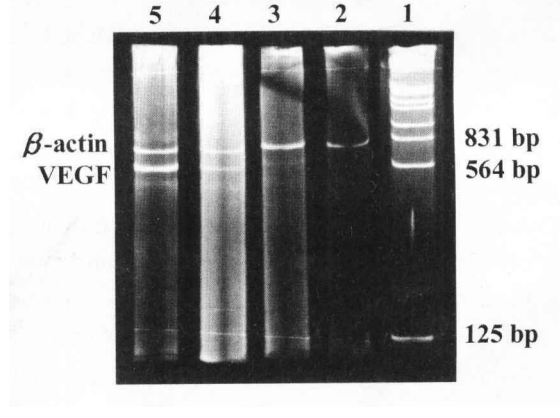

Fig 1. VEGF mRNA level in astrocytes treated with bFGF (1, 10, or 100 μg·L^{-1}) for 12 h. Lane 1) marker; 2) Control; 3) 1 μg·L^{-1} bFGF-treated; 4) 10 μg·L^{-1} bFGF-treated; 5) 100 μg·L^{-1} bFGF-treated.

VEGF protein level after bFGF stimulation Immunocytostaining assay showed nearly no signs of positive reaction to VEGF antibody in astrocytes without stimulation of bFGF. After the astrocytes were treated with bFGF (10 μg·L^{-1}) for 24 h, positive reaction (yellow gains) was seen in the cytoplasm of astrocytes (Fig 3).

Tab 1. Effect of bFGF (1, 10, or 100 μg·L^{-1}) on VEGF mRNA expression in astrocytes after incubation for 12 h. The VEGF mRNA level in the cells was expressed by the ratio of VEGF mRNA to β-actin mRNA. The results were obtained by using RT-PCR assay. $n = 3$ (triplicate). $\bar{x} \pm s$. $^cP < 0.01$ *vs* control.

Group	Control	bFGF-treated (1 μg·L^{-1})	bFGF-treated (10 μg·L^{-1})	bFGF-treated (100 μg·L^{-1})
VEGF mRNA/β-actin mRNA	0.16 ± 0.06	0.28 ± 0.09	0.75 ± 0.17^c	0.79 ± 0.15^c
Induction rate (%)	100	175	469	527

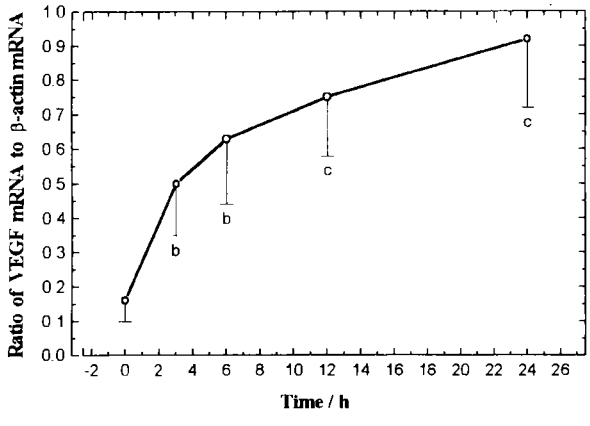

Fig 2. VEGF mRNA expression in astrocytes after treatment with bFGF 10 μg·L^{-1} for 3, 6, 12, or 24 h. Results were got by RT-PCR assay and the experiment was done in triplicate. $n = 3$ (triplicate). $^bP < 0.05$, $^cP < 0.01$ *vs* control group.

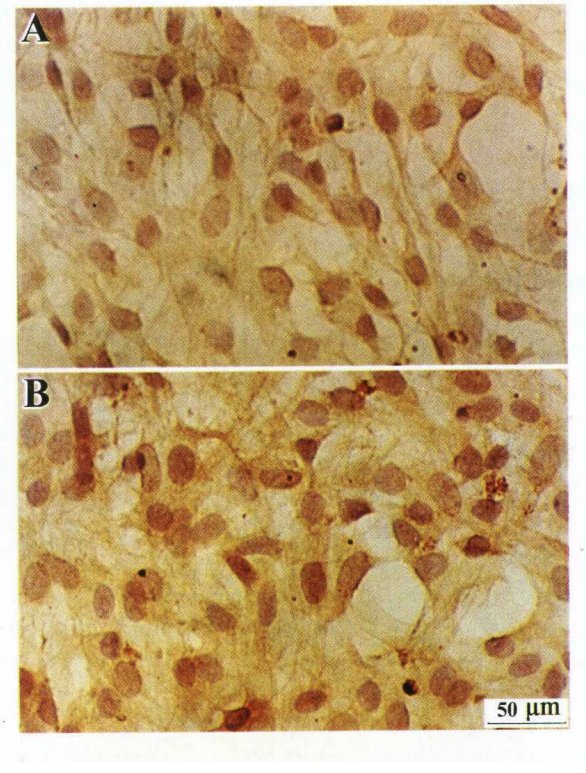

Fig 3. VEGF protein level in astrocytes treated with bFGF (10 μg·L^{-1} for 24 h). DAB stain showed VEGF protein in cytoplasm and then hematoxylin stain showed nuclei of cells. A: Control. B: bFGF-treated. ×200.

DISCUSSION

Basic fibroblast growth factor is a 18 kDa polypeptide with potent survival-promoting and protective effects on central nervous system cells including neurons, glia and endothelial cells. Because of its remarkable neuroprotective effects this factor has been listed as a future candidate for preventing neuronal damage caused by ischemic conditions[1-3]. Although the mechanism by which bFGF induces a protective effect against ischemic damage has not been fully clarified, evidence has shown that this effect may partly result from its capability to promote cerebral angiogenesis in ischemic brain[1-6].

Angiogenesis, the formation of new blood vessels, involves the activation, migration, and proliferation of endothelial cells and is regulated by several peptide and nonpeptide molecules. Among the polypeptide angiogenic factors, VEGF and bFGF have gained much attention because these two peptides are endothelial cell mitogens *in vitro* and have a potent angiogenic activity *in vivo*. Especially, VEGF is a specific mitogen for vascular endothelial cells and plays a pivotal role in angiogenesis. VEGF administration to brain tissue *in vitro* greatly enhanced vascular proliferation in a dose-dependent manner and produced more vessels than bFGF exposure. Direct brain infusion of VEGF also caused substantial vascular growth *in vivo* and appeared to be a far better effector of angiogenesis than intraventricular infusion of bFGF[10].

VEGF and bFGF are not only correlated with the

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development of central nervous system but also participate in the CNS response to injury[11-14]. Evidence has also shown that treatment with topical VEGF application greatly reduces ischemic brain damage. Though VEGF seemed to have no direct neuroprotective effect on neural cells, it reduced the ischemic damage by protecting and inducing the vascular system in the brain[15]. VEGF, as a secreted glycoprotein, could be expressed and secreted by several types of cells including glial cells. Our results that bFGF induced a marked increase in VEGF expression in astrocytes raised the possibility that in addition to its direct angiogenic properties, bFGF may also regulate neovascularization indirectly by modulating VEGF expression in astrocytes.

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碱性成纤维细胞生长因子上调原代培养鼠星形胶质细胞中血管内皮生长因子的表达

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关键词 碱性成纤维细胞生长因子; 星形胶质细胞; 内皮生长因子; 培养的细胞; 遗传转录; 聚合酶链反应; 信使 RNA; 免疫组织化学

目的: 观察碱性成纤维细胞生长因子(bFGF)对鼠星形胶质细胞中血管内皮生长因子(VEGF)表达的影响. **方法**: 用半定量聚合酶链反应(PCR)法和免疫组织化学法分别考察 bFGF 对 VEGF 信使 RNA (mRNA)水平和蛋白质水平的影响. 对不同剂量 bFGF (1, 10, 100 $\mu g \cdot L^{-1}$)和不同孵育时间(3, 6, 12, 24 h)的作用均进行了分析. **结果**: bFGF 可以剂量依赖性升高 VEGF 的 mRNA 水平. bFGF 10 $\mu g \cdot L^{-1}$在孵育 3 h 后即可上调 VEGF mRNA 水平, 孵育 24 h 后细胞内 VEGF mRNA 水平显著高于对照组. 免疫细胞化学分析结果表明 bFGF 也能升高细胞的 VEGF 蛋白质水平. **结论**: 碱性成纤维细胞生长因子可以上调鼠星形胶质细胞中血管内皮生长因子的表达.

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