RNA Interference with EAG1 Enhances Interferon Gamma Injury to Glioma Cells *In Vitro*

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Abstract. *Aim: The aim of this study was to silence Ether à go-go 1 (EAG1) in glioma cells by RNAi in order to further analyze whether silencing this channel would improve injury caused by interferon gamma (IFN-γ). Materials and Methods: EAG1 silencing by the siRNAs EAG1hum_287 and EAG1hum_1727 (sequence targets 5'-GGCCTATTGTGT ACAGCAATT-3' and 5'-GGGACTTCCTGAAGCTCTATT-3', respectively) was determined by reverse transcription real-time quantitative polymerase chain reaction (RT-qPCR). Cell viability was measured by the 3-(4,5)-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide (MTT) assay. U-138MG glioma cells were injured by IFN-γ (25 ng/ml, 24 h) with or without the RNAi for EAG1 by a non-viral vector (pKV10.1-3, 0.2 μg). Results: EAG1hum_287 and EAG1hum_1727 caused 0.46- and 0.52-fold decrease in EAG1 mRNA content, respectively. RNAi for EAG1 by pKv10.1-3 strengthened the reduction in cell viability caused by IFN-γ (11.4% versus 40.4%, p<0.05). Conclusion: The present study reinforces the notion that EAG1 has a role in glioma biology, suggesting that this channel is a relevant player preserving the cell viability during IFN-γ injury.*

Growing evidence has established the ether à go-go 1 (EAG1) potassium channel as a key player for cancer cell competence (1, 2). Clinical tumors of different origins present high EAG1 content (3). The cancer cell type studied here, glioblastoma multiforme, and the respective malignant brain tumor also express EAG1 (4, 5). Indeed, both EAG1 blockage with antibodies or gene silencing by RNA interference (RNAi) can inhibit cancer cell proliferation (6, 7). Although EAG1 has been assigned a definitive place in tumor biology, its precise role in carcinogenesis remains elusive.

Interferons are also relevant molecules mediating tumor-immune system interactions (8, 9). Our study focused on interferon gamma (IFN-γ), a type-II interferon available for brain tumor immunotherapy (10, 11). IFN-γ exerts its actions by triggering a specific signal transduction cascade named the Janus kinases (JAK) - Signal transducer and activator of transcription (STAT) pathway, which ultimately regulates the transcription of several genes (9). Very briefly, IFN-γ binds to high affinity IFN-γ receptors which then activate JAKs. A residue of the IFN-γ receptor-1 subunit is then phosphorylated to form STAT1. STAT1 binds to IFN-γ-activated sites (GAS) sequences to induce gene expression (12). Thus IFN-γ regulates key genes involved in cancer signaling, which will result in cell growth arrest and apoptosis (13, 14). As an example, IFN-γ can trigger p53 protein transcription, causing cellular senescence (15). Apoptosis induced by IFN-γ is related to the co-activation of p53 by STAT1 (16). The *p53* gene also contains GAS sequences, thus it is regulated by the JAK-STAT whis is pathway controlled by IFN-γ (14). Finally, mice that lack sensitivity to IFN-γ and are deficient in the *p53* tumor-suppressing gene, form tumors more rapidly and develop a broader range of tumor types (17).

The cancer cell transcriptome is also regulated by microRNAs, which has brought a new layer of complexity to oncology. IFN-γ can drive the expression of various microRNAs in tumor cells, including miR-34a (18, 19). A recent study revealed that the p53 protein negatively regulates the expression of EAG1 through a pathway involving the miR-34a (20). This finding might explain, at least in part, why the inactivation of p53 could lead to EAG1 overexpression (21). These findings suggest that IFN-γ enhances p53 expression, which in turn may silence EAG1.

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Key Words: EAG1, glioma, glioblastoma multiforme, RNAi, IFN-γ, gene therapy.

As EAG1 contributes to oncogenesis, this potentially suppressive effect of IFN-γ on EAG1 would reinforce the antitumoral activity of this cytokine. Lins *et al*.'s work also pointed to Eag1 as the final effector of the p53-miR-34-E2F transcription factor-1 (E2F1) pathway described for the neuroblastoma SH-SY5Y cell lineage (20). This evidence supports our hypothesis that EAG1 would have a positive role in cancer cell defense against IFN-γ. An RNAi for EAG1 would then impact the viability of glioma cells injured by IFN-γ.

In the present study, we first examined if EAG1 expression in glioma cells could be effectively silenced by RNAi. We then evaluated whether cells silenced for EAG1 would present a different degree of cell viability after IFN-γ treatment.

Materials and Methods

siRNAs and shRNA expression vectors. RNAi targets for *EAG1* mRNA were identified by using the Biopredsi algorithm (22). We selected two target sequences in the coding region of the human EAG1 complementary DNA sequence (NM_172362.2). Target sequences and the synthetic small-interfering RNAs (siRNAs) used in this study were 5'-GGCCTATTGTGTACAGCAATT-3' (exon 2, nt 287–307, siRNA EAG1hum_287) and 5'-GGGACTTCCTGAAG CTCTATT-3' (exon 8, nt 1727–1747, siRNA EAG1hum_1727). Previously described Kv10.1-3 was used as positive control siRNA, targeting the EAG1 sequence 5'-GTCCACTTGGTCCATGTCCAG-3' (exon 8, nt 1793–1813, siRNA Kv10.1-3) (6). We also used the commercial scramble All Stars® as a negative control siRNA (Qiagen, Valencia, CA, USA).This study prepared a plasmid vector to express short-hairpin RNAs (shRNAs) to the same Kv10.1-3 target sequence 5'-GTCCACTTGGTCCATGTCCAG-3'. For this, top and bottom sequences were cloned into pSilencer 3.1-H1 neo vector containing the human H1 RNA polymerase III promoter (Applied Biosystems, Carlsbad, CA, USA). The top and bottom sequences were 5'-ATCCGCCACTTGGTCCATG TCCAGTTCAAGAGACTGGACATGGACCAAGTGGACTTTTT TGGAAA-3', and 5'-AGCTT TTCCAAAAAAGTCCACTT GGTCCATGTCCAGTCTCTTGAACTGGACATGGACCAAGTGG CG-3', respectively. After annealing and cloning the sequences into pSilencer 3.1-H1 neo vector following the manufacturer's instructions, we named this vector pKv10.1-3. We also prepared a vector that express a scrambled sequence (pScramble) as a negative control.

Cell culture and transfection. Human malignant glioma U-138MG cell line was purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 (Gibco BRL, Grand Island, NY, USA), containing 10% fetal bovine serum and 1% Glutamax (Gibco BRL). The cultures were supplemented with 100 U/ml penicillin G, 100 μg/ml streptomycin, 250 ng/ml amphotericin B (Sigma Aldrich, Madrid, Spain). For gene expression analysis, the cells were seeded into flasks at 5.0×10^5. To measure cell viability by the 3-(4,5)-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide (MTT) assay, cells were seeded in 96-well microtiter plates (1.0×10^4/well). Incubations were performed at 37˚C, with 95% humidity and an atmosphere with 5% CO_2.

Transfections were carried out using Lipofectamine (Lipofectamine 2000 Transfection Reagent; Invitrogen, Carlsbad, CA, USA) and Opti-Men I (Invitrogen), according to the manufacturer's instructions. Details of time-course experiments and genetic structure concentrations are reported in the figure legends.

Reverse transcription real-time quantitative polymerase chain reaction (RT-qPCR). In order to test the knocking-down activity of each siRNA, total RNA was isolated from cell culture with a commercial kit (RNeasy® Plus Mini Kit; Qiagen, Hilden, Germany), and quantified by fluorometry (Qubit®; Invitrogen). RNA samples (1.5 μg) were reverse-transcribed by using random primers and commercial kits (SuperScript First-Strand Synthesis System for RT-PCR; Invitrogen). The *EAG1* RT-qPCR amplifications were carried out with the following forward and reverse primers: 5'-TGTGTCCTGTTGCCATATGATGT-3' and 5'-CGGAGCAGCCGG ACAA-3' (6). Poly(A) polymerase alpha (*PAPOLA*) was amplified as a housekeeping gene, with the following forward and reverse primers: 5'-GCTACGAAGACCAGTCCATTG-3' and 5'-TGTT GGTCACAG ATGCTGCT-3' (23).

We performed a SYBR green qPCR (7500 *Fast Real-Time* PCR; Applied Biosystems) to quantify the *EAG1* mRNA content. The qPCR mix contained the following reagents: 2.0 μl of cDNA (1:2), 0.8 μl of each forward and reverse primer (10 pmol/μl), 5.0 μl of Fast SYBR Green Master mix (Applied Biosystems) and milli-Q pure water up to a total volume of 10.0 μl. Quantitative PCR assays were conducted in triplicate according to the following protocol: DNA denaturation at 95˚C for 5 min, followed by 40 amplification cycles at 95˚C for 1 min, and 60˚C for 1 min. We used the $2^{-\Delta\Delta Ct}$ method to express the siRNA silencing effects on *EAG1* mRNA content (24).

Cell viability measurement by the MTT assay. The effects of the *EAG1*-silencing vector on the viability of glioma cells injured by IFN-γ were determined by the MTT colorimetric assay (Invitrogen). The samples were organized into the following experimental groups: I: IFN-γ (25 ng/ml); II: pKv10.1-3 (0.2 μg); III: pKv10.1-3 (0.2 μg) in association with IFN-γ (25 ng/ml); IV: pscramble (0.2 μg); V: pScramble (0.2 μg) in association with pKv10.1-3 (0.2 μg). All cell groups were incubated for 24 h at 37˚C (95% humidity and 5% CO_2 atmosphere). Cells were subsequently exposed to 15 μl/well of the MTT labeling reagent (0.5 mg/ml) and incubated for an additional 3-h period. Dimethylsulfoxide (150 μl/well) was added to dissolve the insoluble formazan product then MTT reduction was measured at 595 nm. Cell viability of treated cells was determined with reference to that of untreated cells, arbitrarily assayed as 100% viability. We carried out seven independent MTT assays for each experimental group, and each assay was performed in triplicate. Absorbance values were averaged.

Statistical analysis. The data were analyzed by using the Statistical Package for Social Sciences (SPSS) version 17 (SPSS Inc, Chicago, IL, USA). To test for intergroup differences, we applied one-way analysis of variance (ANOVA) followed by Tukey's post test. Results are expressed as means±standard error of the means. Differences were considered statistically significant at $p<0.05$.

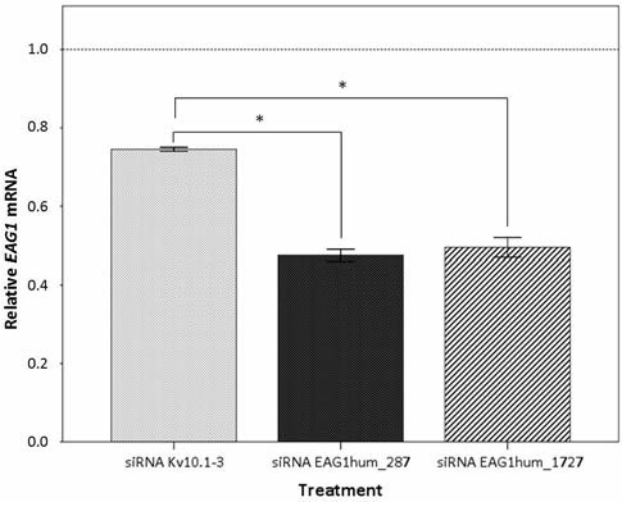

Figure 1. *Knocking-down of ether à go-go 1 (EAG1) gene expression in glioma cells in culture caused by synthetic siRNAs. Bars represent fold difference in EAG1 mRNA content at 8 h post-transfection with each siRNA (18.75 nM, n=3), as determined by RT-qPCR, relative to untreated cells. Data were normalized to the housekeeping gene poly(A) polymerase-alpha (PAPOLA) and compared to the scrambled group (hatched line), by using the $2^{-\Delta\Delta Ct}$ method. Each experimental assay was performed in triplicate. *p<0.05, one-way ANOVA followed by Tukey's test.*

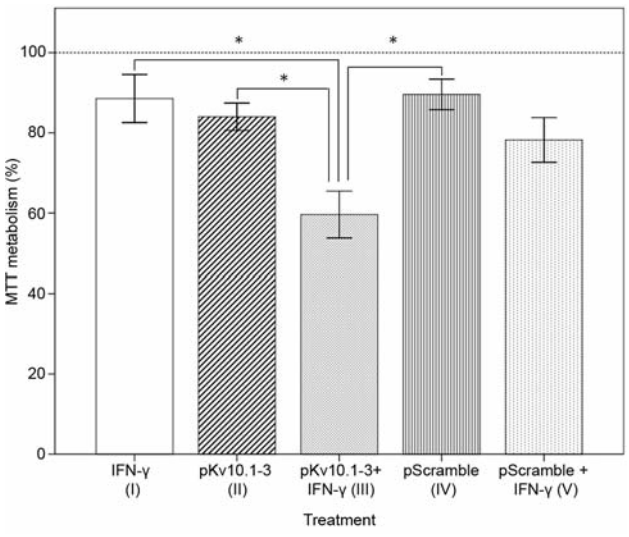

Figure 2. *Viability of glioma cells after treatment with the vectors pKv10.1-3 or pScramble (0.2 μg) alone and in association with interferon gamma (IFN-γ) (25 ng/ml). Bars represent the absorbance values found in the treated groups, normalized to that of the mock control group (hatched line), as determined by the 3-(4,5)-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide (MTT) assay at 24 h. Results are expressed as the mean cell viability of seven independent assays. The samples were organized in the following groups: I: IFN-γ (25 ng/mL); II: pKv10.1-3 (0.2 μg); III: pKv10.1-3 (0.2 μg) in association with IFN-γ (25 ng/ml); IV: pScramble (0.2 μg); V: pScramble (0.2 μg) in association with pKv10.1-3 (0.2 μg). *p<0.05, one-way ANOVA followed by Tukey's test.*

Results

The present work revealed sequences for RNAi in the *EAG1* mRNA that silenced the gene in human glioma cells. Huesken and Hall's artificial neural network algorithm found effective targets placed in distinct exons and spaced by 1440 nucleotides (22). They were present in mRNA sequences coded by exon 2 (siRNA EAG1hum_287, nt 287-307) and exon 8 (siRNA EAG1hum_1727, nt 1727-1747). Our study also evaluated a previously described positive control siRNA, named Kv10.1-3, already tested in other cancer cell lineages (6).

We first examined whether the designed siRNAs would reduce the *EAG*1 mRNA content in glioma cells. Both the siRNA EAG1hum_287 and siRNA EAG1hum_1727 silenced the expression of *EAG1* mRNA at 8 h post-transfection, as determined by RT-qPCR (Figure 1). The highest knocking-down of *EAG*1 was found for siRNA EAG1hum_287 (0.46-fold), followed by siRNA EAG1hum_1727 (0.52-fold). The siRNAs had a significantly higher silencing effect on EAG1 compared to the previously described siRNA Kv10.1-3 (0.75-fold) (Figure 1).

The most relevant aim of this study refers to the effects of *EAG1* gene silencing on the viability of glioma cells injured by IFN-γ. We first built the vector pKv10.1-3 that expresses hairpins for the same *EAG1* target reached by the siRNA Kv10.1-3. pKv10.1-3 had the ability to reduce the viability of glioma cells, as determined by the MTT assay. Glioma cells transfected with the vector (0.2 μg, 24 h, group II) were 16% less viable than the controls (Figure 2). In addition, IFN-γ (25 ng/ml, 24 h, group I) reduced the viability of glioma cells by 11%. We then focused on the critical question of this study - to examine whether RNAi for EAG1 would affect cell injury caused by IFN-γ. For this, cells received simultaneous treatment with IFN-γ (25 ng/ml) and the *EAG1* vector (pKv10.1-3, 0.2 μg) for 24 h (group III). pKv10.1-3 enhanced the injury caused by IFN-γ, reducing cell viability by up to 40% ($p<0.05$).

Treatment with pScramble caused no significant effects on IFN-γ injury (group IV), which reinforces the idea that the effects of pKv10.1-3 were *EAG1* sequence-specific.

Discussion

Glioblastoma multiforme presents an aberrant pattern of cell signaling, resulting from an altered transcriptome (25-27). Despite convincing evidence for the involvement of potassium channels in cancer biology, their precise roles in carcinogenesis, the cell cycle and apoptosis remains yet elusive (2, 28). In the present work, we showed that EAG1 is

a viable target for RNA interference in glioblastoma multiforme cell lines. We also present an early insight into the role of *EAG1* in responses of glioblastoma cells to the apoptotic agent IFN-γ.

Silencing effects of siRNAs on *EAG1* message have been tested in various cell lines, except for glioblastoma by Weber *et al*. (6). Their work examined the effect of RNAi on *EAG1* in breast carcinoma (MDA-MB435S) and cerebellar medulloblastoma (Daoy) cell lines. The Daoy cell line is derived from an original CNS tumor with cells presenting neuronal and glial phenotypes (29). The U-138MG cell line used in our study was also derived from a CNS tumor, human malignant glioma. For both cell lineages, the *EAG1* content reduced after treatment with *EAG1*-targeted siRNAs. At 8 h post-transfection, siRNA Kv10.1-3 reduced *EAG1* mRNA content by more than 0.5 fold in Daoy cells (6). The same siRNA Kv10.1-3 produced a lower reduction in EAG1 message in the cell line tested in this study (0.75-fold). Variations in siRNA knock-down according to cell type frequently occurs, as reported for Daoy and MDA-MB435S cells (6). Gene knock-down may also vary due to each RNAi target sequence. Our study tested two new *EAG1* targets, both selected by the algorithm Biopredsi. It found RNAi sequences coded by exon 2 (EAG1hum_287) and exon 8 (EAG1hum_1727). The previously described siRNA Kv10.1-3 also recognizes a sequence coded by exon 8; however, they are 66 nucleotides apart. In our study, both EAG1hum_287 and EAG1hum_1727 siRNAs were superior to Kv10.1-3 in silencing *EAG1* in glioma cells (0.46-fold and 0.52-fold, respectively, *versus* 0.75-fold) (Figure 1). Previous work on EAG1 gene silencing has also found a striking variation in message knock-down among each tested siRNA. Higher effects were found for Kv10.1-3 (approximately 0.25-fold), followed by Kv10.1-2, Kv10.1-1, and Kv10.1-4 (approximately 0.75-fold). This same order of silencing among each siRNA occurred independently in the cell lineages evaluated, in both Daoy or MDA-MB435S (6).

Tumor growth and metastasis depend on abnormalities in cell proliferation (30). EAG1 potassium channels enable cancer cells to proliferate, contributing to carcinogenesis (1-3, 5). Blocking of EAG1 by monoclonal antibodies inhibited the growth of breast and pancreatic cancer cells (7). Weber *et al*.'s study also showed that *EAG1* knock-down by RNAi may reduce the viability of some cancer cell lines, to different degrees according to each cell type. As an example, the viability of Daoy cells decreased by about 30% after Kv10.1-3 treatment (6). In our study, the expression vector pKv10.1-3 reduced the glioma cell viability by 16% (Figure 2, group II). Both studies corroborated previous findings pointing to the importance of EAG1 for tumor cell growth.

The present study also disclosed a role for EAG1 in injurious effects of IFN-γ on glioblastoma cells. This cytokine reduces tumor cell proliferation, causing programmed cell death or apoptosis (11, 12). At the dosage of 25 ng/ml (250 U/ml) used in our study, IFN-γ alone caused a 11% reduction in U-138MG glioma cell viability at 24 h. Previous work tested a protocol based on RNAi and IFN-γ to control the proliferation of glioblastoma cell lines SNB19 and LN18 (31). A lower dose of IFN-γ alone (10 ng/ml) caused a higher drop in cell viability by up to 50%. In comparison to our study, the authors used a longer period of IFN-γ incubation (48 h). In another study, IFN-γ induced apoptosis of the glioblastoma A172 and LN18 cell lines (32). The IFN-γ dosage (200 U/mL) was close to the dosage used in our study (250 U/mL), but the cells were exposed to the cytokine for 48 h. Our study used a shorter period of IFN-γ exposure (24 h). According to Weber *et al*.'s work, 24 h was enough to cause a significant decrease in both EAG1 mRNA and protein. Regarding siRNA Kv10.1-3 effects on EAG1 function, at 24 h post-transfection the K(+) current was totally abolished (6). Thus the 24 h time point would better reveal whether the reduction in EAG1 message would change the intensity of the IFN-γ injuring actions on glioma cells.

Previous work revealed that IFN-γ affects the activity and expression of potassium channels. The potassium channel gene (*KCNF1*) presents the promoter sequence regulated by IFN-γ through the JAK-STAT pathway, the GAS element (UniGene Hs.23735) (14). IFN-γ also suppresses the activity of K(+) channels of proximal tubule cells (33). To our knowledge, no previous work has addressed the relationship between IFN-γ and the EAG1 voltage-gated potassium channel in glioma cells. Our study shows that EAG1 gene silencing enhances the reduction in glioma cell viability caused by IFN-γ. While the isolated use of IFN-γ or pKv10.1-3 reduced cell viability by about 15%, their use in association almost tripled this effect (Figure 2, group III). These results clearly reveal that EAG1 has a role in the effects of IFN-γ on glioma cell viability.

Interferons strongly affect the biology of tumors by changing patterns of gene expression. Some affected genes play a role in the tightly-adjusted balance between cell death and cell growth. Caspase-1, tumor necrosis factor receptor superfamily, member 6 (Fas, APO-1/CD95), STAT1, STAT3, STAT5, tumour necrosis factor-alpha (TNF-α), interferon regulatory factor-1 (IRF1), BCL2-antagonist/killer 1 (BAK1), breast cancer 1, early onset (BRCA1), tumor necrosis factor (ligand) superfamily, member 10 (TRAIL, TNFSF10), B-cell CLL/lymphoma-2 (Bcl2), BCL2-like-1 (Bcl-x_L, BCL2L1), and p53 are all relevant examples (12, 34). Previous work revealed that IFN-γ and tumor-suppressing p53 protein have a prominent role in controlling tumor development (17). IFN-γ triggers *p53* transcription, and the p53 protein negatively regulates EAG1, considered to be final effector of a signaling cascade that recruits miR-34 and E2F1 (15, 16, 20). The p53 protein would then reduce

EAG1 expression, thus preventing cells from expressing their tumor phenotype. Our data are in agreement with such a proposed final role for EAG1 in tumor biology. Firstly, the pKv10.1-3 vector reduced the viability of glioma cells, confirming the need for EAG1 in glioma cell growth. Secondly, glioma cells silenced for EAG1 exhibited a higher drop in cell viability when exposed to the apoptotic agent IFN-γ (Figure 2, group III).

The present work reinforces the existence of a role for EAG1 in the molecular biology of glioma cells, highlighting its importance in cancer cell defense against the cytokine IFN-γ. Further study would focus on the messages connecting such functionally distinct and important cell membrane signaling proteins – the IFN-γ receptor and the EAG1 potassium channel.

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Received December 19, 2012
Revised February 4, 2013
Accepted February 4, 2013