THE EFFECT OF ANTISENSE EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR) RNA ON THE PROLIFERATION OF HUMAN GLIOMA CELLS AND INDUCTION OF CELL APOPTOSIS

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ABSTRACT

Objective: To study the effect of antisense EGFR RNA on the growth of human glioma cells *in vitro* and evaluate the feasibility of targeting EGFR gene for gene therapy of gliomas. Methods: Southern and Northern blot analysis, *in situ* hybridization and immunohistochemical staining were used to detect the integration and expression of antisense EGFR constructs. MTT assay and the average number of AgNOR for evaluation of cell proliferation, and the TUNEL method and ultrastructural change for observation of cell apoptosis. Results: Exogenous antisense EGFR cDNA was integrated into the genome of glioma cells and highly expressed, which resulted in a dramatic decrease of endogenous EGFR mRNA and GEPR protein levels. Clones with high expression of the antisense construct showed a lower proliferation activity and the induction of apoptosis *in vitro*. Conclusion: This study suggests that EGFR plays an important role in the genesis of gliomas; it may be used as a target for antisense gene therapy of gliomas.

Key words: Glioma cells, Antisense EGFR RNA, Proliferation, Apoptosis.

Malignant glioma is one of the most devastating diseases. The prognosis of patients has not been improved even when the currently available combined therapies are used. As the knowledge of tumor biology and molecular genetics has greatly increased over the past decades, it has been shown that tumorigenesis basically results from the activation of protooncogenes and inactivation of tumor suppressor genes. Since genes encoding growth factors and their receptors are closely related to oncogenes, much attention has been focused on the role of growth factors and their receptors in the process of tumorigenesis in gliomas.

A number of studies have demonstrated that the gene encoding the epidermal growth factor receptor (EGFR), the highly mitogenic normal counterpart of the viral erbB oncogene, is amplified and overexpressed in malignant gliomas.[1–3] Our previous studies on EGFR gene expression in gliomas also showed overexpression of EGFR in about 70% of malignant tumors.[4] These results imply that the EGFR may play an important role in the development and progression of malignant gliomas.

In the present study, the effects of antisense EGFR RNA on the growth of human glioma cells were investigated. Since we have found that the human glioblastoma cell line TJ905 established by our Laboratory[5] highly expresses both EGFR mRNA and EGFR protein, as shown by Northern blot analysis and immunohistochemical staining, we tried to use an antisense approach to block EGFR expression in TJ905 cells and investigated the inhibitory effect of EGFR antisense RNA on the proliferation of glioma cells as well as the effect of induction of apoptosis *in vitro*. A successful outcome would provide an experimental basis for selecting the EGFR gene as a target for gene therapy of malignant gliomas.

MATERIALS AND METHODS

Antisense EGFR RNA Expression Plasmids

The plamids were kindly provided by Dr. Laura Beguinot, Italy.[6] These included pactQsR1, an antisense construct complementary to the full length EGFR mRNA (AS-FL, 4200 bp); pactQsR3, an antisense construct complementary to the 3'-coding region of EGFR mRNA (AS-3', 552 bp); pactQs, the empty vector as control, and pSV-neo, inserted with a neomycin resistance gene, as selective marker. The plasmid DNA was prepared in large scale by alkaline lysis and further purified by

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CsCl/ethidium bromide equilibrium centrifugation according to described procedures.[7]

Cell culture and Transfection

TJ905 glioma cell (G cells) were maintained in DMEM supplemented with 15% fetal calf serum (FCS) and subcultured every other day. G cells (3×10^5) were plated in 35 mm petri dishes and grown overnight at 37°C with 5% CO_2 until about 80% confluency. Transfection of the antisense EGFR constructs into G cells was mediated with Lipofectamine (Life Technologies, USA). The plasmid DNA of pactQsR1, pactQsR3 and pactQs were cotransfected with pSV-neo in a 5:1 ratio. 100 μl of serum free DMEM (DMEM-SF) containing 2 μg of plasmid DNA was mixed gently with 100 μl of DMEM-SF containing 10 μl of Lipofectamine and incubated at room temperature for 40 min. The mixed complex was added to G cells in 35 mm petri dishes. An additional 0.8 ml of DMEM-SF was added to the dishes. The cells were incubated in 5% CO_2 at 37°C for 8 h. 1 ml of DMEM containing 30% FCS was added and the incubation continued for an additional 72 h. Then the culture medium was replaced with fresh medium containing G418 (1 mg/ml). After 14 days, clones resistant to G418 were selected and expanded. These clones were named GPR1 (with AS-FL), GPR3 (with AS-3') and GP (with empty vectro) respectively.

Southern and Northern Blot Analysis

For Southern blot analysis, the genomic DNAs of GPR1, GPR3 and GP cells digested with the restriction enzymes Xba I and Xho I and were analyzed by electrophoresis through 0.7% agarose gels. The digested products were transferred to a nitrocellulose membrane and hybridized to a ^{32}P-labeled antisense EGFR cDNA probe as described previously.[7]

For Northern blot analysis, the total RNA of GPR1, GPR3 and GP cells was extracted by the guanidine isothiocyanate procedure[8] (Chomzynski and Sacchi, 1987), resolved on 0.9% formaldehyde agarose gels, transferred to nitrocellulose membranes and hybridized with ^{32}P-labeled EGFR cDNA. The blots were stripped and rehybridized with a ^{32}P-labeled β-actin cDNA probe as an internal control. The images were quantitated by densitometry.

The Evaluation of EGFR Expression

The expression of the EGFR gene in transfected G cells was detected by *in situ* hybridization and immunohistochemical staining, for *in situ* hybridization, the 40 mer oligonucleotide probe complementary to EGFR cDNA 186–225 bp was used (5'-CGCCA-GGAGCGCTGCCCCGGCCGTCCCGGAGGGTCGCA TC-3'). The oligonucleotide 3'-end labeling kit and DIG nucleic acid detection kit (Boehringer Mannheim, Germany) are used for labeling the oligonucleotide probe and hybridization. The number of cells stained blue and the intensity of staining were evaluated under the light microscope.

EGFR immunostaining was performed with the ABC-peroxidase method. The primary EGFR polyclonal antibody was used in 1:50 dilution (Santa Cruz Biotechnology, USA). The cells not incubated with primary antibody served as negative control. Human placenta which has been reported to be rich in EGFR was similarly stained as a positive control.

Growth Rate Determination

The MTT assay was used for determination of growth rate of transfected and control G cells, according to the procedures described by Mosmann.[9] It was expressed as percentage of control.

The proliferation activity of transfected and control G cells was evaluated by estimating the average number of AgNOR (argyrophilic protein of nuclear organizer region)/cells. The AgNOR staining of the cells was carried out as previously reported.[10] The number of AgNORs in 100–200 cell nuclei were counted under a light microscope at a magnification of × 1000 (oil immersion), and the mean number of AgNORs was determined for each nucleus.

The Detection of Cell Apoptosis

Apoptosis was detected by the TUNEL method (Terminal deoxynucleotide transferase (TdT) mediated dUTP Nick End Labeling) with an *in situ* cell death kit (Boehringer Mannheim, Germany). The free 3'-OH termini of DNA strand breaks were labeled with fluorescein-dUTP using TdT. Then anti-fluorescein antibody conjugated with alkaline phosphatase (AP) was added. After reaction with the substrate AP, the apoptotic cells were stained blue and identified using a light microscope. The procedures for detection were according to the supplier's instruction.

RESULTS

The Integration and Expression of Antisense EGFR RNA in Transfected G Cells

As shown by Southern blot analysis (Figure 1), the control G cells and GP cells had an endogenous EGFR band at 9 kb, but the transfected GPR1 and GPR3 cells had an exogenous extra band of 4.2 kb and 0.5 kb respectively.

Northern blot analysis indicated that the antisense EGFR transcript was strongly expressed at 4.2 kb and 0.5 kb (Figure 2). The expression of the 0.5 kb antisense EGFR RNA resulted in block of the endogenous EGFR expression at 5.8 kb. These results demonstrated that the

antisense EGFR constructs were integrated into genomic DNA of transfected GPR1 and GPR3 cells and highly expressed.

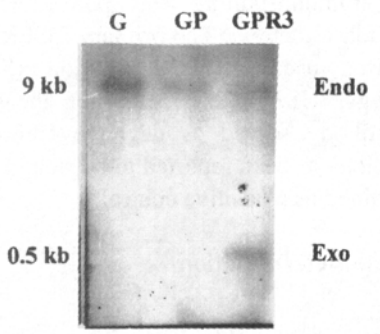

Fig. 1. Southern blot analysis of G cell pooled clones. The 9 kb fragment from the endogenous EGFR gene is indicated as Endo. The 0.5 kb fragment from the exogenously introduced DNA is indicated in GPR3 cells as Exo.

The Proliferation of GPR1 and GPR3 Cells

The transfected GRP1 and GPR3 cells proliferated at a significantly lower rate than control GP and G cells measured by MTT assay (Table 1). The growth rate of GPR3 cells was the lowest. There was no significant difference between the growth rate of control transfected GP cells and of parental G cells. Similarly, the proliferation activity of transfected GPR1 and GPR3 cells assessed by the average number of AgNOR per cell was significantly lower than that of control GP and G cells, and it was lower in GPR3 cells than in GPR1 cells (Table 2).

The Expression of EGFR in Transfected G Cells

By *in situ* hybridization, it was shown that the EGFR mRNA expression in GPR1 and GPR3 cells was lower than that of control GP and G cells (Figure 3). The EGFR immunostaining of GPR1 and GPR3 cells also demonstrated that the number of positive staining cells and the intensity of staining were both decreased (Figure 4).

Apoptosis in Transfected G cells

Using the TUNEL method, it was found that there were many apoptotic cells. By ultrastructural examination, the characteristic features of apoptotic cells, including aggregation of condensed chromatin under the nuclear membrane and apoptotic bodies were found in transfected GPR cells, but not in control G cells (Figure 5).

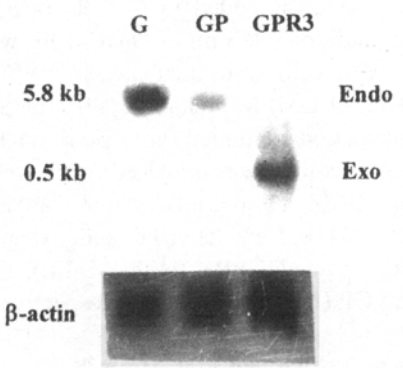

Fig. 2. Northern blot analysis of G cell pooled clones. The 5.8 kb endogenous EGFR mRNA is indicated as Endo. The 0.5 kb antisense EGFR mRNA is indicated as Exo in GPR3 cells as Exo which resulted in block of endogenous EGFR expression.

Table 1. The survival rate of G, GP, GPR1, GPR3 cell clones examined by MTT assay

Culture time (day)	n	G ($\bar{x}\pm s$)	GP ($\bar{x}\pm s$)	GPR1 ($\bar{x}\pm s$)	GPR3 ($\bar{x}\pm s$)	F value	*P* value
1	6	100.0	101.2±13.6	92.1±27.0	86.0±7.2	25.59	<0.0001
2	6	100.0	99.6±7.1	85.1±2.5	74.4±11.9	91.71	<0.0001
3	6	100.0	100.3±29.8	78.9±20.1	71.9±17.2	58.16	<0.0001
4	6	100.0	103.7±6.8	71.2±13.0	66.4±11.4	237.45	<0.0001
5	6	100.0	101.4±24.4	60.7±12.0	49.1±9.4	292.83	<0.0001
6	6	100.0	99.1±22.8	47.7±11.6	33.1±4.8	594.46	<0.0001

Table 2. The average number of AgNOR of G, GP, GPR1 and GPR3 clones

Cell clones (n)	The average number of AgNOR ($\bar{x}\pm s$)	F value	P value
G (6)	14.58±0.60		
GP (6)	13.60±0.99		
GPR1 (6)	10.70±0.49	93.30	<0.0001
GPR3 (6)	7.63±0.45		

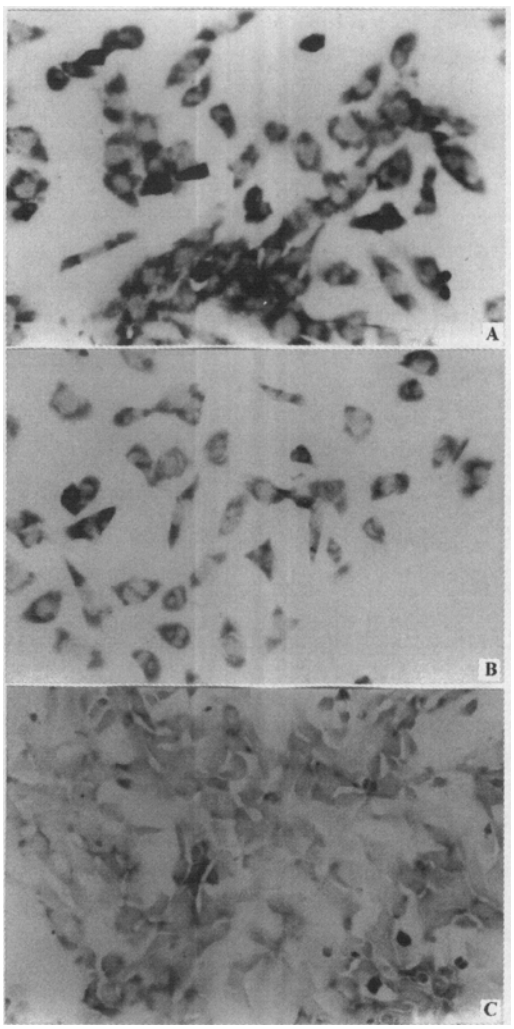

Fig. 3. *In situ* hybridization of EGFR mRNA of G, GPR1 and GPR3 cells, (× 200).
A. G cells B. GPR1 cells C. GPR3 cells

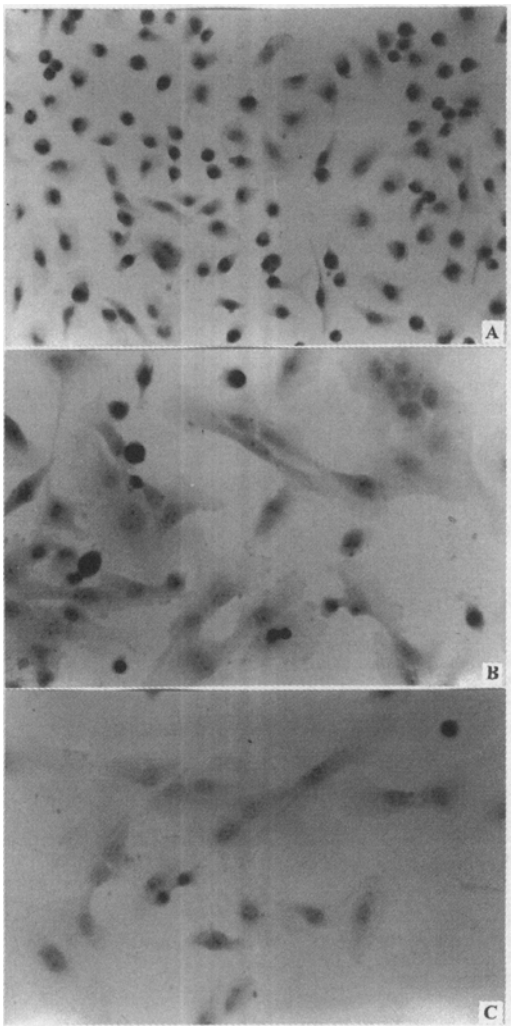

Fig. 4. Immunohistochemical staining of EGFR in G, GPR1 and GPR3 cells (× 400).
A. G cells B. GPR1 cells C. GPR3 cells

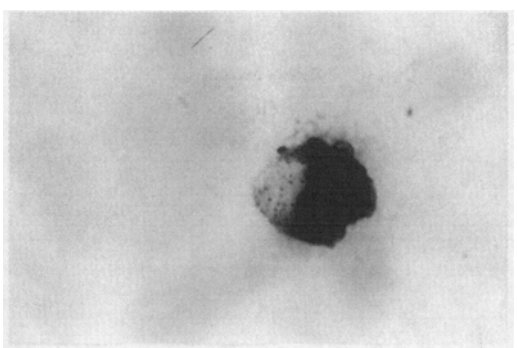

Fig. 5. The apoptotic cell showed condensed chromatin under the nuclear membrane by TUNEL method (×1000).

DISCUSSION

In the present study, we have used an antisense approach to investigate the oncogenic role of EGFR in the development of malignant gliomas as well as the effects of antisense EGFR RNA on the inhibition of glioma cell growth and induction of cell apoptosis. We have demonstrated that glioma cells could be stably transfected with an antisense EGFR construct mediated by Lipofectamine. Antisense EGFR constructs complementary to both the 3' or the full-length coding region of EGFR mRNA (AS-3' and AS-FL) were effective in blocking EGFR gene expression of human glioma cells, but the effect of AS-3' was more prominent. A similar result was obtained by Moroni et al., who demonstrated that microinjection of antisense EGFR cDNA into the cytoplasm of human carcinoma KB cells was able to inhibit EGFR synthesis, and they also noted that AS-3' was the most effective.[6] Maybe it was easier for AS-3' to be carried into tumor cells due to its shorter length.

The growth rate and proliferation of transfected G cells *in vitro*, was greatly reduced in GPR1 and GPR3 cells compared to that in the control GP and G cells. Our

results also showed that apoptosis was induced in the antisense EGFR RNA-expressing cells, but not in control G cells. These findings suggested that EGFR was essential for the growth of transformed cells and decreased expression of EGFR in G cells transfected with antisense EGFR RNA resulted in cell apoptosis.

The present study strongly supports that EGFR represents a logical target through which to effect the growth of glioma cells, and it should be studied further for its antiproliferative effect of glioma *in vivo*.

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