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Induction of Apoptosis in Glioblastoma Cells by Inhibition of Protein Kinase C and Its Association with the Rapid Accumulation of p53 and Induction of the Insulin-like Growth Factor-1-Binding Protein-3

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ABSTRACT. Increased protein kinase Cα (PKCα) expression in glioblastoma cells is associated with proliferation and resistance to drug-induced apoptosis by an undefined anti-apoptotic pathway. To clarify the role of PKC in apoptosis, we have investigated the effect of the selective PKC inhibitor Ro 31-8220 (3-[1-[3-(amidinothio)propyl]-3-indolyl]-4-(1-methyl-3-indolyl)-1*H*-pyrrole-2,5-dione methanesulfonate) in two glioblastoma cell lines whose proliferation is dependent on high levels of PKCα. U-87 and A172 cells treated with an IC_{50} of Ro 31-8220 exhibited nucleosomal DNA fragmentation that coincided with an increase in the number of apoptotic cells. This effect was preceded by the rapid nuclear accumulation of wild-type p53 within 2 hr, and an increased level of the pro-apoptotic protein, insulin-like growth factor-1-binding protein-3, (IGFBP3) but not other p53-regulated proteins such as $p21^{WAF1}$ or Bax. Accumulation of p53 was also associated with the hypophosphorylated and activated form of the retinoblastoma tumor suppressor protein (RB) at later times after treatment. These results suggest that PKCα suppresses apoptosis in glioblastoma cells primarily by restricting the accumulation of p53 and the expression of insulin-like growth factor-1-binding protein, as well as by maintaining RB in an inactive hyperphosphorylated state. BIOCHEM PHARMACOL **55**;10:1711–1719, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. PKC; apoptosis; p53; IGFBP3

PKC† is a multigene family consisting of conventional (α, βI, βII, γ), novel (δ, ϵ, θ, η), and atypical (λ, ζ, ι) isoform classes [1] that participate in signal transduction pathways regulating growth and differentiation [2–4]. Elevated PKC activity is associated with the proliferation of glioblastoma cells [5–7] and the anaplastic phenotype of primary glioblastoma multiforme [6]. PKCα is highly expressed in the glioblastoma cell lines U-87 and A172 [8, 9] and is associated with their proliferation by an autocrine mechanism [10]. PKC has been proposed as a therapeutic target for the treatment of glioblastoma multiforme [10–12], and expression of an antisense PKCα cDNA in U-87 cells or treatment with an antisense PKCα oligonucleotide inhibits proliferation and tumorigenicity and elicits an antitumor response [13, 14].

Apoptosis, or programmed cell death, is closely linked to the proliferative capacity of the cell and can be triggered by a variety of extrinsic and intrinsic signals [15–17]. Although diverse stimuli can induce apoptosis in a wide variety of cell types, a small number of highly conserved genes regulate a final common cell death pathway. In many cases, apoptosis requires the p53 tumor suppressor protein [18, 19], and p53 expression not only is induced by several cell death stimuli, but also is itself sufficient to induce apoptosis following gene transfer to a variety of cell lines [20].

Short-term exposure to phorbol ester activators of PKC protects cells against apoptosis induced by radiation, glucocorticoids, and growth factor deprivation [21–23]. Conversely, PKC inhibitors, either alone or in combination with other chemotherapeutic drugs, promote apoptosis in neuroblastoma, glioblastoma, and gastric cancer cell lines [24–27]. Despite this evidence, the PKC isoform(s) and the downstream effector(s) of PKC involved in preventing apoptosis have not been defined.

During the course of our studies examining the response of glioblastoma cells to PKC inhibition, we noted that the apoptotic effects of the selective PKC inhibitor Ro 31-8220 (3-[1-[3-(amidinothio)propyl]-3-indolyl]-4-(1-methyl-3-indolyl)-1H-pyrrole-2,5-dione methanesulfonate) occurred only in high PKCα-expressing cells. Therefore, we investigated whether this response was associated with a specific complement of anti-apoptotic and proapoptotic genes. In

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† *Abbreviations:* CDK, cyclin-dependent kinase; FACS, fluoresence-activated cell sorting; IGFBP3, insulin-like growth factor-1-binding protein-3; ISEL, *in situ* end-labeling; oligo, phosphorothioate oligodeoxynucleotide; PKC, protein kinase C; and RB, retinoblastoma tumor suppressor protein.

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this report, we show that apoptosis induced by Ro 31-8220 was associated with the rapid nuclear accumulation of wild-type p53 and the subsequent elevation of the pro-apoptotic protein IGFBP3.

MATERIALS AND METHODS

Reagents

Ro 31-8220 was supplied by Dr. Geoffrey Lawton (Roche Products Ltd.). Antibodies were obtained from the following suppliers: PKCα and PKCζ affinity-purified polyclonal antibodies from Life Technologies; PKCβ, PKCδ, and PKCε monoclonal antibodies from Transduction Laboratories; Bcl-2 and RB (IF8) monoclonal and Bcl-X_L and Bax polyclonal antibodies from Santa Cruz; IGFBP3 polyclonal antibody from Upstate Biotechnology Inc.; p53 monoclonal antibody Do-1 from NeoMarkers; and horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse IgG from Bio-Rad Laboratories. Biotinylated anti-mouse IgG, a VECTASTAIN *elite* ABC Kit, a Vector VIP substrate kit, and the diaminobenzidine (DAB) substrate were from Vector Laboratories. ECL detection reagents were purchased from Amersham Life Science.

Cell Culture

Human glioblastoma cell lines U-87 and A172 were obtained from the American Type Culture Collection. Cells were grown at 37° under 5% CO_2 in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum and 50 μg/mL of gentamicin.

Cytotoxicity Assays

Cells were seeded at 7500 cells/well in a 24-well plate for the trypan blue exclusion cytotoxicity assay [13]. Following incubation overnight, cells were exposed to various concentrations of Ro 31-8220 for 24 hr, washed twice with PBS, and incubated with 0.2% trypan blue. Trypan blue exclusion was determined on the total number of adherent and nonadherent cells. Cytotoxicity was also assessed by colony formation after 24 hr of treatment with Ro 31-8220 and determination of colony number 2 weeks after plating [28]. Colony formation is expressed as the number of colonies formed in treated cells/number of colonies formed in control cells × 100.

DNA Fragmentation Assay

Following incubation with Ro 31-8220 for various time intervals, adherent and nonadherent cells were harvested, and soluble DNA was isolated from 1×10^6 cells [29]. Cells were collected by centrifugation, washed twice with PBS, and resuspended in 0.5 mL of lysis buffer [1% NP-40, 50 mM of Tris-HCl (pH 7.5), and 20 mM of EDTA] [29]. Cells were kept on ice for 10 min, mixed gently, and centrifuged in a microcentrifuge for 10 min at 14,000 *g* at 4°; the supernatant was saved. The extraction was repeated once, the supernatants were combined, and SDS and RNase A were added to a final concentration of 1% and 0.5 mg/mL, respectively. Samples were incubated at 56° for 2 hr, proteinase K was added to a final concentration of 2 mg/mL, and the mixture was incubated at 37° for 2 hr. One-half volume of 10 M of ammonium acetate and 2.5 vol. of cold ethanol were added to the supernatant and incubated overnight at −20°. DNA was collected by centrifugation at 14,000 *g* for 20 min at 4°, the DNA pellet was dissolved in 20 μL of TE buffer, [50 mM of Tris-HCl (pH 7.5) and 20 mM of EDTA], separated by electrophoresis in a 1.5% agarose gel containing 0.5 μg/mL of ethidium bromide in TAE buffer [40 mM of Tris-acetate (pH 8.0), and 2 mM of EDTA and visualized by UV fluorescence. Soluble DNA extracted from apoptotic TF-1 leukemia cells served as a positive control [30].

Flow Cytometry

FACS analysis was performed by the Flow Cytometry Core Facility, Lombardi Cancer Center, Georgetown University. Adherent cells were trypsinized and combined with nonadherent cells and fixed with ethanol as described previously [31]. A total of 1–2 × 10^6 cells were collected by centrifugation at 400 *g* for 5 min, and resuspended in 0.5 mL of cold PBS by vortexing gently; single-cell suspensions were verified by microscopy. Three aliquots of 0.5 mL of ice-cold absolute ethanol were added sequentially to the cell suspension, dropwise while mixing gently. Fixed cells were kept on ice for at least 20 min before staining with propidium iodide. Analysis was carried out with a FACsort (Becton-Dickinson), and data analysis and display utilized Reproman computer software.

In Situ *End-Labeling*

Cells were grown in a 6-well plate and incubated with 2.5 μM of Ro 31-8220 for 24 and 48 hr. Adherent and nonadherent cells were collected by centrifugation, and then the cells were washed twice with PBS and fixed with 10% formalin in PBS for 10 min. After washing twice with PBS, 2×10^5 cells were resuspended in 200 μL of PBS, spotted on a polylysine-coated glass slide, and allowed to air dry overnight. Cells were rehydrated in PBS, and DNA strand breakage was detected by ISEL and DAB staining [32].

Western Blot Analysis

Cells were harvested, washed twice with PBS, and suspended in a buffer containing: 50 mM of Tris-HCl (pH 7.5), 2 mM of EDTA, 1 mM of EGTA, and 50 μg/mL of phenylmethylsulfonyl fluoride. The cell suspension was homogenized by sonication, and protein concentrations were determined with Coomassie Protein Assay Reagent (Pierce, Inc.), using BSA as a standard. Fifty micrograms of cell lysate was separated in either 8 or 12% polyacrylamide

gels by SDS–PAGE. Samples were transferred electrophoretically to nitrocellulose membranes, blocked with 5% fat-free dry milk in TBST [50 mM of Tris-HCl (pH 7.5), 0.15 M of NaCl, 0.1% Tween-20] and incubated for 3 hr with the appropriate primary antibody diluted in TBST. After incubation with a horseradish peroxidase-conjugated secondary antibody, immunoreactive proteins were visualized by chemiluminescence with the ECL detection system (Amersham). Membranes were reprobed with different primary antibodies after stripping the membrane in a buffer containing 62.5 mM of Tris-HCl (pH 7.6), 2% SDS, and 100 mM of β-mercaptoethanol for 1 hr at 50°.

Immunohistochemistry

Cells were grown on chamber slides and treated with 2.5 μM of Ro 31-8220 for various time intervals. Cells were fixed for 10 min in 10% formalin in PBS containing 0.1% Triton X-100, washed three times with PBS, incubated for 5 min in 0.5% Triton X-100 in PBS, and washed three times with PBS. Fixed cells were incubated for 30 min at room temperature with either the p53 monoclonal antibody Do-1 or the Rb monoclonal antibody and were washed five times with PBS. Cells were incubated for 30 min with biotinylated anti-mouse IgG, and washed five times with PBS, and the antigen was visualized with the immunoperoxidase-based VECTASTAIN *elite* ABC kit and VIP staining (Vector Laboratories).

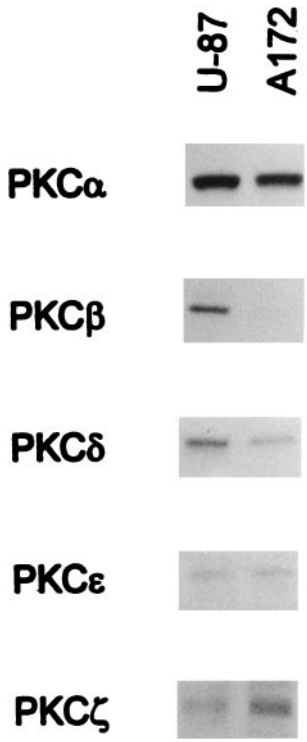

FIG. 1. Immunoblot analysis of PKC isoforms in U-87 and A172 cells. Lysates (50 μg) were separated in 8% polyacrylamide gels by SDS–PAGE and transferred electrophoretically onto nitrocellulose. Blots were developed using isoform-specific primary antibodies, and immunoreactive proteins were visualized by chemiluminescence.

RESULTS

Inhibition of PKC and DNA Fragmentation

The complement of PKC isoforms in U-87 and A172 cells was determined by immunoblotting (Fig. 1). PKCα, β, δ, ε, and ζ were detected in cell lysates from both cell lines, and PKCα was the most abundant isoform present. PKCβ and δ levels were greater in U-87 than in A172 cells, and both cell lines expressed similar levels of PKCε and PKCζ. PKCγ was not detected in either cell line.

The cytotoxicity of the PKC-selective inhibitor, Ro 31-8220 was determined in U-87 and A172 cells by trypan blue exclusion (Fig. 2, left panel) and by colony formation (Fig. 2, right panel). The IC_{50} of Ro 31-8220 for A172 and U-87 cells after 24 hr of exposure was approximately 2.5 and 5 μM, respectively. Colony formation was used to measure cell lethality after treatment with Ro 31-8220 (Fig. 2, right panel). A172 cells were slightly more sensitive to Ro 31-8220 than U-87 cells, and a one log reduction in colony formation was obtained at 1.7 and 2 μM Ro 31-8220, respectively.

To determine if cytotoxicity was associated with apoptosis, flow cytometry was used to analyze the cell population after Ro 31-8220 treatment (Fig. 3). Treatment of U-87 and A172 cells for up to 48 hr with 2.5 μM of Ro 31-8220 resulted in 22 and 31% apoptotic cells, respectively, as indicated by the "A_o" population of cells (Fig. 3). A_o cells reflect the reduced fluorescence emanating from the condensed chromatin in apoptotic cells [31]. A172 cells were more sensitive than U-87 cells as determined by the percentage of A_o cells 16 hr after treatment, and both cell lines accumulated at G_2/M beginning 16 hr after the addition of Ro 31-8220. DNA fragmentation, a hallmark of apoptosis, was assessed by agarose gel electrophoresis (Fig. 4) and ISEL (Fig. 5). Treatment with 2.5 μM of Ro 31-8220 produced a classic 180-bp nucleosomal DNA ladder in U-87 and A172 cells that correlated with the culmination of apoptosis (Fig. 4). DNA fragmentation was

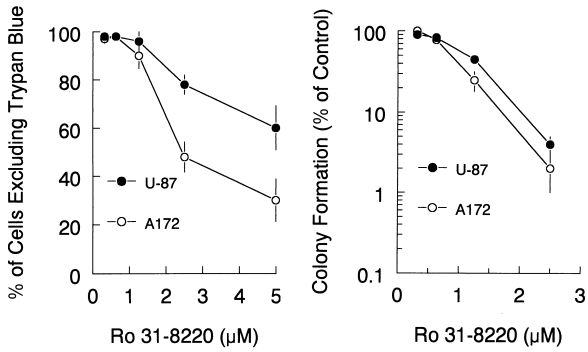

FIG. 2. Cytotoxicity of Ro 31-8220 to U-87 and A172 cells. Cells (7.5×10^3) were exposed for 24 hr to Ro 31-8220, and cell viability was determined by the proportion of cells that excluded trypan blue (left panel), and colony formations (right panel). Each value is the mean ± SEM of three separate experiments.

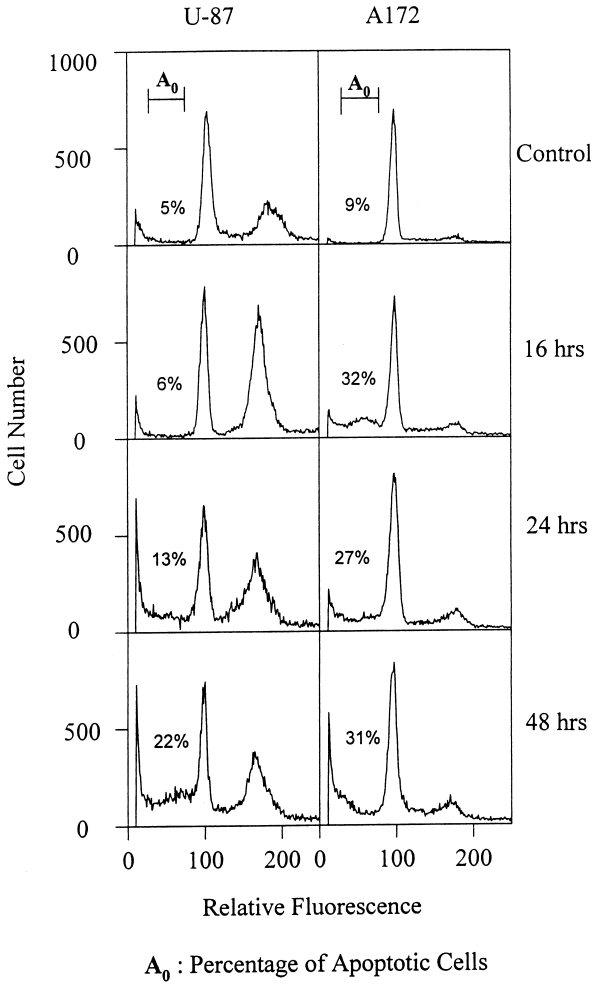

FIG. 3. FACS analysis of Ro 31-8220-treated glioblastoma cells. Cells were treated for 16, 24, and 48 hr with 2.5 μM of Ro 31-8220 and stained with propidium iodide for DNA content.

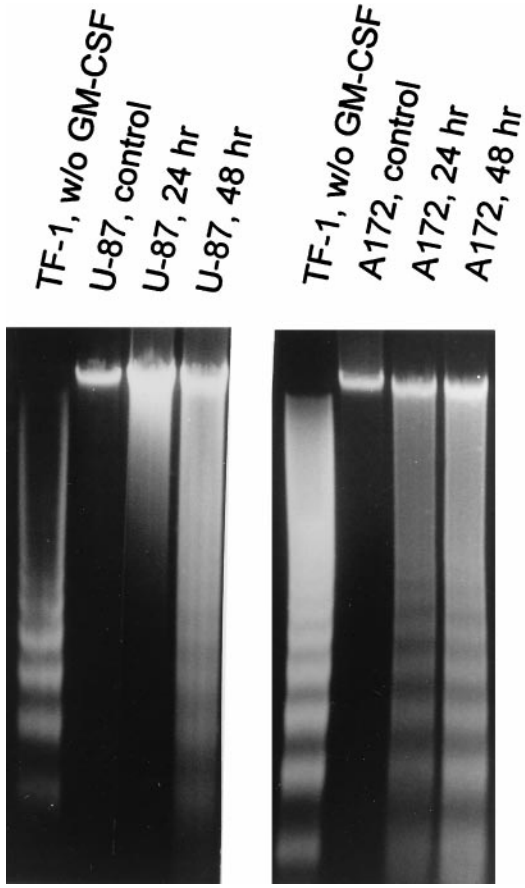

FIG. 4. Induction of DNA fragmentation by Ro 31-8220. Cells were treated for 24–48 hr with either vehicle (control) or 2.5 μM of Ro 31-8220, and soluble DNA was extracted and separated in a 1.5% agarose gel by electrophoresis. Soluble DNA extracted from apoptotic TF-1 leukemia cells served as a positive control [30].

also assessed by ISEL, which detects 3′-end cleavage of DNA (Fig. 5). The percentage of cells exhibiting DNA breakage increased with the length of Ro 31-8220 treatment and also corresponded closely to the percentage of A_o cells, i.e. 11 and 30% apoptotic U-87 cells and 27 and 28% apoptotic A172 cells 24 and 48 hr after treatment, respectively.

Rapid Nuclear Accumulation of p53 and Increased IGFBP3 Expression

p53 is often required for the efficient execution of apoptosis by a variety of adverse growth conditions and genotoxic stress [18, 19, 33], and, therefore, the level and cellular localization of p53 were determined immunohistochemically in U-87 and A172 cells following Ro 31-8220 treatment (Fig. 6). A172 cells contained low but detectable levels of wild-type p53 as shown previously for U-87 cells [34, 35] (Fig. 6, top). Ro 31-8220 produced a dramatic increase in nuclear p53 accumulation in A172 cells within 2 hr after treatment (Fig. 6, top), and similar results were observed in U-87 cells (data not shown). Immunoblotting confirmed that p53 levels were increased dramatically in both cell lines (Fig. 7A).

Because repression of cell cycle-related genes by activation (hypophosphorylation) of RB could also potentially elicit an apoptotic response, the localization, level, and phosphorylation state of RB were examined in A172 cells after Ro 31-8220 treatment. Nuclear accumulation of RB occurred rapidly in A172 cells beginning 2 hr after Ro 31-8220 treatment (Fig. 6, bottom), and similar results were observed in U-87 cells (results not shown). At later times after Ro 31-8220 treatment, the level of hyperphosphorylated RB decreased and eventually disappeared 24–48 hr after drug treatment (Fig. 7A, "ppRB"). The onset of RB accumulation (Fig. 6, bottom) did not correlate with the disappearance of its hyperphosphorylated form (Fig. 7A) but did approximate the commencement of apoptosis in both cell lines (Fig. 3).

p53 is known to induce the expression of the proapoptotic protein, Bax [17], a member of the *Bcl-2* gene family, which also includes the anti-apoptotic proteins Bcl-2 and Bcl-X_L [17]. Therefore, the levels of these proteins were determined before and after Ro 31-8220 treatment (Fig.

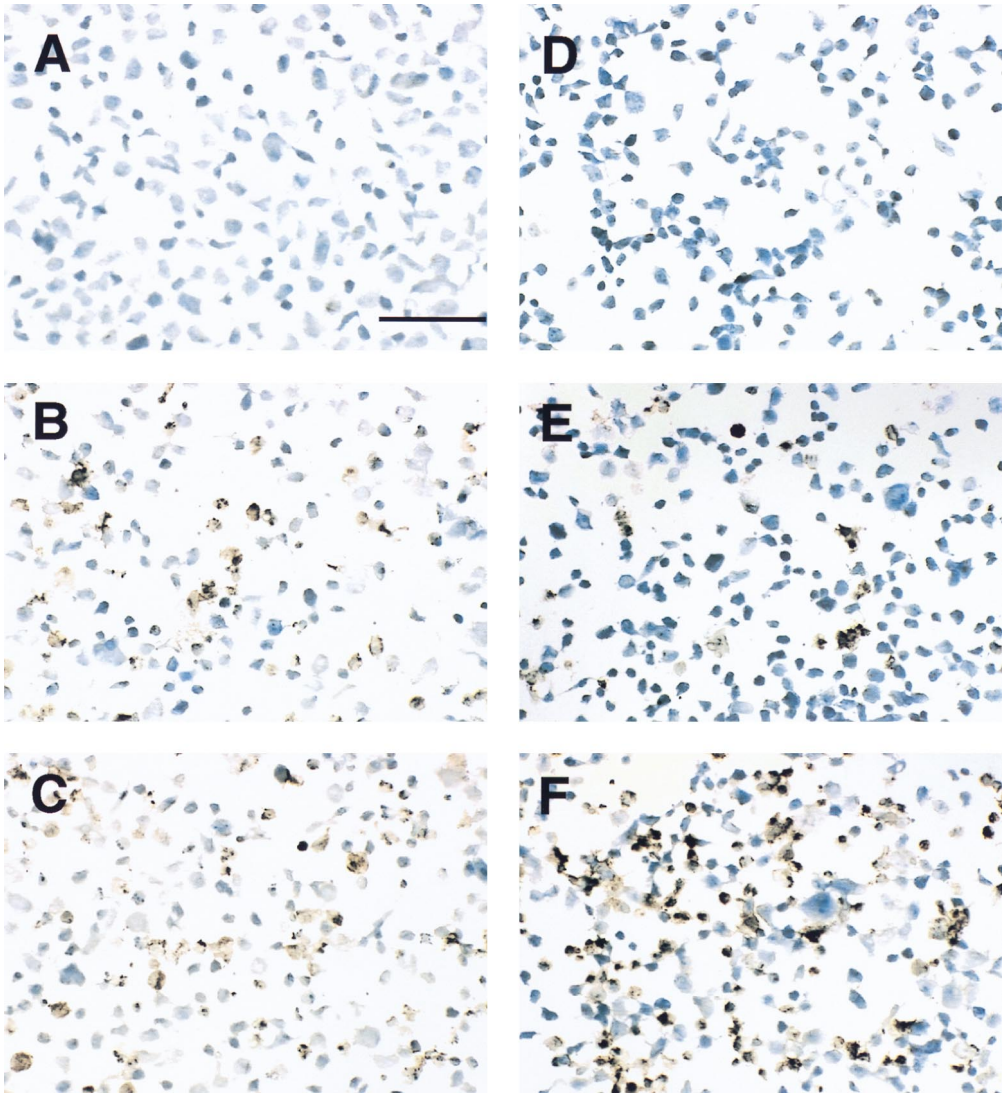

FIG. 5. ISEL detection of DNA strand breakage in U-87 and A172 cells. Cells were treated for 24 (B and E) and 48 (C and F) hr with either vehicle (A and D) or 2.5 μM of Ro 31-8220 (B, C, E, and F), and DNA breakage was detected by ISEL (cells stained brown). Panels A–C represent A172 cells and panels D–F represent U-87 cells.

7A). Neither cell line contained Bcl-2 (data not shown), and the levels of Bcl-X_L and Bax remained relatively constant throughout treatment. In addition, the levels of the p53-regulated inhibitor of the cyclin A- and cyclin E-dependent CDK2, $p21^{WAF1}$, did not increase after Ro 31-8220 treatment (Fig. 7A). Apoptosis may also be induced by the p53-regulated IGFBP3 [36, 37], and, therefore, the time course for p53 induction and IGFBP3 expression was determined in A172 cells after Ro 31-8220 treatment (Fig. 7B). IGFBP3 levels were elevated markedly beginning 16 hr after treatment and coincided with the onset of apoptosis in A172 cells. Similar results were observed in U-87 cells (results not shown).

DISCUSSION

This study provides evidence that the selective PKC inhibitor Ro 31-8220 elicits apoptosis in glioblastoma multiforme cells that express high PKCα levels. Inhibition of PKC was associated with the rapid nuclear accumulation of wild-type p53, elevation of IGFBP3, and RB hypophosphorylation. These results suggest that the dependence of tumor cell proliferation on PKCα-dependent signaling pathways renders these cells susceptible to apoptosis following PKC inhibition. Ro 31-8220 is a selective inhibitor of PKC [38] and, like other staurosporine-derived inhibitors, preferentially inhibits PKCα, β, and γ [39]. Because PKCα is the prevalent isoform with sensitivity to Ro 31-8220 in U-87 and A172 cells, it is likely that the effect of Ro 31-8220 is primarily produced through inhibition of this isoform. This is also supported by the observation that a PKCα antisense oligonucleotide [14] produced the same effects as Ro 31-8220 in these cell lines (unpublished results). U-87 cells, but not A172 cells, also contain PKCβ, and inhibition of $PKC\beta_{II}$ was found previously to lead to cell cycle arrest in the G_2/M phase [40]. Because U-87 cells,

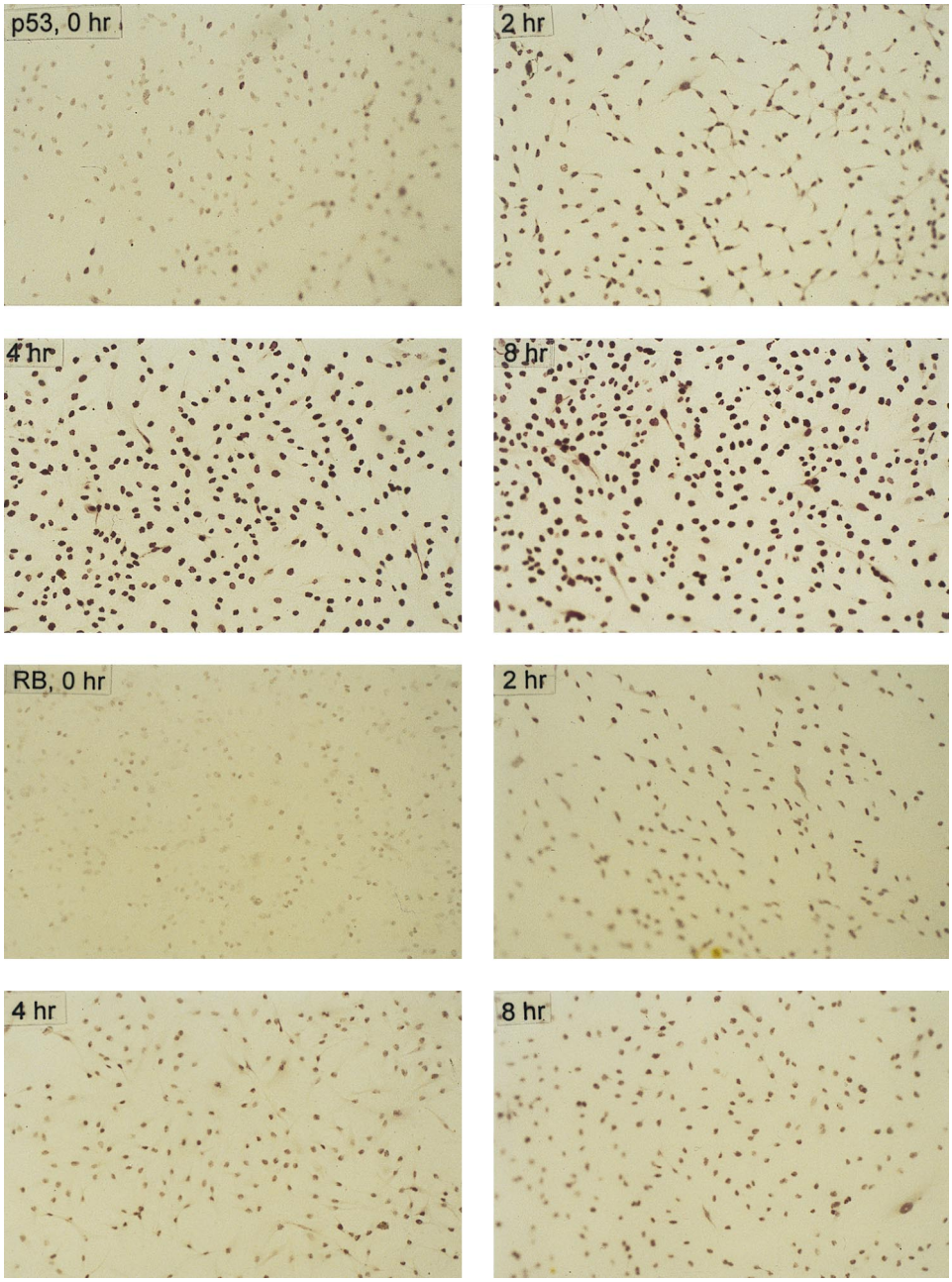

FIG. 6. Immunocytochemical detection of p53 and RB in A172 cells. A172 cells were treated with either vehicle or 2.5 μM of Ro 31-8220 for 2, 4, and 8 hr, and fixed cells were stained for p53 (top) or RB (bottom). Positive cells are stained purple.

showed a greater transient G_2/M arrest after Ro 31-8220 treatment than A172 cells, this effect may have been related to inhibition of PKCβ. G_2 arrest has also been noted in U-87 cells undergoing apoptosis following transfection with wild-type p53, and, therefore, the accumulation of p53 also may have contributed to G_2/M arrest [41].

Overexpression of PKCα markedly reduces the levels of wild-type p53 [42], and this observation is consistent with our findings that inhibition of PKC by Ro 31-8220 produces the converse effect. These results suggest that PKCα is not only a dominant factor associated with tumor proliferation, but is also a determinant of whether the cell will die by apoptosis or necrosis. p53 is involved in the cellular response to genotoxic stress induced by DNA-damaging agents [33], and homozygous deletion of p53 results in resistance to the apoptotic response to chemotherapy [18, 19]. Mutation of p53 is common in brain tumors [43], but approximately one-third of glioblastomas contain wild-type p53 [44]. Therefore, the nuclear localization of p53 following PKC inhibition suggests a mechanism whereby chemotherapeutic agents of this type may induce apoptosis and exert an antiproliferative effect on glioblastoma and other tumors with this phenotype.

PKC inhibition may also result in cell cycle dysregulation through the hypophosphorylation and subsequent activation of RB. RB has been functionally linked to the Ras

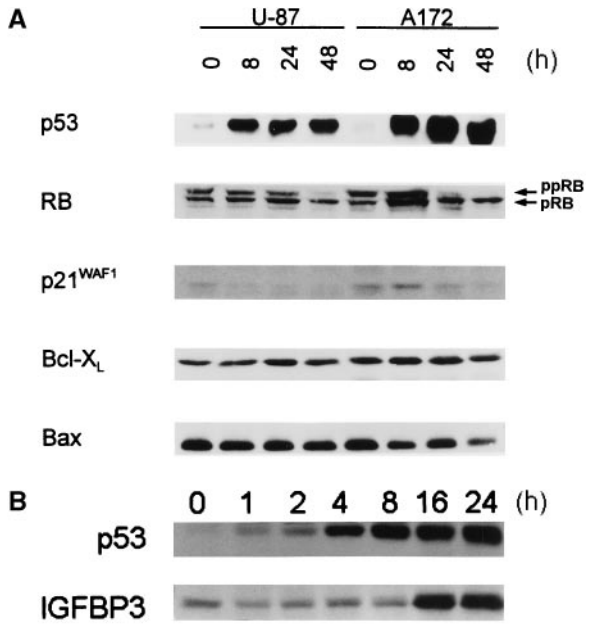

FIG. 7. Effect of Ro 31-8220 on the levels of p53, RB, $p21^{WAF1}$, Bcl-X_L and Bax. Lysates (50 μg) were prepared from U-87 and A172 cells that were treated for 8, 24, and 48 hr with 2.5 μM of Ro 31-8220. Samples were separated in either 8 or 12% polyacrylamide gels by SDS–PAGE and transferred electrophoretically onto nitrocellulose; immunoreactive proteins were visualized by chemiluminescence. (B) Effect of Ro 31-8220 on the levels of p53 and IGFBP3 in A172 cells. Lysates were prepared and separated as described for panel A, and immunoreactive proteins were visualized by chemiluminescence.

signaling pathway, where Ras inactivation leads to a decline in RB phosphorylation and G_1 arrest [45]. Since PKC serves as an effector of Ras/Raf activation [46–48], it is likely that inhibition of PKC results in RB hypophosphorylation, its retention in the nucleus [49, 50], and cell cycle arrest. Additionally, Raf-1 activates the Cdc25A bifunctional phosphatase that activates Cdc2 kinase [51], and, therefore, PKC inhibition would be expected to result in reduced Cdc2 kinase activity and consequently RB hypophosphorylation. It is evident from our data that RB hypophosphorylation occurs subsequent to nuclear localization (Fig. 6, bottom, and Fig. 7A) and is associated with the onset of apoptosis (Fig. 3). Transient cell cycle blockade through this mechanism would be expected to produce conflicting growth signals and possibly an apoptotic response.

Apoptosis caused by a broad range of stimuli is blocked by overexpression of Bcl-2 and Bcl-X_L [52], and reduction in the ratio of Bcl-2 to Bax has been suggested as one mechanism by which apoptosis can be modulated by chemotherapeutic agents [53, 54]. Wild-type p53 negatively regulates Bcl-2 and positively regulates Bax gene expression [55, 56]. However, there was neither a reduction of Bcl-X_L levels nor an increase in Bax levels associated with the up-regulation of p53. Although it has been suggested that PKC inhibition promotes apoptosis through the down-regulation of Bcl-2 [26, 57], U-87 and A172 cells lack Bcl-2. These results do not exclude the possibility that inhibition of PKC may influence the activities of Bcl-X_L Bax, or another gene family member posttranslationally through phosphorylation. Although Bcl-X_L and Bax contain a number of consensus sites for PKC phosphorylation, we have not detected phosphorylation of Bcl-X_L and Bax following immunoprecipitation of these proteins in U-87 and A172 cells (unpublished results).

On the other hand, IGFBP3 was induced at 16 hr after Ro 31-8220 treatment and, therefore, may play a role in the apoptotic process induced by PKC inhibition. Although the IGFBP3 promoter is activated by p53 [58, 59] and recombinant IGFBP3 induces apoptosis in MCF-7 cells [36], it is unclear if IGFBP3-induced apoptosis occurs solely through a p53-dependent mechanism [37]. Similarly, p53-induced apoptosis has been reported to occur independently of *trans*-activation [60], and it cannot be ruled out that a non-transcriptional mechanism is involved. Although p53 accumulation preceded the changes in IGFBP3 levels, it remains to be seen if these two effects are causally related and whether IGFBP3 is responsible for the ensuing apoptotic response.

In summary, our results suggest that high PKCα expression is a predisposing factor for preventing PKC inhibitor-induced apoptosis in glioblastoma cells. The rapid induction of p53 and the p53-dependent gene, *IGFBP3*, suggests at least one complementary pathway that may initiate the onset of apoptosis.

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