Response of malignant glioma cell lines to activation and inhibition of protein kinase C-mediated pathways

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To evaluate the role of protein kinase C-mediated pathways in the proliferation of malignant gliomas, this study examined the effect of a protein kinase C (PKC)-activating phorbol ester (12-O-tetradecanoyl-13-phorbol acetate or TPA) and a protein kinase C inhibitor (polymyxin B) on deoxyribonucleic acid (DNA) synthesis of malignant glioma cells in vitro. A serum-free chemically defined medium, MCDB 105, was employed for all studies. Two established human malignant glioma cell lines (T98G and U138), two rat glioma lines (9L and C6), and two low-passage human glioma lines (obtained from surgical specimens) were studied. With the exception of the C6 line, all tumors responded in a dose-dependent fashion to nanomolar concentrations of TPA with a median effective dose that varied from 0.5 ng/ml for the U138 glioma to 1 ng/ml for the T98G glioma. At optimal concentrations (5 to 10 ng/ml), TPA produced a two- to five-fold increase in the rate of DNA synthesis (p < 0.05) as assessed by incorporation of 3H-thymidine. However, TPA had no additive effect on the mitogenic response produced by epidermal growth factor (EGF) or platelet-derived growth factor (PDGF). Inhibition of PKC using the antibiotic polymyxin B (20 μg/ml) abolished the TPA-induced mitogenic response in the five responsive lines tested. In two tumors (U138 and 9L), polymyxin B also eliminated EGF-, PDGF-, and serum-induced DNA synthesis as well as abolishing baseline DNA synthesis. These cells remained viable, however, as assessed by trypan blue exclusion; after removal of polymyxin B from the medium, they were able to resume DNA synthesis in response to TPA and serum. In the three other tumors (T98G and the two low-passage human glioma lines), growth factor-induced and serum-induced DNA synthesis were inhibited by approximately 25% to 85%.

It is concluded that PKC-mediated pathways affect DNA synthesis in the human malignant glial tumors studied. The response of the glioma cells to TPA is similar to the responses seen in fetal astrocytes, but differs significantly from those reported for normal adult glial cultures. Because the response of the 9L glioma to TPA is similar to the responses seen in the human tumors, the 9L rat glioma model may prove useful for examining the role of PKC-mediated pathways in controlling glioma growth in vitro.

Key Words - growth factor - glioma - phorbol ester - polymyxin B - protein kinase C

UNDE.APPLICATION requires critical analysis of the biochemical events controlling cell proliferation. Recent research in this area has focused on the role of the second messenger protein kinase C (PKC), a calcium-sensitive phospholipid-dependent protein kinase, in regulating normal and abnormal cell differentiation and proliferation. Agents such as the potent phorbol ester, 12-O-tetradecanoyl-13-phorbol acetate (TPA), which binds to and directly activates PKC, or enhances proto-oncogene expression, and induce many characteristics associated with the transformed phenotype including morphological transformation, enhanced deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein synthesis, increased secretion of plasminogen activator, and increased polyamine synthesis. Furthermore, PKC activation is intimately linked to a number of growth factor-mediated pathways. Agents activating PKC, such as phorbol esters, alter the affinity of the epidermal growth factor (EGF) receptor for EGF by inducing phosphorylation of the EGF receptor. Conversely, EGF enhances expression of the c-sis gene encoding platelet-derived growth factor (PDGF), and acts synergistically with EGF and PDGF to enhance proliferation.
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and PDGFn, enhance turnover of phosphatidylinositol, leading to increased production of diacylglycerol, an endogenous ligand for the PKC receptor. Other oncogenes and transforming gene products also have been shown to stimulate phosphatidylinositol turnover. To date, much of the work in this area has been performed in hematopoietic, fibroblastic, and epidermoid cell lines, and relatively little is known about the role of PKC in the growth and development of malignant glial tumors.3,4

Under normal circumstances, expression of PKC is developmentally regulated in the central nervous system (CNS). In the developing CNS, PKC is found in astrocytes, oligodendroglia, and neurons in the adult CNS, PKC expression is normally restricted to neuronal elements. Accordingly, phorbol esters have been reported to have a major impact on the normal differentiation of oligodendrocytes and astrocytes and to be potent mitogens for developing astrocytes; however, they have little effect on the proliferation of adult glia. We questioned whether this developmental regulation of TPA responsiveness was lost in malignant glia and, hence, whether proliferation of malignant glial cells was enhanced by activation of PKC-mediated pathways. For potential therapeutic intervention, we sought to establish whether a growth factor-induced proliferative response could be blocked by inhibitors of PKC. In the past, a major problem in evaluating the response of cultured glioma cells to exogenous growth factors has been the lack of a suitable serum-free culture system. Although many chemically defined media lacking endogenous growth factors have been formulated, cells grown in such media often show decreased viability and respond poorly to added growth factors with DNA synthetic rates only a fraction of those observed in serum-supplemented media. Serum contains numerous naturally occurring growth factors, however, making it impossible to conduct a meaningful analysis of growth factor responsiveness. In the present study we employed the serum-free chemically defined medium MCDB 1054,15 to examine the in vitro response of human and rat malignant glioma lines to the PKC-activating phorbol ester, TPA, and to a PKC inhibitor, polymyxin B.

Materials and Methods

Cell Culture

The human glioblastoma cell lines U138 and T98G (derived from spontaneously occurring human malignant gliomas) and the N-nitrosomethylurea-induced rat glioma lines C6 and 9L were initially grown in modified Eagle's medium (MEM) with 10% fetal calf serum (FCS). Cultures were established in 75-sq cm Costar culture flasks maintained at 37°C in a humid atmosphere with 5% CO2 in air, and were subcultured every 4 to 6 days by treating the monolayers with 0.25% trypsin in Hanks' balanced salt solution.

Low-passage cultures of two human malignant gliomas (BB and PB) were derived from freshly obtained surgical specimens which were finely minced and mechanically dissociated at 37°C in MEM/10% FCS. The BB and PB cells were grown under the same conditions as the established cell lines. The glial nature of the cultured cells was confirmed by immunohistochemistry for glial fibrillary acidic protein (GFAP), a cell type-specific marker for astrocytes; in both cultures, greater than 99% of the cells were positive for GFAP. Cells were used in their second or third passage.

Analysis of Dose-Response to TPA

Log phase cells from the established rat and human glioma lines or low-passage cultures of human malignant gliomas obtained at surgery were plated at 2 × 104 cells/200-μl well in 96-well Costar microtiter trays. After a 6-hour attachment period, the cells were washed extensively with MCDB 105 and maintained for 24 hours under serum-free conditions. Serum (0.5% to 10%) or TPA (0.01 to 100 ng/ml) was then added. The T98G, 9L, and primary human glioma lines were incubated with serum or TPA for 24 hours; because of their longer cell-cycle time, the U138 and C6 cells were incubated for 48 hours. Control cells received no serum or growth factors. All studies were performed in triplicate. Cells were then washed and incubated for 4 hours in serum-free medium with 3H-thymidine (specific activity 5 Ci/mmol; final concentration 1 μCi/ml). After extensive washing to remove unbound radioactivity, cells were detached by 30-minute incubation at room temperature in 2.5% trypsin/0.85% NaCl and treated for 30 minutes with 6% trichloroacetic acid and 0.03% unlabeled thymidine. The acid precipitate was washed three times and solubilized in 0.5 M NaOH with 0.1% bovine serum albumin. Radioactivity was measured in a beta scintillation counter.

Interaction of EGF and PDGF with TPA

In previous studies we have found that the polypeptide growth factors, EGF and PDGF, are each mitogenic for malignant glial cells and, when administered simultaneously, produce an additive response. We questioned whether providing these cells with EGF or PDGF in conjunction with TPA would also produce an enhanced mitogenic response. Log phase cells were plated as described above and, after attachment, were washed extensively with MCDB 105, and maintained for 24

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* Rat glioma cell lines C6 and 9L obtained from American Type Culture Collection, Rockville, Maryland; fetal calf serum obtained from GibCO Laboratories, Grand Island, New York.

† Culture flasks manufactured by Costar, Cambridge, Massachusetts.

‡ MCDB 105 and TPA obtained from Sigma Chemical Co., St. Louis, Missouri.

hours under serum-free conditions. Then EGF (20 ng/ml) or PDGF (50 ng/ml) was added with TPA (0.01 to 100 ng/ml) for 24 or 48 hours. Control cells received EGF, PDGF, or various concentrations of TPA alone. Tritiated thymidine incorporation was measured as described above. All studies were performed in triplicate.

Inhibition of Baseline and Growth Factor-Induced Synthesis

To determine the effect of inhibiting the second messenger, PKC, on growth factor-mediated DNA synthesis, cells were treated for 24 or 48 hours with varying concentrations (0.02 to 20 \( \mu g/ml \)) of the PKC inhibitor polymyxin B along with PDGF, EGF, TPA, or serum. Tritiated thymidine incorporation was determined as described above. To determine baseline DNA synthesis, one group of control cells received neither polymyxin B nor growth factors or serum. Another group of cells received polymyxin B without added growth factors or serum to evaluate the effect of the inhibitor on baseline DNA synthesis.

Results

Response of Cultured Glioma Cells to TPA

Each of the human lines responded in a dose-dependent fashion to nanomolar concentrations of TPA. The median effective dose (ED\(_{50}\)) ranged from 0.5 ng/ml for the U138 line to 1 ng/ml for the T98G line (Fig. 1). The two low-passage human glioma lines responded in a similar fashion as did the 9L glioma. At optimal concentrations of TPA (5 to 10 ng/ml), DNA synthesis was increased two- to fivefold over baseline (\( p < 0.05 \), unpaired Student's t-test) (Fig. 2). This is equivalent to the response seen in 1% FCS and is approximately 30% to 50% of the response seen with 10% FCS. The C6 line showed no response to TPA in concentrations as high as 100 ng/ml, although this line remained profoundly sensitive to serum, with a threefold enhancement of \(^3\)H-thymidine incorporation in 10% FCS.

Simultaneous stimulation with TPA and either EGF (Fig. 2) or PDGF did not produce a significantly additive mitogenic response in any of the five TPA-responsive tumors, although the ED\(_{50}\) for TPA was decreased in the T98G line to 0.5 ng/ml. No mitogenic response to any combination of these agents was noted in the C6 line.

Inhibition of Baseline and Growth Factor-Induced DNA Synthesis

At concentrations ranging from 0.02 to 20 ng/ml, polymyxin B produced a dose-dependent inhibition of the mitogenic response induced by the PKC activator, TPA, in all five responsive lines. At 20 \( \mu g/ml \) polymyxin B inhibited DNA synthesis in the T98G line and in the two low-passage glioma lines to baseline levels obtained in the absence of added growth factors (Fig. 3). However, in the U138 (Fig. 4) and 9L lines, DNA synthesis was inhibited well below baseline levels despite the presence of optimal concentrations of TPA. Polymyxin B also abolished growth factor- and serum-induced DNA synthesis in these tumors (Fig. 5). Even with high concentrations of PDGF, EGF, or serum, the radioactivity incorporated in these cultures was less than 2% of baseline levels obtained in control cells that had received neither polymyxin B nor added growth factors. To determine whether this inhibitory effect was due to a cytotoxic action of the inhibitor, cell viability was assessed by trypan blue exclusion and found to be greater than 98% in all specimens tested. Furthermore, if the inhibited cells were washed extensively in media free of polymyxin B and then incubated for 48 to 72 hours with either TPA or serum to reactivate PKC, cell proliferation resumed.

In the three other human tumors (T98G and the two low-passage glioma lines), growth factor- and serum-induced DNA synthesis was inhibited by approximately 25% to 85%. Polymyxin B had no effect on DNA synthesis in the TPA-unresponsive C6 line.

Discussion

Protein Kinase C in Normal CNS Tissue

The phospholipid- and calcium-dependent protein kinase, PKC, is found ubiquitously in the developing
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**Fig. 2.** A comparison of $^{3}$H-thymidine incorporation in the absence of added growth factors (control) and in the presence of TPA (5 ng/ml), TPA (5 ng/ml) plus epidermal growth factor (EGF) (20 ng/ml), or 10% fetal calf serum (FCS) in six cultured malignant gliomas. Error bars represent standard error of the mean.

Brain and is expressed at high levels in adult neurons. $^{52,53}$ At least seven subtypes of PKC have been found, each with a distinct pattern of localization in different tissues. $^{52,55}$ Protein kinase C forms one element in a cascade of pathways involved in the transduction of extracellular signals to the interior of a cell. Under normal conditions, binding of a ligand to appropriate cell surface receptors initiates phospholipase C-mediated hydrolysis of inositol phospholipids, producing inositol phosphates which mobilize intracellular cal-

**Fig. 3.** Graph showing the effect of varying concentrations of polymyxin B (PMB) on TPA-induced DNA synthesis in the T98G glioma. Baseline $^{3}$H-thymidine incorporation (in the absence of added growth factors) was $7.04 \pm 0.61 \times 10^{3}$ cpm. A similar pattern of inhibition was obtained in the PB and BB low-passage glioma lines. Error bars represent standard error of the mean.

**Fig. 4.** Graph showing the effect of varying concentrations of polymyxin B on TPA-induced DNA synthesis in the U138 glioma. Baseline $^{3}$H-thymidine incorporation (in the absence of added growth factors) was $2.4 \pm 0.2 \times 10^{3}$ cpm. A similar pattern of inhibition was obtained in the 9L rat glioma. Error bars represent standard error of the mean.
cium stores and diacylglycerol which binds to and activates PKC. In turn, PKC phosphorylates a wide range of cellular proteins. Recent studies have confirmed that the tumor-promoting phorbol esters also bind to and activate PKC, and that the majority of their effects on cell proliferation and differentiation are mediated by their potent, long-lasting activation of PKC. These exogenous agents have therefore been extensively employed for studying PKC-mediated pathways in vitro.

It has become increasingly apparent that PKC plays a major role in various aspects of normal cellular differentiation and proliferation in the developing brain. In cultures of normal fetal astrocytes, phorbol esters induce the formation of cellular processes and increased expression of glutamine synthetase, which are associated with the differentiated phenotype. Similarly, in oligodendrocytes in vitro, phorbol esters induce morphological differentiation and increased phosphorylation of myelin basic protein. In addition, these agents produce a pronounced proliferative response in neonatal glia. In the adult brain, in situ hybridization studies have failed to demonstrate PKC messenger RNA (mRNA) associated with glial cells. Accordingly, phorbol esters have no effect on the proliferation of adult glia. Taken together, these findings suggest that PKC plays a role in the growth of developing glial cells but has limited expression in normal mature glia.

**Protein Kinase C Activation in Malignant Glial Cells**

Our data demonstrate that, as with developing astrocytes, TPA is a potent mitogen in vitro for malignant glioma lines. The role of PKC-mediated pathways in glioma proliferation has not been previously described, although several bodies of evidence have suggested that such pathways are involved. It has been shown that TPA activates transcription of the cellular oncogenes c-myc and c-fos in cultured astrocytoma cells, a preliminary step in growth factor-induced mitogenesis. In addition, this agent enhances expression of c-sis mRNA (encoding for PDGF) in certain glioblastoma cell lines while having no significant effect on normal glial cells. Since glioma cells express receptors for PDGF and show a pronounced mitogenic response to exogenous PDGF, PKC-mediated enhancement of PDGF synthesis may provide one mechanism for autocrine growth stimulation in these cells.

The demonstration in the current study of a mitogenic response to TPA in established human and rat glioblastoma lines and in two low-passage human glioma lines was facilitated by the use of a serum-free culture medium. In the presence of even low concentrations of serum (0.5%) the response to TPA was blunted by an increase in the baseline 3H-thymidine incorporation, and with higher concentrations of serum (1% or 10%) no statistically significant mitogenic response could be detected (data not shown). Although a number of media have been described for the serum-free culture of glial cells, the use of MCDB 105 is favored for its low cost and its ability to sustain short-term glioma cell growth in vitro in the absence of added proteins or hormones. Since the cells remain responsive to exogenously administered mitogens, the impact of various agents on glioma cell growth can be readily determined.

The finding that in the TPA-responsive malignant gliomas the mitogenic effects of TPA and EGF or PDGF were not additive suggests that these polypeptide growth factors and TPA may share certain elements of a common pathway leading to the induction of cell proliferation. In other cell systems, both EGF and PDGF have been shown to increase phosphatidylinositol turnover, thus increasing production of the endogenous PKC-activator, diacylglycerol. While both EGF and PDGF are known to initiate a multitude of other effects in vitro, at least a portion of their mitogenic properties in glioma cells may be attributable to enhancement of PKC-mediated pathways. It is of interest in this regard that the C6 line, which showed no response to either EGF or PDGF, also failed to respond to TPA.
Protein Kinase C Inhibition

To further define the role of PKC in glioma proliferation, we examined the effect of selective PKC inhibition on DNA synthesis. Polymyxin B, a cyclic polycationic peptide antibiotic that interacts with the cell membrane, inhibits PKC activity with an inhibition constant (Ki) of 1.8 to 7 μM. This agent has been extensively employed in other cell systems as a PKC inhibitor and has the advantage of relative nontoxicity in tissue culture conditions and comparative specificity for the PKC receptor. Mazzei, et al., and Wrenn and Wooten have shown that, at the concentrations used in this study, polymyxin B had little effect on the calmodulin-sensitive protein kinase and virtually no effect on protein kinases dependent on cyclic adenosine monophosphate and cyclic guanosine monophosphate. In the present study, polymyxin B produced a dose-dependent inhibition of TPA-induced DNA synthesis in each of the five TPA-responsive gliomas. Furthermore, in two of these lines, polymyxin B also completely and reversibly abolished EGFr-, PDGF-, and serum-induced DNA synthesis as well as baseline DNA synthesis. In the other three lines, polymyxin B produced a partial inhibition of EGFr-, PDGF-, and serum-induced DNA synthesis with approximately a 25% to 85% decrease in the mitogenic response to these agents. In the TPA-unresponsive C6 line, polymyxin B had no such effect on DNA synthesis.

Conclusions

Taken together, our data suggest the importance of PKC-mediated pathways in controlling glioma cell division. The response of the established human glioma lines and low-passage glioma lines to exogenously administered TPA is similar to the mitogenic effects of this agent on fetal and neonatal astrocytes grown under serum-free conditions. Presumably, these cells have acquired immature features during the transformation that enable them to proliferate in response to PKC activation. However, the observation that DNA synthesis in the C6 rat line is unaffected by PKC activation or inhibition indicates that sensitivity to TPA and polymyxin B is not an invariable property of malignant glial cells in culture, but rather is a feature that may be present in only a portion of malignant glioma lines. The finding that (like the four human tumors studied) the chemically induced rat glioma line 9L is also sensitive to PKC activation and inhibition, suggests that the rat 9L glioma model may prove useful for evaluating the effect of selective PKC inhibition on glioma growth in vivo.

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