Gamma Irradiation Results in Phosphorylation of p53 at Serine-392 in Human T-Lymphocyte Leukaemia Cell Line MOLT-4

(phosphorylation / p53 / radiation-induced apoptosis)

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Abstract. Exposure of human leukaemia MOLT-4 cells to ionizing irradiation led to apoptosis, which was detected by flow cytometric analysis and degradation of the nuclear lamina. The multiple signalling pathways triggered by either membrane or DNA damage play a critical role in radiation-induced apoptosis. The response to DNA damage is typically associated with the p53 protein accumulation. In this study, we proved that the transcriptionally active p53 variant occurs in the MOLT-4 cells and its abundance alteration is triggered in the γ-irradiated cell population concomitantly with phosphorylation at both the serine-392 and serine-15 residues. The p21 upregulation followed the p53 phosphorylation process in irradiated MOLT-4 cells.

Apoptosis, a form of controlled cell death, represents a major regulatory mechanism in embryonal development, growth and differentiation (Wyllie et al., 1980). Apoptosis in haematopoetic cells can be induced by a variety of stimuli such as ionizing radiation. The mitochondrial pathway leading to apoptosis is activated in response to extracellular cues and internal insults such as DNA damage (Hengartner, 2000). The activation of ATM kinase by autophosphorylation is evidently an initiating event in cellular response to irradiation (Bakkenist and Kastan, 2003).

Human T-lymphocyte leukaemia MOLT-4 cells are highly radiosensitive and die by apoptosis after exposure to ionizing irradiation (Nakano and Shinohara, 1994). Another group of authors (Endlich et al., 2000) verified apoptosis induction after irradiation of MOLT-4 cells by anexin V binding, TUNEL assay and formation of DNA ladders in agarose gels. Computerized video time-lapse microscopy of X-irradiated MOLT-4 cells demonstrated that these cells exhibit a wide disparity in the timing of induction and execution of radiation-induced cell death that included rapid interphase apoptosis, delayed apoptosis and postmitotic apoptosis. Coelho et al. (2000) examined cell death in X-irradiated MOLT-4 cells pretreated with Ac-DEVD-CHO, an inhibitor of caspase-3-like activity. This inhibition prevented internucleosomal DNA fragmentation and partially the externalization of phosphatidylserine. However, it did not affect the cell survival of irradiated MOLT-4 cells. Instead, these cells treated by the inhibitor exhibited characteristics of necrotic cell death.

The accumulation of p53 as a response to DNA damage was described in irradiated MOLT-4 cells (Zhao et al., 1999). The p53 protein is stabilized and accumulated in the nucleus after exposure to DNA-damaging agents. The functional activity of p53 is effectively regulated by post-translational modifications such as phosphorylation after exposure to stress signals (Kastan et al., 1991; Lakin and Jackson, 1999). While phosphorylation at serine-15 leads to p53 stabilization (Shieh et al., 1997), phosphorylation at serine-392 increases the p53-specific DNA binding as a prerequisite for its transcriptional activity (Hupp and Lane, 1994). Lu et al. (1998) have shown that murine p53 is selectively phosphorylated at serine-389 (homologue of serine-392 in human p53) after UV but not after γ-irradiation. There

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Abbreviations: 1-D – one-dimensional, 2-D – two-dimensional, DTT – dithiothreitol, FASAY – functional analysis of separated alleles in yeast, IPG – immobilized pH gradient, PVDF – polyvinylidendifluoride.

is still controversy regarding the role of the p53 molecule in radiation-induced MOLT-4 cell apoptosis. A recent study demonstrated p53-independent apoptosis in X-irradiated MOLT-4 cells (Inanami et al., 1999). On the contrary, Nakano et al. (2001) indicated that X-ray-induced apoptosis in MOLT-4 cells is fully p53-dependent.

In this report, we examined the effect of ionizing radiation on the induction of apoptosis in MOLT-4 cells and then we evaluated radiation-induced alterations at the p53 level and its phosphorylation status. The transcriptional p53 activity was verified by FASAY (functional analysis of separated alleles in yeast) and indirectly via modulation of the p21 expression.

Material and Methods

Equipment and chemicals

Immobiline Dry-Strips with nonlinear pH gradient 3–10 (18 cm), pharmalytes pH 3–10 were from Amersham Pharmacia Biotech (Uppsala, Sweden) and 6000 V power supply was from Serva (Heidelberg, Germany). Dithiothreitol (DTT), urea, CHAPS and acrylamide were purchased from USB (Cleveland, OH). TRIS base, n-octyl-β-D glucopyranoside, sodium ortho-vanadate, trichloroacetic acid, ampholytes pH 9–11, thiourea, SB 3–10, sodium dodecylsulphate (SDS) and iodoacetamide were from Sigma (St. Luis, MO). 1, 6-Bis(acryloyl)piperazine, TEMED and ammonium persulphate were purchased from Bio-Rad (Richmond, CA). Polyvinylidendifluoride (PVDF) membrane and Complete ^{TM}Mini were from Boehringer (Mannheim, Germany). Tributylphosphine was from Fluka (Steinheim, Switzerland). Monoclonal antibodies against human p53 (DO1, DO11 and FP31.1) were supplied by Dr. Vojtěšek (Masaryk Memorial Cancer Institute Brno, Czech Republic); p21 monoclonal antibody was purchased from BD Transduction Laboratories (San Jose, CA), phospho-p53 (Ser15) antibody was from Cell Signalling Technology (Beverly, MA) and anti-lamin B antibody was from Oncogene Research Products (Cambridge, MA).

Cell culture and gamma irradiation

Human T-lymphocyte leukaemia MOLT-4 cells (American Type Culture Collection, University Blvd. Manassas, VA) were maintained in Iscove's modified Dulbecco medium (Sigma) supplemented with 20% foetal calf serum (GibcoBRL, Grand Island, NY). Culture conditions were 37°C in a humidified atmosphere buffered by 5% CO_2. The exponentially growing cells were irradiated at room temperature using a ^{60}Co γ-ray source with a dose rate of 0.66 Gy/min. MOLT-4 cells were irradiated with a single dose of 7.5 Gy.

In vitro clonogenic assay

The cell survival was measured by the clonogenic assay. The untreated control (10^2/ml) and irradiated (10^2–10^5/ml) MOLT-4 cells were grown in 0.9% methylcelulose in Iscove's modified Dulbecco medium with 30% foetal calf serum. After 14 days, colonies with > 40 cells were counted. Three independent experiments were performed.

FASAY

FASAY was performed using the procedure described by Flaman et al. (1995) with several modifications described earlier (Smardova et al., 2001). Briefly, harvested cells were immediately homogenized in RLT lysis buffer (Qiagen Inc.) and total RNA was purified using RNeasy Mini Kit (Qiagen Inc.). cDNA was synthesized by SuperScript II (Life Technologies Inc.) using oligo(dT)$_{12}$ as a primer. The central part of the *p53* gene (exons 4–10) was amplified by PCR. PCR was performed using primers P3 (5'-CCT-TGC-CGT-CCC-AAG-CAA-TGG-ATG-AT-3') and P4 (5'-ACC-CTT-TTT-GGA-CTT-CAG-GTG-GCT-GGA-GT-3') and Pfu DNA Polymerase (Stratagene). Yeast cells were co-transformed with the PCR product, linearized pSS16 vector, and the salmon sperm DNA carrier (GibcoBRL) by the lithium acetate procedure, as described by Ishioka et al. (1993). Transformed yeast cells were plated on a minimal medium without leucine and with a low amount of adenine (5 µg/ml), followed by incubation for 2–3 days at 35°C and then for 2–3 days at room temperature.

1-D and 2-D gel electrophoresis

Whole cell extracts were prepared by lysis in 500 µl of lysis buffer (137 mM NaCl, 10% glycerol, 1% n-octyl-β-D glucopyranosid, 50 mM NaF, 20 mM Tris pH 8, 1 mM Na_3VO_4, Complete ^{TM}Mini). Nuclear and cytoplasmic extracts were prepared using a kit from Pierce. Protein concentration was determined by a modified bicinchoninic acid (BCA) assay (Brown et al., 1989). The cell extracts were resuspended in Laemmli's sample buffer and denaturated by heating. Equal amounts of proteins (30 µg) were loaded into each lane of a polyacrylamide gel.

For 2-D electrophoretic separation, MOLT-4 cells were lysed as described above. Extracted proteins were then precipitated overnight in 20% trichloroacetic acid in acetone (-18°C) containing 0.2% DTT (Görg et al., 1997) and then solubilized in buffer for isoelectric focusing - IEF (9 M urea, 4% CHAPS, 70 mM DTT and 2% carrier ampholytes pH 9–11). Immobilized pH 3–10 gradient (IPG) gels were used for the first dimension-isoelectric focusing. Commercial strips were swollen overnight in a rehydration buffer containing 2 M thiourea, 5 M urea, 2% CHAPS, 2% SB 3–10, 2 mM tributyl phosphine, 40 mM Tris base and 0.5%

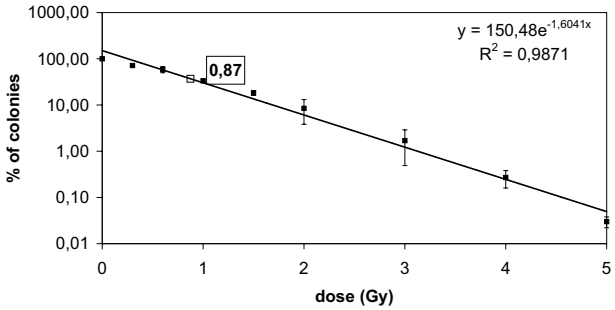

Fig. 1. The dose-response curve for loss of clonogenity in MOLT-4 cells exposed to γ-rays. Surviving fractions are presented as a mean value from three experiments. Colonies with > 40 cells were counted after 14 days.

Ampholine pH 3–10. The IPG separation was carried out overnight using a Multiphor II apparatus (Amersham Pharmacia Biotech). Immediately after being focused, the IPG gels were equilibrated in 6 M urea, 2% SDS, 50 mM Tris-HCl, pH 6.8, 30% v/v glycerol and 1% DTT, then in the same solution except that DTT was replaced by 5% w/v iodoacetamide. In the second dimension gradient, 9–16% SDS polyacrylamide gels were used.

Western blotting

After 1-D or 2-D gel electrophoresis, the proteins were transferred to PVDF membranes. The membranes were blocked in Tris-buffered saline containing 0.1% Tween 20 and 4% non-fat dry milk and then incubated with anti-p53 (DO1+DO11, mixture 1 : 1), anti-$p53^{Ser392}$ (FP31.1), anti-$p53^{Ser15}$, anti-p21 and anti-lamin B. After washing, the blots were incubated with secondary peroxidase-conjugated antibody (Dako, High Wycombe, UK) and then the signal was developed with a chemiluminescence (ECL) detection kit (Boehringer, Mannheim, Germany).

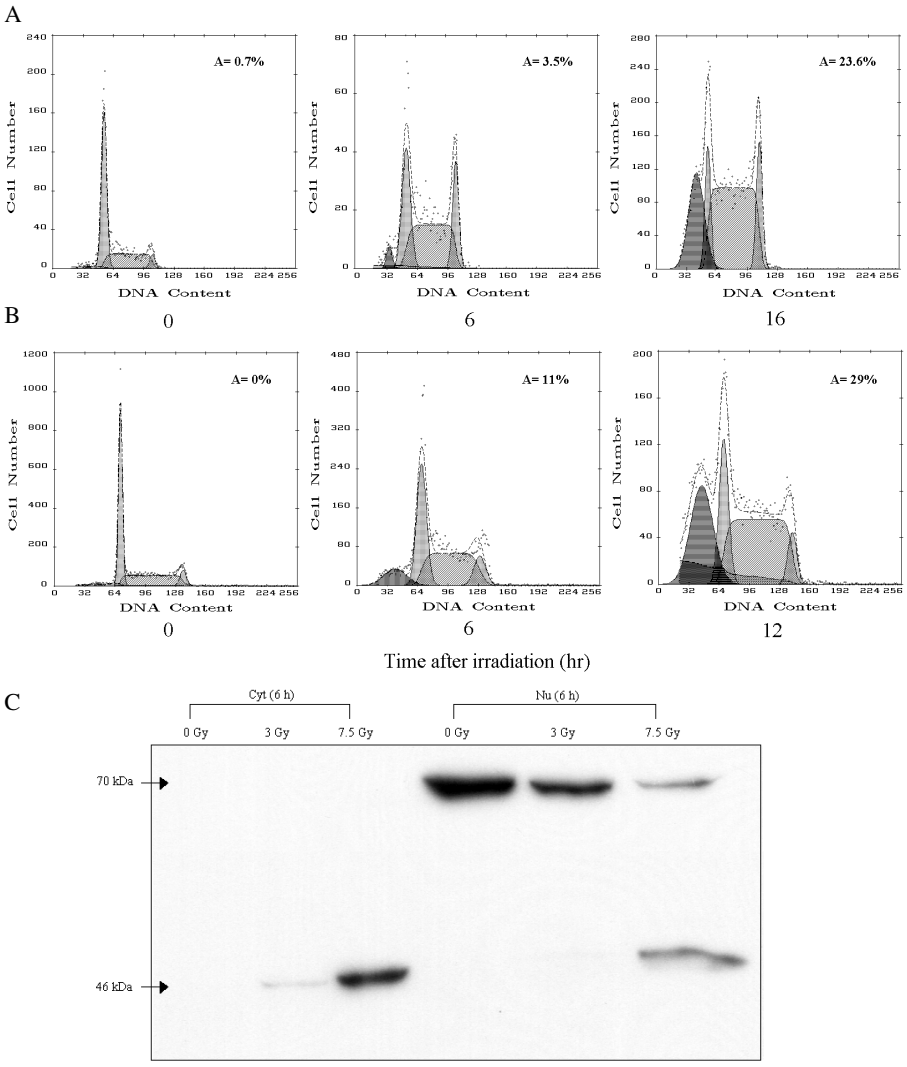

Fig. 2. Flow-cytometric analysis of the cell cycle of MOLT-4 cells at various times after exposure to 3 (A) and 7.5 Gy (B). Apoptotic cells were identified as cells with sub-diploid DNA content, i.e. sub-G_1 peak. (C) Western blot analysis of lamin B cleavage 6 h after MOLT-4 cell irradiation with 3 and 7.5 Gy. Nuclear (Nu) and cytosol (Cyt) fractions were isolated with NE-PER reagents.

Flow cytometry

Flow cytometry was used for cell cycle analysis, as described by Marekova et al. (2000). Briefly, the cells were fixed in 70% ethanol and stained with propidium iodide in Vindelov's solution. Fluorescence (DNA content) was measured using Coulter Electronic (Hialeah, FL).

Results

Cell survival and FASAY

Figure 1 shows the results of a clonogenic assay of MOLT-4 cells exposed to γ-rays. The D_0 value (the dose required to reduce colony survival to 37%) was 0.87 Gy.

FASAY determined less than 10% of red colonies, suggesting the expression of only the functional p53 protein in the MOLT-4 cell line.

Detection of apoptosis

To detect apoptosis in MOLT-4 cells we measured the sub-diploid DNA content after exposure to 3 and 7.5 Gy (Fig. 2A, 2B) and the proteolytic fragments of lamin B (Fig. 2C).

Flow-cytometric analysis revealed a gradual increase in the S and G_2 cell populations

irradiated with both doses of γ-rays. The sub-G_1 fraction, which indicates apoptosis, was observed in both cases 6 h after irradiation. Nevertheless, a dose of 7.5 Gy caused a greater appearance of the apoptotic cells 6 h after treatment than did a lower dose of 3 Gy.

Cleavage of the nuclear lamina was detected at the same time as the sub-G_1 peak was first observed in the irradiated cells. After a dose of 7.5 Gy the proteolytic fragment was present in both the nuclear and cytosol fractions, while no lamina cleavage was detected in the nuclear fraction after 3 Gy and only a weak signal was observed in the cytosol fraction.

γ-irradiation and p53 phosphorylation

DNA damage caused by a dose of 7.5 Gy induced high levels of the p53 protein at 2 h and this increased p53 expression continued until the end of the examined period (Fig. 3A). To examine the p53 level we used amino-terminal p53 specific DO1 antibody and DO11 antibody recognizing the epitope within the amino acids 181–190. This increased p53 expression was followed by phosphorylation of the p53 molecule at serine-392 (Fig. 3B) and serine-15 (Fig. 4). Both the FP31.1 and phospho-p53 (Ser15) antibodies detected p53 phosphorylation starting 2 h post-irradiation and declining at 8 h. To confirm the specificity of FP31.1 we examined $p53^{Ser\text{-}392}$ expression at the same PVDF membrane incubated before detection with ALP (alkaline phosphatase). After ECL no p53-specific bands were observed (results not presented). Furthermore, the p53 level and the existence of p53 charge variants with phosphorylation at serine-392 were examined using 2-D Western blot analysis (Fig. 5A, 5B). We observed five isoforms of $p53^{Ser\text{-}392}$ 5 h after irradiation.

p21 expression in γ-irradiated MOLT-4 cells

The results from FASAY analysis suggested that p53 expressed in MOLT-4 cells was wild-type. To confirm the data from FASAY we examined the p21 expression after γ-irradiation (7.5 Gy). Figure 6 shows the induction of the p21 level that started 3 h after irradiation. The maximal p21 expression was observed between 4 and 6 h post-irradiation.

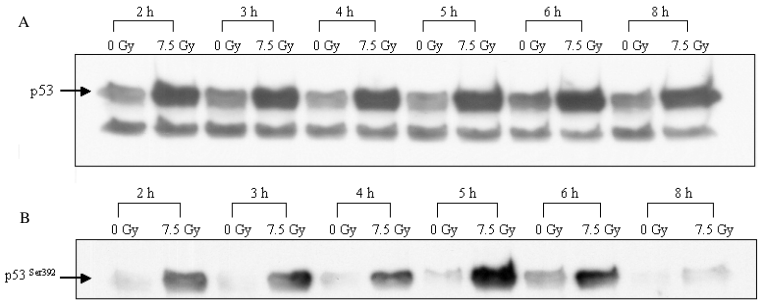

Fig. 3. Western blot analysis of p53 (A) and phosphorylated p53 at serine-392 (B) at various times (2, 3, 4, 5, 6 and 8 h) in MOLT-4 cells exposed to 7.5 Gy.

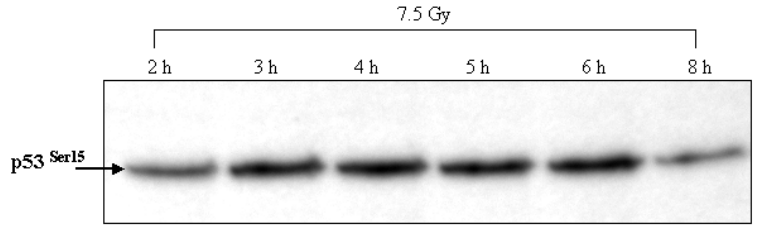

Fig. 4. Western blot analysis of phosphorylated p53 at serine-15 at various times (2, 3, 4, 5, 6 and 8 h) in MOLT-4 cells exposed to 7.5 Gy. For each time interval unirradiated control cells were used. No $p53^{Ser15}$-specific signal was observed in unirradiated cells (data not shown).

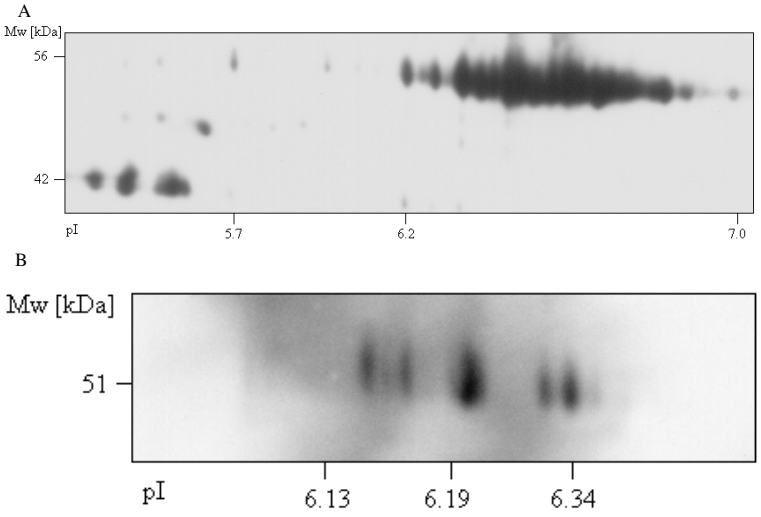

Fig. 5. Two-dimensional Western blot analysis of p53 (A) and phosphorylated p53 at serine-392 (B) 5 h after irradiation of MOLT-4 cells with 7.5 Gy.

Discussion

Exposure of cells to ionizing radiation launches, via induction of the membrane or DNA damage, intracellular signalling pathway molecules that govern the cells into apoptosis. With regard to DNA damage, tumour suppressor protein p53 and ATM protein play key roles in apoptosis (Schmidt-Ullrich et al., 2000).

MOLT-4 cells exhibit the same antigens as T lymphocytes (i.e. CD4 and CD8); hence they represent a good *in vitro* model for studying the molecular mechanisms regulating the radiation-induced apoptotic process in thymocytes (Nakano and Shinohara, 1994). Highly radiosensitive MOLT-4 cells ($D_0 = 0.88$) (Fig. 1)

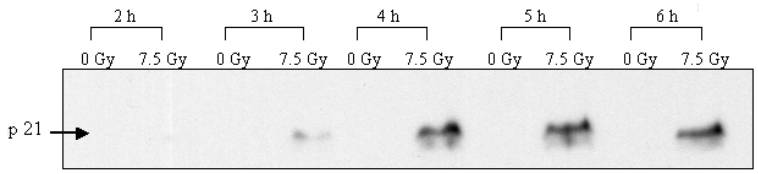

Fig. 6. Western blot analysis of the p21 protein at various times (2, 3, 4, 5 and 6 h) in MOLT-4 cells irradiated with a dose of 7.5 Gy.

die after irradiation by apoptosis (Nakano and Shinohara, 1994). One of the methods used for detecting apoptosis was the nuclear lamina cleavage, which is considered a key aspect of the nuclear phase of apoptosis (Kaufmann, 1989). Proteolytic fragments of lamin B were present 6 h after irradiation with doses of both 3 and 7.5 Gy (Fig. 2C). Using this method we obtained results corresponding to flow-cytometric analysis, where apoptotic cells were first observed 6 h after irradiation both with a dose of 3 Gy and with a dose of 7.5 Gy. However, irradiated MOLT-4 cells were especially accumulated in S phase and G_2 phase of the cell cycle (Fig. 2A, 2B). Our results may be supported by other published findings describing the induction of apoptosis after accumulation of X-irradiated MOLT-4 cells in G_2 phase (Aldridge and Radford, 1998). Schwartz et al. (1997) showed that p53 has a key function in G_1/S arrest and also in G_2 phase, where the cells repairing DNA damage are accumulated. In a previous study (Vávrová and Filip, 2002), the apoptotic MOLT-4 cells were also detected using monoclonal APO2.7 antibody that was developed against mitochondrial membrane 7A6 antigen.

To determine the transactivation ability of the p53 protein we performed FASAY analysis, which revealed the expression of wild-type p53 in MOLT-4 cells. These data correspond with other published findings describing undetected p53 gene mutations in human leukaemia MOLT-4 cells (Cheng and Haas, 1990; O'Connor, 1997).

The p53 upregulation in irradiated MOLT-4 cells has already been described (Zhao et al., 1999). Similarly, we also observed γ-irradiation-induced p53 accumulation 2–8 h after irradiation (Fig. 3A). One of the main functions of p53 is cell cycle regulation, where its upregulation results in cell cycle arrest in G_1 phase (Kuerbitz et al., 1992). It has been suggested that p21 mediates this p53-induced cell cycle arrest (El-Deiry et al., 1993). Our results showed induction of p21 expression 3–6 h after irradiation (Fig. 6). This result stands in contrast with the study documenting no p21 induction in X-irradiated MOLT-4 cells (Inanami et al., 1999). However, in a more recent publication p53-induced p21 expression in X-irradiated MOLT-4 was described and, furthermore, the authors proved that p21 production was dependent on p53 mutant/wild-type ratio in these cells (Nakano et al., 2001). Nevertheless, despite p21 production, we did not observe a typical G_1 arrest after irradiation (Fig. 2A, 2B). This finding seems to corroborate the function abrogation of additional G_1-block modulating factors by exposure to ionizing radiation. Similar results were obtained for other human tumour cell lines that exhibited a normal p53 and p21 expression pattern (Li et al., 1995).

Recently, it has been shown that posttranslational p53 modification at serine-15, which can be phosphorylated by ATM, occurred in response to ionizing irradiation (Siliciano et al., 1997). In our study, p53 phosphorylation at serine-15 was also found after MOLT-4 cell irradiation (Fig. 4). Shieh et al. (1997) described how p53 phosphorylation at serine-15 leads to reduced p53 interaction with its negative regulator MDM2.

Besides posttranslational modifications and protein stabilization, tetramer formation is also important for p53's ability to activate transcription. Sakaguchi et al. (1997) published results showing that p53 phosphorylation at serine-392 enhances tetramer formation. Our results demonstrate for the first time the induction of $p53^{Ser392}$ modification in γ-irradiated MOLT-4 cells (Figs. 3B, 5B). Previous studies suggested that only UV but not γ-radiation is able to induce phosphorylation of the p53 protein at serine-392 (Kapoor and Lozano, 1998; Lu et al., 1998).

As discussed above, the p53 levels are increased in response to DNA damage. It is achieved through posttranslational modifications of the p53 polypeptide (Kastan et al., 1991). The existence of a great number of p53 charge variants is well demonstrated by the 2-D Western blot analysis of irradiated MOLT-4 cells (Fig. 5A). Furthermore, five p53 isoforms differing in size and pI were phosphorylated at serine-392 (Fig. 5B).

In this study we have used FASAY analysis for determining the p53 status in human leukaemia MOLT-4 cells. Current results, including our own, describe apoptosis and p53 accumulation in γ-irradiated MOLT-4 cells. Radiation-induced p21 expression is in accordance with the results from FASAY analysis documenting wild-type p53 expression in MOLT-4 cells. In addition to radiation-induced p53 phosphorylation at serine-392, which could act as a critical switch activating p53, we also found in irradiated MOLT-4 cells the phosphorylation of p53 at serine-15 that may contribute to p53 stabilization.

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