

Involvement of PKC- ι in glioma proliferation

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Received 18 May 2007; revision accepted 28 July 2007

Abstract. Atypical protein kinase C- ι (PKC- ι) protects cells against apoptosis and may play a role in cell proliferation. However, *in vivo*, the status and function of PKC- ι in human normal brain tissue, gliomas, benign and malignant meningiomas as well as its *in vitro* status in proliferating and confluent glioma cells, remains unknown. **Objectives:** The objectives of our research were to determine whether expression of PKC- ι is altered either in gliomas or in benign and malignant meningiomas, compared to normal brain. In addition, we wished to establish the expression of PKC- ι in proliferating plus in cell cycle-arrested glioma cell lines, as well as the relationship between PKC- ι siRNA on PKC- ι protein content and cell proliferation. **Materials and Methods:** Western blot analyses for PKC- ι were performed on 12 normal brain biopsies, 15 benign meningiomas, three malignant meningiomas and three gliomas. **Results:** Results demonstrated no ($n = 9$) or very weak ($n = 3$) detection of PKC- ι in normal brain tissue. In comparison, PKC- ι was robustly present in the majority of the benign meningiomas. Similarly, PKC- ι was abundant in all malignant meningiomas and gliomas. Western blotting for PKC- ι in confluent or proliferating glioma cell lines depicted substantial quantities of PKC- ι in proliferating T98G and U-138MG glioma cells. In contrast, confluent cells had either 71% (T98G) or 21% (U-138MG) less PKC- ι than proliferating cells. T98 and U-138 MG glioma cells treated with 100 nM PKC- ι siRNA had lower levels of cell proliferation compared to control siRNA-A and complete down-regulation of PKC- ι protein content. **Conclusion:** These results support the concept that presence of PKC- ι may be required for cell proliferation to take place.

INTRODUCTION

Protein kinase Cs (PKCs) compose a family of 14 known isozymes found in varying ratios in the cytosolic and membrane fractions of cells, depending on the type of tissue and its physiological state (Nishizuka 1992). PKC isozymes can be classified into three groups. Group I includes Ca^{2+} -dependent isozymes: cPKC- α , cPKC- β I, cPKC- β II and cPKC- γ . Isozymes in group II, nPKC- ϵ , nPKC- δ , nPKC- η and nPKC- θ are Ca^{2+} -independent. Group III includes the atypical PKC: aPKC- ι (Selbie *et al.* 1993), aPKC- ζ , aPKC- ζ II (Hirai *et al.* 2003), aPKC- μ (protein

kinase D) and aPKC- ν (Hayashi *et al.* 1999) that are insensitive to both diacylglycerol and calcium and neither bind to nor are activated by phorbol esters. PKC regulates cellular functions, metabolism and proliferation by phosphorylating proteins in response to transmembrane signals from hormones, growth factors, neuro-transmitters and pharmacological agents.

Literature summarizing PKCs in general in normal brain tissue (Tanaka & Saito 1992), gliomas (da Rocha *et al.* 2002) and meningiomas (Johnson & Toms 2005) have been published.

Of special interest is atypical PKC- ι that does not contain a Ca²⁺-binding region, has one zinc finger-like motif and is the human homologue of the mouse PKC- λ (Diaz-Meco *et al.* 1996). PKC- ι may play a role in development of malignancy at the cellular level, as shown by its association with the transformed phenotype of human melanomas *in vivo* and *in vitro* (Selzer *et al.* 2002) and that PKC- ι protects cells against drug-induced apoptosis (Murray & Fields 1997; Xie *et al.* 2000). In cells of some types of human lung cancer, PKC- ι is a Bad kinase that can phosphorylate and inactivate the pro-apoptotic BH3-region containing protein, leading to enhanced survival and chemoresistance (Jin *et al.* 2005). In glioblastoma cells, PKC- ι protects against cisplatin cytotoxicity by attenuation of p38 mitogen-activated protein kinase signalling (Baldwin *et al.* 2006). Additionally, in glioma cells a link between PKC- ι and cyclin-dependent kinase 7 (Cdk7) activation has been reported previously (Acevedo-Duncan *et al.* 2002; Bicaku *et al.* 2005). PKC- ι is also required for oncogenic Ras- and carcinogen-mediated colon carcinogenesis *in vivo* (Murray *et al.* 2004).

This study investigates the content of PKC- ι in cells of normal brain tissue, gliomas, benign and malignant meningiomas and in the T98G and U-138MG cell lines.

MATERIALS AND METHODS

Brain tissue

Human autopsy-derived brain tissue and meningiomas were obtained from the Cooperative Human Tissue Network (Southern Division) at the University of Alabama (Birmingham, AL, USA). Tissue specimens were obtained from both males and females of varying ages (23–80 years of age). Normal brain tissue included specimens from the frontal lobe, brain cortex, cerebellum, hippocampus, pons, corpus collosum and basal ganglia. Tumours labelled 'benign' were meningiomas. Malignant tumours were either meningiomas or gliomas. Brain tissue protein lysates (50 μ g) were subjected to gel electrophoresis and Western blotting was performed using monoclonal antibodies against PKC- ι (cat. no. 610176, BD Transduction, San Diego, CA, USA) at a 1 : 2000 dilution (5 μ g). Secondary antibodies were obtained from Accurate (JOM035146, Westbury, NY, USA), and were used at 1.5 : 10 000 dilution (48 μ g). Western blots were probed for actin with a goat polyclonal antibody to actin (SC-1616) at 2.5 : 2000 dilution (10 μ g) and secondary antibodies SC-2350 at a 1 : 2000 dilution (8 μ g, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Positive control for PKC- ι immunoreactivity was U-373MG cell lysate (81 μ g) that is known to contain PKC- ι .

Passage of T98G and U-138MG glioma cells

Human glioma U-138MG (ATCC no. HTB-16) and T98G (ATCC no. CRL-1690) cells were obtained from the American Tissue Culture Collection (Rockville, MD, USA). U-138MG cells were originally isolated and established into a stable cell line by Ponten & MacIntyre (1968), having been derived from an astrocytoma-glioblastoma (grade III) of a 47-year-old Caucasian male. These cells have oligodendroglia histological features and a hyperdiploid to pentaploid

karyotype. They grow in semisolid medium but are not tumourigenic in immunosuppressed mice. In our laboratory, U-138MG cells have a doubling time of 36 h. The T98G cell line was isolated as a spontaneous variant of the parental T98G cells by Stein (1979), derived from a glioblastoma multiforme tumour of a 61-year-old Caucasian male. T98G appeared sometime between population doubling level 25–300 and the growth characteristics have been stable. The cells have a hyperpentaploid chromosome count, display anchorage independence and immortality but are not tumourigenic in nude mice. The reported doubling time of T98G cells is 26 ± 2 h (Stein 1979); however, in our conditions T98G cell populations double every 18 h. We are not certain why this happens, because we use ATCC media to grow the cells and they are subcultured according to ATCC procedures. The one parameter that distinguishes T98G cells from fully transformed cells is that they behave similarly to normal cells and can become arrested and stationary in G₁ phase (Stein 1979). T98G and U-138MG glioma cells were grown as adherent cultures. Cells were seeded into 0.22 mm filter 75 cm² flasks containing 90% minimal essential media (MEM), 10% fetal calf serum, and standard antibiotics. Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO₂ until they became no more than 70–80% confluent. Medium was changed (level of 5 mL medium/25 cm² growth area) every other day, because these cells metabolize it quickly as judged by its yellow colouration if left for a longer period.

Inhibition of gene expression with siRNA

RNA interference functions by a regulatory mechanism for sequence-specific gene silencing through double stranded (ds)RNA. Sequence specific RNA 19–25 nucleotides in length were synthesized by Santa Cruz Biotechnology against PKC- ϵ . PKC- ϵ siRNA were transfected into glioma cells (U-138MG and T98G) using lipid based siRNA transfection reagent (Santa Cruz Biotechnology). Sequence-specific PKC siRNA were as follows: PKC- ϵ siRNA is a pooled sequence that consists of three combined RNA sequences – mRNA location. Gene accession number for PKC- ϵ is NM_002740. PKC- ϵ siRNA: 663: 5'-CAAGCCAAGCGUUUCAACA-3' and 5'-UGUUGAAACGCUUGGCUUG-3'; 739: 5'-GGAACGAUUGGGUUGUCAU-3' and 5'-AUGACAACCCAAUCGUUCC-3'; and 2137: 5'-CCCAUAUCUUCUCUUGUA-3' and 5'-UACAAGAGAAGAUUUGGG-3'.

In addition to these siRNAs, negative controls containing a scrambled sequence (which does not lead to specific degradation of any known cellular mRNA) was synthesized; this is proprietary and Santa Cruz Biotechnology does not reveal it.

Cell viability assay

Effects of PKC- ϵ siRNA were determined in exponentially growing T98G or U-138MG glioma cells in complete media over 72 h. Cells were plated on 75 cm² vessels at a density of 3.75×10^5 cells/flask. Twenty-four hours after plating, cells were incubated with either siRNA-A or PKC- ϵ siRNA (100 μ M) according to the manufacture's instructions (Santa Cruz Biotechnology). Following initial exposure to siRNA, no additional siRNA was applied or removed during the 3-day incubation period. Following treatments, cells were washed with phosphate-buffered saline (PBS), were trypsinized and re-suspended in 3 mL of PBS. Cell viability was quantified using the trypan blue exclusion assay. Two hundred microlitres of the cell suspension was added to 50 μ L of trypan blue and numbers of unstained and stained cells were counted. For Fig. 3, the same cultures from cell proliferation assays were harvested after counting and were prepared for Western blot analysis. Equal amounts of cellular protein (15 μ g) were loaded per well. Anti-PKC- ϵ (P20520; Transduction Laboratories, Lexington, KY, USA) was used at 1 : 12 000 dilution (0.83 μ g). Western blots were also performed to verify that protein loading (15 μ g) and protein integrity were equal. Western blots were probed with a monoclonal antibody to

β -actin (Santa Cruz Biotechnology; SC-8432) at 5 : 1000 dilution (40 μ g). Secondary antibodies were obtained from Accurate (JGM035146, Westbury) and were used at 1 : 15 000 dilutions (1 μ g).

Tissue fractionation

Brain tissue, T98G or U-138MG glioma cells were re-suspended and sonicated in 2 mL homogenization buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 0.1% Tween-20, 1 mM EDTA (ethylenediaminetetraacetic acid) and 2 mM EGTA (glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid), 0.1 mM orthovanadate, 1 mM NaF, 2 mM PMSF (phenylmethylsulphonyl fluoride), 2.5 μ g/mL leupeptin, 1 mM DTT (dithiothreitol), 0.15 U/mL aprotinin; Agrawal *et al.* 1995]. The suspension was sonicated for 3 \times 15 s, cycles on ice. Brain tissue suspensions or cell lysates were centrifuged at 100 000 *g* for 30 min to obtain cell extracts. Protein content was measured according to Bradford (1976).

Cell cycle analysis by flow cytometry

Cell cycle analysis was performed as previously described (Acevedo-Duncan *et al.* 1997). Confluent cell cultures were either rapidly proliferating or semisynchronized by contact inhibition and serum starvation for 48 h. Subsequently, cells were collected and were washed twice with PBS and then were trypsinized. Cells in the suspension were centrifuged then the trypsin was decanted. To fix the cells, first, 3 mL of ice-cold PBS was added and the pellet was re-suspended. While vortexing gently, 7 mL of ethanol was added drop wise. The day before analysis, the 70% ethanol was decanted off and PBTB (PBS, 0.2% Triton and 1% bovine serum albumin) was added. Cells were counted and diluted to 1 \times 10⁶ cells/mL with PBTB. Then, they were filtered and 50 μ L of RNase was added. Nuclei were analysed for DNA content using a propidium iodide (10 μ L) staining protocol plus flow cytometry (Carlton *et al.* 1991). Distributions of 40 000 nuclei were quantified using a FAC STAR^{Plus}, flow cytometer (Becton Dickinson, San Jose, CA, USA) and ModFitLT cell cycle analysis program (version 2.0; Verity Software House Inc., Topsham, ME, USA). Mean separation was by using Student's *t*-test (Minitab Inc., State College, PA, USA).

Western blot analysis

Cell extracts containing equal amounts of protein in each lane were run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to the protocol of Laemmli (1970); proteins were transblotted according to the method of Towbin *et al.* (1979). Brain tissue or glioma cell lysate PKC- ι was probed with monoclonal antibodies against PKC- ι (BD Transduction Laboratories), secondary antibodies were obtained from Accurate (JOM000003, Westbury). Immunoreactive bands were visualized with enhanced chemiluminescence according to the manufacturer's instructions (Amersham, Piscataway, NJ, USA). For Fig. 2, duplicate cultures from the flow cytometry experiment were harvested at the indicated times and were prepared for Western blot analysis. Equal amounts of cellular protein (20–50 μ g) were loaded per well. Anti-PKC- α , anti-PKC- β 1, anti-PKC- δ , anti-PKC- ϵ and anticaspase 7 (SC-208, SC-209, SC-937, SC-214 and SC-8510; Santa Cruz Biotechnology) were used at 1 : 1300 dilution (3 μ g). Anti-PKC- ι (P20520; Transduction Laboratories) was used at 1 : 12 000 dilution (0.83 μ g). Western blots were also performed to verify that protein loading (20 μ g) and protein integrity are equal; they were then probed with a monoclonal antibody to actin (Santa Cruz Biotechnology; SC-8432) 5 : 1000 dilution (40 μ g). Secondary antibodies were obtained from Accurate (JGM035146 and JG2035744, Westbury) and were used at 1 : 15 000 dilutions (1 μ g).

Table 1. Status of PKC- ι in brain tissues, meningiomas and gliomas^a

Tissue type	Not present	Weakly present	Positively present
Normal brain	9	3	0
Glioma	0	0	3
Benign meningioma	0	1	14
Malignant meningioma	0	0	3

^aNormal brain tissue was obtained from frontal lobe, cortex, cerebellum, hippocampus, pons, corpus collosum or basal ganglia; benign tumours were meningiomas. Malignant tumours were either meningiomas or gliomas.

Densitometry

The intensity of each band was measured using Gel Base/Gel Blot-Pro software (Synoptics Ltd., Cambridge, UK); briefly, background intensity was subtracted from the intensity of each band, to derive the corrected intensity.

RESULTS

The relationship between absence of PKC- ι in normal brain tissue and its robust presence in either benign/malignant meningiomas or gliomas is summarized in Table 1. Western blots probed for PKC- ι in 12 normal brain biopsies, 15 benign meningiomas and 6 malignant tumours revealed complete absence ($n = 9$) or very low detection ($n = 3$) of PKC- ι in normal brain tissue (Table 1). By comparison, PKC- ι was very clearly evident in the majority of benign meningiomas ($n = 14$) but was only weakly present in one. Similarly, PKC- ι was abundant in all malignant meningiomas ($n = 3$) and gliomas ($n = 3$). Table 2 presents the main characteristics (age, gender and World Health Organization classification of tumours) of the patients and tumour specimens. Western blots corresponding to some of the data present in Table 1 are shown in Fig. 1a. PKC- ι was identified in Western blots by a band of molecular weight 67 kDa that corresponded to the immunoreactive signal obtained from U-373MG glioma cells that contain PKC- ι . Western blot controls for PKC- β II did not show a pattern of expression specific to either normal brain tissue, or benign or malignant brain tumours (data not shown). Control actin Western blots showed actin immunoreactive bands at a molecular weight of 42 kDa. Actin immunoreactive bands were of equal intensity, indicating that equal amounts of protein were loaded into each lane. Data presented in Table 1 and Fig. 1a are also presented graphically in Fig. 1b that depicts a 38–46-fold increase in PKC- ι immunoreactivity in glioma, benign and malignant meningiomas when compared to normal brain tissue. These data were subjected to analysis of variance and mean separation by Tukey's honestly significant difference test (Minitab Inc., State College, PA, USA). The level of PKC- ι in normal brain tissue differed from that in gliomas, and benign or malignant meningiomas ($P < 0.001$), while there was no significant difference ($P > 0.05$) between levels of PKC- ι immunoreactivity between gliomas, benign and malignant meningiomas. This study demonstrates that PKC- ι is overexpressed in gliomas, benign and malignant meningiomas compared to normal brain tissues.

To establish whether PKC- ι plays a role in cell cycle progression, T98G and U-138MG glioma cells were plated and samples were taken for flow cytometric analysis or Western blotting, when cells were either serum starved for 48 h and 100% confluent, or when cells were 50% confluent

Table 2. Patient data and WHO classification of brain tissues, meningiomas and gliomas

Specimen ^a	Sex	Age	WHO classification
N1	M	76	N/A
N2	M	54	N/A
N3	M	59	N/A
N4	M	47	N/A
N5	M	44	N/A
N6	M	52	N/A
N7	F	79	N/A
B1	M	53	Meningioma – N/L
B2	F	61	Fibrous meningioma – grade I
B3	F	46	Fibrous meningioma – grade I
M1	F	62	Glioblastoma multiforme – grade IV
M2	M	23	Glioblastoma multiforme – grade IV
M3	M	57	Malignant meningioma – N/L
M4	F	64	Atypical meningioma – grade II
M5	M	36	Astrocytoma – grade IV
M6	M	56	Anaplastic meningioma – grade III
B4	F	46	Meningioma – N/L
B5	F	49	Meningioma – N/L
B6	F	90	Meningioma – N/L
B7	M	73	Meningioma – N/L
B8	F	53	Meningioma – N/L
B9	M	80	Meningothliomatous meningioma – grade I
B10	F	43	Meningothliomatous meningioma – grade I
B11	F	66	Fibrous meningioma – grade I

F, female; M, male; N/A, not applicable; N/L, not listed.

Table 3. Summary of cell cycle phases^a

Cell type	G ₀ /G ₁	S	G ₂ /M
100% Confluent T98G	94 ± 1	4 ± 3	2 ± 3
50% Confluent T98G	64 ± 3	33 ± 3	4 ± 4
100% Confluent U-138MG	74 ± 11	20 ± 10	2 ± 3
50% Confluent U-138MG	54 ± 17	39 ± 18	7 ± 1

^an = 3 experiments per cell line.

and not serum starved. T98G cells and U-138MG glioma cells that were 100% confluent had 94% and 74% of the cells in quiescence/Gap 1 (G₀/G₁), respectively (Fig. 2a,c and Table 3). In contrast, 64% of rapidly dividing 50% confluent T98G cells accumulated in G₀/G₁ phase and 54% of U-138MG glioma cells were in G₀/G₁ phase (Fig. 2b,d). To insure that 48 h serum deprivation did not lead to cell death nor alteration in intracellular cascades, we evaluated caspase 7 activation. Caspase 7 is an executioner/effector caspase that promotes apoptotic morphological alteration by cleaving several death substrates (Araya *et al.* 2002). In cells undergoing apoptosis, 35 kDa caspase 7 is cleaved into 20 kDa and 10 kDa active subunits. Figure 2e shows that 48 h serum deprivation did not induce degradation of caspase 7 into the 20 kDa subunit, as judged by lack of the cleaved fragment on Western blots and invariant levels of the 35 kDa

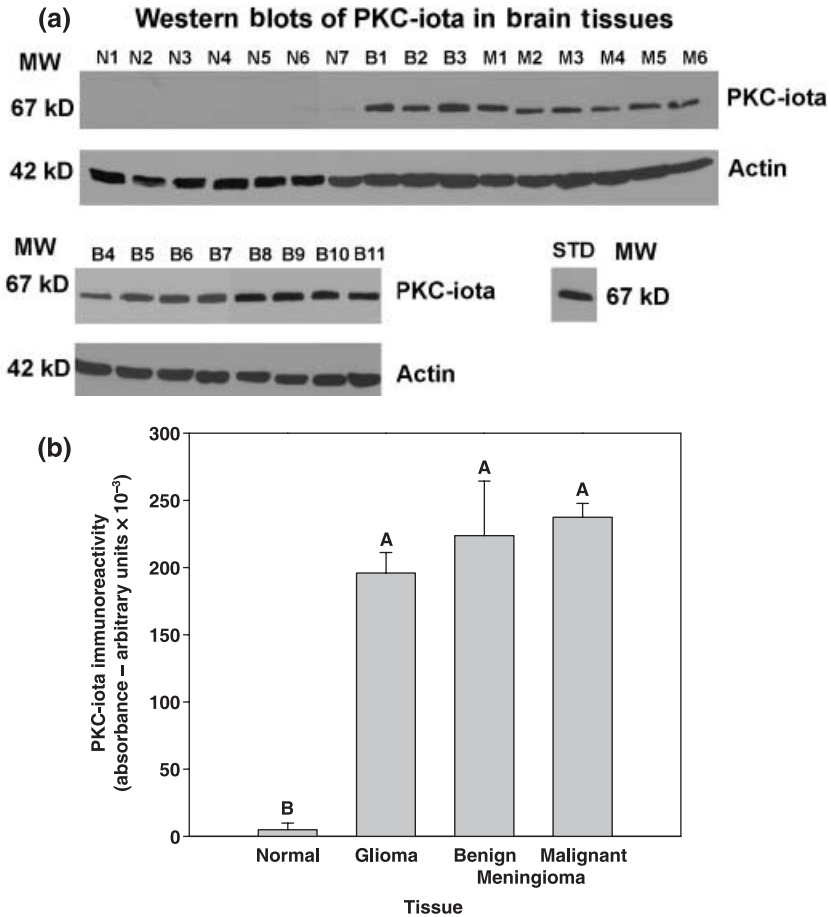


Figure 1. PKC-iota is present in benign and malignant meningiomas, gliomas but not normal brain tissue. (a) Human autopsy-derived normal brain tissue (N1, frontal lobe; N2, cortex; N3 and N4, unspecified brain; N5, cortex; N6, cerebellum), benign tumour tissue (B1, B4, B7, B9 and B10, [WHO grade 1] meningotheial meningioma; B5 and B8, meningioma; B6, fibroblastic meningioma; B2, B3 and B11, fibrous meningioma [WHO grade 1], and malignant tumour tissue; M1 and M2, [WHO grade IV] glioblastoma multiforme; M3, right frontal lobe meningioma; M4, atypical meningioma [WHO grade II]; M5, Astrocytoma [WHO grade IV]; M6, anaplastic meningioma [WHO grade III]). Specimens were obtained from the Cooperative Human Tissue Network. (b) Immunoblots from 12 normal brain specimens, 3 gliomas, 15 benign meningiomas and 3 malignant meningiomas were quantified, and mean plus and minus SE value is presented for each tissue type. Treatments indicated by the same letter do not differ, according to Tukey's honestly significant difference test ($P = 0.001$).

caspase 7 in T98G and U-138MG glioma cells (Fig. 2e). Western blotting for PKC-ι in these cell populations depicted robust quantities of PKC-ι in proliferating 50% confluent T98G and U-138MG glioma cells. In contrast, 100% confluent cells had either 71% (T98G) or 47% (U-138MG) less PKC-ι than 50% confluent cells (Fig. 2e–g). Differences between PKC-ι protein content in 100% confluent and 50% confluent rapidly proliferating cells was significant at $P < 0.05$ ($n = 3$). To establish that variations in PKC-ι may be specific for PKC-ι, we randomly performed Western blots for PKC-α, PKC-β1, PKC-δ and PKC-ε in confluent and proliferating cells. PKC-β1 is known to play a role in angiogenesis and tumourigenesis (Teicher *et al.* 2001). Similarly,

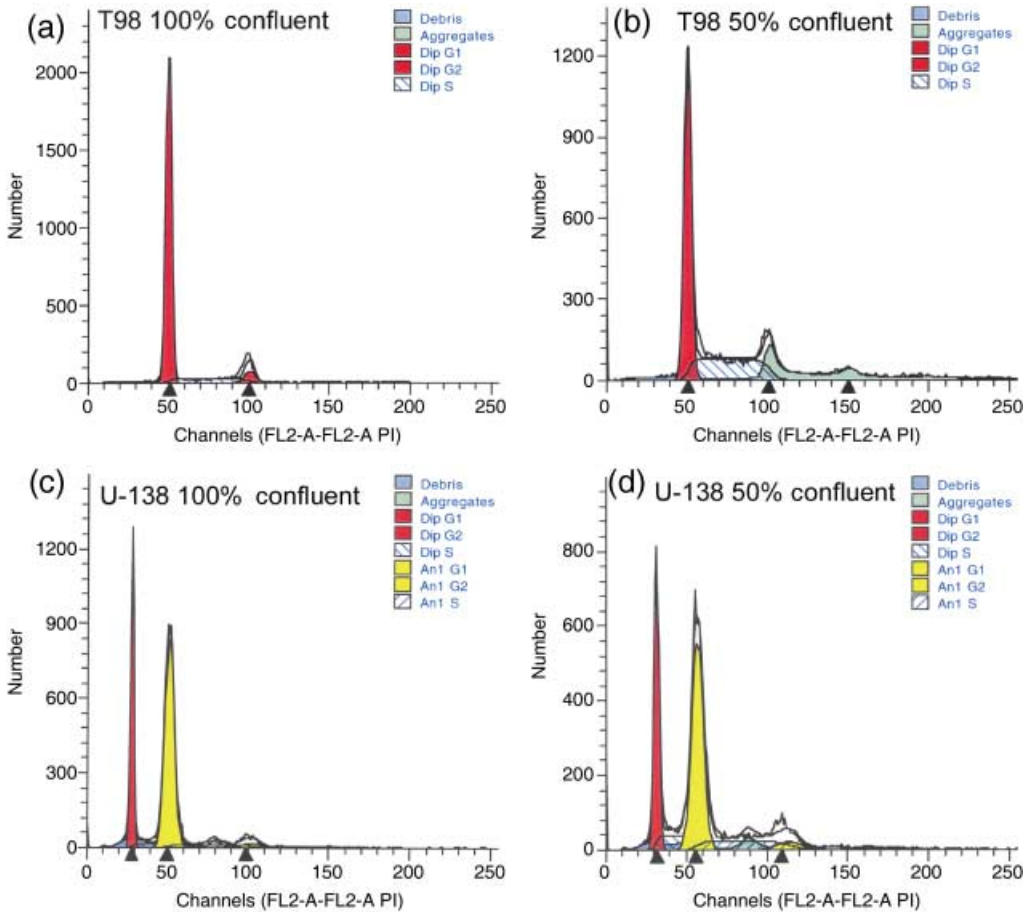


Figure 2. Effects of cell density and cell cycle progression on PKC- ι concentration in T98G and U-138MG glioma cells. FACS analysis of DNA content in 100% confluent T98G (a) or U-138MG cells (c) and 50% confluent T98G (b) or U-138 MG (d) cells. U-138 glioma cells are aneuploid; U-138MG DNA histograms are from one representative experiment and illustrate two cycling populations with a G_0/G_1 peak at 2 N (first red shaded peak) and another at 4 N (second yellow unshaded peak). Total DNA content for G_0/G_1 , DNA synthetic phase (S), gap 2 and mitosis (G_2/M) was quantified by addition of each of the phases in both populations. Forty thousand events were collected per time point and treatment group. Western blots of PKC- α , PKC- β 1, PKC- δ , PKC- ϵ , PKC- ι , and anticaspase 7 present in T98G or U-138MG cells that were at different stages of confluence (e). Band intensity of PKC- ι in T98G (f) and U-138 MG (g) was quantified by densitometry.

PKC- δ (Jane *et al.* 2006) and PKC- ϵ (Sharif *et al.* 2001) are involved in glioma cell proliferation. Westerns blots for PKC- α showed invariant levels of PKC- α in confluent and proliferating cells (Fig. 2e). Of interest, Western blots for PKC- δ did not detect PKC- δ in T98G glioma cells and invariant levels were detected in U-138MGs.

Western blotting for PKC- β 1 depicted decreased levels (35% and 53% reduction) of PKC- β 1 in 100% confluent T98G and U-138MG cultures compared to rapidly proliferating 50% confluent cells, respectively (Fig. 2e). Differences between PKC- β 1 protein content in 100% confluent and 50% confluent rapidly proliferating cells were significant at ($P < 0.05$; $n = 3$) for both cell lines. In contrast, PKC- ϵ was not detected in either T98G or U-138MG cells. Western

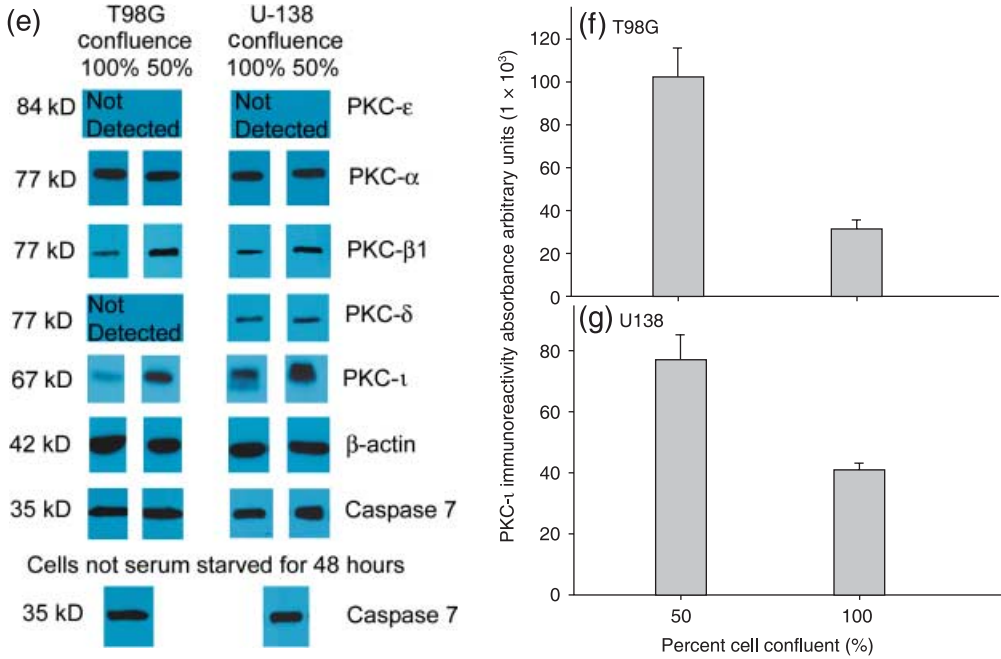


Figure 2. Continued

blots of PKC-α, PKC-β1, PKC-δ and PKC-ε, suggest that the presence or absence of these PKC isozymes may be dependent on cell type. Results depicting a relationship between cell confluence and PKC-ι protein levels suggest that PKC-ι along with PKC-β1, and PKC-δ may play a role in cell cycle progression and/or proliferation. For subsequent studies, we focused on the role of PKC-ι in glioma cell proliferation.

Effects of PKC-ι siRNA on proliferation of T98G and U-138MG glioma cells and appropriate protein content

The effects of exposure to PKC-ι siRNA on T98G and U-138MG glioma cell viability and proliferation were evaluated by trypan blue dye exclusion (Fig. 3a,b). Viable cell number was counted 24–72 h following addition of either control short interfering RNAs (siRNA-A, vehicle control; 100 nM) or PKC-ι siRNA (100 nM) according to manufacturer’s instructions (Santa Cruz Biotechnology). Exposure of T98G or U-138MG glioma cells to PKC-ι siRNA significantly reduced their cell proliferation by 59% ($P = 0.002$) and 69% ($P = 0.03$), respectively, at 72 h post-treatment. Densitometric scanning of Western blots revealed that PKC-ι siRNA decreased PKC-ι protein content by 86% to 77% (T98G) and 66% to 74% (U-138MG) during the 3-day experimental time course (Fig. 3c,d; $n = 3$ experiments). Differences between PKC-ι protein content in control siRNA-A and PKC-ι siRNA treated cells were significant at ($P < 0.05$) for all time points. Control β-actin Western blots produced β-actin immunoreactive bands at a molecular weight of 42 kDa. Actin-immunoreactive bands were of equal intensity, indicating that equal amounts of protein were loaded into each lane. These results indicate that PKC-ι may be required for cell proliferation to take place.

In this study, we examined the PKC-ι protein content in normal brain biopsies, gliomas, benign and malignant meningiomas as judged by Western blotting. Of interest were the results

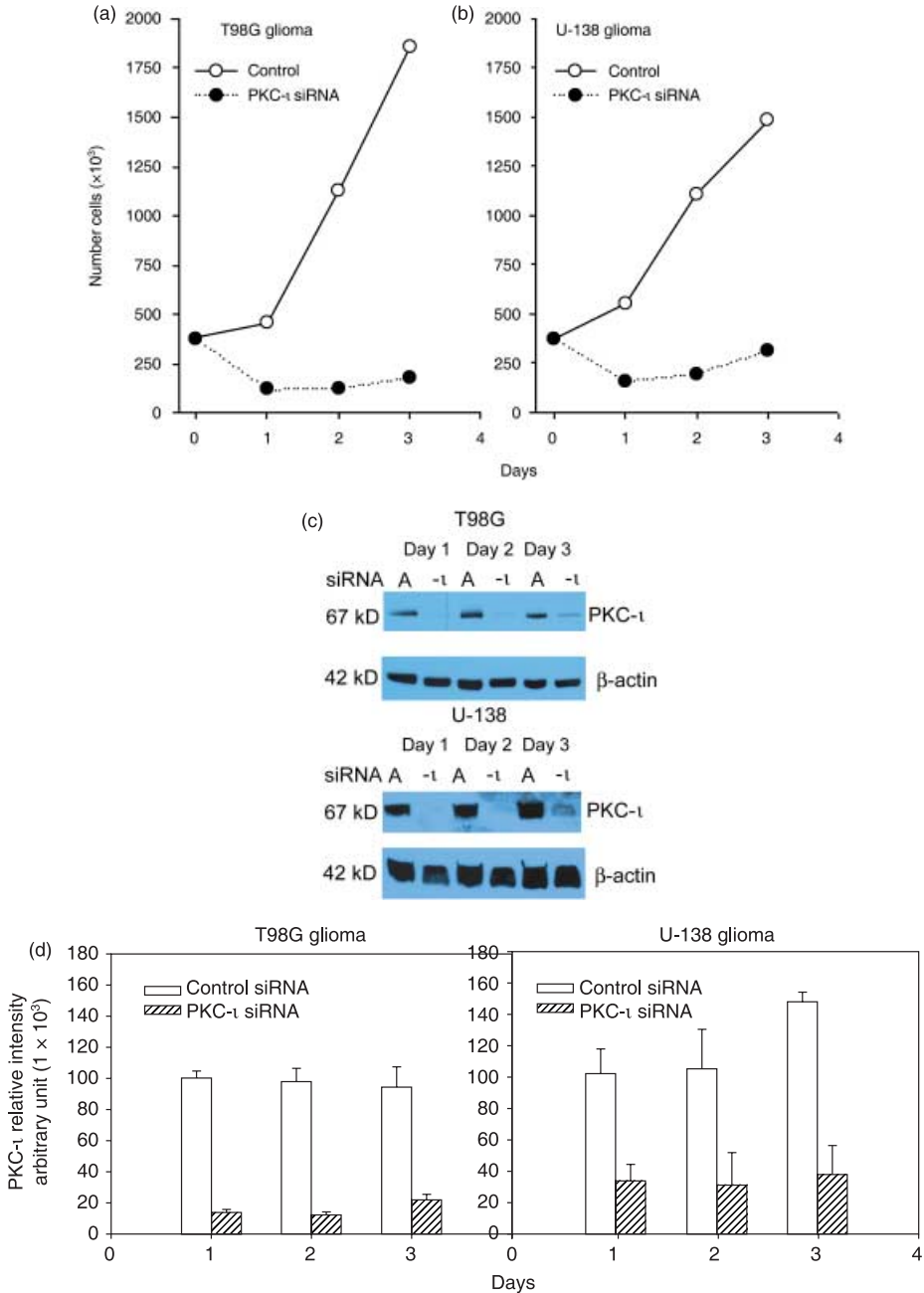


Figure 3. Effects of PKC- ι siRNA on proliferation of T98G and U-138MG glioma cells and PKC- ι protein content. Cells were plated in 75 cm² flasks at a density of 3.75×10^5 cells/flask. Twenty-four hours after plating, cells were incubated with either siRNA-A (100 nM; vehicle-control) or PKC- ι siRNA (100 nM) for 6 h according to manufacturer's instructions (Santa Cruz Biotechnology). During the 3-day incubation viable cells were quantified by trypan blue dye exclusion assay (a, T98G; b, U-138 MG). Open symbols (○) represent control (siRNA-A) treated cells, solid symbols (●) represent cells treated with PKC- ι siRNA (100 nM). Western blots of PKC- ι present in U-138MG and T98G cells, treated with PKC- ι siRNA (100 nM) for three days (c). Band intensity was quantified by densitometry (d). Data are representative of three experiments.

depicting increases in PKC- ϵ abundance in benign or malignant meningiomas and gliomas compared to normal brain tissue. Results from this study indicate that PKC- ϵ may be a characteristic of brain tumourigenesis. We also investigated the effects of PKC- ϵ siRNA on T98G and U-138MG glioma cell lines. Results demonstrated that PKC- ϵ siRNA reduced PKC- ϵ protein content concomitantly with a decrease in glioma cell proliferation. Taken together, these results suggest that PKC- ϵ may play a role in glioma cell proliferation.

DISCUSSION

Typically, high-grade malignant brain tumours are lethal. Despite rigorous therapy, median survival is less than 1 year for patients with high-grade tumours (Allalunis-Turner *et al.* 1992). While post-operative radiation therapy clearly delays tumour re-growth and prolongs survival, total tumour control is rarely achieved. Glioma recurrence and radioresistance may be due to an abundance of hypoxic or tumour stem cells, rapid glioma cell proliferation, low radiosensitivity or the involvement of PKC isozymes in radiation resistance (Baumann *et al.* 1992; Mitsutake *et al.* 2001; Tenzer *et al.* 2001). PKC may be involved in several cell signalling pathways (cell survival including repair of radiation damage) and cell cycle progression (Hallahan *et al.* 1992), thus its inhibition may result in radiosensitization. Additionally, rapid glioma cell growth rate has been attributed to inherently high levels of PKC (Couldwell *et al.* 1990; Pollack *et al.* 1990).

Signal transduction pathways have been studied comprehensively in gliomas, but only lately have they been examined in meningiomas. Roles of growth factor receptors Ras-Raf-1-MEK-1-MAPK, PI3K-Akt/PKB, PLC- γ 1-PKC, phospholipase A2-cyclooxygenase, and TGF- β receptor-Smad pathways on meningioma growth and apoptosis have been reviewed, and sites that can be targeted along these receptor/kinase pathways have been identified (Johnson & Toms 2005). PKC also appears to be involved in growth regulation of low-passage number human meningioma cells *in vitro*, as judged by decreased proliferation of cells following treatment with the PKC inhibitor staurosporine (Todo & Fahlbusch 1994; Huang *et al.* 2000). Additionally, two PKC-activating phorbol esters, 12-O-tetradecanoyl-13-phorbol acetate (TPA) and phorbol-12,13-dibutyrate (PDBu) have been shown to demonstrate different effects on proliferation of low-passage number human meningioma cells in culture (Todo & Fahlbusch 1994). TPA provoked dose-dependent stimulation of cell proliferation while PDBu produced significant inhibition of cell proliferation, suggesting that PKC-mediated signalling pathways may play a role in growth regulation of human meningioma cells. However, immunohistochemical studies investigating the presence of PKC in meningiomas depicted variable immunostaining (Reifenberger *et al.* 1989).

Protein kinase C is the major receptor for tumour-promoting phorbol esters, but the extent of PKC involvement in cellular malignancy is not clearly defined. Various studies indicate that increased tumourigenicity results from deregulation of PKC activity, or changes in PKC concentration, or both (Kamata *et al.* 1987; Housey *et al.* 1988; Mizuguchi *et al.* 1988; Mishima *et al.* 1994; Person *et al.* 1988; Weyman *et al.* 1988). Two PKC isozymes (PKC- β 11 and PKC- ϵ) have been reported to be critical for some forms of cancers, including colon cancer (Murray *et al.* 1999; Gokmen-Polar *et al.* 2001; Murray *et al.* 2002) and chronic myelogenous leukaemia (Murray *et al.* 1993; Murray & Fields 1997; Jamieson *et al.* 1999). PKC- ϵ has also been shown to be overexpressed in serous and nonserous ovarian cancers through a mechanism that results in loss of apical-basal polarity and cyclin E overexpression leading to poor prognosis (Eder *et al.* 2005).

Although, meningiomas arise from arachnoid cells of the meninges of the brain and spinal cord, and gliomas arise from supportive tissues of the brain glial or neuroglial, this study has

demonstrated that PKC- ι is overexpressed in benign and in malignant meningiomas and in gliomas but not in normal brain tissues. Moreover, we have shown that rapidly proliferating T98G and U-138MG glioma cell lines have enhanced levels of PKC- ι compared to confluent cells. Additionally, PKC- ι siRNA reduced PKC- ι protein content and decreased cell proliferation, suggesting a role for PKC- ι in regulating cell proliferation.

ACKNOWLEDGEMENTS

We acknowledge the important contribution of the Flow Cytometry Core, H. Lee Moffitt Cancer Center. This project was supported in part by Inhibition Therapeutics Inc., the Margaret Ewell Dickens Foundation and the Research Service of the Veterans' Administration.

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