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Role of Nuclear PKC δ in Mediating Caspase-3-Upregulation in Jurkat T Leukemic Cells Exposed to Ionizing Radiation

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Abstract The response of Jurkat T cells to ionizing radiation (IR) includes cell cycle arrest and DNA damage, which lead to the occurrence of apoptosis. Here, we try to elucidate some of the early intracellular signals which control the induction of such a process upon IR exposure, addressing to examine the specific role of several PKC isoforms (δ, ε, ζ) and their subcellular distribution. Attention has been focused on the connections between nuclear PKC δ activation and the expression of cell death regulators (Bcl-2 family proteins Bad, Bax and Bcl-2) and cell death effector caspase-3 (CPP32) which lead to the cleavage of cytoskeletal and nuclear proteins and induction of apoptosis. Altogether these results let us to conclude that PKC δ, potentiating the pro-apoptotic effect of caspase 3, plays a key role in the cellular response to IR and thus can be considered a molecular target for therapy. J. Cell. Biochem. 86: 553–560, 2002.

Key words: PKCδ; nucleus; Jurkat; ionizing radiation

One of the signaling pathways activated at plasma membrane in response to apoptotic stimuli, such as ionizing radiation (IR), is under the regulation of the PKCs' superfamily [Kelly et al., 1998; Yuan et al., 1998; Cataldi et al., 2002]. PKC is a family of at least 12 structurally related phospholipid-dependent serine/threonine protein kinases directly involved in the response to many extracellular signals [Stabel and Parker, 1991; Hofman, 1997; Mellor and Parker, 1998]. These are divided into three subgroups: the calcium dependent or cPKCs (α, β_1, β_2, and γ), the novel or nPKCs (δ, ε, η, θ, and μ), and the atypical or aPKCs (ζ, human ι, and mouse λ) [Toker, 1998]. PKCs are ubiquitously expressed and their expression is based on the differentiating or proliferating state of many cell and tissue types [Livneh and Fisherman, 1997; Cataldi et al., 2000]. In addition, their different response is based also on the type of the stimulus administered resulting in an apoptotic or survival signal [Murray and Fields, 1997; Frutos et al., 1999].

Moreover, the mechanism by which IR kills cells is a topic of great interest along with the role played by apoptosis in the response to this genotoxic agent, which is different depending on the cell type and the administered dose rate. In fact, following irradiation, haemopoietic cells undergo rapid apoptosis (within hours), while non-haemopoietic cells are characterized by a delayed apoptosis (within days). In the latter cell models, chromosome damage appears to trigger apoptosis, while in the former, tolerance for DNA damage appears to be reduced independently of cell cycle position and radiation dose administered [Olive and Durand, 1997; Aldridge and Radford, 1998]. Among the human haemopoietic cells sensitive to IR Jurkat can be included. These are lymphoblastic cells deriving from T leukemia extensively studied

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for their sensitivity to this physical agent. Their response to IR includes cell cycle arrest and DNA damage, which lead to the occurrence of apoptosis. While the morphological features of this phenomenon, which discloses a decrease in cell volume, cell shrinkage, blebbing of the cell membrane, nuclear condensation, and fragmentation and internucleosomal DNA cleavage are well-known [Falcieri et al., 1996; Miscia et al., 1997], the early intracellular signals which control the induction of such a phenomenon upon IR exposure need more investigation.

Thus, we have examined the specific role of several PKC isoforms (δ, ε, ζ) in the early induced cellular response and their subcellular distribution in Jurkat T cells exposed to IR. Attention has been focused on the connections between activation of several members of the protein kinase C family and the expression of cell death regulators (Bcl-2 family proteins Bad, Bax and Bcl-2) and cell death effector caspase-3 (CPP32), finally leading to the occurrence of the apoptotic state.

MATERIALS AND METHODS

Cell Culture and IR Exposure

Jurkat T leukemic cells, grown in suspension in RPMI 1640, supplemented with 10% FCS, glutamine, HEPES, penicillin/streptomycin in a controlled atmosphere up to $1{,}5 \times 10^6$/ml were irradiated at room temperature by a Mevatron 74 Siemens linear accelerator (photonic energy: 10 MV) administering 1.5 and 15 Gy (dose rate 3 Gy/min) according to literature [Cataldi et al., 2001]. Cells were then diluted at 3×10^5/ml and viability was followed by the Trypan blue dye exclusion test for 5 days. For fluorescence microscopy and Western blotting analyses, in vitro PKC δ activity, cells were recovered 1 h after radiation exposure. For TUNEL analysis, cells were recovered 6 h after radiation exposure.

TUNEL

Cytospins were fixed in paraformaldehyde (4% v/v in PBS pH 7.4) for 30 min at room temperature and permeabilized in 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 the in situ Cell Death Detection Kit (Boehringer Mannheim, Germany) as described by the manufacturer. Nuclei were counterstained with propidium iodide (PI), mounted in glycerol and analyzed under a fluorescence microscope (Leica Microscopy System, Heidelberg, Germany). The presence of DNA fragmentation was quantified by direct visual counting at light microscopy of fluorescent labeled nuclei on 300 cells.

Fluorescence Microscopy

Cytocentrifuged cells, were fixed in 3.7% paraformaldehyde for 10 min, permeabilized in 0.01% Triton X-100 for 3 min, and covered with 3% normal goat serum (NGS), 2% bovine serum albumin (BSA) in PBS to prevent aspecific binding. Immunolabeling was performed in the presence of 5 μg/ml anti-PKC δ polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted in PBS, 4 mg/ml NGS, and 4 mg/ml human immunoglobulins. Slides were washed and reacted with fluorescein FITC-conjugated anti-rabbit IgG antibody (Boheringer Mannheim,Germany) diluted 1:50 in PBS, 4 mg/ml NGS, and 4 mg/ml human IgG. After several washes in PBS, slides were mounted in glycerol containing 20 μg/ml PI to counterstain nuclei. Internal controls, performed omitting the primary antibody, did not disclose any FITC staining (not shown). The observation was carried out by using a Leica light microscopy equipped with a Coolsnap Videocamera for acquiring computerized images.

Immunoelectron Microscopy

Cells were fixed in a mixture of 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 60 min at 4°C. Samples were dehydrated in alcohol at progressively higher concentrations, embedded in hydrophilic Bioacryl resin, followed by UV polymerization.

Ultrathin sections were cut by using a Reichert ultramicrotome and mounted on 300 mesh nickel grids.

To block non-specific binding sites, grids were treated with a blocking buffer (PBS, 0.1% Tween 20, 0.1% BSA, 1% non-fat dried milk, 3% NGS) and incubated overnight in the presence of anti-PKC δ polyclonal antibody (1:10), followed by incubation in the presence of a secondary antibody conjugated with 15 nm colloidal gold particles (BioCell, Cardiff, UK).

Grids were then counterstained in uranyl acetate and lead citrate to preserve cell morphology and photographed by using a Zeiss electron microscope 109.

Cell Fractionation

Cells (15×10^6), resuspended in 10 mM Tris-HCl pH 7.4, 10 mM NaCl, 2 mM $MgCl_2$, 0.6% Triton X-100, 1.0 mM PMSF, 1 μg/ml leupeptin and aprotinin, 1.0 mM Na_3VO_4 were incubated at room temperature for 2 min, then cooled in ice for 5 min. After four passages through a 22-gauge needle, $MgCl_2$ concentration was adjusted to 5 mM. Nuclei were obtained by centrifuging the suspension at 600*g* for 5 min and cytoplasmic fractions consisted of the postnuclear supernatants.

Western Blotting Analysis

Whole cell, nuclear and cytoplasmic proteins (10 μg) were SDS–PAGE separated and transferred to nitrocellulose. After blocking in 5% non-fat milk, 10 mM Tris pH 7.5, 100 mM NaCl, 0.1% Tween-20, nitrocelluloses were probed with rabbit PKC δ, ε, ζ, Bax and Bad polyclonal or Bcl-2 or CPP-32 monoclonal antibodies (Santa Cruz Biotechnology) and developed with enzyme coniugated anti-rabbit or anti-mouse horseradish-peroxidase. Bands were detected by ECL detection system (Amersham Intl.,UK).

In Vitro PKC δ Activity Assay

Cytoplasmic and nuclear fractions (500 μg) were incubated with 2 μg of rabbit PKC δ polyclonal antibody and 20 μl of protein G-agarose. Pellets collected by centrifugation were washed in PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS. Immunoprecipitated PKC δ were incubated at 30°C for 10 min in 20 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 10 μM ATP, 10 μg of the appropriate synthetic peptide QKRPSQRSKYL, corresponding to sequence derived from myelin basic protein (MBP) (Santa Cruz lab., Santa Cruz, CA), 5 μCi [γ ^{32}P] ATP, in the presence of 40 μg/ml PS and 3.3 μM dioleylglycerol. Proteins, resuspended in 2× sample buffer, were separated by using 18% SDS–PAGE. Gels were then dried and exposed to X-ray films for 16 h at −80°C. Peptide spots were excised and radioactivity was measured in a liquid scintillation counter.

Image Processing and Analysis System

Quantitative analysis of gold granules was performed on immunoelectron micrography by using Leica Quantimet 500 plus software (Leica Cambridge Ltd, Cambridge, UK). Results are expressed as mean ± SD. Statistical analysis was performed by using the analysis of variance (ANOVA). Probability of null hypotesis of $< 0{,}1\%$ ($P < 0{,}05$) was considered statistically significant.

RESULTS

Jurkat cells, lymphoblastic line deriving from T human leukemia, were exposed to 1.5 Gy, suboptimal dose and 15 Gy, dose useful to induce an evident damage in a large number of cells [Di Pietro et al., 2001]. Cells were then assayed for viability by using the Trypan blue dye exclusion test. Cells were then reseeded and interestingly, only 2 h after IR exposure, 15 Gy samples disclosed a reduced viability, with respect to unirradiated and 1.5 Gy irradiated samples, suggesting the occurrence of a serious damage. Viability was followed until the 5th day (120 h). At this time, respectively a 90% reduction of trypan-blue excluding cell number in 15 Gy irradiated sample and a 10% reduction in 1.5 Gy, with respect to unirradiated, was observed. In parallel, a dramatic increase of dead cell number was observed in 15 Gy irradiated samples, with respect to unirradiated and to 1.5 Gy irradiated samples (Fig. 1). Moreover, it must be considered that 120 h after IR exposure the too high cell concentration influenced the viability of unirradiated and, may be, of 1.5 Gy samples, but this high cell concentration did not influence the little amount of 15 Gy irradiated cells.

In order to clarify which kind of damage the irradiated cells had undergone, we sought to perform TUNEL analysis. This method, which detects early breaks occurring even at single DNA strand level, showed changes as early as 6 h, mainly in 15 Gy irradiated samples. An

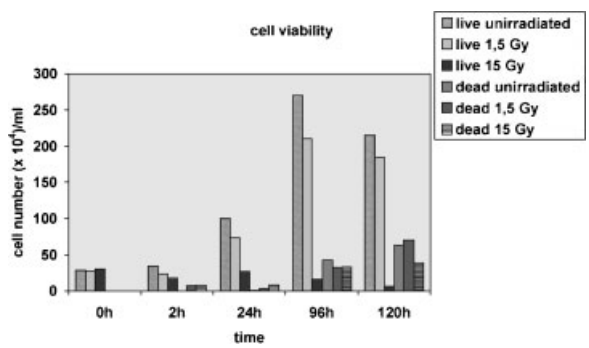

Fig. 1. Assay of viability of Jurkat T leukemic cells followed up to the 5th day after IR exposure. Results are the mean of three different experiments.

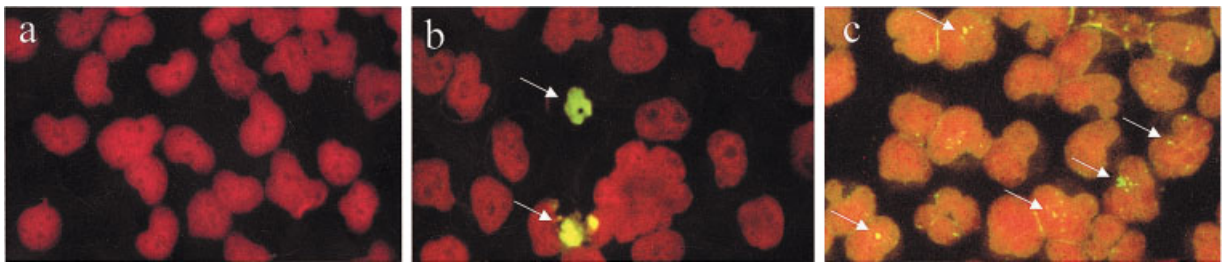

Fig. 2.

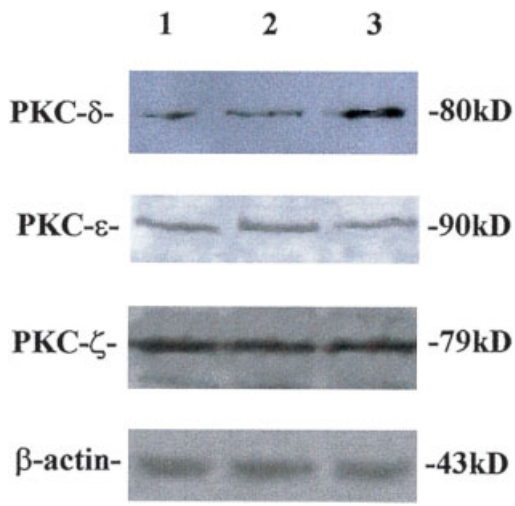

Fig. 3.

evident diffuse and spotlike fluorescence, which can account for an early apoptotic damage, characterized a great amount of positive cells (Fig. 2). Morphological changes, peculiar of this process, like membrane blebbing, cell shrinkage, nuclear condensation and fragmentation were evidenced, later, by electron microscopy (not shown). Since Jurkat T human leukemic cells undergo rapid apoptosis [Olive and Durand, 1997; Aldridge and Radford, 1998], we investigated the early signaling events evoked at plasma membrane, focusing on PKC isoforms δ, ε, ζ, whose involvement in this response has been previously evidenced in other experimental models [Kelly et al., 1998; Yuan et al., 1998; Nakajima and Yukawa, 1999].

Western blotting analysis performed on total cell lysates obtained immediately after IR

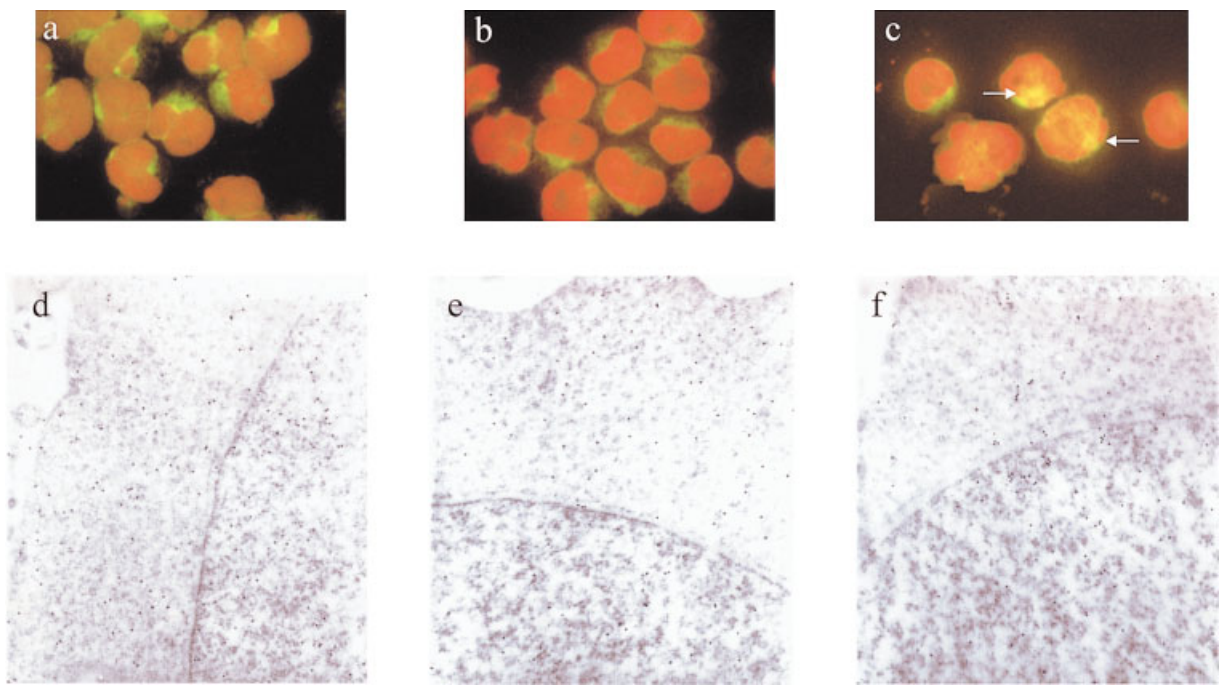

Fig. 4.

TABLE I. Analysis of the Number of PKC δ-Bound Gold Granules Per μm² in Jurkat T Leukemic Cells 1 h After IR Exposure

	Nuclei	Clytoplasms
Unirradiated	19.2 ± 2.19	18.5 ± 3.70
1.5 Gy	18.7 ± 2.25	17.5 ± 2.55
15 Gy	24 ± 1.65*	11.8 ± 3.73*

Data result from the mean ± SD of gold granules distribution analysis performed on 30 different fields at 20,000 × magnification.
15 Gy cytoplasms versus 15 Gy nuclei: $^*P < 0.05$.

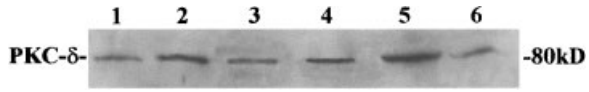

Fig. 5. Western blotting analysis of the subcellular localization of protein kinase C δ in Jurkat T leukemic cells fractions recovered 1 h after IR exposure. Cytosolic and nuclear proteins (10 μg) were run on a 8% polyacrylamide gel, transferred to nitrocellulose and probed with PKC δ specific antibody. **Lane 1**: unirradiated nuclear fraction; **lane 2**: unirradiated cytoplasmic fraction; **lane 3**: 1.5 Gy nuclear fraction; **lane 4**: 1.5 Gy cytoplasmic fraction; **lane 5**: 15 Gy nuclear fraction; **lane 6**: 15 Gy cytoplasmic fraction.

exposure, showed an increased expression of PKC δ in 15 Gy irradiated cells, while no modification was observed concerning the other two isoforms tested, ε and ζ (Fig. 3).

By fluorescence microscopy and immunoelectron microscopy analyses, we have then determined PKC δ distribution. At light microscopy level an increased amount of nuclear δ enzyme after 15 Gy IR exposure was disclosed (Fig. 4a,b,c), more clearly evidenced at ultrastructural level by immunogold technique (Fig. 4d,e,f). Table I shows the different amount of gold granules bound to PKC δ, in nuclear and cytoplasmic compartment, and it numerically documents PKC δ nuclear translocation.

Moreover, biochemical results concerning PKC δ localization evidenced a greater amount of this enzyme in the nuclear compartment with respect to cytoplasm in 15 Gy irradiated sample (Fig. 5e; Table II).

Since nuclear translocation of a protein is a reasonable evidence of activation [Kelly et al., 1998], we checked the "in vitro" activity of nuclear PKC δ, in order to better clarify if nuclear translocation of this enzyme was concomitant with an increased activity and to exclude possible modifications of nuclear membrane permeability. Nuclear immunoprecipitated PKC δ disclosed an increased activity upon 15 Gy IR exposure, with respect to unirradiated nuclear PKC δ immunoprecipitated activity (Table III).

Then we analyzed the expression of the molecular cell death regulators Bax, Bcl_2, and Bad and the molecular cell death effector caspase-3 precursor.

In concomitance to an increased expression of the cell death regulator Bax, no modification concerning the anti-apoptotic Bcl-2 and the proapoptotic Bad concentration was detected (Fig. 6), while Western blotting analysis of the expression of cell death effector caspase-3 precursor, detected by using CPP32 monoclonal antibody, revealed a 20 kD cleavage product corresponding to activated caspase-3 upon 15 Gy IR exposure (Fig. 7).

Taken together these results suggest a role for PKC δ in the early signaling events, switched on at plasma membrane upon IR exposure, transduced inside the cell and accounting in turn, for cleavage of cytoskeletal and nuclear proteins and finally induction of apoptosis.

DISCUSSION

Eukaryotic cells respond to IR with cell cycle arrest [Elledge, 1996], activation of DNA repair [Cataldi et al., 2001], associated DNA damages [Kanaar et al., 1998], which result in mutagenesis, transformation, and apoptosis. In this

Fig. 2. TUNEL detection of apoptotic cells in Jurkat T leukemic cells 6 h after IR exposure. Five slides were examined per sample. The presence of DNA fragmentation was quantified by direct visual counting at light microscopy of fluorescent labeled nuclei at 40× magnification (arrows). (**a**) unirradiated; (**b**) 1.5 Gy; (**c**) 15 Gy.

Fig. 3. Western blotting analysis of protein kinase C δ, ε, and ζ expression in total homogenates obtained from Jurkat T leukemic cells 1 h after 1.5 and 15 Gy IR exposure. Each electrophoretic lane was loaded with equal amount of protein as shown by goat β-actin polyclonal antibody incubation. **Lane 1**, unirradiated; **lane 2**, 1.5 Gy; **lane 3**, 15 Gy.

Fig. 4. (**a,b,c**) Fluorescence light microscopy analysis of protein kinase C δ expression in Jurkat T leukemic cells recovered 1 h after IR exposure. Protein kinase C δ is revealed by green fluorescence. PI counterstains nuclei (red fluorescence). Note the clustering of green fluorescence inside nuclei upon 15 Gy IR exposure (arrows). (**a**) unirradiated; (**b**) 1.5 Gy; (**c**) 15 Gy. (**d,e,f**) Immunoelectron microscopy analysis of protein kinase C δ distribution in Jurkat T leukemic cells recovered 1 h after IR exposure. Gold granules number per square micrometer were counted in 30 different fields at 20000× magnification.

TABLE II. Densitometric Analysis of Protein Kinase C δ Expression in Nuclear and Cytoplasmic Fractions Deriving From Jurkat T Leukemic Cells Recovered 1 h After IR Exposure

	Cytoplasmic fraction	Nuclear fraction
Unirradiated	0.50 ± 0.05	0.25 ± 0.15
1.5 Gy	0.52 ± 0.04	0.30 ± 0.04
15 Gy	0.35 ± 0.03*	0.66 ± 0.05*

Data are the mean of five separate experiments ± SD. $P < 0.005$. 15 Gy cytoplasmic PKC δ versus 15 Gy nuclear PKC δ: *$P = 0.00$.

study, we report that in Jurkat T human leukemic cells high dose IR induces an early lowering of viability concomitant with an increase of the early apoptotic cell damage in a great amount of cells, as determined by the TUNEL method. Several lines of evidence indicate that PKCs are involved in radiation-induced signal transduction pathways in other experimental models [Kelly et al., 1998; Yuan et al., 1998; Nakajima and Yukawa, 1999]. As already underlined, PKCs can mediate either the rescue from IR induced apoptosis [Frutos et al., 1999] or the establishment of this process [Haimovitz-Friedman et al., 1994; Palayoor et al., 1995; Lee et al., 1999]. In Jurkat cells, the evidence that high dose IR, inducing increased expression and activation of PKC δ mediate the occurrence of apoptosis, is in accordance with literature which suggests a role for PKC δ in delivering an apoptotic signal [Ghayur et al., 1996; Mizuno et al., 1997]. In addition, being often the translocation of a protein from the cytoplasm to the nucleus, a reasonable indicator of activation [Kelly et al., 1998], we tested nuclear PKC δ and found that both its expression and activity were enhanced

TABLE III. "In Vitro" Specific Nuclear and Cytoplasmic Immunoprecipitated Protein Kinase C δ Activity Deriving From Jurkat T Leukemic Cells Recovered 1 h After IR Exposure

	Nuclear activity	Cytoplasmic activity
Control	5089 ± 52*	3526 ± 27
1.5 Gy	5125 ± 56	3344 ± 22
15 Gy	7607 ± 61*	3366 ± 24

Values indicate dpm/mg protein. Results are the mean of five separate experiments ± SD. $P < 0.05$.
Unirradiated nuclear PKC δ activity versus 15 Gy nuclear PKC δ activity: *$P = 0.00$.

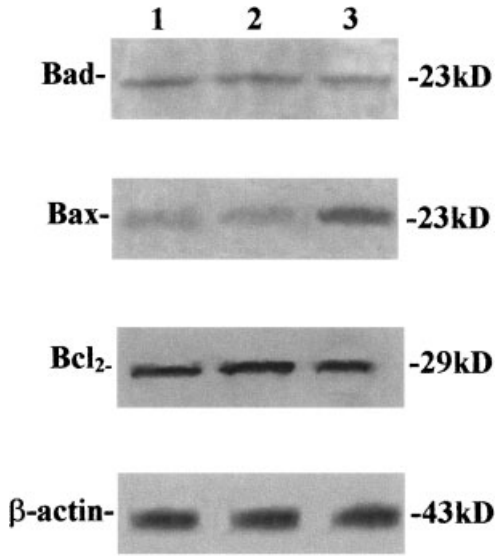

Fig. 6. Western blotting analysis of Bad, Bax and Bcl-2 expression in Jurkat T leukemic cells recovered 1 h after IR exposure. Each electrophoretic lane was loaded with equal amount of protein as shown by goat β-actin polyclonal antibody incubation. **Lane 1**: unirradiated; **lane 2**: 1.5 Gy; **lane 3**: 15 Gy.

in nuclei deriving from 15 Gy IR cells. Moreover, since in the response to IR, like to other apoptotic stimuli, the activation of molecular events inducing such a process is regulated by an upstream step, which determines whether cell survives or dies [Henkart, 1996], we have hypothesized that this decision may be addressed by Bcl-2 gene [Chinnaiyan et al., 1996]. In fact the anti-apoptotic Bcl-2 protein expression did not modify along with proapoptotic Bad, while a consistent increase was

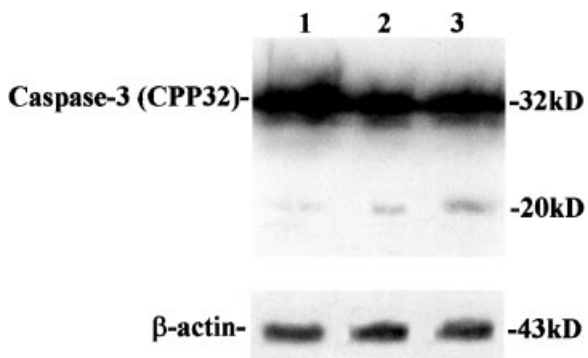

Fig. 7. Western blotting analysis of caspase-3 (CPP32) expression in Jurkat T leukemic cells recovered 1 h after IR exposure. Note full length expression of 32 kD caspase-3 and of the cleaved 20 kD subunit produced after IR exposure. Each electrophoretic lane was loaded with equal amount of protein as shown by goat β-actin polyclonal antibody incubation. **Lane 1**: unirradiated; **lane 2**: 1.5 Gy; **lane 3**: 15 Gy.

evidenced in Bax concentration upon 15 Gy IR. Thus, being Bcl-2 and Bad expression not modified and Bad displacing Bax from heterodimerization with Bcl2, Bax homodimerization should increase. The increased level and homodimerization of Bax protein would induce the activation of the cell death effector caspase-3 finally leading to apoptosis [Yang et al., 1995; Yang and Korsmeyer, 1996; Yu and Little, 1998]. Moreover this evidence is in accordance with other results suggesting that the effect of PKC on Bcl-2 levels determines the entry into pathway for apoptosis [Chen and Faller, 1995; Meinhardt et al., 2000a,b; Reyland et al., 2000; Takahashi and Shibuya, 2001].

Thus, we can conclude that being PKC δ a molecular target for IR which modulates, in turn, cell death regulators, Bcl-2, Bad and Bax, and cell death effector caspase-3, its early activation can justify the dramatic increase of the apoptotic cell number and, in turn, the sensitivity displayed by Jurkat cells to IR. Moreover, further experiments will be performed on PKC δ Jurkat deficient lines addressed to demonstrate the possible clinical significance of this molecule as target for pharmacological strategy directed to amplify the signal evoked by IR in order to improve the remission of leukemia disease.

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