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IONIZING RADIATION-INDUCED APOPTOSIS AND DNA REPAIR IN MURINE ERYTHROLEUKEMIA CELLS

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Abstract

A morphological study of DNA repair and apoptotic patterns in relationship with cell cycle events was performed on murine erythroleukemia cells. The presence and distribution of DNA replicon sites were evaluated through the BrdU-anti BrdU immunofluorescence and immunogold techniques in light and electron microscopy. Different patterns of labelling and percentages of BrdU positive cells were observed depending on irradiation dose (up to 60 Gy) and time in post-irradiation culture (up to 24 hours). An enlargement of the S phase of the cell cycle was evidenced 18 hours post-irradiation as determined by flow cytometry analysis. The high resolution approach showed that, in spite of several morphological alterations, BrdU labelling was present even in cells displaying early and late apoptotic features.

Key Words: Gamma radiation, murine erythroleukemia cells, high-dose radiation exposure, cell death, apoptosis, DNA repair, bromodeoxyuridine, immunofluorescence, immunogold, flow cytometry.

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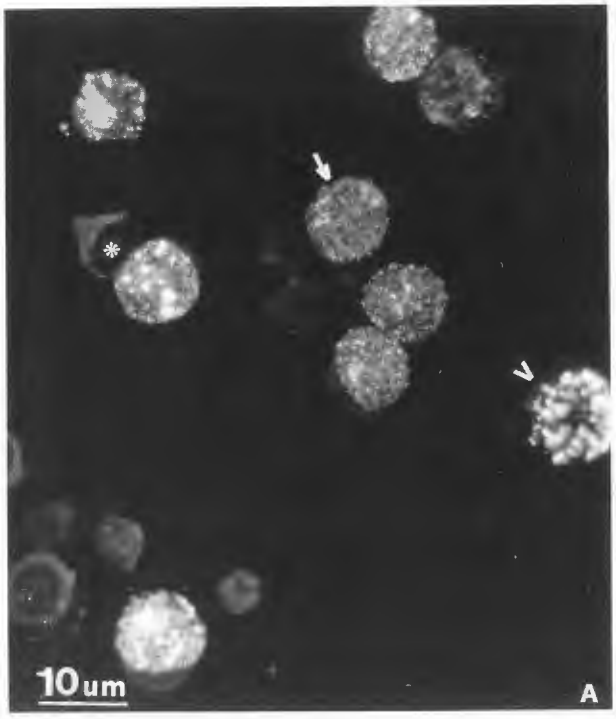
Introduction

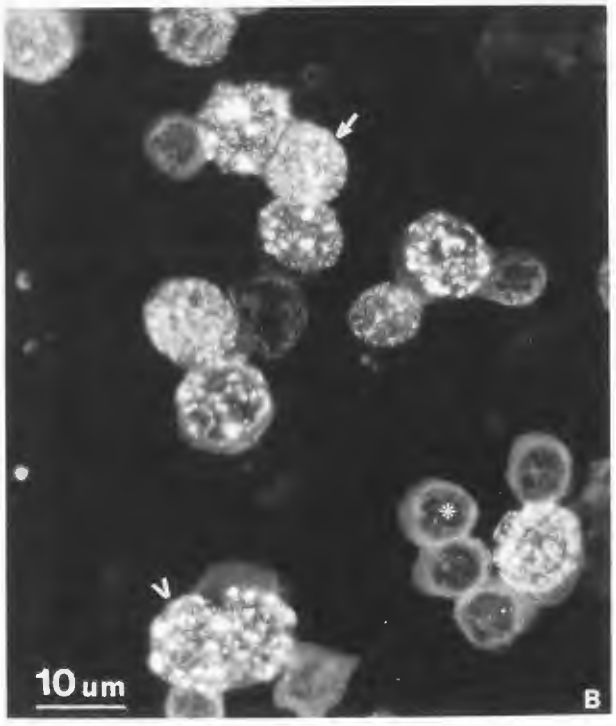
Mammalian cells respond to ionizing radiation with cell cycle arrest, activation of DNA repair and induction of early response genes [9]. Although much has been reported about the mechanisms of DNA repair after radiation damage [7, 8], the possible interaction between DNA repair processes and cell cycle events is still under investigation. Recently, the involvement of topoisomerase II has been proposed in some rapid DNA repair pathways operating during all the phases of the cell cycle and even in DNA repair acting within the radiation-induced G2 block [6]. In a recent work on irradiated murine erythroleukemia cells (MEL) [15], we demonstrated the occurrence of a marked increase in DNA synthesis mainly related to β DNA polymerase activity, known as a radiation inducible enzyme involved in the repair of single strand DNA breaks [14]. In this cell system, the amount and severity of damage appeared to be time- and dose-dependent and resulted in both necrosis and apoptosis [3]. It is known that in susceptible cells the radiation-induced apoptosis is related to the number of DNA strand breaks produced, to the rate at which they occur, and to the rapidity and effectiveness of DNA repair mechanisms [2]. Therefore, the aim of this investigation was to make a correlation between early and late DNA repair patterns, apoptosis and cell cycle events in murine erythroleukemia cells after high dose ionizing radiation exposure.

Materials and Methods

Cell cultures, BrdU incorporation and irradiation protocol

Murine erythroleukemia cells (FLC-745) were grown in RPMI 1640 medium (Gibco Laboratories, Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal calf serum and antibiotics in a 5% CO_2 atmosphere. Asynchronous exponentially growing cells were irradiated at room temperature by a Mevatron 74 Siemens linear accelerator (photonic energy: 10 MV) administering 15 and 60 Gy (dose rate 3 Gy/min). Both





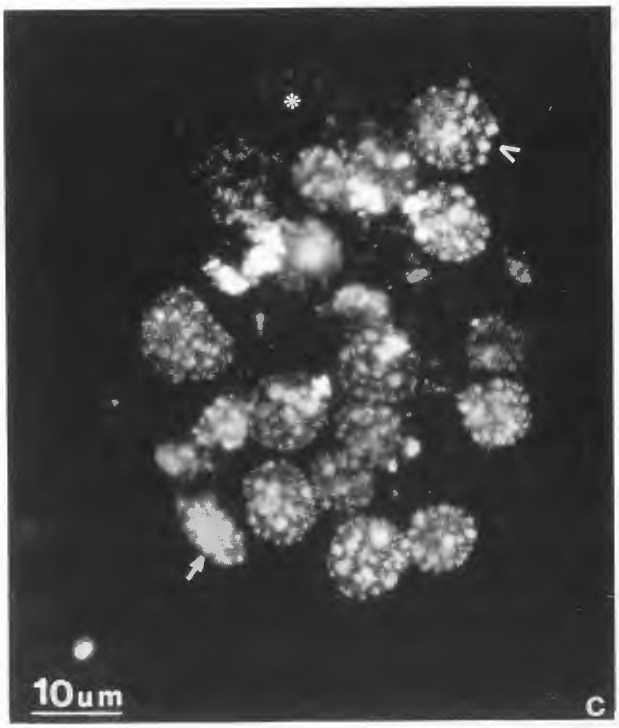


Figure 1. Patterns of BrdU incorporation in 18 hour samples: control (A), 15 Gy (B) and 60 Gy (C); spot (>), diffuse fluorescent labelling (→), and negative nuclei (*) are indicated. Bars = 10 μm. Mean percentage ± SD of: (1) BrdU positive cells: control: 61.2 ± 1.2; 15 Gy: 78.0 ± 1.5; 60 Gy: 82.0 ± 2.5 ($p < 0.05$); and (2) viable cells: 1 hour control, 15 Gy, 60 Gy: 96.3 ± 3.2; 18 hour control: 95.1 ± 2.2; 15 Gy: 94.2 ± 4.7; 60 Gy: 95.2 ± 5.4; 24 hour control: 97.0 ± 3.0; 15 Gy: 96.7 ± 4.1; 60 Gy: 86.7 ± 6.0.

control and irradiated samples were kept at 37°C for 1, 18 and 24 hours and during the last hour pulsed with 50 μM 5-bromo-2'deoxyuridine (BrdU, Sigma, St. Louis, MO). Cell viability was checked with the trypan blue exclusion test.

Immunofluorescence microscopy

The samples were washed twice in phosphate buffered saline (PBS), cyto-centrifuged and fixed in 70% ethanol for 30 minutes at room temperature. For DNA denaturation, the glass slides were incubated with 0.07 N NaOH for 2 minutes at room temperature and thoroughly rinsed with PBS. The subsequent incubations with the primary anti-BrdU mouse IgG monoclonal antibody (Becton Dickinson, Palo Alto, CA) and the secondary goat anti-mouse fluorescein isothiocianate (FITC) labelled IgG (Sigma) were performed as previously detailed [12].

Immuno-electron microscopy

The cells were fixed for 30 minutes in 1.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2, postfixed in 1% OsO_4 and embedded in Spurr resin. Immunocytochemical labelling was performed with the post embedding immunogold technique as previously reported [16].

Table 1. Mean percentage of cells in different phases of cell cycle as determined in flow cytometry.

	1 hour			18 hours			24 hours		
	G1	S	G2/M	G1	S	G2/M	G1	S	G2/M
C	40.9 ± 1.0	46.2 ± 1.5	8.5 ± 0.3	34.0 ± 0.9	61.9 ± 2.0	5.0 ± 0.2	28.0 ± 0.5	52.0 ± 1.8	20.0 ± 0.7
15 Gy	40.0 ± 0.8	50.0 ± 1.1	10.0 ± 0.3	2.0 ± 0.1	92.0 ± 2.0	7.0 ± 0.2	88.0 ± 1.8	2.0 ± 0.1	10.0 ± 0.3
60 Gy	34.2 ± 0.7	47.9 ± 1.5	9.2 ± 0.2	1.0 ± 0.1	94.0 ± 2.1	5.0 ± 0.2	89.0 ± 1.7	2.0 ± 0.1	10.0 ± 0.3

Controls consisted of cells not irradiated, not exposed to BrdU, and not incubated with the primary antibody.

Flow cytometry

Samples were treated as described by Dolbeare *et al.* [4]. Briefly, cells were washed twice in PBS, incubated 30 minutes on ice with 70% cold ethanol and 30 minutes at room temperature in 4 N HCl. Samples were washed in PBS, incubated in $Na_2B_4O_7$ pH 8.5 and briefly treated with 0.5% Triton X-100. After washing, the cells were incubated with FITC-conjugated anti-BrdU moAb (Becton Dickinson) for 30 minutes at 4°C, washed again and finally, counterstained with 5 μg/ml propidium iodide (PI). The cell cycle analysis was performed by a FACStar Plus flow cytometer (Becton Dickinson) equipped with an argon ion laser tuned at 488 nm, 200 mW output, for excitation of FITC and PI [19]. Data were collected in list mode with a Hewlett-Packard Consort 32 minicomputer. F12 fluorescence was collected in log scale to include, in a single acquisition, debris, apoptotic and non-apoptotic cells.

Statistics

Experimental data are expressed as mean percentage ± standard deviation (SD). Comparisons were made by means of the Student's two-tailed t-test. Values of $p < 0.05$ were considered significant.

Results

Immunofluorescence microscopy

Two main patterns of labelling were observed in immunofluorescence: (a) a diffuse fluorescent labelling except for the nucleolar area (early S phase); (b) a peripheral spot fluorescent labelling (late S phase) (Fig. 1). No differences between control and irradiated samples could be pointed out in the percentage of BrdU incorporating cells 1 hour after exposure (control: 60.0 ± 2.2; 15 Gy: 60.5 ± 3.1; 60 Gy: 61.0 ± 2.5) while 18 hours post-irradiation, this percentage increased 1.3 fold in both 15 Gy (78.0 ± 1.5) and 60 Gy (82.0 ± 2.5; $p < 0.05$) irradiated samples compared with controls (61.2 ± 1.2); moreover, if we consider the reduced viability deriving from irradiation, this percentage increases further especially after the higher dose. At this stage, the main pattern of localization of DNA replicon units was the spot reaction. After 24 hours, newly synthetized DNA was detected in just a small cellular pool (16.0 ± 1.5) in irradiated samples, while the controls were mainly negative.

Immuno-electron microscopy

The ultrastructural analysis showed several morphological changes compatible with the already reported necrotic, apoptotic and multinucleate patterns [3]. Most of the apparently viable cells displayed enough BrdU labelling to divide the S phase into three main stages (early S, middle S, late S), identified by the peculiar distribution of gold particles in the corresponding active DNA replicon units. As already described [17], early S was characterized by the labelling of interchromatin spaces (Fig. 2A), middle S by the labelling of the nucleolar periphery and of the interchromatin at the border between condensed and uncondensed chromatin (Fig. 2B) and late S by the labelling of peripheral condensed chromatin (Fig. 2C). Remarkable was the observation that in all apoptotic cells, isolated gold particles were localized to marginated or cap-shaped chromatin areas as well as within high electron-dense micronuclei containing compact chromatin that were mostly surrounded by a nuclear envelope (Fig. 2D).

As shown in Figure 3, the number of labelled cells changed with time of post-irradiation culture. After 1 hour, the three different patterns were present nearly in the same proportions in control and irradiated samples, while 18 hours post-irradiation, the middle-late S features were predominant, the labelling was diffuse and highly intense due to the presence of many clusters of gold particles and almost all the cells were labelled. After 24 hours, the labelling was hard to observe due to the very low number of gold particles. Apoptotic cells were all BrdU positive with labelling noted both in marginated chromatin (specifically in heterochromatin domains) and in apoptotic bodies. However, the amount of labelling was lower compared with that of viable cells (unpublished observations). Obviously, the three stages of S phase were not identified in these cells because of the strong nuclear rearrangements. In addition, necrotic cells displayed only non-specific labelling.

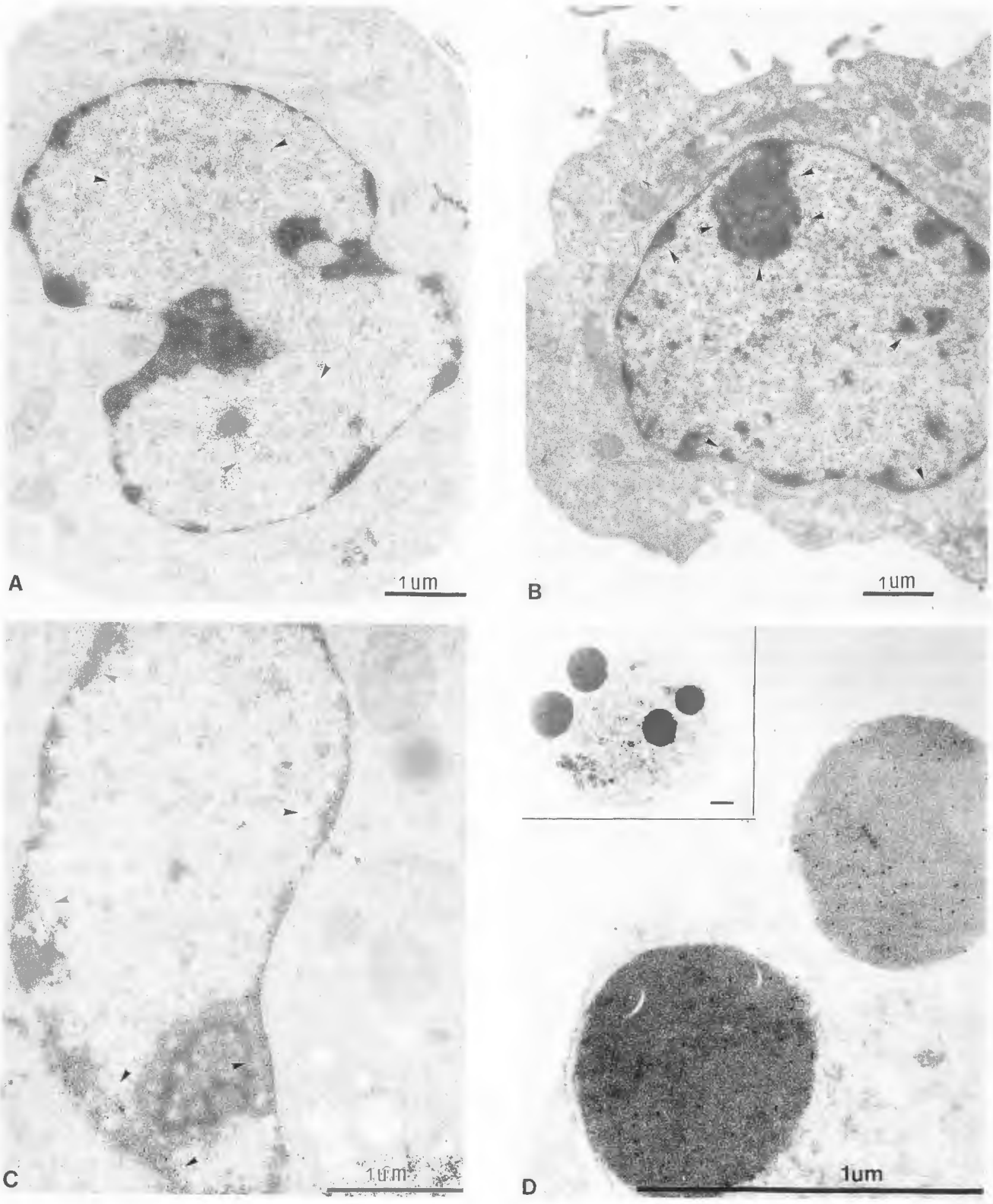


Figure 2. 15 Gy, 18 hours. (**A**) A diffuse labelling is observable on inner interchromatin regions (early S); (**B**) clusters of gold particles are visible at the border between heterochromatin and interchromatin (middle S); (**C**) BrdU labelling is evident in heterochromatin domains (late S); and (**D**) immunogold labelled micronuclei of an apoptotic cell (inset represents the whole apoptotic cell; inset bar = 2 μm). Arrowheads point at immunogold labelling. Bars = 1 μm.

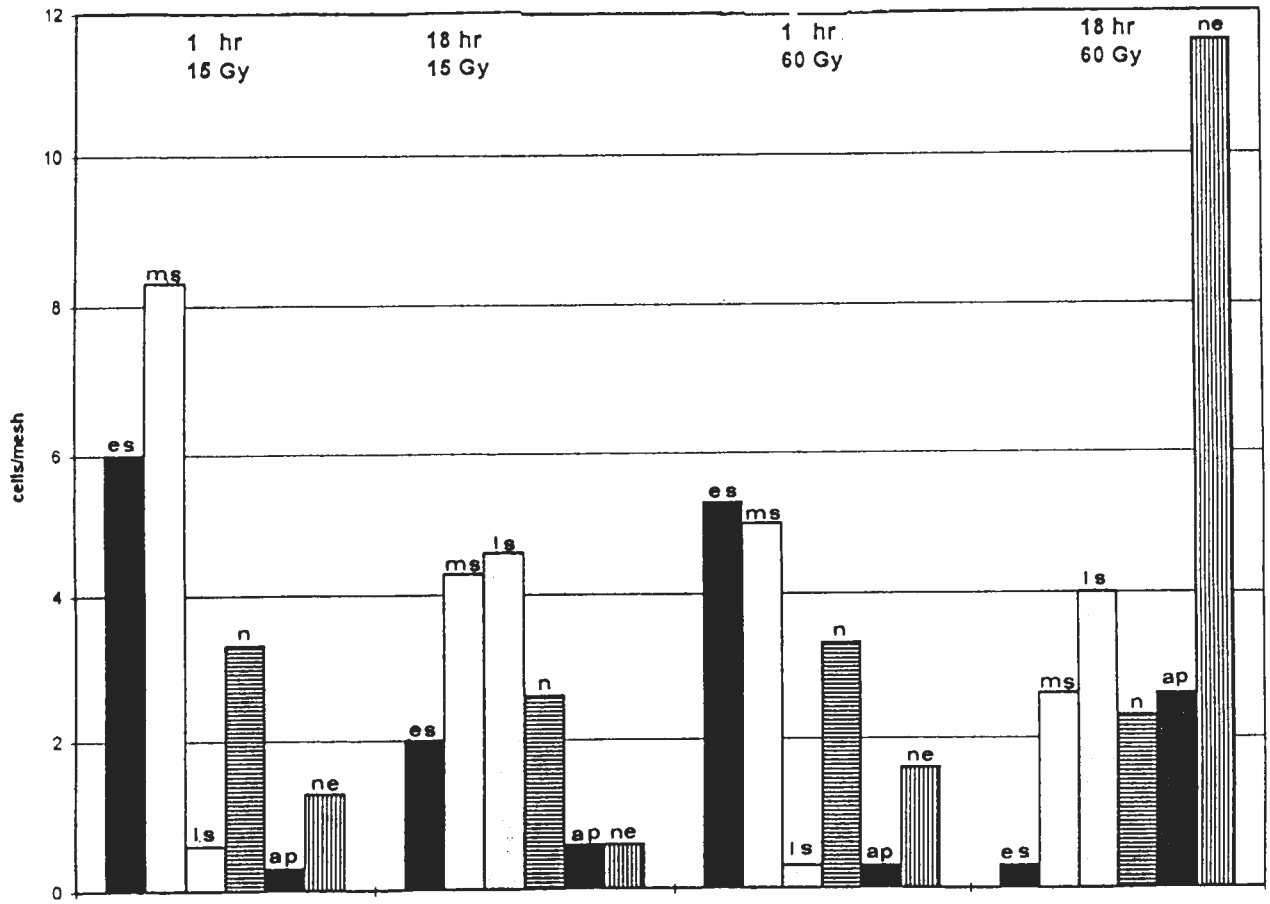


Figure 3. BrdU-antiBrdU immunogold technique in electron microscopy showing mean values of cells in early S phase (es), middle S phase (ms), late S phase (ls); n: negative cells; ap: apoptotic; ne: necrotic cells. Counting was performed on three different meshes on 200 mesh nickel grids (mean number of cells: 20).

Flow cytometry

As illustrated in Table 1, flow cytometry analysis showed alterations in cell cycle progression whatever the dose employed. After 1 hour, the percentage of cells within the different phases was similar to that of controls. After 18 hours, cells mostly accumulated in middle/late S. After 24 hours, nearly 88% of the cells were found in G1 phase, while 2% was detected in S phase. Moreover, the analysis of PI fluorescence revealed a small sub-diploid peak corresponding to cells in late apoptosis (nearly 15%) (Figs. 4A and AB).

Discussion

A morphological study of ionizing radiation-induced apoptosis and early and late DNA repair events was performed on MEL cells and related to cell cycle progression. The timing of observations (1, 18, and 24 hours)

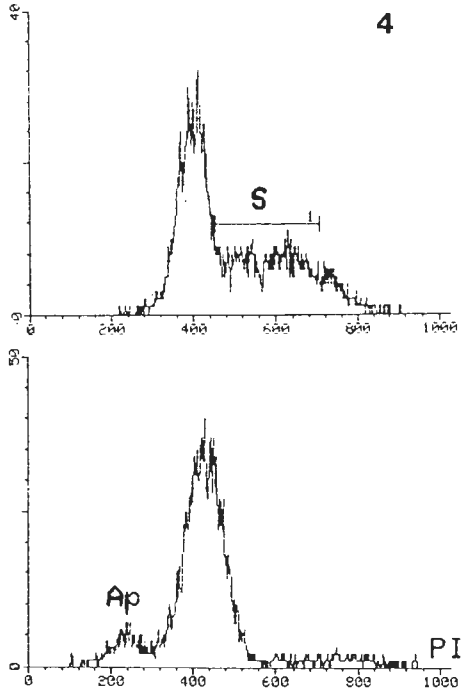


Figure 4. Flow cytometric analysis of PI (propidium iodide) fluorescence emission. Ap: subdiploid peak corresponding to apoptotic cells; S: S phase.

was chosen on the basis of MEL cell cycle length (24 hours) and on previous reports [17] and personal findings in relation to the crucial time for β DNA polymerase repair activity [1, 15]. The dose range was restricted to high doses (15 and 60 Gy) to induce severe damage within a great number of cells. Previous methods for the assessment of unscheduled or repair DNA synthesis were based on the principle of measuring cell survival after damage to DNA by low doses of gamma radiation [1] or by inhibiting scheduled DNA synthesis and then measuring the residual activity with [^{3}H]-TdR incorporation [10]. The morphological study of DNA replication sites has already been reported by a number of investigators [13, 18] and the dynamic localization of newly synthetized DNA obtained with anti-BrdU antibodies [18]. The recently developed BrdU immunogold method does not require DNA denaturation and allows the overcoming of the low resolution power of light microscopy, providing a good morphological preservation and a precise and reproducible localization of the incorporation sites. In spite of the high doses employed and the expected defects in DNA repair [11], new synthesis of DNA occurred in MEL cells and reached the maximal expression 18 hours after exposure, presumably when the β DNA polymerase repair enzyme had increased greatly, clearly overshadowing activity of the α replicative enzyme [15]. This result entitled us to ascribe to repair functions the sequential labelling of specific chromatin regions like the interchromatin spaces, followed by the heterochromatin domains. The presence of these three main patterns of localization was confirmed by the parallel analysis in flow cytometry, whose quantitative determinations had good correlations with both light and electron microscopy observations. More difficult to interpret, given the asynchronous model employed, was the finding of isolated clusters of gold particles 24 hours after the irradiation insult. This labelling was only detectable with the high resolution approach and in a synchronized model could represent single activated replicon units that have just started DNA replication [16]. At this stage, the β polymerase repair activity returns to control levels and is overridden by the α replicative activity in the same manner as after 1 hour post-irradiation [15]. Finally, remarkable was the finding of isolated clusters of gold particles inside areas of compact chromatin, like cap-shaped marginations or even micronuclei. To our knowledge, no data are yet available about the possible functions of compact chromatin areas of early and late stages of apoptosis. It has recently been stated [5] that, in the apoptotic nucleus, the marginated areas contain most of the nuclear DNA and that the DNA concentrated in these areas is cleaved prior to condensation, while nucleoli are rather unaffected by the rearrangements of the other nuclear domains. By means of flow cytometry, the incidence of apoptosis was related to cell cycle phase. A typical sub-diploid peak was detected after 24 hours, when high-dose radiation-induced apoptosis reaches a plateau [3] and late apoptotic stages are the most frequent. These data agree with previous reports showing the dependency of apoptosis on cell cycle events [6, 20]. The expression of apoptosis would not occur until cells reach the G1 phase of a new cycle after irradiation, when nuclear DNA degradation can take place, following possible defects in post replication repair [1]. It is not clear why the same *in vitro* system displayed such a discrepant behaviour with cells capable of DNA repair synthesis and cells probably unable to repair. It is also known that radiation-induced apoptosis is limited to cells genetically "programmed" to respond to environmental stimuli in this manner. Certainly, further investigations are needed to explain the increase in the fraction of irradiated cells in the S phase of the cell cycle and the apparent incorporation of BrdU into cells during the process of apoptosis. Hopefully, a synchronized *in vitro* system will be useful to clarify the role and relationship between the presence and distribution of DNA replicon sites, cell cycle events and apoptosis.

Acknowledgement

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Discussion with Reviewers

Reviewer II: Are there any changes in the presence and distribution of DNA replicon sites during, and/or in the early phase of, induction of apoptosis? What is the role of the described later changes of DNA replicon sites and cell cycle in apoptosis? What is the possible biological meaning of the BrdU labelling of micronuclei?

Author: In the early apoptotic cell, BrdU labelling is very low and not detectable, probably due to an impaired capacity to synthetize DNA. Moreover, the expression of apoptosis would not occur until cells reach the G1 phase of a new cycle after irradiation, when nuclear DNA degradation can take place, following possible defects in post replication repair. The BrdU labelling in micronuclei could be either the expression of the residual capacity of the nucleus to synthetize DNA, and so incorporate BrdU, or the result of a very quick progression towards the late phase of apoptosis in which the BrdU remain captured inside micronuclei.