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Prevention of intima hyperplasia by mitogen-activated protein kinase antisense oligodeoxynucleotide[1]

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KEY WORDS Ca^{2+}-calmodulin dependent kinase; antisense oligonucleotides; vascular smooth muscle; cultured cells; hyperplasia; vascular endothelium; thymidine; Western blotting; angioplasty

ABSTRACT

AIM: To investigate the preventive effect of Ca^{2+}-calmodulin dependent kinase (CCDPK) (formerly: mitogen-activated protein kinase or MAPK) antisense phosphorothioate oligodeoxynucleotide (ODN) on vascular smooth muscle cell (VSMC) proliferation *in vitro* and on intima hyperplasia after injury *in vivo*. **METHODS:** Liposomal transfection was used to introduce phosphorothioate-protected 17-mer antisense CCDPK ODN directed against the initiation of translation sites of the p42 and p44 CCDPK isoforms into cultured rat VSMC to deplete CCDPK and DNA synthesis induced by endothelin-1 (ET) or platelet derived growth factor (PDGF). A 17-mer sense and a random sequence CCDPK ODN were used as controls. CCDPK protein p44 and p42 levels were measured by Western blot. DNA synthesis was measured by [^{3}H]thymidine incorporation. In *in vivo* study, rat balloon angioplasty was performed by a 2F Fogarty catheter. The antisense CCDPK ODN 200 μg was administered to the adventitial surface of the injured carotid artery by pluronic gel 30 % (w/v) solution. Two weeks after vascular injury, carotid arteries were removed and cross sections were made and stained with hematoxylin/eosin for patho-histological examination. Fluorecein isothiocynate (FITC)-labeled and phosphorothioate-protected ODN was used to detect the uptake of ODN *in vitro* and *in vivo*. **RESULTS:** CCDPK antisense ODN (0.4 $\mu mol \cdot L^{-1}$) reduced p42/p44 protein expression and inhibited VSMC [^{3}H]thymidine incorporation stimulated by ET and PDGF. Antisense CCDPK ODN treatment at 2 wk after injury resulted in a significant inhibition of intima hyperplasia, compared with untreated vessels. **CONCLUSION:** The p42/p44-CCDPK antisense ODN inhibits *in vitro* stimulated rat VSMC proliferation and *in vivo* injured arterial intima hyperplasia.

INTRODUCTION

Coronary angioplasty can successfully dilate the narrowed arteries of patients with coronary atherosclerosis, but restenosis occurs in up to 30 % – 50 %[1]. Vascular smooth muscle cell (VSMC) proliferation and migration are essential components in this process. A number of pharmacological agents have been shown to inhibit VSMC proliferation *in vitro*, but only a few have proven their efficacy, usually in specific clinical setting[2]. This is because the multiplicity of the mediators and the redundancy of signal transduction processes prevent the successful targeting of one or two factors.

Ca^{2+}-calmodulin dependent kinase (CCDPK) (formerly: mitogen-activated protein kinase or MAPK) cascade is a major intracellular signaling pathway involved in the regulation of VSMC growth in response to all known growth factors[3]. In experimental models, the involvement of CCDPK signal pathway has been shown to be one of the key events responsible for restenosis after balloon angioplasty[4]. Therefore, it is our opinion that strategy aimed at blocking the common pathway CCDPK activity is better than blocking selective biological mediators considering the limitations caused by the multiplicity of the mediators and the plurality of the cell surface receptors.

Antisense method is an innovative and attractive strategy to block the transcription or translation of specific genes[5]. Antisense against c-*myc*, c-*myb*, proliferating

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cell nuclear antigen, cyclins and nonmuscle myosin has successfully suppressed VSMC proliferation[6].

The present study aimed to determine the effect of antisense p44 and p42 CCDPK ODN on stimulated VSMC proliferation *in vitro*, and to investigate its effect on injured arterial intima hyperplasia, when delivered locally at the site of angioplasty.

MATERIALS AND METHODS

Drugs and reagents Anti-mitogen activated protein kinase, lipofectin, ET-1, PDGF-AB (human; recombinant), leuptinin, pluronic gel F127, HEPES, Triton X-100, pluronic gel, bovine serum albumin (BSA), egtazic acid, HRP-conjugated anti-rabbit secondary antibody were purchased from Sigma Co. Western blot chemiluminescence reagent plus was purchased from NEN™ Life Science Products.

Synthesis and purification of oligonucleotides Phosphorothioate-protected oligonucleotides were used to inhibit ET-1 or PDGF-AB-stimulated VSMC proliferation. The sequences of the oligonucleotides were as follows: 17-mer rat antisense CCDPK (5′-GCC GCC GCC GCC GCC AT) directed against the initiation of translation site of rat p42 and p44 CCDPK mRNA. This ODN has been used successfully to downregulate both isoforms of CCDPK in VSMC[7], rat cardiac myocytes[8] and rat cardiac fibroblast[9]. 17-mer rat sense CCDPK (5′ AT GGC GGC GGC GGC GGC) and 17-mer mismatch sequence (5′-CGC GCG CTC GCG CAC CC) were used as controls. ODN were synthesized and purified at the University of Cincinnati DNA Core.

Cell culture[10] VSMC were routinely isolated from aortas of Sprague-Dawley rats by explant method (supplied by the Animal Center of Hu-nan Medical University, grade Ⅱ, Certificate № 20-009) and maintained in DMEM supplemented with 10 % fetal bovine serum. Cells used in experiments were from passages 4 to 10.

Liposomal transfection ODN 0.8 $\mu mol \cdot L^{-1}$ in antibiotic- and serum-free DMEM were vortex-mixed for 30 s, then mixed with equal volume of DMEM containing lipofectin 80 $mg \cdot L^{-1}$ and incubated at room temperature for 20 min. VSMC were washed gently 3 times in serum and antibiotic free DMEM. ODN/lipofectin mix 200 μL was added to each 12-well plates, or 75 μL to each 24-well plates, with equal volume of serum and antibiotic free DMEM. VSMC were incubated at 37 ℃ in 95 % O_2 – 5 % CO_2 for 8 h, the plates agitated gently every 2 h. Medium was then replaced with the same volume of liposome-free DMEM containing the same concentration of ODN supplemented with 10 % fetal bovine serum. Cells were incubated for another 24 h, before adding growth factor.

Intracellular uptake of FITC-labeled antisense ODN Phosphorothioate-protected antisense ODN was synthesized with an FITC linked to the 5′ end. 60 % confluent VSMC were incubated with FITC labeled antisense ODN for 1 and 8 h, then they were washed extensively with PBS. The incorporate ODN was visualized by fluorescence microscopy.

Measurement of DNA synthesis Cell proliferation in terms of DNA synthesis was determined by measuring [^{3}H]thymidine incorporation. VSMC were plated in 24-well plates at 1×10^4 cells per well. Antisense, sense, and random ODN were transfected by liposomal for 24 h. Following stimulation with 20 % serum for 16 h, cells, labeled with [^{3}H]thymidine 37 $MBq \cdot L^{-1}$ for 8 h, were washed with cold PBS, trypsinized, resuspended in 20 % trichloroacetic acid (TCA), and vortexed vigorously to lyse the cells. The cell lysate was vacuum-filtered through a glass-fiber filter. After washing with cold 5 % TCA followed by 70 % ethanol, the filter was dried. The radioactivity of incorporated [^{3}H]thymidine was measured in a liquid scintillation counter. Experiments were performed 6 times in duplicate.

Western blot After being stimulated for 24 h, cell lysates were prepared using lysis buffer[7]. Protein concentration was estimated by Bradford method[11]. SDS sample buffer [Tris-HCl 0.33 $mol \cdot L^{-1}$, SDS 10 % (wt/vol), glycerol 40 % (vol/vol), and dithiothreitol 20 % (vol/vol) containing bromophenol blue 0.4 % (wt/vol)] of 1/4 volume were added to cell lysates. After boiling for 5 min, 20 μg of the sample was subjected to SDS-PAGE in a 10 % SDS gel, and the protein was transferred to nitrocellulose membrane, which was then blocked for 1 h with 5 % BSA in TBST (Tris-HCl 20 $mmol \cdot L^{-1}$, NaCl 100 $mmol \cdot L^{-1}$ containing 0.1 % Tween-20). The blots were incubated for 1 h at 25 ℃ separately with the primary antibodies of p44 and p42 MAP kinase, at a 1∶10 000 dilution, followed by incubation for 1 h with secondary antibody (horseradish peroxidase conjugated) at a 1∶10 000 dilution. Immunoreactive bands of p42/p44 CCDPK were visualized with enhanced chemiluminescence reagents.

Transfection *in vivo*[6] Antisense ODN was dissolved in a 30 % pluronic gel solution at 4 ℃ at a

concentration of 2 $g \cdot L^{-1}$. Nine male Sprague-Dawley rats (350 – 400 g) were divided into three groups. Intimal hyperplasia was induced by a № Ⅱ Fogarty catheter after anesthetizing with pentobarbital sodium and the catheter was introduced into the left common carotid artery via the external carotid artery. Immediately after vessel injury, 200 μg of the antisense ODN was applied to the adventitial surface of the left common carotid artery. The neck wounds were then repaired and the animals allowed to recover. Fourteen days after injury, the rats were reanesthetized and killed by exsanguination through an aortic cannula. The left and right common carotid arteries were excised and placed in fixative solution for 10 h. Two-μm sections were stained with hematoxylin/eosin and examined microscopically.

Uptake of FITC-labeled ODN *in vivo* Animal surgery was performed and FITC-labeled phosphorothioate ODN was transferred as described above. The vessels were cut at 1 and 12 h after transfection and visualized by fluorescence microscopy.

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

RESULTS

Effect of antisense CCDPK on ET-1 or PDGF-AB-induced VSMC DNA synthesis Pretreatment of rat VSMC with p42 and p44 CCDPK antisense ODN resulted in a significant reduction of ET or PDGF-induced [^{3}H] thymidine incorporation. In contrast, random and sense ODN showed no effect. The proliferative response to ET or PDGF was not altered in cells treated with lipofectin alone (Tab 1).

Effect of antisense CCDPK ODN on CCDPK protein expression Pretreatment of VSMC with p42/p44 antisense ODN resulted in reduction of total p42 and p44 CCDPK protein expression. VSMC exposed to lipofectin in the absence of ODN had no inhibitory effect on CCDPK expression compared with that of ET or PDGF-stimulated VSMC. Sense and random sequence ODN had no inhibitory effect (Fig 1).

Intracellular uptake of FITC-labeled ODN One hour after liposome transfection, among the intracellular uptake of FITC-labeled ODN over 30 % VSMC exhibited fluorescence. At 8 h after transfection, over 90 % VSMC exhibited fluorescence (Fig 2).

Effect of antisense CCDPK ODN on intima hyperplasia As compared with the normal vascular inner layers (Fig 3A), histological examination of stained sections obtained from balloon injured untreated arteries showed substantial intima hyperplasia (neointima) at 2 wk after transfection, as evidenced by the invasion of the tunica intima by proliferating vascular SMC. The marked narrowing lumen of the vessel was also observed (Fig 3B). In contrast, injured arterial segments that were treated with antisense CCDPK ODN 200 μg resulted in a significant reduction in neointima formation and a widening of the vessel lumen (Fig 3C).

Tab 1. Effect of antisense CCDPK ODN pretreatment on ET-1 10 $nmol \cdot L^{-1}$ or PDGF 10 $ng \cdot L^{-1}$-stimulated DNA synthesis in rat smooth muscle cells. $n = 6$ experiments, average of duplicate constitutes one determination. $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$ *vs* stimulated group. $^dP > 0.05$, $^eP < 0.05$ *vs* control.

Treatment	[^{3}H] Thymidine incorporation (kBq per well)
Control	0.64 ± 0.09
ET-1 10 $nmol \cdot L^{-1}$	4.7 ± 0.4^e
ET-1 10 $nmol \cdot L^{-1}$ + lipofectin	4.9 ± 0.4^a
ET-1 10 $nmol \cdot L^{-1}$ + antisense 0.2 $\mu mol \cdot L^{-1}$	0.87 ± 0.14^{bd}
ET-1 10 $nmol \cdot L^{-1}$ + sense 0.2 $\mu mol \cdot L^{-1}$	4.4 ± 0.5^a
ET-1 10 $nmol \cdot L^{-1}$ + random 0.2 $\mu mol \cdot L^{-1}$	4.3 ± 0.3^a
PDGF-AB 10 $ng \cdot L^{-1}$	12 ± 3^e
PDGF-AB 10 $ng \cdot L^{-1}$ + lipofectin	11.2 ± 2.6^a
PDGF-AB 10 $ng \cdot L^{-1}$ + antisense 0.2 $\mu mol \cdot L^{-1}$	0.78 ± 0.23^{bd}
PDGF-AB 10 $ng \cdot L^{-1}$ + sense 0.2 $\mu mol \cdot L^{-1}$	13.6 ± 2.1^a
PDGF-AB 10 $ng \cdot L^{-1}$ + random 0.2 $\mu mol \cdot L^{-1}$	11.7 ± 1.6^a

Fig 1. Effect of antisense CCDPK ODN treatment on rat aortic smooth muscle cell p42/p44 CCDPK protein expression by Western blot. 1) Control; 2) ET-stimulated; 3) ET + lipofectin; 4) ET + sense ODN. 5) ET + random ODN; 6) ET + antisense ODN; 7) PDGF-stimulated; 8) PDGF + lipofectin; 9) PDGF + random ODN; 10) PDGF + sense ODN; 11) PDGF + antisense ODN.

Uptake of FITC-labeled phosphorothioate ODN *in vivo* Pluronic gel-mediated transfer of FITC phosphorathioate ODN into rat left common carotid arteries exhibited fluorescence in the medial vascular layer at 1 h and throughout the medial and intimia of the vascular wall at 12 h after local ODN delivery (Fig 4).

DISCUSSION

The major finding of the present study is that the

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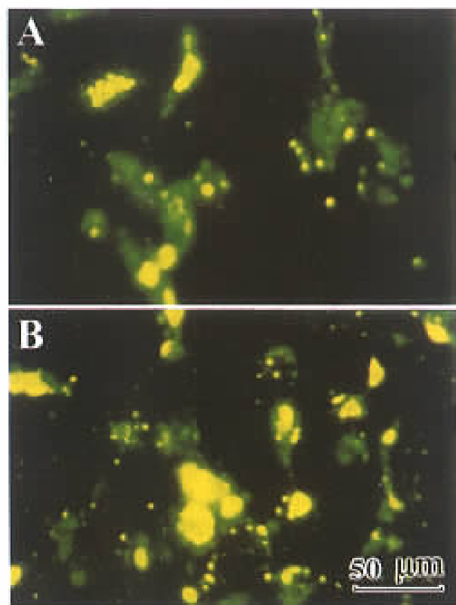

Fig 2. Uptake and intracellular distribution of FITC-labeled phosphorothioate-protected antisense CCDPK ODN transferred by liposome for A : 1 h and B : 8 h in cultured rat VSMC (× 100).

CCDPK is directly involved in VSMC proliferation mediated by vascular injury *in vivo*, and that the local delivery of antisense p44 and p42 CCDPK ODN inhibits neointima formation after balloon injury.

Transfection of VSMC with liposomes occurs with high efficiency. In this study, using an FITC-labeled ODN proved that VSMC treated with as little as 0.2 μmol $\cdot L^{-1}$ for 8 h incorporated the ODN into both the nucleus and the cytoplasm. Transfection of ODN 0.1 – 0.8 $\mu mol \cdot L^{-1}$ with liposome resulted in a specific depletion of CCDPK protein and proved the high efficiency of transfection.

Previously, we have demonstrated that antisense oligodeoxynucleotides (ODN) directed against the initiation of translation sites of the p42/p44 CCDPK inhibited EGF-stimulated rat vascular smooth muscle cell proliferation and the effect of the antisense ODN was sequence-specific[7]. In this study we further proved antisense CCDPK ODN anti-proliferative effect in rat carotid artery injury model. When delivered in pluronic gel, it produced an obvious decrease in neointima formation. Antisense ODN have been delivered effectively by the adventitial application of pluronic gel in the experimental setting[12]. Systemic antisense ODN administration in the dose required to achieve VSMC growth suppression may be toxic. In particular, there is a potential for sequence-specific side effects on healthy proliferating tissues. There are also nonspecific, predominantly, cardio-vascular side effects, such as, hypotension[13]. Therefore, local delivery to the intended site of action has been used as a method of administering ODN. In this study, we applied pluronic gel as a carrier for antisense CCDPK ODN, an uptake of FITC-labeled phosphorothioate ODN appears in the medial layer and intima of the vascular cell.

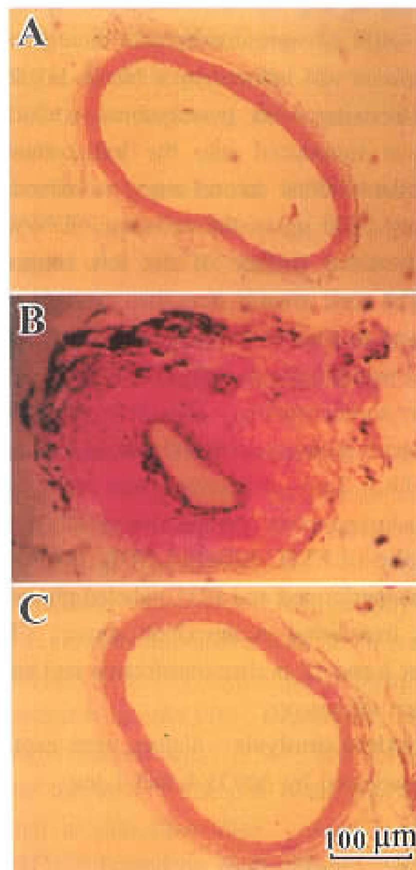

Fig 3. Effect of antisense CCDPK ODN on intima hyperplasia in rat carotid arteries subjected to balloon angioplasty. Representative cross-sections : A) Normal rat carotid artery ; B) Injured rat carotid artery without any treatment ; C) Injured rat carotid artery treated with pluronic gel-antisense CCDPK ODN (× 100).

In conclusion, this study represents a fundamental examination of antisense CCDPK ODN upon stimulated VSMC proliferation and injured arterial intima hyperplasia in a rat model of restenosis after PTCA. Antisense CCDPK ODN inhibits VSMC proliferation and the neointima formation when delivered locally at the time of PTCA by pluronic gel. These data support the utility of antisense CCDPK ODN therapy to combat vascular restenosis.

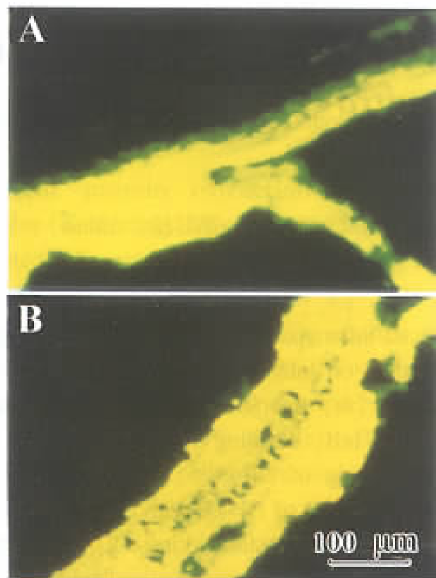

Fig 4. Uptake of FITC-labeled phosphorothioate-protected antisense CCDPK ODN transferred *in vivo* by pluronic gel for A：1 h and B：12 h in rat carotid arterial wall（×100）.

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丝裂素活化的蛋白激酶反义寡脱氧核苷酸防治血管内膜增殖[1]

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关键词　Ca^{2+}-钙调蛋白依赖性蛋白激酶；反义寡核苷酸类；血管平滑肌；培养的细胞；增生；血管内皮；胸苷；蛋白质印迹；血管形成术

目的：探讨 Ca^{2+}-钙调蛋白依赖性蛋白激酶（丝裂素活化的蛋白激酶）（CCDPK）在生长因子诱导体外培养大鼠血管平滑肌细胞增殖中的作用及反义 CCDPK 寡脱氧核苷酸（ODN）对球囊损伤后大白鼠血管内膜增生的抑制作用．方法：利用脂质体转染 17-mer CCDPK 反义 ODN 进入培养的血管平滑肌细胞以抑制 CCDPK 活性，设正义及随机 ODN 作对照．用蛋白质印迹法测定 CCDPK 表达．[³H]胸腺嘧啶核苷酸掺入测定平滑肌细胞 DNA 合成．用 2F 球囊导管造成大白鼠颈动脉再狭窄模型，利用多聚胶 F127-ODN 系统由血管外膜部位给药．于损伤后 2 周取样，固定及 HE 染色观察内膜增生情况．FITC 标记的 ODN 观察体内外给药方法的分布及吸收情况．结果：CCDPK 反义 ODN 能明显抑制 PDGF 及 ET 诱导的 CCDPK 蛋白表达及[³H]胸腺嘧啶核苷酸掺入．在大鼠颈动脉再狭窄模型，能明显抑制血管内膜增生．结论：CCDPK 介导了 PDGF 及 ET 诱导的血管平滑肌细胞增殖．针对 p42-和 p44-CCDPK 起始部位设计的 17-mer 反义 ODN 能有效抑制生长因子诱导的血管平滑肌细胞的增殖及球囊损伤大鼠血管内膜增生．

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