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Genetics

RNA interference targeting EphA2 inhibits proliferation, induces apoptosis, and cooperates with cytotoxic drugs in human glioma cells

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Abstract

Background: Overexpression of EphA2 was detected in low- and high-grade glioma. To examine the role of EphA2 in human glioma cells, we studied its effects on proliferation and apoptosis using gene silencing through RNA interference.

Methods: One siRNA targeting EphA2 gene was synthesized in vitro and was transfected into the glioma U251n cells. Expression of EphA2 proteins was detected by Western blots and immunofluorescence. Cell apoptosis and mitochondrial membrane potential were analyzed by flow cytometry and annexin-V/fluorescein isothiocyanate/propidium iodide, respectively. Caspase-3 activity was measured by a spectrofluorometer. MTT assay was used to examine changes in cell proliferation.

Results: After treatment with sequence-specific siRNA targeting EphA2, the protein level of the transfected group decreased significantly. As compared to non-siRNA transfected cells, the transfected group showed lower proliferation, higher apoptosis, and loss of mitochondrial membrane potential. Caspase-3 activity increased in cells treated with siRNA and downregulated when treated with caspase-3 inhibitor. And the effects were clearly additive when siRNA transfected cells treated with the anticancer agents.

Conclusions: The results suggest that EphA2-siRNA inhibit U251n cell proliferation and induce their apoptosis. It is possible that EphA2 via mitochondrial and caspase-3 inhibits U251n cell apoptosis. And EphA2-siRNA transfection enhances U251n cells' sensitivity to chemotherapy. EphA2 may be an effective therapeutic target in patients with glioma. Silencing the receptor EphA2 gene is a novel approach for the containment of growth and migration of tumor in patients with malignant glioma.

Keywords: Proliferation; Apoptosis; RNA interference; EphA2; Glioma cells; Chemotherapy

1. Introduction

Among all types of intracranial tumors, gliomas are the most frequent and lethal. Although a small portion of low-grade astrocytic tumors displays benign features, most of these tumors are malignant and usually dictate a poor prognosis. Despite modern diagnostics and treatments, the median survival time does not exceed 15 months. Surgical therapy is frequently not curative; it has long been observed that after surgical removal, tumors recur predominantly within 1 cm of the resection cavity. This is mainly due to the fact that at the time of surgery, cells from the bulk tumor have already invaded normal brain tissue. Nonsurgical treatments are often unsuccessful because one of the most important hallmarks of malignant gliomas is their invasive behavior. Currently, some efforts including siRNA technique are directed toward a treatment based on the molecular biology of this disease, which may improve survival.

Abbreviations: Ac-DEVD-AMC, acetyl-Asp-Glu-Val-Asp-amino-methylcoumarin; ACNU, nimustine hydrochloride; CDDP, cisplatin; DEVD-CHO, DEVDaldehyde; MMP, mitochondrial membrane potential; PBS, phosphate-buffered saline; PI, propidium iodide; Rh123, rhodamine 123; RTK, receptor tyrosine kinases; sc-siRNA, scrambled siRNA; siRNA, small interfering RNA; VP16, etoposide.

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Eph receptors are the largest family of transmembrane proteins with an extracellular domain that is capable of recognizing signals from the cell microenvironment and of influencing cell-cell interaction and migration. EphA2 is a member of the family of RTKs and is a widely expressed transmembrane RTK in epithelial tissues [20] and has been implicated in neuronal development [17], as well as regulating cell migration and adhesion [18]. As to brain gliomas, overexpression of EphA2 was detected in low-grade astrocytic tumors and advanced tumors, such as glioblastoma multiforme and anaplastic astrocytomas [4,11,23]. However, to our knowledge, the direct effects of EphA2 on glioma cells have not been investigated. The overexpression of receptor EphA2 has been implicated in tumor growth, angiogenesis, and metastasis [5,6,24]. Therefore, EphA2 appears to be a potential therapeutic target for the containment of tumor growth.

In the current study, we demonstrate that the inhibition of EphA2 expression by using siRNA inhibits tumor cell proliferation and induces their apoptosis, and that EphA2-siRNA transfection enhances U251n cells' sensitivity to chemotherapy.

2. Materials and methods

2.1. Materials

All standard culture reagents were obtained from Gibco BRL, Inc. (NY). Propidium iodide, Rh123, mouse antihuman EphA2, horseradish peroxidase–conjugated secondary antibody, doxorubicin, cisplatin, topotecan, and paclitaxel were purchased from Sigma Chemical Co. (St. Louis). Acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin and DEVD-CHO were from Peptide Institute (Osaka, Japan).

2.2. Cell culture

One established human glioblastoma cell line was used in this study: U251n (Central Chinese Type Culture Collection, Wuhan, China). Cells (approximately $1.0\text{-}1.5\times10^6$) were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum and 0.238% *N*-2-hydroxyethlpiperazine-*N*-2-ethanesulfonic acids, 100 U/mL penicillin G, and 0.1 mg/mL streptomycin. Cells were incubated at 37°C in 5% CO_2.

2.3. Transient transfection

The siRNA targeting the receptor EphA2 was obtained from Santa Cruz Biotechnology (Santa Cruz, Calif) in 2 formulations. A nonsilencing siRNA sequence, shown by BLAST search to not share sequence homology with any known human mRNA (target sequence 5′-AATTCTCC-GAACGTGTCACGT-3′), was used as control for EphA2-targeting experiments. siRNA with the target sequence 5′-AATGACATGCCGATCTACATG-3′, designed and shown [3] to target mRNA of the RTK EphA2, was used to downregulate EphA2 in vitro. U251n cells were plated into 6-well plates or 24-well plates as required for the experiments. The cells were allowed to adhere for 24 hours. The transfection of siRNA was performed using lipofectamine-2000 (Invitrogen) according to the manufacturer's recommendation. After 4 hours of transfection, the culture medium containing 10% serum was added. The assays were carried out 48 hours posttransfection.

2.3.1. Western blot analysis

U251n cells were cultured on 6-well tissue culture plates to confluence. The cells were lysed in lysis buffer, as reported previously [15]. Total protein was estimated by using the bichloroacetic acid method (Pierce, Rockford, Ill). Equal amounts of protein (20 mg per lane) were loaded. Proteins in the samples were separated onto denaturing sodium dodecyl sulfate–7.5% polyacrylamide gels (Bio-Rad) and were transferred electrophoretically onto polyvinylidene difluoride membranes (Immobilon-P; Millipore). The blots were blocked overnight at 4°C with BSA and were incubated with mouse antihuman EphA2 at 1:500 dilution for 1 hour at room temperature (Zymed Laboratories). After washing, they were incubated with the second antibody (horseradish peroxidase–conjugated antimouse immunoglobulin G antibody) at a dilution of 1:1000 for 1 hour. EphA2 receptors were detected by using enhanced chemiluminescence (Amersham Pharmacia Biotech). Prestained protein markers were included for molecular mass determination (Bio-Rad). Each experiment was performed 3 times.

2.4. Hoechst staining

At 48 hours after transfection, Hoechst 33342 (Sigma) was added to the culture medium of living cells; changes in nuclear morphology were detected by fluorescence microscopy using a filter for Hoechst 33342 (365 nm). For quantification of Hoechst 33342 stainings, the percentages of Hoechst-positive nuclei per optical field were counted. To investigate the involvement of caspase-3, caspase-3 inhibitor (Z-DEVD-CHO) was added 24 hours after siRNA transfection at a final concentration of 50 μmol/L. Each experiment was performed 3 times.

2.5. MTT Assay for cell proliferation

Cell proliferation was evaluated by MTT reduction. Briefly, U251n cells were transfected with EphA2 siRNA after 48 hours, MTT solution in PBS was added to attain a final concentration of 0.5 mg/mL, and incubation was continued for 4 hours. Finally, an equal volume of a lysis buffer containing 50% dimethyformamide and 20% sodium dodecyl sulfate (pH 4.8) was added. The mixtures were kept overnight and then the amount of MTT formazan present was quantified by determining its absorbance at 570 nm using an ELISA plate reader (Hua Dong Electronic Co, Nanjing, China). Each experiment was performed 3 times.

2.6. Caspase-3 activity analysis

Caspase-3 activity was measured by its ability to cleave Ac-DEVD-AMC. Cleavage was monitored by measuring the