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Is neuroglial antigen 2 a potential contributor to cilengitide response in glioblastoma?

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Background: Determining the expression levels of neuroglial antigen 2 (NG2) in glioma cell lines and to evaluate the potential contribution of NG2 to cilengitide response were aimed. Materials and Methods: Endogenous expression level of NG2 was determined using quantitative reverse transcription polymerase chain reaction and immunoblotting. Cilengitide responses of the cells were monitored to determine half maximal inhibitory concentration values. Whether the suppression of NG2 expression alters the response of A172 cells to cilengitide was examined. Results: The effect of cilengitide on inducing apoptosis of the cells was determined by TUNEL staining. High mRNA and protein expression of NG2 was detected in A172 and U-87MG cells, while T98G, M059K and M059J cells demonstrated low levels of NG2. A172, U-87MG and positive control MG-63 were relatively sensitive to cilengitide compared to T98G, M059K and M059J. MG-63, A172 and U-87MG were unexpectedly found to be more susceptible to cilengitide. In addition, NG2 knock-down showed no significant difference in cell death between small interfering RNA (siRNA)-transfected and cilengitide-treated groups. The results showed that cilengitide caused detachment and subsequently initiated apoptosis. Glioma cell lines express variable levels of NG2 and differ in their responses to cilengitide. Although increased numbers of apoptotic cells were found in untransfected cells compared to siRNA-transfected cells upon exposed to cilengitide, the difference was not documented to be significant between two groups. Conclusion: It may be proposed that the combination therapy of NG2 suppression and cilengitide treatment showed no considerable effect on glioblastoma compared to cilengitide therapy alone. Response to therapy may be further improved by targeting other factors act in concert in this signaling pathway.

Introduction

Glioblastomas represent formidable challenges in terms of therapy, quality of life, and survival. Prognosis of patients diagnosed with glioblastoma confers a median survival time of just 15 months, even after aggressive therapy with multiple modalities including total tumor resection by surgery, radiotherapy, and chemotherapy, specifically temozolomide (TMZ).[sup][1] The majority of patients are unresponsive to chemotherapy regimens and resistance to treatment portends a dismal prognosis. A small portion of the patients responds to treatment eventually experience tumor recurrence. Yet, there is no standardized treatment protocol for patients with relapsed tumor. Second-line or salvage chemotherapy regimens and targeted therapies such as erlotinib,[sup][2] gefitinib,[sup][3] enzastaurin,[sup][4] imatinib [sup][5] and bevacizumab [sup][6],[7],[8] provided additional treatment options. The response of patients to these diverse classes of anticancer drugs and chemotherapy regimens is not uniform. The underlying mechanisms involved in the differential drug response of glioblastoma are not sufficiently elucidated. Therefore, we investigated the response of the glioma cells to cilengitide treatment and the potential role of neuroglial antigen 2 (NG2) expression in cilengitide response mechanism.

Cilengitide is a cyclic RGD peptide that selectively binds ai[sz]1 and ai[sz]5 integrins and blocks their signaling to downstream targets.[sup][9] Colocalization of NG2 with [sz]1 integrins and their direct interaction have been reported to contribute to increased proliferation of the cells and motility.[sup][10],[11] NG2 by either its own signal transduction capacity or cooperating with [sz]1 integrins contributes to enhanced cell motility, survival, and proliferation of the tumor cells.[sup][11]

Involvement of NG2 in multiple pathways associated with cell motility, proliferation, survival, angiogenesis, and chemoresistance indicates its diverse functional potential and can be critical for glioma progression and tumor cell resistance.[sup][12] Al-Mayhani et al . provided some evidence that NG2-positive glioma cells had a higher proliferation and tumorigenic potential than NG2-negative cells.[sup][13] Aggressive tumor growth and angiogenic activity were reported in nude rat xenografts implanted with NG2-overexpressing U251 cells transfected with NG2 in comparison to those rats implanted with the nontransfected U251 cells.[sup][14] The treatment of xenografted tumors with lentiviral vectors encoding NG2-shRNAs resulted in a reduction in tumor growth,

angiogenesis, and normalized tumor vascularity.[sup][14]

The results of a North American Brain Tumor Consortium study confirmed the improved delivery efficacy of cilengitide and its modest activity when used as a single therapeutic agent in recurrent gliomas.[sup][15] In an in vitro study, cilengitide combined with TMZ has been demonstrated to induce cell detachment, and apoptosis in O [sup]6-methylguanine DNA methyltransferase (MGMT) gene promoter methylated glioma cells.[sup][16] However, a preclinical study using integrin inhibitors S 36578 and cilengitide in an animal model with Lewis lung carcinoma and melanoma showed a controversial effect of these inhibitors at nanomolar concentrations whereby tumor growth and angiogenesis were stimulated while antiangiogenic effects of the inhibitors were observed at high dose.[sup][17] In Phase I/IIa trial, a survival benefit was reported for newly diagnosed glioblastoma patients with MGMT promoter methylation treated with cilengitide (500 mg twice a week) plus concurrent TMZ.[sup][18] However, most recently CENTRIC, Phase III clinical trial designed to test dose-intense cilengitide (2000 mg twice a week) in addition to the standard treatment in newly diagnosed patients with methylated MGMT promoter announced no improved survival with combination treatment.[sup][19]

The antitumorigenic effects of cilengitide and the factors that contribute to cilengitide resistance are not fully understood. In this study, we investigated the inhibitory effects of cilengitide in glioma cell lines and the potential association between NG2 expression levels and response characteristics of the cells. NG2 was selected due to its potential critical roles in mechanism of action of cilengitide employed in salvage therapy for glioma treatment. To the best of our knowledge, this is the first study that has investigated the combined effects of NG2 downregulation and cilengitide treatment in glioma cells. The general aim of the study is to determine the expression levels of NG2 in a panel of five glioma cell lines and to evaluate potential contribution of NG2 to drug resistance.

Methods

Maintenance of cell lines

Glioma cell lines (T98G, A172, M059K, M059J and U-87 MG) were obtained from the American Tissue Culture Collection. An osteosarcoma cell line, MG-63, was kindly provided from Hormones and Cancer Laboratory, Kolling Institute of Cancer Research, Sydney, Australia, and used as a positive control for NG2 proteoglycan expression. All cell lines were maintained according to the supplier's instructions. Recommended cell culture media were supplemented with 10% fetal bovine serum (Sigma - Aldrich, Castle Hill, Australia). Cells were incubated in a humidified atmosphere with 5% CO[sub]2 at 37[degrees]C.

Cell density optimization and chemosensitivity assays

Optimal cell density for each cell line was determined for the drug cytotoxicity assays. Dilutions of cell suspensions from 100 to 5 x 10[sup]4 cells were seeded in 96-well plates (Thermo Scientific, Waltham, MA, USA) in triplicate, and the half maximal inhibitory concentration (IC[sub]50) values were assessed. CellTiter 96[sup][R] AQueous Assay (MTS; Promega Corporation, Madison, WI, USA) was performed at 24, 48, and 72 h posttreatment.

Preparation of cilengitide

Cilengitide (EMD 121974) (Merck, Darmstadt, Germany) was supplied in physiological saline solution at 25 mM (15 mg/ml) concentration and stored at 4[degrees]C. Cilengitide concentrations ranging from 0.25 to 500 [micro]M were tested to determine IC[sub]50 of cilengitide for each cell line.

RNA extraction from cultured cells

RNA from the cells was isolated using TRI Reagent (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. RNA was quantified by NanoDrop spectrophotometer (NanoDrop [sup][R] ND-1000, Wilmington, DE, USA), and the sample was stored at −80[degrees]C.

cDNA synthesis

The reverse transcription was performed using SuperScript [sup][R] III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). RNAs (1000 ng/[micro]l) extracted from cell lines and commercial Human Normal Brain Reference RNA samples (Applied Biosystems/Ambion, Austin, TX, USA); (1 mg/ml) Human Parietal Cortex Superior Brain Total RNA (NBPCS, Cat #6790), Human Brain Reference RNA (NBTot, Cat #6051), Human Orbital Frontal Cortex Brain Total RNA (NBORF, Cat #AM6786), and Human Parietal Cortex Posterior Brain Total RNA (NBPCP, Cat #AM6788) were prepared for cDNA conversion.

Neuroglial antigen 2 gene expression using quantitative reverse transcription polymerase chain reaction

The TaqMan gene expression assay Hs00426981_m1 (CSPG4) (Applied Biosystems, Foster City, CA, USA) was used to amplify NG2 and RNA quantity was expressed relative to Eukaryotic 18S rRNA Endogenous Control (VIC/MGB Probe, Applied Biosystems, Mulgrave, VIC, Australia). Samples were prepared in triplicate, and each assay was repeated three times. All reactions were performed on a RotorGene 3000 (Corbett Research, San Francisco, CA, USA). The fold-change was calculated using ?? Ct method on Relative Expression Software Tool Software (Corbett Research, San Francisco, CA, USA).

Protein extraction from glioma cell lines

Cells were harvested in a cell lysis buffer (dithiothreitol and bromophenol blue added immediately before electrophoresis). The cell lysate was sonicated for 15–30 s and denaturated at 95[degrees]C for 10 min then centrifuged at 10,000 xg for 5 min. The

supernatant was then stored at −80[degrees]C. DC ™ Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA) was used to measure the concentration of cell extracts. The absorbances were measured at 650 nm.

Immunoblotting

The proteins (30 [micro]g) were separated on NuPAGE Nove x 3–8% Tris-Acetate Gel (Invitrogen, Carlsbad, CA, USA) and transferred onto nitrocellulose membrane (GE Healthcare, Buckinghamshire, UK) (90 V for 3 h at 4[degrees]C). NG2 protein expression was detected using an NG2 rabbit antihuman (polyclonal) (1:750, Overnight at 4[degrees]C), and a goat antirabbit (polyclonal) (1:3000, 1 h at RT) antibodies. Monoclonal anti-a-tubulin clone DM1A (Sigma-Aldrich, St. Louis, MO, USA) was used as a loading control. Blots were then developed and imaged on an LAS-4000 (Fuji Photo Film Co., Ltd., Tokyo, Japan). Densitometric analyses were performed using Multi Gauge software V3.0 (Fuji Photo Film Co. Ltd., Tokyo, Japan).

Transfection of cells using nucleofection technique

A172 cells were grown until the monolayer reached 80% confluency. The cells were collected, centrifuged at 200 xg for 10 min, and transfected with 2.5 [micro]g Silencer Select Predesigned small interfering RNA (siRNA) (CSPG4; s3651 (siRNA-51), s3652 (siRNA-52), s3653 (siRNA-53)), Reference Sequence NM_001897.4, Applied Biosystems/Ambion, Austin, TX, USA) or 2.5 [micro]g allstars™ negative control siRNA (Qiagen, Hilden, Germany) for determining unspecific effects of siRNA transfection in the experiments, according to the manufacturer's instructions (Cell line Nucleofector [sup][R] Kit T, Lonza Australia, Pty. Ltd., VIC, Australia). The cells were transferred into plates and allowed to incubate in a humidified atmosphere at 37[degrees]C for 24 h before addition of cilengitide.

Cell apoptosis analysis by TUNEL assay

A cytospin method was used to produce a monolayer of cells (1 x 10[sup]5 cells/200 [micro]l, centrifuged at 800 rpm for 3 min) on poly-l-lysine coated slides. The cells were fixed with 4% paraformaldehyde and stained with In situ cell detection kit, POD (Roche Diagnostics, QC, Canada) following manufacturer's instruction. TUNEL-positive color development was performed by incubating the slides with substrate, liquid DAB + Chromogen Kit (Dako, Glostrup, Denmark) for 5 min. All samples were stained with hematoxylin. The cells stained dark brown were regarded as TUNEL-positive and counted for analysis.

Statistical analysis

Data were expressed as mean [+ or -] standard error of the mean from at least three independent experiments. Statistical analysis (Student's t -test) of the data was performed using GraphPad Prism software (version 4, Graphpad Software, San Diego, Califonia USA). Asterisk rating system (* P < 0.05, ** P < 0.01, *** P < 0.001) was used for representing significance.

Results

A172 cells express high levels of neuroglial antigen 2

The expression of NG2 at both total protein and mRNA levels was determined in T98G, A172, M059K, M059J, U-87 MG, and MG-63 cells. While high levels of NG2 protein were detected in A172, U-87 MG, levels of NG2 in M059K, M059J, and T98G was negligible [Figure 1]a and [Figure 1]b. The same trends were not observed at the mRNA level with highly variable expression detected. Consistent with A172 protein expression, NG2 mRNA was also higher than that in normal brain. However, significantly low NG2 mRNA expression was measured in T98G cells [Figure 1]c.{Figure 1}

A172, U-87 MG, and MG-63 cells show remarkable sensitivity to cilengitide

The proliferative responses of the cell lines were screened to determine IC[sub]50 doses of cilengitide at 24 h after treatment. The viability of A172 and MG-63 cells was effectively inhibited by 50% at 7 and 1.5 [micro]M cilengitide concentrations, respectively. U-87 MG cells were found to be moderate with an IC[sub]50 of 100 [micro]M. However, T98G, M059K, and M059J were resistant to cilengitide, with IC[sub]50 values up to 500 [micro]M [Table 1].{Table 1}

RNA interference suppresses neuroglial antigen 2 expression remarkably

We next assessed whether the knockdown of NG2 interferes with the sensitivity of glioma cells to cilengitide. Knockdown of NG2 was performed using NG2 siRNA in the A172 cells which is a high expressor of NG2. Because the siRNA against NG2 was not validated, 3 potential siRNAs were tested. Among all siRNAs (siRNA-51, siRNA-52, siRNA-53) examined, siRNA-52 suppressed the NG2 expression at a high level [Figure 2]. NG2 protein expression was inhibited by 50% [Figure 2]a and [Figure 2]b and NG2 mRNA levels suppressed by 84% [Figure 2]c at 24 h following transfection. No significant effects on protein and mRNA were observed when transfecting cells with allstars and transfection media only [Figure 2]c.{Figure 2}

Inhibition of NG2 with siRNA-52 was assessed at 24, 48, and 72 h posttransfection. At the mRNA level, NG2 expression was markedly low, with 89%, 60%, and 56% silencing efficiency compared to controls at 24, 48, and 72 h posttransfection, respectively. The total protein levels of NG2 were inhibited by 15% at 24 h, 65% at 48 h, and 62% at 72 h after transfection compared with the controls.

Cilengitide causes increased cell detachment in glioma cells

The morphology of cells treated with siRNA-52, allstars, and nucleofection reagent transfected and untransfected A172 cells at 24 h posttreatment with cilengitide were examined [Figure 3]a. A172 cells treated with 3 [micro]M cilengitide induced the detachment of the cells from the monolayer within 24 h. Increasing the cilengitide dose from 3 to 7 [micro]M resulted in an enhanced detachment of the cells in a concentration-dependent manner. Untransfected and all transfected cell groups formed clumps in response to increased concentration of cilengitide (7 [micro]M).{Figure 3}

The effect of neuroglial antigen 2 suppression on the viability of small interfering RNA-52-transfected glioma cells

Gradually, increased cytotoxicity in response to increased cilengitide concentrations was determined using MTS assay. The viability of the siRNA-52-transfected cells was found to be slightly lower compared to the observed cell viability of the untransfected and other negative control groups at both 3 [micro]M (68% cell viability) and 7 [micro]M (42% cell viability) cilengitide concentrations [Figure 3]b.

Membrane permeabilization of the cells in cell clumps was also assessed with the vital stain, trypan blue. Cilengitide treatment (7 [micro]M) resulted in reduced number of live cells (66%) at 24 h posttreatment in siRNA-52-transfected cells. Despite the lack of statistical significance of difference, the mortality rate of the cells in siRNA-52-transfected cell group was 34% and 18% for transfection control groups treated with cilengitide (7 [micro]M) compared to the untransfected (not shown). The results confirmed that not all cells detached were demonstrated to be nonviable, and reduction in NG2 expression levels resulted in a slight decrease in cell viability.

Cilengitide (7, 10, 15 [micro]M)-induced apoptosis in untransfected and small interfering RNA-52-transfected A172 cells in a dose-dependent manner

DNA fragmentation induced by three different cilengitide doses, 7, 10, 15 [micro]M, was measured in untransfected and siRNA-52 transfected cells [Figure 4]. The rate of TUNEL-positive apoptotic cells was increased in a cilengitide dose-dependent manner in both untransfected and siRNA-52-transfected groups. Cilengitide (15 [micro]M)-induced apoptosis in 14% of the cells in untransfected control group, and 20% of apoptotic cells were present in the siRNA-52-transfected group at the same drug concentration. Although the rate of apoptotic cells was detected to be slightly higher in untransfected cells (7.31-fold) treated with 15 [micro]M cilengitide compared to siRNA-52-transfected cells (6.67-fold) exposed to the same dose, the difference was not statistically significant.{Figure 4}

Discussion

We have examined the relationship between the expression levels of NG2 and its potential impact on drug resistance. Therefore, the knockdown of NG2 expression in a cell line expressing high levels of NG2 was thought to alter the aggressiveness of the glioma cells, and the combination of NG2 knockdown with cilengitide treatment was intended to show a collective impact on survival of glioma cells.

High mRNA and protein expression of NG2 was detected in A172 and U-87 MG cell lines while T98G, M059K, and M059J cell lines demonstrated low levels of NG2 expression. Chekenya et al . reported high endogenous expression levels of NG2 in A172 and U-87 MG cell lines while no expression of NG2 was detected in the U251 cell line, which confirms variable NG2 expression in glioma cell lines.[sup][20] A172, U-87 MG, and MG-63 were relatively sensitive to cilengitide compared to T98G, M059K, and M059J. The results obtained using cilengitide, a small molecule inhibitor, showed comparable and contradictory response patterns with the data used traditional chemotherapeutic agents, previously. A study demonstrated an association of high NG2 expression with increased chemoresistance to etoposide and vincristine.[sup][20] The same researchers also reported higher resistance to doxorubicin, etoposide, and carboplatin in glioma spheroids high in NG2.[sup][20] Cilengitide-resistant cell lines, T98G, M059K, and M059J, were also resistant to etoposide treatment as previously reported.[sup][21] Hovinga et al. reported that U-87 MG cell line expressing NG2 endogenously characterized with a significantly higher radioresistance than U251 and U251-NG2-transfected cell lines which demonstrated no difference between their degrees of resistance to radiotherapy.[sup][22]

NG2 expression in A172 cells was suppressed using NG2 siRNA to establish the contribution of NG2 to response to cilengitide in glioma cells. Although an increase in cell death was observed in siRNA-52-transfected and cilengitide-treated (7 [micro]M, IC[sub]50 for A172) cells, the differences between siRNA-transfected and untransfected groups were not found to be statistically significant [Figure 3]b. All cell groups, transfected with NG2 siRNA-52, allstars, nucleofection reagent, and untransfected cells indicated a dose-dependent detachment of the cells by increasing cilengitide concentrations. This finding was consistent with a number of studies which have previously demonstrated the effect of cilengitide on detachment of endothelial cells [sup][16],[23] and glioma cell lines.[sup][16],[24]

Cilengitide significantly triggered apoptosis of untransfected A172 cells compared to untreated group. Untransfected and siRNA-52-transfected cells were also exposed to higher doses of cilengitide and treatment with cilengitide and all doses examined, 7, 10 and 15 [micro]M, increased the number of apoptotic cells in untransfected A172 cells at slightly higher rates when each group was compared with untreated control cells. When the results obtained from each drug concentration were compared with each other, the apoptosis rate of A172 cells indicated no significant differences between untransfected and siRNA-52-transfected groups [Figure 4]. Cilengitide-caused detachment and subsequently cilengitide-initiated apoptosis of the cells have been shown in a few previous studies.[sup][25],[26]

Conclusion

The results of this study showed that glioma cell lines express variable levels of NG2 and differ in their response to cilengitide treatment. No significant contribution of NG2 overexpression to chemoresistance was documented. The suppression in NG2

expression levels in cilengitide-treated and siRNA-transfected cells gave rise to a slightly reduced the number of nonviable cells compared to the endogenously NG2 expressing cells. Future studies would be needed to elucidate the complex functional interactions between NG2 and other possible signaling molecules in chemotherapy response.[sup][9],[10],[11],[27],[28] Because the suppression of NG2 expression did not substantially increase the response of the cell groups to cilengitide, the evaluation of the function of NG2 associated with response mechanism to cilengitide might need to be pinpointed extensively in a model including the other members of this signaling pathway as well. Because the relationship between integrins, NG2, and cilengitide response has not been evaluated in this study, a future research investigating these mechanisms would be warranted so as to understand the complex nature of the drug-protein interactions. The combination of advanced modalities with available effective therapies targeting multiple signaling pathways which involve cellular proliferation, migration, invasion, and angiogenesis of tumor and its microenvironment may further improve survival of glioma patients in future.

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Conflicts of interest

There are no conflicts of interest.

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