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PHA-induced cell proliferation rescues human peripheral blood lymphocytes from X-ray-induced apoptosis

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Human peripheral blood G_0 lymphocytes were X-irradiated and allowed to recover for different periods both in the presence and absence of phytohemagglutinin (PHA). For each experimental condition the induction of apoptosis was investigated by nuclear morphology and formation of both DNA laddering and high molecular weight DNA fragments by pulsed field gel electrophoresis. The results showed that PHA cell growth stimulation could rescue peripheral blood lymphocytes (PBLs) from X-ray-induced apoptotic cell death. Instead, most X-irradiated lymphocytes held in G_0 phase, once they were committed to apoptosis, inexorably executed the process. These data indicate that the proliferative status of PBLs can influence apoptotic cell death: PHA-stimulated PBLs appear to be more radioresistant as a result of a less efficient apoptotic process. Therefore, in current tests for mutagenicity or cytotoxicity and in biodosimetrical studies the possible role of apoptosis has to be considered.

Introduction

The ability of cells to maintain genome integrity is critical to ensure the long-term survival of the individual. Repair, growth arrest and cell suicide through apoptosis are therefore all possible strategies in response to DNA damage, although each different event will depend on cell type, location, environment and extent of damage.

Apoptosis may be the prudent option in heavily damaged cells that retain substantial replicative potential and therefore constitute a neoplastic risk. Investigation of the factors regulating the apoptotic cell death programme is important both to optimize cancer therapy and to design strategies to improve therapeutic outcomes. Furthermore, the role of apoptosis has to be taken into account to correctly evaluate the DNA damage induced by cell exposure to physical and chemical agents. The outcome of genotoxic damage in cells that are resistant to apoptosis will be entirely different from that in cells that are prone to apoptosis.

Apoptosis is a controlled form of cell death characterized by early morphological signs of chromatin condensation and fragmentation, followed by membrane blebbing and formation of apoptotic bodies (Kerr *et al*., 1972; Wyllie *et al*., 1980; Khodarev *et al.*, 1998). At the biochemical level, the apoptotic process is characterized by a number of different events, including intense nucleolytic activity (McConkey *et al.*, 1990; Salvesen and Dixit, 1997; Masato *et al.*, 1998; Thornberry and Lazebnik, 1998), yielding internucleosomal DNA fragments of 180–200 bp which form a peculiar ladder pattern when separated by conventional agarose gel electrophoresis (Zhivotovsky *et al.*, 1994). This event is temporally and hierarchically preceded by the so-called large scale DNA cleavage that leads to formation of 50–300 kb DNA fragments that can only be detected by pulsed field gel electrophoresis (PFGE) (Filipski *et al.*, 1990; Cohen *et al.*, 1994; Walker *et al.*, 1994). Since it is still unclear which type of DNA fragmentation is the best marker of apoptosis (Oberhammer *et al.*, 1993a,b; Falcieri *et al.*, 1993), DNA degradation remains a good indicator of apoptosis when combined with morphological characterization.

Ionizing radiation is known to induce a variety of DNA lesions. DNA double-strand breaks (DSB) are considered to be the major DNA lesion, which if not repaired or mis-repaired is most likely to lead to cell death (Iliakis, 1991). Thus, DSB should play an important role in radiation-induced apoptosis and in radiation sensitivity (Radford, 1991; Warters, 1992; Olive, 1998); accordingly, cells defective in repair of DSB are sensitive to ionizing radiation (Zdzienicka, 1995).

Radiobiologists often distinguish between radiation-induced cell death with regard to the ability to enter mitosis, i.e. interphase or mitotic cell death. Interphase cell death occurs before entry of the cells into the next division and also occurs in non-cycling cells (Umansky *et al.*, 1981; Yamada and Ohyama, 1988; Shinohara and Nakano, 1993), while mitotic cell death occurs one or even several divisions after irradiation and is characteristic of actively proliferating cells (Radford and Murphy, 1994; Radford *et al.*, 1994). Interphase and mitotic cell death were both shown to involve apoptosis (Tauchi and Sawada, 1994).

It has long been known that culture conditions, cell cycle position and differentiation stage can influence human lymphocyte sensitivity to radiation-induced cell death and, in particular, addition of mitogens can enhance the radioresistance of these cells (Lowenthal and Harris, 1985; Stewart *et al.*, 1988). Some authors have shown that mitogen-stimulated cells immediately after irradiation have a higher frequency of chromosome aberrations than cells resting in G_0 phase before addition of mitogens (Santos Mello *et al.*, 1974) and data in the literature report that radiation-induced cell death was reduced by mitogens (Schrek and Stefani, 1964; Sato, 1970; Meijer *et al.*, 1999). A mechanistic explanation is given by Holmstrom *et al.* (1998), who showed that elevation by PHA of the mitogen-activated protein kinase (MAPK) cascade is a negative regulator of Fas-mediated apoptosis.

The present study has the aim of investigating whether X-ray-induced apoptosis is modified during the process of phytohemagglutinin (PHA)-induced cell proliferation, comparing the extent and kinetics of apoptotic DNA fragmentation during apoptotic cell death in quiescent and proliferating human peripheral blood lymphocytes (PBLs). These cells represent the most common tissue for biological dosimetry

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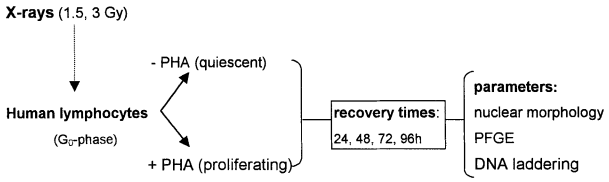

Fig. 1. Experimental scheme. G_0 lymphocytes, isolated from fresh peripheral blood, were exposed to 1.5 or 3 Gy X-rays and allowed to recover for different periods either in the presence or absence of PHA. For each experimental condition the nuclear morphology and the formation of both DNA laddering and HMW DNA fragments were analysed.

studies, therefore, among other factors, induction of apoptosis could negatively affect estimation of radiation exposure both *in vitro* after PBL stimulation and after *in vivo* exposure, where most cells are quiescent.

Materials and methods

Preparation of cells

Lymphocytes were obtained from blood banks and peripheral blood mononuclear cells were isolated by separation in Histopaque 1077 (Sigma) from freshly collected buffy coats. Diluted blood was carefully layered onto the Histopaque 1077 and centrifuged at 400 *g* for 30 min. After centrifugation the opaque interface containing mononuclear cells was collected and washed with phosphate-buffered saline (PBS). To reach the high number of cells needed to perform the experiment, isolated lymphocytes from two or three donors were pooled. After isolation, lymphocytes were seeded at a cell density of 1×10^6 cells/ml in Ham's F10 medium (Bio-Whittaker), 2 mM L-glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin and 20% heat-inactivated fetal calf serum (Bio-Whittaker) and incubated at 37°C in a 5% CO_2 atmosphere at 80% humidity.

Irradiation procedure and experimental protocol

The experimental protocol is shown in Figure 1. G_0 lymphocytes were X-irradiated at 37°C with a 250 kV and 6 mA Gilardoni MGL200/8D X-ray apparatus, at a dose rate of 60 cGy/min. Cells were exposed in complete medium without PHA (Wellcome) to 1.5 and 3 Gy. Immediately after irradiation half of the cell cultures were resuspended in growth medium supplemented with 2% PHA to induce cell proliferation and the other half were kept in medium without PHA. Then the cells were allowed to recover for periods of 24, 48, 72 and 96 h and for each experimental condition the nuclear morphology, the formation of DNA laddering by conventional agarose gel electrophoresis and high molecular weight (HMW) DNA degradation were analysed by PFGE. The experiment was repeated three times.

Analysis of nuclear morphology

For morphological analysis $3–5\times10^5$ cells were fixed in 4% paraformaldeyde. Cells were then seeded on a gelatinized slide, stained with 0.1% Mayers hematoxylin (Sigma) and analysed by optical microscopy. Apoptosis was quantified by scoring cells with condensed and fragmented nuclei. In random selected fields 500 cells from PBLs from three donors were scored at a magnification of 1000×. For each experimental condition the data were evaluated statistically by the χ^2 test.

Conventional agarose gel electrophoresis

To analyse internucleosomal DNA fragmentation, 2.5×10^6 lymphocytes were processed as described by Zhivotovsky *et al.* (1993) with minor modifications. The cell suspensions were mixed with an equal volume of ice-cold lysis buffer (10 mM Tris–HCl, pH 8.0, 20 mM EDTA, 0.5% Triton X-100) and incubated at 4°C for 20 min. The resulting suspension was centrifuged at 12 000 r.p.m. for 15 min to separate cell debris containing intact chromatin (pellet) from DNA fragments (supernatant). The DNA from supernatant was ethanol precipitated followed by RNA digestion with RNase A (200 µg/ml) (Sigma) and subsequently incubated with proteinase K (100 µg/ml) (Sigma). DNA samples were loaded onto a 1.5% neutral agarose gel, subjected to electrophoresis (60 V for 1 h) in 1× TAE buffer (40 mM Tris–acetate, 10 mM EDTA) and photographed using Polaroid 667 positive film under UV (312 nm) illumination.

Pulsed field gel electrophoresis

Agarose plugs were prepared as previously described (Zhivotvsky *et al.*, 1994). Briefly, 6×10^5 lymphocytes were resuspended in 50 µl of a solution containing 0.15 M NaCl, 2 mM KH_2PO_4/KOH, pH 6.8, 1 mM EGTA and 5 mM $MgCl_2$. An equal volume of liquefied 1% low melting point agarose was added to the cell suspension and the mixture was aliquoted into gel plug casting forms. The resulting agarose blocks were placed in lysis buffer (10 mM NaCl, 10 mM Tris–HCl, pH 9.5, 25 mM EDTA, 1% laurylsarcosine) supplemented with proteinase K (1 mg/ml) and incubated at 50°C for 24 h. The agarose blocks were then introduced onto a 1% agarose gel and contour clamped homogeneous electric field electrophoresis was performed using a Gel Navigator horizontal chamber and Pulsator 2015 controller (Pharmacia LKB). Total run time was 19 h at 180 V at 9°C in 0.5× TBE (45 mM Tris, 1.25 mM EDTA, 45 mM boric acid, pH 8.0). Pulses applied were 180 s for 4 h, 90 s for 3 h, 45 s for 6 h and 20 s for 6 h. After the run the gel was stained with ethidium bromide (1 µg/ml) (Sigma), visualized under a 312 nm light source and photographed using Polaroid 667 positive film.

Results

The results presented in this work are representative of three repeated experiments, all showing the same trend and good reproducibility.

Figure 2 shows the data derived from nuclear morphology analysis of control and X-irradiated cells after recovery periods of 24, 48, 72 and 96 h, both in the presence and absence of PHA. In the control sample the yield of apoptotic cells was enhanced with time. In particular, in proliferating lymphocytes the apoptotic cell death percentage was higher and statistically significant at all sampling times except at 24 h, when compared with quiescent lymphocytes. In X-irradiated samples the nuclear morphology evaluation showed a higher induction of apoptosis after X-ray treatment in quiescent cells when compared with proliferating lymphocytes. In fact, apoptotic cell death decreased following direct exposure to medium containing PHA. The effect was statistically significant at recovery times of 48 and 72 h in 1.5 Gy X-irradiated lymphocytes, while in cells exposed to a higher dose (3 Gy) a significant difference in apoptosis induction was already evident at 24 h recovery time. When the nuclear morphology was analysed at 96 h after X-irradiation the incidence of apoptosis was the same in both proliferating and quiescent lymphocytes, as well as in proliferating control cells, when compared with X-irradiated ones.

Moreover, there was no cell death dose–response effect induced by X-rays in proliferating lymphocytes when compared with control cells, while it was present in quiescent ones.

The analysis performed by conventional agarose gel electrophoresis only detected 180–200 bp internucleosomal DNA fragments at 48 h recovery time in quiescent lymphocytes at both X-ray doses (Figure 3).

Figure 4 shows an example of the formation of HMW DNA fragments analysed by PFGE on the same cell cultures. In each agarose plug precisely the same number of cells was embedded and the repeated experiments showed good reproducibility. PFGE (Figure 4A) revealed DNA fragmentation only at the 72 and 96 h recovery times in control proliferating lymphocytes and no degradation at all in control quiescent cells. In quiescent X-ray-treated lymphocytes (Figure 4B) a progressive increase at later times of DNA fragments with a molecular size corresponding to 50 kb and their further cleavage to lower molecular weight fragments was detected. The PFGE results obtained with proliferating X-irradiated cells (Figure 4B) at the 48 and 72 h recovery times showed a decrease in the 50 kb fragments coupled with formation of HMW DNA with a more varying molecular size range than the typical 300 kb. The pattern of DNA degradation did not reveal qualitative differences between the two X-ray doses used. At the 96 h recovery time the control proliferating lymphocytes showed a HMW DNA fragmentation similar to proliferating X-ray-treated cells.

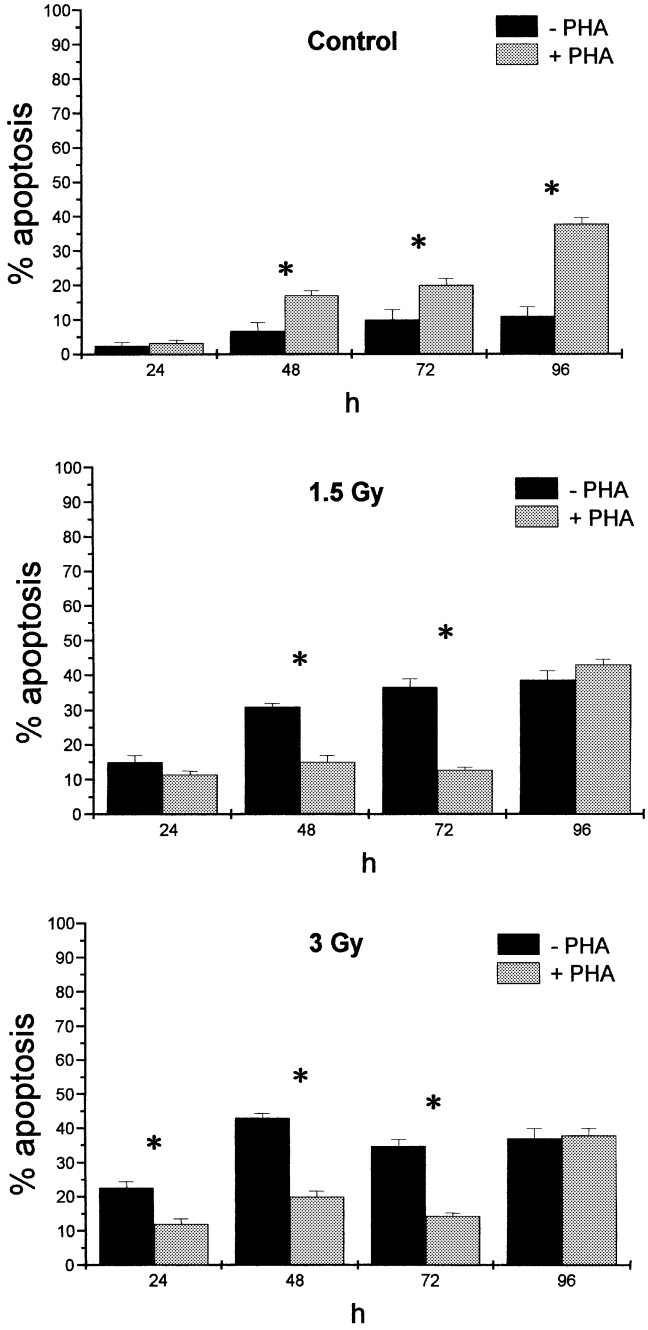

Fig. 2. Analysis of nuclear morphology. Percentage of apoptotic nuclei in proliferating (+PHA) and quiescent (–PHA) PBLs at different times of recovery after X-irradiation. Data points represent the means ± SEM of three independent experiments unless otherwise indicated. *, Values compared between the +PHA and –PHA PBLs are statistically significant (χ^2 test, $P < 0.01$).

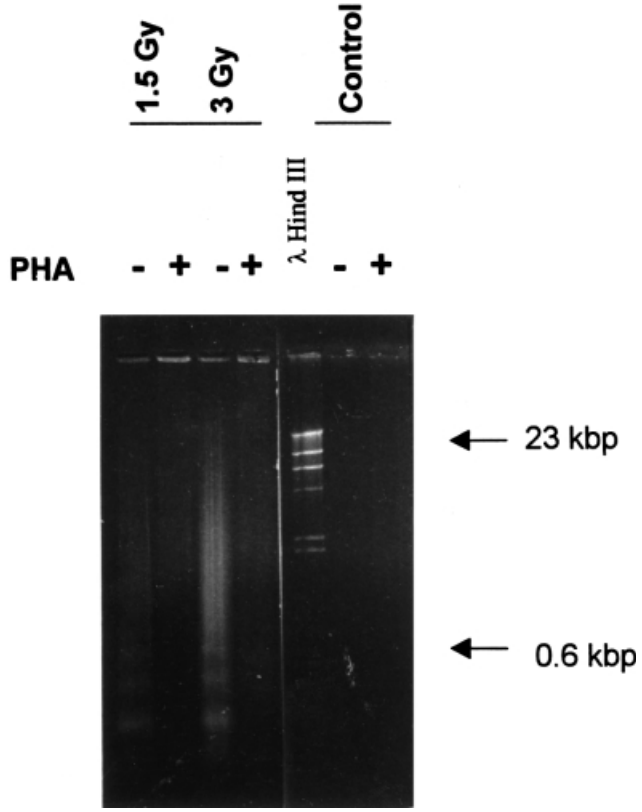

Fig. 3. Agarose gel electrophoresis. Internucleosomal DNA fragmentation (DNA laddering) in proliferating (+PHA) and quiescent (–PHA) PBLs at 48 h recovery after X-irradiation with 1.5 or 3 Gy. DNA size marker, *Hin*dIII-digested λ DNA. The photograph is an example of the results obtained in three repeated experiments showing good reproducibility and comparable outcomes.

Discussion

The aim of this work was to investigate whether X-ray-induced apoptosis in human PBLs could be influenced by PHA-induced cell proliferation, with particular regard to DNA fragmentation.

X-ray-induced apoptosis was reduced in proliferating PBLs, where no cell death dose–response effect was observed, when compared with control cells (Figure 2). Possibly, apoptotic signals may not be generated by specific types or amounts of DNA lesions but could depend on the relative persistence of DNA damage (i.e. irreparable or slowly repaired DNA lesions) (Cregan *et al.*, 1999). This hypothesis could explain the inexorable commitment to apoptosis of X-irradiated lymphocytes held in G_0 phase, as they probably carry persistent DNA lesions.

These conclusions are derived from the data obtained with the nuclear morphology and the DNA laddering analyses, both supporting the hypothesis that proliferating lymphocytes were rescued from cell death. In a recent paper Holmstrom *et al.* (1998) showed suppression of Fas/Apo-1-mediated apoptosis by MAPK signalling. This suggests that PHA-induced protection is most probably linked to PHA stimulation of an anti-apoptotic programme rather than to proliferation itself.

Previous work has already revealed such a mitogen protection effect, but only in terms of an increase in both cell viability and chromosomal aberrations (Harms-Ringdahl *et al.*, 1996; Holmberg *et al.*, 1998; Meijer *et al.*, 1999). The authors suggested that mitogen stimulation of irradiated cells may lead to increased survival at the expense of genomic fidelity and therefore gives rise to a conflict between growth stimulation and radiation-induced growth arrest.

In the present study these hypotheses are reinforced by the analysis of HMW DNA fragment degradation through PFGE. In fact, proliferating X-ray-treated lymphocytes showed HMW DNA fragmentation that did not completely reflect the typical apoptotic DNA degradation pattern and that could derive from DNA repair or mis-repair events, eventually giving rise to cytogenetic damage (Evans, 1977). Our PFGE data indicate that X-ray-induced DSBs involved in mis-repair events could

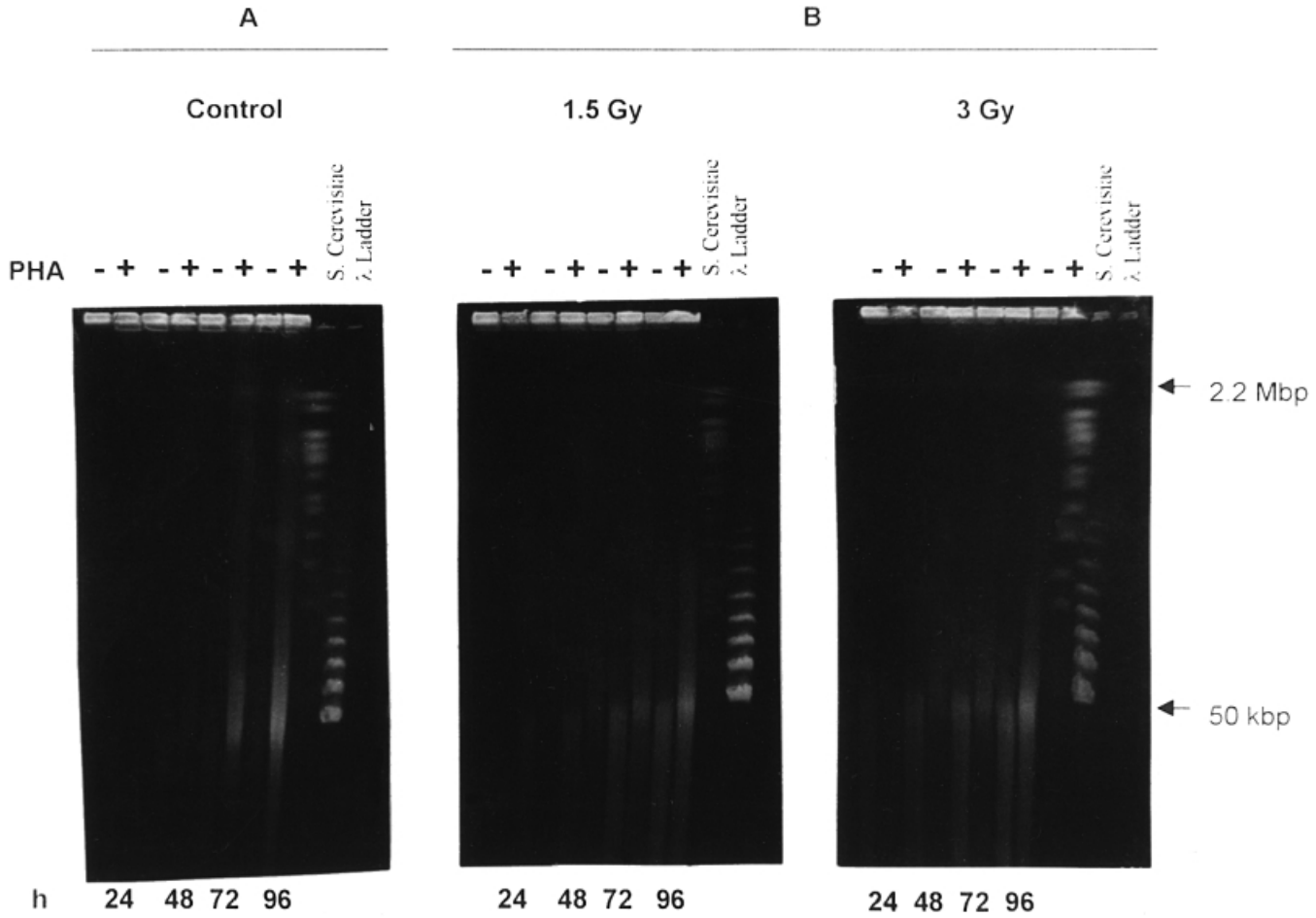

Fig. 4. PFGE. Formation of HMW DNA fragments in proliferating (+PHA) and quiescent (–PHA) PBLs at different recovery times after X-ray exposure. (**A**) Control. (**B**) X-ray-treated PBLs. DNA size markers, yeast chromosomes (2.2 Mb–0.225 kb) and λ ladder (50–1000 kb). The photographs are examples of the results of three repeated experiments showing good reproducibility and comparable outcomes.

reseal in HMW DNA fragments corresponding to a more varied molecular size range than the 300 kb DNA degradation exclusive to the apoptotic process.

In contrast, in quiescent X-irradiated lymphocytes DNA degradation revealed the formation of fragments with a molecular size corresponding to 50 kb, the typical hallmark of apoptosis (Oberhammer *et al*., 1993b), and their further cleavage to lower molecular weight DNA.

Moreover, proliferating treated lymphocytes and control stimulated cells showed similar PFGE DNA degradation patterns at the latest recovery times. The lack of difference in DNA fragmentation could derive from two distinct and independent events: on the one hand, processing of X-ray-induced DNA damage and, on the other, prolonged post-irradiation proliferating culture conditions itself producing DNA degradation. The last effect was clearly evident in the PFGE DNA fragmentation pattern of control proliferating lymphocytes. In agreement with this conclusion, in control stimulated lymphocytes apoptotic cell death was enhanced as recovery time increased, as usually observed in primary cell cultures which undergo the process of senescence and degeneration (Hayflick, 1965; Harman, 1991). Moreover, the mitogen protection effect was absent in irradiated cells at 96 h recovery time. In fact, DNA degradation analysis performed by conventional agarose gel electrophoresis no longer revealed internucleosomal DNA cleavage but a smear (data not shown), probably due to secondary necrosis.

Nevertheless, the appearance of 180–200 bp internucleosomal DNA fragments does not seem to be a necessary event for execution of the apoptotic process since it can be cell type- or agent-specific (Marini *et al.*, 1996). In particular, our data indicated that PBLs did not show DNA laddering until they reached ~30–35% apoptotic morphological changes, as confirmed by other authors (Marini *et al*., 1996). However, the application of PFGE to the study of apoptosis remains a more valid and informative parameter since it shows the formation of 50–300 kb DNA fragments as an invariant feature of early apoptotic stages, independent of the cell type and the apoptotic stimulus (Filipski *et al.*, 1990; Beere *et al.*, 1995). Furthermore, HMW DNA fragmentation precedes formation of apoptotic morphology, as demonstrated by time–course experiments (Oberhammer *et al.*, 1993b; Cohen *et al.*, 1994).

The overall results confirmed, at the biochemical level, how the culture conditions, in particular the presence of factors stimulating progression of the cell cycle, could increase human lymphocyte radioresistance in terms of a reduced rate of cell death. The proliferative status of the cells probably interferes with repair processes in terms of enhanced repair capability of proliferating cells when compared with the level of basal transcription of resting ones (Obe *et al*., 1980; Obe and Beek,

1984). In this context, some DNA repair pathways have been demonstrated to be regulated as a function of cell proliferation and, in particular, increased DNA repair activity in stimulated PBLs irradiated with ionizing radiation has been described (Lavin and Kidson, 1977; MacWilliams *et al.*, 1983).

The data presented here indicate that a less efficient apoptotic process can cause radioresistance. Consequently, it is to be expected that cells carrying lesions would survive even if the risk of persistence of mutated cells and of proliferation of pre-cancer cells was increased.

Efficient execution of the apoptotic process could also affect the outcome of biological dosimetry studies since some circulating G_0 lymphocytes exposed to *in vivo* irradiation might be removed by this cell death mechanism. Thus, chromosomal aberration frequency could be affected in relation to the time lapse between exposure to the clastogenic agent and conventional PBL cytogenetic analysis. Therefore, the role of apoptosis has to be considered in current mutagenicity and cytotoxicity testing.

In general, a detailed knowledge on how the radiation-induced apoptotic process can be counteracted by proliferating agents (Berchem *et al*., 1995; Wang and Hang, 1995; Kiess and Gallaher, 1998) may serve to give us a better understanding of the origin of intrinsic differences in radiosensitivity in terms of chromosomal aberrations and apoptosis, as well as to reveal the genetic control of the process. The possible anti-apoptotic action of agents in the environment may have a serious impact on the removal of cells with damaged and/or eroded genetic information.

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