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

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Received 23 March 2008; accepted 16 April 2008

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.

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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.
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 and regulated 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 antibodies, 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 1.0–1.5×10^6) were maintained in Dulbecco’s Modified Eagle’s Medium supplemented with 10% fetal bovine serum and 0.238% sodium bicarbonate. The cells were washed, trypsinized, and resuspended in serum-free medium containing 10% serum was added. The assays were carried out 48 hours posttransfection.

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′-AAATTCTCC-GAACGTGTCACGT-3′), was used as control for EphA2-targeting experiments. siRNA with the target sequence 5′-AATGACATGCGCAGATCAGTACG-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, III). 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 secondary 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% dimethylformamide 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
fluorescence of the cleaved-AMC. U251n cells were transfected with EphA2 siRNA for 48 hours. After treatment, the cells were collected; washed; suspended in buffer containing 50 mmol/L Tris/HCl (pH 7.4), 1 mmol/L EDTA, and 10 mmol/L EGTA; and lysed by 3 successive freeze-thaw cycles in dry ice at 37°C. Cell lysates were centrifuged at 20,000 \times g for 5 minutes, and the resulting supernatants were stored at −70°C. The protein concentration of each sample was estimated using the Bio-Rad protein assay. A total of 50 μg of protein was incubated with 50 μmol/L of enzyme substrate Ac-DEVD-AMC at 37°C for 30 minutes. Levels of released 7-amino-4-methylcoumarin were measured using spectrofluorometers with excitation at 360 nm and emission at 460 nm. In some experiments using caspase-3 inhibitor (Z-DEVD-CHO), it was directly added to the medium 2 hours before the transfection of siRNA. Each experiment was performed 3 times.

2.7. Measurement of MMP

Mitochondrial membrane was monitored using the fluorescent dye Rh123, a cell permeable cationic dye, which preferentially partitions into mitochondria based on the highly negative MMP. Depolarization of MMP results in the loss of Rh123. Rh123 was added to cell cultures to attain a final concentration of 10 μM for 30 minutes at 37°C after the cells were transfected with siRNA as described above. The cells were collected and washed twice with PBS and then analyzed by flow cytometry. The laser was adjusted to emit at 480 nm, and a 530-nm long-pass filter was used. Each experiment was performed 3 times.

2.8. Detection of apoptosis by annexin-V and PI staining

U251n cells at 80% confluence were transfected with siRNA for EphA2 and sc-siRNA. After 48 hours, detached
cells in the medium were collected, and the remaining adherent cells were harvested by trypsinization. The cells (1×10^5) were washed with PBS and resuspended in 250 μL of binding buffer (annexin-V/fluorescein isothiocyanate kit; Becton Dickinson, Mountain View, CA) that contained 10 μL of 20 μg/mL PI and 5 μL of annexin-V/fluorescein isothiocyanate. The data were collected on FACSCalibur (Becton Dickinson). Fluorescein isothiocyanate and PI emissions were detected in the FL-1 and FL-2 channels, respectively. Subsequent analyses were done with CellQuest software (Becton Dickinson). Each experiment was performed 3 times.

2.9. Anticancer drugs

We chose 3 chemotherapeutic drugs that are routinely administered to patients with glioma. They are CDDP, VP16, and ACNU. Forty-eight hours posttransfection, U251n cells were incubated with these drugs for 48 hours. MTT assay was used to examine changes in cell proliferation. The statistical analysis was done using the Student t test. Each experiment was performed 3 times.

2.10. Statistical analysis

All results were expressed as mean ± SE. The q test and Student t test were used. P < .05 was considered statistically significant.

3. Results

3.1. EphA2 protein after transfection with siRNA EphA2

To assess the effect of siRNA EphA2 on EphA2 protein, U251n cells were transfected with varying concentrations of siRNA EphA2 (10, 50, and 100 nmol/L). Subsequently, Western blot analysis was performed to detect expression of EphA2 protein. A significant downregulation of EphA2 protein was observed in U251n cells in a concentration-dependent manner. siRNA EphA2 at concentrations of 50 and 100 nmol/L knocked down EphA2 protein expression by 60% and 75%, respectively, compared with controls and sc-siRNA (P < .05) (Fig. 1).

3.2. Hoechst staining

Hoechst 33342 staining of U251n cells at 48 hours after transfection with siRNA targeting EphA2 (Fig. 2B) showed the presence of nuclear fragmentation and chromatin condensation, typical hallmarks of apoptosis, which were not observed in sc-siRNA–transfected U251n cells (Fig. 2A). To confirm the involvement of caspase-3, we examined the effects of Z-DEVD-CHO, a caspase-3 inhibitor, on the internucleosomal degradation of DNA. To this end, U251n cells were treated with Z-DEVD-CHO 24 hours after transfection with siRNA targeting EphA2. Hoechst 33342 staining at 48 hours after transfection revealed that apoptosis induced by inhibition of EphA2 was significantly suppressed by Z-DEVD-CHO (Fig. 2C). With fluorescence microscopy, quantitative analysis of Hoechst 33342 stainings at 48 hours after transfection was done by counting the percentages of Hoechst-positive cells per optical field: 4.4% ± 1.9% for sc-siRNA, 23.5% ± 7.1% for EphA2 siRNA, and 6.3% ± 3.1% for EphA2 siRNA supplemented with Z-DEVD-CHO (EphA2 siRNA conditions in the absence of Z-DEVD-CHO were significantly different [P < .05] from the control [sc-siRNA]; n = 7-13).

3.3. MTT Assay

An MTT assay was performed to assess the effect of siRNA on cellular proliferation in the U251n line. U251n cells were transfected with 50 nmol/L EphA2 siRNA, sc-siRNA, or medium alone, and the proliferation of cells was evaluated. Cells transfected with siRNA EphA2 displayed a significant reduction in cell proliferation by 30% (P < .05). A slight decrease in cell proliferation was also found in cells transfected with sc-siRNA group, but these differences were not statistically significant in comparison with the control group (P > .05) (Fig. 3).

3.4. Annexin-V and PI staining detection of apoptotic cells

When U251n cells were transfected with siRNA EphA2 (50 nmol/L), a significant increase in apoptotic cells (34.48%;
P < .05) was observed with annexin-V staining compared with sc-siRNA (5.32%) (Fig. 4). These results indicated that transfection with siRNA EphA2 induced apoptosis of U251n cells.

3.5. Mitochondrial membrane potential

Mitochondria play a critical role in apoptosis caused by drugs such as chemotherapeutics and DNA-damaging agents. However, it is not clear whether EphA2 mediates mitochondrial dysfunction in cells. We determined mitochondrial membrane potential using a fluorescent dye Rh123. Rh123 is a cationic, lipophilic dye that selectively stains mitochondria according to their negative transmembrane potential. Rh123 localizes to mitochondria as a consequence of mitochondrial membrane potential. The higher the mitochondrial membrane potential, the more polarized is the mitochondrial membrane, and more Rh123 is taken up into the mitochondrial matrix. Loss in Rh123 staining indicates disruption of the mitochondrial inner transmembrane potential associated with apoptosis. siRNA EphA2 produced a reduction in retention of Rh123, a result of the loss of the mitochondrial membrane potential, compared with the control group (Fig. 5).

3.6. Caspase-3 activity

Cleavage of the enzyme substrate Ac-DEVD-AMC was used as an indicator of caspase-3 activity. To test caspase-3 activation, cell lysates from U251n cells that were transfected with sc-siRNA were incubated with the substrate, and the increase in fluorescence due to enzymatic cleavage of the peptides was measured with a fluorometer. Fig. 6 shows that treatment of the cells with siRNA EphA2 strongly enhances caspase-3 activity. To confirm that the caspase activity observed is specific to caspase-3, we used a caspase-3-specific inhibitor, Z-DEVD-CHO. Treatment of cells with the inhibitor before the transfection of sc-siRNA reduced the degree of caspase-3 activation.

3.7. Modulation of the cytotoxic effect of anticancer drugs by siRNA EphA2

Our study showed that, in vitro, the siRNA EphA2 was cytotoxic for the glioma cells tested. In the clinical setting, however, it may not be able to kill all the cells present in a tumor. Therefore, we examined whether enhanced cytotoxicity could be achieved by transfecting U251n cells with siRNA and adding to other chemotherapeutic agents. IC_{50} concentrations were 1 µg/mL (CDDP and VP16) and 100 µg/mL (ACNU). Alone, the antisense oligonucleotides manifested almost the same cytotoxic effects as CDDP, VP16, and ACNU. When it was added to cultures treated with the anticancer agents, the effects were clearly additive (Fig. 7).
4. Discussion

A major characteristic of malignant gliomas is their striking neovascularization, proliferation, and invasion. These are generally associated with cerebral edema, vein thrombosis, and tumor necrosis. The mean survival time of patients with very malignant glioma is 1 year. One of the reasons for this dismal prognosis is the resistance or insensitivity of glioma cells to anticancer treatments, such as radiotherapy, immunotherapy, or chemotherapy [16]. Recently, it is generally acknowledged that chemotherapeutic agents exert their cytotoxic effects through activation of apoptosis and promotion of apoptosis. This can increase the chemosensitivity of cancer cells. For this reason, inhibition of some specific genes that inhibit apoptotic cell death can cause malignant cells to be relatively sensitive to the cytotoxic effects of chemotherapeutic agents [7].

Appearance of novel strategies of cancer treatment is based on the selective downregulation of specific targets involved in the neoplastic progression. EphA2 seems to be a relevant target for such therapeutic intervention. EphA2 was detectable in most types of cancer, and its presence was associated with a poor prognosis in many malignant tumors [8]. Previous studies have demonstrated that decreased expression of EphA2 in various cancer cells, such as human osteosarcoma cells [25], malignant mesothelioma cells [14], and ovarian cancer cell lines [10], is associated with inhibited resistance of these cells toward chemotherapy or other apoptosis stimuli. Accordingly, low expression of EphA2 can evoke an apoptotic response. For example, silencing the receptor EphA2 suppresses the growth and haptotaxis of malignant mesothelioma cells [14], and increased expression of EphA2 correlates with adverse outcome in patients with primary and recurrent glioblastoma multiforme [22].

In the present study, we have shown that EphA2 expression levels were significantly downregulated in U251n cells, after transfection with siRNA EphA2, whereas no significant changes in sc-siRNA–treated cells were observed. These results indicate that siRNA EphA2 used in the present study effectively downregulated EphA2 expression in U251n cells. Cell proliferation was significantly decreased in U251n cells after transfection with siRNA EphA2. Relatively high susceptibility to siRNA transfection was observed in U251n cells. Annexin-V and PI staining revealed that apoptotic cell death was abundant in cells transfected with siRNA EphA2 but almost completely absent in cells transfected with sc-siRNA.

The level of expression of EphA2 correlated with cell apoptosis. Nasreen et al [15] reported that silencing the receptor EphA2 suppresses the growth and haptotaxis of malignant mesothelioma cells. The study by Duxbury et al [3] demonstrated that EphA2 siRNA decreases focal adhesion kinase phosphorylation, and caspase 3 activity was increased in pancreatic adenocarcinoma cells. These results showed that there was a connection between EphA2 and caspase-3.

siRNA EphA2 also induced increases in caspase-3 activity on U251n cells. Early in the apoptotic process, the initiator caspases (eg, caspase-8, caspase-9) are activated [2]. They activate other caspases (eg, caspase-3, caspase-7) downstream, which are primarily responsible for the morphological characteristics of apoptosis. Caspase-3 is identified as a key mediator of apoptosis in mammalian cells, which cleaves major structural elements of the cytoplasm and nucleus, DNA components, and protein kinases, causing cells to become round in shape, detach from the plate, and undergo nuclear fragmentation [9]. Although initiation of the caspase cascade in apoptosis remains to be explained, the increase of caspase-3 activity by siRNA promotes apoptosis of cancer cells.

Mitochondrial dysfunction has been reported to play a role in apoptosis [12]. Our observation shows that siRNA induced loss of mitochondrial membrane potential. The observation, together with the previous studies that a loss of mitochondrial membrane potential reflects mitochondrial release of cytochrome c, suggests that transfection of siRNA induces mitochondrial dysfunction [21]. At the same time, siRNA induced an increase in caspase-3 activity and we confirmed the involvement of caspase-3. It is not known whether siRNA induces the caspase-3 directly or indirectly. Taken together, it is likely that the induced-apoptotic effects of siRNA EphA2 are upstream of mitochondrial dysfunction and caspase-3 activation.

In a clinical setting, transfection with siRNA EphA2 alone may not be effective enough to kill all the glioma cells in a tumor. Therefore, we investigated whether it could enhance the cytotoxicity of other widely used chemotherapeutic agents. We chose the anticancer drugs VP16, CDDP, and ACNU because their antitumor effects have been well documented [1]. The usefulness of these drugs in combating glioma cells has also been established by in vitro studies [13]. We found that, in the presence of these drugs, transfection with siRNA EphA2 had additive cytotoxic effects in vitro. Consistent with our results, the use of combined approaches in cancer therapy seems to be more efficient than a single type of treatment [19]. Based on these findings, our study supports the idea that combined cancer treatment approaches such as inhibition of EphA2 would substantially augment the sensitivity of glioma cells to chemotherapy. These data suggest that its addition to these widely used antitumor agents may lead to improved treatment regimens for patients with EphA2-dependent tumors.

In conclusion, our observation indicates that transfection with siRNA EphA2 effectively reduced the expression level of EphA2 and caused apoptosis in U251n cells, suggesting that EphA2 expression in vitro plays an important role in apoptosis modulation. However, the mechanism is complicated and still unclear. It is likely that EphA2 via mitochondrial and caspase-3 signaling pathway inhibits U251n cell apoptosis. Our findings demonstrate that reduction of EphA2 in U251n cells by transfection with
siRNA EphA2 enhances their sensitivity to chemotherapy, which might be an effective therapeutic approach in the clinical management of gliomas.

Acknowledgments

We thank Professor Hong Wang at Henry Ford Hospital for critical reading of the manuscript. This work was supported by a grant (JX1B019) from the Hubei Provincial Health Department Foundation of China.

References


Commentary

The standard therapeutic approach for glioma includes combined therapies such as surgery, radiation therapy, and chemotherapy. The prognosis of high-grade glioma remains poor, even with combined treatments. There are also experimental approaches for glioma such as vector therapies, cell- and peptide-based immunotherapeutic approaches, and anti-angiogenic therapies [1,3]. A recent development for cancer treatment is the identification of the RTKs of the Eph family. These receptors bind to cell surface–associated ephrin ligands on neighboring cells. It is suspected that an imbalance of Eph/ephrin function may contribute to a variety of diseases, including cancer [2]. Overexpression of Eph receptors has been detected in glioma and may be involved in tumor growth, angiogenesis, and metastasis [4]. The authors used a novel gene silencing method—via RNA interference technology (siRNA) —to target EphA2 in human glioma cells and to determine how this may affect proliferation and apoptosis.