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NF-κB Activation Plays an Antiapoptotic Role in Human Leukemic K562 Cells Exposed to Ionizing Radiation

Amelia Cataldi,[1]* Monica Rapino,[2] Lucia Centurione,[1] Nadia Sabatini,[1] Giovanna Grifone,[2] Francesco Garaci,[3] and Rosalba Rana[1]

[1]Dipartimento di Biomorfologia, Università G.D'Annunzio, Chieti, Italy
[2]Istituto di Citomorfologia Normale e Patologica del CNR, Chieti, Italy
[3]Dipartimento di Diagnostica per Immagini e Radiologia Interventistica, Policlinico Tor Vergata, Università degli Studi di Roma,Tor Vergata, Italy

Abstract Exposure of cells to ionizing radiation (IR) determines cellular lesions, such as DNA and membrane damage, which involve a coordinate network of signal transduction pathways responsible for resistance to or delay of apoptosis, depending on cell type and administered dose. Since, after IR exposure, the apoptotic profile appeared different in the two chosen cell lines K562 and Jurkat along with caspase-3 activation, we paid attention to the influence exerted by Protein kinase C δ on transcription factor NF-κB activation. Interestingly, K562 resist to IR carrying out a survival strategy which includes PKC δ/NF-κB pathway activation, probably mediated by novel IKKs and a role for PI-3-kinase in activating PKC δ at Thr 505 by PDK-1 phosphorylation is suggested. In addition, since caspase-3 is not activated in these cells upon ionizing radiation exposure, it could be supposed that NF-κB antagonizes apoptosis induction interfering with pathways which lead to caspase activation, may be by inducing expression of IAP, caspases 3, 7, 9, inhibitor. Thus NF-κB activation explains the resistance displayed by K562 to IR and drug potential interference directed to this protein could overcome apoptosis resistance in clinical settings. J. Cell. Biochem. 89: 956–963, 2003.

Key words: PKC δ; NF-κB; survival; K562; ionizing radiation

Exposure of cells to ionizing radiation (IR) gives rise to a variety of cellular lesions including both DNA and membrane damage. DNA damage leads to a coordinate network of signal transduction pathways involved in cell-cycle arrest, apoptosis, stress response, and activation of DNA repair processes [Cataldi et al., 2001]. DNA damage monitoring and signaling systems are responsible for both resistance to or delay of apoptosis depending on the cell type and on the dose administered [Watters, 1999].

Among the molecules contributing to the respective signaling cascades in response to IR, the PKC family of serine/threonine kinases has been extensively studied [Meinhardt et al., 2000]. This family of proteins include at least 12 members and several of them, namely PKC α, δ, ϵ, ζ, have been recognized to be involved in the response to IR [Balaban et al., 1996; Aldridge and Radford, 1998; Billis, 1998; Haimovitz-Friedman, 1998; Kelly et al., 1998; Yuan et al., 1998; Lee, 1999; Cataldi et al., 2003]. Their response can mediate both the occurrence of or the rescue from apoptosis, activating different regulators inside the cell [Berra, 1997]. One of the potential regulators of these responses is the transcription factor NF-κB, which participates in the control of cell proliferation and survival, as well as in inflammatory response and viral gene expression [Bauerle and Baltimore, 1996]. Under basal conditions, it usually exists in the cytoplasm as a heterodimer of 50 and 65 kDa subunits bound to an inhibitor 41 kDa protein IκB-α. Upon cell stimulation by agents of different nature, IκB-α becomes phosphorylated on serine 32 or 36 and dissociates from NF-κB, allowing free NF-κB to translocate to the nucleus, to bind to DNA sequences and to activate gene expression. The rapid phosphorylation of

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*Correspondence to: Amelia Cataldi, Dipartimento di Biomorfologia, Università G.D'Annunzio, 66100 Chieti, Italy. E-mail: cataldi@unich.it

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IκB-α represents a possible signal for its proteolysis [Brown et al., 1995; Ceren et al., 1996].

Since several PKCs are involved in NF-κB activation in response to a variety of agents [Folgueira et al., 1996; Das and White, 1997; Santag et al., 1997; MacDonald et al., 1999], we investigated the role played by δ, ϵ, and ζ isoforms of this enzyme in influencing this signal transduction pathway in response to IR in K562 compared to Jurkat cells, to shed more light on the mechanisms underlying the occurrence of radioresistance during therapeutical settings and to consider the pathway PKC δ/NF-κB possible target of combined pharmacological therapy. K562 erythroleukemic cells have been chosen for their resistance to IR-induced apoptosis due to the chimeric $p110^{bcr/abl}$ oncoprotein, deregulated protein tyrosine kinase activity (PTK) which is thought to counteract apoptosis induction and cell death, promoting survival of these cells [Papazisis et al., 2000]. Otherwise Jurkat are acute lymphoblastic cells deriving from T leukemia extensively studied for their sensitivity to IR [Syljuasen and McBride, 1999]. A 1.5 Gy has been chosen as suboptimal dose and 15 Gy as the useful dose to induce an evident damage in a large number of cells [Cataldi et al., 2002].

MATERIALS AND METHODS

Cell Culture and IR Exposure

K562 and Jurkat T leukemic cells, grown in suspension in RPMI 1640, supplemented with 10% FCS, glutamine, HEPES, penicillin/streptomycin in a controlled atmosphere, 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). Irradiated and unirradiated cells were reseeded in fresh RPMI and viability was assessed by the Trypan blue dye exclusion test up to the 5th day. When required, wortmannin, well-known PI-3-kinase inhibitor, was added in culture 1 h prior to irradiation at 100 nm concentration. This concentration has been chosen as the optimal to inhibit PI-3-kinase activity, but not expression, without interfering with other ATP requiring enzymes, such as PKCs, Camp dependent protein kinase and Ca^{++} calmodulin dependent PKII [Cantrell, 2001]. For fluorescent microscopy and Western blotting analyses, in vitro PKC δ activity assay, cells were recovered 1 h after IR exposure. For TUNEL analysis, cells were recovered 4 h after IR exposure.

TUNEL Analysis

Cytospinned cells 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 cytrate 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, mounted in glycerol, and analyzed under a fluorescent microscope (Leica Microscopy System, Heidelberg, Germany). Negative controls were performed according to the manufacturer's protocol. The extent of DNA fragmentation was quantified by direct visual counting at light microscopy of fluorescent-labeled nuclei on 300 cells.

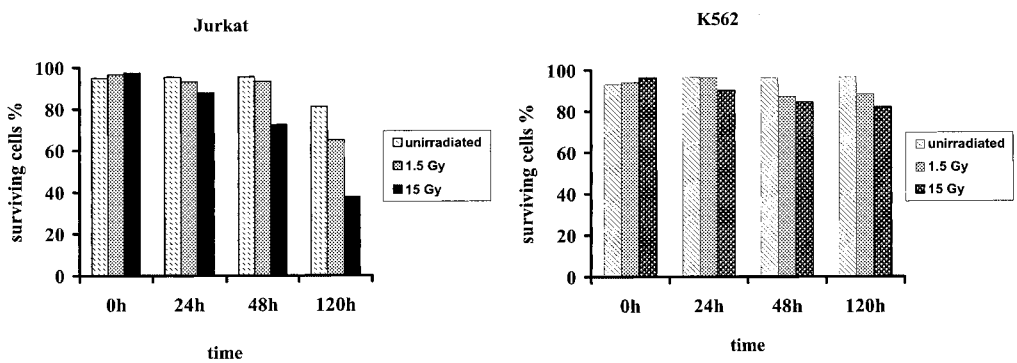

Fig. 1. Percentage of Jurkat and K562 surviving cells after ionizing radiation (IR) exposure. Irradiated cells were reseeded and viability, assessed by Trypan blue dye exclusion test, was followed up to the 5th day. Data are the mean of three separate experiments ± SD.

Fluorescence Microscopy

Cytocentrifuged cells were fixed in 4% paraformaldehyde for 10 min, washed in PBS, and saturated in NET GEL for 30 min. Immunolabeling was performed in the presence of 5 μg/ml anti-phospho-IκB-α polyclonal antibody (Cellular Signaling, New England BioLabs, Hitchin, UK) diluted in NET GEL for 1 h. Slides were washed in NET GEL and reacted with fluorescein FITC-conjugated anti-rabbit IgG antibody (Boheringer Mannheim, Germany) diluted 1:50 in NET GEL for 45 min. After several washes in NET GEL and PBS, slides were mounted in glycerol-DABCO containing 5 μg/ml DAPI (4-6,diamidino-2-phenylindol) to counterstain nuclei. Internal controls, performed by omitting the primary antibody, did not disclose any FITC staining (data not shown). The observation was carried out by using a Leica Light Microscope equipped with a Coolsnap Videocamera for acquiring computerized images.

Cell Fractionation

Cells, 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, and 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 post-nuclear supernatants. Nuclear purity was assessed by detection of β-actin, and only those nuclei showing a complete absence of β-actin in Western blots were used in the reported experiments.

Western Blotting Analysis

Whole cell, nuclear and cytoplasmic protein amounts (10 μg) were normalized by Bio-Rad Dc Protein Assay (Bio-Rad Laboratories, Hercules, CA). After SDS–PAGE proteins were transferred to nitrocellulose. Blots, blocked in 5% non-fat milk, 10 mM Tris, pH 7.5, 100 mM NaCl, 0.1% Tween-20, were probed with rabbit PKC δ, ϵ, ζ, NF-κB, p85 α subunit of PI-3-kinase polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit phospho-IκB-α polyclonal antibody (Cellular Signaling, New England Biolabs, Hitchin, UK) and developed with specific enzyme conjugated horseradish-peroxidase. Bands were detected by ECL detection system (Amersham Intl., UK). When required, blots were stripped of bound antibodies by incubating membranes in wash buffer containing 2% SDS at 50°C for 30 min, blocked and reprobed with anti-phosphotyrosine mono-

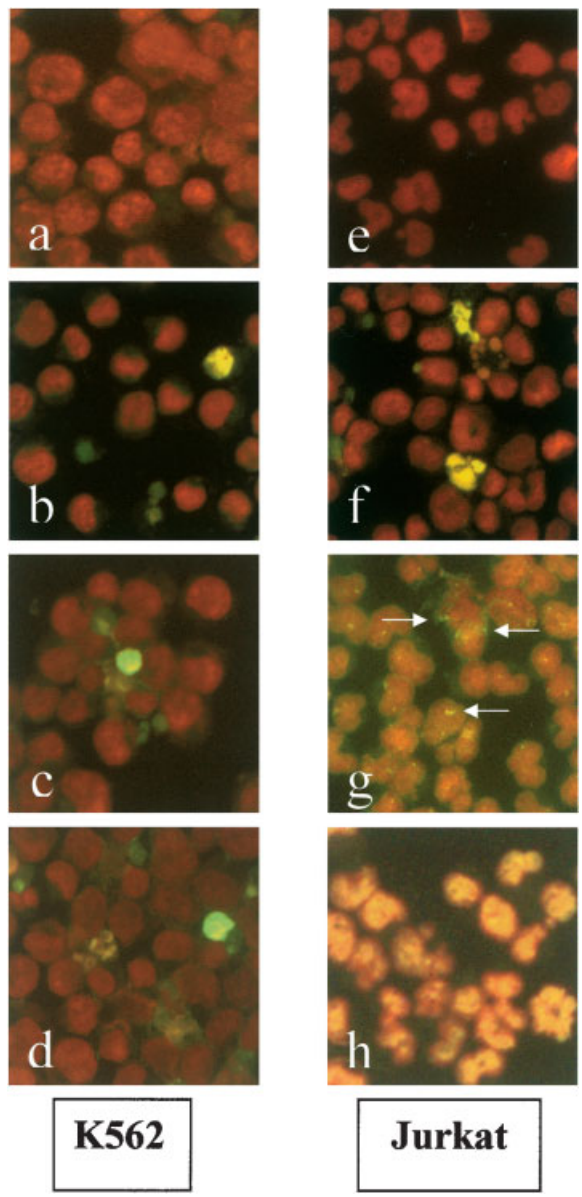

Fig. 2. TUNEL detection of apoptosis in K562 and Jurkat leukemic cells exposed to IR. Five slides were examined for sample. The extent of DNA fragmentation was quantified by direct visual counting of fluorescent labeled nuclei at light microscopy. In g arrows indicate early spot-like DNA strand breaks, while in h a more evident green fluorescence accounts for a wider damage at DNA level. Magnification: 40×; **a–e**: Unirradiated; **b–f**: 1. 5 Gy; **c–g**: 15 Gy; **d–h**: 15 Gy + WM.

clonal antibody (PY-99) (1:2000) (Santa Cruz Biotechnology).

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 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.

Statistical Analysis

Statistical analysis was performed using the analysis of variance (ANOVA). Results were expressed as mean ± SD. Probability of null hypothesis of <0.1% ($P < 0.05$) was considered statistically significant.

RESULTS

Since the effect of IR on the cells determines the induction of the apoptotic response, rate of which is different depending on the cell type and the dose administered, we firstly evaluated cell viability and apoptotic fraction. Cell viability, followed until the 5th day after IR exposure, disclosed no modification concerning K562 cells, while in Jurkat the percentage of viable cells dramatically declined to 22% in concomitance to a 40% of dead cells (data not shown), with respect to unirradiated sample (Fig. 1). As far as TUNEL analysis, method able to recognize early stages of apoptosis and even single DNA strand breaks, in Jurkat T sample detected in almost the total number of cells early damage at nuclear level with respect to K562, at the higher

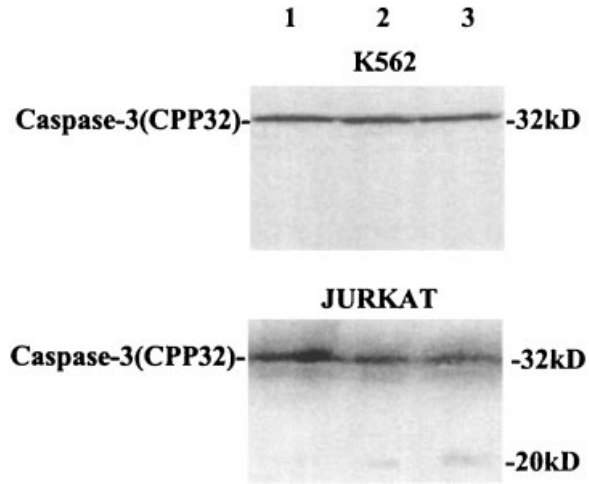

Fig. 3. Western blotting analysis of caspase 3 (CPP32) expression in K562 and Jurkat cells exposed to IR. Note in K562 samples the absence of 20 kDa subunits, accounting for caspase-3 activation, which are represented in irradiated Jurkat cells. **Lane 1**: unirradiated; **lane 2**: 1.5 Gy; **lane 3**: 15 Gy.

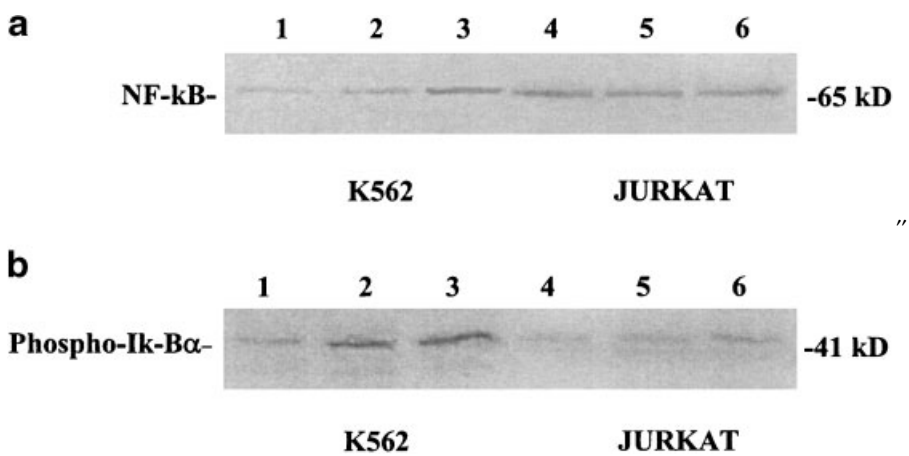

Fig. 4. Western blotting analysis of NF-κB nuclear expression (**a**) and Phospho-IκB-α cytoplasmic expression (**b**) in K 562 and Jurkat leukemic cells exposed to IR. **Lane 1**,**4**: unirradiated; **lane 2**,**5**: 1.5 Gy; **lane 3**,**6**: 15 Gy.

dose of IR (15 Gy), while no significant response appeared upon 1.5 Gy in both cell lines (Fig. 2). In parallel, the cell death effector caspase-3 disclosed a different pattern in the two reported cell lines. In particular, after IR exposure in K562 no activation was detected, with respect to irradiated Jurkat cells, which disclosed 20 kDa fragments production, accounting for caspase-3 activation (Fig. 3) [Prasad et al., 1994; Cataldi et al., 2003]. These evidences induced us to check the possible signaling network leading to the occurrence of these different responses, paying attention to some of the molecular mechanisms underlying cell survival. Among these, NF-κB increased nuclear expression was revealed in 15 Gy irradiated K562, with respect to control, while no modification was observed in corresponding Jurkat T nuclear fractions at Western blotting level (Fig. 4). In addition, an enhanced IκB-α phosphorylation, detected by using a phospho-IκB-α antibody, which recognizes endogenous levels of Ser 32 or Ser 36 phosphorylated inhibitory IκB-α protein, was revealed only in irradiated K562 cytoplams (Fig. 4), confirmed by fluorescence microscopy (Fig. 5b,d), while in the presence of the well-known PI-3-kinase inhibitor wortmannin, IκB-α was not activated (Fig. 5c,e). Altogether, these evidences support the hypothesis of NF-κB transduction to the nucleus upon IR exposure. This kind of response was concomitant to PI-3-kinase activation only in nuclei deriving from K562 cells, further suggesting an attempt of survival carried out by these cells (Fig. 6). Being NF-κB a survival transcription factor, more elucidation needed the molecular signaling events occurring at plasma membrane which determined the nuclear response to IR. Since several isoforms of PKC have been recognized to be involved in NF-κB activation, we checked the expression of δ, ϵ, ζ isoforms, among which only PKC δ showed modifications in terms of expression in both cell lines (Fig. 7). In addition, Jurkat and K562 cells disclosed PKC δ translocation to the nucleus (Fig. 8) paralleled by an increased "in vitro" nuclear specific activity (Table I), according to literature reporting the effect of IR on the activation of this enzyme in other experimental models [Yuan et al., 1998].

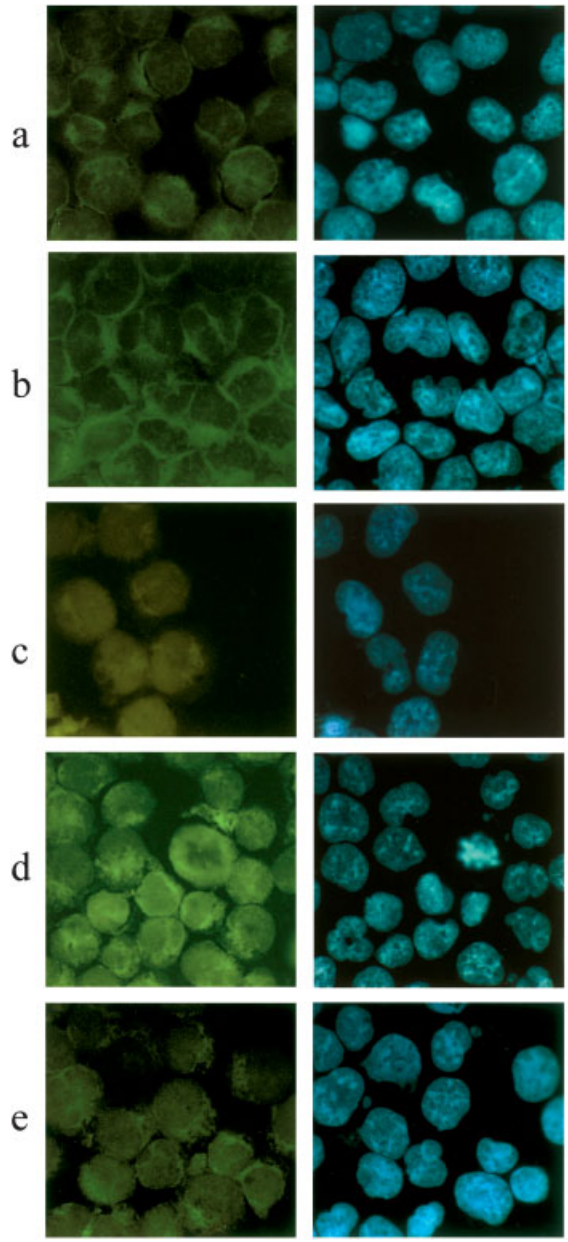

Fig. 5. Fluorescent light microscopy analysis of phospho-IκB-α in K562 leukemic cells exposed to IR. Note that phospho-IκB-α antibody (green fluorescence) detects phosphorylation of cytoplasmic IκB-α at Ser 32 and Ser 36 level resulting in the release and nuclear translocation of NF-κB. In c and in e wortmannin reduces IκB-α phosphorylation. Blue fluorescence of DAPI (4-6,diamino-2-phenyl-indol) counterstains nuclei. Magnification: 100×; **a**: unirradiated; **b**: 1.5 Gy; **c**: 1.5 Gy + WM; **d**: 15 Gy; **e**: 15 Gy + WM.

DISCUSSION

In this report, we tried to elucidate the molecular mechanisms which underlie the different responses shown by two human erythroleukemic (K562) and Acute Lymphoblasitc Leukemic (Jurkat) cell lines to IR exposure. In fact, while Jurkat cells disclosed an high radiosensitivity, demonstrated by the increased number of

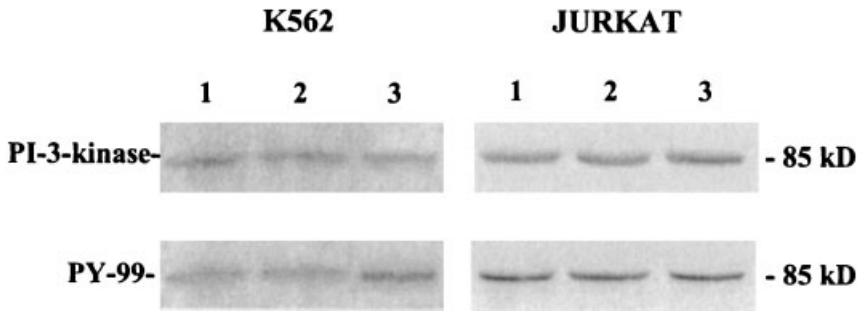

Fig. 6. Western blotting analysis of PI-3-kinase activation in nuclei deriving from K562 and Jurkat leukemic cells exposed to IR. PI-3-kinase activation was determined by stripping the nitrocellulose of bound PI-3-kinase polyclonal antibody and incubating in the presence of PY-99 polyclonal antibody, which detects tyrosine phosphorylated proteins. **Lane 1**: unirradiated; **lane 2**: 1.5 Gy; **lane 3**: 15 Gy.

apoptotic cells, K562 resist to this genotoxic agent carrying out a survival strategy which included activation of the pathway PKC δ/NF-κB. In particular, K562 showed NF-κB activation, which evidenced the detachment and the phosphorylation of IκB-α at cytoplasmic level, as disclosed by fluorescence microscopy analysis, resulting in NF-κB active form nuclear translocation and, presumably, activation of target genes upon 15 Gy. Since the activation and the removal of IκB-α from the NF-κB complex might be due to phosphorylation of IκB-α, we suggest a role for PKC δ in the activation of NF-κB upon IR exposure. PKCs, in fact, have been shown to be involved in the activation of NF-κB upon exposure to a variety of agents [Prasad et al., 1994; Traenckner et al., 1995; Wang et al., 1999]. In addition, as elsewhere reported [Lu et al., 2001], the atypical protein kinase C iota mediates the resistance displayed by the same cell line to antineoplastic drug (taxol)-induced apoptosis and requires the transcription factor NF-κB to confer cell survival. However, the mechanisms by which, in this experimental model, PKC δ activates NF-κB remains to be elucidated. Because PKCs are unable to directly phosphorylate IκB-α, it is possible that the signals generated by PKC δ could be mediated by the novel IKKs, as reported in other experimental models [Diaz Meco et al., 1994; Lallena et al., 1999; Moscat et al., 2001]. Moreover, these results are in accordance with

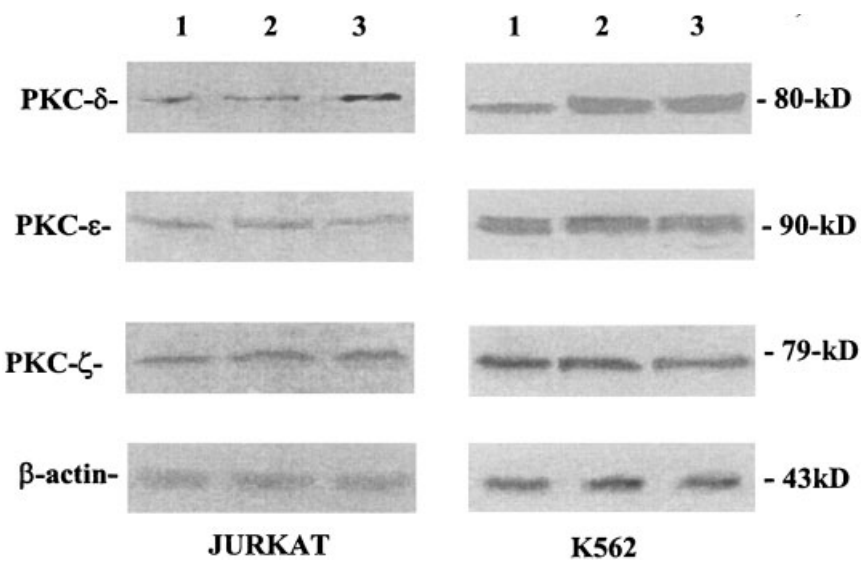

Fig. 7. Western blotting analysis of PKC δ, ϵ, and ζ expression in total homogenates deriving from Jurkat and K562 leukemic cells exposed to IR. Samples were normalized as shown by goat β actin polyclonal antibody incubation. **Lane 1**: unirradiated; **lane 2**: 1.5 Gy; **lane 3**: 15 Gy.

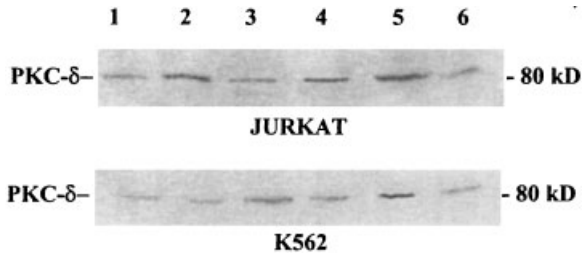

Fig. 8. Western blotting analysis of subcellular localization of PKC δ in Jurkat and K562 exposed to IR. Cytoplasmic and nuclear proteins (10 μg) were run on an 8% polyacrylamide gel, transferred to nitrocellulose and probed with PKC δ specific antibody. **Lane 1**: unirradiated nuclear fraction; **lane 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.

data concerning PI-3-kinase activation, detected in an analogous experimental model [Cataldi et al., 2001] and with the evidence that the addition in cell culture of wortmannin, well known PI-3-kinase inhibitor, prior to irradiation, should selectively interfere with NF-κB activation, by increasing the number of apoptotic cells and decreasing IκB-α phosphorylation. In this case, it could be suggested a role for PI-3-kinase in activating PKC δ, as elsewhere reported also for PKC ζ [Nakanishi et al., 1993; Toker et al., 1994; Cataldi et al., 2000]. In particular, PKC δ could be activated by a PDK-1 phosphorylation at Thr505 in a PI-3-kinase dependent fashion [Le Good et al., 1998]. Moreover, the mechanisms by which activation of NF-κB antagonizes induction of apoptosis upon IR exposure remain to be elucidated. In any case, since caspase 3 is not activated in K562 cells, it could be suggested that NF-κB-dependent proteins might interfere with pathways leading to the activation of this protein, may be by inducing the expression of IAP, an inhibitor of caspases 3, 7, 9 [Bonni et al., 1999] and might generate cross-resistances between different apoptosis inducing agents. Thus NF-κB activation could explain the resistance displayed by K562 cells to IR and drug potential interference directed to this protein could overcome apoptosis resistance in clinical settings.

TABLE I. "In Vitro" Specific Nuclear Immunoprecipitated PKC δ Activity Deriving From K562 and Jurkat Leukemic Cells Exposed to Ionizing Radiation

	K562	Jurkat
Unirradiated	4075 ± 43*	3575 ± 34*
1.5 Gy	4224 ± 37	4045 ± 39
15 Gy	6345 ± 41*	5350 ± 47*

Values indicate dpm/mg protein. Results are the mean of three separate experiments ±SD. PKC δ imorradoated vs. PKC δ 15 GY.
*$P < 0.05$.

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