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Genetics

C-Met antisense oligodeoxynucleotide inhibits growth of glioma cells

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Abstract

Background: C-Met, a receptor tyrosine kinase, and its ligand, hepatocyte growth factor, are critical in cellular proliferation, motility, and invasion and are known to be overexpressed in gliomas. The aim of our study was therefore to investigate the effect of transfected caroboxyfluorescein-5-succimidyl ester (FAM)–labeled c-Met antisense oligonucleotide (ASODN) on growth of glioma cells.

Methods: Conjugated FAM-labeled c-Met ASODN was encapsulated by LIPOFECTAMINE PLUS Reagent and then added into the human glioma cell line U251. Cultured cells were divided into 5 groups: control group, 500 nmol/L nonsense oligonucleotide (NSODN) group, 250 nmol/L ASODN group, 500 nmol/L ASODN group, and 750 nmol/L ASODN group. The intracellular distribution of c-Met ASODN was observed with fluorescence microscopy; cell growth was detected by methyl thiazole tetrazolium assay. The apoptosis of U251 cells was also examined with a flow cytometer. Semiquantitative reverse transcriptase polymerase chain reaction and Western blot examinations were carried for expression of c-Met messenger RNA (mRNA) and protein.

Results: The blue fluorescence was seen in the cytoplast and nuclei of cells of FAM-labeled c-Met ASODN groups with fluorescence microscopy after the cells were treated with FAM-labeled c-Met ASODN–LIPOFECTAMINE PLUS Reagent complex for 3 hours. Antisense (AS) oligonucleotide caused a statistically significant reduction of cell viability ($P < .05$), whereas NSODN had no such changes. The cell growth was also significantly inhibited by ASODN ($P < .05$). After transfection, 250, 500, and 750 nmol/L ASODN induced significant apoptotic response, about 4.67% ± 2.86%, 8.65% ± 3.18%, and 12.76% ± 3.15% for 24 hours ($P < .05$) and 7.79% ± 1.92%, 11.43% ± 1.54%, and 15.78% ± 1.86% for 48 hours ($P < .01$), respectively. However, 500 nmol/L NSODN did not induce any significant apoptotic response until 48 hours after transfection ($P > .05$). A significant loss of c-Met mRNA was presented in ASODN-treated cells, and this was not seen in treatment with NSODN. Protein level was significantly decreased 48 hours after c-Met ASODN trasfected.

Conclusions: Antisense oligonucleotide targeting c-Met can be identified as a most potent AS compound, which can inhibit cell growth and induce cell apoptosis. This provides evidence that c-Met plays a role in tumor progression of glioma by acting as an oncogene and suggests that c-Met ASODN may provide a novel approach to therapy for human glioma.

Keywords: c-Met; Apoptosis; Gene therapy; Glioma; Antisense oligonucleotide

1. Introduction

A variety of growth factors such as epidermal growth factor, transforming growth factor α, and transforming growth factor β appear to play a crucial role in human carcinogenesis. Recently, attention has been focused on the role of the HGF/receptor system because of its multifunctional properties such as cell proliferation [14], cell movement [13], and morphogenesis [27]. The receptor for HGF is a protein product of a proto-oncogene, c-Met [20],

Abbreviations: HGF, hepatocyte growth factor; c-Met, hepatocyte growth factor receptor; ASODN, antisense oligonucleotide; ODNs, oligodexynucleotides; NSODN, nonsense oligonucleotide; FCM, flow cytometry; MTT, methyl thiazole tetrazolium; FAM, caroboxyfluorescein-5-succimidyl ester; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide; RT-PCR, peverse transcriptase polymerase chain reaction; EGF, epidermal growth factor; TGF-α, transforming growth factor-α; TGF-β, transforming growth factor-β; AS, antisense; NS, nonsense; Fig., figure.

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which encodes a transmembrane tyrosine kinase ($P^{190\ c\text{-}Met}$) with structural and functional features of a growth factor receptor [11,25]. Autophosphorylation of this receptor by ligand binding stimulates its intrinsic tyrosine kinase activity with resultant changes in cellular morphology, motility, and growth.

Overexpression of this oncogene was shown in different human solid tumors such as hepatomas and carcinomas of the colon, rectum, stomach, pancreas, thyroid, kidney, ovary, endometrium, bladder, breast, and prostate [2,5,10, 12,15,17,19,23,28,30,32]. We have previously found that HGF/c-Met played an important role in the formation and progression of the brain astrocytoma, can promote tumor proliferation and intratumoral microvascular formation, and was closely related to the prognosis of the patients [9]. Recently, manipulating apoptosis gene by antisense ODN might provide new therapeutic strategies in treating human diseases [18]. Antisense oligonucleotide is a single-strand DNA that is complementary to specific regions of mRNA and capable of inhibiting the antiapoptotic gene, and it is noticeable that ASODN holds great promise as a pharmaceutical agent. Therefore, we investigated whether growth inhibition and induced apoptosis of glioma cell line U251 could be achieved by targeting c-Met with ASODN.

2. Materials and methods

2.1. Cell culture

U251 cell (human glioma cells from Wuhan University of China) was incubated at 37°C in RPMI-1640 medium supplemented with 10% calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin, in an atmosphere of 5% CO_2 at saturation humidity. The cell line was subcultured 2 to 3 days later with an initial concentration of 5×10^4 cells per milliliter. Cells in logarithmic growth were used in all experiments.

2.2. Immunohistochemistry

Cells were plated in 60-mm tissue culture dishes containing cover glasses overnight. The cover glasses were washed in PBS, fixed for 30 minutes in 70 mL/L ethanol, and washed twice in PBS. Quenching of the endogenous peroxidase activity was obtained by treatment with 0.3 mL/L H_2O_2 in methanol. The sections were blocked with 10 mL/L goat serum in PBS and incubated with 3 μg anti–c-Met polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) at 4°C overnight, then incubated with horseradish peroxidase (HRP)–conjuncted antibody for 30 minutes at room temperature. After 3 washes of 5 minutes in PBS, they were developed in a substrate solution of HRP. Negative control omitting c-Met antibody was also performed to confirm the absence of nonspecific reactions. Expression of c-Met was classified into 3 levels: no or weak staining (−), less than 50% positive nuclei staining (+), and greater than 50% positive nuclei staining of the total nuclei (++).

2.3. Oligodeoxynucleotide

Caroboxyfluorescein-5-succimidyl ester (FAM)–labeled phosphorothioate ODNs corresponding to the human c-Met open reading frame were synthesized using an applied biosystems 3900 DNA synthesizer (Shenggong, Shanghai, China). The antisense (AS) and nonsense sequences used were 5′-ACAGCGGGGGCCTTC ATTAT-3′ and 5′-TCGGCTACAAGCTACGGTTG-3′, respectively [4]. After the synthesis, ODNs were purified by use of high-pressure liquid chromatography system, dissolved with PBS, and frozen in aliquots at −20°C until use.

2.4. Transfection

According to the manufacturer's instructions, ODNs were delivered into cells in the form of complexes with LIPOFECTAMINE PLUS Reagent (GIBCO Laboratories, Grand Island, NY).

2.5. Measurement of cell growth

In vitro growth inhibitory effects of ODNs on U251 were assessed by (Roswell Park Memorial Institute) MTT assay performed according to the previously described protocol [31] with a slight modification. Cultured cells were divided into 5 groups: control group, 500 nmol/L NSODN group, 250 nmol/L ASODN group, 500 nmol/L ASODN group, and 750 nmol/L ASODN group. In each well of 96-well micrometer plates, 2×10^4 cells were seeded and allowed to attach overnight. The cells were then treated with ODN–LIPOFECTAMINE PLUS™ Reagent complex for 24 hours and incubated for another 48 hours. Subsequently, 20 μL of MTT (5 g/L; Sigma, St Louis, MO) in PBS was added to each well, followed by incubation for 4 hours at 37°C. Formazan crystals were dissolved in dimethyl sulfoxide. Absorbance was determined with an enzyme-linked immunosorbent assay reader (model318; Shanghai, China) at 540 nm. Each assay was performed 9 times. The results were expressed as mean ± SE of controls with no ODN.

2.6. Reverse transcriptase polymerase chain reaction analysis

Total RNA was extracted from U251 cells by modification of guanidinium thiocyanate–acid phenol method [8,30] and quantified based on the measured absorbance at 260 nm. Complementary DNA (cDNA) was synthesized using 2 μg of RNA, 10^6 U/L reverse transcriptase (GIBCO, Inc), and 0.5 g/L oligodeoxythymidine, in a total volume of 20 μL. Reaction was performed at 42°C for 60 minutes and terminated by heating at 99°C for 5 minutes. The sequences of ODNs primers for RT-PCR were as follows: c-Met-S5′-ACAGTGGCATGTCAACATCGCT-3′, c-Met-A5′-GCTCGGTAGTCTACAGA TTC-3′ [24], G3PDH-S5′-ACCACAGTCCATGCCATCAC-3′, and G3PDH-A5′-