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Target selectivity of MAPK phosphorothioate antisense ODN on p42/p44, p38 MAPK, and JNK protein expression and its inhibitory effect on VSMC DNA synthesis

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KEY WORDS protein kinases; antisense oligonucleotides; vascular smooth muscle; cultured cells; Western blotting; thymidine

ABSTRACT

AIM: To analyze the target selective and sequence-specific inhibitory effect of mitogen-activated protein kinase (MAPK) phosphorothioate antisense oligodeoxynucleotides (ODN) on p42/p44, p38 MAPK, c-*jun* NH_2-terminal protein kinases (JNK) protein expression, and DNA synthesis in vascular smooth muscle cell (VSMC). **METHODS**: Using a phosphorothioate-protected 17-mer antisense MAPK ODN directed against the initiation of translation sites of the p42/p44 MAPK isoforms by liposomal transfection to deplete cultured rat, rabbit, and fetal calf VSMC MAP kinases. The 17-mer sense and random sequence MAPK ODN were used as controls. After liposomal transfection, cells were exposed to 20 % serum for 24 h, and then harvested in lysis buffer. P42/p44, p38 MAPK, and p46/p58 JNK protein expression were measured by Western blot. DNA synthesis was measured by [^{3}H]thymidine incorporation. **RESULTS**: Treatment with MAPK antisense ODN (0.1 - 0.8 $\mu mol \cdot L^{-1}$) for 48 h reduced phosphored p42/p44 MAPK protein expression but without effect on p38 MAPK and JNK expression, and inhibited cultured rat, rabbit, and fetal calf VSMC [^{3}H]thymidine incorporation stimulated by 20 % serum in a concentration-dependent manner. **CONCLUSION**: The MAPK antisense ODN target-selectively and sequence-specifically reduces the p42/p44 MAPK protein expression and concentration-dependently inhibits proliferation of rat, rabbit and fetal calf VSMC.

INTRODUCTION

Restenosis remains a significant clinical problem after coronary angioplasty. The restenosis is implicated by the proliferation of vascular smooth muscle cells (VSMC) and hyperplasia of extracellular matrix and collagen[1]. Several growth factors and components of the cascade have been hypothesized to be directly involved in the promotion of intimal thickening as a result of SMC proliferation[1,2]. Many pharmacological interventions in VSMC proliferation have been identified *in vitro*, such as angiotensin converting enzyme inhibitors, interferon-γ, and heparin. However, none of them has yet been found to successfully inhibit angioplasty restenosis in human trials[3].

The 42- and 44-kDa MAPK are currently found serine/threonine protein kinases that are activated by many stimuli involved in cell growth and as a common pathway to transmit signal into the nucleus regulating cell proliferation[4]. There are 3 subfamilies of MAPK, p42/p44 MAPK, c-*jun* NH_2-terminal protein kinases (JNK), and p38-MAPK, were regulated by distinctive signal transduction pathways and exhibited different functions[5]. The kinase of MAPK cascades is highly conserved and homologous existed in yeast, *Drosophila*, and various species of mammalian cells.

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Exposure of cultured cells to diverse mitogens, including serum, growth factors, and phorbol ester, results in rapid phosphorylation of MAPK[6]. Previously, we have demonstrated that antisense oligodeoxynucleotides (ODN) directed against the initiation of translation sites of the p42/p44 MAPK inhibited epidermal growth factor-stimulated rat proliferation[7]. In this study, we analyzed the target-selective and sequence-specific inhibitory effect of this antisense ODN on p42/p44, p38 MAPK, JNK protein expression, and DNA synthesis in VSMC.

MATERIALS AND METHODS

Drugs and reagents Anti-mitogen activated protein kinase, lipofectin, leuptinin, HEPES, Triton X-100, fetal calf serum albumin, egtazic acid, *S. aureus* protein A, HRP-conjugated anti-rabbit secondary antibody were purchased from Sigma Co. p38 MAPK, JNK, and phosphor-specific p42/44 MAP kinase antibodies were purchased from New England Biolabs Inc. Western blot chemiluminescence reagent plus was purchased from NEN™ Life Science Products.

Synthesis and purification of oligonucleotides Phosphorothioate-protected oligonucleotides were used to inhibit VSMC proliferation induced by 20 % fetal calf serum. The sequences of the oligonucleotides were as follows: 17-mer rat antisense MAPK (5′-GCC GCC GCC GCC GCC AT) directed against the initiation of translation site of rat p42/p44 MAPK mRNA. This ODN has been used successfully to downregulate both isoforms of MAPK in 3T3 cells[8] and rat cardiac myocytes[9]. 17-mer rat sense MAPK (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 isolated separately from aortas of Sprague-Dawley rats, New Zealand rabbit and fetal calf (Supplied by the Experimental Animal Center of Hu-nan Medical University, Grade Ⅱ, Certificate № 20-009) and maintained in DMEM supplemented with 10 % fetal calf serum. Cells used in experiments were passages 4 to 10.

Liposomal transfection[9] VSMC were seeded at 5×10^4 cells per well. ODN at desired concentration in antibiotic- and serum-free DMEM were vortex-mixed for 30 s, then mixed with equal volume of DMEM containing lipofectin 80 mg · L^{-1} and incubated at 25 ℃ for 20 min. VSMC were washed gently in serum and antibiotic-free DMEM 3 times. ODN/lipofectin mixture 200 μL was added for each 12-well plates, or 75 μL for 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, shaking the plates gently every 2 h. Medium was then replaced with the same volume of liposome-free DMEM containing the same concentration of ODN supplemented with 1 % fetal calf serum. Cells were incubated for another 48 h, before adding 20 % serum.

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 5×10^4 cells per well. Antisense, sense and random ODN were transfected for 48 h in DMEM. Following stimulating with 20 % serum for 16 h, cells labeled with [^{3}H]thymidine 37 MBq · 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 independently in duplicate.

Preparation of cell lysates[11] After treatment with ODN, cells were stimulated with 20 % serum for 24 h for MAPK protein expression or 10 min for the phosphorylation of MAPK. 0.1 mL of lysis buffer containing: NaCl 50, NaF 50, sodium pyrophosphate 50, egtazic acid 5, edetic acid 5, Na_3VO_4 2, phenylmethylsulfonyl fluoride 0.5, and HEPES 10 mmol · L^{-1} at pH 7.4, along with 0.1 % Triton X-100 and leupeptin 10 mg · L^{-1} was added. Cell lysates freezing on ice, scraping and sonicating were centrifuged at 18 000 × g at 4 ℃ for 15 min. Protein concentration was estimated by Bradford method[12].

Western blot SDS sample buffers [tris/HCl 0.33 mol, 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, 30 μL of the immunoprecipitation complex 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 PBST (Na_2HPO_4 80 mmol · L^{-1}, NaH_2PO_4 20 mmol · L^{-1}, NaCl 100 mmol·L^{-1} containing 0.05 % Tween-20). The blots were incubated for 1 h at 25 ℃ separately with the primary antibodies of p44 and p42 MAP kinase, phosphor-specific p44 and p42 MAP kinase, p38 MAP kinase and JNK at a 1∶1000 dilution, followed by incubation for 1 h with secondary antibody (horseradish peroxidase conjugated) at a 1∶10 000 dilution. Immunoreactive bands of p42/p44 MAPK were visualized with enhanced chemiluminescence reagents.

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

RESULTS

Selective inhibition of MAPK protein expression in rat VSMC after treatment with antisense ODN Pretreatment of VSMC with p42/p44 antisense ODN resulted in a concentration-dependent reduction in total p42/p44 MAPK protein and phosphor-MAPK protein expression. VSMC exposed to lipofectin in the absence of ODN had no inhibitory effect on phosphor-MAPK content compared with that of serum-stimulated VSMC. Sense and random sequence ODN had no inhibitory effect (Fig 1).

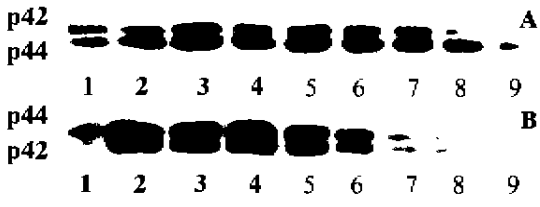

Fig 1. Effect of MAP kinase antisense ODN treatment on rat aortic smooth muscle cell p42/p44 MAPK(A) and phosphored p42/p44 MAPK protein (B) expression by Western blot. 1) Control. 2) Serum-stimulated. 3) Serum + lipofectin. 4) Serum + sense ODN. 5) Serum + random ODN. 6) Serum + antisense ODN 0.1 μmol·L^{-1}. 7) Serum + antisense ODN 0.2 μmol·L^{-1}. 8) Serum + antisense ODN 0.4 μmol·L^{-1}. 9) Serum + antisense ODN 0.8 μmol·L^{-1}.

Pretreatment with p42/p44 MAPK antisense ODN had no effect on p38 MAPK and the p46 and p54 JNK expression (Fig 2).

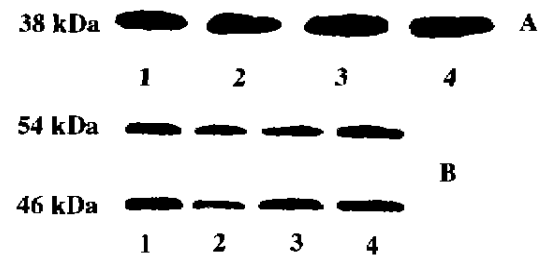

Fig 2. Effect of MAP kinase antisense ODN treatment on rat aortic smooth muscle cell p38 MAPK p46 (A) and p54 JNKs protein (B) expression by Western blot. 1) Control. 2) Sense ODN. 3) Random ODN. 4) Antisense ODN.

Effect of antisense MAPK on VSMC DNA synthesis Pretreatment of rat VSMC with p42/p44 antisense ODN resulted in a reduction of serum-induced [^{3}H]thymidine incorporation in a concentration-dependent manner (Tab 1).

Tab 1. Effect of MAP kinase antisense pretreatment on serum-stimulated DNA synthesis in rat aortic smooth muscle cells. Average of duplicate constitutes one determination. $n = 6$. $\bar{x} \pm s$.
$^aP > 0.05$, $^bP < 0.05$ *vs* serum-stimulated group.
$^dP > 0.05$, $^eP < 0.05$ *vs* control.

Treatment	$10^{-3} \times$ [^{3}H]Thymidine incorporation/Bq per well
Control	2.6 ± 0.3
20 % Serum	15 ± 4^e
20 % Serum + lipofectin	14.7 ± 1.3^a
20 % Serum + antisense 0.1 μmol	5.6 ± 0.5^{be}
20 % Serum + antisense 0.2 μmol	2.4 ± 0.7^{bd}
20 % Serum + antisense 0.4 μmol	1.2 ± 0.5^{bd}
20 % Serum + sense	18 ± 8^a
20 % Serum + random	14 ± 3^a

Random and sense ODN were without effect. The proliferate response to serum was not altered in cells treated with lipofectin alone. The rat antisense ODN also showed inhibitory effect on cultured rabbit and fetal calf VSMC [^{3}H]thymidine incorporation (Tab 2).

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Tab 2. Effect of MAP kinase antisense pretreatment on serum-stimulated DNA synthesis in fetal calf and rabbit aortic smooth muscle cells. Average of duplicate constitutes one determination. $n = 6$. $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$ *vs* serum-stimulated group. $^dP > 0.05$, $^eP < 0.05$ *vs* control.

Treatment	$10^{-3} \times$ [^{3}H]-Thymidine incorporation/ Bq per well	
	Fetal calf	Rabbit
Control	5.6 ± 2.1	3.1 ± 0.3
20 % Serum	22 ± 7^e	28 ± 6^e
20 % Serum + lipofectin	21 ± 6^a	25 ± 9^a
20 % Serum + antisense 0.2 μmol	7.6 ± 2.3^{bd}	2.4 ± 0.7^{bd}
20 % Serum + sense 0.2 μmol	21 ± 7^a	28 ± 10^a
20 % Serum + random 0.2 μmol	19.9 ± 2.6^a	26 ± 4^a

DISCUSSION

Previously, we have demonstrated that the p42/p44 MAPK antisense ODN inhibited VSMC DNA synthesis induced by EGF[7], assumpting that the effect of antisense ODN was sequence-specific on p42/p44 MAPK. In this present study, we further analyzed the possibility that non-sequence-specific anti-protein synthesis effect of antisense ODN on three subfamilies of MAPK, p42/p44, p38 MAPK and JNK protein expression and employed sense and random as controls. We found that the p42-and p44-MAPK antisense ODN concentration-dependently reduced p42/p44-MAPK while no effects were seen on p38 and p46/p54 JNK expression. Random and sense controls had no effect on MAPK expression or on serum-induced proliferation. A rat MAPK antisense ODN sequence also had an inhibitory effect on rabbit and fetal calf VSMC. Our results demonstrated that inhibition of p42/p44 MAPK protein expression was associated with a reduction of SMC proliferation, indicating that the effect of MAPK antisense ODN was conservative and target-selective and sequence-specific on p42/p44 MAPK.

Of note, the p42/p44 MAPK cascade is a major intracellular signaling pathway involved in the regulation of SMC growth in response to all known growth factors. Recent *in vivo* studies have linked upstream components in the MAPK pathway to neointimal proliferation in the rat in response to local injury[13]. After growth stimulation, p42/p44 MAPK require phosphorylation of one tyrosin and one threonine residue in the TEY-1 motif. Activated MAPK translocates into the nuclus to regulate gene expression through the phosphorylation of transcription factors. In the present stydy, we examined both the expression of p42/p44 MAPK and phosphored p42/p44 MAPK and found that after treatment with MAPK antisense ODN the expression of phosphorylated MAPK was decreased, indicating that the inhibitory effect of p42/p44 MAPK antisense on phosphored MAPK expression.

The target-selective and sequence-specific effect of p42/p44 antisense MAPK to inhibit VSMC proliferation suggests that these antisense compounds could serve as a useful tool to understand the pathogenesis of vascular disease and provides a therapeutic strategy to suppress VSMC hyperproliferative disorders such as restenosis after angioplasty.

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丝裂素活化的蛋白激酶反义寡核苷酸对 p42/p44, p38 和 JNK 激酶的靶向选择性及其对血管平滑肌细胞 DNA 合成的抑制作用[1]

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关键词 蛋白激酶类; 反义寡核苷酸类; 血管平滑肌; 培养的细胞; 蛋白质印迹; 胸苷

目的: 探讨丝裂素活化的蛋白激酶(MAPK)反义寡核苷酸对血清诱导的培养大鼠血管平滑肌细胞增殖的选择性及序列依赖性抑制作用. **方法**: 用脂质体将 p42-和 p44-MAPK 反义寡核苷酸转染入大鼠血管平滑肌细胞, 设正义及随机寡核苷酸对照, 20 % 血清刺激后, 用 Western Blot 法测定总 p44/p42-MAPK、p38 MAPK 及 JNKs 蛋白水平及磷酸化 MAPK 表达. [^{3}H]胸腺嘧啶核苷酸掺入测定平滑肌细胞 DNA 合成. **结果**: MAPK 反义寡核苷酸能明显抑制血清诱导的血管平滑肌细胞总 MAPK 蛋白水平及磷酸化 MAPK 蛋白表达, 对 p38 MAPK 及 JNKs 表达无影响, 并能明显抑制[^{3}H]胸腺嘧啶核苷酸掺入. **结论**: 针对 p42-和 p44-MAPK 起始部位设计的 17-mer 反义寡核苷酸能选择性及序列依赖性地抑制血清诱导的血管平滑肌细胞的增殖.

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