Protein kinase C-η regulates resistance to UV- and γ-irradiation–induced apoptosis in glioblastoma cells by preventing caspase-9 activation[1]

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Both increased cell proliferation and apoptosis play important roles in the malignant growth of glioblastomas. We have demonstrated recently that the differential expression of protein kinase C (PKC)-η increases the proliferative capacity of glioblastoma cells in culture; however, specific functions for this novel PKC isozyme in the regulation of apoptosis in these tumors has not been defined. In the present study of several glioblastoma cell lines, we investigated the role of PKC-η in preventing UV- and γ-irradiation–induced apoptosis and in caspase-dependent signaling pathways that mediate cell death. Exposure to UV or γ irradiation killed 80% to 100% of PKC-η–deficient nonneoplastic human astrocytes and U-1242 MG cells, but had little effect on the PKC-η–expressing U-251 MG and U-373 MG cells. PKC-η appears to mediate resistance to irradiation specifically such that when PKC-η was stably expressed in U-1242 MG cells, more than 80% of these cells developed resistance to irradiation-induced apoptosis. Reducing PKC-η expression by transient and stable expression of antisense PKC-η in wild-type U-251 MG cells results in increased sensitivity to UV irradiation in a fashion similar to U-1242 MG cells and nonneoplastic astrocytes. Irradiation of PKC-η–deficient glioblastoma cells resulted in the activation of caspase-9 and caspase-3, cleavage of poly (ADP-ribose) polymerase (PARP), and a substantial increase in subdiploid DNA content that did not occur in PKC-η–expressing tumor cells. A specific inhibitor (Ac-DEVD-CHO) of caspase-3 blocked apoptosis in PKC-η–deficient U-1242 MG cells. The data demonstrate that resistance to UV and γ irradiation in glioblastoma cell lines is modified significantly by PKC-η expression and that PKC-η appears to block the apoptotic cascade at caspase-9 activation. *Neuro-Oncology 4, 9-21, 2002 (Posted to Neuro-Oncology [serial online], Doc. 01-032, November 28, 2001. URL <neuro-oncology.mc.duke.edu>)*

GBM[3] as a biologically aggressive neoplasm has an elevated, often aberrant, proliferative capacity with a diffuse pattern of brain invasion. These features preclude complete tumor resection and make the formulation of effective adjuvant therapies imperative. Although these tumors invariably have increased numbers of proliferating cells, apoptosis, while always present, appears to be highly variable (Heesters et al., 1999; Takekawa et al., 1999; Yew et al., 1998). A better understanding of how the apoptotic cascade is regulated in glioblastoma cells could be helpful in designing more efficacious adjuvant therapies.

Although radiation is the most common adjuvant treatment for malignant astrocytomas and glioblastomas, the therapeutic responses of these tumors are typically heterogeneous and incomplete and are frequently accompanied by development of tumor resistance (Yount et al., 1998). Two distinct cellular responses to radiation have been identified in glioblastoma cells (Miyashita et al.,

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[3]Abbreviations used are as follows: GBM, glioblastoma multiforme; α-MEM, α-minimal essential medium; PARP, poly (ADP) ribose polymerase; PKC, protein kinase C.

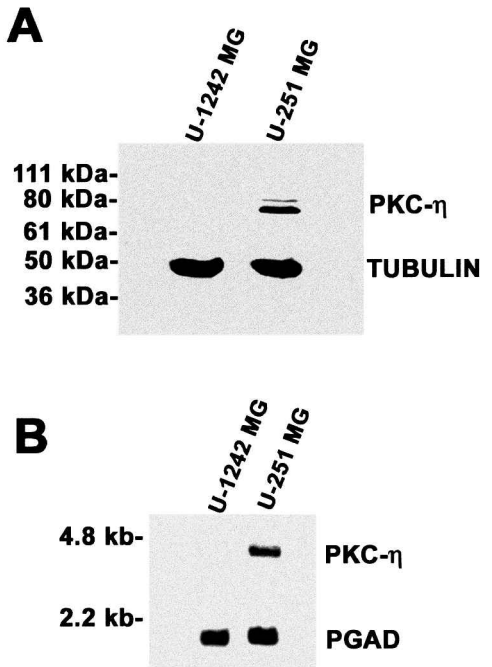

Fig. 1. PKC-η expression in glioblastoma cells. A. Western blot analysis of cellular protein (200 μg/lane) after sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE). PKC-η protein was detected with polyclonal antibody (sc-215). DM1A monoclonal antibody was used to detect tubulin as a control for protein loading. PKC-η was detected in U-251 MG cells but not in U-1242 MG cells. B. Northern blot analysis of U-1242 MG and U-251 MG cells. Total RNA (20 μg) from each clone was subjected to Northern blot analysis using [α-^{32}P]dCTP-labeled probes for PKC-η and phosphoglyceraldehyde dehydrogenase (loading control).

1994). One is p53-independent apoptosis and the other is p53-dependent G_1-arrest. Previous studies correlating radiation resistance with loss of the tumor suppressor gene *TP53* yielded conflicting results (McIlwrath et al., 1994; Slichenmyer et al., 1993). Mutation of *TP53* has been associated with increased, decreased, or no change in radiation resistance of cancer cells (Biard et al., 1994; Brachman et al., 1993; Dee et al., 1995; Jung et al., 1992). The regulation of p53-independent apoptosis in glioblastoma cells is not well understood.

Apoptosis is a physiologic form of cell death characterized by distinct morphologic and biochemical changes such as an increase in the cellular reactive oxygen intermediates (Lee and Um, 1999), internucleosomal DNA degradation (Sakahira et al., 1998), and nuclear condensation (Janicke et al., 1998). Several studies suggest that there are 2 distinct signaling pathways mediating apoptosis, one involving cell surface death receptors and the other mediated via the release of mitochondrial cytochrome *c* (Li et al., 1997; Earnshaw et al., 1999; Rodriguez and Lazebnik, 1999) and apoptosis induction factor (AIF) (Susin et al., 2000; Daugas et al., 2000). The ligation of the so-called death receptors (Fas, tumor necrosis factor-α receptor, and DR3-6) by their ligands (FasL and tumor necrosis factor-α) leads to activation of one of the major initiator caspases, caspase-8 (Earnshaw et al., 1999). This in turn causes cleavage and activation of caspse-3 and caspase-7; and, finally, active caspase-3 cleaves and activates caspase-6 (Earnshaw et al., 1999). The most well-studied mitochondrial pathway is through the release of cytochrome *c* that interacts with apoptotic protease-activating factor-1 (Apaf-1) to activate one of the other initiator caspases, caspase-9, which in turn cleaves downstream effector caspases-3, -6, and -7. The latter then cleave various death substrates, such as PARP, mouse double-minute 2 (MDM2), focal adhesion kinase (FAK), and others to induce apoptosis (Earnshaw et al., 1999; Rodriguez and Lazebnik, 1999). One of the cytosolic death substrates for caspase-3 is a novel PKC (PKC-δ) that is proapoptotic in keratinocytes (Denning et al., 1998).

PKC is a family of phospholipid-dependent kinases that are involved in various cellular responses, including apoptosis. There are 3 major classes of PKC isozymes: conventional (α, βI, βII, and γ), which are activated by acidic phospholipids, calcium, and diacylglycerols or phorbol esters; novel (δ, η, ε, and θ), which lack the calcium-binding segment; and atypical (λ and ζ), which are independent of calcium and phorbol ester (reviewed in Ron and Kazanietz, 1999). PKC inhibitors, administered either alone or in combination with other chemotherapeutic agents, induced apoptosis in neuroblastomas and GBM (Behrens et al., 1995; Couldwell et al., 1994; Ikemoto et al., 1995), and some PKC inhibitors, including bryostatin and derivatives of staurosporine such as UCN-01 and CGP41251, have been evaluated as anticancer agents in patients (Caponigro et al., 1997).

Conversely, a number of PKC isozymes have been implicated in apoptosis in other cell types. In human keratinocytes, PKC-δ is cleaved by UV irradiation in a caspase-dependent proteolysis (Denning et al., 1998). Similarly, some other PKC isozymes (α, βI, ε, θ, and ζ)

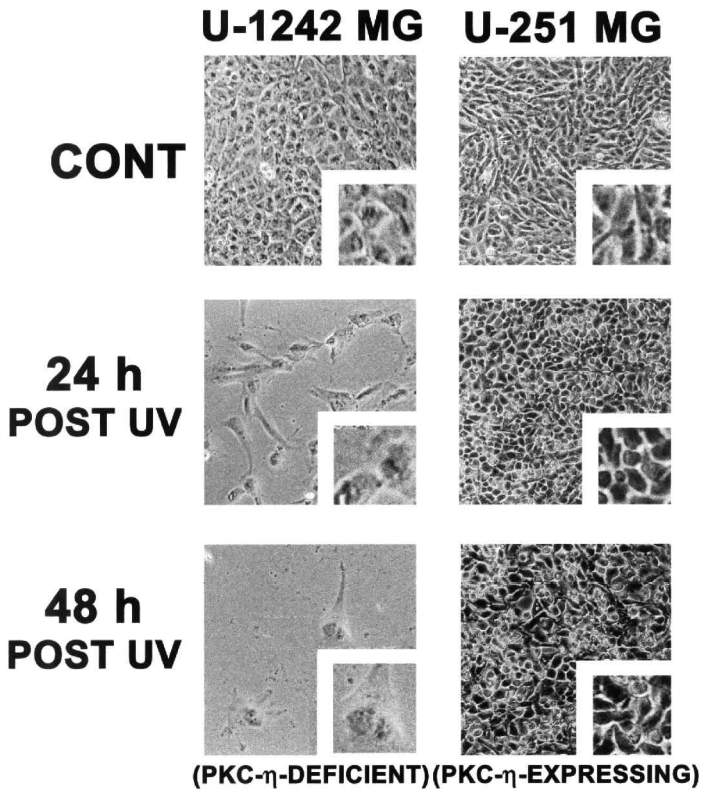

Fig. 2. Irradiation-resistant PKC-η–expressing glioblastoma cells. Glioblastoma cell lines (U-1242 MG and U-251 MG) were grown to 100% confluence and exposed to UV light (87.3 mJ/m^2) for 15 s in serum-free α-MEM (n = 3). Cells were kept in the incubator for 24 h, washed 3 times in serum-free α-MEM, and photographed. Original magnification x100.

undergo limited proteolysis during apoptosis (Datta et al., 1997; Emoto et al., 1996; Mizuno et al., 1997; Shao et al., 1997). Ceramide, an apoptotic product, induces the redistribution of PKC-δ and PKC-ε from a particulate to a cytosolic compartment in human leukemia cells (Verheij et al., 1996). In contrast, Jamieson et al. (1999) demonstrated that the activity of PKC-ι is necessary for BCR-Abl–mediated resistance to drug-induced apoptosis in K562 chronic myelogenous leukemia cells. Recently, Akkaraju and Basu (2000) reported that the expression of PKC-η in a breast cancer cell line (MCF-7 cells) attenuates tumor necrosis factor–induced cell death by preventing activation of caspase-7 and caspase-8.

In this study, we investigated the role of the novel PKC isoform, PKC-η, in the response of glioblastoma cells to irradiation. Both human nonneoplastic astrocytes and the U-1242 MG glioblastoma cell line, which lack PKC-η, were sensitive to either UV or γ irradiation, whereas U-251 MG and U-373 MG cell lines, which express PKC-η, were resistant to irradiation. Expression of PKC-η in the U-1242 MG cells and the knockout of PKC-η expression in U-251 MG cells conferred and abrogated radiation resistance, respectively. Both UV and γ irradiation activated caspase-9 and caspase-3 and cleaved PARP in PKC-η–deficient U-1242 MG cells but not in PKC-η–expressing cells. These data suggest that expression of PKC-η by glioblastoma cells may affect the sensitivity to radiation therapy by blocking caspase-9 activation.

Materials and Methods

Materials

Antibody (DMA1) to tubulin was purchased from Sigma (St. Louis, Mo.). A cDNA probe (GDB 129009) and a polyclonal antibody specific for human *PKC-η* were from the American Tissue Culture Collection (ATCC) (Rockville, Md.) and Santa Cruz Biotechnology, Inc. (Santa Cruz, Calif.), respectively. PKC-δ antibody was from Transduction Laboratories (San Diego, Calif.). Antibody against PARP was from Kamiya Biomedical Co. (Seattle, Wash.), and antibodies against caspases-3, -6, -7, and -9 are products of Oncogene Research Products (Boston, Mass.). Antibodies that recognize cleaved and active caspase-3 were purchased from BD PharMingen (San Diego, Calif.). A specific caspase-3 inhibitor (Ac-DEVD-CHO) was from Alexis Biochemicals (San Diego, Calif.). Lipofectamine is a product of GIBCO BRL (Gaithersburg, Md.).

Cell Culture

Human U-251 MG (mutant *TP53*) and U-1242 MG (wild-type *TP53*) cell lines were originally isolated from astrocytic tumors that were designated as glioblastomas, and their PKC profiles were described previously (Hussaini et al., 2000). U-373 MG cells were purchased from ATCC. Although monolayer cultures of the cell lines

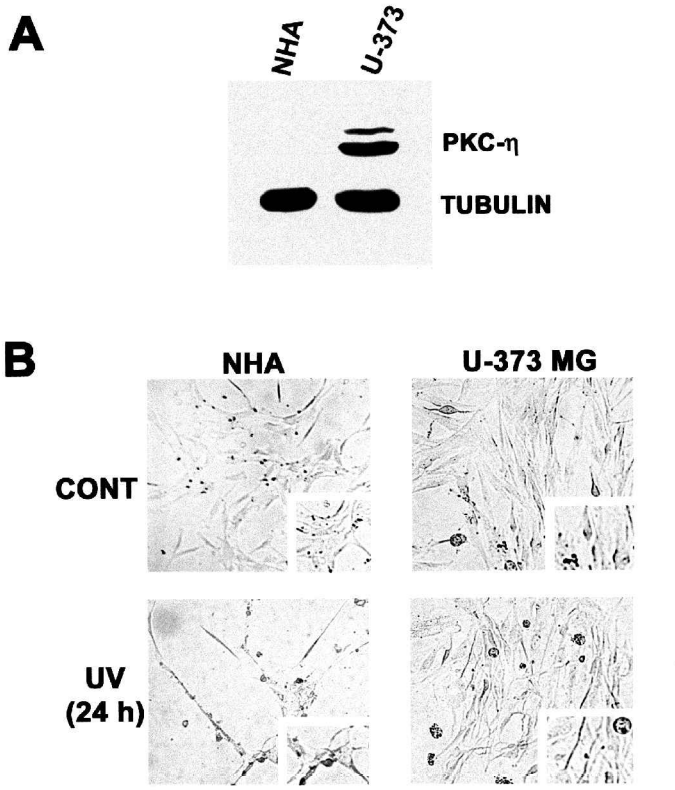

Fig. 3. Differential expression of PKC-η between normal human astrocytes (NHA) (UV sensitive) and glioblastoma cells, U-373 MG (UV resistant). A. Western blot analysis of cellular protein (200 μg/lane) was performed by SDS-PAGE. PKC-η protein was detected with polyclonal antibody (sc-215). DM1A monoclonal antibody was used to detect tubulin as a control for protein loading. B. NHA and U-373 MG cells were grown to 60% confluence and exposed to UV light (87.3 mJ/m^2) for 15 s in serum-free α-MEM. Cells were kept in the incubator for 24 h, washed 3 times in serum-free α-MEM, and photographed. Original magnification x100.

have significant proliferative fractions in nonconfluent growth conditions, U-1242 MG cells demonstrate a relative contact inhibition at confluence that U-251 MG or U-373 MG cells lack (data not shown). The lines were regularly determined to be free of mycoplasma with reagents from Gen-Probe, Inc. (San Diego, Calif.). Cells were grown in minimal essential medium-α modification (α-MEM with 10% defined fetal bovine serum [Hyclone, Logan, Utah]) and 20 μg/ml bovine zinc-insulin (25.7 IU/mg) (Sigma). Primary cultures of human fetal astrocytes (NHA) were obtained from Clonetics (San Diego, Calif.) and cultured in a growth medium containing 25 μg/ml bovine insulin, 20 ng/ml epidermal growth factor, 5% fetal bovine serum, 25 ng/ml progesterone, and 50 μg/ml transferrin. The cells were cultured to 100% confluence and passaged every 4 to 5 days from an initial concentration of 6 to 8 × 10^3/cm^2 in T flasks, 6- or 24-well plates, and cultured at 37°C in 4.8% CO_2, 90% relative humidity.

Transfection of PKC-η cDNA into U-1242 MG Cells and Expression of an Antisense RNA Construct in U-251 MG Cells

A 2.2-kb human PKC-η cDNA fragment with a complete coding sequence was inserted into a pCI-neo expression vector with a cytomegalovirus promoter and an SV40-neoresistance gene cassette. U-1242 MG cells were transfected with 5 μg/ml of the human PKC-η cDNA or with empty vector (pCI-neo that did not contain a cDNA insert) by incubation for 6 h with lipofectamine (4 μl/ml in serum-free α-MEM). The cells were then washed twice with serum-free α-MEM and cultured in 10% fetal calf serum–supplemented medium containing the aminoglycoside G418 (300 μg/ml). After 4 weeks, the cultures were screened for *PKC-η* expression and single-cell clones were derived by limiting dilution.

PKC-η antisense RNA expression constructs were designed for stable integration and constitutive RNA synthesis. A *Xho*I-*Eco*RI restriction fragment of 408 bp (PKC-η cDNA 2-410 bp) was excised and ligated, in reversed orientation, into eukaryotic expression vector pCI-neo. U-251 MG cells were transfected with the human PKC-η antisense construct (4, 8, and 10 μg/ml) in serum-free α-MEM for 4 h with lipofectamine (4 μl/ml in serum-free α-MEM). The cells were then washed twice with serum-free α-MEM and cultured in 10% fetal calf serum–supplemented medium. Stable PKC-η knockout clones were screened with G418 as described above.

Western Blot Analysis

For the detection of PKC isozymes, caspases, PARP, or tubulin, the rinsed cultured cells were extracted with 1%

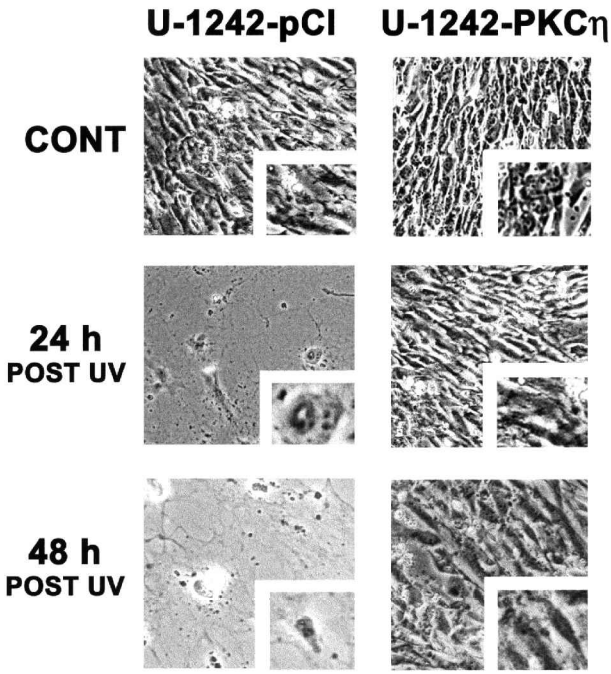

Fig. 4. U-1242 MG cells stably transfected with PKC-η and showing no apoptosis. Cultures of U-1242-pCI or U-1242- PKC-η were exposed to UV for 15 s in 10% serum containing α-MEM. Cells were kept in the incubator for either 24 h or 48 h, washed 3 times in serum-free α-MEM, and photographed. The experiment was carried out in triplicate. Original magnification x100.

Triton X-100, 0.2% Nonidet P-40 (NP-40) in the presence of 2 mM EDTA, 100 μM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, and 1.0 μg/ml aprotinin. Proteins were boiled for 5 min in sodium docecyl sulfate/polyacrylamide gel electrophoresis buffer and separated by sodium docecyl sulfate/polyacrylamide gel electrophoresis on 10% or 12% polyacrylamide slabs. They were then electroblotted onto nitrocellulose-1 (GIBCO BRL) and reacted with polyclonal and monoclonal antibodies specific to PKC isozymes and tubulin, respectively. The antibodies were detected with antirabbit or antimouse peroxidase conjugates, and final detection was carried out with chemiluminescence enhancement (Amersham, Piscataway, NJ) as described by the manufacturer. Densitometer and ImageQuant software (Molecular Dynamics, Sunnyvale, Calif.) were used to quantitate the protein bands.

Northern Blot Analysis

Total RNA was isolated from adherent cells using TRIZOL reagent (GIBCO BRL) as a monophasic solution of phenol and guanidine isothiocyanate, as modified from the single-step RNA isolation method developed by Chomczynski and Sacchi (1987). All total RNA samples were quantified by UV spectrophotometry (200 ± 25 μg/10^7 cells; the 260/280 ratio ranged between 1.6 and 1.75) and stored at –70° C before use. Total RNA (20 μg) from control and treated glioblastoma cells was electrophoresed on 1% (wt/vol) agarose gels and electroblotted onto Zeta probe nylon membranes (Bio-Rad Laboratories, Richmond, Calif.). A cDNA probe specific for human PKC-η was cut with *Xba*I and separated on low-melting Seaplaque agarose (FMC BioProducts, Rockland, Maine). The probe (25-50 ng) was labeled with [α-^{32}P]dCTP (50 μCi) using a random prime DNA labeling kit (GIBCO BRL). Electroblotted RNA on nylon membranes was hybridized with the labeled cDNA fragment at 42°C for 24 h. As a control for loading, membranes also were hybridized with labeled human-specific phosphoglyceraldehyde dehydrogenase cDNA. PhosphoImager (Molecular Dynamics) analysis was used to quantitate the radioactive bands.

Flow Cytometry

Control and UV-irradiated glioblastoma cells were analyzed for DNA content using flow cytometry. Cells were washed twice with 1% bovine serum albumin/phosphate-buffered saline solution by centrifugation (1500 × g for 5 min), and 1×10^6 cells were resuspended in 1 ml staining solution (0.1% sodium citrate, 0.1% Triton X-100, 50 μg/ml propidium iodide, and 100 U/ml RNAse [1 mg/ml]). Cells were stained for 30 min at room temperature before DNA content was analyzed. Analytical cytometry was performed on a FACScaliber system using CellQuest software (Becton Dickenson, San Jose, Calif.). Gating to exclude debris was based upon DNA fluorescence, and a minimum of 40,000 events were collected per sample for analysis.

Irradiation

Both subconfluent (60%-80%) and confluent (100%) cultures were used for radiation studies. Cells were exposed to UV (87.3 mJ/m^2) for 15 s or γ irradiation (2 doses of 20 Gy

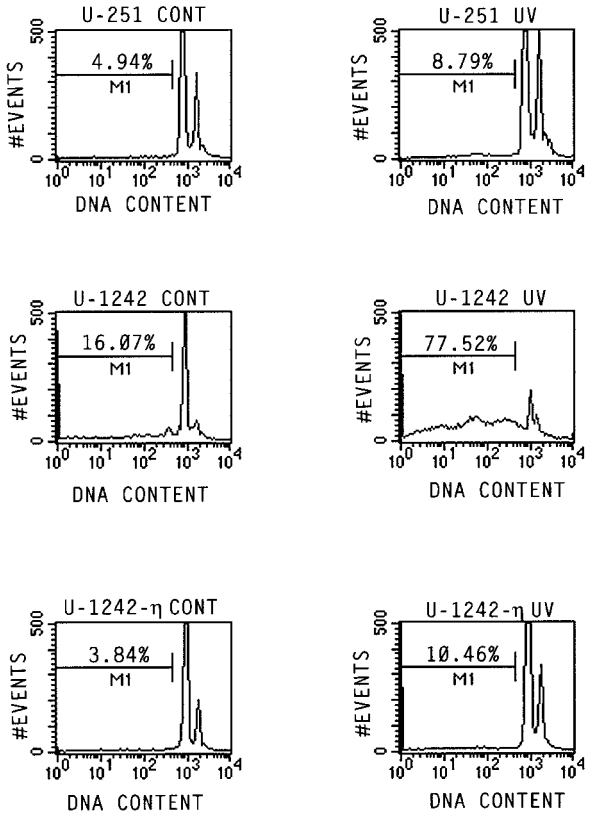

Fig. 5. Propidium iodide fluorescence (logarithmic scale) of U-251 MG, U-1242 MG, and U-1242-PKC-η cells. Control and UV-irradiated cells were stained with propidium iodide (50 μg/ml) and analyzed by fluorescence-activated cell sorting.

separated by 24 h) in serum-containing or serum-free α-MEM. After either the single UV exposure or the second γ irradiation, cells were washed 3 times with serum-free α-MEM and photographed or lysed for protein or DNA analysis at 3, 6, or 24 h. Preliminary experiments were performed to determine the doses of γ irradiation that were cell-killing. When cultures were exposed to a single dose of 10, 20, or 50 Gy, only the 50-Gy dose was sufficient to kill U-1242 MG cells (PKC-η–deficient). When the dose was lowered to 20 Gy, 2 doses at 24 h intervals had the same effect as the single 50-Gy treatment. The single 20-Gy dose with a total of 40 Gy is similar to tumor irradiation doses achieved at the margins and center of tumors with gamma knife radiosurgery (Kim et al., 1999; Niranjan et al., 2000).

Cell Number

Viable cell numbers were determined by trypan blue exclusion. Glioblastoma cells were released from the plate with 0.05% wt/vol trypsin-EDTA (0.53 mM) for 2 min, centrifuged, and resuspended in serum-free α-MEM. Trypan blue stain (0.4% wt/vol) was added, and cells were counted under the microscope with a hemocytometer.

Statistics

Results show mean ± SE with n as the number of observations. Statistical analysis of results was performed using unpaired Student's t test. A P value (2-tailed) of ≤ 0.05 indicated statistical significance.

Results

PKC-η Expression in Glioblastoma Cells

The basal and phorbol 12-myristate 13-acetate (PMA)-regulated expression of 8 PKC isozymes was previously determined by Western blot analysis in U-251 MG and U-1242 MG cells (Hussaini et al., 2000). Both astrocytic cell lines expressed multiple conventional (α, βI, βII, and γ) as well as novel (δ, ε, and θ) PKC isozymes. A doublet of PKC-η–reactive bands was detected only in U-251 MG cells, and not in U-1242 MG cells (Fig. 1). In U-251 MG cells, a PKC-η mRNA transcript of 4.3 kb was detected, whereas no detectable mRNA was observed in U-1242 MG cells (Fig.1).

Effect of PKC-η Expression on UV Resistance in Glioblastoma Cells

Because the differential expression of PKC-η increases the proliferative capacity of glioblastomas (Hussaini et al., 2000), we examined the role of PKC-η expression in blocking the apoptosis in response to UV irradiation. Subconfluent or confluent cultures in 10% serum-containing α-MEM were exposed to UV irradiation (87.3 mJ/m^2). A single exposure killed 80% to 100% of PKC-η-deficient U-1242 MG cells in 24 h and > 95% in 48 h, but did not significantly affect

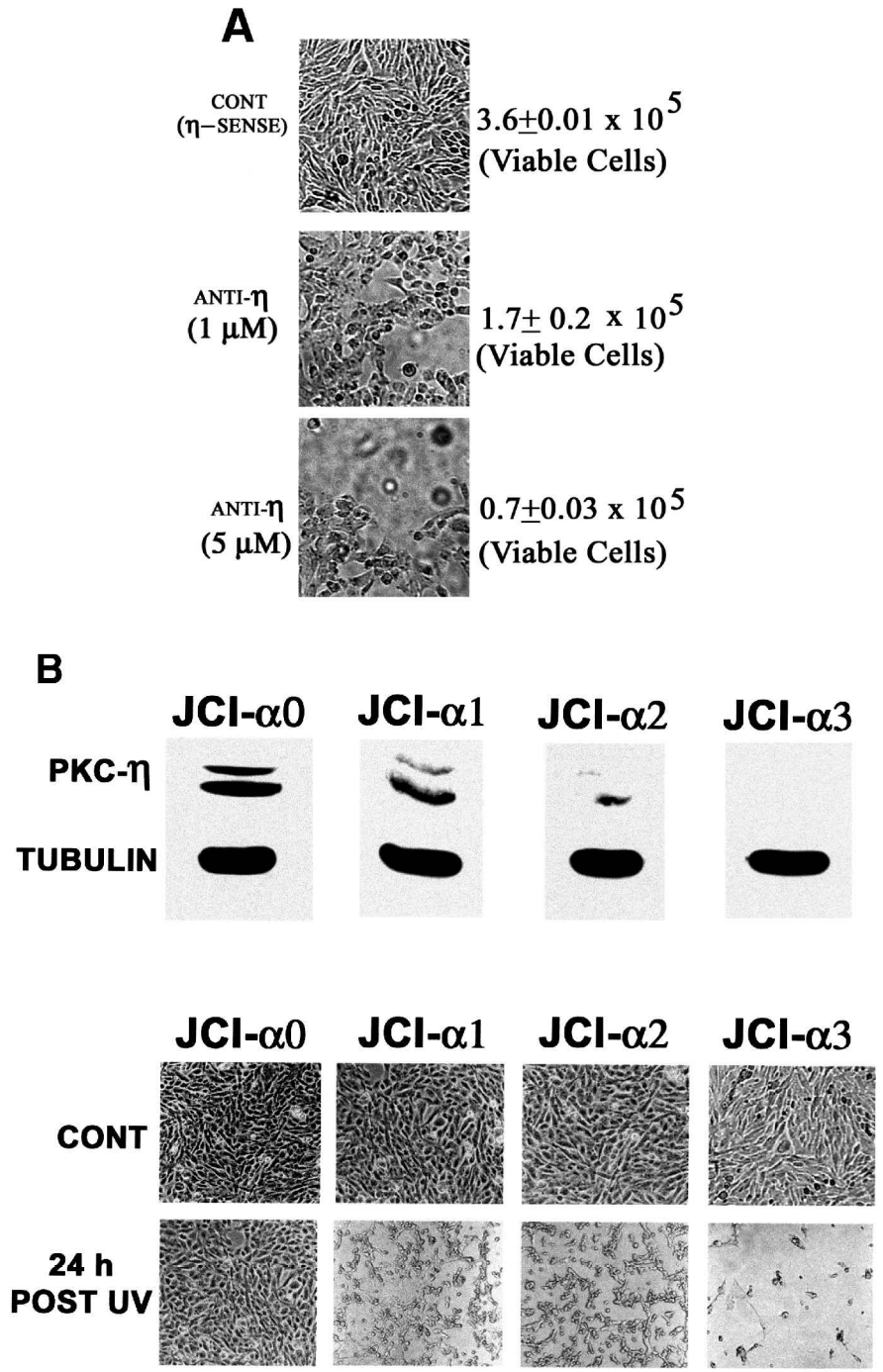

Fig. 6. Loss of UV resistance in U-251 MG cells by PKC-η antisense transfection. A. Transient PKC-η antisense oligonucleotide transfection. Cells were pretreated with PKC-η oligonucleotide antisense (1 or 5 μM) or sense (5 μM) for a total of 20 h and exposed to UV for 15 s. Twenty-four hours after irradiation, cells were washed 3 times and photographed. Adherent cells were trypsinized, stained with trypan blue dye, and counted under the microscope. B. Cells transfected with PKC-η antisense construct. Stable single cells were cloned from mixed cultures and exposed to UV for 15 s. Twenty-four hours after irradiation, the adherent and floating cells were used for Western blot analysis, or the adherent cells were washed 3 times and photographed.

the viability of PKC-η–expressing U-251 MG cells (Fig. 2). All experiments were performed in triplicate. Although U-251 MG cells did lose the spindle shape and become more rounded with irradiation, the cells could be successfully subcultured. UV irradiation induced DNA laddering, an indication of apoptosis, in U-1242 MG cells but not in U-251 MG cells (data not shown). Figure 3 also shows that differential expression of PKC-η in normal human astrocytes (PKC-η–deficient) and U-373 MG cells (PKC-η–expressing) confers sensitivity and resistance to UV irradiation, respectively.

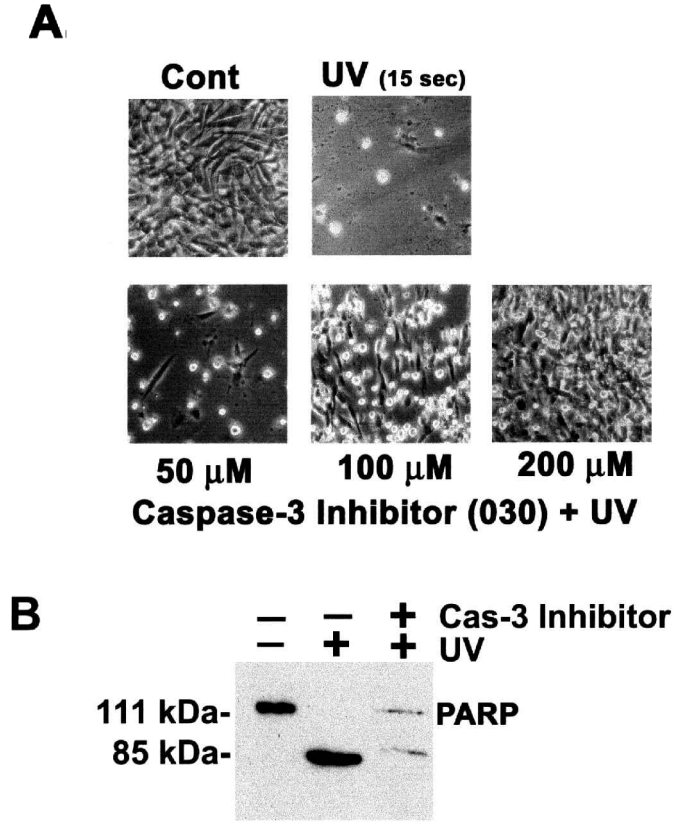

Fig. 7. Effects of caspase-3 inhibition on apoptosis in PKC-η–deficient U-1242 MG cells. A. U-1242 MG cells treated for 1 h with different concentrations of caspase-3 inhibitor (Ac-DEVD-CHO, 030) prior to UV irradiation for 15 s. The cells were then washed 3 times with serum-free α-MEM 24 h postirradiation and photographed. B. Analysis of PARP expression in cells treated with caspase-3 inhibitor. U-1242 MG cells were treated with or without 100 μM of caspase-3 inhibitor (Ac-DEVD-CHO, 030), and PARP expression was analyzed by Western blotting using a polyclonal antibody.

Stable Transfection of PKC-η Blocks UV-Induced Apoptosis in U-1242 MG Cells

To test whether PKC-η can confer resistance specifically to UV irradiation–induced apoptosis, a U-1242 clone (U-1242-PKC-η) that expresses both PKC-η protein and mRNA (Hussaini, et al., 2000) was compared with a non-PKC-η–expressing control (U-1242-pCI) in response to UV irradiation. As shown in Fig. 4, the U-1242-PKC-η had dramatically increased resistance to UV irradiation–induced cell death, with >80% viable cells after irradiation. Similar to the U-251 MG cells, these UV-irradiated U-1242-PKC-η cells also could be subcultured.

Quantitation of Apoptosis in Glioblastoma Cells Using Propidium Iodide DNA Staining

U-251 MG, U-1242 MG, and U-1242-PKC-η cells were exposed to UV irradiation (87.3 mJ/m^2) for 15 s to induce apoptosis in serum-free α-MEM and were analyzed for propidium iodide–stained DNA content by fluorescence-activated cell sorting analysis. Fig. 5 demonstrates typical propidium iodide histograms of cells in cycle (G_0/G_1 and G_2/M) and undergoing apoptosis (sub-$G_{0,}$). After being irradiated, the sub-G_0 apoptotic cells increased by 3.9%, 61.5%, and 6.7% (n = 2) over control (nonirradiated cells) in U-251 MG, U-1242 MG, and U-1242-PKC-η cells, respectively.

PKC-η antisense neutralizes resistance of U-251 MG cells to irradiation-induced apoptosis transient PKC-η knockdown. To determine whether knocking down PKC-η expression would affect the sensitivity of U-251 MG cells to UV irradiation, we incubated the cells with PKC-η antisense oligonucleotide. Fig. 6A shows the effect of PKC-η antisense on sensitivity to UV irradiation. The sense oligonucleotide (5 μM) had no effect on the resistance of U-251 MG cells to UV, but the antisense oligonucleotide dose dependently increased the sensitivity of the cells to UV. Exposure of U-251 MG cells to UV irradiation in the presence of PKC-η oligonucleotide antisense (1 or 5 μM) killed 52% or 82% (n = 3, P < 0.001) of the cells, respectively. We have shown previously that PKC-η antisense oligonucleotide selectively decreased the expression of PKC-η by approximately 80%, but had no significant effect on other PKC isozymes examined (Hussaini et al., 2000).

Stable PKC-η–knockout U-251 MG cells. To further confirm our transient knockdown study, we generated stable PKC-η–deficient U-251 MG cells and exposed them to UV irradiation. None of the cells that were generated from cultures transfected with the empty vector (pCI-neo) control construct demonstrated partial or complete PKC-η defi-

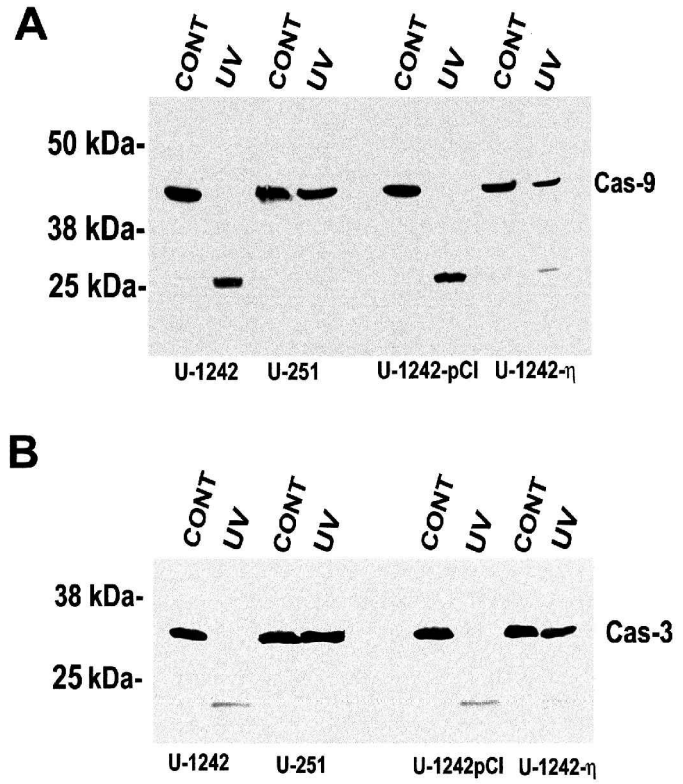

Fig. 8. Caspase cleavage in PKC-η–deficient cells. Western blot analysis of cellular protein (200 μg/lane) was performed after SDS-PAGE. Cleavage of caspase-9 (A) or caspase-3 (B), as a measure of activation, was detected with a monoclonal or polyclonal antibody, respectively. The U-1242-pCI clone was from U-1242 MG cells transfected with an empty vector. The PKC-η clone was from U-1242 MG cells transfected with pCI-neO-PKC-η. U-251 MG cells express endogenous PKC-η. Cells were exposed to UV for 15 s and proteins were extracted for immunoblotting 24 h after irradiation.

ciency (JCI-α0) (Fig. 6B). PKC-η expression was decreased by approximately 40% to 100% in the knockdown (JCI-α1 and JCI-α2) and knockout (JCI-α3) clones, respectively (Fig. 6B). The expression of PKC-η, a novel PKC isoform that has the highest amino acid sequence similarity to PKC-η, was unaffected. In the PKC-η knockout clone, irradiation killed >95% of the cells while the knockdown clones showed approximately 50% survival (compared with 100% survival in wild-type U-251 MG cultures; Fig. 1).

Caspase-3 Inhibition Blocks Apoptosis in PKC-η–Deficient U-1242 MG Cells

The only downstream effector caspase detected in U-1242 MG cells is caspase-3; the other 2 downstream caspases, caspase-6 and caspase-7, were absent (data not shown). Caspase-3 can cleave death substrates both in the cytosol and nucleus of cells during apoptosis (Earnshaw, et al., 1999; Rodriguez and Lazebnik, 1999). To determine whether caspase-3 is involved in apoptosis of U-1242 MG cells, we blocked its activity with a specific inhibitor (Ac-DEVD-CHO; 030) and assessed the effect of UV irradiation. In a concentration-dependent manner, the caspase inhibitor protected the PKC-η–deficient cells from UV-induced apoptosis (Fig. 7A). We also tested the effectiveness of the inhibitor on blocking the cleavage of a nuclear death substrate for caspase-3, PARP. Fig. 7B shows UV-induced cleavage of PARP in U-1242 MG cells, which was partially blocked by Ac-DEVD-CHO (100 μM). PARP (116 kDa) and PKC-η (78 kDa) were cleaved by irradiation to generate their respective fragments (85 kDa and 40 kDa) only in PKC-η–deficient U-1242 MG cells, but not in PKC-η–expressing U-251 MG cells (data not shown).

Caspase Activation–Mediated Apoptotic Pathway in U-1242 MG Cells

Because caspase-3 activation is implicated in UV-induced apoptosis by a specific inhibitor (see above), we investigated the activation of both caspase-3 and its upstream activator (caspase-9) in response to UV by the detection of cleavage products. Both caspase-3 (32 kDa) and caspase-9 (48 kDa) were activated by UV irradiation in U-1242 MG cells and not in U-251 MG cells (Fig. 8), as determined by the generation of cleavage products of 17 kDa and 31 kDa, respectively. The cleavage of both caspases was detected as early as 6 h after UV irradiation. In U-242 MG cells transfected with the *PKC-η* gene, the activation of both caspase-3 and caspase-9 was substantially decreased (Fig. 8). Some of the U-1242-PKC-η cells (U-1242 MG transfected with PKC-η) might not be

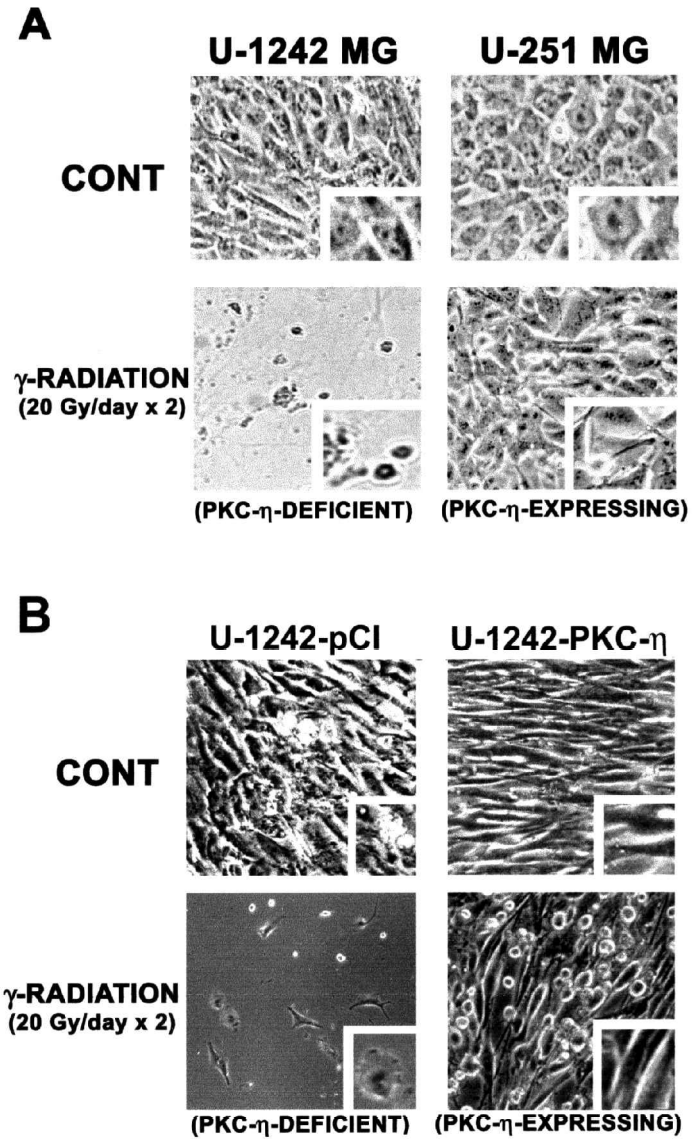

Fig. 9. A. PKC-η–expressing astrocytic tumor cells showing resistance to γ irradiation. Glioblastoma cell lines (U-1242 MG and U-251 MG) were grown to 100% confluence and exposed to γ irradiation (20 Gy/day) for 2 days in serum-free α-MEM. Cells were kept in the incubator for either 24 h or 48 h, washed 3 times in serum-free α-MEM and photographed. Original magnification X 100. B. U-1242 MG cells in which apoptosis is blocked after stable transfection with PKC-η. Cultures of U-1242-pCI or U-1242-PKC-η were exposed to γ irradiation (20 Gy/day) for 2 days in serum-free α-MEM. Cells were kept in the incubator for either 24 h or 48 h, washed 3 times in serum-free α-MEM and photographed. Original magnification x100.

expressing high enough levels of the PKC isozymes to protect them from irradiation. We always see some (10%-20%) cell death with this clone. That may explain the partial cleavage of caspase-3 and caspase-9 in the U-1242-PKC-η clone.

PKC-η Expression and Resistance to γ Irradiation

To determine whether PKC-η expression conferred resistance to γ irradiation as it did to UV irradiation, we exposed U-251 MG and U-1242 MG cells to γ irradiation. First, exposure of the PKC-η–deficient U1242 MG cells to γ irradiation killed only 30%, but a second exposure of the cells to the same dose 24 or 48 h after the first exposure killed almost 100% of U-1242 MG cells. The γ irradiation protocol did not significantly affect the viability of PKC-η–expressing U-251 MG cells (Fig. 9). The number of control U-251 MG cells was $8.35 \pm 0.8 \times 10^6$ compared with $6.7 \pm 0.5 \times 10^6$ γ-irradiated cells (mean ± SE; $P > 0.05$; $n = 4$). Similarly, the PKC-η–expressing U-1242 MG cells (U-1242-PKC-η) were also resistant to γ irradiation (Fig. 9). PARP and caspase-9 were cleaved in response to γ irradiation in U-1242 MG cells, but not in U-251 MG or U-1242-PKC-η cells (Fig. 10).

Discussion

Cellular proliferation in glioblastomas is elevated and often aberrantly regulated. Likewise, the regula-

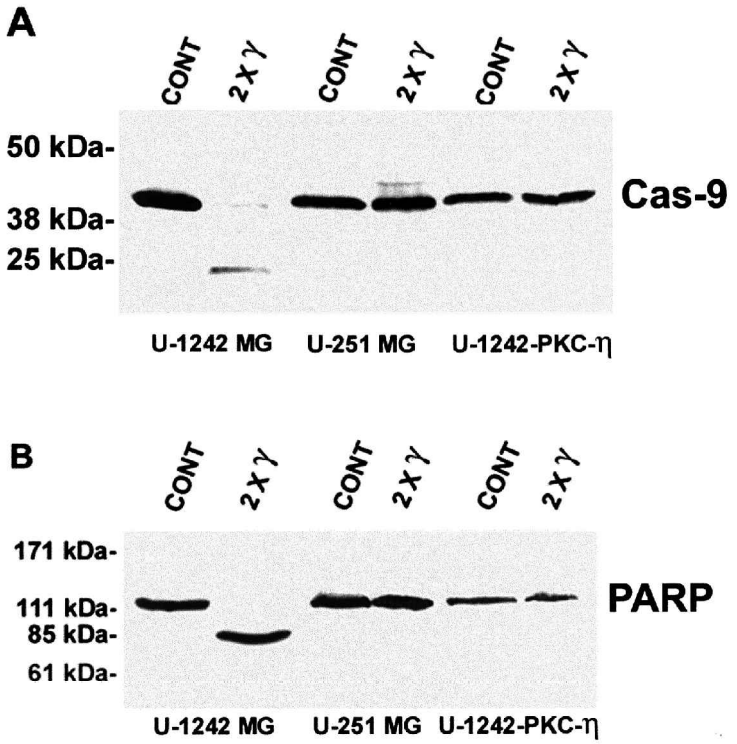

Fig. 10. Effect of γ irradiation on caspase-9 and PARP in glioblastoma cells. Western blot analysis of cellular protein (200 μg/lane) was performed after SDS-PAGE. Cleavage of caspase-9 (A) or PARP (B) was detected with a monoclonal or polyclonal antibody, respectively. The PKC-η clone was from U-1242 MG cells transfected with pCI-neo-PKC-η. U-251 MG cells express endogenous PKC-η. Cells were exposed to γ irradiation (2 x 20 Gy), and proteins were extracted for immunoblotting 24 h after the second dose of irradiation.

tion of apoptosis appears to be atypical, but overall levels appear to be highly variable (Heesters et al., 1999; Takekawa et al., 1999; Yew et al., 1998). The relationship between increased proliferative capacity and the underlying mechanisms that regulate apoptosis in glioblastomas is not well understood. Previous studies demonstrated that the differential expression of PKC-η increases the proliferative capacity of glioblastomas (Hussaini et al., 2000). In this study, we demonstrated that PKC-η–deficient nonneoplastic astrocytes and glioblastoma cells (U-1242 MG) are sensitive to UV and γ irradiation, whereas PKC-η–expressing cells are resistant to both forms of irradiation.

Several experiments suggest that resistance to UV and γ irradiation in glioblastoma cell lines in culture is specifically affected by the expression of PKC-η. First, stable transfection of PKC-η into PKC-η–deficient U-1242 MG cells renders them resistant to both forms of irradiation-induced apoptosis (Figs. 5 and 9B). Second, reduction or abrogation of PKC-η expression with a specific antisense construct causes UV- and γ-radioresistant U-251 MG cells to become more sensitive to irradiation-induced death (Fig. 6). Third, the apoptotic signaling pathway involving activation of caspase-9 and caspase-3 and proteolysis of PARP was activated by irradiation in U-1242 MG cells, but not in wild-type U-251 MG cells or in U-1242 MG cells stably transfected with the PKC-η expression construct (Figs. 7B and 8). We observed similar protection against UV irradiation in another independent glioblastoma cell line (U-373 MG) that expresses PKC-η (Fig. 3).

The UV- and γ-irradiation–induced apoptosis is mediated via caspase-9 and caspase-3 in PKC-η–deficient glioblastoma cells (Fig. 8) and also involves the cleavage of the death substrate, PARP (Fig. 7B). Blocking the cleavage of death substrates with a specific caspase-3 inhibitor (Ac-DEVD-CHO) increased the resistance of the glioblastoma cells deficient in PKC-η to irradiation (Fig. 7A).

It is not clear how PKC-η expression blocks UV- and γ-irradiation–induced apoptosis. Loss of irradiation-induced cleavage of caspase-9 and caspase-3 with PKC-η expression suggests that PKC-η may interfere with a mitochondria-dependent apoptotic pathway. Cytochrome *c*, released from the intermembrane space of the mitochondria, binds to the cytoplasmic scaffolding protein Apaf-1 and to procaspase-9 to generate the active caspase-9 (Zou et al., 1999). Although some of the events leading to the release of cytochrome *c* from the mitochondria still are not clear, changes in the conformation and/or activity of proapoptotic Bcl-2 family members Bak and Bax may be involved. The antiapoptotic Bcl-2 family members, Bcl-2 and Bcl-x_L, localize to the outer mitochondrial membrane and tend to prevent the release of cytochrome *c* from the mitochondria (Boise and Thompson, 1997). Bcl-2 has been shown to suppress cell death induced by a number of stimuli including chemotherapy and irradiation (Deng et al.,

2000; Sentman et al., 1991). The mechanism of Bcl-2 antiapoptotic activity is not clear. It is suggested that Bcl-2 helps maintain mitochondrial integrity (Green and Reed, 1998) and/or blocks the activation of the downstream caspase cascade that induces apoptosis. Thus, the putative regulation of the expression and/or activity of either antiapoptotic or proapoptotic Bcl-2 members by PKC-η expression could affect the release of mitochondrial cytochrome *c* and subsequently alter activation of caspase-9 and caspase-3. In HL60 cells, mitochondrial PKC-α has been shown to phosphorylate Bcl-2 (Ruvolo et al., 1999). Another possibility is that PKC-η may prevent the translocation of cytoplasmic PKC-δ to mitochondria where PKC-δ has been shown to induce cytochrome *c* release (Majumder et al., 2000).

Radiation therapy is used extensively in the primary management of patients with GBM. Results still remain poor due to the heterogenous clinical response and the development of radioresistance by this group of astrocytomas. Because 40% to 60% of GBM have *TP53* mutations, the development of radioresistance has been previously attributed to abnormal or absent *TP53* function (Chen et al., 1995; Haas-Kogan et al., 1996; Wu et al., 1993). However, no strong correlation between a functional *TP53* and the radiosensitivity of GMB has been identified (Haas-Kogan et al., 1996; Yount et al., 1996). Thus, there is a strong need to identify other cellular components that contribute to radioresistance in experimental systems and that could be targeted to improve tumor responsiveness to radiation therapy. Although understanding the role of PKC-η expression in the development of γ-radioresistance in primary glioblastomas (in situ) awaits additional studies, the characterization of glioblastoma cells exhibiting dramatic differences in radiation sensitivity provides an excellent model system for elucidation of mechanisms of resistance to radiation therapy. These cells will help establish the importance of the mitochondrial/caspase-9 and caspase-3 pathway in the development of resistance to radiation therapy and the role of PKC-η in the pathway. Specific inhibition of PKC-η expression or activity may provide critical adjuvant therapy as a sensitizer to radiation therapy for glioblastomas.

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