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Antisense Ca^{2+}-calmodulin dependent protein kinase oligonucleotide inhibits bFGF-induced proliferation of rat vascular smooth muscle cells[1]

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KEY WORDS Ca^{2+}-calmodulin dependent protein kinase; basic fibroblast growth factor; antisense oligonucleotides; vascular smooth muscle; cultured cells; hyperplasia; thymidine; Western blotting; nucleic acid synthesis inhibitors; p42 (Mapk) kinase

ABSTRACT

AIM: To investigate the role of Ca^{2+}-calmodulin dependent protein kinase (CCDPK) on basic fibroblast growth factor (bFGF)-induced vascular smooth muscle cell (VSMC) proliferation and the inhibitory effect of antisense CCDPK oligonucleotides (ODN). **METHODS**: Before being exposed to bFGF, cultured rat VSMC CCDPK activity was inhibited by pretreatment with either a phosphorothioate-protected 17-mer antisense CCDPK ODN-directed against the initiation of translation sites of the p42 and p44 CCDPK isoform or with CCDPK kinase inhibitor PD98059. All ODN were introduced into cells by liposomal transfection. DNA synthesis was measured by [^{3}H]thymidine incorporation. P44- and p42-CCDPK protein expression and phosphorylation were measured by Western blot. **RESULTS**: PD98059 inhibited bFGF-induced phosphorylation of CCDPK and DNA synthesis. Antisense CCDPK ODN 0.2 - 0.8 $\mu mol \cdot L^{-1}$ reduced both p44- and p42-CCDPK expression and phosphorylation of CCDPK in a concentration-dependent manner and DNA synthesis induced by bFGF. Lipofectin alone or sense and random CCDPK ODN did not affect p44- and p42-CCDPK protein expression or bFGF-induced phosphorylation of CCDPK or DNA synthesis. **CONCLUSION**: bFGF-stimulated rat VSMC proliferation is mediated by CCDPK. The antisense CCDPK ODN can inhibit bFGF-induced VSMC proliferation through down-regulating p44- and p42-CCDPK level.

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INTRODUCTION

Excessive proliferation of vascular smooth muscle cells (VSMC) is a critical process in the development of atherosclerotic and restenotic lesions[1]. The processes are regulated by several growth factors that may be released by cells within the injured vessel or from circulating cells, in which basic fibroblast growth factor (bFGF) plays an important role[2]. bFGF regulates proliferation by binding to receptors with intrinsic protein tyrosine kinase activity, resulting in the initiation of intracellular signaling cascades[3,4]. Activation of p44- and p42-CCDPK is a common pathway for cell proliferation induced by growth factors[5]. But the effect of p44- and p42- CCDPK on bFGF-induced VSMC growth signal pathway is unclear.

An early study demonstrated that a phosphorothioate-protected antisense oligonucleotides (ODN), directed against the initiation of translation sites of rat p42 and p44 CCDPK mRNA, has been used successfully to inhibit rat cardiac myocyte hypertrophy[9], EGF[10], and serum-induced VSMC proliferation[16], but nothing is known about the inhibitory effect of this antisense on bFGF-induced VSMC proliferation. Antisense method is an effective gene therapeutic strategy to block expression of specific genes. ODN-mediated inhibition of gene expression is also being evaluated as a paradigm for therapy of human disease. The potential impact of this technology on the treatment of vascular disease has been recently reported[6,7].

We assumed that the strategies aimed at blocking the common pathway, CCDPK, by antisense ODN can inhibit bFGF-induced VSMC proliferation. This study was to

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examine this assumption and to investigate the effect of CCDPK on bFGF-induced VSMC proliferation.

MATERIALS AND METHODS

Drugs and reagents Anti-mitogen activated protein kinase, bFGF, M199 medium, trypsin, lipofectin, HEPES, Triton X-100, egtazic acid were purchased from Sigma Co. PD98059 was purchased from BioLabs Inc. Western blot chemiluminescence reagent plus was purchased from NEN™ Life Science Products. Horseradish peroxidase conjugated secondary antibody was purchased from Wuhan Boster Biological Technology Co. Low-range protein molecular weight markers, nitrocellulose membrane were purchased from Beijing Tianxiangren Biotechnology Co.

Cell culture[8] VSMC were isolated from aortas of 200 – 250 g Sprague-Dawley rats by explant method Rats were supplied by the Animal Center of Hunan Medical University (Grade Ⅱ, Certificate № 20-009) and VSMC were maintained in M199 supplemented with 10 % fetal bovine serum (FBS). Cells used were passages 4 to 10.

Measurement of DNA synthesis[10] Cell proliferation in terms of DNA synthesis was determined by measuring [^{3}H]thymidine incorporation. VSMC were plated in 24-well plates at 5×10^4 cells per well and grown to 80 % confluence. Following pretreatment with antisense ODN for 48 h or PD98059 for 1 h, cells were stimulated with bFGF for 16 h, and labeled with [^{3}H]thymidine 37 $MBq \cdot L^{-1}$ for 8 h. The radioactivity of incorporated [^{3}H]thymidine was measured in a liquid scintillation counter. Experiments were performed 6 times in duplicate.

Synthesis and purification of oligonucleotides The sequences of the oligonucleotides were as follows: 17-mer rat antisense CCDPK (5′-GCC GCC GCC GCC GCC AT-3′) directed against the initiation of translation sites of rat p42 and p44 CCDPK mRNA. Seventeen-mer rat sense CCDPK (5′AT GGC GGC GGC GGC GGC-3′) and 17-mer mismatch sequence (5′-CGC GCG CTC GCG CAC CC-3′) were used as controls. Phosphorothioate ODN was synthesized and purified at the University of Cincinnati DNA Core.

Liposomal transfection[9] ODN 0.8 $\mu mol \cdot L^{-1}$ in antibiotic- and serum-free M199 was vortex-mixed for 30 s, then mixed with equal volume of M199 containing lipofectin 80 $mg \cdot L^{-1}$ and incubated at 25 ℃ for 20 min. VSMC were washed gently 3 times in serum- and antibiotic-free M199. ODN/lipofectin mixture 200 μL was added to each 12-well plate, or 75 μL to each 24-well plate, with equal volume of serum- and antibiotic- free M199. VSMC were incubated at 37 ℃ in 95 % O_2 + 5 % CO_2 for 8 h, agitating the plates gently every 2 h. Medium was then replaced with the same volume of liposome-free M199 containing the same concentration of ODN supplemented with 10 % FBS. Cells were incubated for another 24 h before adding bFGF.

Western blot Cell lysates were prepared by previous method[10]. Protein concentration was estimated by the Bradford method[11]. The extracted protein 10 μg was subjected to SDS-PAGE in a 10 % SDS gel, and the protein was transferred to nitrocellulose membrane, which was then blocked with 5 % BSA in TBST for 1 h. The blots were incubated for 1 h at 25 ℃ with the primary monoclonal antibodies of p44 and p42 CCDPK at a 1:10 000 dilution or with phosphored p44 and p42 CCDPK at a 1:1 000 dilution, followed by incubation with secondary antibody (horseradish peroxidase conjugate) at a 1:10 000 dilution for 1 h. Immunoreactive bands of p42 and p44 CCDPK were visualized by using enhanced chemiluminescence reagents. Quantification of p42 and p44 CCDPK activity was done by scanning densitometry of autoradiographs.

Statistical analysis Values were expressed as $\bar{x} \pm s$ and assessed by ANOVA and t-test.

RESULTS

PD98059 inhibited bFGF-stimulated CCDPK activation and DNA synthesis Rat aortic VSMC were made quiescent by 24-h treatment in 0.1 % serum. Quiescent VSMC exhibited low CCDPK phosphorylation as showed by Western blot using monoclonal antibodies directly against the phosphored 42- and 44-kDa CCDPK protein bands. bFGF induction of CCDPK phosphorylation was in a concentration-dependent manner after 10-min treatment with bFGF. This effect was regulated by a synthetic inhibitor of CCDPK kinase (MEK). PD98059 10 $\mu mol \cdot L^{-1}$ inhibited the bFGF-induced activation of CCDPK in rat VSMC. At the maximal tested concentration of 50 $\mu mol \cdot L^{-1}$, PD98059 inhibited bFGF-induced CCDPK activity nearly to the baseline CCDPK activity seen in quiescent cells (Fig 1, Tab 1).

Pretreatment of cultured VSMC with PD98059 10 $\mu mol \cdot L^{-1}$ elicited a reduction in [^{3}H]thymidine incorporation stimulated by bFGF (Tab 2).

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Tab 1. Effect of bFGF and PD 98059 on the expression of rat aortic smooth muscle cell (1×10^9 cells·L^{-1}) phosphorylation of p44- and p42- CCDPK measured by Western blot and scanning densitometry of autoradiographs. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* corresponding bFGF group.

bFGF/ ng·L^{-1}	PD98059/ μmol·L^{-1}	Phosphored-p44/p42 CCDPK expression 10^{-3} × Peak area/mm^2
0	0	54 ± 8
0.025	0	93 ± 15
0.25	0	166 ± 67
2.5	0	233 ± 43
25	0	276 ± 27
0.25	10	70 ± 6^b
2.5	10	132 ± 23^c
25	10	176 ± 11^c
25	30	88 ± 9^c
25	50	42 ± 8^c

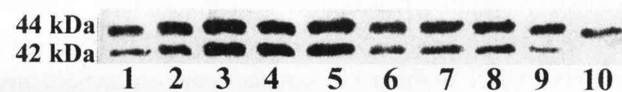

Fig 1. Inhibitory effect of PD98059 on bFGF-induced phosphorylation of CCDPK by Western blot. 1) Control. 2) bFGF 0.025 ng·L^{-1}. 3) bFGF 0.25 ng·L^{-1}. 4) bFGF 2.5 ng·L^{-1}. 5) bFGF 25 ng·L^{-1}. 6) PD98059 10 μmol·L^{-1} + bFGF 0.25 ng·L^{-1}. 7) PD98059 10 μmol·L^{-1} + bFGF 2.5 ng·L^{-1}. 8) PD98059 10 μmol·L^{-1} + bFGF 25 ng·L^{-1}. 9) PD98059 30 μmol·L^{-1} + bFGF 25 ng·L^{-1}. 10) PD98059 50 μmol·L^{-1} + bFGF 25 ng·L^{-1}.

Tab 2. Inhibitory effect of PD98059 10 μmol·L^{-1} on bFGF-stimulated DNA synthesis in rat aortic smooth muscle cells (1×10^8 cells·L^{-1}). $\bar{x} \pm s$ of 6 experiments. $^bP < 0.05$, $^cP < 0.01$ *vs* corresponding bFGF group.

bFGF/ ng·L^{-1}	PD98059/ μmol·L^{-1}	[^{3}H]thymidine incorporation/Bq per well
0	0	279 ± 88
0.025	0	550 ± 85
0.25	0	810 ± 67
2.5	0	1052 ± 239
25	0	1659 ± 277
0.025	10	363 ± 61^b
0.25	10	461 ± 65^c
2.5	10	623 ± 95^c
25	10	943 ± 209^c

Antisense ODN against CCDPK mRNA down-regulation in CCDPK protein expression and inhibition in bFGF-mediated DNA synthesis Before exposure to bFGF for 24 h, cultured rat VSMC were pretreated with a 17-mer antisense ODN targeting the initiation site for p44- and p42 CCDPK mRNA for 24 h. Antisense CCDPK ODN reduced p44- and p42 CCDPK protein levels in a concentration-dependent manner (Fig 2A, Tab 3). Lipofectin alone or control ODN, both sense and scrambled, did not affect p44- and p42 CCDPK protein levels.

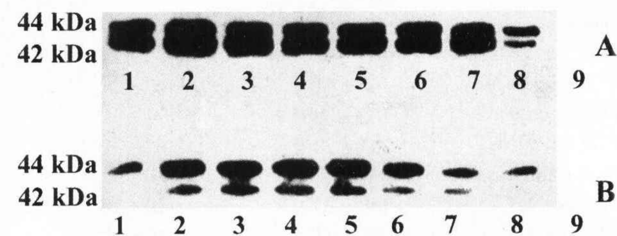

Fig 2. Effect of MAP kinase antisense treatment on the expression of rat aortic smooth muscle cell: A) p42 and p44 CCDPK protein; B) phosphorylation of p44- and p42- CCDPK by Western blot. bFGF concentration is 25 ng·L^{-1}. 1) Control. 2) bFGF-stimulated. 3) bFGF + sense. 4) bFGF + lipofectin. 5) bFGF + random. 6) bFGF + antisense 0.1 μmol·L^{-1}. 7) bFGF + antisense 0.2 μmol·L^{-1}. 8) bFGF + antisense 0.4 μmol·L^{-1}. 9) bFGF + antisense 0.8 μmol·L^{-1}.

Treatment with the antisense ODN for 48 h also inhibited phosphorylation of p44- and p42- CCDPK protein induced by bFGF stimulation for 10 min. At concentration of 0.4 μmol·L^{-1}, antisense ODN inhibited phosphorylation of CCDPK to baseline. Sense and random showed no effect (Fig 2B, Tab 3).

Liposomal transfection of rat VSMC with the CCDPK antisense ODN 0.4 μmol·L^{-1} also attenuated bFGF-mediated proliferation. Liposomal transfection alone and with control ODN did not alter the DNA synthesis response of rat VSMC to bFGF (Tab 4).

DISCUSSION

The present investigation provides strong evidence for the involvement of the CCDPK signaling pathway in bFGF-mediated proliferation of VSMC. bFGF is a potent activator of the CCDPK pathway and acts by triggering RAS-RAF activation, which activates MEK, and then phosphorylates p44- and p42- CCDPK in VSMC. To investigate the role of the CCDPK pathway in the mitogenic effect of bFGF, synthetic inhibitor of the MEK, PD98059

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Tab 3. Inhibitory effect of CCDPK antisense ODN (0.4 μmol·L^{-1}) on the expression of rat aortic smooth muscle cell (1×10^9 cells·L^{-1}) p42 and p44 CCDPK protein and phosphorylation of p44- and p42- CCDPK measured by Western blot and scanning densitometry of autoradiographs. $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ vs bFGF group. $^dP > 0.05$, $^eP < 0.05$ vs control.

Treatment	p42/p44 CCDPK expression $10^{-3} \times$ Peak area/mm^2	Phosphored-p44/p42 CCDPK expression $10^{-3} \times$ Peak area/mm^2
Control	88 ± 16	48 ± 6
bFGF	169 ± 33^e	154 ± 24^e
bFGF + lipofectin	$163 \pm 41^{a,e}$	$163 \pm 11^{a,e}$
bFGF + sense	$158 \pm 38^{a,e}$	$174 \pm 12^{a,e}$
bFGF + random	$164 \pm 28^{a,e}$	$167 \pm 12^{a,e}$
bFGF + antisense		
0.1 μmol·L^{-1}	$148 \pm 23^{b,e}$	$112 \pm 18^{b,e}$
0.2 μmol·L^{-1}	$97 \pm 11^{b,d}$	$46 \pm 6^{b,d}$
0.4 μmol·L^{-1}	$24 \pm 9^{c,e}$	$29 \pm 4^{c,e}$
0.8 μmol·L^{-1}	0	0

Tab 4. Inhibitory effect of CCDPK antisense ODN (0.4 μmol·L^{-1}) on bFGF (25 ng·L^{-1})-stimulated DNA synthesis in rat aortic smooth muscle cells (1×10^8 cells·L^{-1}). $\bar{x} \pm s$ of 6 experiments. $^aP > 0.05$, $^bP < 0.05$ vs bFGF group. $^dP > 0.05$, $^eP < 0.05$, $^fP < 0.01$ vs control.

Treatment	[^{3}H]thymidine incorporation/Bq per well
Control	255 ± 59
bFGF	2565 ± 551^e
bFGF + lipofectin	2101 ± 477^{af}
bFGF + antisense	302 ± 117^{bd}
bFGF + sense	2274 ± 372^{af}
bFGF + random	2239 ± 558^{af}

was used. The effect of bFGF on the phosphorylation of CCDPK and DNA synthesis in PD98059-treated VSMC was examined. Treatment of the cells with PD98059 10 μmol·L^{-1} resulted in obvious reduction of the DNA synthesis but not completely. This may be explained by the observation that PD98059 10 μmol·L^{-1} failed to inhibit bFGF (25 ng·L^{-1})-induced CCDPK activity completely, and the remaining of the phosphorylated CCDPK isoforms may be sufficient for the mitogenic response to bFGF.

In this study, bFGF induced an increase in phosphorylation of CCDPK after 10-min stimulation and an increase on p44- and p42- CCDPK protein level. Others have proved that an increase in CCDPK protein level but not CCDPK activity is associated with cell growth[12]. We used an antisense against rat p44- and p42- CCDPK mRNA to suppressed CCDPK protein expression and phosphorylation of CCDPK induced by bFGF. This antisense ODN also inhibits bFGF-mediated rat VSMC DNA synthesis. To confirm the sequence specificity, two control ODN, sense and random were used and results showed that both sense and scrambled control ODN had no effect on CCDPK expression or bFGF-mediated proliferation. These data provide more conclusive proof that the CCDPK pathway plays an important role in bFGF-induced proliferation and indicate that selective blockade of p44- and p42-CCDPK by antisense can effectively inhibit VSMC proliferation.

Transfection of VSMC with liposomes occurs with high efficiency. Pickering *et al*[13] recently, using a fluorescein-tagged ODN, proved that VSMC treated with as little as 0.2 μmol·L^{-1} for 1 h incorporated the ODN into both the nucleus and the cytoplasm . In present study, transfection of ODN 0.1 – 0.8 μmol·L^{-1} with liposomes resulted a specific depletion of CCDPK protein, which proves the high efficiency of transfection. The ability of antisense ODN to bind to complementary sequences in mRNA or its precursor serves as a useful tool for probing gene function[14]. Oligonucleotide-mediated inhibition of gene expression is also being evaluated as a new strategy for therapy of human disease[15]. In this regard, antisense CCDPK has been shown to block proliferation stimulated by serum, EGF, and bFGF, this may underscore the importance of targeting components of the signaling cascade that are shown by many factors as therapeutic approach.

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CCDPK反义寡核苷酸抑制bFGF诱导的大鼠血管平滑肌细胞增殖

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关键词 Ca^{2+}-钙调蛋白依赖性蛋白激酶；碱性成纤维细胞生长因子；反义寡核苷酸类；血管平滑肌；培养的细胞；增生；胸苷；蛋白质印迹；核糖核酸合成抑制剂；p42 (Mapk)激酶

目的：探讨Ca^{2+}-钙调蛋白依赖性蛋白激酶(CCDPK)在碱性成纤维细胞生长因子(bFGF)诱导体外培养大鼠血管平滑肌细胞(VSMC)增殖中的作用及反义CCDPK寡核苷酸的抑制作用. **方法**：利用CCDPK特异性抑制剂PD98059或17-mer CCDPK反义寡核苷酸抑制CCDPK活性，用Western blot法测定p44-/p42-CCDPK蛋白表达及磷酸化. [^{3}H]胸腺嘧啶核苷酸掺入测定SMC DNA合成. **结果**：PD98059及CCDPK反义寡核苷酸明显抑制bFGF诱导的CCDPK蛋白表达和CCDPK活性以及[^{3}H]胸腺嘧啶核苷酸掺入. **结论**：CCDPK介导了bFGF诱导的VSMC增殖. 针对p42-和p44-CCDPK起始部位设计的17-mer反义寡核苷酸使p44-和p42-CCDPK蛋白表达下调，能有效抑制bFGF诱导的VSMC的增殖.

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