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CASPASE-3 IS DUALLY REGULATED BY APOPTOGENIC FACTORS MITOCHONDRIAL RELEASE AND BY SAPK/JNK METABOLIC PATHWAY IN LEUKEMIC CELLS EXPOSED TO ETOPOSIDE-IONIZING RADIATION COMBINED TREATMENT

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Ionizing radiation induces a series of multiple intracellular events which can lead to activation of caspases, cytoplasmic proteases involved in the occurrence of apoptosis. The response of leukemic cells to ionizing radiation is amplified when they have been pre-treated with the anticancer drug etoposide, therefore the aim of this work has been to establish the lowest etoposide concentration combined with the lowest ionizing radiation dose to obtain the best antineoplastic response. Two leukemic cell lines, HL-60 and Jurkat, employed in this study, demonstrated different sensitivities to ionizing radiation and to etoposide treatment, with Jurkat T cells requiring a higher dose (1 μM) to display cell cycle perturbation and apoptotic DNA damage similar to those seen in HL-60. We hypothesize that this kind of response could be mediated by mitochondrial release of apoptogenic factors and by SAPK/JNK metabolic pathway activation, both leading to caspase-3 cleavage. All in all these results provide insight into the sensitivity or resistance of leukemic cells to antineoplastic agents and identify molecular targets for rational therapeutic intervention strategies.

Apoptosis is a cell suicide program essential for development and for maintaining adult tissue homeostasis in all metazoan animals. It eliminates redundant cells during development and exhausted cells in aging, while in pathological conditions it promotes the formation of damaged or mutated cells. Moreover many pathologies imply a dysregulation of apoptosis (1-4). Since this process provokes morphological alterations affecting the nucleus, it has long been assumed that it was controlled at nuclear level (5-7). Later on, studies performed on the nematode Caenorhabditis elegans have pointed out that a specific class of cytoplasmic proteases called "caspases" (cysteine aspartate specific proteases) are involved in the occurrence of apoptosis (8). This family of proteases cleaves polypeptides on the carboxyl site of aspartate residues such as PARP (poly-ADP-ribose polymerase) and when DNA damage is too great for survival, apoptosis is initiated (9-10). Caspases can be activated by multiple signalling pathways depending on the cell type and on the stimulus administered (11-16). Among the signalling pathways activating caspase-3 is the mitochondrial apoptogenic factor cytochrome c, a well known component of the mitochondrial respiratory chain (17). It is bound to the outer surface of the inner mitochondrial membrane where it functions as an electron shuttle between complex III and IV of the mitochondrial respiratory chain. It is released in

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the cytosol at the early stages of apoptosis and, combining with some cytosolic proteins, like Apaf 1 (apoptosis activating factor 1) forms a high molecular weight complex with caspase-9, which leads to the formation of "apoptosome" and activates caspase-3 (18). In addition, much evidence suggests that Stress Activated Protein Kinases (SAPK) are activated by many stimuli (19-21) and that this activation leads to the occurrence of apoptosis. In particular, SAPK/JNK pathway activation is mediated by apoptosis signal regulating kinase (ASK-1) which, in response to various stresses, translocates from the cytoplasm to the nucleus (22). Upon ASK-1 translocation, activated SAPK/JNK can interact with mitochondrial antiapoptotic Bcl2, determining release of cytochrome c, caspase-3 activation and induction of apoptosis. Since the occurrence of apoptosis plays a key role in the treatment of neoplastic diseases such as leukemia, and since this response is cell type dependent (23-24), we have tested some of the molecular mechanisms carried out by Jurkat and HL-60 cells against ionizing radiation. Concerning ionizing radiation doses administered to the cells, 1.5 Gy has been chosen as suboptimal dose, since it is the daily fraction delivered in human tumour radiotherapeutic protocols, while 15 Gy has been chosen as a dose useful to induce evident damage in a large number of cells and normally reached at the end of the treatment (25, 26).

The second purpose of this study was to establish the lowest etoposide concentration combined with the lowest ionizing radiation dose to obtain the best antineoplastic response. To this aim, a human promyeloid leukemia cell line, HL-60, and a T leukemic cell line, Jurkat, both exquisitely sensitive to ionizing radiation, were irradiated after pretreatment with etoposide in the range 0.1-5 μM. Etoposide, a common antineoplastic agent useful for a wide range of cancers, is an inhibitor of Topoisomerase II, which makes double-stranded cuts in DNA. Etoposide does not kill cells by blocking Topoisomerase catalytic function, but poisons this enzyme by increasing the steady state concentration of covalent DNA cleavage complexes. For this reason Topoisomerase II becomes a physiological toxin that introduces high levels of transient protein-associated breaks in the genome of treated cells. When these breaks become permanent and are present at sufficient concentration they trigger a series of events that ultimately culminate in cell death by apoptosis (27).

MATERIALS AND METHODS

Cell culture and ionizing radiation exposure

HL60 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). Cells were then reseeded in fresh RPMI and viability was assessed by Trypan blue dye exclusion test. When required, etoposide was added in culture 1 hr prior to irradiation, at concentrations ranging between 0.1 μM and 5 μM. For fluorescent microscopy and western blotting analyses cells were recovered 1h after ionizing radiation exposure, while for cell cycle analysis and apoptosis cells were recovered 24 hr later.

Evaluation of cell cycle and apoptosis

Samples containing 2-5x10^5 cells were harvested by centrifugation at 200g for 10 min at 4°C, fixed with 70% cold ethanol for at least 1hr at 4°C, and treated as previously detailed (28). Analysis of PI fluorescence was performed with an EPICS Coulter flow cytometer with FL2 detector in a linear mode using the Expo 32 analysis software. For each sample, 10000-20000 events were collected. Multicycle sotfware was used for cell cycle phases analysis. For quantitative evaluation of apoptosis, subdiploid (less than 2n) DNA content was calculated as described (29) and expressed as percentage of apoptotic versus non apoptotic cells, regardless of the specific cell-cycle phase.

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 5 min, then cooled on ice for 5 min. After 5 passages through a 22-gauge needle, $MgCl_2$ concentration was adjusted to 5 μM. Nuclei were obtained by centrifuging the suspension at 1200 g for 15 min and cytoplasmic fractions consisted of the postnuclear supernatants. Nuclear purity was assessed by detection of beta-actin and only nuclei showing a complete absence of beta-actin in western blots were used in the reported experiments (26).

Immunoprecipitation

Total cellular lysates (500 μg as protein) were supplemented with 2 μg of rat Apaf-1 monoclonal antibody (Apotech, San Diego, CA) for 1 hr at 4°C and incubated with 20 μl of Protein G-Agarose at 4°C on a rocker platform for 1 hr. Immunoprecipitated proteins were collected by centrifugation at 2500 rpm, washed 4 times in RIPA buffer. After the final wash, cell pellets were resuspended in 2x sample buffer.

Western blotting analysis

Total cell lysates, nuclear or immunoprecipitated proteins were electrophoresed and transferred to nitrocellulose. Blots were blocked in 5% non-fat milk, 10 mM Tris pH 7.5, 100 mM NaCl, 0.1% Tween-20, and probed with rabbit PKCδ, ASK-1, rat Apaf-1 polyclonal or mouse Bax, Bcl-2, and cytochrome c monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), mouse Caspase-3 and mouse p-SAPK/JNK monoclonal antibodies (Cellular Signalling, New England Biolabs, Hitchin, U.K.), mouse PARP monoclonal antibody(Oncogene Research Products, La Jolla, CA) and developed with specific enzyme coniugated horseradish-peroxidase. Bands were detected by ECL detection system (Amersham Intl.,U.K.). 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 other primary and secondary antibodies.

Fluorescence microscopy

Cytocentrifuged cells were fixed in 4% paraformaldehyde for 10 min, washed in PBS and saturated in NET GEL for 30 min.Immunolabelling was performed in the presence of 5 μg/ml rabbit ASK-1 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted in NET GEL for 1 hr. Slides were washed in NET GEL and reacted for 45 min with fluorescein FITC-conjugated anti-rabbit IgG antibody (Boheringer Mannheim, Germany) diluted 1:50 in NET GEL. After several washes in NET GEL and PBS, slides were mounted in glycerol-DABCO containing 5 μg/ml DAPI (4-6,diamidino-2-phenyl-indol) to counterstain nuclei. Internal controls, performed omitting the primary antibody, did not disclose any FITC staining (not shown). The observations were carried out by means of a Leica Light Microscope equipped with a Coolsnap Videocamera for acquiring computerized images.

Immunoelectronmicroscopy

Cytocentrifuged cells were fixed in 4% paraformaldehyde / 0.1 % glutaraldehyde in 0.1 mol/L cacodylate buffer, pH 7.6, for 3 hrs at 4°C and then processed according to literature (30). In order to block non-specific binding sites, grids were blocked in PBS 0.1% Tween, 0.1% BSA,1% non-fat milk, 3 % NGS, incubated in the presence of mouse cytochrome c monoclonal antibody (Santa Cruz Biotechnology,Santa Cruz, CA) and rat Apaf-1 monoclonal antibody (Apotech, San Diego, CA), and then in the presence of a secondary antibody conjugated to 20 nm colloidal gold particles (anti-cytochrome c) or to 10 nm colloidal gold particles (anti-Apaf-1) (Biocell, Cardiff, U.K.). Uranyl acetate and lead citrate counterstained grids were observed using a Zeiss 109 electron microscope.

Analysis system

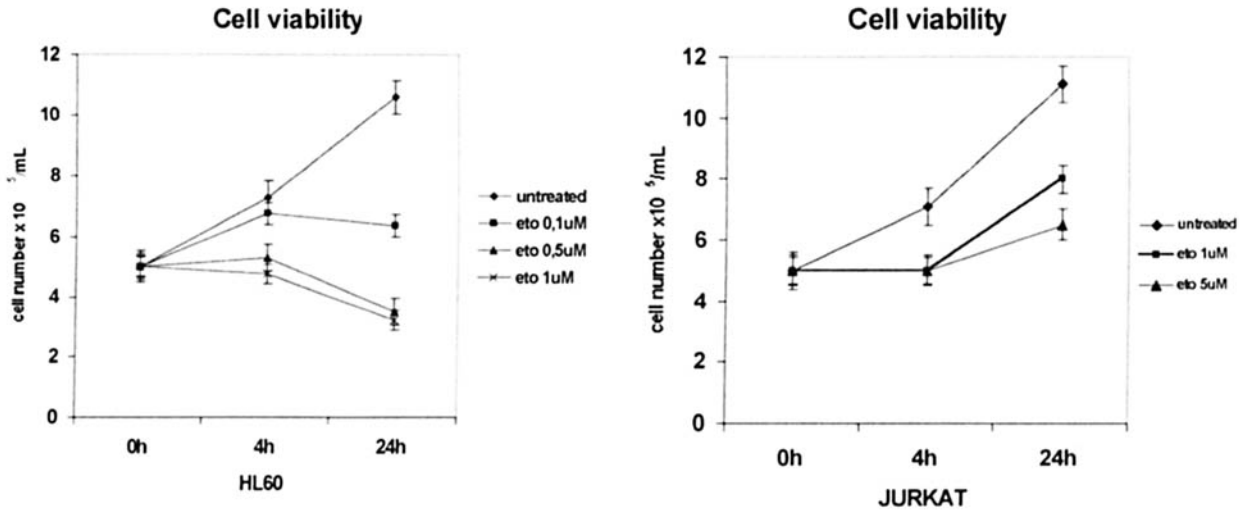
Statistical analysis was performed using the analysis of variance (ANOVA). Results were expressed as mean ± S.D. Probability of null hypothesis of < 0.1% ($p < 0.01$) was considered statistically significant.

RESULTS

For determining optimal etoposide concentration, both cell lines have undergone a preliminary treatment with concentrations ranging between 0.1 μM and 5 μM. Cells were treated for 1 hr with etoposide, reseeded in fresh medium and 24 hr later processed for Trypan blue dye exclusion test and cell cycle analysis in flow cytometry. Fig. 1 shows the different response shown by the two cell lines against etoposide. It is evident that HL-60 are already responsive to 0.5 μM while Jurkat, as expected since these cells are resistant to etoposide (31), need a higher concentration (1 μM) to display a similar rate of cell growth inhibition. Note that concentrations employed in this study are lower than those used by other authors (32, 33).

Once both etoposide concentrations and ionizing radiation doses had been established, experiments were performed to check the best response of each cell line to the treatment, focusing attention on some molecular mechanisms underlying such response. First of all cell cycle analysis gave evidence of a G2/M accumulation in HL-60 or Jurkat T cells upon either etoposide or radiation single treatment (Tab. I), compared to untreated samples. This accumulation was particularly evident upon the higher radiation dose when the highest

Fig.1. *Cell viability analysis performed on HL-60 and Jurkat cell lines at 4 and 24 hr after 1 hr exposure to the indicated concentrations of etoposide. Data are representative of three independent experiments ± S.D.*



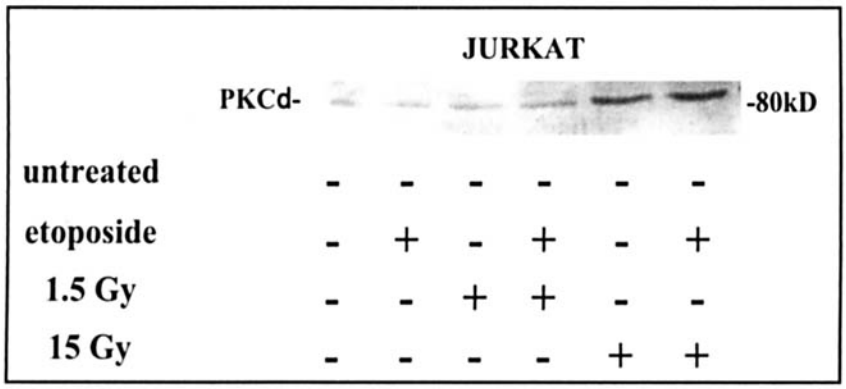


Fig.2. *Effect of 1 μM etoposide associated to 1.5 and 15 Gy ionizing radiation on nuclear PKC δ expression in Jurkat cells. Normalized amounts of nuclear proteins (20 μg) were resolved by SDS-PAGE, transferred to nitrocellulose membrane and probed with anti-PKC δ antibody. Data are representative of three independent experiments ± S.D.*

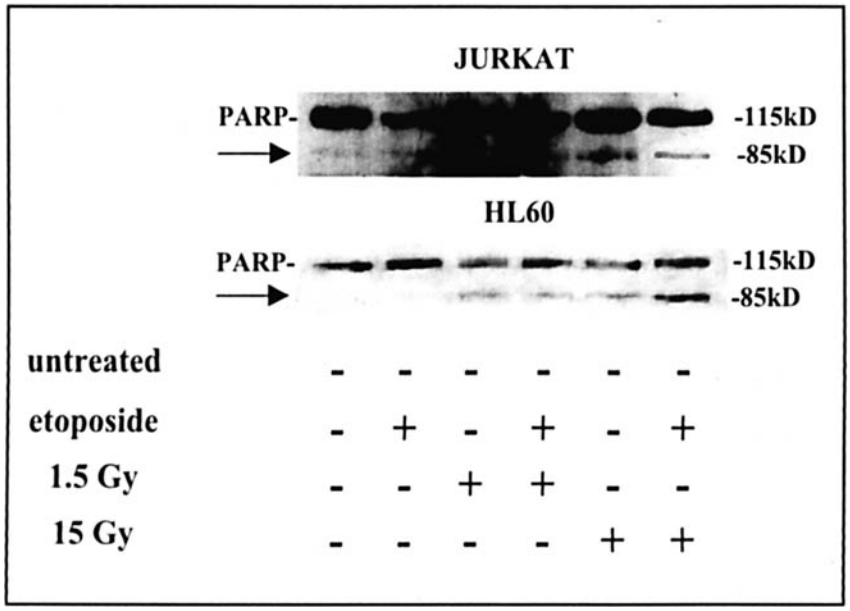


Fig.3. *Effect of 0.5 and 1 μM etoposide associated to 1.5 and 15 Gy ionizing radiation on PARP cleavage in Jurkat and HL-60 cells. Arrow indicates 85 kD cleavage product. Normalized amounts of whole cell proteins (20 μg) were resolved by SDS-PAGE, transferred to nitrocellulose membrane and probed with anti-PARP antibody. Data are representative of three independent experiments ± S.D*

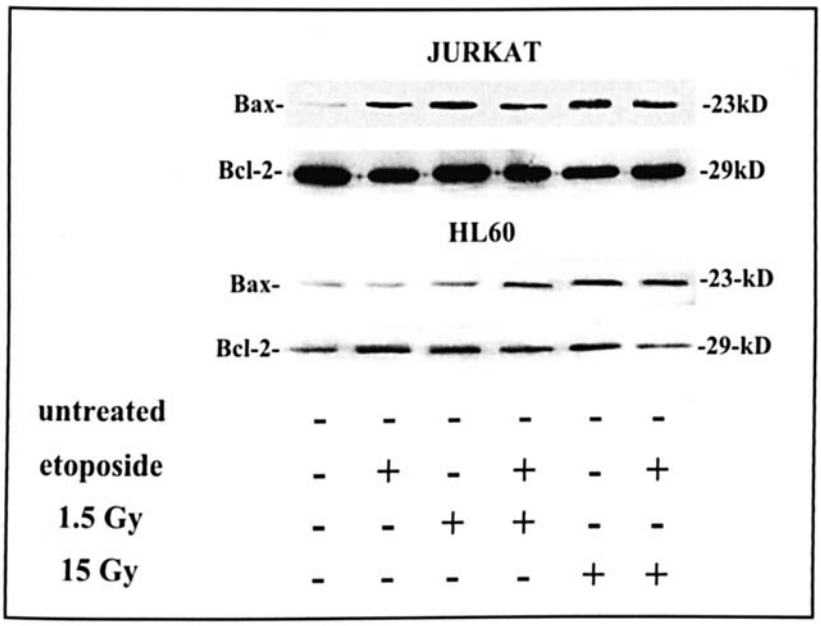


Fig.4. *Effect of 0.5 and 1 μM etoposide associated to 1.5 and 15 Gy ionizing radiation on Bax and Bcl2 levels in Jurkat and HL-60 cells. Normalized amounts of whole proteins (20 μg) were resolved by SDS-PAGE, transferred to nitrocellulose membrane and probed with mouse anti-Bax and anti-Bcl2 monoclonal antibodies. Data are representative of three independent experiments ± S.D.*

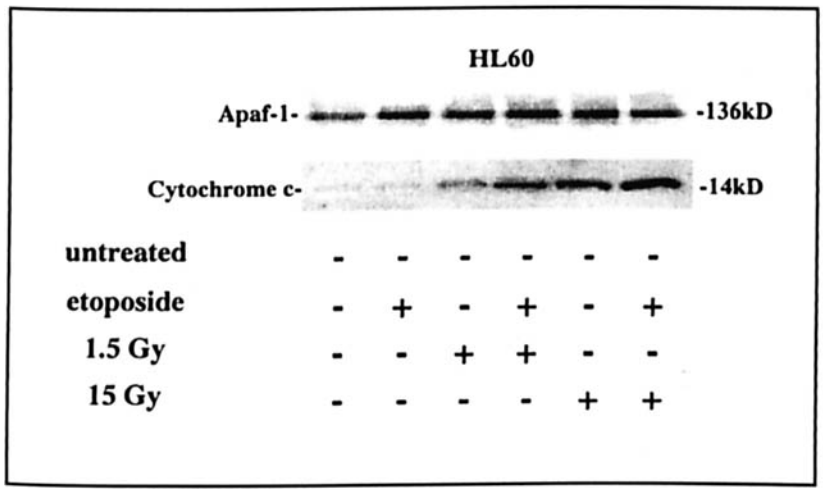
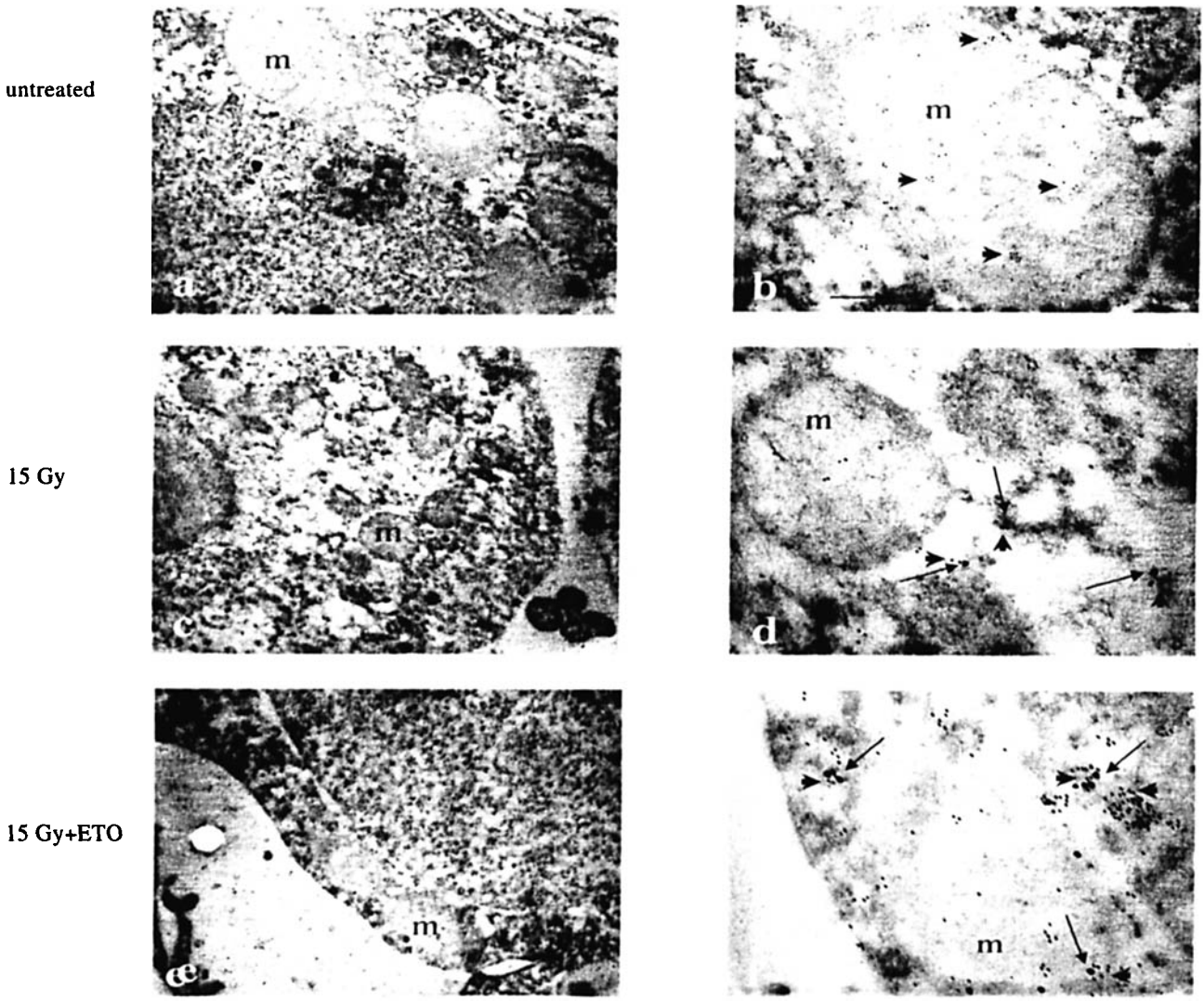


Fig.5. *Effect of 0.5 μM etoposide associated to 1.5 and 15 Gy ionizing radiation on coimmunoprecipitation of cytochrome c and Apaf-1. Apaf-1 immunoprecipitated from 10^6 HL-60 cell lysates was blotted against mouse anti-cytochrome c. Data are representative of three independent experiments ± S.D.*

incidence of apoptosis was detected. Since previous results by our group (34) suggested for Jurkat cells a PKC δ mediated caspase-3 upregulation in response to ionizing radiation, we tried to verify that the same signalling system could be activated upon etoposide treatment. Fig. 2 showed an increased nuclear expression of PKC δ upon 15 Gy exposure, further amplified in the presence of etoposide, while 1.5 Gy also associated to etoposide, did not determine significant changes compared to untreated Jurkat cell nuclei. HL-60, on the other hand, showed no PKC _ nuclear modification (not shown). Moreover caspase-3 was activated upon 1.5 Gy in HL-60 cells and upon 15 Gy in Jurkat, as documented by PARP cleavage (Fig. 3), along with no modifications concerning Bcl2 but Bax expression upon ionizing radiation or etoposide treatment (Fig. 4). Thus, in light of these data, we wanted to check whether caspase-3 was activated by mitochondrial release of apoptogenic factors (cytochrome c, Apaf-1, AIF) and/or by SAPK/JNK pathway. In HL-60 cells cytochrome c and Apaf-1 coimmunoprecipitated upon 1.5 Gy alone or associated to etoposide (Fig. 5), activating in turn, in the same experimental conditions, caspase-9 and caspase-3. Jurkat cells showed the same response as evidenced by immunoelectronmicroscopy co-localization of cytochrome c and Apaf-1 outside

Fig.6. *Immunoelectronmicroscopy analysis of cytochrome c and Apaf-1 colocalization. Jurkat T cells were immunolabelled using mouse anti-cytochrome c monoclonal antibody plus 10 nm gold conjugated mouse IgG and rat anti-Apaf-1 monoclonal plus 20 nm gold conjugated goat anti-rat IgG. Note that in untreated cells (a-b) cytochrome c is inside mitochondrion (arrowheads), Apaf-1 in the cytoplasm (arrow); in 15 Gy (c-d) and 15 Gy+etoposide (e-f) samples cytochrome c (arrowheads) and Apaf-1 (arrows) colocalize in the cytoplasm. m = mitochondrion ; cy = cytoplasm Magnification: a,c,e x12000; b,d,f x 50000.*



the mitochondrion both in 15 Gy and 15 Gy + etoposide exposed samples (Fig. 6). It is known that caspase-3 can be activated also by the SAPK/JNK metabolic pathway which is triggered by a variety of environmental stresses including ionizing radiation (19, 21). In both of our experimental systems, we observed the activation of SAPK/JNK upon etoposide treatment in combination with both ionizing radiation doses employed (Fig. 7). These data have a good correlation with the occurrence of ASK-1 translocation, documented in light microscopy for HL-60 (Fig. 8) and in western blotting for Jurkat cells and particularly evident upon etoposide-ionizing radiation combined treatment (Fig. 9).

DISCUSSION

An increase in apoptotic cells may be observed upon chemoterapeutic treatments and many authors have assumed that anticancer drugs kill cells by inducing apoptosis. The most relevant endpoint of

cell death following treatment of tumour cells is loss of reproductive ability. Since cells with limited reproductive potential cannot regenerate a tumour (35) we wanted to investigate the relationship between apoptosis and reproductive cell death following *in vitro* treatment of mammalian cell lines with both the anticancer drug (etoposide) and ionizing radiation in order to establish the lowest dose of both agents without inducing toxicity. If anticancer drugs or radiation activate pathways that can lead to either cell death or survival then these pathways can be manupulated to influence sensitivity to these agents.

Activation of caspase-3 is the downstream event which leads to the occurrence of apoptosis in a large variety of cells (12, 36-37). This protein is usually activated by pro-apoptotic Bcl2 family members (Bax, Bad) or by other molecular mechanisms among which SAPK/JNK metabolic pathway is included (20, 38). In addition, many reports suggest that mitochondria, releasing cytochrome c in the cytoplasm, play an essential role in the apoptotic program (39). Here we report that two exquisitely radiosensitive leukemia cell

Tab.I. *Cell cycle analysis in flow cytometry of HL-60 and Jurkat cell lines at 24 hr post irradiation*

	HL-60				JURKAT			
	G0/G1	*S*	*G2/M*	*Apoptosis*	*G0/G1*	*S*	*G2/M*	*Apoptosis*
Untreated	38 ± 4.9	50 ± 2.1	13 ± 2.8	4 ± 4.2	46 ± 3.4	41 ± 5.6	13 ± 5.7	3 ± 1.4
Eto 0.5 μM	25 ± 12.7	40 ± 3.5	36 ± 9.2	7 ± 6.4	n.d.	n.d.	n.d.	n.d.
Eto 1 μM	35 ± 4.0	25 ± 8.5	34 ± 3.2	8 ± 0.7	42 ± 7.5	39 ± 6.1	19 ± 6.4	5 ± 2.3
1.5 Gy	35 ± 3.3	48 ± 4.7	17 ± 1.5	11 ± 1.1	39 ± 3.7	29 ± 2.6	32 ± 3.3	7 ± 4.2
1.5 Gy + Eto 0.5 μM	36 ± 3.8	48 ± 4.8	16 ± 1.4	14 ± 1.2	n.d.	n.d.	n.d.	n.d.
1.5 Gy + Eto 1 μM	n.d.	n.d.	n.d.	n.d.	33 ± 3.2	45 ± 4.2	22 ± 1.9	5 ± 0.4
15 Gy	68 ± 6.7	1 ± 0.2	32 ± 3.0	28 ± 2.5	28 ± 4.8	15 ± 0.7	58 ± 4.5	10 ± 0.9
15 Gy + Eto 0.5 μM	65 ± 6.6	18 ± 1.5	17 ± 1.6	30 ± 2.8	n.d.	n.d.	n.d.	n.d.
15 Gy + Eto 1 μM	n.d.	n.d.	n.d.	n.d.	48 ± 4.8	26 ± 2.4	26 ± 2.8	11 ± 1.2

Values are expressed as mean % ± SD
Eto: Etoposide
n.d.: not determined

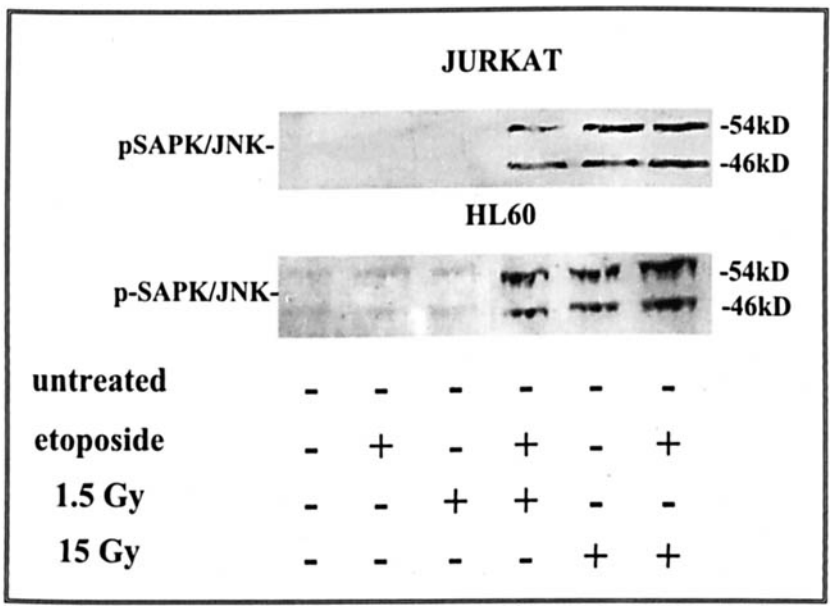
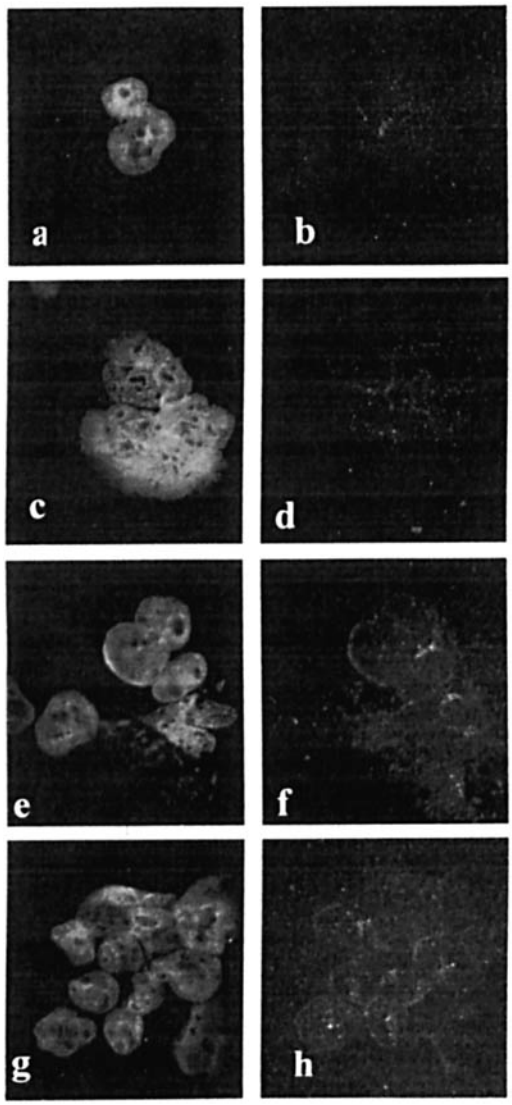


Fig.7. *Effect of 0.5 and 1 μM etoposide associated to ionizing radiation on SAPK/JNK activation in Jurkat and HL-60 cells. Normalized amounts of whole cell proteins (20 μg) were resolved by SDS-PAGE, transferred to nitrocellulose membrane and probed with mouse anti-SAPK/JNK monoclonal antibody, which detects endogenous levels of p46 and p54 SAPK/JNK dually phosphorylated at threonine 183 and tyrosine 185. Data are representative of three independent experiments ± S.D.*

lines respond to ionizing radiation-etoposide exposure with caspase-3 activation, mediated by mitochondrial release of apoptogenic factors as well as by SAPK/JNK activation. In particular HL-60, which are responsive already to 0.5 μM etoposide associated to 1.5 Gy disclose caspase-3 activation, documented by PARP fragment production concomitant with ASK-1 nuclear translocation and SAPK/JNK phosphorylation. Jurkat T cells, on the other hand, need a higher etoposide concentration (1 μM) associated to a higher radiation dose (15 Gy) to show the same response. In this case PKC δ-mediated caspase-3 upregulation, also in the presence of etoposide, is paralleled by ASK-1 nuclear translocation which initiates SAPK/JNK activation, regulating, in turn activity of several transcription factors such as c-jun, ATF2 and p53. HL-60 not only show a larger number of apoptotic cells upon 1.5 Gy +0.5 μM etoposide, but also an accumulation of cells in G2/M cell cycle phase, compared to untreated cells. This kind of response is not PKC δ mediated, but the apoptotic stimuli determined by ionizing radiation and etoposide directly regulate ASK-1 which, in turn, reduces Bcl2 expression, promoting cytochrome c release, binding of Apaf-1 and caspase-9-caspase-3 activation .

Thus, these results suggest that SAPK/JNK and the mitochondrial route may contribute to the positive regulation of drug and ionizing radiation induced apoptosis. This in turn adds to the understanding of complex interactions between different cellular programs, providing insights into the sensitivity or resistance of tumour cells and identifies molecular targets for rational therapeutic intervention strategies.

Fig. 8. *Fluorescent light microscopy analysis of ASK-1 expression in HL-60 cells. Note that ASK-1 translocates into the nucleus upon 1.5 Gy ionizing radiation exposure, mainly upon 1.5 Gy ionizing radiation associated to etoposide. Fluorescence (right column) detects ASK-1; fluorescence (left column) of DAPI (4-6 diamino-2-phenyl-indol) counterstains nuclei.*
***a- b:** untreated; **c-d:** etoposide 0.5 μm; **e-f:** 1.5 Gy; **g-h:** 1.5 Gy+ etoposide 0.5 μm. Magnification :100 x.*



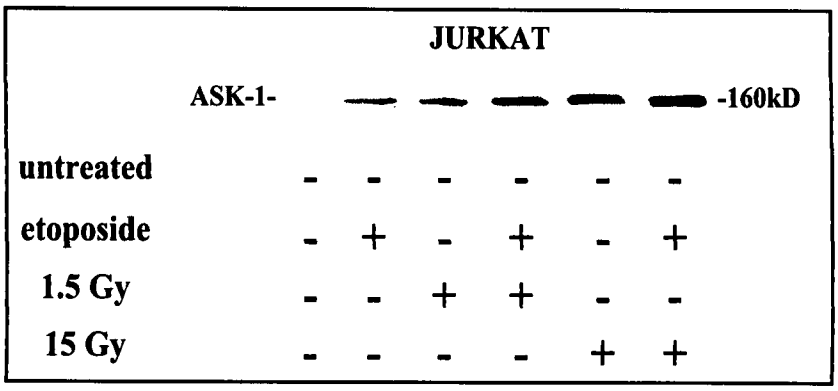


Fig.9. *Effect of 1 μM etoposide associated to ionizing radiation on ASK-1 nuclear expression in Jurkat cells. Normalized amounts of nuclear proteins (20 μg) were resolved by SDS-PAGE, transferred to nitrocellulose membrane and probed with mouse anti-ASK-1 monoclonal antibody. Data are representative of three independent experiments ± S.D.*

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