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Effect of antisense mitogen-activated protein kinase oligonucleotides on rat vascular smooth muscle cell proliferation induced by EGF *in vitro*

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KEY WORDS protein kinases; epidermal growth factor-urogastrone; antisense oligonucleotides; vascular smooth muscle; cultured cells

AIM: To study the preventive effect of down-regulating mitogen-activated protein kinase (MAPK) on vascular smooth muscle cell (VSMC) proliferation. **METHODS:** Cultured rat VSMC was pretreated with a phosphorothioate-protected 17-mer antisense MAPK oligodeoxynucleotides (ODN) directed against the initiation of translation sites of the p42- and p44-MAPK isoforms by liposomal transfectinon. A 17-mer sense and a random sequence MAPK ODN were used as control. After liposomal transfection, cells were exposed to epidermal growth factor (EGF) 1 nmol · L^{-1} for 10 min and then harvested in lysis buffer. MAPK activity was measured by Western blot and P-81 phosphocellulose filter papers method by using [γ-^{32}P] ATP and myelin basic protein as substrate. DNA synthesis was measured by [^{3}H] thymidine incorporation. **RESULTS:** Antisense ODN 0.2 μmol · L^{-1} reduced EGF-induced MAPK activities by 84 %, and inhibited VSMC [^{3}H] thymidine incorporation stimulated by EGF. **CONCLUSION:** A 17-mer MAPK antisense oligonucleotide directed against the initiation of translation sites of the p44- and p42-MAPK inhibited EGF-stimulated rat VSMC proliferation.

Percutaneous transluminal coronary angioplasty (PTCA) has become a well-established therapy in the management of coronary artery disease. However, in the last 15 years, the frequent occurrence of restenosis following 30 % - 40 % of initially successful procedures remained the major limitation of the technique and little has been achieved in the prevention of the restenosis. In experimental models, vascular smooth muscle cell (VSMC) proliferation has been shown to be a key event responsible for restenosis after balloon angioplasty[1,2], and the involvement of a complex interaction between multiple growth factors that promote VSMC proliferation including epidermal growth factor (EGF), platelet-derived growth factor, and fibroblast growth factor[3]. These factors activate membrane tyrosine kinase-coupled receptors or heptahelical G protein-coupled receptors. Receptor binding 'in turn' activates a protein kinase cascade linking extracellular signal events present at the cell membrane with changes in gene expression in the nucleus. Critical enzymes in this cascade are the 42- and 44-kDa mitogen-activated protein kinases (MAPK). The 42- and 44-kDa MAPK are serine/threonine protein kinases that are activated by many stimuli involved in cell growth and as a common pathway to transmit signal for proliferation into the nucleus regulating cell proliferation[4]. On the basis of these findings, we hypothesized that inhibition of the MAPK activity by its antisense oligodeoxyneuleotides (ODN) might suppress VSMC proliferation and hence restenosis. The purpose of this study therefore is to examine the inhibitory effect of the translation of p44- and p42-MAPK mRNA on VSMC proliferation.

MATERIALS AND METHODS

Chemicals EGF (from mouse submaxillary glands), anti-mitogen-activated protein kinase, lipofectin, myelin basic protein, protein kinase inhibitor (TTT AAP IAS GAT GAA AAI HA), leuptinin, HEPES, Triton X-100, bovine serum albumin, and egtazic acid were purchased from Sigma. P-81 filter paper was purchased from Waterman Co. Western blot chemiluminescence reagent plus was purchased from NEN™ Life Science Products.

Synthesis of oligonucleotides Phosphorothioate-protected oligonucleotides were used to

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inhibit proliferation of EGF-stimulated rat SMCs *in vitro*. The sequences of the phosphorothioate-protected oligonucleotides were 17-mer rat antisense MAPK (5′-GCC GCC GCC GCC GCC AT-3′) directed against the initiation of translation site of rat p42- and p44-MAPK mRNA. This ODN has been used successfully to down-regulate both isoforms of MAPK in 3T3 cells[5] and rat cardiac myocytes[6]. 17-Mer rat sense MAPK (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. ODN were synthesized and purified at the DNA Core, University of Cincinnati.

Cell culture[7] VSMC were isolated from aorta of 200 – 250-g Sprague-Dawley rats (supplied by the Experimental Animal Center of Hunan Medical University, grade Ⅱ, Certificate №20-009) and maintained in DMEM supplemented with 10 % fetal calf serum. Cells were 4 to 8 passages.

Liposomal transfection[6] 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 incubatd at 25 ℃ for 20 min. VSMC were washed gently 3 times in serum and antibiotic free DMEM. ODN/lipofectin mix ture 200 μL was added for each well of 12-well plates, or 75 μL for each well of 24-well plates, with equal volumn of serum and antibiotic-free DMEM. VSMC were incubated at 37 ℃ in 95 % O_2 + 5 % CO_2 for 8 h, 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 calf serum. Cells were incubated for another 24 h, before adding EGF.

[^{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 for 48 h in DMEM. Cells stimulated with EGF for 16 h, 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 washed with cold 5 % TCA followed by 70 % ethanol, the filter was dried. The radioactivity of incorporated [^{3}H]thymidine was measured using liquid scintillation counter. Experiments were performed 6 times in duplicate.

Preparation of cell lysates[8] After treated with ODN, cells were washed with PBS, and 0.1 mL 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 $mmol \cdot L^{-1}$ at pH 7.4, along with 0.1 % Triton X-100 and leupeptin 10 $mg \cdot L^{-1}$ was added. Cell lysates were frozen on ice, scraped, sonicated, and centrifuged at 18 000 × *g* for 15 min (4 ℃), protein concentration was estimated by the dye method[9].

Western blot SDS sample buffer containing 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 was added to cell lysates. 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 for 1 h with 5 % BSA in PBST (Na_2HPO_4 80 $mmol \cdot L^{-1}$, NaH_2PO_4 20 $mmol \cdot L^{-1}$, NaCl 100 $mmol \cdot L^{-1}$ containing 0.05 % Tween-20). The blots were incubated at 25 ℃ with the primary monoclonal antibodies of p44- and p42-MAPK at a 1∶10 000 dilution for 1 h, followed by incubation with secondary antibody (horseradish peroxidase conjugated) at a 1∶800 dilution for 1 h. Immunoreactive bands of p42- and p44-MAPK were visualized by using enhanced chemiluminescence reagents. Quantification of p42- and p44-MAPK activity by a scanning densitometry of autoradiographs.

P-81 filter paper kinase assay[10] Fifty μL of the cell lysates were mixed with 10 μL of assay buffer {HEPES 120, $MgCl_2$ 60, $MnCl_2$ 12, DTT 12, Na_3VO_4 3 $mmol \cdot L^{-1}$, pH 7.2, protein kinase inhibitor (TTT AAP IAS GAT GAA AAI HA) 12, BSA 3.6 $g \cdot L^{-1}$, MBP 1 $g \cdot L^{-1}$, [γ-^{32}P]ATP 74 kBq 50 μmol} which is six times of final concentration, then incubated at 30 ℃ for 10 min. The reactions, terminated by spotting 40 μL of the reaction mixture onto P-81 papers, which were immediately immersed in ice-cold H_3PO_4 75 $mmol \cdot L^{-1}$, and washed in H_3PO_4

for 10 min for 6 times and counted. The reaction blank was a mixture containing all of the reagents but without cell lysate.

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

RESULTS

Effect of oligonucleotides on VSMC DNA synthesis Pretreatment of cultured VSMC with antisense ODN 0.2 $\mu mol \cdot L^{-1}$ reduced [^{3}H]thymidine incorporation stimulated by EGF, but neither sense nor random ODN did (Tab 1).

Tab 1. Effect of MAP kinase antisense oligodeoxynucleotides pretreatment on EGF-stimulated [^{3}H] thymidine incoporation in rat aortic smooth muscle cells. $\bar{x} \pm s$ of 6 independent experiments in cultured rat VSMC. Average of duplicate constitutes one determination. $^aP > 0.05$, $^bP < 0.05$ *vs* EGF group. $^dP > 0.05$, $^eP < 0.05$ *vs* control.

Treatment	[^{3}H]Thymidine incorporation, Bq/well
Control	256 ± 33
EGF	2 205 ± 350^e
EGF + lipofectin	2 040 ± 495^a
EGF + antisense	259 ± 75^{bd}
EGF + sense	2 840 ± 414^a
EGF + random	2 073 ± 404^a

Western blot analysis The monoclonal antibodies were directed against MAPK protein identified the 42- and 44-kDa bands in extracts of rat SMC. Antisense ODN 0.2 $\mu mol \cdot L^{-1}$ reduced MAPK activity by 84 % after liposomal transfection. VSMC exposed to lipofectin without ODN had no inhibition effect on MAPK content compared with EGF-stimulated VSMC. Sense and random sequence ODN also had no inhibition effect (Fig 1, Tab 2).

P-81 filter paper kinase assays Pretreatment with antisense ODN 0.2 $\mu mol \cdot L^{-1}$ inhibited EGF stimulated-phosphorylation activity of MAPK. Neither Sense nor random ODN had a significant effect on the MAPK phosphorylation activity (Tab 3).

DISCUSSION

Antisense ODN complementary to sequences in specific genes are useful probes for expression of those genes in various biological processes.

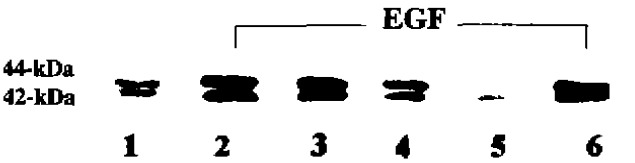

Fig 1. Effect of MAP kinase antisense treatment on the expression of rat aortic smooth muscle cell p42- and p44-MAPK proteins by Western blot. 1) control. 2) EGF-stimulated. 3) EGF + sense. 4) EGF + lipofectin. 5) EGF + antisense. 6) EGF + random.

Tab 2. Effect of MAPK ODN treatment on expression of rat aortic smooth muscle cell p42- and p44-MAPK proteins by Western blot. $\bar{x} \pm s$ of 6 independent experiments in cultured rat VSMC. Average of duplicate constitutes one determination. $^aP > 0.05$, $^bP < 0.05$ *vs* EGF group. $^dP > 0.05$, $^eP < 0.05$ *vs* control.

Treatment	$10^{-3} \times$ Peak area/cm^2
Control	35 ± 8
EGF	154 ± 33^e
EGF + lipofectin	132 ± 17^{ae}
EGF + antisense	26 ± 5^{bd}
EGF + sense	175 ± 10^{ae}
EGF + random	125 ± 16^{ae}

Tab 3. Effect of MAPK antisense pretreatment on MAPK activity in rat aortic smooth muscle cells. $\bar{x} \pm s$ of 6 independent experiments in cultured rat VSMC. Average of duplicate constitutes one determination. $^aP > 0.05$, $^bP < 0.05$ *vs* EGF group. $^dP > 0.05$, $^eP < 0.05$ *vs* control.

Treatment	[γ-^{32}P]ATP incorporation, Bq·min^{-1}/g protein
Control	2 550 ± 1159
EGF	25 657 ± 5512^e
EGF + lipofectin	21 010 ± 4 765^a
EGF + antisense	3 015 ± 1 172^{bd}
EGF + sense	22 741 ± 3 723^a
EGF + random	22 393 ± 55 80^a

Antisense strategies have been successfully usedto investigate single gene function in VSMC and inhibition of VSMC proliferation and hence of restenosis[13]. Cell proliferation involves the complex interactions of mitogen binding to receptors, intracellular signal transduction path-

ways, and changes in the expression of specific genes. Block of a single receptor or signaling pathway will not be sufficient to suppress proliferation. A large number of antisense oligonucleotides targeting the cell cycle mRNA such as nonmuscle myosin heavy chain, proliferating-cell nuclear antigen(PCNA), and c-*myc*[11], c-*myb* were used to inhibit VSMC proliferation. In the present study, we selected a critical final common mediator of growth factor signal transduction, MAPK as the target, and used MAPK antisense ODN to inhibit VSMC proliferation. Recent evidence indicated that many growth factors which caused VSMC proliferation was mediated by MAPK cascade. Transient early activation of MAPK occured within 5 to 10 min of agonist administration, whereas a sustained late activation appeared after 1 to 2 h and was required for cell cycle progression[12]. Specific growth related events that are regulated by MAPK include induction of mRNA for proto-oncogenes (*c-myc*, *c-fos*, and *c-myb*).

In the present study, the ODN are phosphorothioate protected to be resistant to nucleases. To confirm the sequence specificity, two control ODN sense and random were used. The activity of MAPK are identified by P-81 filter paper kinase assay combined with the Western blot method. The mechanism by which antisense ODN inhibited expression of target genes within cells was thought to involve interference with translation of the target mRNA and/or induction of cleavage of DNA/mRNA hybrids by RNAase H[13].

In conclusion, the results of the present study demonstrated that the MAPK pathway played an important role in EGF-induced proliferation of rat VSMC and implied that a 17-mer antisense MAPK oligodeoxynucleotides directed against the initiation of translation sites of the MAPK mRNA could inhibit EGF-stimulated rat VSMC proliferation and thus might be a possible strategy for inhibition of VSMC proliferation and hence of restenosis.

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489-492 (24)

丝裂素活化的蛋白激酶反义寡核苷酸抑制表皮生长因子诱导体外培养大鼠血管平滑肌细胞增生

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关键词 蛋白激酶类；表皮生长因子-尿抑胃激素；反义寡核苷酸类；血管平滑肌；培养的细胞

目的：探讨丝裂素活化的蛋白激酶(MAPK)反义寡

核苷酸(ODN)对表皮生长因子(EGF)诱导的培养大鼠血管平滑肌细胞增生的抑制作用. **方法**: 用脂质体将 p42-和 p44-MAPK ODN 0.2 μmol·L^{-1}转染入大鼠血管平滑肌细胞, 设正义及随机 ODN 为对照, 用 Western Blot 法结合 P-81 滤纸法以髓磷脂碱性蛋白为底物测定 MAPK 活性. [^{3}H]胸腺嘧啶核苷酸掺入测定平滑肌细胞 DNA 合成. **结果**: MAPK ODN 能明显抑制 EGF 诱导的 MAPK 蛋白表达及 MAPK 活性, 并明显抑制血管平滑肌细胞的[^{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 ODN 能有效抑制 EGF 诱导的血管平滑肌细胞的增生.

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Effect of histidine on myocardial mitochondria and platelet aggregation during thrombotic cerebral ischemia in rats[1]

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KEY WORDS photochemistry; thrombosis; cerebral ischemia; platelet aggregation; myocardium; heart mitochondria; histidine

AIM: To study the effect of histidine on cerebral thrombosis and possible mechanism. **METHODS**: Cerebral-cardiac stroke was produced by photochemically induced thrombotic cerebral ischemia in rats. **RESULTS**: Platelet aggregation in whole blood increased markedly, peak heights at 4 and 24 h were (5.1 ± 0.5) Ω and (4.3 ± 0.5) Ω, respectively. Heart mitochondria volume (V), volume density (V_v), surface density (N_m), and surface density of outer membrane (S_{vl}) increased (8.2 ± 5.5, 0.59 ± 0.16, 0.11 ± 0.03, and 0.22 ± 0.05, respectively, $P < 0.01$), but numerical density (N_v), specific surface of inner membrane (δ_2) and of the cristae (δ_3) decreased (0.07 ± 0.02, 2.8 ± 0.8, and 2.4 ± 0.7, respectively, $P < 0.01$) after cerebral thrombosis. The myocardial histopathologic characteristics were different from those of ischemic necrosis and myocardial damage caused by ischemic reperfusion. In rat treated with histidine after photochemical reaction, platelet aggregation decreased markedly [(2.93 ± 1.08) Ω, $P < 0.01$], reversible change often went with parameters related to the inner mitochondrial membrane but not the outer mitochondrial membrane. **CONCLUSION**: Histidine depressed platelet aggregation and reduced myocardial mitochondrial damage resulted from cerebral ischemia.

In "cerebral-cardiac stroke" induced by photochemical reaction[1], we observed that cardiac systolic and diastolic dysfunctions recovered completely in 5 - 7 d. Histidine shortened the reversible injury, known as "stunning"[2] by improving the myocardial contractility[1], suggesting that the functional heart depression but not morphologic changes occurred at cerebral thrombosis. To further elicit the nature of cardiac complication of stroke, we observed the changes of myocardium ultrastructure and whole blood aggregation after cerebral thrombosis, and it might be helpful to clarify the possible mechanism of histidine in improving myocardial stunning.

MATERIALS AND METHODS

Thrombotic cerebral ischemia The cerebral thrombosis was produced in 45 ♂ Wistar rats (Grade Clean) weighing (280 ± 20) g under anesthesia with 2.5 % thiopental sodium (40 mg·kg^{-1}) ip. The rats were given rose bengal (10 mg·kg^{-1}) in 7.5 g·L^{-1} saline solution and the intact skall was irradiated with green light (560

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