Ionizing Radiation Induces Apoptotic Signal Through Protein Kinase Cδ (delta) and Survival Signal Through Akt and Cyclic-Nucleotide Response Element-Binding Protein (CREB) in Jurkat T Cells

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Abstract. Although ionizing radiation induces a loss of proliferative capacity as well as cell death by apoptosis and necrosis, cells can oppose the damaging effects by activating survival signal pathways. Here we report the effect of 1.5- and 6-Gy doses of ionizing radiation on apoptotic protein kinase Cδ (PKCδ) and survival cyclic-nucleotide response element-binding protein (CREB) signal in Jurkat T cells. Cell cycle analysis, performed by flow cytometry, showed a significant G2M arrest 24 h after exposure to 6 Gy. This arrest was accompanied by dead cells, which increased in number up to 7 days, when cell viability was further reduced. The response was apparently promoted by caspase-3-mediated PKCδ activation, and thus apoptosis. Moreover, the presence of viable cells up to 7 days in samples exposed to 6 Gy is explained by Akt activation, which may influence the nuclear transcription factor CREB, leading to resistance to ionizing radiation. Thus, the knowledge of apoptotic and survival pathways activated in tumor cells may help in establishing specific therapies by combining selective inhibitors or stimulators of key signaling proteins with conventional chemotherapy, hormone therapy, and radiotherapy.

Introduction

Ionizing radiation induces a loss of proliferative capacity as well as cell death by apoptosis and necrosis. Apoptosis can occur during interphase before division after the G2 block induced by radiation (fast apoptosis) or after one or more divisions (late apoptosis). Some radiosensitive cells, such as thymocytes, lymphocytes, and intestinal crypt cells, undergo fast apoptosis without cell division after ionizing radiation, while irradiated mouse leukemia cells undergo a block at G2 and the apoptotic fraction begins to increase on the release from this G2 block (Radford *et al.,* 1994; Tauchi and Sawada, 1994; Merritt *et al.* 1997).

Moreover, the cell can respond by trying to counteract radiation-induced damage to the membrane and to repair single- and double-stranded breaks in DNA by activating survival-signal pathways. Whether apoptosis ensues or the cell survives, genes controlling complex pathways are activated (Cohen-Jonathan *et al.,* 1999; Miura, 2004).

Among the proteins involved in cell responses to ionizing radiation, a role has been assigned to members of the protein kinase C (PKC) family (Haimovitz-Friedman, 1998; Cataldi *et al.,* 2002; Dent *et al.,* 2003) and to the cyclic-nucleotide response element-binding (CREB) nuclear transcription factor (Shaywitz and Greenberg, 1999; Shi *et al.,* 2004; Dodson and Tibbetts, 2006; Cataldi *et al.,* 2006). PKCs belong to a class of serine-threonine kinases comprising at least 12 closely related isozymes that have distinct, and in some cases opposing, roles in cell growth, differentiation, apoptosis, and survival (Nishizuka,1995; Dempsey *et al.,* 2000; Musashi *et al.,* 2000). Among these 12 isozymes, PKCδ (delta) is known to be activated in response to a variety of genotoxic stresses (Kronfeld *et al.,* 2000; Kikkawa *et al.,* 2002; Brodie and Blumberg, 2003; Jackson and Foster, 2004; Nakajima, 2008).

PKCδ is regulated not only by binding diacylglycerol or phorbol esther, but also by molecular mechanisms such as phosphorylation and proteolysis. In particular, it shows an activation loop, a turn, and a hydrophobic motif site at Thr 505, Ser-643, and Ser-662, respectively, and these sites are substantially phosphorylated *in vivo* (Konishi *et al.,* 2001). It can be further phosphorylated on tyrosine residues such as Tyr-52, Tyr-155, Tyr-187, Tyr-311,Tyr-332, and Tyr-565, depending on the cell stimulus (Li *et al.,* 1994). In addition, a catalytically active 40-kDa fragment of PKCδ is generated by proteolysis in cells exposed to ionizing radiation, DNA-damaging drugs, or anti-FAS antibody (Emoto *et al.,* 1995; Ghayur *et al.,* 1996; Mizuno *et al.,* 1997; Takahashi *et al.,* 1998; Lewis *et al.,* 2005).

CREB protein is a 43-kDa basic leucine zipper (bzip) transcription factor composed of a C-terminal basic DNA-binding domain, an adjacent leucine zipper dimerization domain, and a kinase-inducible transcriptional activation domain. It is implicated in numerous cell functions including proliferation, apoptosis, survival, differentiation, and adaptive response (Wilson *et al.,* 1996; De Cesare *et al.,* 1999; Saeki *et al.,* 1999; Andreatta *et al.,* 2004; Di Pietro *et al.,* 2007; Caravatta *et al.,* 2008). CREB activation depends not only on the cell type but also on the stimulus administered (Wilson *et al.,* 1996; Du and Montminy, 1998; Walton *et al.,* 1999; Choi *et al.,* 2003; Dodson and Tibbetts, 2006; Di Giulio *et al.,* 2007), and it results from post-translational modifications such as phosphorylation of serine-133 (Ser-133) by several factors (Muthusamy and Leiden, 1998; Shi *et al.,* 2004). The signaling molecules responsible for CREB Ser -133 phosphorylation, which differ among cell types, include Akt/protein kinase B (PKB) (Du and Montminy, 1998; Pugazenthi *et al.,* 2000; Das *et al.,* 2005; Yano *et al.,* 2005). Akt stimulates target gene expression *via* CREB in a phospho-(ser-133)–dependent manner, promoting cell survival in response to growth factor stimulation (Uchiyama *et al.,* 2004).

In the light of this evidence, the present study addressed the effect of ionizing radiation on apoptotic PKCδ signal and survival Akt/CREB signal activation in Jurkat T human lymphoblastoid cells, which are sensitive to ionizing radiation (Dahlberg *et al.,* 1999; Di Pietro *et al.,* 2004). A 1.5-Gy dose was chosen as suboptimal, and 6 Gy is the threshold above which cells lose clonogenicity and the apoptosis rate increases (Cataldi *et al.,* 2006). In addition, 6 Gy is the suboptimal dose delivered in radiotherapeutic protocols for human tumors and induces evident damage in many cancer cell lines (Dahlberg *et al.,* 1999).

Materials and Methods

Cell culture and ionizing radiation exposure

Jurkat T leukemic cells, grown in suspension in RPMI 1640 medium, supplemented with 10% FCS (fetal calf serum), glutamine, HEPES, and penicillin/streptomycin in a controlled atmosphere, were irradiated at room temperature by a Siemens Mevatron 74 linear accelerator (Munich, Germany) (photonic energy: 10 MV) with doses of 1.5 and 6 Gy (dose rate 3 Gy/min). To ensure that the effects of ionizing radiation observed were specifically attributable to the activation of PKCδ, 3 μmol l^{-1} rottlerin (Sigma Co., St. Louis, MO), a highly selective inhibitor of such enzymes (Gschwendt *et al.,* 1994), was added to some cultures 1 h before irradiation. The cells were diluted and then reseeded in fresh RPMI, and viability and death were assessed using a Trypan blue dye exclusion test until day 7. For fluorescence and electron microscopy and western blotting, cells were recovered within 1 h after exposure to the radiation, a reasonable time interval to study molecular signaling activation; TUNEL analysis and cell cycle were evaluated 24 h later.

Evaluation of cell cycle and apoptosis

Samples containing $2–5 \times 10^5$ cells were harvested by centrifugation at $200 \times g$ for 10 min at 4 °C, fixed with 70% cold ethanol for at least 1 h at 4 °C, and resuspended in 20 μg/ml propidium iodide (PI) and 100 μg/ml RNAse (final concentration). Cell cycle profiles (10,000 cells) were analyzed by an EPICS Coulter flow cytometer (Fullerton, CA) with an FL2 detector in a linear mode using the Expo 32 analysis software (Beckman Coulter, Miami, FL). For each sample, 10,000–20,000 events were collected. Multicycle software (Phoenix Flow Systems, San Diego, CA) was used for analysis of cell cycle phase. For quantitative evaluation of apoptosis, subdiploid (less than 2n) DNA content was calculated and expressed as the percentage of apoptotic *versus* non-apoptotic cells, regardless of the specific phase of the cell cycle.

Electron microscopy analysis

Cells were fixed in a mixture of 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 mol l^{-1} cacodylate buffer, pH 7.4, for 60 min at 4 °C. Samples were dehydrated using an alcohol series and embedded in hydrophilic Bioacryl resin, followed by UV polymerization. Ultrathin sections, cut with a Reichert ultramicrotome and mounted on 300-mesh nickel grids, were counterstained in uranyl acetate and lead citrate to preserve cell morphology, and then photographed using a Zeiss electron microscope 109.

TUNEL analysis

After centrifugation in a cytospin, cells were fixed in paraformaldehyde (4% v/v in phosphate buffered saline [PBS], pH 7.4) for 30 min at room temperature and permeabilized with 0.1% Triton, 0.1% sodium citrate for 2 min on ice. DNA strand breaks, characteristic of apoptotic cells,

were identified by labeling the free 3′-OH nucleotide termini with fluorescein–dUTP with an In Situ Cell Death Detection kit (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany). Slides were mounted in glycerol and examined under fluorescence microscope (Leica Microscopy System, Heidelberg, Germany). Negative and positive controls were performed according to the manufacturer's protocol (not shown).

Western blotting analysis

Samples, 20 μg each, of total cellular protein lysates were subjected to electrophoresis on a 10% sodium dodecyl sulphate(SDS)-polyacrylamide gel. The gel was then electroblotted onto nitrocellulose. Blots were blocked with 5% nonfat milk, 10 mmol l^{-1} Tris (pH 7.5), 100 mmol l^{-1} NaCl, 0.1% Tween-20; probed with rabbit polyclonal antibodies against PKCδ, Akt-1, p-Akt-1 (Ser473), CREB, and p-CREB (Ser133) or with mouse monoclonal antibodies against cleaved caspase-3 (Santa Cruz Biotechnology, Santa Cruz, CA), PARP (Poly(ADP-Ribose)Polymerase) (Oncogene Research Products, La Jolla, CA); and developed with specific enzyme-conjugated horseradish-peroxidase. Bands were detected by an ECL detection system (Amersham Intl., UK). When required, the blots were stripped of bound antibodies by incubating the membranes in wash buffer containing 2% SDS at 50 °C for 30 min, blocked, and re-probed with other primary and secondary antibodies.
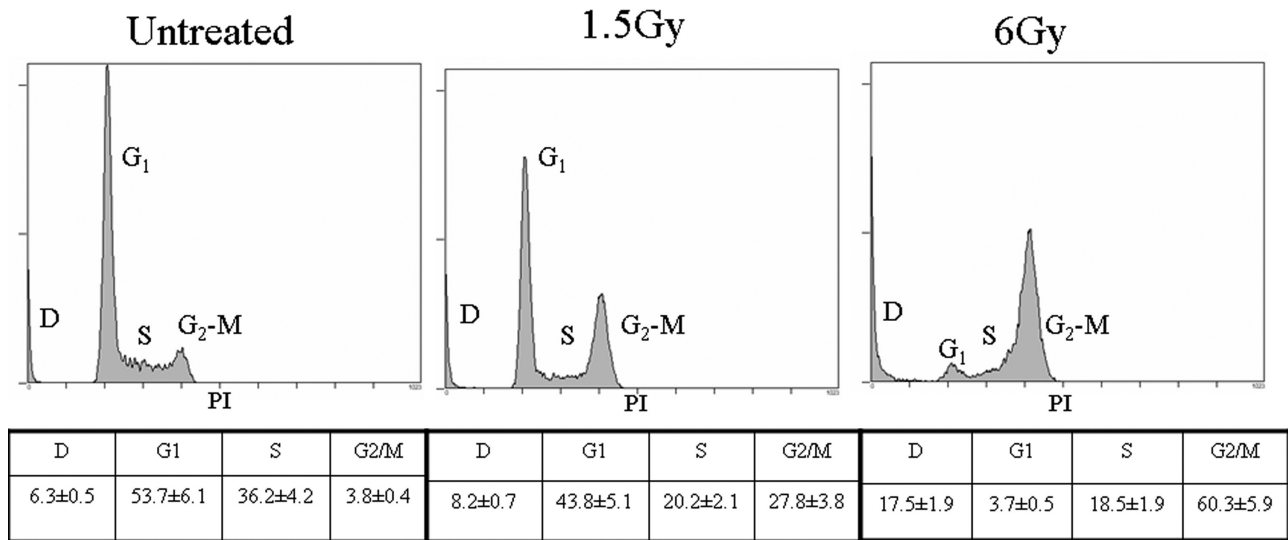
Fluorescence microscopy

After centrifugation in a cytospin, cells were fixed in 4% paraformaldehyde for 10 min, washed in PBS, and treated with 5% donkey serum in PBS for 20 min at room temperature. Immunolabeling was performed for 1 h at 37 °C in the presence of 5 μg/ml rabbit polyclonal antibodies (diluted in PBS, 5% Tween-20, and 2% bovine serum albumin) against p-PKC (Thr 507) and p-Akt (Ser 473) (Santa Cruz Biotechnology, Santa Cruz, CA). The p-PKC, which represents the activation loop of PKC, identifies the catalytic domain of the protein (Cheng *et al.,* 2007). The slides were washed in PBS and treated for 45 min at 37 °C with FITC (fluorescein-isothiocyanate)-conjugated anti-rabbit IgG (immunoglobulin) antibody or TRITC (tri-rhodamine-isothiocyanate)-conjugated anti-goat IgG antibody (Boheringer Mannheim, Germany) diluted 1:50 in PBS, 5% Tween-20, 2% BSA. After several washes in PBS, the slides were mounted in glycerol-DABCO (1-4-diazabicyclo[2-2-2]octane) containing 5 μg/ml DAPI (4-6,diamidino-2-phenyl-indol) to counterstain nuclei. Internal controls, performed by omitting the primary antibody, showed no FITC staining. The labeled slides were examined under a Leica light microscope (Heidelberg, Germany) equipped with a Coolsnap videocamera (RS Photometrics, Tucson, AZ) to acquire computerized images.

Densitometry analysis and statistics

Densitometric values of protein bands, expressed as integrated optical intensity, were estimated in a CHEMIDOC XRS System by the QuantiOne 1-D analysis software, ver. 22 (BIORAD, Richmond, CA). Values obtained were normalized with reference to densitometric values of internal beta tubulin. Data were analyzed using the two-tailed, two-

D	G1	S	G2/M		D	G1	S	G2/M		D	G1	S	G2/M
6.3±0.5	53.7±6.1	36.2±4.2	3.8±0.4		8.2±0.7	43.8±5.1	20.2±2.1	27.8±3.8		17.5±1.9	3.7±0.5	18.5±1.9	60.3±5.9

Figure 1. Flow cytometry analysis of cell cycle performed 24 h after exposure of Jurkat T cells to ionizing radiation. Letters indicate the different phases of the cell cycle. D: dead cells. The most representative of three independent experiments whose results were consistent is shown. Tables show cell percentages quantified by the mean (±SD) of three different peak analyses.

LIVE CELLS

DEAD CELLS
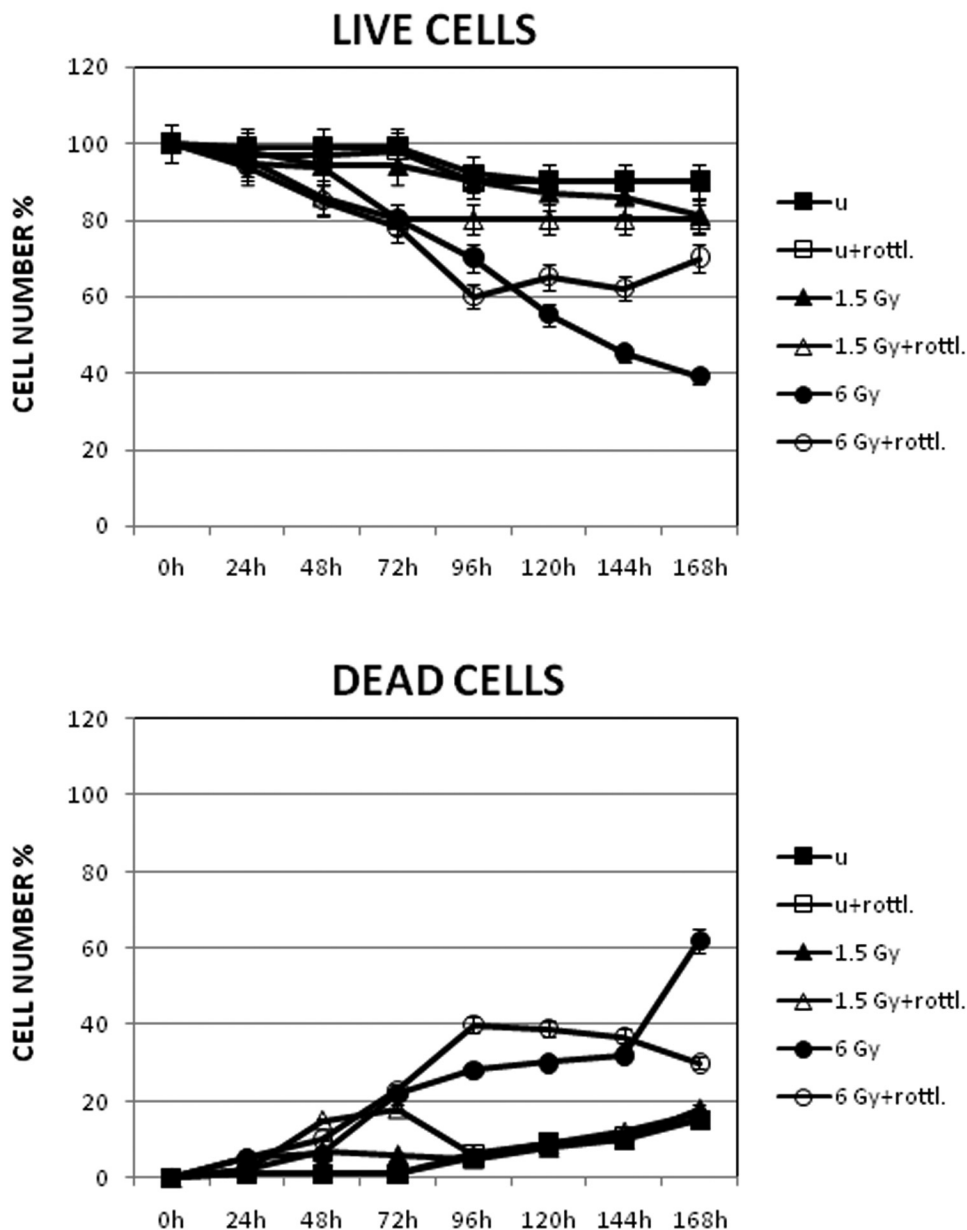
Figure 2. Cell viability and death assessed by Trypan blue dye exclusion test on Jurkat T cells up to 7 days after exposure to 1.5- and 6-Gy ionizing radiation. Data, presented as percentages, are the mean ($\pm$SD) of three independent experiments whose results were consistent.

sample Student's *t* test. Results were expressed as mean $\pm$ SD. Values of $P < 0.05$ were considered significant.

Results

Cell cycle analysis, performed 24 h after the exposure to ionizing radiation, showed that 27.8% of the cells congregated in G2-M phase after 1.5 Gy, and 60.3% after 6 Gy; the corresponding percentages of dead and early apoptotic cells were 8.2% and 17.5%, respectively (Fig. 1). Decreased cell viability was also apparent for up to 7 days after 6-Gy exposure (Fig. 2), even though compensatory cellular proliferation occurred during the course of the assay, which may affect the results of such assessment. The percentage of proliferation in unexposed and 1.5-Gy-exposed cells decreased by 10%–20%, while the rate after exposure to 6 Gy decreased to 60% instead of 38% as reported in Figure 2 (live cells). Lastly, the percentage of TUNEL-positive apoptotic cells, the characteristic features of which are evidenced by electron microscopy analysis (Fig. 3), increased up to 40% in the 6-Gy-exposed sample (Table 1).

a

b
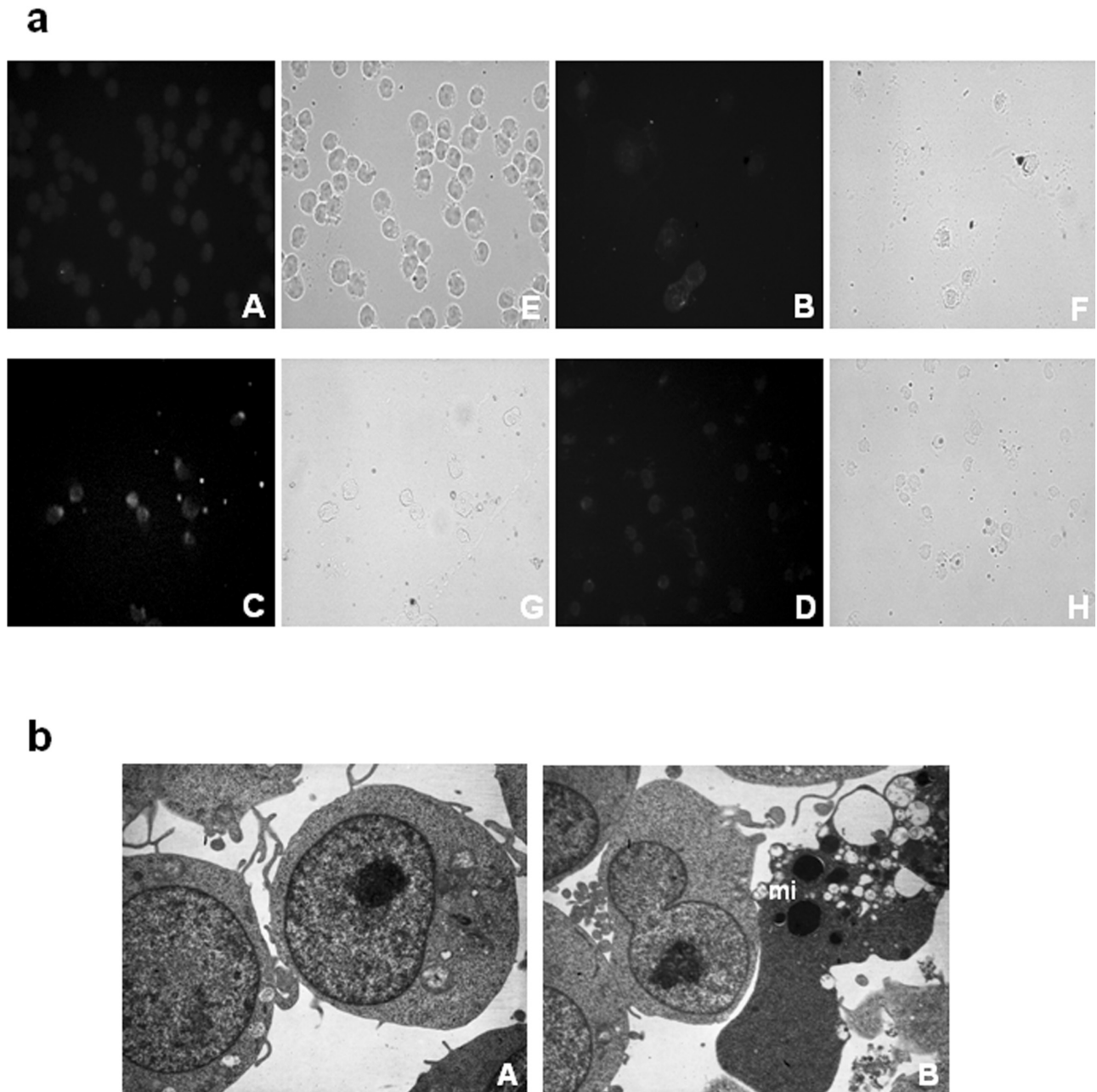
Figure 3. (a) TUNEL detection of apoptosis and phase contrast light microscopy (E–H) of Jurkat T cells exposed to 6-Gy ionizing radiation (A-D). When required, 3 μmol l^{-1} rottlerin, a highly selective PKCδ inhibitor, was added to the culture 1 h prior to irradiation. The extent of DNA fragmentation was quantified by direct visual counting of fluorescent-labeled nuclei by light microscopy, as shown in Table 1. Phase contrast light microscopy shows cells with apoptotic features. Magnification: 40$\times$. A, E: unexposed; B, F: unexposed + rottlerin; C, G: 6 Gy; D, H: 6 Gy + rottlerin. **(b)** Morphological features of apoptosis revealed by electron microscopy in Jurkat T cells exposed to ionizing radiation. Note the presence of membrane blebbing and micronuclei (mi) with highly compact, electron-dense chromatin, which are typical features of the apoptotic state. Magnification: 4400$\times$. A: unexposed; B: 6 Gy.

In view of these preliminary results, we next investigated key signal transduction pathways involved in the apoptotic and survival responses of Jurkat T cells to ionizing radiation. Because the percentage of dead cells was significant after exposure to 1.5 Gy and especially after 6 Gy, we investigated the signaling pathway likely to be involved in this death response. Since our group, among the PKC isoforms checked (δ, ε, ζ), previously assigned a role to PKCδ

Table 1

TUNEL detection of apoptosis in Jurkat cells exposed to ionizing radiation

Experimental points	Apoptotic cells
Unexposed cells	4.0 ± 0.09
Unexposed cells + rottlerin	2.0 ± 0.07
6 Gy	40.0 ± 3.08*
6 Gy + rottlerin	20.0 ± 1.12*

Five slides were examined per sample within 5 h after exposure to ionizing radiation. The presence of DNA fragmentation was quantified by direct visual counting of fluorescent-labeled nuclei at 40× magnification (light microscopy). Apoptotic cells were counted out of a total of 100 cells. Values (expressed as %) are means ± SD; $n = 3$ for all groups.

* Values of $P < 0.05$ are considered statistically significant.

in the apoptotic response of Jurkat cells to ionizing radiation (Cataldi *et al.,* 2002) and since cleavage by caspase-3 is reasonable evidence of activation of this protein (Emoto *et al.,* 1995; Lewis *et al.,* 2005), we checked the effects of 1.5- and 6-Gy ionizing radiation on caspase-3 mediated PKCδ activation. When PKCδ expression was checked after exposure to ionizing radiation, western blotting revealed a cleaved fragment of 40 kDa, coinciding with the 20-kDa
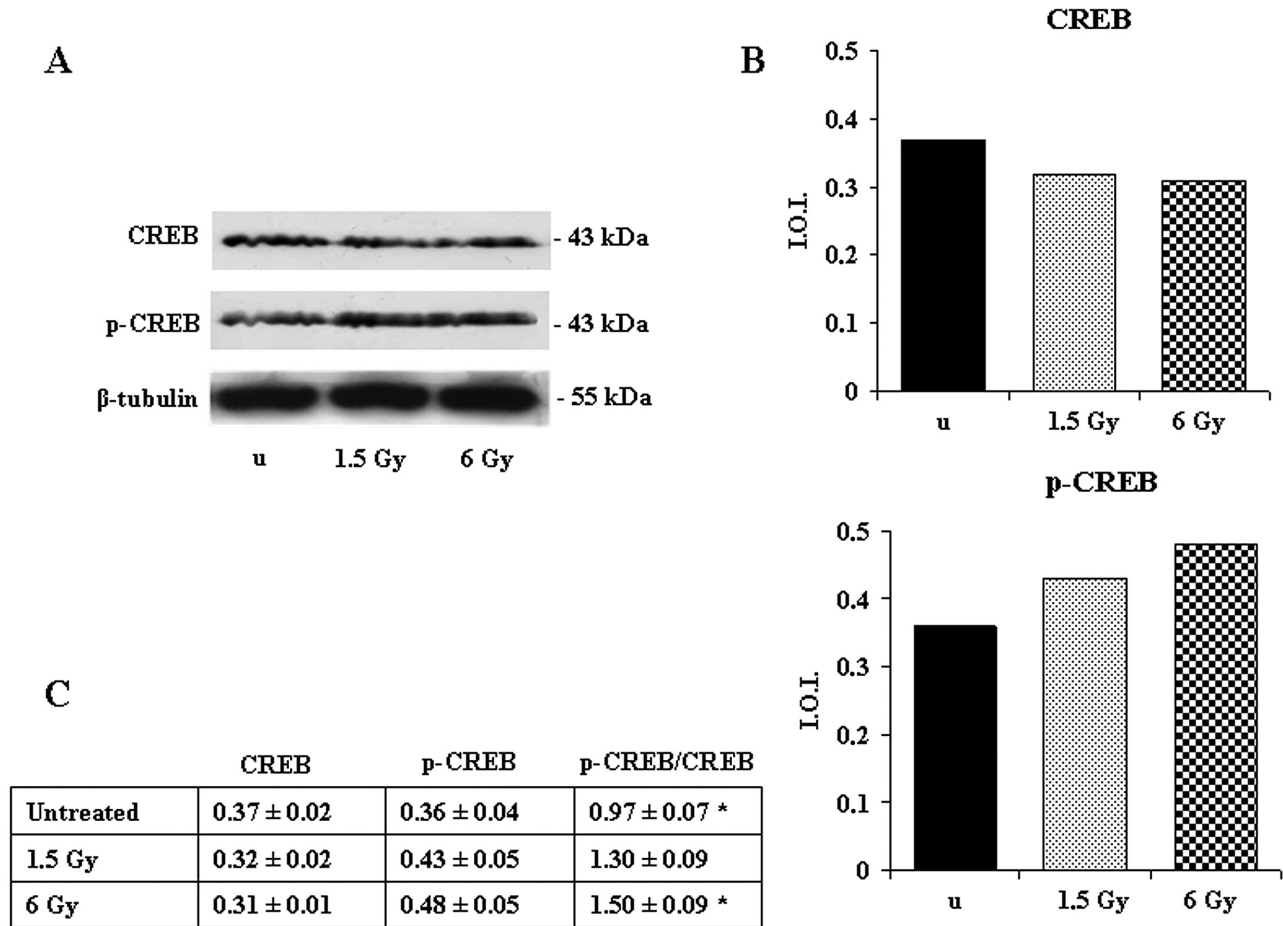
caspase-3 cleaved fragment and the 85-kDa PARP cleaved fragment (Fig. 4), suggesting a new role for proteolytic activation of PKCδ by caspase-3. This biochemical response was corroborated by the effects observed in this cell line when the PKCδ highly selective inhibitor, rottlerin, was added to the cultures. In the presence of rottlerin, the number of dead cells decreased, as shown by the Trypan blue dye exclusion test and TUNEL analysis (Figs. 2–3 and Table 1), even though cell viability also decreased.

Since the CREB nuclear transcription factor is an important element in the cAMP-dependent survival signaling (Shaywitz and Greenberg, 1999), we then checked its phosphorylation state by western blotting analysis. CREB expression was unchanged, but its phosphorylation on Ser 133 increased after irradiation with 1.5 Gy and particularly after 6 Gy (Fig. 5). In addition, given that CREB is considered a regulatory target for the protein kinase Akt/PKB (Du and Montminy, 1998), we investigated the response of Akt to the ionizing radiation. Akt expression was unchanged, but its phosphorylation on Ser 473 increased after exposure to 6 Gy (Fig. 6), suggesting that its activation could determine CREB phosphorylation.

Lastly, we performed a double immunolabeling of p-PKCδ (Thr 507) and p-Akt (Ser 473). 1.5-Gy-exposed sam-
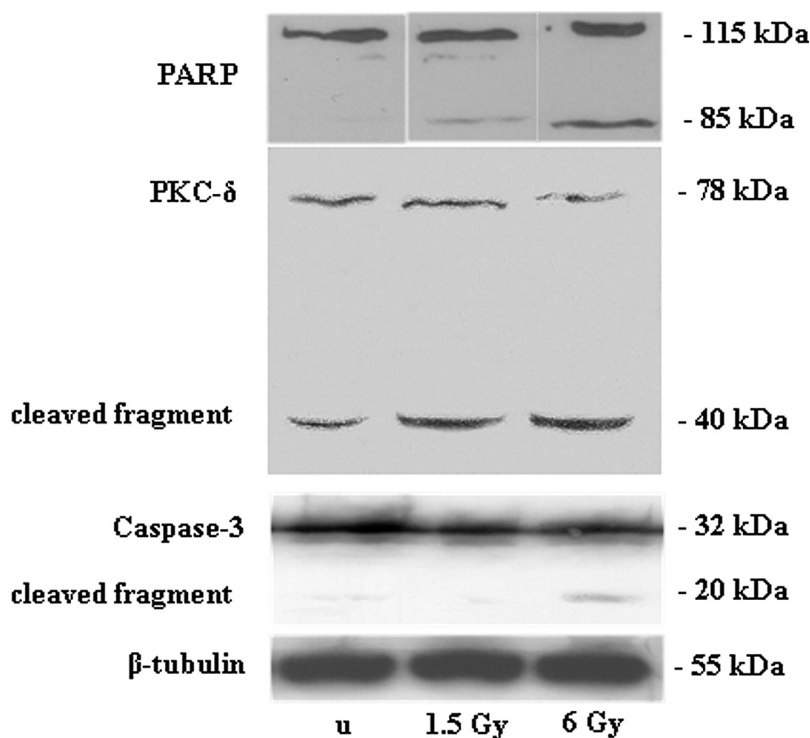
Figure 4. Effect of 1.5- and 6-Gy ionizing radiation on PARP, PKCδ, and caspase-3 expression and cleavage in Jurkat T cells. The 85-kDa PARP cleaved subunit, 40-kDa PKCδ fragment, and 20-kDa caspase-3 fragment are apparent after exposure to ionizing radiation. Immunostaining with a monoclonal antibody against β-tubulin confirms equal loading (20 μg). The blots shown are the most representative of three independent experiments whose results were consistent. u = unexposed.

A

B

C

	CREB	p-CREB	p-CREB/CREB
Untreated	0.37 ± 0.02	0.36 ± 0.04	0.97 ± 0.07 *
1.5 Gy	0.32 ± 0.02	0.43 ± 0.05	1.30 ± 0.09
6 Gy	0.31 ± 0.01	0.48 ± 0.05	1.50 ± 0.09 *

Figure 5. Effect of 1.5- and 6-Gy ionizing radiation on CREB and p-CREB expression in Jurkat T cells. (A) Western blotting analysis of CREB and p-CREB (Ser 133) expression. Immunostaining with a monoclonal antibody against β-tubulin confirms equal loading (20 μg). Blots are the most representative of three independent experiments whose results were consistent. (B) Densitometric analysis of CREB and p-CREB (Ser 133) expression of the blot shown. (C) In the table, the ratio between the densitometric values of p-CREB (Ser133) and CREB is given $\pm$ S.D. u = unexposed. *6 Gy pCREB/CREB *vs.* unexposed pCREB/CREB: $P < 0.05$
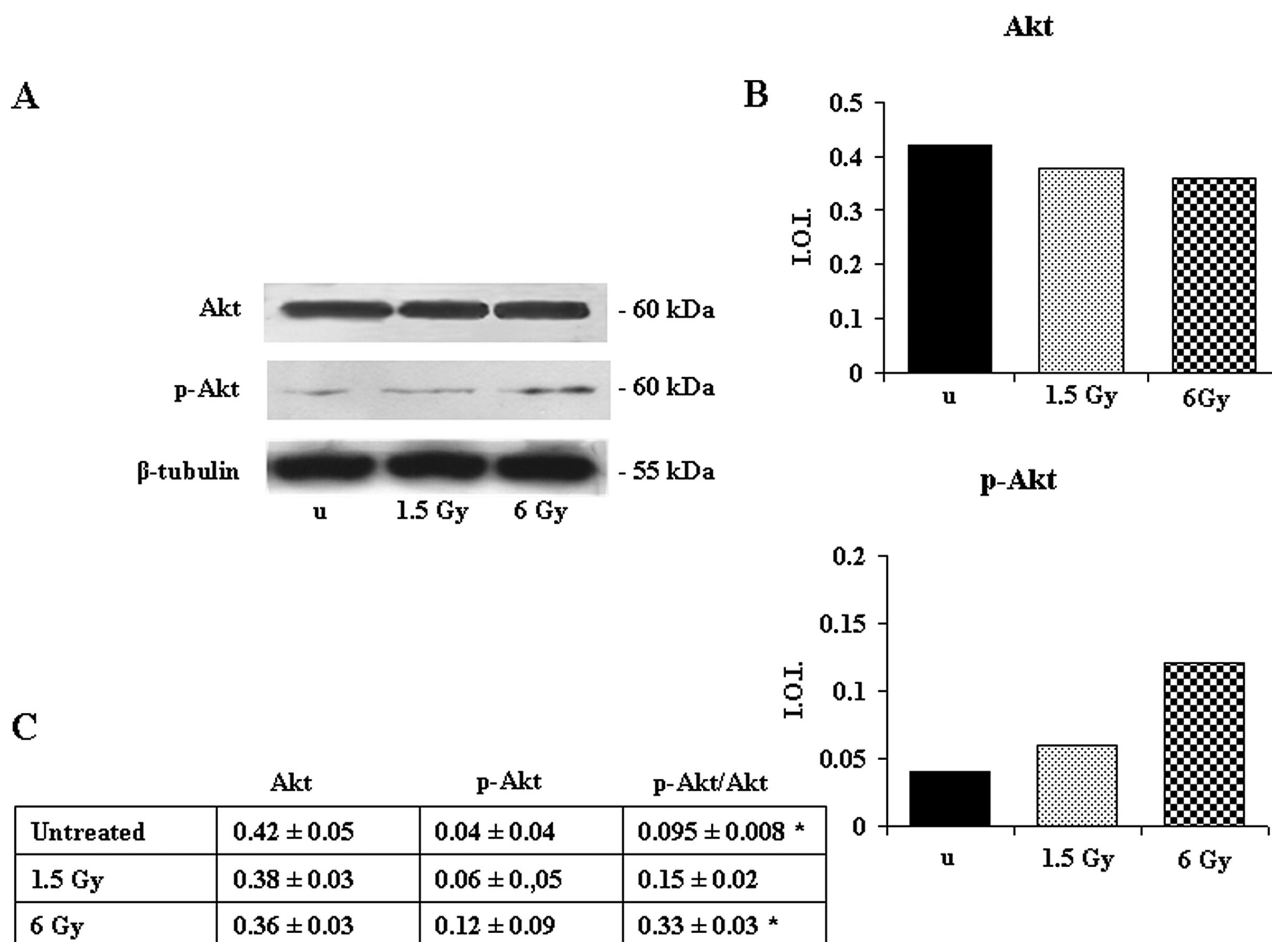
ples showed an increased level and nuclear translocation of p-Akt, while 6-Gy-exposed samples showed more p-PKCδ-positive than p-Akt-positive nuclei (Fig. 7), thus explaining the presence of a large number of dead cells, evidenced by TUNEL analysis (Fig. 3a). Interestingly, the number of apoptotic cells decreased in the presence of rottlerin (Table 1), while phosphorylation of the Akt/CREB signaling machinery increased after exposure to a 6-Gy dose of ionizing radiation (Table 2).

Discussion

Ionizing radiation is known to cause cell cycle arrest, allowing cells to repair some of the damages induced to DNA by this oxidative stress. Such damage occurs in G1 prior to DNA replication, in S phase, and also in G2 before mitosis.

Moreover, the cells are activated against ionizing radia-

tion mechanisms, allowing them to repair membrane damage and single- and double-stranded breaks in DNA. In this study we wanted to investigate the effect of ionizing radiation on apoptotic and survival molecular response in Jurkat T lymphoblastoid cells in order to identify molecular targets for rational therapeutic intervention strategies. The threshold over which ionizing radiation induces considerable damage at the cellular level seems to be 6 Gy, even though a proportion of the cells activate repair mechanisms and still survive. In fact, significant G2 arrest occurs 24 h after exposure, along with generation of dead cells whose number increases in parallel to the decrease in cell proliferation for up to 7 days. These responses seem to be promoted by caspase-3-mediated PKCδ activation, as indicated by the appearance of a 40-kDa PKCδ fragment along with a phosphorylated active form of the enzyme translocated into the nucleus, shown by immunofluorescence microscopy, espe-

A

B

Akt

C

	Akt	p-Akt	p-Akt/Akt
Untreated	0.42 ± 0.05	0.04 ± 0.04	0.095 ± 0.008 *
1.5 Gy	0.38 ± 0.03	0.06 ± 0.,05	0.15 ± 0.02
6 Gy	0.36 ± 0.03	0.12 ± 0.09	0.33 ± 0.03 *

Figure 6. Effect of 1.5- and 6-Gy ionizing radiation on Akt and p-Akt (Ser 473) expression in Jurkat T cells. (A) Western blotting analysis of Akt and p-Akt (Ser 473) expression. Immunostaining with a monoclonal antibody against β-tubulin confirms equal loading (20 μg). The blots are the most representative of three independent experiments whose results were consistent. (B) Densitometric analysis of Akt and p-Akt(Ser 473) expression of the blot shown. (C) In the table, the ratio between the densitometric values of p-Akt and Akt is given ±SD. u = unexposed. *6 Gy pAkt/Akt *vs.* unexposed pAkt/Akt: $P < 0.05$.

cially after exposure to 6 Gy. This result is consistent with publications reporting that phosphorylation at Tyr 311 promotes degradation of PKCδ, presumably after ubiquination (Lu *et al.,* 1998; Cataldi *et al.,* 2002). Death-activated PKCδ transiently accumulates in the nucleus where it is cleaved by caspase-3 to generate the constitutively activated PKCδ catalytic fragment. Nuclear accumulation of pPKCδ thus commits a cell to undergo apoptosis (De Vries-Selmon *et al.,* 2007; Sun *et al.,* 2008). If caspase-3 is not active it cannot cleave PKCδ and thus induce apoptosis. In fact, the sequential regulation of PKCδ and caspase-3 may assure that sustained nuclear accumulation of p-PKCδ occurs only when caspase-3 is activated (Reyland, 2007). Nevertheless, a smaller relative amount of PKCδ fragment is also present in untreated cells, as elsewhere reported in other experimental models (Lewis *et al.,* 2005). The absence of detectable proteolytic cleavage of other PKC isoforms (not shown)

provides further support for the selectivity of PKCδ cleavage induced by ionizing radiation.

Moreover, the cells seem to activate a survival signal system driven by Akt soon after exposure to ionizing radiation, and this, in turn, could activate the nuclear transcription factor CREB. The transient activation of this signaling system is further confirmed by the presence of viable cells in the samples exposed to 6 Gy up to 3 days after treatment, accompanied by a number of dead cells similar to that present in the 1.5-Gy-exposed sample. It is evident that the cells try to counteract the effect of 6 Gy, but they are less viable after 7 days. In fact, when the dead cell population was evaluated, more apoptotic cells were apparent in the 6-Gy-exposed samples.

Cell death was reduced when the PKCδ highly selective inhibitor rottlerin was added to the culture 1 h before exposure to the ionizing radiation. Rottlerin does not di-
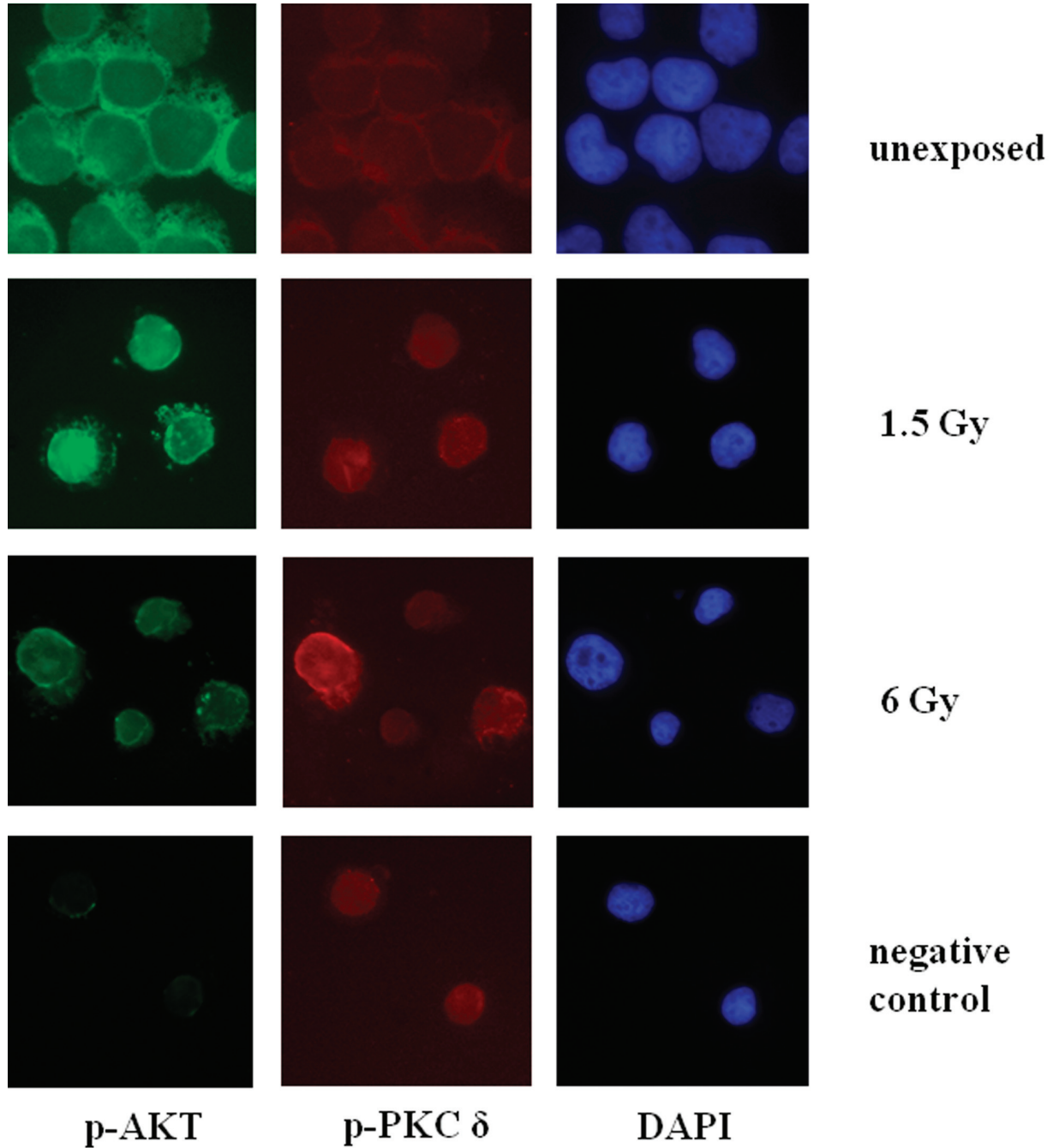
Figure 7. Light microscopy immunofluorescence analysis of p-PKCδ (Thr 507) and p-Akt (Ser 473) expression in Jurkat T cells exposed to 1.5- and 6-Gy ionizing radiation. Green fluorescence shows p-Akt (left column), red fluorescence shows p-PKCδ, blue fluorescence represents DAPI (4-6-diamino-phenyl-indol) counterstaining nuclei (right column). Negative controls were performed by omitting the primary antibody (bottom row). Magnification: 40×

rectly block PKCδ activity but—by uncoupling mitochondria, depolarizing their membrane potential, reducing cellular ATP levels, activating 5′-AMP-activated protein kinase(AMPK), and affecting mitochondrial production of reactive oxygen species (ROS)—it ultimately precludes PKCδ tyrosine phosphorylation and activation (Soltoff,

2007). Lastly, since it has been reported that BCR-induced CREB phosphorylation on Ser 133 in B lymphocytes requires the novel PKCδ isoform (Blake *et al.,* 1999; Blois *et al.,* 2004), we checked the phosphorylation state of Akt and CREB in the presence of rottlerin. The phosphorylation levels of both Akt and CREB increased in the presence of

Table 2

Quantitative analysis by western blotting of CREB and Akt phosphorylation in Jurkat T cells exposed to ionizing radiation

Experimental points	p-CREB	p-Akt-1
Unexposed cells	0.14 ± 0.03	0.04 ± 0.006
Unexposed cells + rottlerin	0.28 ± 0.06	0.13 ± 0.010
1.5 Gy	0.18 ± 0.06	0.08 ± 0.002
1.5 Gy + rottlerin	0.30 ± 0.09	0.14 ± 0.020
6 Gy	0.35 ± 0.12*	0.12 ± 0.010*
6 Gy + rottlerin	0.46 ± 0.16*	0.28 ± 0.030*

Data are the mean ±SD of three different experiments whose results were consistent.

* Values of $P < 0.05$ are considered statistically significant.

rottlerin, and the number of dead cells decreased concomitantly (Tables 1–2), suggesting that Akt/CREB phosphorylation follows a PKC-independent route and constitutes part of the survival pathway.

Overall, our results are consistent with the response showed by other types of cells *"in vivo"* (Chong *et al.,* 2005) and confirm the importance of Akt as a vital and broad cytoprotectant against cellular stresses in parallel to pro-apoptotic PKCδ activator (Nakajima *et al.,* 2006).

Thus, knowledge about some of the molecular mechanisms that drive cellular survival or death responses to ionizing radiation can help to establish therapies for several types of cancer by combining selective inhibitors or stimulators of key signaling proteins with conventional options for chemotherapy, hormone therapy, and radiotherapy.

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