Response of low-passage human malignant gliomas in vitro to stimulation and selective inhibition of growth factor-mediated pathways

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The proliferation of many nonglial tumors in vitro depends on the presence of nanomolar concentrations of one or more growth factors. To define the growth factor requirements of malignant gliomas, the authors examined the response properties of four low-passage human malignant glioma lines to the following mitogens: epidermal growth factor (EGF), acidic and basic fibroblast growth factors (FGF's), insulin-like growth factor I (IGF-I), nerve growth factor (NGF), platelet-derived growth factor (PDGF), 12-0-tetradecanoyl-13-phorbol acetate (TPA), and serum. Each of the tumors showed increased deoxyribonucleic acid (DNA) synthesis (assessed by acid-precipitable [3H]-thymidine incorporation) in response to PDGF with a maximum effect at 50 ng/ml. Three tumors responded to EGF, three to IGF-I, two to acidic FGF, two to basic FGF, and two to TPA with maximum effects at 10, 50, 1, 1, and 10 ng/ml, respectively. None of the tumors responded to NGF. In the responsive tumors, optimum concentrations of EGF, IGF, TPA, acidic FGF, and basic FGF induced, at most, a two- to fourfold increase in [3H]-thymidine incorporation, which was only 30% to 50% of the response seen in 10% serum. In contrast, PDGF increased DNA synthesis eight- to 10-fold, equaling the effect of 10% serum. Measurements of cell proliferation also demonstrated a significant response to PDGF in each of the tumors. Appropriate concentrations of an anti-PDGF neutralizing antibody inhibited baseline DNA synthesis and proliferation in the absence of added growth factors, suggesting the possible role of PDGF in autocrine stimulation of these cells. However, this antibody produced only slight inhibition of serum-induced mitogenesis. Trapidil, an agent reported to inhibit the effects of PDGF, and polymyxin B, an inhibitor of protein kinase C, strongly inhibited baseline as well as PDGF- and serum-induced mitogenesis. It is concluded that, in the malignant gliomas studied, PDGF may be acting as a dominant mitogen to enhance DNA synthesis, and may function in autocrine stimulation. However, other factors contained in serum can also contribute to cell division.

KEY WORDS  •  glioblastoma multiforme  •  platelet-derived growth factor  •  protein kinase C  •  trapidil

The prognosis for patients with malignant gliomas treated with the currently available modalities of surgery, radiotherapy, and chemotherapy remains poor. Further improvements in patient survival may depend upon a deeper understanding of the growth factor pathways that regulate glioma proliferation. During the last several years, increasing interest has been focused on the role of various polypeptide growth factors such as epidermal growth factor (EGF), insulin-like growth factor I (IGF-I), nerve growth factor (NGF), platelet-derived growth factor (PDGF), fibroblast growth factors (FGF's), and the insulin-like growth factors (IGF's) as well as the role of protein kinase C-mediated pathways in the growth of malignant gliomas.

Several lines of evidence support the involvement of these polypeptide mitogens and protein kinase C in glioma proliferation. First, amplification and possible rearrangement of the EGF receptor gene, increased transcription of EGF receptor messenger ribonucleic acid (mRNA), and enhanced EGF receptor expression have been found in some malignant glioma specimens. Second, the observation that many malignant gliial lines not only have receptors for PDGF but also may express the cellular oncogene that codes for the cellular counterpart of the viral transforming gene that is present in many transformed cells.
Definitions of Abbreviations

BSA = bovine serum albumin
EGF = epidermal growth factor
FGS = fetal calf serum
FGF = fibroblast growth factor
FGF(a) = acidic FGF
FGF(b) = basic FGF
GFAP = glial fibrillary acidic protein
HBSS = Hanks' balanced salt solution
IGF = insulin-like growth factor
NGF = nerve growth factor
PBS = phosphate-buffered saline
PDGF = platelet-derived growth factor
TCA = trichloroacetic acid
TPA = 12-O-tetradecanoyl-13-phorbol acetate

for PDGF\textsuperscript{3,10,21} and synthesize and secrete a PDGF-like mitogenic factor\textsuperscript{20,44,45,46} suggests the occurrence of autocrine stimulation.\textsuperscript{6,10} Third, glial tumors are known to synthesize FGF\textsuperscript{2} and IGF\textsuperscript{9,52} and to have receptors for these hormones.\textsuperscript{7,14,15,28,56} suggesting the presence of multiple autocrine loops. Finally, EGF, FGF,\textsuperscript{2,12,32,49,58} PDGF,\textsuperscript{46,75} IGF\textsuperscript{19,31,36} and the protein kinase C-activating phorbol ester, 12-O-tetradecanoyl-13-phorbol acetate (TPA),\textsuperscript{40,75} have each been shown to lead to a pronounced mitogenic response in fetal and neonatal astrocytes, indicating that immature glia possess the capacity to respond to these hormones.

Since it has been reported that none of these agents is mitogenic in normal adult glia,\textsuperscript{75} this responsiveness is apparently lost during maturation but may be reacquired in neoplastic glia as these cells assume an undifferentiated phenotype. However, direct evidence supporting a role for one or more of these growth factor pathways in glioma proliferation has been presented in only a handful of reports.\textsuperscript{7,11,13,15,56,69} To address this issue in detail, we examined the effect of various mitogens, neutralizing antibodies directed against selected growth factors, and inhibitors of growth factor-mediated pathways on deoxyribonucleic acid (DNA) synthesis and cell division of low-passage human glioma lines grown in a chemically defined medium.

Materials and Methods

Cell Culture

Cultures of human malignant glial cells were derived from freshly obtained surgical specimens. The histological diagnosis of the tumors used was glioblastoma multiforme. Tumor tissue was finely minced, passed through 100-μm nylon mesh, and plated in modified Eagle's medium supplemented with Earle's salts, trace elements, and 10% fetal calf serum (FCS), and with the following antibiotics: penicillin G, 80 U/ml; streptomycin, 80 μg/ml; and fungizone, 0.20 μg/ml. Cells were maintained at 37°C in a humidified atmosphere with 5% CO\textsubscript{2} in air. After reaching confluence, the cells were treated with 0.25% trypsin in Hank's balanced salt solution (HBSS) and subcultured for immunohistochemical and growth factor studies. All studies were performed on cells in their second to fourth passages.

Immunohistochemical Examination

Immunohistochemical staining for glial fibrillary acidic protein (GFAP) was performed to confirm the glial nature of the cultured lines. Cells were plated onto glass slides, at 5 × 10\textsuperscript{5} cells/slide, in the above serum-supplemented medium and, on the 3rd day after seeding, were fixed with ice-cold 70% ethanol for 1 hour. The cells were then washed in phosphate-buffered saline (PBS), rendered permeable by treatment with 0.1% Triton-X in PBS for 10 minutes, washed again, and incubated for 1 hour in 10% horse serum/PBS to minimize nonspecific binding of the primary antibody. The monolayer was then incubated with a rabbit polyclonal antibody to GFAP,\textsuperscript{*} 1:1000 dilution in 10% horse serum/PBS, for 2 hours at room temperature. Control cells were incubated with horse serum/PBS alone. After extensive washing in PBS, cells were labeled using a horseradish peroxidase-conjugated avidin-biotin peroxidase complex kit.* Antibody binding was detected using an intensified diaminobenzidine reaction.\textsuperscript{25} Each of the four lines used in this study had greater than 98% GFAP-positive cells.

Measurement of Growth Factor-Induced DNA Synthesis

Cells were seeded onto 96-well round-bottomed microtiter trays (2 × 10\textsuperscript{5} cells in 200 μl of serum-supplemented medium/well), and after a 6-hour attachment period, were washed three times in serum-free MCDB 105 medium\textsuperscript{22,37} with antibiotics and grown for 24 hours under serum-free conditions. This medium has been previously shown to maintain the viability of malignant glial cells for up to 7 days in culture. Cells were then incubated for 24 hours with the various concentrations of the following mitogens in MCDB 105 with 0.1% bovine serum albumin (BSA): EGF (0.1 to 50 ng/ml), acidic FGF (0.1 to 50 ng/ml), basic FGF (0.1 to 50 ng/ml), IGF factor I (IGF-I, 1 to 200 ng/ml), nerve growth factor (NGF, 1 to 500 ng/ml), PDGF B chain dimer (1 to 200 ng/ml), TPA (0.1 to 50 ng/ml), or FCS (1% and 10%). Control cells were incubated without exogenous mitogens in MCDB with BSA. All studies were performed in triplicate. Cells were then washed and incubated for 4 hours in serum-free medium with [\textsuperscript{3}H]-thymidine (1 μCi/ml). After extensive washing to remove unbound radioactivity, cells were detached by a 30-minute incubation at room temperature in 2.5% trypsin/0.85% NaCl and centrifuged for 20 minutes at 2000 G. The pellet was suspended in 6%

* Avidin-biotin peroxidase complex kit manufactured by Vector Laboratories, Burlingame, California.
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Fig. 1. [3H]-thymidine incorporation in four human malignant gliomas (F.M., R.E., L.C., and E.S.) in response to growth factors and serum. Concentrations of growth factors shown are: EGF, 10 ng/ml; FGF(a), 1 ng/ml; FGF(b), 1 ng/ml; IGF, 50 ng/ml; NGF, 100 ng/ml; PDGF, 50 ng/ml; and TPA, 10 ng/ml (see Definitions of Abbreviations table). Significant difference from control, unpaired Student's t-test: * = p < 0.05; ** = p < 0.01; *** = p < 0.001.

trichloroacetic acid (TCA) and 0.03% unlabeled thymidine. Following a 30-minute incubation period, the acid precipitate was centrifuged; the pellet was then suspended and incubated in fresh TCA solution, and centrifuged again. The precipitate was solubilized in 200 µl of 0.5 N NaOH with 0.1% BSA, transferred to scintillation vials, and mixed with 5 ml of Ecolite scintillation cocktail. Incorporated radioactivity was measured in a beta scintillation counter.

Proliferative Response to Added Growth Factors

Cells from three of the tumors (LC, ES, and RE) were seeded into 35-mm Petri plates (5 × 10⁶ cells/plate) in serum-supplemented medium, and after a 6-hour attachment period, were grown under serum-free conditions (MCDB 105 with 0.1% BSA) for 24 hours. (The fourth tumor did not survive long enough in culture for these studies to be performed.) Cell counts were rechecked 24 hours after plating to determine the total number of cells present before the addition of growth factors. Growth factors or serum were then added, and the cells were grown for an additional 96 hours. Control cells received no growth factors or serum. All studies were performed in triplicate. Cells were then detached with 0.25% trypsin in HBSS and counted in a hemacytometer.

Effect of Growth Factor Antibodies and Inhibitors

To examine the effect of selective blockade of growth factor-mediated pathways on control (no added growth factors) and PDGF- and serum-stimulated DNA synthesis in the LC, ES, and RE tumors, we repeated the [3H]-thymidine incorporation studies in the presence of the following neutralizing antibodies to various growth factors and inhibitors of selected growth-mediated pathways: 1) anti-EGF, 2) anti-PDGF, 3) anti-acidic FGF, 4) anti-basic FGF, 5) anti-NGF, 6) trapidil (5-methyl-7-diethylamino-s-triazol [1,5a] pyrimidine),‡ an inhibitor of PDGF,³⁰,⁴⁷,⁵⁰,⁶³ and 7) polymyxin B, an inhibitor of protein kinase C activation.²⁹,³⁶,⁶⁴,⁷²,⁷⁴,⁷⁸ Anti-growth

† Ecolite scintillation cocktail manufactured by ICN Biomedicals, Cleveland, Ohio.

‡ Trapidil was kindly provided by Squibb Inc., Princeton, New Jersey.
factor antibodies were used at concentrations of 2.5, 10, or 40 μg/ml; 10 to 40 μg/ml of each has previously been found to inhibit by more than 90% the responses to exogenous administration of optimal concentrations of their respective growth factors in the EGF-, PDGF-, and FGF-responsive T98G malignant glioma line (data not shown). In this cell line, the anti-PDGFAntibody blocked the effects of both recombinant B chain homodimeric PDGF and platelet-derived (presumably A-B heterodimeric) PDGF. Trapidil was used at concentrations of 10 and 100 ng/ml, and 1, 10, and 100 μg/ml. Polymyxin B was used at concentrations of 1, 5, 10, 20, and 50 μg/ml.

Cell proliferation studies in control and PDGF- and serum-stimulated cells from the LC, ES, and RE gliomas were also performed in the presence of selected concentrations of the above inhibitors.

Results

The responses of the four human gliomas to optimum concentrations of the different growth factors and to serum are shown in Fig. 1. Platelet-derived growth factor produced a significant enhancement of DNA synthesis (p < 0.05, unpaired Student’s t-test) in all four tumors, with maximum effects at 50 ng/ml. Three tumors showed a significant dose-dependent response to EGF, three to IGF-I, two to acidic FGF, two to basic FGF, and two to TPA, with maximum effects at 10, 50, 1, 1, and 10 ng/ml, respectively. None of the tumors responded to NGF. In the responsive tumors, optimum concentrations of EGF, IGF, TPA, and FGF induced a two- to fourfold increase in [3H]-thymidine incorporation, which was only 30% to 50% of the response seen in 10% serum. In contrast, PDGF increased DNA synthesis eight- to 10-fold over control, generally approximating the effect of 10% serum. The dose-response curves for each of the tumors to PDGF are illustrated in Fig. 2.

Studies of cell proliferation in each of the three tumors tested confirmed the pronounced mitogenic effect of PDGF (Fig. 3). Whereas control cells increased in number by 10% to 74% in the absence of added growth factors, cells treated with PDGF demonstrated a 110% to 230% increase in number. In each of the tumors, the response to PDGF approximated that seen with 10% serum. Epidermal growth factor also produced a significant increase in cell number over control levels in each of the three tumors, but the magnitude of the response was much smaller than that obtained with PDGF. The responses to the other mitogens varied among the different cell lines and, in general, were comparatively small.

As expected, the anti-PDGFAntibody neutralizing antibody strongly inhibited DNA synthesis and proliferation induced by exogenously administered PDGF (data not shown). In addition, this antibody significantly inhibited baseline DNA synthesis (Fig. 4) and cell proliferation (Fig. 5) in cells not receiving exogenous growth factors or serum. However, it only slightly inhibited the effect of serum on DNA synthesis (Fig. 6) and cell proliferation (Fig. 7). Although the degree of inhibition was statistically significant in each of the lines tested,
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Fig. 3. Cell proliferation in three human malignant gliomas in response to growth factors and serum. Twenty-four hours after plating (prior to the addition of growth factors), mean cell counts (± standard error of the mean) for the three tumors were: L.C. = 6.08 ± 0.64 × 10⁴; R.E. = 5.46 ± 0.66 × 10⁴; and E.S. = 6.88 ± 0.44 × 10⁴. Growth factor concentrations are as described in the legend to Fig 1. See Definitions of Abbreviations table.

Fig. 4. Effect of an anti-platelet-derived growth factor antibody (PDGF Ab), polymyxin B (PMB), and trapidil (Trap.) on baseline DNA synthesis in three human malignant gliomas (L.C., R.E., and E.S.).

Discussion

Effect of PDGF on Low-Passage Tumor Lines

The data presented here suggest that PDGF is a dominant mitogen for low-passage human malignant gliomas in vitro. Platelet-derived growth factor causes a substantial enhancement of both DNA synthesis and cell proliferation which, at optimum concentrations, approximates the response obtained with 10% serum. A similar effect of PDGF on the proliferation of low-passage cultures of malignant glial tumors has recently been noted by Frappaz, et al.¹¹ and Westphal, et al.⁶³ Other evidence supporting a major role for PDGF in glioma proliferation is provided by the observations of several groups that gliomas not only possess receptors for PDGF, but also secrete a PDGF-like mitogenic factor,⁴⁴,⁵⁰,⁵¹,⁵⁴ creating a potential PDGF-mediated...
autocrine loop. Our finding that both an antibody against PDGF and trapidil, an agent reported to inhibit the action of PDGF, inhibited proliferation of control cells is consistent with the hypothesis that a PDGF-mediated autocrine loop does indeed contribute to the basal mitotic rate we observed in our cells when they were maintained in a serum- and growth factor-free environment.

It remains uncertain whether the A-A, B-B, or A-B chain forms of PDGF are responsible for this autocrine stimulation since glioma-derived cell lines are known to express mRNA encoding both the A and B chains of PDGF. Since all three forms of PDGF may activate glioma PDGF receptors, more selective inhibitors targeted individually at the A-A, B-B, and A-B chain dimers are needed to elucidate their relative contributions to the autocrine growth stimulation. Furthermore, it remains uncertain whether the autocrine activation in these tumors occurs strictly extracellularly or both extracellularly and intracellularly. Huang, et al. reported that in simian sarcoma virus-transformed NRK and NIH 3T3 cells, which secrete p28, a transforming protein homologous to the PDGF B chain, [3H]-thymidine incorporation was inhibited (but not eliminated) by the administration of an anti-PDGF antibody. This suggests that some of the autocrine stimulation in these cell lines resulted from interaction of p28 with cell-surface receptors. However, other studies have indicated that the major component of autocrine activation in some lines may occur intracellularly. Our finding that baseline DNA synthesis and cell proliferation in three human glioma lines was largely, but not totally, eliminated by the PDGF anti-

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body suggests that in these tumors a significant component of the autocrine activation occurs by extracellular interaction of PDGF with its receptor; however, it leaves open the possibility that intracellular activation may also be occurring.

The Effect of Inhibition of PDGF Pathways

Although PDGF appears to play a major role in controlling glioma proliferation in a chemically defined medium, our finding that serum-induced DNA synthesis and proliferation was only slightly affected by the anti-PDGF antibody indicates that other components of serum also modulate glioma growth. Other investigators have shown that EGF, FGF, and IGF are all mitogens for certain human gliomas in vitro. Although the responses to these other agents varied among the four tumors examined in this study and exerted, at best, only a fraction of the effect noted with PDGF, the composite effect of the different growth-stimulating constituents of serum is apparently sufficient to support an intense mitogenic response in the absence of PDGF. The fact that trapidil was able to strongly inhibit both baseline and serum-stimulated DNA synthesis and proliferation indicates that the inhibition of mitogenesis caused by this agent involves more than the PDGF-mediated pathways.

The Role of Protein Kinase C

Our finding that the protein kinase C inhibitor, polymyxin B, produced a significant dose-dependent inhibition of control, as well as PDGF- and serum-stimulated DNA synthesis and proliferation, suggests that protein kinase C-mediated pathways are involved in the proliferation of these low-passage tumors. This is in agreement with our previous work on established human glioma lines, in which we found a pronounced, reversible cytostatic effect of polymyxin B (with no alteration of cell viability, as assessed by trypan blue exclusion). Although it cannot be established with certainty that the inhibitory effects of polymyxin B are due solely to its effect on protein kinase C, the concentrations used in this study have been shown to produce fairly selective inhibition of protein kinase C with little effect on the protein kinases sensitive to circulating adenosine monophosphate, circulating glucose monophosphate, or calmodulin, and have been found to inhibit other protein kinase C-dependent processes in cultured cells. Although two of the tumors inhibited by polymyxin B did not show a significant response to direct activation of protein kinase C by the phorbol ester TPA, it is conceivable that protein kinase C-mediated pathways may be involved indirectly as a part of the mitogenic signal initiated by the binding of other growth factors such as PDGF and EGF to their respective receptors. In this regard, both EGF and PDGF are known to induce tyrosine phosphorylation of phospholipase C and to stimulate phospholipase C activity, which this leads to enhanced turnover of phosphatidylinositol and increased production of diacylglycerol, an endogenous ligand for the protein kinase C receptor. It has therefore been suggested that the mitogenic effects of agents such as PDGF may result, in part, from protein kinase C-mediated phosphorylation of appropriate substrates.

Conclusions

Human glioblastoma cell lines in culture show a consistent and dramatic mitogenic response to PDGF. Although these tumors also responded to a number of other growth factors, the magnitude of the responses was comparatively small and the pattern of responsiveness varied significantly among different gliomas. In addition, our glioma lines were able to proliferate in vitro in the absence of exogenous growth factors or
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serum, probably because of autocrine stimulation by mitogens such as PDGF. Unfortunately from a therapeutic standpoint, the selective blockade of the binding of a single growth factor, such as PDGF, to its receptor may not prove useful for inhibiting glioma growth in vivo, given that the effect of serum could not be inhibited completely by any one of the anti-growth factor antibodies tested. Other strategies that capitalize on the presence of high numbers of certain growth factor receptors on malignant gliomas, such as the use of toxin- or radionuclide-conjugated ligands, may be more useful. An encouraging observation is the finding that inhibition of protein kinase C appears to strongly inhibit baseline as well as growth factor- and serum-induced DNA synthesis and cell proliferation. It remains to be seen whether protein kinase C inhibition will prove clinically effective, without unacceptable levels of toxicity.

References


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