目的：研究石杉碱甲对基底核大细胞部(NBM)损毁诱导的工作记忆障碍的影响. **方法**：采用八臂迷宫延迟插板的程序研究空间记忆. 胆碱乙酰转移酶(ChAT)活力测定采用[^{3}H]乙酰辅酶 A 转变成[^{3}H]乙酰胆碱的方法. **结果**：单侧损毁 NBM (卡因酸 0.02 μmol)导致空间记忆障碍. 在不同的延迟间隔，大鼠完成程序产生的正确数减少和错误数增多. 损毁侧大脑皮层 ChAT 酶的含量下降了大约 40 %. 石杉碱甲(0.2 mg·kg^{-1}实验前 30 min ip)改善这种空间记忆障碍. 毒扁豆碱(0.2 −0.3 mg·kg^{-1}实验前 20 min ip)也有改善作用. **结论**：完整的 NBM 是空间记忆形成的关键. 石杉碱甲有效的改善 NBM 损毁导致的空间记忆障碍.

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Inhibitory effect of antisense basic fibroblast growth factor oligonucleotides on proliferation of cultured aortic smooth muscle cells induced by angiotensin Ⅱ in SHR rats[1]

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KEY WORDS basic fibroblast growth factor; antisense oligonucleotides; hyperplasia; vascular smooth muscle; cultured cells; thymidine; angiotensin Ⅱ; inbred SHR rats

AIM: To study the effect of antisense basic fibroblast growth factor (bFGF) oligonucleotides (ODN) transfection on the growth of cultured aortic smooth muscle cells (SMC) in spontaneously hypertensive rats (SHR). **METHODS**: Using cationic liposome-mediated method, antisense bFGF ODN were introduced into SMC, bFGF gene expression was detected by Northern blotting, cell hyperplasia was evaluated by [^{3}H] thymidine incorporation and cell counting. **RESULTS**: Transfection of antisense bFGF ODN (5 μmol·L^{-1}) almost completely inhibited enhanced bFGF mRNA expression and inhibited cell proliferation induced by angiotensin Ⅱ (Ang 1 μmol·L^{-1}). In basal state and Ang-stimulated state, [^{3}H]thymidine incorporation was inhibited by 26.5 % ($P<0.01$) and 42.0 % ($P<0.01$) and cell number was inhibited by 17.3 % ($P<0.01$) and by 22.2 % ($P<0.01$), respectively. **CONCLUSION**: The transfection of antisense bFGF ODN into cultured SMC effectively suppressed bFGF mRNA expression and inhibited the SMC proliferation induced by Ang.

Abnormal growth of vascular smooth muscle cells (VSMC) is critical to the pathophysiology of hypertension. Angiotensin Ⅱ (Ang) played a major role in the regulation of VSMC growth in hypertensive animal models[1].

The inhibition of VSMC hyperplasia by antisense oligonucleotides (ODN) transfer maybe lead to new therapy approaches for certain cardiovascular diseases[2] such as hypertension, atherosclerosis, and vascular restenosis following balloon angioplasty.

The delivery of antisense proto-oncogene *c-myb* ODN effectively suppressed intimal accumulation of rat carotid smooth muscle cells after catheter-mediated injury *in vivo* for at least 2 wk[3].

The delivery of antisense cell cycle regulatory genes (such as cell division cycle 2 kinase, cyclin-dependent kinase, and proliferating cell nuclear antigen) inhibited intimal formation in the rat carotid injury model for as long as 8 wk after a single transfer[4]. Previously, we had demonstrated that Ang enhanced basic fibroblast growth factor (bFGF)

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mRNA expression and stimulated spontaneously hypertensive rats (SHR) VSMC proliferation in culture and this effect could be obviously inhibited by anti-bFGF antibody[1,5]. In this study, we investigated the effect of antisense bFGF (ODN) on the bFGF gene expression and cell hyperplasia.

MATERIALS AND METHODS

Cell culture The thoracic aorta was excised from male 10-wk-old SHR (NIH strain, purchased from Cardiovascular Institute, Chinese Academy of Medical Sciences, Beijing, clean, Certificate № 01-3080) weighing 320 – 350 g (333 ± 5 g, $n = 6$) and the VSMC (passage 4 – 5) were isolated and cultured at 37 ℃, 5 % CO_2 in Dulbecco's modified Eagle's medium (DMEM, Gibcol/BRL) containing 10 % fetal calf serum (FCS, Sigma)[6]. The VSMC were plated into 24-well tissue culture dishes (Nunc, Denmark) or 25-cm^2 flasks (Nunc, Denmark). Morphology of VSMC was verified routinely by phase-contrast light microscopy (Nikon/diaphot, Dusseldorf, Germany). At confluent, the cells exhibited a typical hill-valley pattern. The cells were made quiescently by incubation at 37 ℃, 5 % CO_2 for 48 h in DMEM containing 0.2 % FCS.

Synthesis and purification of antisense bFGF ODN and selection of sequence targets The sequence[7] of antisense and sense bFGF ODN directed at the translation initiation sites of human bFGF mRNA used in this study were: 3'-CCCTGGTACCGTCGG-5' and 5'-GGGACCATGGCAGCC-3', respectively. The synthesized ODN (provided by Biochemistry Department of Cardiovascular Institute, Chinese Academy of Medical Sciences, Beijing) were purified by ethanol precipitation, lyophilized, dissolved in 0.2 % FCS/DMEM before use, and quantitated by spectrophotometry (Model 200-20, Japan). A complex containing a cationic liposome and ODN was prepared according to the method of Itoh *et al*[7]. The final concentration of ODN and liposomes (Sigma) in the complex was 25 mg·L^{-1} (5 μmol·L^{-1}) and 4 mg·L^{-1}, respectively.

Transfection of antisense bFGF ODN VSMC were seeded on 24-well tissue culture dishes or 25-cm^2 flasks at about 7.0×10^4 cells/well or 2.5×10^5 cells/flask in 10 % FCS/DMEM. After confluence, cells were made quiescent for 48 h before transfection. Cells were washed 3 times with PBS (pH 7.4), 0.2 mL complex was added to each well and 2 mL complex was added to each flask. The cells were incubated at 4 ℃ for 5 min followed at 37 ℃ for 6 h, and then washed 3 times with DMEM. Then, the solution was changed to fresh 0.2 % FCS/DMEM with or without Ang (Sigma) 1 μmol·L^{-1}.

Northern blot Total RNA was extracted according to the method of Chomczynski and Sacchi[8]. The RNA content was quantified by ultraviolet absorbance at $\lambda_{260\ nm}$. RNA 10 μg of each group sample was electrophoresed on 1 % agarose/formaldehyde 1 mol·L^{-1} gel electrophoresis. Northern blotting was performed by capillary transfer in 20 × standard saline citrate (SSC) [1 SSC = NaCl 0.15, and sodium citrate 0.015, (mol·L^{-1}) pH 7.0] to nylon membrane (Amersham), which were later baked at 80 ℃ for 2 h. The membranes were prehybrided with 100 μg·L^{-1} sonicated and denatured salmon sperm DNA at 68 ℃ for 2 h in a hybridization buffer containing 6 × SSC, 0.5 % sodium dodecyl sulfate (SDS), and 5 × Denhardt's solution (50 × Denhardt's solution = 1 % Ficoll, 1 % polyvinyl pyrrolidine, and 1 % bovine albumin wt/vol in water). Hybridization with fresh hybridization solution containing [^{32}P]-labeled bFGF cDNA (0.45 kb) probe 1 GBq·L^{-1} (provided by Biochemistry Department of Chinese Academy of Military Medical Sciences, Beijing), 6 × SSC, 0.5 % SDS, 50 % formamide and salmon sperm DNA 100 mg·L^{-1} for 20 h at 42 ℃. The cDNA probe was labeled by a random priming methodology (Amersham-Frence Kit) in the presence of 1.11 MBq [α-^{32}P]dCTP (> 111 PBq·mol^{-1}, 370 GBq·L^{-1}) and a specific activity was up to 2.8 TBq/g DNA. The blot was performed with Kodak XAR-5 film (Eastman Kodak Co, Rochester, New York, USA) with intensifying screens at – 70 ℃.

[^{3}H] Thymidine incorporation VSMC were plated into 24-well tissue culture dishes at about 1.0×10^6 cells·L^{-1}. After the transfection, 1.0 mL 0.2 % FCS/DMEM containing Ang 1 μmol·L^{-1} was added to each well for 20 h in Ang-treated groups whereas cells of Ang-untreated groups were incubated in 0.2 % FCS/DMEM without Ang. The media were aspirated and cells were washed 3 times with 1.0 mL DMEM and incubated for additional 8 h in 1.0 mL DMEM containing [^{3}H]thymidine 37 kBq/well. The media were removed and the cells were washed 3 times with 1.0 mL ice cold 10 % trichloroacetic acid (TCA). After one addition wash with 10 % TCA at 4 ℃ for 30 min, cells were rinsed in 95 % ethanol and dissolved in NaOH 1 mol·L^{-1}, neutralized with HCl 1 mol·L^{-1} and radioactivity was determined by scintillation spectrometry (Model 1215 LKB-RackBeta, Sweden, efficiency 60 %).

Cell counting VSMC were seeded on 24-well tissue culture dishes at about 2.0×10^7 cells·L^{-1} in 1.0 mL 10 % FCS/DMEM. After 24 h, the media were changed to 0.2 % FCS/DMEM for 24 h. Thereafter, the ODN transfection and Ang stimulation were performed as mentioned above. The media were replaced every 2 d and 6 d after the stimulation by Ang. The number of cells was measured using a cell counter.

Statistic analysis All values were expressed as $\bar{x} \pm s$. Unpaired student's t test was employed for comparisons between the 2 groups. The experiments presented were representive of 2 to 3 separate experiments.

RESULTS

bFGF gene expression Ten hours after

stimulation by Ang 1 $\mu mol \cdot L^{-1}$, the level of bFGF mRNA expression in Ang-stimulated group was much higher than that in control group (without Ang). The enhanced bFGF mRNA expression induced by Ang was almost completely inhibited by the transfection of antisense bFGF ODN 5 $\mu mol \cdot L^{-1}$ (Fig 1).

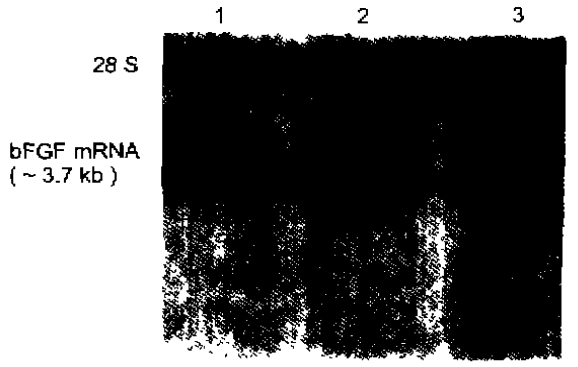

Fig 1. Detection of bFGF mRNA expression by Northern blots. 1. Ang-treated VSMC; 2. Ang-treated VSMC with the transfection of antisense bFGF ODN; 3. untreated VSMC.

VSMC hyperplasia Ang (1 $\mu mol \cdot L^{-1}$) markedly stimulated the [^{3}H]thymidine incorporation and cell proliferation. The [^{3}H] thymidine incorporation and cell proliferation in basal state was inhibited by 26.5 % ($P < 0.01$), 17.3 % ($P < 0.01$) and in Ang-stimulated state inhibited by 42.0 % ($P < 0.01$) and 22.2 % ($P < 0.01$) after the transfection of antisense bFGF ODN 5 $\mu mol \cdot L^{-1}$, respectively (Tab 1).

Tab 1. Effects of antisense bFGF ODN on cultured VSMC hyperplasia. Control: untreated VSMC; Ang: Ang-treated VSMC; $n = 6$ wells, $\bar{x} \pm s$.
$^cP < 0.01$ *vs* control. $^fP < 0.01$ *vs* Ang group.

Antisense/ $\mu mol \cdot L^{-1}$	Ang/ $\mu mol \cdot L^{-1}$	[^{3}H]Thymidine (Bq/well)	Cell number $10^{-3} \times$ cells/well
0	0	285 ± 44	46 ± 4
5	0	210 ± 29^c	38 ± 3^c
0	1	768 ± 109	72 ± 4
5	1	445 ± 145^f	56 ± 4^f

DISCUSSION

Antisense ODN have shown clear promise as agents for blocking specific gene expression and for gene therapy for certain human diseases[9]. In present study, using cationic liposome-mediated transfer method, antisense bFGF ODN nearly completely inhibited bFGF mRNA expression induced by Ang and markedly inhibited cultured SHR VSMC hyperplasia, especially in Ang-stimulated state. In contrast, control sense bFGF ODN could not inhibit bFGF mRNA expression and only had little effect on VSMC hyperplasia. The size of bFGF mRNA detected in this study was approximately 3.7 kb. These results indicated that autocrine bFGF might play an important role in VSMC hyperplasia and that Ang-induced enhanced bFGF production might be an important mechanism of cultured VSMC hyperplasia in SHR. In addition, antisense bFGF ODN may offer a new potential gene therapy approach for certain cardiovascular diseases associated intimately with VSMC hyperplasia.

ODN are very susceptible to nucleolytic activity and their rapid degration in various cells and in culture media or sera is well known[10]. In this study, to reduce ODN degration and to enhance ODN transfer efficiency, we introduced ODN into VSMC by liposome-mediated method. Liposomes are mixture of cationic and neutral lipids that form micellar structures in an aqueous environment. These regents are capable to complex with ODN and, upon exposure to cells, fusing with the plasma membrane, thereby delivering ODN to the intracellular milieu[1,9]. Although liposomes might possess toxic effect on cells to a certain extent[2,9], however, in this study, we did not demonstrate that liposomes (4 $mg \cdot L^{-1}$) had not obviously toxic effects on the cultured VSMC morphologically examined every two hours during the period of transfection by phase-contrast microscopy.

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反义碱性成纤维细胞生长因子寡核苷酸对血管紧张素Ⅱ诱导培养的自发性高血压大鼠主动脉平滑肌细胞增殖的抑制效应

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关键词 碱性成纤维细胞生长因子；反义寡核苷酸；增生；血管平滑肌；培养的细胞；胸腺嘧啶；血管紧张素Ⅱ；近交自发性高血压大鼠

目的：研究转染反义碱性成纤维细胞生长因子(bFGF)寡核苷酸(ODN)对培养的自发性高血压大鼠(SHR)主动脉平滑肌细胞(SMC)生长的影响. **方法**：用脂质体介导法将反义 bFGF ODN 转入 SMC 内，用 Northern 杂交检测 bFGF 基因表达，并测定[^{3}H]thymidine 掺入和细胞计数. **结果**：转染反义 bFGF ODN (5 μmol·L^{-1})几乎完全抑制血管紧张素Ⅱ (Ang Ⅱ，1 μmol·L^{-1})诱导增高的 bFGF mRNA 表达和明显抑制 SMC 增殖，在基础状态和 Ang Ⅱ 刺激条件下，[^{3}H]thymidine 掺入分别被抑制 26.5 % ($P<0.01$)和 42.0 % ($P<0.01$)，细胞数分别被抑制 17.3 % ($P<0.01$)和 22.2 % ($P<0.01$). **结论**：反义 bFGF ODN 能有效抑制 Ang Ⅱ 诱导的 bFGF 基因表达和 SMC 增殖.

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