Effects of Antisense Glial Fibrillary Acidic Protein Complementary DNA on the Growth, Invasion, and Adhesion of Human Astrocytoma Cells

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ABSTRACT

Gial fibrillary acidic protein (GFAP)-positive astrocytoma cells were stably transfected with an expression vector carrying a murine complementary DNA for GFAP in the antisense orientation. Three stably transfected GFAP-negative transformants were identified by indirect immunofluorescence and expanded in vitro. The stably transfected and control cell clones were analyzed for morphological alterations, growth in monolayer and soft agar, adhesiveness, and in vitro invasive potential. In contrast to control astrocytoma cells which retained an astrocytic phenotype with polygonal or triangular cells extending multiple long and thin processes, the antisense GFAP-transfected cells demonstrated marked morphological alterations in the form of flat, epithelioid cells devoid of long, astrocytic glial processes. The antisense GFAP-transfected clones demonstrated a greater degree of cell crowding and piling at confluence than did controls. By titrated thyminidine analysis, the antisense GFAP-transfected cell clones demonstrated a 2-3-fold increase in incorporation of the radiolabel, suggesting an enhanced proliferative potential over controls. Antisense GFAP-transfected astrocytoma clones formed larger and more numerous colonies than did controls when tested for anchorage-independent growth in soft agar. Following a time-course adhesion assay, antisense GFAP-transfected astrocytoma clones were found to be less adherent to their substratum than controls. When assessed in an in vitro invasion assay system, antisense GFAP-transfected astrocytoma cells more readily penetrated Matrigel-coated filters than did controls. These data have shown that eliminating GFAP expression from astrocytoma cells has affected astrocytoma cell morphology and adhesion. The data also suggest that the growth and invasive potential of the antisense GFAP-transfected astrocytoma cells have been significantly enhanced by altering the expression of this glial-specific cytoskeletal protein in this experimental cell system.

INTRODUCTION

Anaplastic astrocytomas are the commonest neoplasms to arise in the CNS. Highly malignant and invasive neoplasms, anaplastic astrocytomas are a phenotypically and genotypically heterogeneous group of neoplasms for which, in the clinical setting, there are few real cures. The inability to find effective treatments for patients with anaplastic astrocytomas stems in part from our limited knowledge of the basic biology and molecular genetic changes that accompany these neoplasms.

Our current understanding of the biology of human astrocytomas has been increased to some extent by detailed analyses of experimental astrocytoma model systems. To this end, a large number of anaplastic astrocytoma cell lines have been established and characterized which continue to serve as useful models (1–5). A valuable feature of some anaplastic astrocytoma-derived cell lines is the ability of the tumor cells to retain some of the phenotypic properties of the original tumor. In 1968, Ponten and Westermark established a unique astrocytoma cell line, U 251 MG, which has been extensively studied and characterized (6, 7). In vitro, U 251 MG has retained many phenotypic features characteristic of astrocytes observed in vivo (1); U 251 MG astrocytoma cells are polygonal or triangular with thin and long cytoplasmic extensions (8). The cytoplasm is filled with coarse filibrillary filamentous network which stains positively for GFAP. Interestingly, only a paucity of astrocytoma cell lines have retained the ability to express GFAP as permanent cultures (1, 2, 4, 9–11).

GFAP is an astrocyte-specific intermediate filament thought to provide structural support to normal astrocytes. In our previous report, we transfected GFAP-negative astrocytoma cells with an expression vector containing a GFAP cDNA in the sense orientation and measured the effects of GFAP expression in stably transfected cell clones. Our results suggested that the expression of GFAP in previously GFAP-negative astrocytoma clones was responsible in part for the observed reduction in astrocytoma proliferation and tumorigenicity (12). To further understand the role of this glial specific intermediate filament in regulating astrocytoma cell growth and morphology, in the present report we describe the transfection of U 251 MG astrocytoma cells with an expression vector containing a GFAP cDNA in the antisense orientation and the effects of eliminating GFAP expression on the morphology, growth rate, adhesion, and invasion of human astrocytoma cells. Our results further support the notion that GFAP per se provides structural cytoskeletal support to maintain certain astrocytic and morphological features such as cytoplasmic process formation. By eliminating GFAP expression using antisense techniques, we show that GFAP may also play an important role in growth control and cell/cell or cell/substratum interactions in this experimental cell system.

MATERIALS AND METHODS

Astrocytoma Cell Lines and Culture Conditions. Human astrocytoma cell lines U 251 MG and U 343 MG-A are two previously well-characterized permanent astrocytoma cell lines derived from patients with malignant astrocytomas (3, 5, 7, 13) and are the generous gifts of Dr. Bengt Westermark, University of Uppsala. Both cell lines express GFAP in high titer over serial passages. U 251 MG grows in monolayer, is anchorage independent in soft agar, and grows as xenografts in athymic mice (1, 5, 8); by comparison, U 343 MG-A is an obligate, anchorage-dependent tumor cell line (13). The astrocytoma cell lines were grown as monolayers and passed weekly in αMEM supplemented with 10% FCS (GIBCO, Grand Island, NY), 100 units/ml penicillin, 100 μg/ml streptomycin sulfate, and 0.25 μg/ml fungizone (all GIBCO). The astrocytoma cell lines were grown in a humidified environment containing 5% CO2 at 37°C.

Expression Vector Construct. The expression vector pSV2neo/aGFAP is a 7-kilobase plasmid which was used to introduce the antisense GFAP gene into human astrocytoma cells. This plasmid carries a 1.2-kilobase fragment from the coding region of the murine GFAP gene in the antisense orientation driven by a viral SV-40 early promoter sequence. In addition, the plasmid contains the neomycin resistance selectable marker driven by a separate viral
SV-40 promoter. A bacterial ampicillin resistance gene found downstream from the antisense GFAP gene facilitates growth of the plasmid in bacteria culture medium. This plasmid has been previously well characterized and is the generous gift of Dr. R. Lien, Columbia University (14).

**Astrocytoma Cell Transfection.** Approximately 1.6 x 10^7 U 343 MG-A and U 251 MG human astrocytoma cells were trypsinized in 0.25% trypsin (GIBCO), pelleted in a clinical centrifuge, washed in 5 ml of sterile Ca^{2+}- and Mg^{2+}-free PBS, repelleted, and resuspended in sterile Ca^{2+}- and Mg^{2+}-free PBS. Astrocytoma cells were then added to 20 μg sterile pSV2 neo/AsGFAP in a 0.45-cm electrode gap electroporation cuvette (Bio-Rad, Richmond CA) and incubated on ice for 15 min. Specific electroporation voltage and capacitance values required to produce 50% cell death were determined and used. U 251 MG and U 343 MG-A astrocytoma cells required 250 V and 500 μF for efficient transfection. Following electroporation, astrocytoma cells were incubated on ice for 30 min before 2 x 10^6 cells from a single cell suspension were plated onto 100 mm^2 tissue culture dishes (Corning, Corning, NY). Twenty-four h after electroporation, the sparsely seeded and adherent astrocytoma cells were washed once with Ca^{2+}- and Mg^{2+}-free PBS before 6 ml of aMEM containing 600 μg/ml of G418 (Sigma Chemical Co., St. Louis, MO) were added. Colony formation was observed over time by phase microscopy. When individual colonies were comprised of 50—100 cells (approximately 2 weeks post electroporation), they were lifted separately from the 100-mm^2 plates with a P-200 micropipette tip (Starstedt, Montreal, Quebec) under microscopic guidance. These colonies were then expanded as separate clones of cells in G418-containing media in new tissue culture dishes. Control transfections for all studies which follow include the transfection of the pSV2neo/asGFAP plasmid minus the antisense GFAP cDNA.

**Immunocytochemistry**. Successfully transfected G418-resistant astrocytoma clones were examined for GFAP expression by indirect immunofluorescence as described previously (15, 16). Astrocytoma clones were grown and stained in Lab-Tek eight-well chambers (Nunc, Naperville, IL). Cells were fixed in a solution of methanol and ethanol (1:1) for 10 min. Non-specific background staining was eliminated by incubating the cells with nonimmune swine serum (Dako, Santa Barbara, CA; dilution, 1:5). Rabbit anti-cow GFAP antisera was used as the primary antibody (Dako; dilution, 1:100). Rhodamine-conjugated swine anti-rabbit immunoglobulins were used as the secondary antibody (Dako; dilution, 1:32). Normal rabbit immunoglobulins (Dako; dilution, 1:100) were used as negative controls for each of the clones. The U 343 MG-A parent astrocytoma line which displays an exuberant network of GFAP-positive intracytoplasmic cytoskeletal filaments was used as a positive control. Fluorescence microscopy was performed with a Leitz Orthoplan system microscope equipped for incident-light immunofluorescence. The intensity of staining and the percentage of cells that stained positively were recorded in each case. Negative controls were examined for each astrocytoma clone.

**Astrocytoma Cell Proliferation Assay.** To determine the effects of antisense GFAP gene expression on the proliferation of U 251 MG astrocytoma cells on culture adhesiveness was determined as described previously (15). Briefly, antisense GFAP-transfected astrocytoma cells and controls were cultured in 100 mm^2 dishes, rinsed with 3 ml of PBS, and then placed in 1.5 ml of EDTA (0.2 g/liter in PBS). The flask were then shaken in a Tek-Tator V variable speed rotator at 110 rpm for 5 min at room temperature. The detached cells were removed and trypsinized to produce a single cell suspension; the adherent cells were also trypsinized into a single cell suspension. Both adherent and detached cells were counted in a hemocytometer. Trypan blue exclusion was used to determine cell viability. The flask were set up so that the assay could be performed on day 3 (lag phase), day 6 (exponential growth phase), and day 9 (plateau growth phase) of the growth curve in antisense GFAP-transfected and control clones.

**Invasion Assay.** To determine if antisense GFAP expression among stably transfected clones affects astrocytoma cell invasion, we tested both antisense GFAP astrocytoma clones and controls in an *in vitro* invasion assay as described by Repesh (18). Briefly, transfwll chambers (Costar, Cambridge, MA) with 6.5 mm diameter polyvinylpyrrolidone-free polycarbonate filters of 8 μm size were coated with 100 μl of 1:20 dilution (30 μg/filter) of Matrigel (Collaborative Research, Cambridge, MA) in cold DMEM and allowed to air-dry overnight. The Matrigel was then reconstituted the following day with 100 μl of serum-free DMEM at room temperature for 90 min with constant rotation. Prior to the addition of the radiolabeled astrocytoma cells, the excess media was removed from the filters.

Stably transfected antisense U 251 MG astrocytoma control and control cells were radiolabeled for 24 h with (15 μCi/ml) 45 μCi of tritiated thymidine (3 ml; Amersham). Labeled cells were washed three times with PBS to remove unbound label and then trypsinized and resuspended in DME/F and 10% FCS. Approximately 2 x 10^4 cells were added to 200 μl of medium to triplicate upper chamber wells. After 72 h incubation, the number of astrocytoma cells traversing the filter into the lower wells (containing 800 μl of DME/F) at 37°C was quantified by assessing the radioactivity associated with astrocytoma cells in the media below the polycarbonate filter. The radioactivity in the media present in the lower well and in several rinses was assessed in a Beckman scintillation counter. Typically, less than 0.1% of the original label bound to the filter. The percentage invasion was calculated from the amount of radioactivity in the lower wells (including all the washings) expressed as a percentage of the sum of radioactivity in all the wells plus the membrane.

**RESULTS**

**Isolation and Characterization of Stably Transfected GFAP-negativ1 Astrocytoma Clones.** Following electroporation, transfection, and selection of U 251 MG astrocytoma cells in G-418-containing medium, three cell clones (asGFAP U 251 MG clones 6, 10, and 11) were identified, which were GFAP-negative by immunocytochemistry (Fig. 1). A control cell clone, U 251 MG N4, derived from transfecfion of U 251 MG with pSV2 neo alone, was found to be GFAP-positive and was isolated and expanded for further studies. Despite several attempts during which electroporation voltage and capacitance parameters were altered, we were unable to grow long-term cultures of cells from astrocytoma cell line U 343 MG-A after transfection (data not shown).

Stably transfected asGFAP U 251 MG astrocytoma cell clones demonstrated marked morphological changes when compared to control cells (Fig. 2). Whereas control U 251 MG cells had many cells which extended long cytoplasmic processes rich in GFAP and which were similar to the parent cell line, all asGFAP U 251 MG astrocytoma clones were epithelioid or cuboidal in appearance with loss of process formation. At confluence, asGFAP U 251 MG astrocytoma cells packed together tightly with an increased nuclear:cytoplasmic ratio and a tendency to overgrow the culture, whereas control U 251 MG cells maintained their stellate morphology and were maintained longer in monolayer culture without being shed into the medium than were asGFAP-transfected clones.
Antisense GFAP Astrocytoma Clones Form Larger and More Numerous Colonies in Soft Agar than Do Controls. Control U 251 MG N4 astrocytoma cells are capable of forming colonies in soft agar with a colony-forming efficiency similar to the nontransfected, parental cell line. By way of contrast, all asGFAP U 251 MG cell clones formed colonies that were not only more numerous but also larger than controls (Fig. 3). In general, asGFAP U 251 MG cell clones formed two times as many colonies that were approximately twice as large as control colonies (Table 1).

Antisense GFAP Astrocytoma Clones Show Enhanced Proliferative Potential. When compared to control cell clone U 251 MG N4, all stably transfected asGFAP U 251 MG astrocytoma cell clones demonstrated increased uptake of tritiated thymidine in a standard monolayer assay (Fig. 4). The increased uptake of tritiated thymidine varied from 165% of control for asGFAP-transfected clone 10 to 260% of control for asGFAP-transfected clone 6.

Antisense GFAP Astrocytoma Clones Are More Invasive than Controls. When tested in an *in vitro* invasion assay using Matrigel as the barrier for analyzing invasive potential amongst astrocytoma cells, asGFAP U 251 MG clones demonstrated a greater ability to penetrate Matrigel than did controls (Fig. 5).

Antisense GFAP Astrocytoma Clones Are Less Adherent to Substratum than Controls. In a time-course adhesion assay, asGFAP U251 MG clones were less adherent to their substratum than were controls. At days 3, 6, and 9 of analysis, during which the astrocytoma cells were in lag, early exponential, and early plateau phases of culture growth, asGFAP U 251 MG clones were more readily detached from their substratum than were controls (Fig. 6). The percentage detachment of all asGFAP U 251 MG clones and controls was greatest on day 9 with the exception of asGFAP clone 11 in which detachment was greatest on day 6.

DISCUSSION

The intermediate filaments form a family of 8–10-nm cytoskeletal proteins, which provide structural support in most cell types in which they are expressed. Not only are they developmentally regulated, but they are also tissue specific in expression so that desmin is produced almost exclusively by muscle cells, cytokeratins by epithelial cells, vimentin by mesenchymal cells, and GFAP by astroglial cells. Aside from their postulated role in providing structural support to a variety of cell types, intermediate filaments may interact with other key proteins of the cytoskeleton which form links between the cytoskeleton and the plasma membrane. As a result, disturbances of intermediate filament synthesis or structure in a given cell may lead to profound changes in cell morphology, growth, proliferation, and invasiveness (19).

In this report, we have stably transfected GFAP-positive human astrocytoma cells with an expression vector which contains the GFAP gene in the antisense orientation. Expression of the antisense GFAP gene in this model system has eliminated GFAP expression amongst U 251 MG astrocytoma cells by indirect immunofluorescence analysis. GFAP-negative U 251 MG astrocytoma cells adopted a flattened, epithelial morphology strikingly different from control GFAP-positive control cells which were stellate and bipolar. The loss of GFAP expression was associated with a modestly enhanced proliferative potential and growth in soft agar when compared to controls; the antisense GFAP transfected astrocytoma clones demonstrated increased invasive potential over controls when their ability to cross a Matrigel-coated filter membrane was measured. Taken together, these results suggest that the loss of GFAP expression has played an important role in the morphoregulatory responses observed in this experimental cell system.

In our study, we attempted transfection of two human astrocytoma cell lines that express GFAP over serial passages but were able to stably transfect only one cell line successfully, U 251 MG. We had hoped to transfet GFAP-positive U 343 MG-A cells to determine if elimination of GFAP gene expression would facilitate growth of this anchorage-dependent cell line in soft agar, but our current technique of transfection by electroporation even at low voltages and capacitances led to early cell death. We are currently exploring the efficacy of other transfection methods, such as calcium/phosphate and liposome transfection, to determine the effects of antisense GFAP gene expression on this particular cell line.

Using calcium:phosphate coprecipitation, Weinstein et al. (14) stably transfected human U 251 MG astrocytoma cells with the same anti-sense GFAP expression vector. In their report, the authors were primarily concerned with the effect of eliminating GFAP expression on the formation of stable astrocytic processes in response to neurons or neuronal membranes in coculture experiments (14). They determined that antisense GFAP transfected clones did not form long complex processes in response to neurons as did control cells, and that these clones continued to support neuronal survival and neurite extension. In their study, the effects of antisense GFAP expression on astrocytoma growth in soft agar, colony formation and morphology at subconfluence and confluence, invasive potential, and adhesion were not studied.
In our study, we sought to determine how the loss of GFAP expression altered certain morphological and growth properties of the U 251 MG astrocytoma cell line. Although our method of transfecting this cell line was different than that described by Weinstein et al. (14) and although our study involved an analysis of different parameters than that previously described, our results are complementary to those reported previously (14): we, too, observed loss of astrocytoma cytoplasmic process formation concomitant with the suppression of GFAP production. However, whereas Weinstein et al. (14) observed persistence of cell cycle withdrawal in U 251 MG cells post antisense GFAP transfection (14), we noted a modest growth enhancement among our antisense GFAP-transfected clones over controls. The apparent discrepancy in growth rates among transfected astrocytoma cells in the two studies may relate to the use of neuronal cell membranes by Weinstein et al. (14). Neurons are potent inhibitors of astrocytoma cell growth (20). The growth inhibition of astrocytoma cells by neurons appears to be mediated by cell/cell interactions rather than by soluble factor release (20).

Interestingly, GFAP and vimentin intermediate filaments have been colocalized to the same filamentous network in U 251 MG astrocytoma cells (21–23). Weinstein et al. (14) demonstrated that the removal of GFAP from U 251 MG cells by antisense mRNA did not appear to affect vimentin expression. While we cannot state definitively from our analysis that the perturbations observed among antisense GFAP-transfected glioma cells are specific for the elimination of GFAP per se, ongoing studies of vimentin expression at molecular, light, and ultrastructural levels will help us determine if vimentin intermediate filaments are functionally and structurally conserved in our system.

The seminal work by Stephenson and Zamecnik (24) in 1978 demonstrated that an antisense oligonucleotide against the Rous sarcoma virus could abort Rous sarcoma virus infection. Since this finding, a number of strategies have been designed by which antisense oligonucleotides can be used to artificially regulate eukaryotic genes (25–28). And, a number of endogenous genes have now been successfully suppressed in a variety of experimental cancers (25–28). The power of antisense technology enables cell surface-mediated events to be bypassed such that antisense sequences can directly influence the expression of a given gene of interest (25). As we wished to study the effects of continuous and long-term suppression of GFAP synthesis and filament formation in human astrocytoma cells, we used a GFAP cDNA in the antisense orientation contained with a mammalian expression vector system.

The mechanism by which the antisense suppression of GFAP expression has enhanced the invasive potential of U 251 MG astrocytoma cells in our study is unclear. In general, tumor cell invasiveness is a complex, multistep process involving cell attachment, proteolysis of matrix components, and migration of cells through the defect in the matrix (29). We have previously shown that human fetal astrocytes and astrocytoma cell lines express matrix metalloproteinase...
Table 1 Correlation between loss of GFAP expression and growth in soft agar

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<tr>
<th>Cell clone</th>
<th>GFAP immunoreactivity</th>
<th>Soft agar colonies/cm²</th>
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<tr>
<td>U251 MG</td>
<td>+</td>
<td>550 ± 52</td>
</tr>
<tr>
<td>U251 MG N4</td>
<td>+</td>
<td>550 ± 52</td>
</tr>
<tr>
<td>asGFAP 6</td>
<td>−</td>
<td>800 ± 56</td>
</tr>
<tr>
<td>asGFAP 10</td>
<td>−</td>
<td>909 ± 44</td>
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<tr>
<td>asGFAP 11</td>
<td>−</td>
<td>1345 ± 83</td>
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Fig. 3. Effect of antisense GFAP expression on the growth of U251 MG astrocytoma colonies in soft agar 14 days after seeding. a, control U251 MG cells (N-4) showing well-formed colonies in soft agar. b, growth of antisense GFAP-transfected U251 MG clone 11 in soft agar. When compared to controls, antisense clones demonstrate enhanced growth in soft agar with some colonies that are larger than colonies formed by controls. The number of colonies is increased 2.5-fold over controls. Bar, 40 μm.

The mechanism by which antisense suppression of GFAP has altered astrocytoma growth and morphology in our study is also unclear. However, it is known that cell/cell and cell/ECM adhesion is mediated through several different families of plasma membrane receptors which also have structural links with proteins of the cytoskeleton. In a recent study, Narayanan et al. (32) showed that expression of antisense RNA to the DCC gene caused the transformation of rat-1 fibroblasts. DCC is a candidate tumor suppressor gene on chromosome 18 q which encodes for a cell adhesion molecule thought to be important in regulating cell/cell and cell/substratum interactions. Using a dexamethasone-inducible antisense RNA expression vector, the authors showed that DCC expression could be eliminated from Rat-1 fibroblasts. Such DCC-negative cells grew faster and had increased tumorigenicity in athymic mice and soft agar than did DCC-positive control fibroblasts. Loss of DCC expression led to clones having a difficult time attaching to substratum due to the loss of cell/ECM interactions. In a manner perhaps analogous to this mechanism, loss of GFAP expression by U251 MG astrocytoma cells may lead to cytoskeletal collapse and loss of critical cell/cell or cell/substratum interactions. Such cytoskeletal changes may lead to inability to extend processes, alter cell/cell interactions, and enhance growth under anchorage-independent conditions.

Another possible mechanism by which inhibition of GFAP expression by antisense GFAP could conceivably lead to enhanced growth of U251 MG astrocytoma cells in soft agar is through a down-regulation in expression of other important cell surface molecules which are physically linked to the cytoskeleton and which mediate cell/extracellular matrix adhesion. In support of this possibility, Giancotti and Ruoslahti (33) showed that up-regulating the α5β1 integrin (fibronectin receptor) in Chinese hamster ovary cells by gene transfer...
suppressed the tumorigenicity and transformed phenotype of these cells. In normal astrocytes within the CNS, just as in U 251 MG astrocytoma cells, the highest concentration of GFAP is found in the glial filaments within the long astrocytic cytoplasmic processes. By ultrastructural analysis, astrocytic foot processes in the CNS adhere in great numbers to the glial limitans externa and the basal lamina of the cerebral microvasculature. Both the glial limitans externa and the basal lamina of the capillary endothelial cells are well-defined basement membranes known to be enriched in laminin, type IV collagen, fibronectin, and heparan sulfate proteoglycan (34). Paulus et al. (35) have recently demonstrated that astrocytes at the glial limitans externa express α2, α3, α6, β1, and β4 integrin receptor chains. It is perhaps reasonable in this context to propose that loss of GFAP-enriched glial filaments from cytoplasmic processes in U 251 MG astrocytoma cells may significantly alter the code and requirement for the expression of integrins at the cell surface leading to altered cell/ECM or cell/cell binding.

From our previous report in which we stably transfected human astrocytoma cells with a sense-oriented GFAP cDNA expression vector, human astrocytoma cells were growth inhibited and exhibited stellate process formation indicative of a more differentiated astrocytic phenotype. From the present work in which we have eliminated GFAP expression in a different astrocytoma cell line by antisense techniques, our data further support the role of GFAP in the maintenance of normal astrocytic morphology and in the regulation of certain aspects of astrocytoma cell growth. Future studies are being directed toward the mechanisms by which alterations in GFAP gene regulation exert such profound effects in astrocytoma morphoregulatory responses through an examination of integrin and cell adhesion molecule expression.

REFERENCES


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