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c-Met Antisense Oligodeoxynucleotides as a Novel Therapeutic Agent for Glioma: *In Vitro* and *In Vivo* Studies of Uptake, Effects, and Toxicity

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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 to investigate the uptake and effects of c-Met antisense oligodeoxynucleotides (ASODNs) on rat and human glioma cells *in vitro* and the uptake and toxicity of these nucleotides in rat carcinomatosis and brain tumor models.**

Materials and methods. **The three human cell lines (U87, BT325, SHG44) and the C6 rat glioma cell line were cultured. To study the uptake of oligodeoxynucleotides (ODNs) by glioma cells *in vitro*, cultured glioma cells readily incorporated caroboxyfluorescein-5-succimidyl ester (FAM) labeled phosphorothioate oligodeoxynucleotides, as demonstrated by immunofluorescence microscopy and flow cytometry. To study the effect of ASODNs treatment on c-Met expression *in vitro*, Expression of c-Met was assessed by immunofluorescence microscopy and reverse transcriptase polymerase chain reaction (RT-PCR) analysis. For animal studies of ODNs toxicity and uptake, eight rats underwent placement of cisternal catheters, under general anesthesia. Four rats were given 24 μg FAM-labeled ASODNs while the others were given a saline control injection. After a 24 h observation period, rats were sacrificed by barbiturate overdose, and their brains were studied.**

Results. **For all cell lines, fluorescence was seen to increase with increasing ASODNs concentration. Cells treated in similar fashion were also analyzed by flow cytometry to graphically illustrate the differing fluorescence. Multiple glioma cell lines were tested, with similar results. c-Met ASODNs was found to be successfully incorporated from the media into cultured human glioma cells, even at concentrations as low as 2 μM. In addition, maintenance of the pH-dependent green fluorescence color, as seen by immunofluorescence microscopy and by using flow cytometry, indicated that the FAM was not contained within lysosomes. Immunofluorescence microscopy and RT-PCR analysis showed decreases in c-Met expression with oligodeoxynucleotides treatment. Uptake into tumor cells was also demonstrated *in vivo*, with no detectable toxicity at concentrations exceeding expected therapeutic levels.**

Conclusion. **These data are encouraging for further study of c-Met antisense oligodeoxynucleotides as a therapeutic modality for glioma.**

Key Words: **antisense oligodeoxynucleotides; c-Met; glioma; therapy.**

INTRODUCTION

We have previously found that HGF/c-Met played an important role in the formation and progression of brain astrocytoma, could promote tumor proliferation and intratumoral microvascular formation, and was closely related to the prognosis of the patients [1–3]. Recently, we have also found that recombinant human hepatocyte growth factor could resist apoptosis of U251 glioma cells induced by mitomycin C, and the sensitivity of U251 glioma cells to mitomycin C could be enhanced by the blockage of the c-Met mRNA [4, 5]. We have also found the c-Met antisense oligodeoxynucleotides (ASODNs) could potentiate the cytotoxic effect of radiation on human U251 gliomas *in vitro* and *in vivo* [6]. Recently, we found that antisense oligodeoxynucleotide targeting the c-Met gene also down-regulated or inhibited c-Met expression, which could inhibit U251

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glioma cell growth and induce cell apoptosis [7]. These findings prompted us to investigate whether c-Met is an appropriate target for CNS ASODNs therapy.

The goals of this study, therefore, were (1) to determine whether or not c-Met ASODNs would be incorporated into cultured glioma cells simply through addition to the culture media; (2) to study the effect such treatment would have on c-Met expression as protein and mRNA; (3) to determine whether or not rats injected intracisternally with ASODNs would show detectable adverse effects; and (4) to see whether or not intracranial tumor cells would incorporate ASODNs injected intracisternally or directly into tumor.

MATERIALS AND METHODS

Cell culture, ODN Production, and Treatment

The three human cell lines (U87, BT325, SHG44) and the C6 rat glioma cell line were obtained from the Fourth Military Medical University of China. All cells were grown in a humidified atmosphere with 5% CO_2 under standard sterile culture conditions. Cells were harvested with trypsin-EDTA solution and counted on a hemacytometer. 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 and nonsense sequences used were 5′-ACAGCGGGGGCCTTCATTAT-3′ and 5′-TCGGCTACAAGCTACGGTTG-3′, respectively [8]. After synthesis, ODNs were purified by use of high-pressure liquid chromatography system, dissolved with PBS, and frozen in aliquots at −20°C until use.

For c-Met ASODNs uptake studies, cells at a concentration of 1×10^5 cells/chamber were plated into eight-chambered slides (NUNC, Inc., Naperville, IL). Twenty-four h after plating, cells were washed twice in serum-free RPMI 1640 media; then, FAM-labeled ASODNs at concentrations of 2 μM, 20 μM and 100 μM in serum-free media was added. After 24 h, cells were rinsed twice with PBS, fixed in 100% methanol for 5 min at 4°C, then observed (and photographed) using a Leitz immunofluorescence microscope (Melbourne, Australia). Similar studies were done on a larger scale in 25 cm^2 flasks. ODNs-treated cells were harvested with trypsin-EDTA and cytospins were made, observed, and photographed. For ODNs treatment of cultured cells used for (RT-PCR) analysis, cells were plated at a concentration of 2.4×10^5 cells/well in 6-well culture plates (Falcon; Becton Dickinson Labware, Bedford, MA). After 24 h, cells were rinsed twice in serum-free RPMI 1640 media, then fresh serum-free media was added containing c-Met ASODNs or nonsense ODNs (*versus* control) at concentrations of 2 μM, 20 μM, and 100 μM. Cells were harvested after incubating for 48 h at 37°C.

Flow Cytometry

Flow cytometric determinations of FAM-ODNs uptake were made on the EPICS Profile (Coulter Electronics Inc., Hialeah, FL) instrument. Argon laser excitation at 488 nm was used. A 515 nm longpass filter was placed in front of the green photomultiplier tube. Full-Bright microspheres (Coulter) were used to aid in alignment. All cell suspensions were filtered through a 37 micron nylon monofilament mesh filter prior to analysis; 2.5×10^4 cells were collected for each data point. During flow cytometry, cells were maintained at 4°C.

Anti-c-Met Staining

For c-Met staining, cells either in monolayer or in suspension after trypsin-EDTA harvest were washed with PBS, fixed with 0.5% paraformaldehyde (30 min, 4°C), then permeabilized for 3 min (0.1% Triton-X). Cells were then suspended in 20 μg/mL anti-c-Met monoclonal antibody (no. 394; American Diagnostica, Stamford, CT) (or nonspecific isotype control antibody) for 1 h at 4°C, washed twice, then suspended in FAM-conjugated goat anti-mouse $F(ab')_2$ antibody fragment (Tago Corp., Burlingame, CA) for 1 h. Cells were washed twice before observation and photography with a Leitz immunofluorescence microscope. Cytospins were made or cells stained in suspension.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) Analysis

Tota1 RNA was extracted from cells by modification of guanidium-thiocyanate acid phenol method [9] and quantified based on the

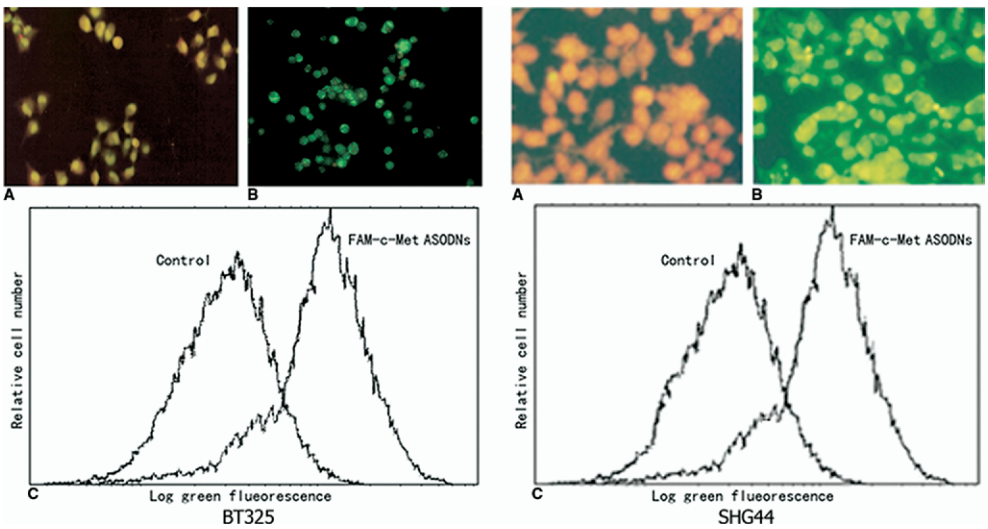

FIG. 1. Immunofluorescence photomicrographs of cytospins of control BT325 cells (A); BT325 and SHG44 cells treated for 24 h with 100 μM FAM-labeled ASODNs (B). Cellular uptake of ASODNs is indicated by the green staining. Flow cytometry histograms (C) of cells as in (A) and (B) above. X-axis: log green fluorescence. Y-axis: relative number of cells. The shift of the histogram peak to the right indicates the cellular uptake of ASODNs. (Color version of figure is available online.)