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# Malignant Glioma-Derived Soluble Factors Regulate Proliferation of Normal Adult Human Astrocytes

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**Abstract.** Malignant gliomas are characteristically surrounded by marked gliosis. To assess whether glioma-derived products contribute to the proliferation of astrocytes, a feature of the gliosis response, we evaluated the influence of culture supernatants from malignant human glioma lines and tumor cyst fluids collected from two patients with glioblastoma multiforme on the proliferation of non-transformed adult human astrocytes. Both the culture supernatants and cyst fluids significantly increased DNA synthesis in astrocytes as assessed by a double immunofluorescence glial fibrillary acidic protein-bromodeoxyuridine technique. The net proliferative effect mediated by glioma cell line supernatants was tumor growth phase-dependent, being preferentially expressed during the logarithmic phase of glioma cell growth. Specific growth factor molecules and cytokines known to be secreted by gliomas (epidermal growth factor, fibroblast growth factor, platelet-derived growth factor, transforming growth factor-$\beta$, interleukin-6, and tumor necrosis factor-$\alpha$) could not reproduce the mitogenic effects of the glioma-derived soluble factors. Cytokines which can induce DNA synthesis by adult human astrocytes *in vitro*, gamma-interferon and interleukin-1, were not detected in the culture supernatant of glioma lines used in this study. In conjunction with the documented effects of glioma products on endothelial and lymphoid cells, the current study suggests that soluble glioma products can contribute to the production of surrounding gliosis observed *in vivo*.

**Key Words:** Astrocyte; Glioma; Gliosis; Proliferation; Soluble factors.

## INTRODUCTION

Reactive gliosis, where astrocytes undergo hypertrophy and proliferation, is a pathologic feature of tissue that surrounds malignant brain tumors (1–4). In many cases the glial reaction forms a pseudocapsule that facilitates surgical removal of the tumor; in other instances, the periphery of the neoplasm is difficult to distinguish from reactive glial proliferation (3, 4). The cause of the reactive gliosis has been attributed to the "irritative" effect of an invasive tumor on the surrounding brain tissue. However, since gliomas themselves can secrete a range of soluble molecules (5–16), the potential role of these products also needs to be considered. Among growth factor molecules known to be produced by gliomas, epidermal growth factor (EGF), acidic or basic fibroblast growth factor (aFGF and bFGF, respectively) and platelet-derived growth factor (PDGF) have been shown to promote proliferation of neonatal rodent or fetal human astrocytes *in vitro* (17–22).

Although patients with malignant gliomas exhibit a profound decrease in systemic immunity (10, 23–26), glioma cells *in vitro* have the capability to synthesize cytokines such as transforming growth factor-$\beta$ (TGF-$\beta$), tumor necrosis factor (TNF), interleukin (IL)-1 and IL-6 (10, 11, 14–16). These cytokines, if locally released by gliomas, may play a role in mediating the reactive gliosis observed around brain tumors *in vivo*, especially in view of reports that IL-1 (27–29), IL-6 (30) and TNF-$\alpha$ (30, 31) are mitogens for astrocytes *in vitro*. We have recently reported that gamma-interferon ($\gamma$-IFN) induces proliferation of cultured non-malignant adult human astrocytes (32).

The present study was undertaken to examine the overall effect of soluble molecules secreted by malignant gliomas on the proliferative rate of non-malignant astrocytes, one feature of the reactive gliosis response. It is noted that the process of gliosis can involve many types of changes to the astrocyte, including increases in immunoreactivity and/or content of glial fibrillary acidic protein (GFAP; the astrocyte intermediate filament), hypertrophy, proliferation and other metabolic alterations. Indeed, we have recently suggested that the mediators for many of these changes, which are not necessarily correlated, may be different (33). In the present study, we have chosen to examine one aspect of the reactive gliosis response, that of proliferation. Cultured adult human astrocytes served as proliferation targets for cell-conditioned supernatants of glioma cell lines, as well as for tumor cyst fluids extracted from two patients with glioblastoma multiforme. For the glioma cell line-conditioned media, these were collected at different stages of the glioma growth *in vitro* in order to examine whether the secretion of soluble astrocyte mitogen(s) was growth phase-specific. Further experiments involved assessing whether the astrocyte mitogenic effect could be accounted for by known glioma secretory products (EGF, FGF,

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PDGF, TGF-$\beta$, TNF-$\alpha$ and IL-6) or by mitogens described for adult human astrocytes ($\gamma$-IFN and IL-1) (32).

## MATERIALS AND METHODS

### Human Glioma Cell Lines

Glioma cell lines A172 and U563-MG are well-characterized lines established elsewhere (34, 35). These lines are passaged regularly (consisting of gentle trypsinization at 0.05% for 10 minutes followed by serum inactivation of trypsin and then repeated washes with phosphate buffered saline [PBS] and replating) and are maintained in 25 $cm^2$ tissue culture flasks in medium consisting of Eagle's minimum essential medium supplemented with 10% fetal bovine serum (FBS), gentamicin (20 $\mu$g/ml), glutamine (2 mM), pyruvate (1 mM), dextrose (0.1%) and essential amino acids buffered to a pH of 7.0. Cells are grown at 37°C in a humidified 5% $CO_2$ incubator.

### Collection of Glioma Supernatants

Glioma cell lines were seeded at a density of $1 \times 10^5$ cells in 5 ml of tissue culture medium per 25 $cm^2$ culture flask and permitted to grow to confluency. Cell-conditioned medium (supernatant) was replaced every 2 days to avoid nutrient depletion or accumulation of metabolites that might influence the bioassays described below. For the purposes of this study, three time points for each culture were chosen to represent different stages in the growth phase of each tumor: early logarithmic growth phase, late logarithmic growth phase, and immediately following the attainment of confluency. Since the U563-MG tumor grows more slowly than the A172 (36), supernatant from days 4–6 post-seeding of U563-MG represented the early logarithmic growth phase in contrast to A172 glioma where this phase was achieved between days 2–4 post-plating. Similarly, the two later time points were obtained at intervals of days *in vitro* 4–6 and 12–14 for A172; for U563-MG, corresponding intervals were days *in vitro* 8–10 and 23–25, respectively. Supernatants were centrifuged to eliminate cellular debris and fast frozen to −70°C for storage prior to use.

### Collection of Glioma Cyst Fluids and Control Cerebrospinal Fluids

Cyst fluids were collected from two patients with glioblastoma multiforme proven histologically; the fluids were aspirated to achieve decompression. Repeated aspiration through an Ommaya reservoir system was done in one patient three times at intervals of approximately 2 weeks. Cyst fluid was aspirated at time of surgical excision in the second patient. A third set of cyst fluid was collected from a patient with hemangioblastoma, a non-glial tumor that is usually of vascular origin.

As controls for the cyst fluids, cerebrospinal fluid was obtained by lumbar puncture from two subjects being investigated for herniated lumbar discs.

### Astrocyte Cell Cultures

Our technique of isolation of non-malignant astrocytes has been published previously (37,38). Non-malignant human brain tissue was obtained from young adults undergoing surgical resection to ameliorate intractable epilepsy. Tissue adjacent to the epileptogenic focus was removed by Cavitron ultrasonic aspiration. For cell isolation, meninges and visible blood vessels were removed and brain tissue was cut into cubes of 1 mm or less. Viable dissociated cells were then obtained by previously established protocol using trypsin digestion and Percoll centrifugation (37, 38). Cells were suspended in feeding medium and placed in 25 $cm^2$ Falcon flasks for 24 hours (h) after which the floating cells (mostly oligodendrocytes) were removed for other studies. Adherent cells (mostly astrocytes and microglia) were left undisturbed and allowed to differentiate for a period of 7 days. By immunohistochemical identification, the majority of the cells were either astrocytes as assessed by GFAP immunofluorescence or presumed microglia/macrophage cells (leu-M5-positive). We have been unable to enrich for astrocytes beyond 70% purity; however, we have previously demonstrated that the mitogenic effect of $\gamma$-IFN on adult human astrocytes did not depend on the relative amounts of microglial cells that were present in culture (32). As will be indicated in the Results section, the amount of microglial cells in culture did not appear to be a factor for cyst fluids from glioma patients to elicit a proliferative response on astrocytes.

Feeding medium was Eagle's minimum essential medium supplemented with 5% FBS, 20 $\mu$g/ml gentamicin, and 0.1% dextrose. The cultures used in this study ranged in age from 2 to 4 weeks post-dissociation.

### Astrocyte Proliferation Assays

*GFAP-Bromodeoxyuridine (BrdU) double labeling technique:* Since the non-malignant cultures contained microglial cells in addition to astrocytes, $^3$H-thymidine incorporation would not have yielded information as to the cell type that has incorporated the proliferation label. For this reason, a double immunofluorescence technique that allows direct visualization of the cell type that has incorporated the proliferation label (BrdU) was used (17, 39). The astrocyte cultures described above were removed from their flasks by 0.05% trypsin and seeded on poly-l-lysine-coated 9 mm Aclar plastic coverslips at a density of $10^4$ cells per coverslip. Cells were incubated with test supernatants (1:1 concentration with feeding medium) for 4 days, and 10 $\mu$M BrdU was added during the last 48 h to allow proliferating cells to incorporate this label. Preliminary experiments had indicated that this time frame of treatment was optimal for assessment of proliferation of adult human astrocytes. The cells were then immunostained using antibodies to GFAP and BrdU by a method that has been described in detail elsewhere (17, 39); in a recent report (33) we have shown that this method yielded results reflective of changes in actual cell numbers. In cultures treated with cyst fluids or human cerebrospinal fluids, these fluids were used at 20% final concentration (v/v) in feeding medium. All immunolabeled samples were coded and subsequently counted blindly to obtain the percentage of GFAP-positive cells that have incorporated BrdU. In cases where results were pooled from multiple series of human astrocyte cultures (Fig. 1), each with slightly varying control rates of proliferation, results were expressed as the proliferation index (PI). The PI was the percentage of GFAP and BrdU double-positive cells (proliferating astrocytes) in test cultures divided by similar results from untreated controls in the same experiment.

To attempt to account for the identity of the astrocyte mitogen in glioma-derived supernatants or cyst fluids, the following were added for 4 days to adult human test cultures for GFAP and

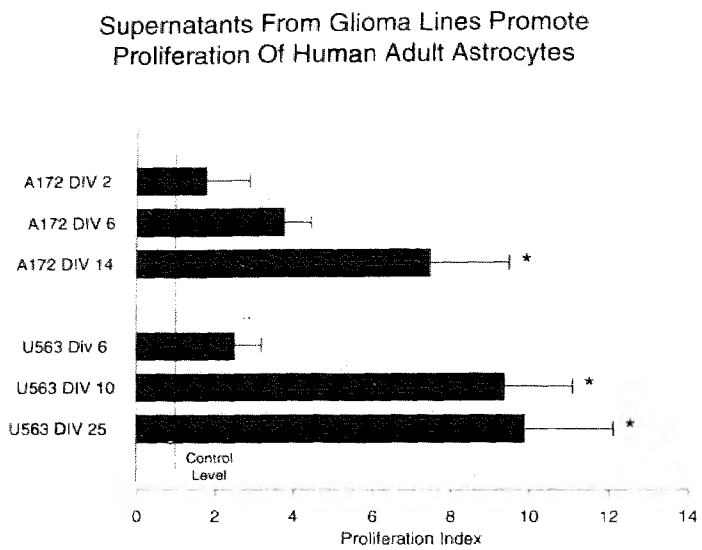


**Fig. 1.** Glioma supernatants promote proliferation of adult human astrocytes. Proliferation indices are mean ± SEM of four samples following a 4 day treatment period. Supernatants were collected from glioma lines A172 and U563-MG at the days *in vitro* (DIV) indicated following replating of cells (time 0). The percentage of astrocytes in control cultures that had incorporated BrdU was 2.8 ± 1.1%. ★Significantly different from control cultures using a one-way analysis of variance (ANOVA) with Duncan's multiple comparison, $p < 0.05$. All analyses were performed blindly on coded specimens.

BrdU immunofluorescence: EGF (Boehringer Mannheim, Germany; 20 ng/ml), acidic or basic human FGF (UBI, Waltham, MA; 20 ng/ml), human PDGF (Genzyme, Boston, MA; 200 mU/ml), human TGF-$\beta$ (Calbiochem, La Jolla, CA; 20 ng/ml), recombinant human IL-1, IL-6 and TNF-$\alpha$ (all from Genzyme, Boston, MA; 100 U/ml). These were optimal concentrations that were selected based on previous studies to generate dose-response curves for these agents on neonatal mouse, fetal human or adult human astrocytes (17, 32, unpublished observations). In addition, a mixture of EGF, aFGF, bFGF (all at 20 ng/ml) and PDGF (at 200 mU/ml) was also assessed.

All experiments were conducted in feeding medium where the final concentration of FBS was 5%.

*Flow Cytometric Cell Cycle Analysis:* To confirm the results of the GFAP-BrdU incorporation assay, cell cycle analysis was performed by propidium iodide staining with analysis by flow cytometry. As above, the cultures were maintained for a period of 4 days with the glioma supernatants (1:1 concentration with feeding medium), after which the cells were trypsinized, washed three times in PBS, and fixed by resuspending $1 \times 10^6$ cells per ml in a solution of PBS:ice cold methanol (1:2 final concentration, vortexing the cells to avoid clumping) for a period of 1 h at 4°C. The propidium iodide staining protocol was adapted from that of Hurley (40). After fixation, cells were centrifuged and the supernatant decanted. Immunocytochemical GFAP staining was performed in 1.8 ml Ependorf tubes using 30 $\mu$l of rabbit anti-GFAP primary antibody (volume to achieve saturation of antigen) followed by equivalent volumes of goat anti-rabbit secondary antibody conjugated with fluorescein isothiocyanate (FITC), each for an incubation period of 1 h at 4°C. Each stain was followed by three washes with PBS. Following GFAP labeling, 0.5 ml propidium iodide stain solution was added. This stain solution contained 10 mg propidium iodide (Calbiochem), 0.1 ml Triton X-100 (Sigma, St. Louis, MO), and 3.7 mg EDTA (Sigma) in 100 ml PBS. Immediately following addition of the stain solution, 0.5 ml ribonuclease (RNase) solution (10 mg RNase [Sigma] mixed with 5 ml PBS and heated to 75°C for 30 minutes before use) was added to eliminate double-stranded RNA which would interfere with DNA quantification. The samples were then analyzed by flow cytometry (FACScan$^M$, Becton Dickinson, San Jose, CA) after a 1 h staining period at 37°C in the dark. To specifically determine the proliferation of astrocytes in the mixed population, the cytometer was gated to analyze the DNA content of cells labeled with the GFAP-specific marker. Cellular DNA events were acquired using CELLFIT software (Becton Dickinson), with estimation of percentage of cells in particular phases performed by the RFIT model. Proliferation indices for flow cytometry, defined by the sum %S + %$G_2$/M phases, were calculated for each of the various treatments.

### Measurements of $\gamma$-IFN and IL-1 in Glioma Culture Supernatants

Culture supernatants of A172 glioma cells from days 7–9 post-seeding were collected. Twenty $\mu$l, in triplicates, were analyzed for $\gamma$-IFN using a radioimmunoassay kit (Centrecore, Malvern, PA) following the manufacturer's instructions. As a positive control for the assay, 20 $\mu$l of culture medium of human CD8$^+$ T-lymphocytes activated by OKT3 and IL-2 (32) were used.

Interleukin-1 was measured in A172 culture supernatant (7–9 days post-seeding) by its ability to stimulate thymocyte proliferation in the presence of phytohemagglutinin, following published protocols (41). As a positive control for the IL-1 bioassay, 20 $\mu$l of culture supernatant collected from a highly enriched (over 95%) adult human microglia culture and treated for 24 h with 5 $\mu$g/ml bacterial lipopolysaccharide were used.

## RESULTS

### Effects of Glioma Cell Line Supernatants on Astrocyte Proliferation

*GFAP-BrdU Double Labeling Proliferation Assay:* Figure 1 shows the relative PI obtained following the incubation of glial cultures for a period of 4 days in the presence of glioma supernatants from various growth phases. Supernatants from both the A172 and U563-MG glioma lines increased the PI of the human astrocyte cultures

**Fig. 2.** GFAP-BrdU double immunolabeling technique. Adult human glial cultures in control feeding medium (upper) and following the addition of glioma supernatant (lower). Cultures exposed to the glioma supernatants show an increased number of BrdU-staining GFAP-positive cells (arrows). A, C: GFAP. B, D: BrdU.

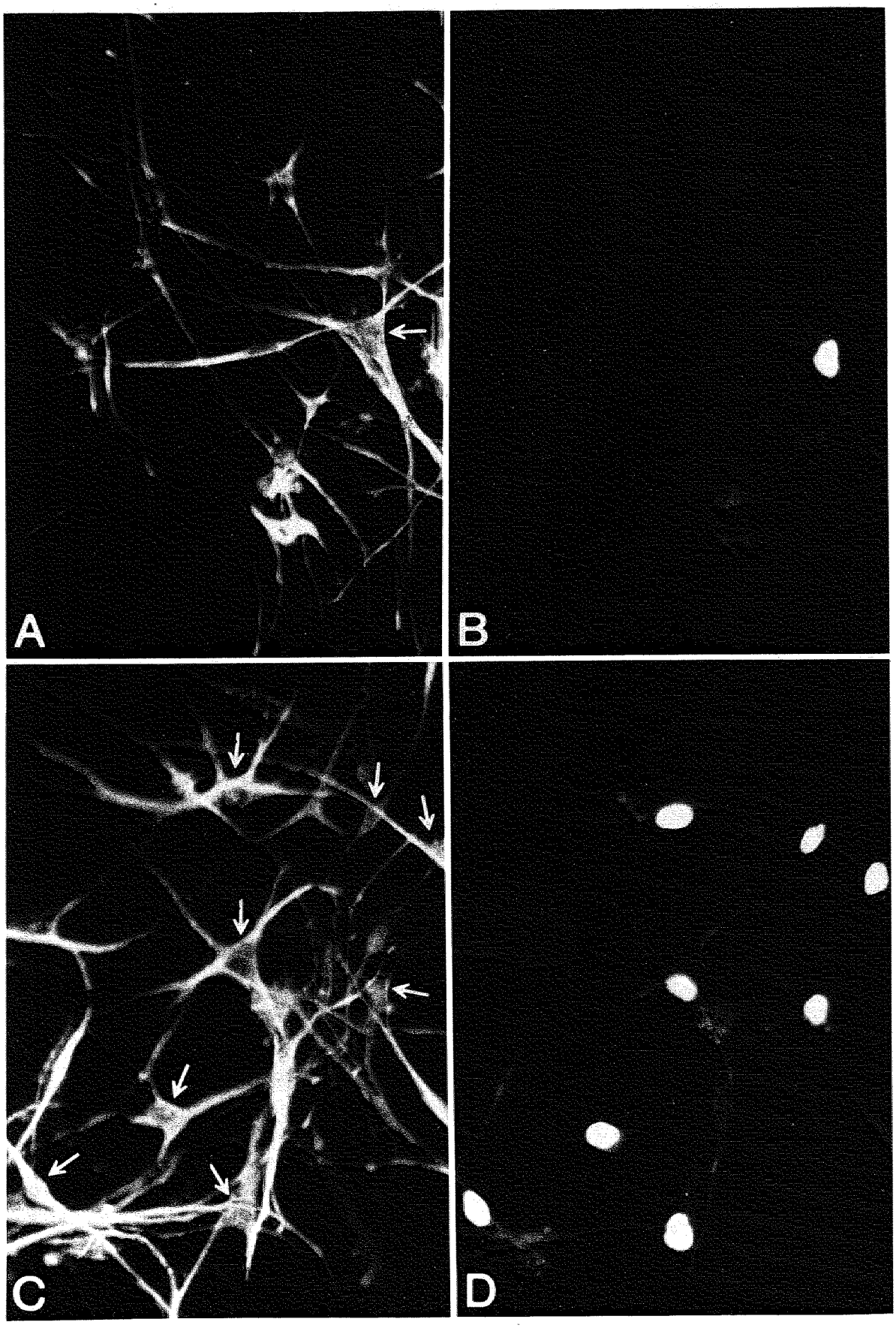
A
B
C
D

TABLE 1
Flow Cytometric Analysis of Cell Cycle Kinetics of Astrocytes Following Addition of Glioma Supernatants

| Treatment | %DNA synthesis (S-phase) | Proliferation index (%S + %$G_2$/M) |
|---|---|---|
| Control | 0.7 | 6.8 |
| A172 DIV 3 | 3.9 | 9.2 |
| A172 DIV 11 | 10.2 | 16.2 |
| PDB 100 nM | 12.7 | 19.9 |

Values of S-phase and proliferation index were determined using the RFIT model (CELLFIT software, Becton Dickinson) from DNA histograms. Incubation of cultures with glioma supernatants produced an increase in the proliferation index of astrocytes. Positive control in this assay was the phorbol ester 4-beta-phorbol-12,13-dibutyrate (PDB), an identified mitogen for adult human astrocytes (36).
DIV = days *in vitro*.

Supernatants derived from tumors in later stages of tumor growth were more effective mitogens. Supernatants from the slower growing U563-MG cell line were more effective at promoting proliferation of the cultured adult astrocytes than were those derived from the faster growing A172 glioma line. Figure 2 illustrates visual results from the GFAP and BrdU immunofluorescence technique; more double-positive cells are evident in cultures treated with the glioma supernatants.

Control astrocyte cultures not exposed to glioma products had very low basal rates of proliferation. Over a 48 h incubation period with 10 $\mu$M BrdU, 0.1–3% of astrocytes were GFAP and BrdU double-positive. This low rate is reflective of the slow turnover of adult astrocytes *in vivo* (42). In contrast, over a 48 h pulse with BrdU, 47% and 57% of fetal human and neonatal mouse astrocytes had BrdU in their nuclei, respectively (33).

*Cytofluorometric DNA Analysis:* To confirm the GFAP and BrdU assays, and to determine the specific cell cycle kinetics of the astrocyte cultures, PI (defined as the sum of %S + %$G_2$/M phases) were determined from DNA histograms (43) following incubation of the astrocyte cultures with glioma supernatants. Supernatants from the glioma line A172 increased the PI of astrocyte cultures; supernatants from cultures in later stages of growth increased the PI more effectively (Table 1). Positive assay control was the phorbol ester and astrocyte mitogen 4-beta-phorbol-12,13-dibutyrate (PDB) (36).

## Effects of Glioma Cyst Fluid on Astrocyte Proliferation

Cyst fluids collected from the patient with glioblastoma multiforme at three time points all induced proliferation of human adult astrocytes (Fig. 3). Similarly, cyst fluid from the second glioma patient resulted in an astrocytic proliferative response. The magnitude of the proliferative

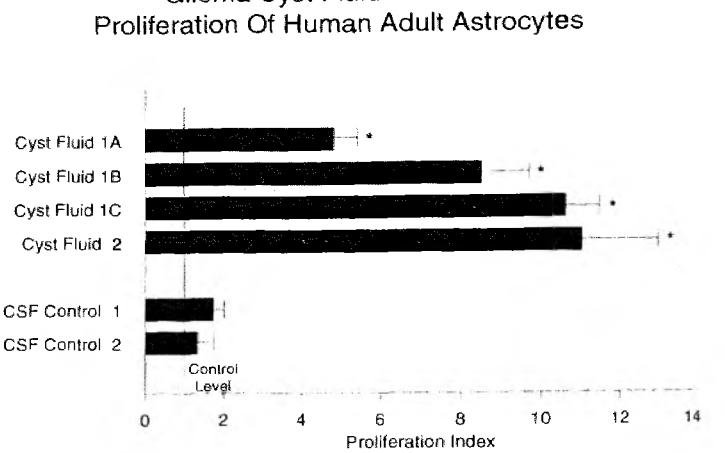


**Fig. 3.** Glioma cyst fluid increases proliferation of adult human astrocytes. Cyst fluid was collected from patient 1 at three time points, A, B and C at two week intervals. Cyst fluid was drained from patient 2 once. Values are mean ± SEM of four samples pooled from two adult human culture series, one with less than 20% microglial cells, and the other between 20–50% microglial cells. Control cultures (eight samples) had 3.2 ± 0.5% GFAP and BrdU double-positive cells. ★Significantly different from controls by a one-way analysis of variance (ANOVA), $p < 0.05$. Control level was proliferation index of 1 where no cyst fluid or cerebrospinal fluid (CSF) was added to sister cultures. Cerebrospinal fluid from two glioma-free and neurologically normal subjects served as additional controls.

effect (approximately tenfold) was equivalent to that produced by glioma culture supernatants at the concentrations used (compare Fig. 1 to Fig. 3). Cyst fluid from the patient with hemangioblastoma similarly enhanced proliferation of astrocytes (PI of 13.4 ± 1.1). In contrast, human cerebrospinal fluids from non-neoplastic subjects did not promote proliferation of adult human astrocytes.

## Effects of Purified Growth Factors on Astrocyte Proliferation

To attempt identification of the soluble mitogen(s) in glioma cell supernatants or cyst fluids, adult human astrocyte cultures were treated with factors known to be produced by gliomas. However, none of these (EGF, aFGF, bFGF, PDGF, a mixture of these, TGF-$\beta$, IL-6 and TNF-$\alpha$) enhanced proliferation of astrocytes (Table 2).

## Failure to Detect $\gamma$-IFN and IL-1 in Glioma Culture Supernatants

We have previously described $\gamma$-IFN (10–1,000 U/ml) and IL-1 (0.5–500 U/ml) to enhance DNA synthesis (PI of sevenfold at best) in adult human astrocytes (32). When the A172 glioma cell culture supernatant was analyzed for the presence of $\gamma$-IFN, none was detected; the positive control used for the assay, activated $CD8^+$ T-lymphocyte supernatant, had 107 U/ml. Similarly, no IL-1 was de-

tected in the A172 culture supernatant. Adult human microglial cells treated with lipopolysaccharide for 24 h released 202 U/ml of IL-1 into the culture medium.

## DISCUSSION

Malignant gliomas are known to produce a range of soluble factors which can influence their own growth. These molecules include PDGF, EGF and FGF (5–8, 12, 44), which can enhance glioma proliferation via autocrine and/or angiogenic mechanisms. The presence of insulin-like growth factor (IGF)-I and -II receptors (13) and amplification of the gene coding for the EGF receptor (5) have also been observed in higher grade gliomas. Furthermore, even though the immune status of patients with malignant gliomas is one of immunosuppression, glioma cells, at least *in vitro,* have the capacity to synthesize cytokines that include TGF-$\beta$, IL-1, IL-6 and TNF-$\alpha$ (10, 11, 14–16).

In addition to modulating their own growth, malignant gliomas can influence the function of non-glioma cells such as endothelial and lymphoid cells (9, 23–26, 45). In the present study, we provide evidence that glioma cells secrete soluble factors that can increase the proliferation of non-transformed astrocytes. This is seen not only in tissue culture-maintained glioma cell lines but also in tumor cyst fluids derived from two patients with glioblastoma multiforme. It is unclear why the cyst fluid from the glioma patient collected at three different time points promoted proliferation to different extents (Fig. 3); this may relate to the evolution of the glioma as is suggested by the glioma culture supernatant results (Fig. 1) where the mitogenic activity produced by glioma lines is more evident at later growth phases.

Candidate molecules for the mitogenic effect would include the glioma-produced growth factors (EGF, FGF, PDGF, IGF-I) which have been shown to promote proliferation of astrocytes (**neonatal rodent** or **fetal human**) by many laboratories including our own (17–22). However, these non-cytokine growth factors, alone or in combination, did not stimulate proliferation of **adult human** astrocytes (Table 2); we have previously shown that IGF-I was not a mitogen for adult human astrocytes (32). The failure of TNF-$\alpha$ in this report (in 5% FBS-containing medium) to enhance proliferation of adult human astrocytes in contrast to the report of Barna et al (31) (in 10% FBS-containing conditions) could be explained by different amounts of FBS in the test conditions; we have determined that TNF-$\alpha$ can be a mitogen for adult human astrocytes, but only if the culture medium contains a concentration equal to, or in excess of, 10% FBS (Tejada-Berges, Antel and Yong, manuscript in preparation).

Identified mitogens for adult human astrocytes include $\gamma$-IFN and IL-1, but these were not detected in the culture supernatant of A172 glioma cells. Heterogeneity among different glioma cell lines in the synthesis of cytokines has been documented (46), which may explain the non-detection of IL-1 in the culture supernatant of A172 while a different glioma line has been reported to synthesize IL-1 (15). Furthermore, some glioma cell lines do not synthesize cytokines *de novo* but will secrete these in response to activating agents, e.g. IL-1 (47). Thus, the identity of the astrocyte mitogen(s) released by gliomas in this study remains unknown. The current results do not rule out particular combinations of the test factors used in this study.

Interestingly, while glioma cell line-derived supernatants promoted proliferation of adult human astrocytes, we have previously reported that these supernatants suppressed lymphocyte functions at all stages of the glioma cell growth (9, 48). Furthermore, when culture supernatants from glioma cells at different stages of growth were placed on **glioma cells** (of the same cell line or on other glioma lines) in logarithmic growth phase, a biphasic response was observed: supernatants from cells in early or mid-logarithmic growth enhanced glioma growth rates while supernatants from cells at post-confluency inhibited cellular proliferation (48). These results indicate a capacity for gliomas to modulate their own growth in both a positive and negative manner, and also to affect alterations in the biologic properties of surrounding non-malignant glial cells and of tumor-infiltrating lymphocytes.

TABLE 2
Proliferative Response (BrdU Incorporation) of Human Adult Astrocytes (GFAP-positive Cells) to Test Factors

| Test factor | Concentration | % of GFAP and BrdU double-positive cells |
|---|---|---|
| Control | — | 0.8 ± 0.4 (18) |
| Epidermal growth factor | 20 ng/ml | 0.8 ± 0.5 (4) |
| Acidic fibroblast growth factor | 20 ng/ml | 0.3 ± 0.2 (4) |
| Basic fibroblast growth factor | 20 ng/ml | 0.4 ± 0.2 (4) |
| Platelet-derived growth factor | 200 mU/ml | 0 ± 0 (4) |
| Mixture* | | 0.1 ± 0.1 (4) |
| Transforming growth factor-$\beta$ | 20 ng/ml | 1.9 ± 1.0 (6) |
| Interleukin-6 | 100 U/ml | 2.1 ± 0.7 (4) |
| Tumor necrosis factor-$\alpha$ | 100 U/ml | 1.3 ± 0.1 (4) |

Values are mean ± SEM with number of coverslips analyzed shown in parentheses. On each coverslip, an average of 145 GFAP-positive astrocytes was counted. * Mixture refers to a combination of epidermal growth factor, acidic and basic fibroblast growth factor and platelet-derived growth factor at the concentrations indicated. None of the experimental values are statistically significant from controls (a one-way analysis of variance [ANOVA] with Duncan's multiple comparisons). We have previously reported that biopsy-derived cultured adult human astrocytes have a low rate of proliferation, as indicated by the 0.8% value in controls above (32).

The net functional autocrine and paracrine effects of gliomas vary with the growth phase of the tumor and presumably reflect complex variations in the wide array of soluble molecules which can be produced by the glioma.

It should be noted that proliferation of astrocytes is only one aspect of the process of gliosis, which includes other alterations such as astrocyte hypertrophy, increase in GFAP immunoreactivity and/or content, and several metabolic changes such as enhancement of various mitochondrial enzyme activities. Not all of these may occur in all injury models. For example, in the facial nerve resection model, increase in the number of GFAP-immunoreactive astrocytes is observed without any documented astrocytic proliferation (49); the rise in the number of GFAP-immunoreactive astrocytes is likely due to exposure of previously hidden epitopes as the astrocyte undergoes hypertrophy. Thus, it remains to be proven that proliferation of reactive astrocytes occurs in brain regions surrounding gliomas, but the data presented here would suggest that this occurs. The data that cyst fluid from a patient with hemangioblastoma also enhances proliferation of non-malignant astrocytes *in vitro* raise the possibility that gliosis surrounding an array of non-glial neoplasms possibly including metastatic lesions may also reflect, at least in part, soluble factor effects.

In conclusion, the data from this study expand the reported effects of soluble factors derived from glioma cells on the local environment, including endothelial, lymphoid and non-malignant glial cells. We suggest that the soluble astrocyte mitogen(s) released by glioma cells can contribute to the production of surrounding gliosis in the brain.

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