RESEARCH COMMUNICATION

VEGF-C Antisense Oligoxydeonucleotide Suppression of Invasive Ability of the A-549 Lung Carcinoma Cell Line

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Abstract

<u>Objective</u>: Vascular endothelial growth factor C (VEGF-C), a member of the VEGF family, has been reported to promote angiogenesis and tumor cell growth. In this study, we analyzed inhibitory action of a VEGF-C antisense oligoxydeonucleotide (ASODN) on a lung carcinoma cell line A-549 and its invasive ability in vitro. <u>Methods</u>: Liposomes, liposome-mediated sense oligoxydeonucleotide (SODN) and ASODN were transfected into A-549 cells, with a blank control group. The expression of VEGF-C was examined by western blotting and invasive ability of cells was detected at four levels. <u>Results</u>: Lower expression of VEGF-C and lower invasive ability to recombinate basal membranes were apparent in the ASOND group (P<0.01). However, there were no significant differences among the other three groups (P>0.05). In the invasion assay, the number of transmembrane cells was significantly reduced in the ASODN group after 48 hours (58.6±9.2 P<0.01), but there was no variation amongcontrol, LIP and SOND groups (132.5±15.6, 129.7±16.1, 118.2±12.5, P>0.05). <u>Conclusions</u>: VEGF-C ASODN can downregulate the expression of VEGF-C in cell lines and can obviously inhibit invasive ability in vitro.

Keywords: Vascular endothelial growth factor C - antisense oligoxydeonucleotide - lung carcinoma - in vitro

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Introduction

Lung cancer, with high morbidity and mortality and poor prognosis, is a worldwide problem. Though rapid progress has been made in operation and other comprehensive treatment recently, the 5-year survival rate of lung cancer is not satisfactory. So doctors are aiming to find a new way of treatment, that is gene therapy (Tangney, 2010). VEGF-C is a specific lymphatic endothelial cell growth factor, its roles of promoting tumor cell growth and angiogenesis have been reported in more literatures(Mandriota et al., 2001; Benke et al., 2010). In this study, VEGF-C ASODN was transfected into A-549 cell lines, its invasive ability and expression of VEGF-C were examined, maybe the gene VEGF-C will be a new target in treatment of lung cancer.

Materials and Methods

Human lung cancer cell line A-549 cells were purchased from Shanghai Cell Institute of Chinese Academy of Sciences, ASODN and SODN were from Shanghai Bio-Engineering Company, and were phosphorothioate-modified. RPMI-1640 culture medium (containing L-glutamine) was from Gibco BRL Company. A-549 cells were maintained in RPMI-1640 with 10% heat-inactivated FBS at 37°C in a humiditied atmosphere of 5% CO₂. Cells were inoculated at a density 3×10⁵ cells and cultured under the above conditions. After 15h when cells reached to 80% confluence, they were transfected in serum-free medium using Lipofectamine-plus in accordance with the manufacturer's instructions.Sequence of ASODN is 5'-AAGAAGCCCAGCAAGTGCAT-3', and SODN is 5'-ATGCACTTGCTGGGCTTCTT-3'. Some oligonucleotides have been fluorescently labeled. Oligonucleotides were mixed with lipofectamine by the proportion of 1:2.5 according to manual. Four groups were divided into according to different transfection complexes: control group (blank control group),LIP group (lipofectamine transfection), SOND group (SODN transfection) and ASODN group(ASODN transfection). The final concentration is 1333ng/ml.We observed the transfection process under fluorescence microscope, the labeled oligonucleotides began to appear in cells within half an hour. The fluorescence intensity in cells grew with the time increasing, which could reach peak after 6 to 12 hours. Transfection rate was about 70%.

The 96-well plates, in which were added different pretreated cell lines ($5x10^4$ per well), was coated by 5μ g Matrigel and sealed by 2% BSA, and then was put in a humiditied atmosphere of 5% CO₂ at 37°C. Culture medium was discarded after 30min, 60min, 90min and 120min, respectively. Non-adhesive cells were washed to remove with PBS. 20 μ l MTT and 200 μ l serum-free RPMI-1640 were added to each well, and supernatant were aspirated 4 hours later, then 200 μ l DMSO were added

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and oscillation was done for 10min.Absorbance (A570) of each well was measured with automatic microplate and the rate of cell adhesion was calculated three times. Adhesion rate = (experimental group A570 value / control group A570 value) \times 100%.

The matrix plastic $(40\mu l \text{ per well})$, were covered onto small chamber polycarbonate membrane, turned into gel after 30 min and irradiated with UV lamp overnight. Different pretreatment cells were inoculated into small chamber, NIH/3T3 cell supernatant (chemotactic factor),600 μ l each well, were added. After 24-hour inoculation, the gel were wiped by cotton swabs, and the small chamber were washed with PBS three times, fixed with 95% ethanol and subsequent HE staining. The polycarbonate membrane were peeled off and attached to the glass slide. We countered transmembrane cells in five field randomly under 400 times light microscope, the experiment was repeated three times and means could be got.

For Western blotting analysis , the medium was then replaced with fresh RPMI-1640 containing insulin (10 μ g/ml), transferrin (5.5 μ g/ml), sodium serenite (6.7 ng/ml), and heparin (20 μ g/ml). After 16 h of incubation, conditioned medium was collected for analysis. Proteins in conditioned medium were separated by electrophoresis on 15% SDS-PAGE gels and transferred electrophoretically onto nitrocellulose membranes. Blots were blocked with 5% nonfat dried milk and incubated with anti-VEGF-C antibody and horseradish peroxidase-conjugated goat antirabbit IgG. Protein bands were visualized using an enhanced chemiluminescence detection kit (Amersham Pharmacia). Quantitative analysis of the blots was performed with an imaging densitometer.

Statistical Methods

The results were expressed as mean \pm standard deviation. One-way analysis of variance (ANOVA) was used to determine the levels of difference between all groups. Comparisons for all pairs were made using Student-Newman-Keuls (SNK) test. p < 0.05 was considered statistically significant.

Results

The procession of transfection was smooth, and transfection rate was about 70% after 6 to 12 hours. The adhesion of the cells in ASODN group was suppressed obviously after 30min, though it increased with time in four groups, at the same time point, the adhesion rate in ASODN group was significantly lower than the other three groups (P<0.01), and there is no significant difference in these three groups(P>0.05) (Table 1).

In the invasion assay, the number of the transmembrane cells was significantly reduced in ASODN group compared with other three groups after 48 hours (58.6+9.2 P<0.01), cell structure was not clear under light microscope, and invasion on the reconstituted basement membrane weakened (Figure 1). However, there is no difference in control group, LIP group and SOND group (132.5+15.6,129.7+16.1,118.2+12.5 P>0.05). In Western blot experiment, The expression of VEGF-C protein is lower **2098** Asian Pacific Journal of Cancer Prevention, Vol 12, 2011

Table 1. Adhesion Rates in the Four Groups by MTT(Mean±SD)

Group	30min	60min	90min	120min
Control	0.32±0.03	0.58±0.02	0.79±0.03	0.96±0.03
LIF	0.28 ± 0.02	0.56 ± 0.03	0.77 ± 0.02	0.95 ± 0.02
SODN	0.26 ± 0.02	0.45 ± 0.02	0.76 ± 0.02	0.92 ± 0.02
ASODN	0.15±0.01	0.29 ± 0.02	0.49 ± 0.02	0.76 ± 0.01



Figure 1. Invasion in the Control (upper) and ASODN (lower) Groups (HE X400)

in ASODN group than the other three groups $(0.15\pm0.01 \text{ P}<0.01)$. there is also no difference in control group, LIP group and SOND group $(0.48\pm0.03, 0.45\pm0.02, 0.36\pm0.02 \text{ P}>0.05)$.

Discussion

Lung carcinoma is the most common cause of disease-related death worldwide, and the outcome of patient with lung carcinoma remains poor. Lymph node metastasis specifically is recognized as an important prognostic factor (Suzuk et al., 1999). Lymphatic invasion and blood vessel invasion are additional indicators of disease recurrence or poor overall survival. VEGF-C is believed to be the only lymphangiogenic factor in the VEGF family. The structure of lymphangiogenesis is in favor of tumor cells into the lymphatic channels, thus the formation of lymphatic metastasis and distant metastasis (Ishikawa et al., 2003; Neuchrist et al., 2003). Current studies suggest a clinicopathologic role for VEGF-C in various malignancies, Positive correlations between lymph node metastasis or lymphatic vessel invasion and VEGF-C expression have been reported in patients with lung carcinoma (Bo et al., 2009). Overexpression of VEGF-C and components of their signaling systems is observed frequently in lung carcinoma and serves as the principal cause of metastasis (Saharinen and Petrova, 2004; Taniguchi et al., 2006). The growth and metastasis of tumor cells can be slow after inhibition of VEGF-C activity and protein expression (Lin et al., 2005;Roberts et al., 2006). Thus, VEGF-C and their receptors have become one of the major targets gene to develop new modes of

therapy for lung carcinoma.

Gene therapy, showing a great hope, gradually becomes one of the most prospective methods in biologic treatment of tumor (Edelstein et al., 2007). It is the fifth treatment modality from Flotte (2007) after surgery, radiotherapy, chemotherapy and immunotherapy. It transfects the external functional gene into the patient's body by certain ways so that it could decrease and/or increase expressions of certain genes. Because of the rapid development of tumor molecular pathology as well as recombinant DNA technology, the clinical application of gene therapy in lung carcinoma is practicable.

Antisense nucleic acid is a naturally existing or synthetic nucleotide sequence. VEGF-C ASODN hybridizes with target genes through Watson Crick principle of complementary base pairing to prevent gene expression, inhibit cell proliferation, promote apoptosis, and achieve the purpose of preventing or treating tumors. The natural oligonucleotide is easily degraded, but phosphorathioate modifying can increase the capacity of its tolerance to nucleic acid hydrolysis, with good solubility and hybridization properties. The effectiveness and safety have been universally accepted by researchers. ASODN targeting the gene has yielded beneficial results to suppress tumor growth in vitro. Typical examples are basic fibroblast growth factor and its receptor in melanoma (Oku T et al., 1998), and insulin-like growth factor-Ireceptor, hepatocyte growth factor, and tumor necrosis factor in glioblastoma (Aggarwal et al., 1996; Rininsland et al., 1997; Abounader et al., 1999). However, it is still at the initial stage.

In the current study, we found that the VEGF-C ASODN with phosphorothioate modification was successfully transfected in A549 cells. As time passed, the transfection rate increased. Adhesion experiments and invasion assay showed low adhesion rate and invasive in group of ASODN transfection. The expression of VEGF-C protein in A549 cells with VEGF-C ASODN transfection were lower than the other three groups. The signal translation of VEGF-C is broken, so the growth and infiltration of tumor are inhibited.

In conclusion, we demonstrated potent growth inhibitory effects of VEGF-C ASODN on lung carcinoma growth in vitro. However, Much work remains to be performed before VEGF-C ASODN can be applied to treatment of human lung carcinoma.

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