Disparity in Expression of Protein Kinase C \( \alpha \) in Human Glioma versus Glioma-derived Primary Cell Lines: Therapeutic Implications

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ABSTRACT

Intracellular signal transduction by the protein kinase C (PKC) family of enzymes plays a critical role in carcinogenesis and cellular growth regulation. Recent studies have suggested that the PKC isoform \( \alpha \) may be a critical target for antiglioma therapy in humans (G. H. Baltuch et al., Can. J. Neurol. Sci., 22: 264–271, 1995).

We studied the expression and subcellular distribution of the PKC \( \alpha \) isoform in human high- and low-grade gliomas and also in glioma-derived cell lines with immunoblot analyses. Cell lines derived from high-grade gliomas expressed higher levels of PKC \( \alpha \) than did cell lines derived from low-grade gliomas. In glioblastoma-derived cell lines, PKC \( \alpha \) was mainly expressed in the soluble (cytosolic) fraction, indicating an inactive state of the enzyme. When analyzed in freshly frozen samples from human gliomas, the expression of PKC \( \alpha \) was at similar levels in high- and low-grade tumors and was also similar to the levels in normal brain tissue controls. The PKC partial antagonist bryostatin 1, currently undergoing Phase II testing in patients with malignant gliomas, was capable of specifically down-regulating PKC \( \alpha \) in vitro in glioblastoma-derived cell lines. However, this was not associated with significant growth inhibition.

We conclude that the observed overexpression of PKC \( \alpha \) in glioblastoma-derived cell lines may be an artifact of in vitro growth. Furthermore, we conclude that expression of PKC \( \alpha \) in glioma-derived cell lines is not essential for cellular growth in vitro because down-regulation of PKC \( \alpha \) following treatment with bryostatin 1 was not associated with growth inhibition.

INTRODUCTION

PKC\( ^\alpha \) is an enzyme family consisting of at least 12 different isoforms with serine/threonine kinase function. The isoforms are closely related structurally and consist of a single polypeptide chain divided into two domains: a regulatory domain at the NH\(_2\) terminus and a catalytic domain at the COOH terminus. They can be categorized in three groups: conventional or calcium-dependent PKC (\( \alpha, \beta1, \beta2, \gamma \)); novel or calcium-independent PKC (\( \delta, \epsilon, \eta, \theta, \mu \)); and atypical PKC (\( \lambda, \tau, \zeta \); Ref. 1). PKC isoforms mediate the normal cellular signaling pathways for the lipophilic second messenger sn-1,2-diacylglycerol, which is involved in many biological responses, including modulation of cell growth and differentiation (2). The conventional and novel PKC isoforms also serve as primary receptors for tumor-promoting phorbol esters, as well as for sn-1,2-diacylglycerol, which is produced at elevated levels in cells transformed by products of oncogenes, such as the epidermal growth factor receptor homologue erb-B2, ras, and src (2). The potent tumor promoter TPA activates PKC allosterically and causes a concurrent translocation to the membrane fraction of cells. Treatment of cells with TPA ultimately leads to the down-regulation of PKC activity, a loss of immunologically detectable PKC protein, and reduced biological function (2, 3). Both activation and down-regulation of PKC may be important in regulating cellular functions (1). Bryo 1, a macrocyclic lactone, is a partial antagonist of PKC acting through the regulatory domain (3). Bryo 1 activates conventional and novel PKC isoforms, but it also antagonizes many effects that are produced by TPA, perhaps by isoform-specific down-regulation of PKC (3). Various pharmacological inhibitors of PKC have been shown to inhibit tumor promotion and to inhibit the proliferation of tumor cells (3). Although the specific functions of the individual PKC isoforms are still unclear, there is evidence that different isoforms exert specific biological effects and that these effects are cell type specific. Prior studies of the expression of various PKC isoforms in human gliomas and the correlation with tumor grade and biological behavior have suggested that PKC may be involved in the high proliferative capacity and tumorigenicity of glioma cells (4, 5). Human or rat glioma cells had high PKC enzyme activity, compared to their nontransformed glia counterparts (6). Pharmacological inhibitors of PKC (tamoxifen, staurosporin, UCN-01, hypericin, and CGP 41251)

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3 The abbreviations used are: PKC, protein kinase C; TPA, 12-O-tetradecanoylphorbol-13-acetate; Bryo 1, Bryostatin 1; Mab, monoclonal antibody.
Expression of PKC α in Human Glioma

Low-grade (astrocytoma- and oligodendroglioma-derived; LGO-MN, LGA-SW, and LGO-AN) cell lines. (i.e., fractionation studies show a high expression of PKC α in the soluble (Lanes S) or cytosolic fraction in unstimulated high-grade glioma cell lines. B, a significant translocation (i.e., activation) of the PKC α isoform to the particulate (Lanes P) or membrane fraction after treatment with TPA (10 nM) for 1 h in the glioblastoma-derived cell lines. Similar results were obtained in two independent experiments.

Fig. 1 Immunoblots showing high PKC α expression in high-grade (glioblastoma-derived; U-87, GBM-CC, and GS-1) versus low expression in low-grade (astrocytoma- and oligodendroglioma-derived; LGO-MN, LGA-SW, and LGO-AN) cell lines. NB, normal brain tissue. A, subcellular fractionation studies show a high expression of PKC α in the soluble (Lanes S) or cytosolic fraction in unstimulated high-grade glioma cell lines. B, a significant translocation (i.e., activation) of the PKC α isoform to the particulate (Lanes P) or membrane fraction after treatment with TPA (10 nM) for 1 h in the glioblastoma-derived cell lines. Similar results were obtained in two independent experiments.

block basal or mitogen-enhanced proliferation of glioma cells (7–9).

The PKC α isoform has been implicated as a specific positive regulator of cellular growth and tumorigenesis in human gliomas (6, 10, 11). Stable expression of antisense PKC α DNA in the human glioblastoma cell line U-87 resulted in growth inhibition and decreased tumorigenicity (10). Administration of PKC α antisense oligodeoxynucleotides inhibited the growth of human glioblastoma cells in vitro and in vivo (11). These results have prompted a clinical trial of PKC α antisense oligodeoxynucleotides in patients with progressive or recurrent high-grade astrocytomas.

The purpose of this study was to assess more accurately the role of the PKC α isoform in the growth regulation of human gliomas. Therefore, we studied the differential expression and subcellular distribution of the PKC α isoform in human high- and low-grade gliomas and also in human glioma-derived cell lines, as compared to normal brain tissue controls. We also investigated the effects of Bryo 1, a potent partial PKC antagonist, on the expression of PKC α and on in vitro growth in human glioblastoma multiforme-derived cell lines.

**MATERIALS AND METHODS**

**Tumor Cell Lines.** The following human glioma-derived cell lines were cultured in DMEM supplemented with 20% fetal bovine serum, 1% L-glutamine, 1% penicillin (100 units/ml), and 1% streptomycin (100 μg/ml); U-87, GS-1, and GBM-CC (glioblastoma derived); LGA-SW (low-grade astrocytoma derived); and LGO-MN and LGO-AN (low-grade oligodendroglioma derived). With the exception of U-87, which is an established, commercially available human glioblastoma-derived cell line, all glioblastoma cell lines represent primary cell lines that were established by investigators in the Department of Neurosurgery, Columbia-Presbyterian Medical Center (New York, NY), and they have been described and characterized previously (9). The morphology and growth characteristics of the low-grade glioma-derived primary cell lines were not characteristic of fibroblasts (data not shown).

**Tumor and Normal Brain Tissue Specimens.** Tumor samples from nine different gliomas (GMB-LT, GBM-MS, and GBM-DG from glioblastomas; LGA-SF, LGA-HS, and LGA-WR from low-grade astrocytomas; and LGO-MN, LGO-KN, and LGO-CN from low-grade oligodendrogliomas) were immediately frozen and stored at −80° C after surgery. Normal brain tissue (cortex and white matter) was stored at −80° C after autopsy (8 h postmortem) of a 30-year-old male with cardiac arrest.

**Reagents.** PKC α Mab (M6) was purchased from Upstate Biotechnology (Lake Placid, NY) and used in a dilution of 1:2000. PKC λ and μ Mabs (Transduction Laboratories, Lexington, KY) were diluted 1:500, and secondary sheep antimouse antibody (Amersham Corp., Arlington, IL) was diluted 1:2000. PKC α band was confirmed when this band was not detected on blots reprobed with the Santa Cruz PKC λ-specific antibody after the diluted antibody had been preabsorbed with the peptide antigen to eliminate epitope-specific binding (data not shown).

Bryo 1 was provided by the Division of Cancer Treatment, Diagnosis and Centers at the National Cancer Institute (Bethesda, MD), and by Bristol-Myers Squibb Corp. (Wallington, CT). TPA (LC Laboratories, Woburn, MA) and Bryo 1 were dissolved in DMSO. In all experiments, the final concentration of...
DMSO in the medium was ≤0.01%, which had a negligible effect on cell growth.

PKC α Translocation and Down-Regulation Studies. Each tumor cell line was grown in 150-mm plates to subconfluency. To evaluate the activation/translocation of the isofrom, one set of plates was treated with 10 nM TPA dissolved in DMSO, and the other set was treated only with DMSO as a control 1 h before the cells were harvested. TPA and DMSO were dissolved in serum-free medium to avoid growth factor-mediated activation.

For the PKC down-regulation studies, all plates were incubated with 100 ng/ml Bryo 1 in complete DMEM medium over various time periods (30 min, 1 h, 6 h, 24 h, 48 h, 72 h, and 96 h) and harvested at the same time.

Protein Extraction, Subcellular Fractionation, and Immunoblot Assay. Freshly frozen tumor samples were thawed with a few drops of lysis buffer and minced with a scalpel for 2 min until a homogeneous tissue pulp could be pipetted into 2–3 ml of lysis buffer. The cultured cells were washed three times with PBS at 4°C and scraped into 2–3 ml of lysis buffer [20 mM Tris (pH 7.5), 100 μM Na3VO4, 2 mM EDTA, 2 mM EGTA, 50 mM sodium fluoride, 1.25 mM phenylmethylsulfonyl fluoride, 5 mM DTT, 10 μg/ml leupeptin, and 20 μg/ml aprotinin]. The lysates were sonicated on ice with two pulses of 5–10 s each, placed on ice for 20 min, and then centrifuged at 42,000 rpm for 60 min at 4°C. The supernatant was called soluble (cytosolic) protein fraction. Two to 3 ml of lysis buffer containing 1% Triton X-100 were added to the pellet and sonicated with pulses of 5–10 s until the pellet was completely dissolved. This lysate was placed on ice for 20 min and was designated the particulate (membrane) protein fraction. The protein content in these extracts was determined according to the method of Bradford (Bio-Rad, Hercules, CA). The equivalent of 100 μg was fractionated by electrophoresis in 8% polyacrylamide gels (SDS-PAGE) and transferred to nitrocellulose membranes. After the filters were blocked over night with 3% nonfat dry milk in TTBS buffer [50 mM Tris (pH 7.5) and 150 mM sodium chlo-

**RESULTS AND DISCUSSION**

PKC α is considered a significant target in antglioma therapy. It is currently being evaluated in clinical trials using PKC α antisense oligodeoxynucleotides and Bryo 1 to treat patients with malignant gliomas. However, rather inconsistent results have been reported previously regarding the expression and histopathological correlation of this isofrom in human gliomas. Although Benzil et al. (5) demonstrated in an immuno-histochemical study that PKC α expression was high in well-differentiated astrocytomas and low in glioblastomas, Todo et al. (4) found no correlation between the intensity of staining for PKC α and the grade of glial tumors. When assayed by immunoblotting, PKC α expression was at least 10-fold higher in C6 rat gliomas than in nonneoplastic astrocytes (6), whereas no difference between human glioblastoma cell lines and "normal human glial cultures" was reported by Xiao et al. (12).

Here, we studied the expression of PKC α in different high- and low-grade human glioma-derived primary cell lines and in freshly frozen tumor samples of human high- and low-grade gliomas, compared to frozen samples of normal human brain by

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**Fig. 2** Expression of PKC α in nine freshly frozen tumor samples from glioblastomas (GBM-LT, GBM-DG, and GBM-MS), low-grade oligodendrogliomas (LGO-MN, LGO-KN, and LGO-CN), and low-grade astrocytomas (LGA-HS, LGA-WR, and LGA-SF), compared with samples from normal brain (NB-WM, white matter; NB-CX, cortex). Data from two independent immunoblot experiments (A and B) are shown. No significant differences between high- and low-grade tumors and normal brain tissue were observed.
Fig. 3  Down-regulation of PKC α in glioblastoma-derived cell lines U-87 (A) and GBM-CC (B) after incubation with Bryo 1 at 100 ng/ml over various time periods (30 min, 1 h, 6 h, 24 h, 48 h, 72 h, and 96 h). Although PKC α is rapidly activated and translocated from the soluble (S) to the particulate (P) fraction and is completely down-regulated after 24 h, the isoforms λ and μ are neither translocated nor down-regulated by Bryo 1. Similar results were obtained with two different glioblastoma-derived cell lines (data not shown).

immunoblot analyses. Because activation of PKC can be monitored by the translocation of the isoform from the cytosol (soluble fraction) to the cell membrane (particulate fraction), we performed subcellular fractionation studies to estimate the state of PKC α activation in each tumor or cell line. Cell lines derived from high-grade gliomas expressed higher levels of PKC α relative to cell lines derived from low-grade gliomas (Fig. 1). Subcellular fractionation studies demonstrated a prevalent overexpression in the soluble (cytosolic) fraction of glioblastoma-derived cell lines, indicating that this isoform is highly expressed without being translocated (i.e., activated) during in vitro growth (Fig. 1A). Fig. 1B illustrates a significant translocation of the PKC α isoform to the particulate (membrane) fraction in the glioblastoma-derived cell lines after treatment for 1 h with TPA (10 nm). This demonstrates that the abundance of PKC α that is present in the inactive state in glioma cell lines is rapidly activated by a PKC agonist. In cell lines derived from low-grade tumors, PKC α was not translocated in response to TPA treatment.

In contrast to the cell lines, PKC α expression in freshly frozen tumor tissues was not increased in glioblastomas relative to low-grade gliomas, including both astrocytomas and oligodendrogliomas, and was also not elevated compared to normal brain tissue controls (Fig. 2). The degree of activated PKC α, as estimated by the relation of soluble and particulate fraction, was not significantly different from normal brain controls either. Therefore, in freshly frozen tumor tissues, which may more truly represent the state of the in vivo tumor mass, no significant differences in PKC α expression and activation between high- and low-grade glial tumors and between tumors compared to normal human brain tissue could be found.

Taken together, these results suggest that the relatively high levels of PKC α expressed in glioblastoma-derived cell lines may reflect an artifact of in vitro cellular growth that occurs during the establishment of primary tumor-derived cell lines.

Bryo 1, a macrocyclic lactone, is a partial PKC antagonist and has several potent antineoplastic activities when assayed in vitro and in vivo (13, 14). Both, the phorbol ester TPA and Bryo 1 can substitute for the endogenous PKC activator sn-1,2-diacetylglycerol through their ability to bind to PKC at the regulatory domain (15). Bryo 1, after short-term activation, is capable of specifically down-regulating and depleting PKC α in human breast and lung carcinoma cells and human neuroblastoma cells (16–18). Our immunoblot analyses of subcellular extracts of glioma-derived cell lines revealed expression of the PKC isoforms α, λ, and μ. Short-term (<6 h) incubation with 100 ng/ml Bryo 1 activated PKC α, as measured by translocation from the cytosolic to the membrane subcellular fraction, whereas incubation for ≥24 h produced a complete down-regulation of immunodetectable PKC α in the glioblastoma-derived cell lines U-87 (Fig. 3A) and GBM-CC (Fig. 3B). This depletion of PKC α was not associated with significant growth inhibition in the same cell lines incubated with the same concentration of Bryo 1 (100 ng/ml; Fig. 4). The PKC isoforms λ and μ were neither activated nor down-regulated by Bryo 1 (Fig. 3). It is not surprising that PKC λ is not affected by Bryo 1 treatment because it lacks the phorbol ester-binding site and, thus, is
exception for PKC γ (20), there are no inhibiting drugs with the PKC α isoform with inhibition of in vitro PKC α, demonstrated a selective down-regulation of encoding glioblastoma cell line U-87 with antisense complementary DNA regarding the results of these prior studies (6, 10, 11), one explanation may be that other factors, for example, the oligodeoxyribonucleotide delivery vehicle Lipofectin or nonspecific antisense effects, possibly in combination with PKC α down-regulation, contribute to the growth-inhibitory effect in these studies, but this remains to be investigated.

We also tested Bryo 1 in an additional seven glioblastoma-derived cell lines, and no effects on cellular growth were observed at any concentrations we tested (data not shown). However, because the reduction of PKC α expression has been shown to attenuate the multidrug resistance phenotype (21), it seems imperative to analyze the antitumor effects of combinations of Bryo 1 with established chemotherapeutic agents.

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**REFERENCES**

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