Antisense oligodeoxynucleotide inhibits vascular endothelial growth factor in human glioma cells[1]

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KEY WORDS antisense oligodeoxynucleotides；glioma；endothelial growth factor；reverse transcriptase polymerase chain reaction；immunohistochemistry；enzyme-linked immunosorbent assay；cultured tumor cells；messenger RNA

ABSTRACT

AIM：To study the effects of antisense vascular endothelial growth factor (VEGF) oligodeoxynucleotide (ODN) on the expression of VEGF in human glioma cell line (A172 cells). METHODS：VEGF mRNA level was measured by semiquantification reverse transcriptase polymerase chain reaction (RTPCR). VEGF protein expression in the cells was determined by immunohistochemistry. VEGF protein level in the media was measured by ELISA. RESULTS：When the cells were treated with antisense VEGF ODN (6.25 – 50 μmol·L^{-1})，VEGF mRNA level in the cells decreased remarkably in a concentration-dependent manner. No change was found when the cells were treated with sense or missense ODN. When the cells were treated with antisense VEGF ODN 25 μmol·L^{-1}，VEGF protein level decreased greatly both in the cells and the media. CONCLUSIONS：Antisense VEGF ODN inhibited VEGF expression specifically in A172 cells *in vitro* and thus the results provided the basis for the further studies *in vivo*.

INTRODUCTION

Malignant gliomas constitute 40 % – 50 % of all brain tumors. They cause approximately 2 % of all cancer deaths. The median survival time from diagnosis is less than 2 years[1]. Thus the development of an efficient therapy is vital. It is well known that tumor angiogenesis is closely related to carcinogenesis，tumor growth and metastasis. So it sounds reasonable that antiangiogenic agents have elicited interest as potential cancer therapeutic agents[2]. Our previous studies revealed that several antisense oligodeoxy- nucleotides (ODN) of VEGF synthesized in our lab could inhibit angiogenesis in a tumor model on the cornea of rat[3]，and that the medium from the antisense VEGF ODN-treated S180 cells could inhibit the growth of calf vascular endothelials[4]. In the present study，the effect of antisense VEGF ODN，with a large loop through base paring between 5′ and 3′ ends，on the levels of VEGF mRNA and protein were investigated in cultured human glioma cell line (A172 cells).

MATERIAL AND METHODS

ODN The sequence of the antisense VEGF ODN was 5′-GCAGTA GCTGCGCTGA TAGTGC-3′，which was complementary to the third extron of human VEGF mRNA. A large loop through base paring between 5′and 3′ends was formed. For control，sense and missense ODN were used in the experiment. The sequences were 5′-CTA TCA GCG CAG CTA CTG C-3′ and 5′-CCT CGT CAT GAG ACA CGT C-3′respectively. All ODN were synthesized in our lab with Beckman Oligo1000 DNA/RNA synthesizer and purified by PAGE.

Transfection of ODN to A172 cells A172 cells 5 $\times 10^5$，maintained in DMEM/10 % FCS，were seeded in a 6-well plate overnight. The media was replaced and the cells were then treated with different concentration of antisense VEGF ODN (0，3.125，6.25，12.5，25.0，50.0 μmol·L^{-1}) in serum-free DMEM for 24 h. FCS was then added into the media in a final concentration of 10 %，and the cells were cultured for another 48 h. Cells were then used for VEGF semiquantification reverse

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transcriptase polymerase chain reaction (RTPCR) and immunostaining, and the supernatants were collected for ELISA.

RTPCR Total RNA was isolated from the cells by using Trizol reagent (GIBCO/BRL). Reverse transcriptase polymerase chain reaction (RTPCR) was performed on total RNA as described previously[5]. Five μL each of the PCR reaction products was separated on PAGE (8 %) and visualized using silver salts. For quantification of VEGF mRNA, the bands were scanned morphometrically with densitometer.

Immunohistochemistry Cells were fixed in 4 % paraformaldehyde solution at 4 ℃ for 20 min. Streptavidin-biotin-peroxidase complex (SABC) method was used for VEGF staining as described previously[5]. SABC kit was supplied by Wuhan Boster Bioengineering Co. To determine the amount of VEGF protein expression in the cells, the optical densities of 3 areas of each haematoxylin-uncounterstained slide were measured on a 400× field. The average absorption was used as the total VEGF protein expression in the cells.

ELISA for VEGF level in the supernatants The supernatants as well as VEGF standards (0, 0.25, 0.5, 1.0, 2.0, 4.0 μg/L) were added to the microtiter wells precoated with mouse monoclonal anti-human VEGF antibody (Sigma, USA) and incubated overnight at 4 ℃. The immunologic procedures were the same as those in immunohistochemistry method. Tetramethyl-benzidine-H_2O_2 mixture was used as substrate and the optical density was determined using a microplate spectrophotometer (BioRad, USA) with a 490-nm wave length. The linear regression analysis of standard concentrations with the values of optical densities was carried out. The concentration of each sample was calculated according to the standard curve.

Statistical analysis All the experiments were carried out in triplicate, the results were reported as $\bar{x} \pm s$ and compared by t test. All Ps were two-tailed.

RESULTS

Effect of antisense VEGF ODN on VEGF mRNA level To investigate the effect of antisense VEGF ODN on target mRNA production, RTPCR was used. The result was expressed as a ratio relative to the level of β-actin. Among 25 – 35 PCR cycles, satisfactory bands were obtained at 28 cycles (Fig 1).

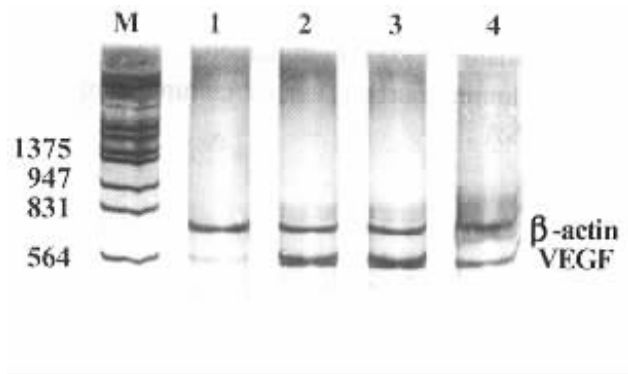

Fig 1. Expression of VEGF mRNA in A172 cells treated with various ODN 25 μmol·L^{-1}. **PCR was carried out for VEGF and β-actin in the same reaction after 28 PCR cycles. M: marker λDNA/Hind Ⅲ + EcoR Ⅰ 1: antisense ODN 2: sense ODN 3: missense ODN 4: control.**

When the cells were treated with antisense VEGF ODN 6.25 – 50 μmol·L^{-1} in serum-free DMEM for 24 h and then cultured in DMEM/10 % FCS for another 48 h, the level of VEGF mRNA in A172 cells decreased in a concentration-dependent manner (Tab 1). No change was found in the VEGF mRNA level when the cells were treated with sense VEGF ODN (3.125 – 50 μmol·L^{-1}) or missense VEGF ODN (3.125 – 50 μmol·L^{-1}).

Effects of antisense VEGF ODN on VEGF

Tab 1. Effects of VEGF ODN on VEGF mRNA expression. $n = 3$ experiments. $\bar{x} \pm s$.
$^bP < 0.05$, $^cP < 0.01$ *vs* control.

Group	Concentration of VEGF ODN/μmol·L^{-1}				
	3.125	6.25	12.5	25.0	50.0
Control			2.35 ± 0.33		
Antisense ODN	2.08 ± 0.34	1.63 ± 0.25^b	0.76 ± 0.14^c	0.15 ± 0.03^c	0.12 ± 0.04^c
Sense ODN	1.88 ± 0.21	1.74 ± 0.53	1.99 ± 0.25	1.73 ± 0.27	1.92 ± 0.13
Missense ODN	2.05 ± 0.28	2.05 ± 0.35	1.93 ± 0.46	2.10 ± 0.39	1.88 ± 0.21

protein level When VEGF protein was detected using immunohistochemistry method, the positive VEGF immunoreaction mainly located in the cytoplasm of the cells decreased (Fig 2).

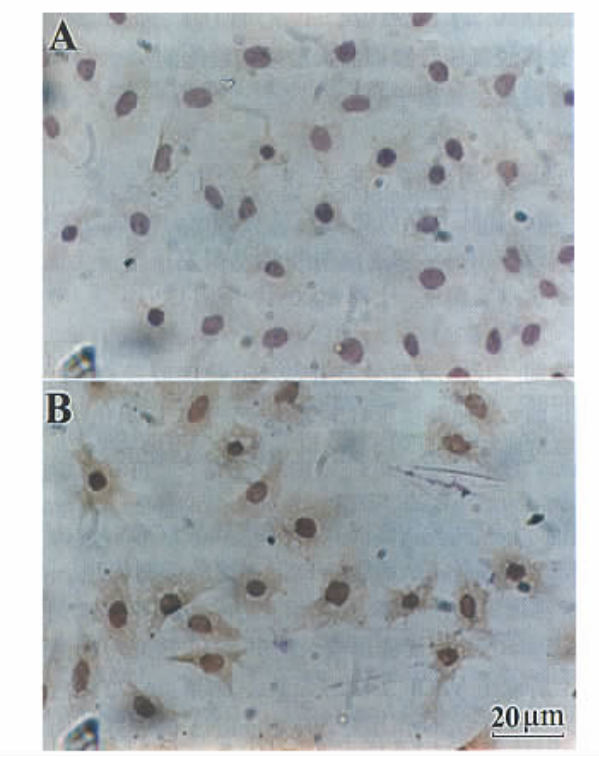

Fig 2. Expression of VEGF protein in A172 cells. VEGF protein was showed as yellow gains at the cytoplasm of the cells. (Immunohistochemistry, SABC, counterstained with haematoxylin, 400×).
A, control. B, treated with antisense VEGF ODN.

After the cells were treated with 25 μmol·L^{-1} antisense ODN, VEGF protein expression in the cells decreased as shown morphometrically. When VEGF level in the medium was measured by ELISA, a great decrease was found after the cells were treated with the antisense ODN 25 μmol·L^{-1}. The treatment of the cells with sense or missense VEGF ODN had no effect on VEGF protein level (Tab 2).

Tab 2. Effects of various VEGF ODN 25 μmol·L^{-1} on VEGF protein expression in A172 cells. $n = 3$ experiments. $\bar{x} \pm s$. $^bP < 0.05$ *vs* control.

	Morphometrical analysis (A)	ELISA/ μg·L^{-1}
Control	0.46 ± 0.10	2.39 ± 0.42
Antisense ODN	0.16 ± 0.07^b	1.45 ± 0.22^b
Sense ODN	0.46 ± 0.08	2.21 ± 0.40
Missense OND	0.41 ± 0.07	2.27 ± 0.33

DISCUSSION

Tumor growth and invasion are dependent on angiogenesis[6]. Tumor angiogenesis is believed to be mediated by some soluble factors such as bFGF, angiogenin, tumor necrosis factor α, VEGF, and so on[7]. Among these factors, VEGF has gained much attention because it is an endothelial cell mitogen *in vitro* and has a potent angiogenic activity *in vivo*. Evidence has shown that the antiangiogenetic strategy is an efficient way for cancer therapy. Thus, inhibition of VEGF in tumor cells may be an important candidate for therapeutic intervention.

Recent studies have shown that inhibition of tumor-secreted VEGF limited primary tumor growth of sarcoma cell line by inhibiting host angiogenesis[8]. Treatment of animals with VEGF-neutralizing antibodies not only inhibited primary tumor growth but also suppressed metastasis *in vivo*[9]. Recently gene transfer strategies have also been used to minimize tumor antiangiogenesis[10]. By constructing an eukaryotic expression vector bearing an antisense VEGF165 cDNA and then transfecting it to rat C6 glioma cell, a reduced level of VEGF in culture was detected[1]. As antisense ODN is relatively simple to design and easy to control, it thus shows a broad potential applicability for therapeutic proposal[11]. It has been found that antisense VEGF ODN inhibited Kaposi sarcoma (KS) cells growth not only *in vitro* but also in nude mice model owing to their blocking of VEGF production specifically[12]. Although antisense VEGF ODN have shown great promise as a sequence-specific inhibitor of VEGF expression, a barrier that must be overcome in a successful therapy *in vivo* is its stability. Phosphodiester ODN are known to be rapidly degraded in serum-containing tissue liquid, due to its 3′-exonuclease activity[13].

In the present study, a 19-mer antisense VEGF ODN with a large loop through base paring between 5′ and 3′ ends was used. The results revealed that it could not only specifically reduce VEGF mRNA and protein levels in A172 cells, but also decrease VEGF protein level in the medium. The results provided a further evidence for the potential use of antisense VEGF ODN in cancer gene therapy. However, further studies should be carried out to determine its usefulness *in vivo*.

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反义寡脱氧核苷酸抑制人胶质瘤细胞的血管内皮生长因子[1]

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关键词 反义寡脱氧核苷酸类；神经胶质瘤；内皮生长因子；逆转录聚合酶链反应；免疫组织化学；酶联免疫吸附测定；培养的肿瘤细胞；信使 RNA

目的：了解反义 VEGF 寡脱氧核苷酸(ODN)对人胶质细胞瘤系(A172 细胞) VEGF 表达的抑制作用. 方法：应用半定量 PCR、免疫组化方法分别了解细胞内 VEGF mRNA 和蛋白的变化，并利用 ELISA 检测培养上清液中 VEGF 蛋白的含量. 结果：A172 细胞经反义 VEGF ODN 作用后 VEGF mRNA 的表达量明显减少，且随其浓度的增加而明显减少，但其表达量不受正义和错义 VEGF ODN 的影响. 当 A172 细胞经 50 $\mu mol \cdot L^{-1}$ 反义 VEGF ODN 作用后，细胞内及上清液内 VEGF 蛋白水平较对照显著减少. 结论：反义 VEGF ODN 特异地抑制 A172 细胞 VEGF mRNA 和蛋白的表达.

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