PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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Inhibitory effects of EGFR antisense oligodeoxynucleotide in human colorectal cancer cell line

Yong He[1], Jun Zhou[2], Jin Sheng Wu[3] and Ke Feng Dou[1]

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INTRODUCTION

Epidermal-growth-factor receptor[1] (EGFR) is a polypeptide with 1186 amino acids, which binds to EGF family growth factors. Two major natural ligands in the family interact with EGFR: one is EGF, the other is transforming growth factor-α (TGF-α)[2]. When EGF or TGF-α, binds to EGFR, tyrosine kinase activity is induced which in turn triggers a series of events regulating the cell growth[3-8]. The importance of EGFR in growth regulating pathways was confirmed by the fact that enhanced expression of this receptor was found in brain glioblastomas, breast, lung, ovarian, colorectal, and renal carcinomas[9,10]. Elevated EGFR levels correlated with poor prognosis in human tumors[11-17], for this reason, it seemed to be that EGFR would be a logical target for cancer therapy.

Previous reports had shown that monoclonal antibodies to EGFR were effective in the treatment of many human carcinoma cells[18-20]. Other drug thera pies which targeted the EGFR had also been successful. Kunkel had shown a drug that inhibited EGFR tyrosine kinase activity could inhibit the growth of A431 cells in nude mice[10]. Yoneda also reported that selective inhibitors of EGFR tyrosine kinase activity, such as tyrphostins, could inhibit the growth of squamous carcinoma in nude mice[21].

Antisense oligodeoxynucleotides inhibit gene expression on a highly selective and target sequence in a specific manner[22-25]. Specific oligonucleoti des hybridize to complementary mRNA and decrease protein expression[26-29] . Antisense oligonucleotides against proto-oncogenes of growth factors had already been shown to be successful in cell lines[18,30]. For example, an antisense oligonucleotide to the erB2 gene product had been shown to inhibit protein production in a breast cancer cell line[31]. Akino reported inhibition of *in vivo* growth and metastases in malignant pituitary tumors with an antisense compound to the PTHrp (parathyroid hormone-related peptide)[32]. An oligonucleotide to the c-myc gene inhibited the growth of thyroid carcinoma cell lines[33]. Phosphorothioate antisense oligodeoxynucleotides targeted against human c-raf-1 kinase producing potent antiproliferative effects on cell culture and *in vivo* antitumor effects against a variety of tumor types[34].

The co-expression of EGFR along with TGF-α in human colon cancer cell lines, also in colon carcinoma tissue, had led to the suggestion that the autocrine stimulation of EGFR by its ligands could be a mechanism for tumor cells to escape from normal growth controls[35]. Previous studies in our laboratory confirmed over-expression of EGFR in HR8348 cells[36]. In this investigation, we hypothesized that growth and proliferation of HR8348 could be inhibited by EGFR ASODN. In this report 15-mer EGFR ASODN was synthesized and the effects of EGFR ASODN on cell proliferation and tumorigenic rate of HR8348 cells were observed.

MATERIALS AND METHODS

Cell line

The liver metastasis of human colorectal cancer cell line HR8348 was developed by Zhang *et al*[37] and cultured at 37℃ in a humidified at mosphere containing 50mL/L CO_2 in RPMI1640 medium (Gibco) supplemented with 100mL/L heat-inactivated fetal calf serum (FCS), penicillin (50×10^3 units/ mL), and streptomycin sulfate (50mg/L) unless otherwi se specified.

Synthesis of oligonucleotides

The AEGFR oligonucleotide sequence, 5'-CCGTGGTCATGCTCC-3 ' is complementary to EGFR cDNA 3811-3825, which contains the opal translation termination codon at residues 3817-3819. The control oligonucleotide sequence, a randomized phosphodiester 15-mer oligonucleotide with the sequence 5'-GCTGACGCACTGACT- 3'(RC 15) is not complementary to any cDNA. Oligodeoxynucleotides were synthe sized on an automated DNA synthesizer.

Formation of the lipid-ODN complex

ODN and liposome, Lipofectamine (Gibco-BRL), were each diluted to 0.1mL with RPMI1640 (serum and antibiotic free) and then mixed together, following the manufacturer 's protocols. The lipid-

[1]Department of Hepatobiliary Surgery, Xijing Hospital, the Fourth Military Medical University, Xi'an 710032, China
[2]Department of Pathology, Qindu Hospital, the Fourth Military Medical University, Xi'an, China
[3]Department of General Surgery, Tangdu Hospital, the Fourth Military Medical University, Xi'an China
Dr. Yong He, graduated from the Fourth Military Medical University as a Bachelor of Medicine in 1980, and got MD in 2000, now working in Department of Hepatobiliary Surgery as abdominal surgeon, specializing in the genetic diagnosis and therapy of gastrointestinal tumor, having 11 papers published.
Correspondence to: Dr. Yong He, Department of Hepatobiliary Surgery, Xijing Hospital, the Fourth Military Medical University, No.17 Changle Xilu, Xi'an, 710032, Shaanxi Province, China
Tel. 0086-29-3375259, Fax, 0086-29-3375261
Email. heyong007@yahoo.com
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ODN complexes were used in gene transfection immediately after its formation.

Treatment of cells

To determine the effect of anti-EGFR oligonucleotides on HR8348 cell proliferation, MTT method was adopted. Fourty μL HR8348 cells (1×10^4) in 96-hole culture dishes were treated at 37℃ for 5h with either free or lipid-ODN mixture and then added 200μL fresh medium with 100 mL/L fetal calf serum for a further 48h. At this point, the cells were washed twice with (serum free) RPMI1640, and RPMI1640 200μL, added MTT (5g/L) 20μL and the cells were incubated at 37℃ for 4h, then added and quantified the DMSO.

Flow cytometry analysis

Cells of 0.8mL (1.5×10^6) were plated in 35mm tissue culture plates and added 0.2mL of the lipid-ODN mixture. The cells were incubated for 5h at 37℃, and then 4mL of RPMI1640 medium with 100mL fetal bovine serum was added for 48h, then cells were harvested, and analyzed for cell-cycle distribution by a FACScan flow cytometer.

Assay for tumorigenicity in nude mice

HR8348 cells (1×10^7) treated with or without ODN were injected subcutaneously in 6-week-old nude mice (Swiss nu/nu). The animals were monitored for tumor formation every week.

RESULTS

Antiproliferative activity of AEGFR on HR8348 cell line

A short exposure of HR8348 cells (5h) to the oligonucleotides was followed by an additional 3-day growth in maintenance medium with 10% FCS. MTT assa y showed that treatment of HR8348 cells with liposome encapsulated AEGFR resulted in a 82.5% reduction in proliferation as compared with untreated cells, whereas RC15 group resulted in a 12.6% reduction in proliferation compared with untreated cells (Table 1).

Cell cycle assay

The HR8348 cells treated with AEGFR displayed an increased percentage of cells in the G_1/G_0 phase and a decreased percentage of cells in the S phase (Table 2).

Decreased tumorigenicity in AEGFR-treated HR8348 cells

The AEGFR cells displayed a marked inhibition on tumorigenicity rate in nude mice as compared with control cells(Table 3).

Table 1 Inhibitory effects of liposome-ODN

Group	48h		72h	
	MTT value ($\bar{x}\pm s$)	Inhibition rate (%)	MTT value ($\bar{x}\pm s$)	Inhibition rate (%)
Control	0.445±0.016		0.337±0.003	
Liposome-RC15	0.389±0.015	12.6	0.091±0.008	12.2
Liposome-AEGFR	0.078±0.022	82.5	0.079±0.005	76.6

Table 2 Cell cycle assay

Group	G_0/G_2(%)	S(%)	G_2+M(%)	PI
Control	21.6	59.9	18.5	0.784
Liposome-RC15	29.5	54.9	15.6	0.705
Liposome-AEGFR	57.1	32.5	10.1	0.329

Table 3 Inhibition of subcutaneous HR8348 adenocarcinoma growth by ASODN

Group	Diameter of tumor (cm)	Rate of tumorigenicity (%)
Control	1.00±0.08	100(10/10)
Liposome-RC15	0.95±0.07	100(10/10)
Liposome-AEGFR	0.80	20(2/10)

DISCUSSION

Colorectal carcinomas generally show a poor response to conventional chemothe rapeutics[38]. Several growth factors are involved in the control of colon carcinoma cell proliferation. In particular, the epidermal growth factor (EGF), transforming growth factor-alpha (TGF-alpha) and their receptor EGFR which are frequently overexpressed. EGF and TGF-α are structurally related peptides that stimulate DNA synthesis and cell growth. Both EGF and TGF-α recognize and compete the same cell membrane receptor (EGFR) through which they mediate their biological action. It has been recently demonstrated that autocrine secretion also exists in the human colon carcinoma tissue and which enables uncontrollable growth of the tumor cells. In the autocrine hypothesis[39], the increased EGFR activity during tumorigenesis is attributed to autocrine stimulation by TGF-α. TGF-α produced by transformed cells acts on the cell surface EGFR to promote unrestrained cell proliferation. Being consistently amplified in human tumors of ectodermal origin, EGFR is shown to be an active factor in development and proliferation of neoplasia. Over-expression of EGFR can pro mote transformation of cells.

In this study, EGFR ASODN could inhibit the proliferation of human colorectal carcinoma cell lines. The antisense compound could also inhibit significantly the cell growth *in vivo* when compared to a scrambled oligonucleotide control. This inhibition is specific since the control oligonucleotide had no effect on cell proliferation and cell growth *in vivo*.

At this moment, we do not know the precise mechanism of inhibition. It could simply be that interfering with the initial step in the cell cycle or mRNA expression, disrupting the cascade of events leading to cell proliferation. The mechanism, however, could be much more complicated. The antisense compounds could decrease levels of cyclin-dependent kinases (CDK) which are essential to cell cycle progression. Treatment of human prostatic and colon carcinoma cells with EGFR antibody could decrease CDK levels resulting in G_1 arrest[11,40]. Another possibility is that treatment with the EGFR antisense oligonucleotides induces apoptosis, a phenomenon observed in human colon carcino ma cells when the EGFR was blocked by its monoclonal antibody[11,40].

Antisense oligodeoxyribonucleotides have

shown great efficacy in the selective inhibition of gene expression. In fact, antisense oligonucleotides directed aga inst TGF-alpha, EGFR, are able to inhibit growth and transformation of several human carcinoma cell lines. These data suggest that the EGF-like growth factor s and their receptors offer potential usefulness as targets for experimental therapy of human colon carcinoma. In our study, both cell growth and DNA synthesis of human colorectal carcinoma cell line HR8348 could be inhibited by EGFR ASODN, perhaps due to blockage of autocrine stimulation cycle of EGFR by its ligands. Cell cycle analysis indicated that with AEGFR-treatment, the proportion of cells in G_0/G_1 increased, and proliferation index (PI) was lower than that in the control group. This inhibition effect of cell proliferation by specifically repressing growth-receptor productions strongly indicated the presence of this autocrine hypothesis of tumor-cell growth.

Lipofectamine[41] was cationic liposomes. Cationic liposomes represent synthetic genetic delivery systems that avoid the potential infectious complicat ions of viral vectors. Due to its absence of cellular toxicity, convenience and high efficiency, liposome is used widely in gene transfection. It can provide a complex with a net positive charge that can associate with the negatively charged surface of the cell and it can be taken up by cells, and this method is useful for introducing large DNA molecules, oligonucleotides, and RNAs into mammalian cells. *In vivo* gene transfer with DNA-cationic liposome co mplexes has been proven to be safe to the host, low in cost and relatively easy in preparation, if can be used in the treatment of cancer.

This study demonstrates that it is possible to identify oligonucleotides which selectively inhibit the expression of EGFR by an antisense mechanism, provided that a careful search for optimal target sites on the mRNA is conducted and delivery of the oligonucleotides to cells in culture is facilitated by the use of cationic liposomes. These results warrant further assessment of the compo unds in inhibition of cell growth *in vivo* and in their possible use as therapeutic agents in the treatment of human cancer.

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