Radiation-induced apoptosis in MOLT-4 cells requires de novo protein synthesis independent of de novo RNA synthesis

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Abstract We investigated the effects of inhibition of de novo RNA and protein synthesis in ionizing radiation (IR)-induced apoptosis in the human T cell line MOLT-4. We observed that pretreatment with cycloheximide inhibited IR-induced apoptosis. However, pretreatment with actinomycin D did not inhibit apoptosis induced by IR. These results suggest that apoptosis induced by IR in MOLT-4 cells requires de novo protein synthesis but not de novo RNA synthesis. This finding suggests that the mRNA encoding the proapoptotic protein(s) is stabilized to facilitate translation independent of de novo gene transcription in response to IR. Our results also indicate that translation of the required proapoptotic protein(s) occurs upstream of mitochondrial depolarization and after 2 h post-IR.

Key words: Apoptosis; Transcription; Translation; Ionizing radiation; Phosphatidylserine; Mitochondrial transmembrane potential

1. Introduction

Programmed cell death (apoptosis) can be induced by a variety of stimuli including growth factor withdrawal, ligand binding, cytotoxic chemicals, and physical stress such as ionizing radiation (IR) [1–4]. Because of the ability of IR to induce apoptosis in malignant cells, it is frequently used in the treatment of many cancers [5].

Apoptosis occurs through various molecular pathways. Many of these pathways overlap in their cascade of events, but all terminate with the same result: cell death. Implicit in this is that different pathways incorporate different proteins, post-translational modifications, and membrane alterations [6–9]. Similarly, some apoptotic pathways are dependent on de novo gene expression while others are not. This has been demonstrated by the inability or ability of cells to undergo apoptosis in the presence of actinomycin D (act-D) or cycloheximide (CHX), which inhibit RNA and protein synthesis, respectively [10–16].

In MOLT-4 cells, IR causes increased expression of apoptosis inducing ligand (Apo2L), p53, type 3 inositol-1,4,5-trisphosphate receptor, Fas, DR5, and Bax [17,18]. These genes are believed to play critical roles in IR-induced apoptosis. However, of these genes, only Apo2L, Fas, and DR5 are specifically known to be transcriptionally upregulated in response to IR in MOLT-4 cells [17]. Apoptosis induced by IR in MOLT-4 cells is characterized by multiple molecular events which include phosphorylation of stress-activated protein kinase/c-Jun NH_2-terminal kinase (SAPK/JNK) [19], depolarization of the mitochondrial transmembrane potential ($\Delta\psi_m$) [18], externalization of phosphatidylserine (PS) [20], caspase activation [18], cleavage of poly-ADP-ribose polymerase [8], and fragmentation of chromosomal DNA [21].

While it is clear that in MOLT-4 cells IR causes increased expression of specific genes involved in apoptosis, whether or not the altered expression patterns are critical for apoptosis remains unknown. To address this question, we investigated the effects of inhibition of de novo RNA and protein synthesis in IR-induced apoptosis of MOLT-4 cells. We blocked RNA and protein synthesis using act-D and CHX, respectively, prior to apoptotic induction by IR. We demonstrate that IR-induced apoptosis occurs in the presence of act-D but not CHX. These results suggest that in MOLT-4 cells, IR-induced apoptosis is independent of de novo RNA synthesis and dependent on de novo protein synthesis. These findings also suggest that the mRNA encoding the proapoptotic protein(s) is stabilized. Similarly, we examined when the required translation occurs in the apoptotic process. We show that the required de novo protein synthesis occurs after 2 h post-IR and precedes $\Delta\psi_m$ depolarization in the apoptotic cascade.

2. Materials and methods

2.1. Reagents and chemicals

CHX, act-D, RNase A, and propidium iodide (PI) were obtained from Sigma Chemical Co. Act-D stock solutions were prepared in dimethyl sulfoxide (DMSO). CHX stock solutions were prepared in ethanol. All other solutions were prepared in distilled water. Tris, sodium chloride, DMSO and EDTA were obtained from Fisher Scientific.

2.2. Cell culture

MOLT-4 cells were purchased from ATCC and cultured in RPMI 1640 supplemented with 10% bovine calf serum, 2 mM L-glutamine, 1.5 g/l sodium bicarbonate (Hyclone), penicillin (100 U/ml), and 100 μg/ml streptomycin (Sigma). The cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Cells were maintained

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Abbreviations: IR, ionizing radiation; act-D, actinomycin D; CHX, cycloheximide; $\Delta\psi_m$, mitochondrial transmembrane potential; PS, phosphatidylserine; FITC, fluorescein isothiocyanate; PI, propidium iodide

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in exponential growth (4–8×10^5 cells/ml) and viability was monitored periodically by trypan blue exclusion.

2.3. Cell treatment

Act-D or CHX was added to cell cultures 1 h prior to irradiation to inhibit RNA and protein synthesis, respectively [10–16]. Following addition of the inhibitors, the cell cultures were returned to a 37°C incubator. The inhibitors were removed from the cell cultures immediately prior to sample analysis. Apoptosis was induced in MOLT-4 cells by exposure to 10 Gy IR. Approximately 10^6 cells were collected and irradiated using a MARK I γ-irradiator ^{137}Cs source at a dose rate of 2.65 Gy/min (Shepard and Associates, San Fernando, CA, USA). After irradiation the cells were seeded in 24 well plates.

2.4. Measurement of apoptotic events

Externalization of PS was determined by measuring annexin V-FITC binding using a kit purchased from Immunotech. Briefly, 5×10^5 cells were harvested and stained with annexin V-FITC and PI. The samples were then analyzed by flow cytometry with a Coulter Epics XL flow cytometer. Fluorescence of PI and FITC was measured on logarithmic scales. Cell viability was assessed by the cells' ability to exclude PI. In Fig. 1A, cells appearing above and below the horizontal axis are considered PI positive and negative, respectively. Similarly, cells appearing to the right and left of the vertical axis are considered annexin V positive and negative, respectively.

Cell cycle distributions were determined by measuring DNA content. Approximately 10^6 cells were collected and fixed in 70% ethanol. The cells were then washed in phosphate buffered saline and resuspended in a solution containing 10 mM Tris, 10 mM NaCl, 30 μM PI, and 20 μg/ml RNase A. The samples were then incubated at 37°C for 30 min prior to analysis by flow cytometry. Hypodiploid DNA content is associated with apoptotic cell death and is thought to be the result of nuclear chromatin fragmentation. While this method is not a direct measure of DNA fragmentation, it clearly demonstrates the initiation of this late apoptotic event. Therefore, we determined the percentage of cells with fragmented nuclear chromatin by quantifying the percentage of cells with hypodiploid DNA content (sub-G_0/G_1) as described previously [20].

Loss of $\Delta\psi_m$ was measured using a MitoTag JC-1 kit purchased from Intergen. JC-1 accumulates in healthy mitochondria and fluoresces red. The dye fails to accumulate in depolarized mitochondria and remains in the cytosol fluorescing green. Treated cells were analyzed by flow cytometry.

2.5. Statistical analysis

The data shown represent mean values ± S.E.M. (error bars) of experiments repeated at least three times. Statistical significance was determined using Student's *t*-tests. *P* values less than 0.05 are considered statistically significant.

3. Results

3.1. IR induces apoptotic cell death in MOLT-4 cells

To characterize the apoptotic cell death of MOLT-4 cells

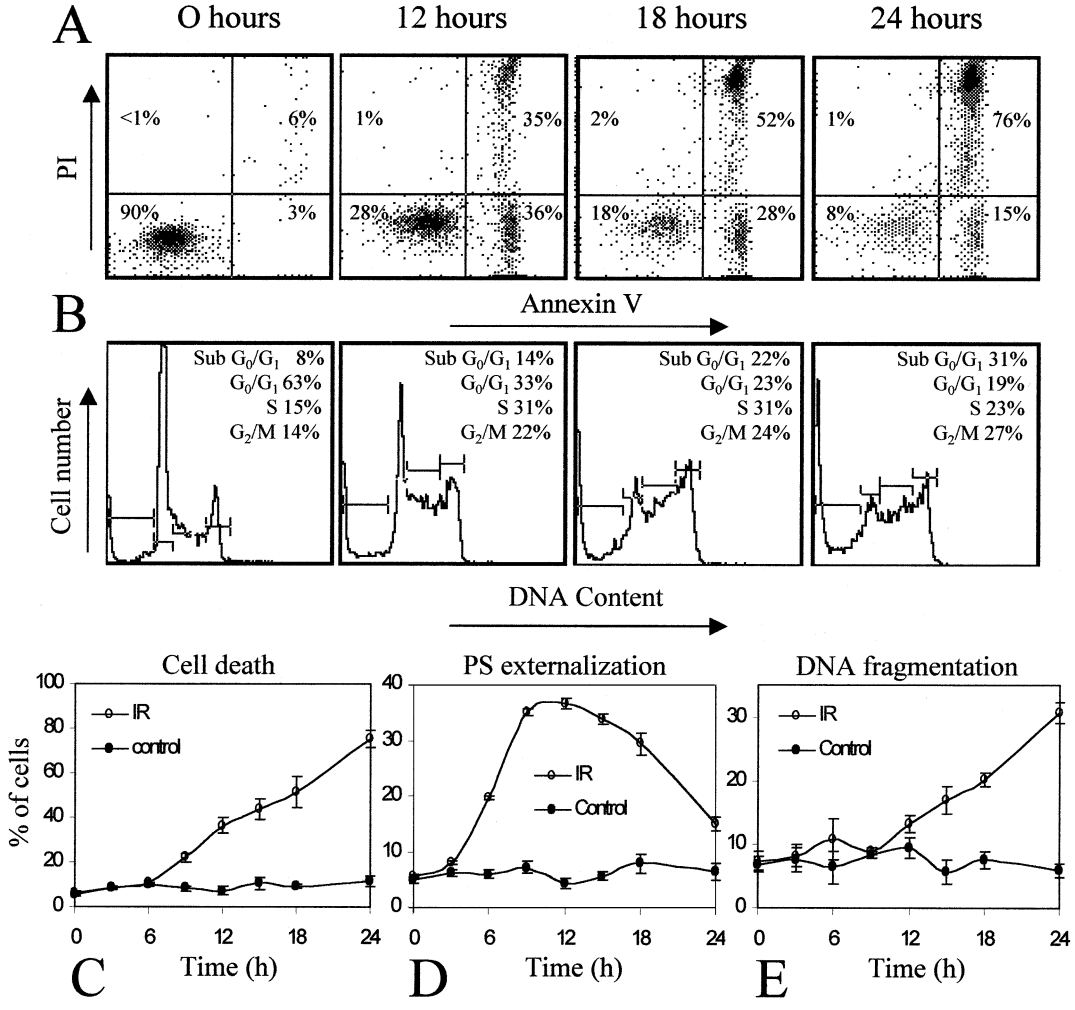

Fig. 1. Analysis of apoptotic events following induction by IR. A,B: Time course of (A) PS externalization and cell death and (B) DNA fragmentation in MOLT-4 cells. C: Cell death of irradiated (empty) and non-irradiated (filled) cells. D: PS externalization of irradiated (empty) and non-irradiated (filled) cells. E: DNA fragmentation of irradiated (empty) and non-irradiated (filled) cells.

following IR, we conducted a series of time course experiments. We measured cell death, PS externalization, and DNA fragmentation at 3 h intervals after IR. In Fig. 1A, dead cells are represented in the upper quadrants (PI positive), while live cells are represented in the lower quadrants (PI negative). Cell death of MOLT-4 cells began to increase 6 h after IR (Fig. 1C). It is important to note, however, that while the uptake of PI indicates cell death, it does not differentiate between apoptotic and necrotic modes of death. Staining the cells with annexin V-FITC in addition to PI makes this distinction clear. The lower left quadrants of Fig. 1A (PI negative/annexin V negative) represent live cells and the lower right quadrants (PI negative/annexin V positive) represent live cells undergoing early stages of apoptosis (PS externalization). We observed that the percentage of cells with externalized PS reached a maximum of 36.5% 12 h after IR (Fig. 1D). Based on these results, we measured PS externalization and cell death at 12 h post-IR on all subsequent experiments. Similarly, in response to IR, the percentage of cells with fragmented nuclear chromatin increased in a time-dependent manner and was maximal 24 h after IR (Fig. 1B,E). Therefore, we measured DNA fragmentation at 24 h post-IR in all subsequent experiments. We also observed that following IR, the percentage of cell death (PI positive cells) was greater than DNA fragmentation throughout the time course (Fig. 1C,E).

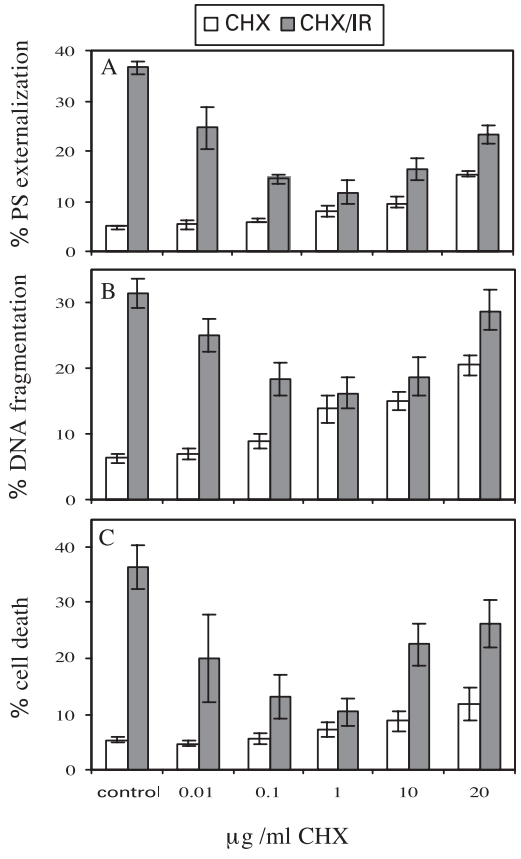

Fig. 2. Effects of protein synthesis inhibition on IR-induced apoptosis. MOLT-4 cells were treated with CHX alone (light) or in combination with IR (dark) as described in Section 2 and assayed for PS externalization at 12 h post-IR (A), DNA fragmentation at 24 h post-IR (B), and cell death at 12 h post-IR (C).

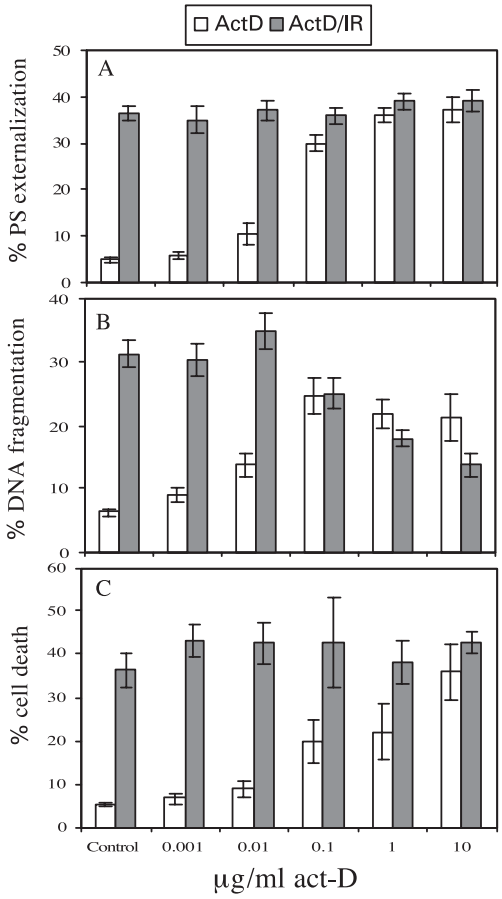

Fig. 3. Effects of RNA synthesis inhibition on IR-induced apoptosis. MOLT-4 cells were treated with act-D alone (light) or in combination with IR (dark) as described in Section 2 and assayed for PS externalization 12 h post-IR (A), DNA fragmentation at 24 h post-IR (B), and cell death at 12 h post-IR (C).

These results suggest that in MOLT-4 cells, IR may cause a decrease in plasma membrane integrity that precedes fragmentation of nuclear chromatin.

3.2. *De novo protein synthesis is required for IR-induced apoptosis*

Treatment with CHX alone resulted in dose-dependent increases in PS externalization (Fig. 2A), DNA fragmentation (Fig. 2B), and cell death (Fig. 2C). Pretreatment of irradiated cells with CHX inhibited PS externalization in a dose-dependent manner up to a concentration of 1 μg/ml. Treatment with IR alone resulted in PS externalization of 36.5% of the cells while pretreatment with 1 μg/ml CHX inhibited PS externalization to 11.8% of the population ($P < 0.001$). Concentrations greater than 1 μg/ml CHX resulted in diminished inhibition of IR-induced PS externalization (Fig. 2A). Similarly, DNA fragmentation was inhibited maximally by pretreatment with 1 μg/ml CHX. IR alone resulted in DNA fragmentation of 31.2% of MOLT-4 cells while pretreatment with 1 μg/ml CHX inhibited DNA fragmentation to 16.2% of the population ($P < 0.01$). Cell death was also significantly abrogated in a dose-dependent manner and inhibited maximally with 1 μg/ml CHX ($P < 0.02$). Collectively, these results suggest that de novo protein synthesis is required for IR-induced apoptosis.

3.3. De novo RNA synthesis is not required for IR-induced apoptosis

Next, we investigated whether or not de novo RNA synthesis is required for IR-induced apoptosis. Treatment with act-D alone caused dose-dependent increases in PS externalization and cell death (Fig. 3A,C). However, blocking de novo RNA synthesis with act-D did not inhibit IR-induced PS externalization or cell death at any concentration. Similarly, treatment with act-D alone caused increased DNA fragmentation in a dose-dependent manner up to 0.1 μg/ml. Higher concentrations resulted in moderately decreased values of DNA fragmentation from that obtained with 0.1 μg/ml act-D. Interestingly, concentrations of act-D less than 0.1 μg/ml did not inhibit IR-induced DNA fragmentation, while concentrations equal to and greater than 0.1 μg/ml resulted in decreased DNA fragmentation. However, these higher concentrations induced significant cell death and PS externalization in cells treated with act-D alone (Fig. 3A,C). Therefore, the decrease in DNA fragmentation with increasing concentrations of act-D is likely artifactual and not the result of inhibited apoptotic processing. These results suggest that de novo RNA synthesis is not required for IR-induced apoptosis in MOLT-4 cells.

3.4. IR-induced $\Delta\psi_m$ disruption is dependent on de novo protein synthesis

We then investigated how IR-induced $\Delta\psi_m$ depolarization is affected by inhibition of de novo protein and RNA synthesis. IR resulted in significant $\Delta\psi_m$ depolarization 12 h after irradiation ($P < 0.001$). However, pretreatment of cells with CHX prior to IR resulted in dramatic inhibition ($P < 0.001$) of IR-induced $\Delta\psi_m$ disruption (Fig. 4A). These results suggest that de novo protein synthesis is required for $\Delta\psi_m$ depolarization in IR-induced apoptosis. We then sought to determine the involvement of de novo RNA synthesis in IR-induced $\Delta\psi_m$ disruption. Pretreatment of irradiated cells with act-D had no inhibitory effect ($P > 0.1$) on $\Delta\psi_m$ depolarization (Fig. 4A). This suggests that de novo gene transcription is not required for $\Delta\psi_m$ depolarization in IR-induced apoptosis.

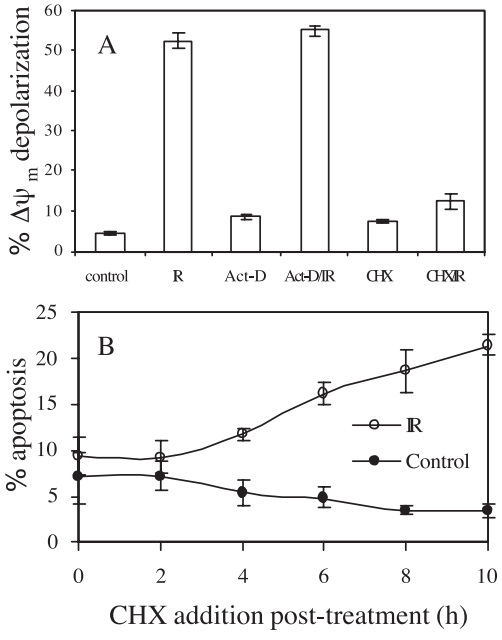

Fig. 4. Effects of RNA and protein synthesis inhibition on IR-induced $\Delta\psi_m$ depolarization. A: MOLT-4 cells were treated with various combinations of IR and inhibitors (10 Gy IR, 1 μg/ml CHX, 0.01 μg/ml act-D) and then assayed for $\Delta\psi_m$ depolarization. Treated cells were analyzed by flow cytometry. B: Cells were irradiated (empty) or not (filled) followed by addition of 1 μg/ml CHX at various times after IR. Apoptosis was determined by measuring PS externalization 12 h after IR.

3.5. The required de novo protein synthesis occurs after 2 h post-IR

Next, we investigated when the required translation occurs in the apoptotic process. We conducted time course experiments in which we added CHX to irradiated cells at 2 h intervals after IR (Fig. 4B). Addition of CHX as late as 2 h after IR significantly ($P < 0.001$) inhibited apoptosis to the same extent as pretreatment with CHX 1 h prior to IR. Addition of CHX at later intervals resulted in progressively diminished inhibition of apoptosis. These results suggest that translation of the proapoptotic protein(s) occurs after 2 h post-IR.

4. Discussion

Depending on the cell type and stimulus, some apoptotic pathways require de novo gene expression while others do not. For example, IR-induced apoptosis in rat thymocytes requires both de novo RNA and protein synthesis [12]. However, in F9 teratocarcinoma cells, IR-induced apoptosis is independent of both de novo RNA and protein synthesis [13]. In this study, we report that IR-induced apoptosis in MOLT-4 cells is dependent on de novo protein synthesis and independent of de novo RNA synthesis. This finding is significant because it suggests that the mRNA encoding the proapoptotic protein(s) is stabilized after synthesis. This type of cellular mechanism would facilitate induced translation of the stabilized mRNA independent of de novo gene transcription [22]. Interestingly, it has been demonstrated that treatment with the transcription inhibitor act-D causes increased translation of p53, Bax, Bcl-X(s), and c-Myc [23–25]. These findings support our observations by demonstrating that increased translation of these proapoptotic proteins can occur in the absence of de novo gene transcription. Similarly, while IR causes increased transcription of proapoptotic genes in MOLT-4 cells [17], our results suggest that de novo gene transcription is not required for IR-induced apoptosis.

In addition to altering gene expression, IR induces numerous apoptotic events [17–21]. Depolarization of $\Delta\psi_m$ is an early event in IR-induced apoptosis [26]. We found that inhibition of de novo protein synthesis, but not RNA synthesis, prevented IR-induced $\Delta\psi_m$ depolarization. These findings suggest that de novo protein synthesis is required for IR-induced $\Delta\psi_m$ disruption in MOLT-4 cells. We also observed that translation of the required proapoptotic protein(s) occurs after 2 h post-IR. This finding is significant in light of the fact that SAPK/JNK is phosphorylated 1 h after treatment with 10 Gy IR in MOLT-4 cells [19]. Collectively, these results suggest that translation of the proapoptotic protein(s) may occur downstream of SAPK/JNK phosphorylation and upstream of $\Delta\psi_m$ depolarization in IR-induced apoptosis in MOLT-4 cells.

In summary, we report a unique phenomenon in which

apoptosis induced by IR requires de novo protein synthesis but not de novo RNA synthesis. These findings suggest that the mRNA of the gene(s) involved is stabilized to facilitate translation independent of de novo gene transcription in response to IR. Additionally, the proapoptotic protein(s) involved acts upstream of $\Delta\psi_m$ depolarization and is translated after 2 h post-IR. Further studies are necessary to identify the specific gene(s) involved and the mechanism by which the mRNA is stabilized.

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