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Sensitivity of human glioma U-373MG cells to radiation and the protein kinase C inhibitor, calphostin C

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Abstract. We assessed the radiosensitivity of the grade III human glioma cell line U-373MG by investigating the effects of radiation and the specific protein kinase C inhibitor, calphostin C on the cell cycle and cell proliferation. Irradiated glioma U-373MG cells progressed through G_1-S and underwent an arrest in G_2-M phase. The radiosensitivity of U-373MG cells to graded doses of either photons or electrons was determine by microculture tetrazolium assay. The data was fitted to the linear-quadratic model. The proliferation curves demonstrated that U-373MG cells appear to be highly radiation resistant since 8 Gy was required to achieve 50% cell mortality. Compared to radiation alone, exposure to calphostin C (250 nM) 1 h prior to radiation decreased the proliferation of U-373MG by 76% and calphostin C provoked a weakly synergistic effect in concert with radiation. Depending on the time of application following radiation, calphostin C produced an additive or less than additive effect on cell proliferation. We postulate that the enhanced radiosensitivity observed when cells are exposed to calphostin C prior to radiation may be due to direct or indirect inhibition of protein kinase C isozymes required for cell cycle progression.

INTRODUCTION

Recently, considerable attention has been given to the possibility of utilizing protein kinase C (PKC) inhibitors such as calphostin C, UNC-01, tamoxifen, hypericin and PKC antisense as chemotherapeutic adjuncts for the treatment of malignant gliomas (Ikemoto *et al.* 1995; Baltuch *et al.* 1993; Couldwell *et al.* 1993; Couldwell *et al.* 1994a, 1994b; Dean *et al.* 1994a; Dean & McCay 1994b; Pollack, Kawecki & Lazo 1996; Pollack & Kawecki 1997; Ahmad *et al.* 1994). PKC has been targeted in malignant gliomas since their rapid proliferation rates compared to nontransformed astrocytes have been functionally linked to inherently high levels of glioma PKC, resulting in excessive activation of PKC-mediated pathways (Couldwell *et al.* 1991; Couldwell *et al.* 1994c; Baltuch & Yong 1996). Anti-PKC therapy (Couldwell *et al.* 1994a) in combination with radiation may prove to be promising for controlling

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glioma proliferation since radiation therapy is used for the early treatment of localized gliomas. However, heterogeneity in intrinsic and acquired radiation resistance in gliomas is a major obstacle to the efficacy of radiation therapy (Yang *et al.* 1991) making adjunct therapies necessary.

Previous work with nonspecific PKC inhibitors (i.e. tamoxifen, isoquinoline sulphonamide H7 and staurosporine) revealed the possibility that PKC inhibitors may act as radiation sensitizers (Hallahan *et al.* 1992a, 1992b; Zhang *et al.* 1992; Couldwell *et al.* 1993; Zhang *et al.* 1993a; Zhang *et al.* 1996). However, nonselective protein kinase inhibitors have an IC_{50} (concentration required for 50% inhibition) for PKC in the micromolar concentration range and possible inhibition of multiple kinase(s) could not be excluded (Mori *et al.* 1980). Consequently, these studies are being confirmed with specific PKC inhibitors, i.e. sangivamycin (Hallahan *et al.* 1992a), hypericin (Zhang *et al.* 1996), chelerythrine (Chumra *et al.* 1997) and UCN-01 (Tsuchida & Urano 1997).

Inhibition of PKC may be beneficial since PKC activation is one of the initial events following ionizing radiation exposure and PKC is involved in radiation induced cell cycle arrest at checkpoints associated with genome integrity (Hallahan *et al.* 1991a; Hallahan *et al.* 1992b). The G2 phase arrest is important for cell survival since irradiated cells repair their DNA during this checkpoint and PKC inhibitors increase radiosensitivity by attenuating the radiation-induced G2 arrest (Hallahan *et al.* 1992b; Zhang *et al.* 1993a; Bernhard *et al.* 1994a; Bernhard, McKenna & Muschel 1994b; Maity *et al.* 1997).

Current postulates for radiation-induced PKC activation are that DNA damage may initiate PKC activation, or that radiation directly induces the oxidation of PKC (Hallahan *et al.* 1992b) or that radiolysis of cytosolic and bound water produces hydrogen peroxide, hydroxyl and superoxide radicals (Hutchinson 1985). These oxidants stimulate tyrosine kinase and lipid peroxidation (Nakajima & Yukawa 1996), i.e. hydrolysis of phospholipase C resulting in arachidonate, diacylglycerol and inositol triphosphate causing calcium mobilization and PKC activation (Nicotera *et al.* 1986). Radiation-mediated signal transduction also provokes disturbances in the PKC-phospholipase A2 pathway and affects the transcription of early response genes (i.e. TNF-α, Egr-1 and jun; Hallahan *et al.* 1991a, 1991b; Hallahan *et al.* 1992b) whose transcription can be blocked with PKC inhibitors. Ionizing radiation also promotes PKC-α mRNA expression in Syrian hamster embryo cells in a dose and radiation type dependent manner (Woloschak *et al.* 1990).

Given the growing evidence for the potential therapeutic application of PKC inhibitors and the unknown radiosensitivity of human glioma U-373MG cells, we investigated the effects of radiation and the PKC inhibitor, calphostin C on U-373MG cell cycle and proliferation. Our studies focused on the U-373MG cell line since we have previously characterized its total PKC activity (Acevedo-Duncan & Zhang, 1994), ultrastructural movement of PKC-α and PKC-β (Acevedo-Duncan *et al.* 1995), and effects of calphostin C on the U-373MG cell cycle (Acevedo-Duncan *et al.* 1997). Calphostin C, a specific perylene quinone, is derived from the fungus *Cladosporium cladosporioides* and is a selective and potent PKC irreversible inactivator with an IC_{50} of 0.05 μM (Kobayashi *et al.* 1989). The mechanism by which calphostin C inhibits PKC and its effects on various glioma cell lines has been described (Gopalakrishna, Chen & Gundimeda 1992; Ikemoto *et al.* 1995; Pollack & Kawecki 1997). Calphostin C inhibits both phorbol ester binding and phosphotransferase activity by binding to the regulatory domain of PKC without competing for phospholipids (Gopalakrishna *et al.* 1992). Calphostin C is activated by brief (min) exposure to light and may be of value in photodynamic therapy (Burns *et al.* 1991).

METHODS

Passage of human glioma cells

The U-373MG cell line was obtained from the American Tissue Culture Collection (Rockville, MD, USA). Cells were seeded (1×10^6) and grown as monolayers in 75 cm^2 flasks containing 90% minimal essential media (MEM), 10% fetal calf serum (FCS), 2 mM L-glutamine and antibiotics (penicillin 10 U/ml and streptomycin 10 μg/ml) according to Ponten & MacIntyre (1968).

Cell cycle analysis of gliomas treated with electrons

Standard radiation procedures developed at the Moffitt Cancer Institute (Tampa, FL, USA) were used to irradiate U-373MG cells. Flasks were exposed to 6 MeV electrons at a dose rate of 0.45 Gy/sec using a Siemens linear accelerator for the dose ranges and time periods indicated in the result section. Cell cycle analysis was performed as previously described (Acevedo-Duncan *et al.* 1997). Briefly, cells were incubated in serum and removed at specific times, washed in phosphate buffered saline (PBS), trypsinized and fixed in cold ethanol. Single nuclei were prepared by incubating cells with 0.04% pepsin followed by 2N HCL and 0.1 M sodium borate. Nuclei from irradiated U-373MG cells were stained with propidium iodide (PI) by modification of Yang *et al.* (1991). Nuclei were stained with PI to measure DNA content and the distributions of 40 000 nuclei were quantified using a FAC STAR^{Plus}, flow cytometer and CellFIT Cell Cycle Analysis program (Version 2.01.2; Becton Dickinson, San Jose, CA, USA).

Microculture tetrazolium (MTT) assay

The colorimetric assay containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Alley *et al.* 1988) was selected to measure cell proliferation following irradiation. The MTT assay does not quantify cell survival and is not a substitute for the standard clonogenic assay. The rationale for selecting the MTT assay as opposed to the standard clonogenic assay which measures clonogen survival was based on a glioma radiosensitivity study (Ramsey, Ward & Bleehen 1992). The study compared the clonogenic assay with the more rapid MTT assay and concluded that results from the MTT assay can be correlated with the clonogenic assay (Ramsey *et al.* 1992). Additionally, there has been increasing use of MTT assay for radiosensitivity testing (Carmichael *et al.* 1987; Price & McMillian 1990; Ramsey *et al.* 1992; Zhang *et al.* 1992; Zhang *et al.* 1993a; Zhang *et al.* 1996) and the MTT assay has been shown to correlate with viable glioma cell numbers (Zhang *et al.* 1996). The procedures used to measure the effects of radiation and/or calphostin C on cell proliferation have been described (Acevedo-Duncan *et al.* 1997). Briefly, cells were plated on 24-well plates at a density of 10^4 cells/well and irradiated and/or incubated with vari-ous concentrations of calphostin C under fluorescent light. Following, initial exposure to calphostin C, additional calphostin C was not applied or removed during the 7 day incubation period. On day 7, MTT (0.014 mg/ml) was added and after 4 h incubation at 37 °C, media was aspirated and dimethlysulfoxide (DMSO) was added to solubilize the MTT-formazan product. Following mixing, absorbance was read at a wavelength of 570 nm using a plate reader (MR 700 Dynex; Dynatech Laboratories Inc., Chatilly, VA, USA).

Output of linear accelerator

The Moffitt linear accelerator is used for patient radiation therapy and its output is checked daily using an ionization chamber. The dose rate of photons and electrons was verified within 2% and 3%, respectively, which is very acceptable.

Statistics

Statistical determination and fits to the linear quadratic equation (L-Q equation) was by Student's *t*-test using Minitab program (Mininc. State College, PA, USA).

RESULTS

Effects of radiation on U-373MG cell cycle progression

The consequences of cell cycle modulators on the cell cycle depend on the position of the cell cycle phase in which they are applied (Sinclair 1968; Allalunis-Turner, Barron & Day 1997). To establish the cell cycle effects of irradiating U-373MG cells in G_1 we attempted a reversible G_0/G_1 arrest by serum starvation. U-373MG were seeded at 5×10^5 cells/flask and incubated at 37 °C in media plus 10% serum for 71 h. Subsequently, cells were washed with and incubated in serum free media for 24 h. Cells were washed again, incubated in media containing 10% serum and harvested at the indicated times for flow cytometric analysis. As shown in Fig. 1a, serum deprivation for 24 h produced a cell population consisting of G_0/G_1, 75%; S, 12%; and G_2M, 13%. Since serum starvation did not produce a synchronous G_0/G_1 cell population, we examined the cell cycle effects of radiation on a high G_0/G_1 semisynchronous cell population. These results are consistent with published data indicating that it is difficult to completely arrest transformed cells (Cacace *et al.* 1993). Electrons were arbitrarily chosen to generate the radiation data presented in Figs 1 and 3 because the photon and electron proliferation curves in Fig. 2 were similar. Figure 1 illustrates the cell cycle distributions of (85% confluent) U-373MG

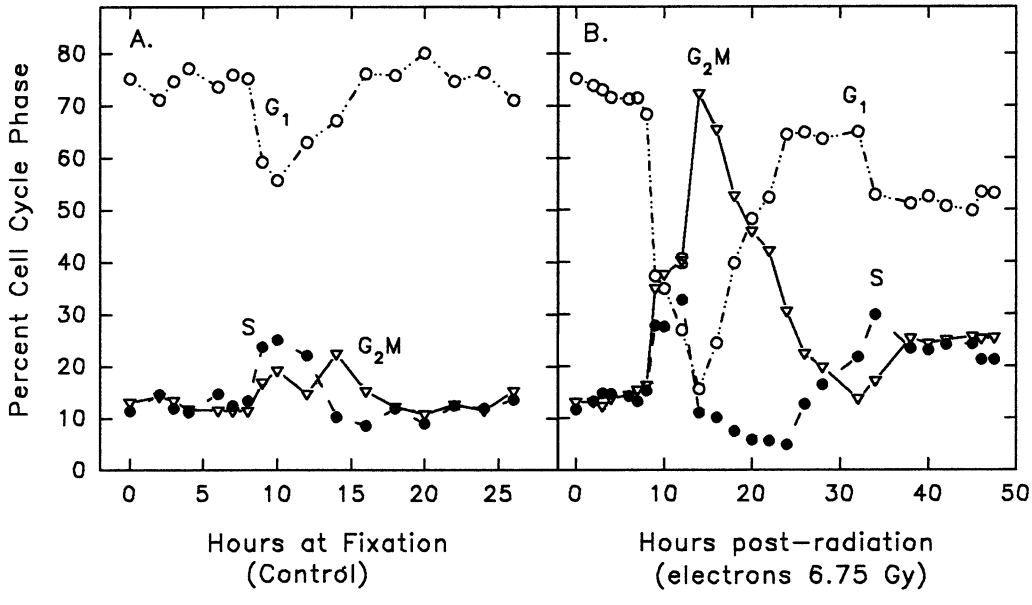

Figure 1. Cell cycle distribution of U-373MG as a function of time. Cells were serum starved for 24 h and serum stimulated to enter the cycle. Subsequently, cells were sham irradiated (control, a) or treated with electrons (6.75 Gy, b). Symbols represent G_1 (○), S (●), and G_2M (▽). Data is representative of two independent experiments. Number of events collected was 40 000 per time point and treatment group.

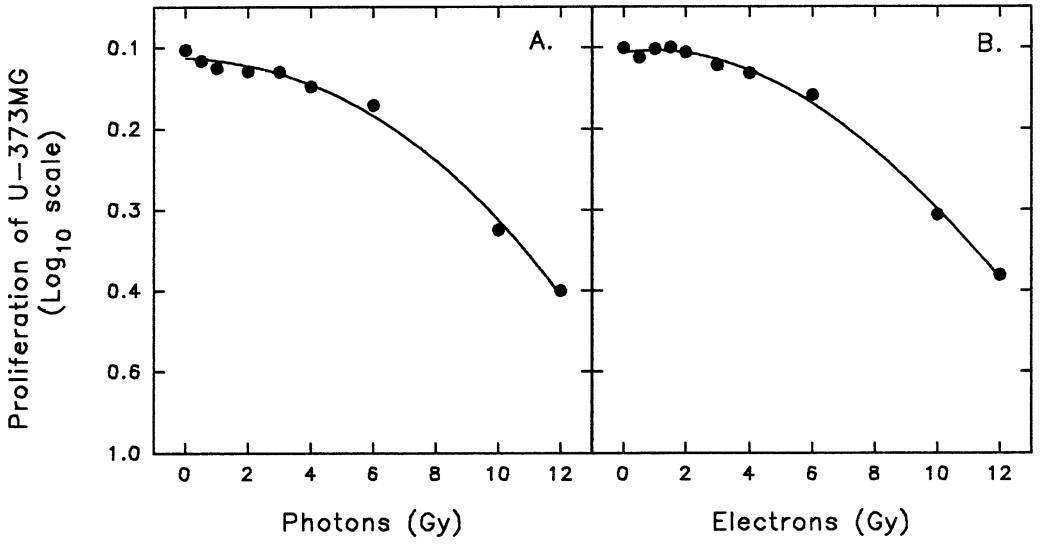

Figure 2. Radiation dose–response curve of U-373MG glioma cells. (a) photons; (b) electrons. Control cells were sham irradiated. The data is a representative experiment and are expressed as the means ± SEM of triplicate determinations. SEM error bars are present but not visible due to the reproducibility of data.

asynchronous control cells and cells treated with electrons (6.76 Gy). As shown in Fig. 1a, radiation did not produce a G_1 arrest. The absence of a radiation induce G_1 arrest is expected since the p53 tumor suppressor is mutated in U-373MG and there is no functional p53 pathway (Russell *et al.* 1995) which contributes in part to the intrinsic radiation resistance found in U-373MG cells. However, compared to controls, radiation (electrons, 6.76 Gy) provoked a G_2M arrest as seen by the prolonged and augmented G_2M phase (Fig. 1b).

Effects of radiation on U-373MG proliferation

The effects of radiation on U-373MG cell proliferation was evaluated by microculture tetrazolium (MTT) assay which quantifies cell number by tetrazolium dye reduction (Alley *et al.* 1988). Glioma U-373MG were seeded into 24-well plates at a density of 10^4 cells/well and cells were irradiated 24 h post cell plating. To compare cell proliferation following irradiation with either photons or electrons, cells were irradiated with either 6 MV or 6 MeV (Siemens linear accelerator; Siemens Medical Systems Inc., Iselin, NJ, USA) using either a dose rate for photons (0.035 Gy/sec) or for electrons (0.45 Gy/sec) and a single-dose exposure ranging from 0 to 10 Gy. Cells were then incubated at 37 °C and on day seven, MTT was added and following a 4-h incubation, cells were solubilized and absorbance measured. Photons and electrons produced the same radiobiological effects on U-373MG cell proliferation (Fig. 2). The proliferation curves demonstrate that the U-373MG are radiation resistant since 50% cell kill was achieved with 8 Gy. The data shown in Fig. 1. fitted a shouldered survival curve and the D_q (quasi-threshold dose), D_o (mean lethal dose; slope = $_1$/Do), *n* (extrapolation number; ability of cells to accumulate sublethal XRT damage), and $\bar{D}$ (mean intrinsic radiation sensitivity), was calculated (Weichselbaum, Hallahan & Chen 1992). For the photon survival curve, we calculated $D_q = 4.4$ Gy, $D_0 = 5.8$ Gy, $\bar{D} = 8$ Gy, $n = 2.14$, $\alpha = 0.03$ Gy^{-1} and $\beta = 0.007$ Gy^{-2}. For the electron survival curve $D_q = 4.5$ Gy, $D_0 = 5.7$ Gy, $\bar{D} = 4.5$ Gy, $n = 2.18$, $\alpha = 0.03$ Gy^{-1} and $\beta = 0.007$ Gy^{-2}. The survival curves were

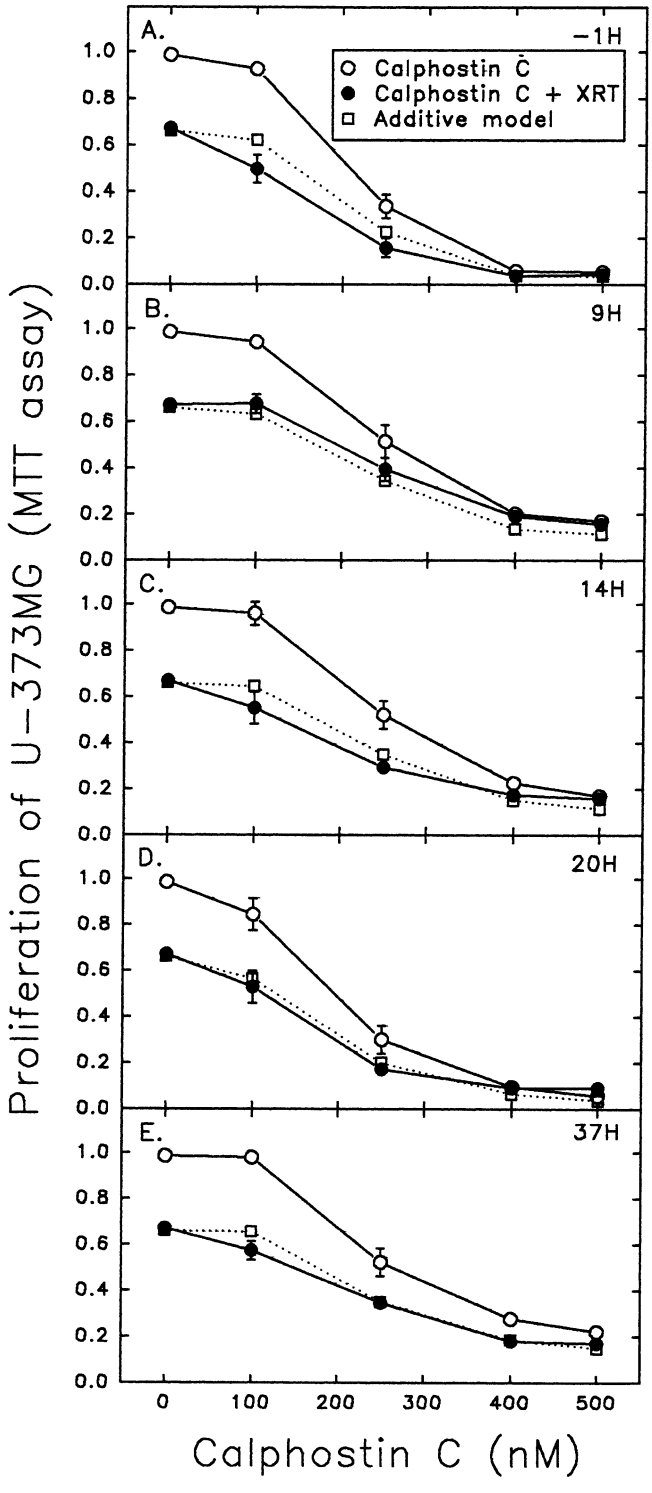

Figure 3. Survival of U-373MG treated with calphostin C and radiation. ○ Represents the survival of U-373MG as a function of calphostin C concentration. ● Represents the survival of U-373MG irradiated with electrons (6.76 Gy) in the presence of varying concentrations of calphostin C. Irradiation was with 6 MeV using a dose rate of 0.45 Gy/sec for a duration of exposure of 15 s. Additive model is depicted by the dotted line and □. Data below the additive model is weakly synergistic. Data are from two independent experiments with 12 control replicates and 3 experimental replicates per experiment. Exposure times were (a) 1 h prior to radiation (b) 9 h (c) 14 h (d) 20 h, and (e) 37 h post radiation, respectively.

constructed using a log 10 scale for proliferation of the U-373MG cells and the dose of electrons and photons is in Gy. The extrapolation number (*n*) was calculated to be 2.14 and 2.18 for the electron survival curve. These *n*-values reflect a relatively wide shoulder of 4.4 Gy (electron; D_q) and 4.5 (photons; D_q). However, the D_0 is much larger than normal (usually 1–2 Gy).

Effects of radiation and calphostin C on U-373MG proliferation

Since the effectiveness of PKC inhibitors may depend on the time of application of the PKC inhibitor following radiation, we examined the effect on radiosensitivity of various calphostin C concentrations at 1 h prior to radiation and at 9, 14, 20 and 37 h post radiation (Fig. 3). Cells were serum deprived for 24 h and prepared as in Fig. 1. The initial cell population at time zero consisted of G_0/G_1, 70% ± 0.828 SEM; S, 14% ± 0.523 SEM; and G_2M, 13% ± 0.832 SEM ($n = 10$ replicates). Calphostin C was activated by exposing calphostin C treated cells to flourescent light for 5 min. Once cells were treated with calphostin C the media was not changed throughout the entire 7 day incubation period.

Figures 3a and 3d show that calphostin C (250 nM) at 1 h prior to radiation and in the 20 h controls decreased proliferation to similar levels (0.34 ± 0.050 SEM and 0.29 ± 0.062, respectively). Another pattern in the effectiveness of calphostin C in reducing proliferation is seen at 9, 14 and 37 h of calphostin C (250 nM) treatment. In these cells proliferation decreased to 0.51 ± 0.07 SEM, 0.52 ± 0.06 SEM and 0.52 ± 0.06 SEM, respectively. As stated in the discussion section, future flow cytometric studies will address the exact cell cycle phase at specific times of calphostin C application.

The radiation data demonstrated that compared to irradiated controls, pretreatment with calphostin C (100 nM) for 1 h decreased cell proliferation by 26% ($P = 0.048$; Fig. 3a). When the proliferation of irradiated controls are compared to the proliferation of irradiated and calphostin C (250 nM) treated cells, calphostin C (250 nM) enhanced cell radiosensitivity by 76% ($P = 0.0001$; Fig. 3a). These results indicate that calphostin C at either 100 nM or 250 nM is weakly synergistic with radiation. An additive model is depicted on the graphs and shows the predicted values for an additive radiosensitivity response. Any data below the additive model is weakly synergistic. The results show that following radiation and calphostin C pretreatment (100 nM or 250 nM), only 0.49 (± 0.06 SEM) and 0.158 (± 0.043 SEM) of the cells survived, respectively. If calphostin C's effect was additive with radiation, the predicted survival at 100 nM calphostin C would be 0.62 [0.92 (100 nM calphostin C alone) X 0.67 (radiation alone)]. At 250 nM calphostin C, the predicted survival would be 0.22 [0.33 (100 nM calphostin C alone) X 0.67 (radiation alone)]. In both cases, calphostin C (100 nM and 250 nM) increased the actual cell radiosensitivity by 26% and 39%, respectively.

In each of the other time points (9, 14, 20 and 37 h post radiation), examination of the overall relationship between calphostin C exposure and radiosensitivity were consistently less than the enhanced radiation sensitivity obtained when cells were pretreated with calphostin C 1 h prior to radiation (Figure 3b–e). The changes in cell proliferation following calphostin C incubation at 9 h (control) or 9 h post radiation are shown in Fig. 3b. Calphostin C (250 nM) at either 9 h (control) or 9 h post radiation was not as effective in killing cells compared to calphostin C pretreatment, 1 h prior to radiation.

DISCUSSION

In this study we investigated the radiosensitivity of U-373MG cells by measuring the effects of radiation and the specific PKC inhibitor, calphostin C on cell proliferation. We found that irradiated

U-373MG cells progressed through G_1-S and underwent a G_2M arrest (Fig. 1). As in many human cancers, the absence of a radiation-induced G_1 arrest in U-373MG cells is due in part to a mutated p53 tumor suppressor protein (Russell *et al.* 1995) whose normal function is to permit apoptosis in cells that have become tumorigenic. The cyclin-dependent kinase inhibitor, p21 and the DNA damage-gene GADD45 also play a role in the G_1 arrest (El-Deiry *et al.* 1994). The G_2M arrest following exposure to genotoxic agents is a checkpoint for cellular repair of DNA damage and may involve suppression of cyclin B mRNA and/or protein and tyrosine phoshorylation of $p34^{cdc2}$ (Maity, McKenna & Muschel 1994).

Our calphostin C proliferation data illustrate that there is a relationship between the time of calphostin C (100 nM and 250 nM) exposure and its antiproliferative effect (Fig. 3). Cells appear to be more sensitive to calphostin C if they receive a single application of calphostin C (100 nM or 250 nM) 1 h prior to radiation or if they are exposed to calphostin C (250 nM) at 20 h post initiation of the experiment. As stated in the Results section, at time zero, cells had a high G_0/G_1 cell population (G_0/G_1, 70% ± 0.828 SEM; S, 14% ± 0.523 SEM; and G_2M, 13% ± 0.832 SEM; $n = 10$ replicates). Therefore, 20 h post commencement of the experiment, cells probably had a high G_0/G_1 cell population and similar results were observed. Future flow cytometric studies from our laboratory will address which exposure time(s) and cell cycle phase(s) are most sensitive to calphostin C.

We previously reported that calphostin C inhibited U-373MG PKC activity, and that high concentrations of calphostin C (400 nM or 500 nM) in the absence or presence of serum blocked progression through either S phase or G_2M, respectively (Acevedo-Duncan *et al.* 1997). In the aforementioned study, a single exposure to calphostin C at 100 nM did not arrest cells in S phase. Additionally, we have shown herein that calphostin C at 100 nM did not completely inhibit U-373MG proliferation (Fig. 3). Our results appear to differ from those of Ikemoto *et al.* (1995) who reported that calphostin C (100 nM) for 16–24 h is capable of producing apoptotic DNA fragmentation and cell death. However, our results cannot be compared to those of Ikemoto *et al.* (1995) due to differences in the experimental methodology. They quantified cell viability using trypan blue exclusion while we used the MTT assay to measured cell proliferation seven days post calphostin C incubation. Alternatively, Pollack & Kawecki (1997) reported that repeated daily exposures to calphostin C (60–125 nM) for 4 days completely blocked glioma proliferation. In our previous study, we also reported that TPA treatment (0.1–1.0 μM; for 27 h with and without serum) elevated the number of cells blocked in S phase and decreased U-373MG cell proliferation (Acevedo-Duncan *et al.* 1997). The difference on the effects of calphostin C at 100 nM and TPA on S phase progression are due to their mechanism of action. Prolonged TPA treatment initially activates PKC isozymes and then depletes PKC (Couldwell *et al.* 1991; Pollack *et al.* 1991), while calphostin C (100 nM) inhibits the regulatory domain of PKC by irreversible oxidative inactivation (Gopalakrishna *et al.* 1992).

Treatment with the PKC inhibitor calphostin C (250 nM) prior to radiation, additively enhanced the radiosensitivity of U-373MG cells by decreasing cell proliferation by 76% (Fig. 3). We speculate that the enhanced radiosensitivity presented herein for cells treated with calphostin C (250 nM) prior to radiation may be due to the temporal inhibition of PKC isozymes required directly or indirectly for cell proliferation or DNA repair following irradiation. Support for the latter postulate comes from data reported by Zhang *et al.* 1993a, 1993b. They showed that inhibition of rat C6 glioma PKC activity by prolonged treatment with TPA or the nonspecific PKC inhibitor staurosporine prior to irradiation, enhanced the radiation-induced DNA damage and attenuated the repair of DNA injury. However, the other studies conflict with this hypothesis. Hallahan *et al.* (1992a) found that treatment of JSQ-3 squamous carcinoma cells with X-irradiation and the PKC inhibitor sangivamycin resulted in no difference in DNA single- and double-strand breaks as

measured by DNA alkaline and neutral elutions. Future work with U-373MG cells will determine if in this glioma cell line, PKC isozymes function directly or indirectly to repair DNA post irradiation.

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