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Publication Date

1997

DOI

10.1038/sj.onc.1201050

Peer reviewed



Dissociation between cell cycle arrest and apoptosis can occur in Li-Fraumeni cells heterozygous for p53 gene mutations

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The radiation response was investigated in two lymphoblastoid cell lines (LBC) derived from families with heterozygous germ-line missense mutations of p53 at codon 282 (LBC282) and 286 (LBC286), and compared to cells with wt/wt p53(LBC-N). By gel retardation assays, we show that p53-containing nuclear extracts from irradiated LBC282 and LBC286 markedly differ in their ability to bind to a p53 DNA consensus sequence, the former generating a shifted band whose intensity is 30–40% that of LBC-N, the latter generating an almost undetectable band. Unlike LBC286, which fail to arrest in G₁ after irradiation, LBC282 have an apparently normal G₁/S checkpoint, as they arrest in G₁, like LBC-N. While in LBC-N, accumulation of p53 and transactivation of p21^{WAF1} increase rapidly and markedly by 3 h after exposure to γ -radiation, in LBC286 there is only a modest accumulation of p53 and a significantly delayed and quantitatively reduced transactivation of p21^{WAF1}. Instead, in LBC282 while p53 levels rise little after irradiation, p21^{WAF1} levels increase rapidly and significantly as in normal LBC. Apoptotic cells present 48 h after irradiation account for 32% in LBC-N, 8–9% in LBC282 and 5–7% in LBC286, while the dose of γ -radiation required for killing 50% of cells (LD₅₀) is 400 rads, 1190 rads and 3190 rads, respectively, hence indicating that the heterozygous mutations of p53 at codon 282 affects radioresistance and survival, but not the G₁/S cell cycle control. In all LBC tested, radiation-induced apoptosis occurs in all phases of the cell cycle and appears not to directly involve changes in the levels of the apoptosis-associated proteins bcl-2, bax and mcl-1. Both basal as well as radiation-induced p53 and p21^{WAF1} proteins are detected by Western blotting of FACS-purified G₁, S and G₂/M fractions from the three cell lines. p34^{CDC2-Tyr15}, the inactive form of p34^{CDC2} kinase phosphorylated on Tyr15, is found in S and G₂/M fractions, but not in G₁. However, 24 h after irradiation, its levels in these fractions diminish appreciably in LBC-N but not in the radioresistant LBC286 and LBC282. Concomitantly, p34^{CDC2} histone H1 kinase activity increases in the former, but not in the latter cell lines, hence suggesting a role for this protein in radiation-induced cell death. Altogether, this study shows that, in cells harbouring heterozygous mutations of p53, the G₁ checkpoint is not necessarily disrupted, and this may be related to the endogenous p53 heterocomplexes having

lost or not the capacity to bind DNA (and therefore transactivate target genes). Radiation-induced cell death is not cell cycle phase specific, does not involve the regulation of bcl-2, bax or mcl-1, but is associated with changes in the phosphorylation state and activation of p34^{CDC2} kinase.

Keywords: p53; p21^{WAF1}; p34^{CDC2}; cell cycle; apoptosis

Introduction

It is well established that the oncosuppressor gene p53 plays a key role in the regulation of cell cycle, apoptosis, and maintenance of genomic stability, mainly through its transcriptional regulation of crucial growth inhibitory genes such as p21^{WAF1}, of genes involved in DNA repair such as GADD45 and PCNA, or of genes involved in cell survival such as bcl-2 and bax (El-Deiry *et al.*, 1993, 1994; Smith *et al.*, 1994; Oren, 1994). These p53 regulated and coordinated functions are thought to represent a basic mechanism which ultimately prevents replication of damaged DNA (Lane, 1992; Hartwell and Kastan, 1994). Agents which produce genetic injury, such as ionising radiation, elicit in cells expressing wild type p53 the rapid accumulation of this protein, which in turn induces the activation or suppression of target genes resulting in the block of cells in late G₁ and the inhibition of DNA replication (Kuerbitz *et al.*, 1993; Hartwell and Kastan, 1994). Both the inhibition of DNA replication and the block at G₁/S transition allow time for the cells to repair their DNA before progressing into cycle, unless the extent of the damage is high, in which case p53 may trigger apoptotic cell death in certain types of cells.

Substantial data indicate that p53 is a direct regulator of apoptosis. For instance, leukaemic cells transfected with a p53 temperature sensitive mutant, when grown at a temperature which allows the p53 protein to assume a wild type conformation, undergo apoptosis (Yonish-Rouach *et al.*, 1991). Additionally, some types of cells from p53 null mice are more radioresistant and less prone to apoptosis induced by various DNA strand break inducing agents than cells from normal mice (Clarke *et al.*, 1993; Lowe *et al.*, 1993a). Although a number of data have led to the assumption that disruption of the G₁/S checkpoint and decreased susceptibility to apoptosis are inseparable

able events associated with p53 mutations, very recently it has been observed that a p53 cell mutant at codon 175 loses the apoptotic function despite preserving the capacity to arrest in G₁ (Rowan *et al.*, 1996). This finding therefore indicates not only that cell cycle arrest and apoptotic activity of p53 can be dissociated, but also that inactivation of the apoptotic function alone can contribute to tumour development.

The mechanism of p53-induced apoptotic cell death involves, at least in certain cell types, upregulation of the pro-apoptotic gene Bax and downregulation of the survival gene Bcl-2 (Miyashita *et al.*, 1994a), both achieved by the functional and/or physical interaction of p53 protein with DNA response elements present in the promoter regions of these genes (Miyashita *et al.*, 1994b, 1995a). It should be noted, however, that p53 may also trigger apoptosis in the absence of transcriptional activation of p53 target genes (Caelles *et al.*, 1994).

EBV-immortalized lymphoblastoid cell lines (LBC) derived from individuals affected by the cancer-predisposing Li-Fraumeni syndrome and carrying germ-line heterozygous mutations of p53 (Birch, 1994) provide an important cell system to study the transdominant effects of mutated p53 over normal p53, as well as the biochemical consequences relevant to tumour development. These studies are also possible because EBV immortalization does not impair the function of p53 (Allday *et al.*, 1995a). In this study we have analysed the contribution of various p53 mutations with regard to cell cycle control and apoptosis. We demonstrate that in a cell line carrying a heterozygous p53 mutation at codon 282, the G₁ arrest is not impaired whereas the apoptotic function is repressed. Additionally, we show that the normal G₁ arrest by this cell line is correlated with a normal upregulation of p21^{WAF1} in response to DNA damaging agents. We also provide data showing expression of p21^{WAF1} in G₂/M cells and that, in cell undergoing apoptosis the cyclin-dependent serine-threonine kinase p34^{CDC2}, whose activity in cell cycle and mitosis is modulated by phosphorylation (Hunter, 1995) and whose role in certain types of cell death has been demonstrated (Shi *et al.*, 1994), shows markedly reduced levels of phosphorylation on Tyr 15 and concomitant increase in kinase activity.

Results

p53 status and DNA-binding activity of the cell lines

The lymphoblastoid cell lines LBC286 and LBC282, derived from probands of two unrelated Li-Fraumeni families, harbour germ-line heterozygous mutations of p53 at codon 286 (GAA→GCA; Glu→Ala) and 282 (CGG→TGG; Arg→Trp), respectively. LBC-N was derived from a normal individual and has normal p53. By RT-PCR analysis for wt and mut alleles (Wada *et al.*, 1994), we have found that LBC286 expresses higher amounts of wt p53(GAA) than mt p53(CGA) allele, while LBC282 co-expresses equal amounts of wt(CG) and mt(TGG) alleles (data not shown). Both the 282 and the 286 mutant alleles derived from these cell

lines were, in the absence of the wtp53 allele, transcriptionally silent when functionally examined in a yeast assay for p53 alleles (Flaman *et al.*, 1995) (data not shown), thus indicating that these mutations abolish the DNA binding activity of p53 protein. This also agrees with the fact that, being amino acids 282 and 286 localized in the H2α helix (aa 278–286) which fits the major groove of the DNA, mutations of these residues profoundly disrupt protein-DNA interactions (Cho *et al.*, 1994).

To determine how, in our p53^{wt/mut} cell lines, the mutant p53 allele affected the activity of the normal allele, we examined the DNA-p53 complex formation in these cells by gel retardation assays. Nuclear extracts were prepared from the cells harvested 3 h after treatment with 400 rads, and tested for binding to a DNA target carrying the p53 consensus binding sequence (El Deiry *et al.*, 1992). In LBC-N, the formation of a p53/DNA complex was quite evident (Figure 1), and appeared specific because of its inhibition by an excess of the same unlabelled oligo,

LBC-282				LBC-N				LBC-286				CELL LINE
-	-	+	-	-	-	+	-	-	-	+	-	Comp.A
-	-	-	+	-	-	-	+	-	-	-	+	Comp.B
-	+	-	-	-	+	-	-	-	+	-	-	Pab421

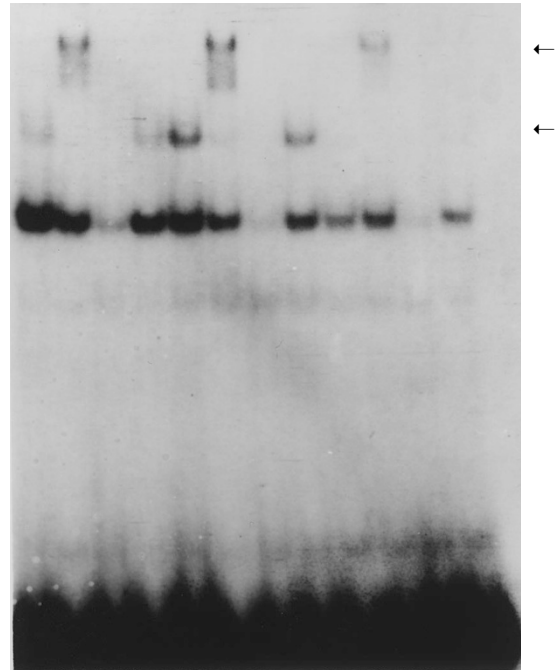


Figure 1 Complex formation between p53-containing nuclear extracts and a p53 consensus DNA oligonucleotide. Nuclear extracts were prepared from cells harvested 3 h after irradiation with 400 rads. The binding reactions contained 1 ng (1.5–2 × 10⁵ c.p.m) of radiolabelled double strand p53 DNA consensus oligonucleotide and nuclear extracts used at appropriate concentrations (between 8 and 12 μg) to normalised for equal amounts of p53 protein (p53 protein levels were determined by densitometric analysis of immuno dot blots performed on nuclear extracts, as reported in Materials and methods). Competition reactions were performed in the presence of 50-fold excess of unlabelled double strand oligonucleotides carrying the p53 DNA consensus sequence (Comp A) or mismatches in this consensus sequence (Comp B) (see Materials and methods). Shifted and supershifted bands relative to sequence specific p53/DNA complexes are indicated by the lower and upper arrow, respectively

but not by a missense oligo. In addition, this complex was supershifted by PAb421, as expected from previous reports showing that this antibody retards the mobility of the complex (Funk *et al.*, 1992). PAb421, in addition restores DNA binding to some mutant p53 proteins (Niewolik *et al.*, 1995). Specific p53/DNA complexes were also observed in nuclear extracts from LBC282, however, according to the intensity of shifted band, the amount of complexes formed was 60–70% less than in LBC-N. By contrast, the formation of p53/DNA complexes in nuclear extracts from LBC286 was almost undetectable, except when in the presence of PAb421. These findings therefore indicate that while p53 heterocomplexes present in LBC282 are capable of binding DNA, though less efficiently than LBC-N, p53 heterocomplexes present in LBC286 are virtually impaired in this DNA-binding activity.

Cell cycle analysis and apoptosis

The p53-dependent G₁ cell cycle checkpoint is functional in cells expressing wild type p53, but usually disrupted in cells carrying p53 mutations. Thus, while cells with normal p53 arrest in G₁ following exposure to DNA damaging agents, cells with alterations of p53 typically fail to arrest in G₁ (O'Connor *et al.*, 1993; Lowe *et al.*, 1993a, b).

To determine if the response to DNA strand break-inducing agents is equally affected by different p53 mutations, we examined the cell cycle response of normal and p53 heterozygous LBC exposed to 400 rads of γ -radiation. It can be seen (Table 1) that in normal LBC, the fraction of cells in G₁ diminished slightly after irradiation, while that of cells in G₂ increased twofold. By contrast, irradiated LBC286 showed a marked reduction of cells in G₁ and a

striking accumulation of cells in G₂. Unexpectedly, in LBC282, the fraction of G₁ cells diminished slightly 24 h post irradiation, but markedly decreased by 48 h. Concomitantly, the fraction of G₂ cells increased twofold at 24 h, and fourfold at 48 h, relative to controls. These findings suggest that mutations of p53 at codon 282, unlike those at codon 286, do not impair the G₁ checkpoint, at least in heterozygous conditions.

In addition to affecting the cell cycle response to DNA-damaging agents, p53 mutations usually decrease the apoptotic sensitivity to these agents (Fan *et al.*, 1994). To determine the relationship between p53 mutations, radiosensitivity and apoptosis, the fraction of live and of apoptotic LBC cells was quantified after 400 rads treatment. It can be seen (Figure 2a) that, upon irradiation, while the viability of normal LBC declined by almost 30% at 24 h and 50% at 48 h, the viability of LBC with wt/mut p53 declined by about 10% at 48 h, irrespective of the type of p53 point mutation. The dose of radiation required for killing

Table 1 γ -Radiation-induced cell cycle phase changes in p53 heterozygous LBC

Cell line	%	Contr	24 h	48 h
LBC-N	G ₁	73.2 ± 14.7	64.6 ± 12.4	70.1 ± 9.9
	G ₂ /M	10.8 ± 3.9	25.4 ± 11.4	22.1 ± 7.6
LBC286	G ₁	61.6 ± 3.2	37 ± 6.2	49.6 ± 3.5
	G ₂ /M	20 ± 4.5	54.6 ± 7.5	41.3 ± 3.4
LBC282	G ₁	72.3 ± 4.2	60.4 ± 5.8	51.5 ± 2.1
	G ₂ /M	14.7 ± 3.5	23.6 ± 8.9	42.5 ± 4.9

The reported percentages (\pm s.d.) of G₁ and G₂/M fractions were calculated from the DNA histograms generated by the flow cytofluorimetric analysis (10 000 acquired events) of propidium iodide-stained samples, 24 or 48 h after exposure to 400 rads of γ -radiation

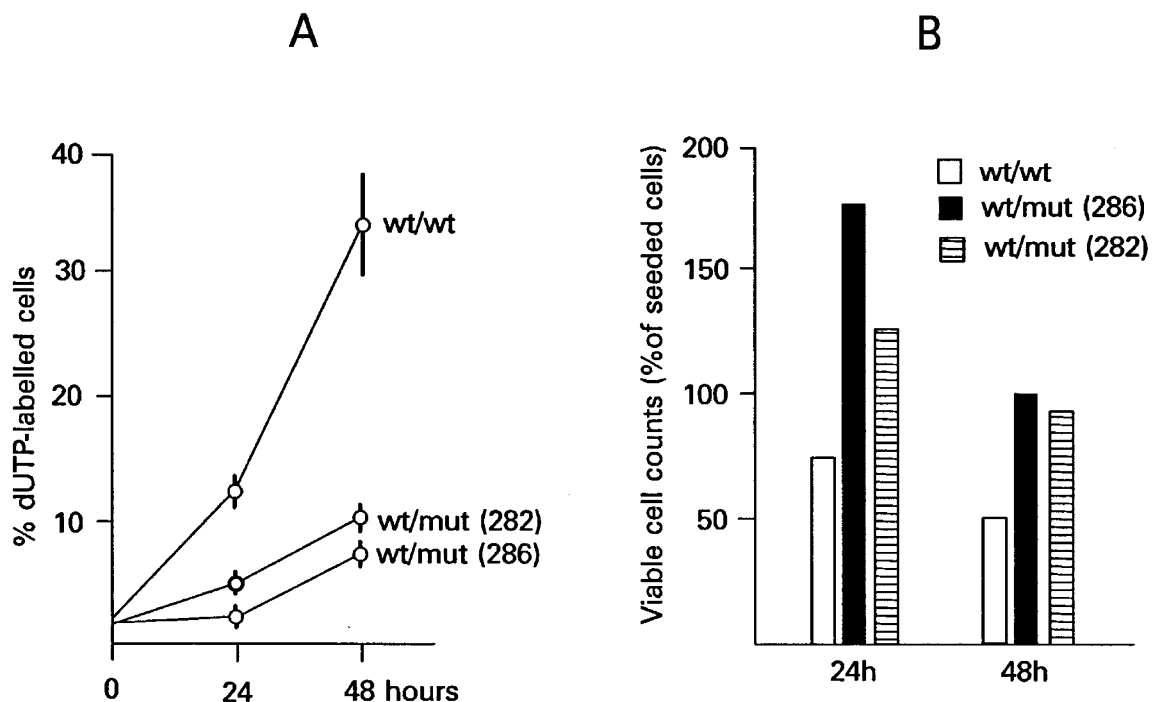


Figure 2 Cell viability and apoptosis in radiation-treated LBC. Twenty-four and 48 h after exposure to 400 rads, the cells were harvested, split into two aliquotes, one analysed for cell viability (b), and the other for apoptosis after staining with FITC-dUTP (a) as indicated in Materials and methods. Samples were examined by fluorescence microscopy and by flow cytofluorimetry. Data are average of at least four independent experiments

50% of cells (LD_{50}) was, at 48 h, 400 rads for LBC-N, 3190 rads for LBC286 and 1190 rads for LBC282, as determined by dose- and time-dependent assays.

The number of apoptotic cells, detected by *in situ* dUTP-labelling of fragmented nuclear DNA, was significantly higher after irradiation in normal LBC cells (12% at 24 h and >30% at 48 h) than in LBC282 or LBC286 cells (3–5% at 24 h, 7–10% at 48 h) (Figure 2b). Altogether, these data emphasize the different contribution of heterozygous p53 mutations at codons 286 and 282 with regard to loss of G_1 arrest, but not with regard to resistance to apoptosis.

Cell cycle specificity of radiation-induced apoptotic cells

In order to determine whether radiation-triggered apoptosis is cell cycle-phase dependent, we employed a recently described flow cytometric method which enables determination of the cell cycle of apoptotic cells through the simultaneous staining of F-actin and DNA with FITC-phalloidin and propidium iodide (PI), respectively (Endresen *et al.*, 1995). Since apoptotic cells bind less to FITC-phalloidin and have a higher side scatter than live cells, they can be easily identified by bivariate flow cytometry. This method allows, in addition, to identify apoptotic cells with no apparent decrease in cell DNA content, as observed in some instances (Fournel *et al.*, 1995).

The results, illustrated in Figure 3, evidence in irradiated normal LBC a time-dependent increase in the number of phalloidin-dim, apoptotic cells (demarcated by region R1), whose cell cycle histogram demonstrates two populations, one with an apparently normal DNA content and with clearly distinguishable G_1 , S and G_2/M events, the other with a subdiploid DNA content, which most likely represent early and late stages of apoptosis, respectively. Similar findings were observed in LBC286 cells, although at a later time post-irradiation. To ascertain that the phalloidin-dim, diploid elements were truly apoptotic, they were sorted out by FACS and examined by fluorescence microscopy, which confirmed that >95% of cells were apoptotic. These data establish that radiation-induced apoptosis occurs independently of the cell cycle phase.

Regulation of p53, p21^{WAF1} and of apoptotic genes

Upon irradiation, normal LBC showed a marked accumulation of p53 protein, whose maximal levels were found at 3 h and began to decline 24 h later (Figure 4). Concomitantly, there was a rapid and striking increase in p21^{WAF1} protein levels, becoming maximal at approximately the same time as p53 (Figure 4). Irradiation of LBC286 resulted in a modest increase of the already elevated constitutive levels of p53 protein, and in a gradual increase in the amount of p21^{WAF1}, with maximal levels being attained at \approx 24 h (Figure 4). Similarly to LBC286 cells, LBC282 cells expressed relatively high basal levels of p53 protein, which increased modestly after irradiation. However, unlike LBC286 cells in these cells the upregulation of p21^{WAF1} in response to radiation was fast, with maximal levels of protein detectable by 3 h (Figure 5). Collectively, these data emphasize the difference between the 286 and 282 heterozygous cell mutants with regard to the kinetics of p21^{WAF1} transactivation,

being significantly delayed in the former and apparently normal in the latter. It is conceivable that these differences actually reflect differences in the p53/DNA binding activity (and therefore transactivation activity) by these cells (see Figure 1).

Since radiation-induced apoptosis is a p53-dependent process (Lowe *et al.*, 1993a; Clarke *et al.*, 1993), and since in certain cell systems p53-triggered cell death involves the downregulation of the survival protein bcl-2 and the upregulation of the pro-apoptotic protein bax (Miyashita *et al.*, 1994a), we sought to determine the involvement of these genes in our cells. Additionally, we analysed mcl-1, another member of the bcl-2 protein family (Kozopas *et al.*, 1993) which antagonizes apoptosis (Reynolds *et al.*, 1994), and whose involvement in radiation-induced cell death is unknown. The steady state levels of these proteins, both in normal and p53 mutated LBC cells, remained unchanged during the 48 h time-course after radiation (Figures 4 and 5), despite the occurrence of apoptosis in the former cells. Curiously, however, although the anti-bax antibody predominantly detected the 21 kDa protein, in irradiated LBC-N, but not LBC286 or LBC282, it evidenced an additional band of \approx 18 kD (Figure 4) whose amount augmented with time and with the number of apoptotic cells (see Figure 2b for comparison). Whether this 18 kD protein represents a proteolytic product of p21 bax or, alternatively, an isoform of bax, remains to be established. A similar finding, however, has been recently observed in drug-induced apoptosis in B-cell chronic lymphocytic leukaemia (Thomas *et al.*, 1996). It should also be noted that Northern analysis of the three cell lines demonstrated a consistent rise in bax mRNA, whose maximal levels (2.6-fold, relative to untreated cells) were observed 9 h post irradiation (Figure 6). No changes in mcl-1, bcl-2 or bcl-x mRNA were observed (data not shown). Collectively, these results suggest that the radiation-induced apoptotic process in our cells is not directly mediated by these genes.

Cell cycle-phase related expression of p53, p21^{WAF1}, Rb, p34^{CDC2}

While it is well established that radiation-induced G_1 arrest in normal cells is selectively regulated by the p53–p21^{WAF1} pathway, the involvement of this pathway in the control of the G_2/M phase arrest has not been fully elucidated. To gain an insight into the role of p53 in G_2/M growth arrest, we examined the cell-cycle-related expression of this protein as well as of p21^{WAF1}, before and after irradiation. For this purpose, cells in G_1 , S and G_2/M were separated by FACS sorting to >95% purity, as described in Materials and methods, and used for Western blot analysis. It should be noted that at least three independent experiments were performed on each cell line and that, for each immunoblot, the relative abundances of the indicated gene products were determined by scanning densitometry and normalization against β -actin levels. In normal LBC, basal levels of p53 were faintly detectable in G_1 , G_2/M (Figure 7) and S (data not shown). Twenty four h after exposure to 400 rads, the accumulation of p53 protein was seen in all phases of the cell cycle, though not to the same extent. There

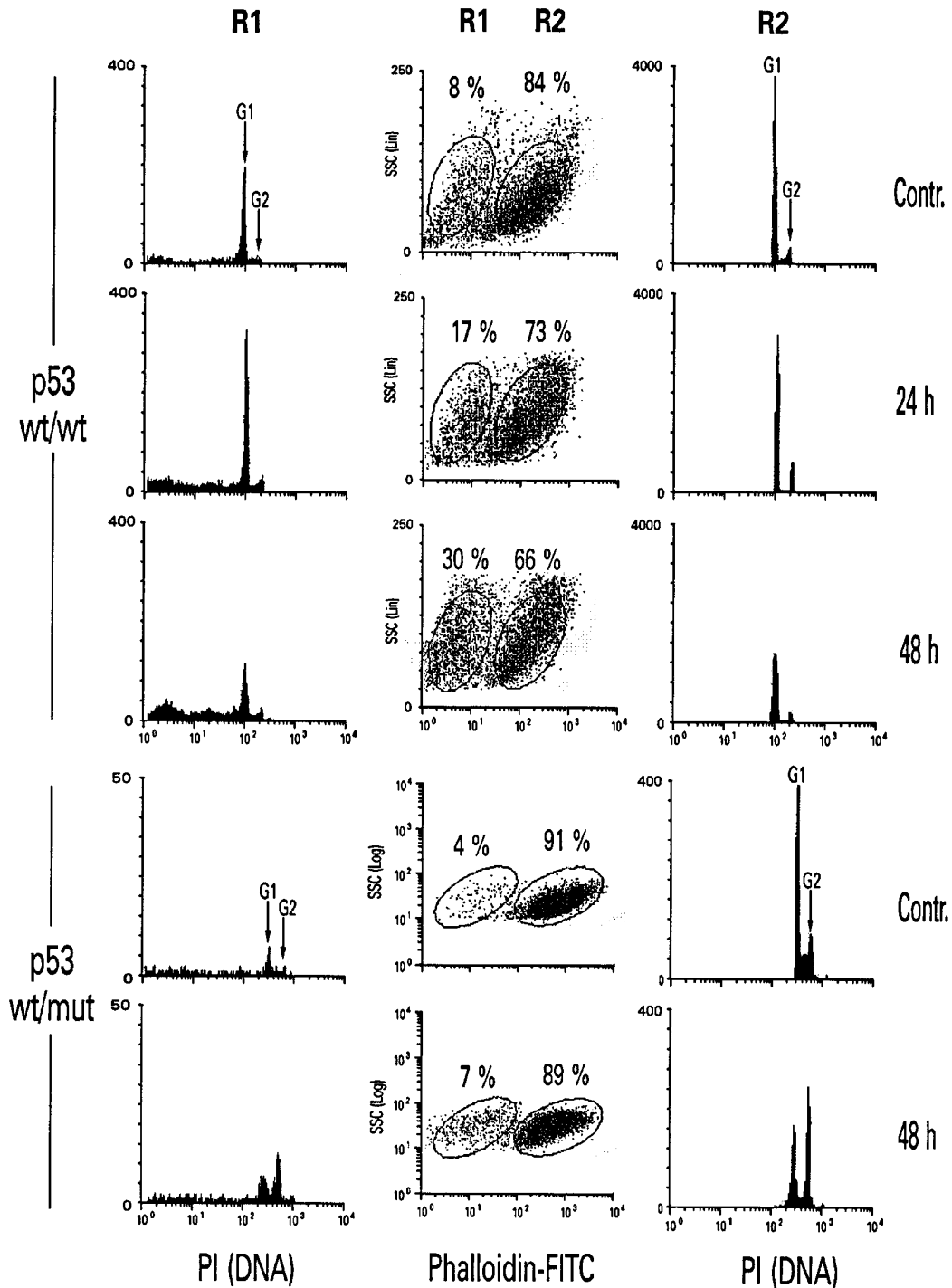


Figure 3 Cell cycle-phase of apoptotic cells. At the indicated time post irradiation, the cells were fixed, stained with FITC-Phalloidin and propidium iodide, and analysed by dual-colour (green/red) flow cytometry. The dot histograms display the intensity of FITC-Phalloidin and the cell size (side scattering) in the horizontal and vertical axis, respectively. R1 and R2 are electronic windows which delineate the apoptotic and non-apoptotic cells, respectively. The DNA histograms of cells gated in R1 and R2 are shown in the left and right panels, respectively

was, in fact, a 6.9-fold increase in p53 protein in G₁, threefold in S and 3.6-fold in G₂/M, relative to the respective control fractions. Basal levels of p21^{WAF1} protein were detectable in all cell cycle phases (Figures 7 and 8), with S-phase cells showing the lowest amounts (3.6-fold less than G₁ cells). Twenty four h after irradiation, p21^{WAF1} increased 6.8-fold in G₁, 7.8-fold in S and 6.4-fold in G₂/M, compared to the respective unirradiated fractions. In LBC286, the

distribution of p53 and p21^{WAF1} along the cell cycle was similar to that of normal LBC, both before and 24 h after irradiation (Figures 7 and 8).

Both the amount and phosphorylation of the retinoblastoma (Rb) protein were examined in G₁ and G₂/M sorted fractions. No significant changes in total Rb protein were found in any of the cell lines after irradiation (not shown). The ratio between the hyper and hypophosphorylated Rb was 0.4 in G₁, 1.5 in G₂/

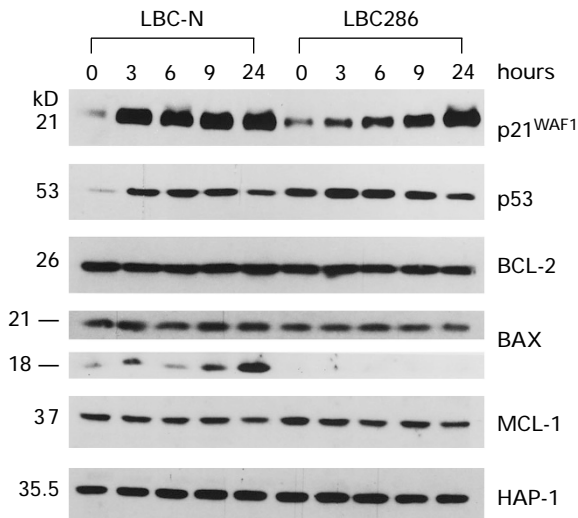


Figure 4 p21^{WAF1}, p53 and apoptosis-related proteins in LBC-N and LBC286 after radiation treatment. Immunoblots were performed on lysates (20 μ g/lane) prepared from LBC-N and LBC286, harvested at the indicated times after exposure to 400 rads. Blots were probed with the reported antibodies, as well as with an anti-HAP1 antibody specific for a 35.5 kD protein whose levels remain constant in irradiated cells (DD unpublished), to normalize for protein content

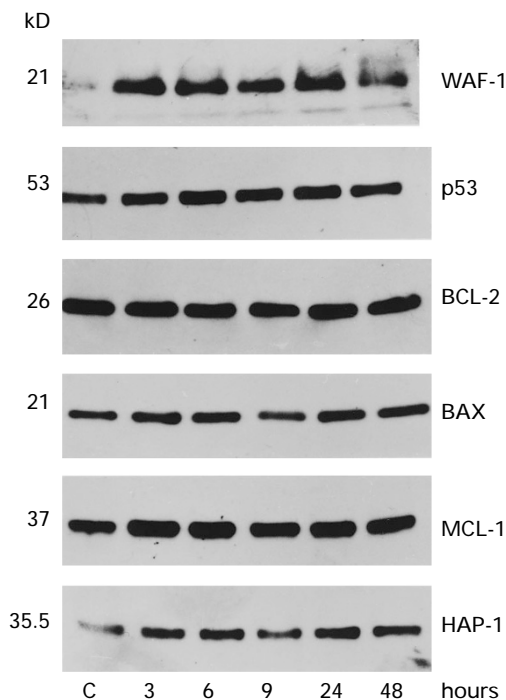


Figure 5 p21^{WAF1}, p53 and apoptosis-related proteins in LBC282 after radiation treatment. Immunoblots were performed on lysates (20 μ g/lane) prepared from LBC282, exposed to 400 rads and harvested at the specified times. Blots were normalized for protein content by probing with anti-HAP1

M cells (Figure 7), and 1.5 in S (not shown), and these values did not change significantly 24 h post irradiation.

Basal levels of the p53-regulated gene MDM2 were detected in untreated normal LBC, both in G₁ and G₂/M, but not in the LBC286 (Figure 7). At 24 h after irradiation the amount of MDM2 in these cells substantially increased in both fractions.

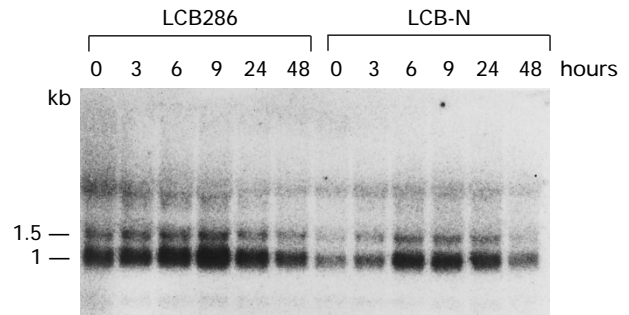


Figure 6 Bax mRNA modulation after irradiation. Northern blots were performed on RNA extracted from the indicated cell lines at various time-points after irradiation with 400 rads. The blots were hybridised using a radiolabelled Bax cDNA probe, and radioactivity detected by phosphoimager

Recently, it was shown that premature activation of the serine-threonine kinase p34^{CDC2} leads to apoptotic cell death (Shi *et al.*, 1994), suggesting that this may be a mechanism by which cells induced to die by apoptosis initiate the dissolution of the nuclear membrane. The kinase activity of p34^{CDC2} is regulated by cyclins and by phosphorylation. In particular, when phosphorylated on Tyr15, p34^{CDC2} is inactive, but becomes active when dephosphorylated at the G₂/M transition (Hunter, 1995 for a review). To determine whether radiation-induced apoptosis somehow involved p34^{CDC2}, its expression throughout the cell cycle was analysed with antibodies specific for p34^{CDC2} phosphorylated on Tyr15. In untreated normal LBC, p34^{CDC2-Tyr15} was expressed (Figures 7 and 8) in S and G₂/M but very faintly in G₁ (about 80-fold difference in signal intensity). Although this distribution was not modified 24 h post irradiation, nevertheless the amount of p34^{CDC2-Tyr15} in both S and G₂/M fractions dropped fivefold and 3.1-fold, respectively. This drop was not observed 3 h post irradiation. The total amount of p34^{CDC2} protein in these cells remained unchanged after irradiation. LBC286 showed a similar pattern of p34^{CDC2-Tyr15} distribution through the cell cycle as normal LBC. However, 24 h after irradiation the G₂/M cells showed virtually unchanged levels of p34^{CDC2-Tyr15}, in contrast to the marked reduction observed in normal irradiated G₂/M cells. It should also be noted that in unfractionated LBC286, p34^{CDC2-Tyr15} levels 24 h after irradiation increased, but this was actually related to the rise in the percentage of G₂/M cells (see Table 1). The amount of p34^{CDC2-Tyr15} in LBC282 remained unchanged 24 h after irradiation (data not shown). To determine whether the decrement in inactive p34^{CDC2-Tyr15} correlated with enhanced kinase activity, total p34^{CDC2} was immunoprecipitated from equal amounts of cytosolic protein from control and 24 h irradiated cells and assessed for histone H1 kinase activity. Figure 9 shows that whereas in LBC282 and LBC286 the p34^{CDC2} kinase activity remained unchanged 24 h after irradiation, in LBC-N it increased substantially.

Altogether, the data demonstrate that the radiation-induced expression of p53 and p21^{WAF1} is not cell cycle phase restricted. In addition, they point out that p34^{CDC2} is involved in radiation-induced apoptosis, as evidenced by the decreased levels of inactive

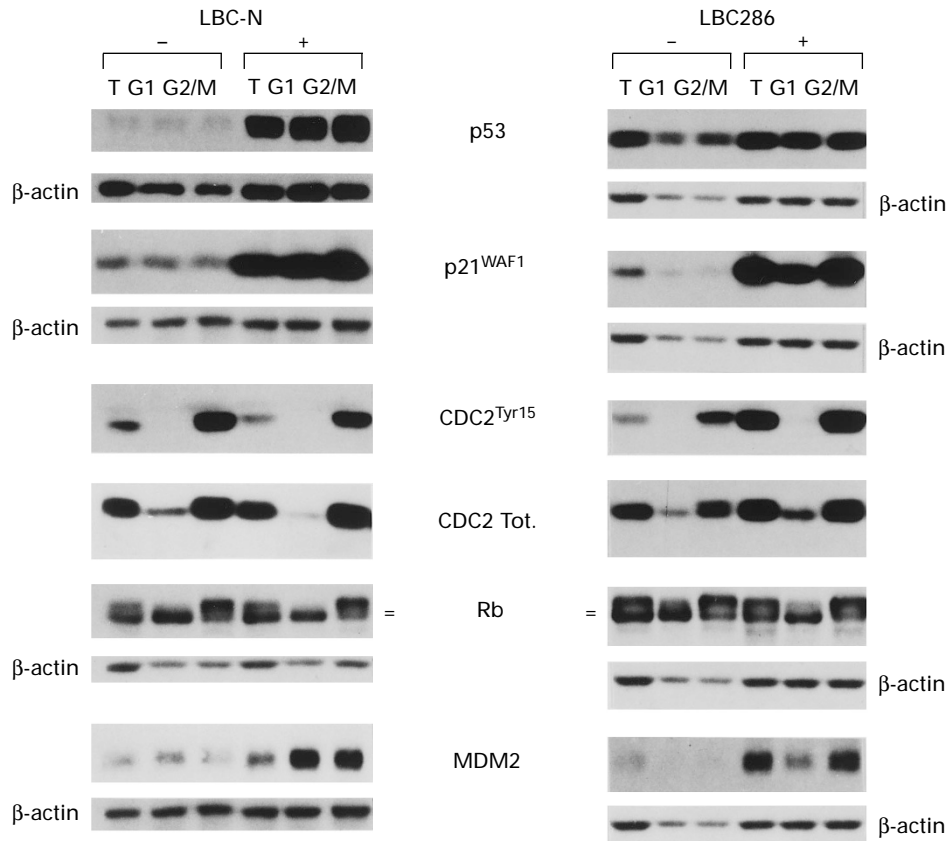


Figure 7 Cell cycle-related expression of p53, p21^{WAF1}, p34^{CDC2} and p34^{CDC2-Tyr15}. Western analysis of total (T), G₁ and G₂/M cells separated by FACSvantage from LBC-N and LBC286 before (–) or 24 h (+) after 400 rads of γ -irradiation

p34^{CDC2-Tyr15} and enhanced histone H1 kinase activity in LBC-N cells undergoing radiation-induced apoptosis, but not in cells resistant to it.

Discussion

We have established lymphoblastoid cells from patients affected by the cancer-predisposing Li-Fraumeni syndrome and carrying heterozygous mutations of p53, to determine the contribution of this genetic abnormality with regard to cell cycle control and cell death. It is important to note that EBV immortalization does not affect the function of p53 (Allday *et al.*, 1995a). In this study we have found, as expected from other studies (O'Connor *et al.*, 1993, Lowe *et al.*, 1993b), that whereas LBC with wt/wt p53 have a functional G₁/S block, LBC with wt/mut p53 at codon 286 are defective in this block. Though not shown, we have observed a similar G₁/S defect in two other related donors with heterozygous mutations of p53 at codon 132 (AAG to GAG). Unexpectedly, however, we have found that LBC with the p53 mutation at codon 282 (CGG to TGG), which abrogates the sequence-specific DNA binding capacity of the encoded p53 mutant protein (Rolley *et al.*, 1995), have a relatively normal G₁ checkpoint since they arrest in G₁ following irradiation. This finding, along with very recent results demonstrating, by gene transfer experiments that p53 mutant at codon 143 does not cause, in contrast to other p53 mutants examined,

disruption of the G₁ checkpoint in a cell line expressing wt p53 (Pocard *et al.*, 1996), and that a p53 175P cell mutant loses the apoptotic function but preserves the G₁ checkpoint (Rowan *et al.*, 1996), emphasizes the view that different p53 mutations can have distinct effects on cell cycle control and cell death, at least when heterozygous.

To try to understand the basis for such phenomena, we sought to establish whether in LBC282 the functional activity of the wt/mut p53 protein complex was any different from that in LBC286. Interestingly, the gel retardation assays have evidenced that whereas p53-containing nuclear extracts from LBC282 bind a p53 consensus DNA sequence, albeit less efficiently than LBC-N, nuclear extracts from LBC286 are almost devoid of this DNA-binding activity. Together with the finding that both the 282 and 286 mutant alleles are transcriptionally inactive when functionally tested in yeast (see Results), these findings argue that the p53 mutant at codon 286 exerts a much stronger dominant negative effect over wild type p53 function than the p53 mutant at codon 282. That certain mutant p53 alleles have little dominant negative effect on endogenous wild type p53 function associated with transcriptional activation has been most recently documented in transient transfection studies (Davis *et al.*, 1996).

We have examined the time-dependent kinetics of p53 accumulation and p21^{WAF1} transactivation after exposure to γ -rays. Compared to normal LBC, in which maximal levels of p53 and p21^{WAF1} are achieved 3 h after irradiation, LBC286 show not only a modest

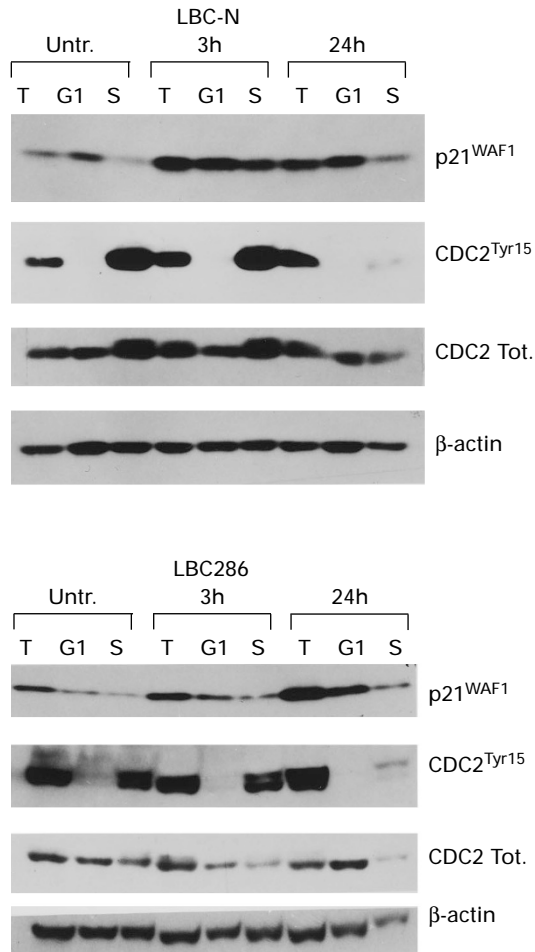


Figure 8 Cell cycle-related expression of p53, p21^{WAF1}, p34^{CDC2} and p34^{CDC2-Tyr15}. Western analysis of total (T), G₁ and S-phase cells separated by FACSvantage from LBC-N (top) and LBC286 (bottom) 3 and 24 h after exposure to 400 rads

accumulation of p53 but also a markedly delayed elevation of p21^{WAF1} protein, which reached maximal levels 24 h post irradiation. LBC282 cells also experienced a modest increase in p53 levels, but, unlike LBC286 and similar to LBC-N, these cells exhibited a rapid and strong induction of p21^{WAF1}, with maximal levels being attained by 3 h post-irradiation. Given that the p21^{WAF1}-dependent G₁ arrest depends on the stoichiometry of this protein with cyclin/CDK complexes (Zhang *et al.*, 1994), the G₁ arrest observed in LBC282 but not LBC286 may be attributed to differences in the effective threshold concentrations of p21^{WAF1} achieved after irradiation, which in the former cells are rapidly attained within 3 h after irradiation. It should be noticed that the rapid p53-dependent transactivation of p21^{WAF1} in LBC282, but not in LBC286, most likely reflects the different functional activity in these cells of the endogenous p53 heterocomplexes, as reported above.

Since DNA damage typically induces apoptosis by a p53-dependent mechanism, and since alterations of p53 normally antagonize this process (Lowe *et al.*, 1993a, b; Clarke *et al.*, 1993), we have also investigated the relationship between p53 heterozygous mutations and apoptosis. We have shown that, in contrast to normal LBC, LBC with wt/mut p53 alleles were significantly less sensitive to radiation-induced apoptosis. Coupled

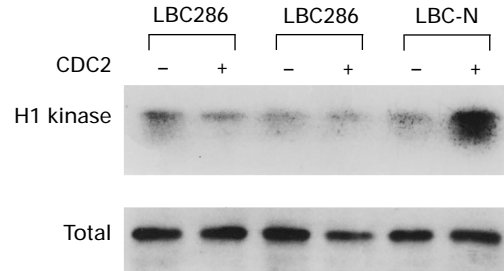


Figure 9 p34^{CDC2} histone H1 kinase activity before and 24 h after 400 rads treatment. Equal amounts of cytosol from control cells (-) or from cells harvested 24 h post irradiation (+) were incubated with a monoclonal antibody to p34^{CDC2}. The formed immunocomplexes were immobilized on sepharose, washed and assayed for histone H1 kinase activity in the presence of radiolabelled ATP, as reported in Materials and methods. The reactions products were resolved on SDS-PAGE and autoradiographed. To normalise for total p34^{CDC2} protein, the radioactive gel was electroblotted onto PVDF membrane, which was then probed with an antibody to p34^{CDC2} and ECL detection.

with the cell cycle results, these data indicate that p53 mutations contribute differently to cell cycle control and apoptosis.

To get an insight into the role of apoptotic genes in radiation-induced cell death, we have analysed the expression of the bcl-2 related family of genes. We have, in particular, examined bcl-2 and bax, two proteins which have opposing effects on cell survival and whose transcription is reciprocally regulated in cells undergoing apoptosis by enforced expression of wt p53 (Miyashita *et al.*, 1994a), apparently by interaction of p53 with regulatory DNA sequences present in these genes (Miyashita *et al.*, 1995a, b).

As shown here, the steady-state levels of Bcl-2 and Bax proteins remained unchanged during the 48 h following irradiation, thus suggesting that these genes are not directly involved in radiation-induced cell death in LBC cells, at least in the experimental conditions used here. These findings are concordant with data by Allday *et al.* (1995b) showing no changes in the bcl-2/bax protein ratio in LBC induced to death by the DNA-damaging agent cisplatin. It should be noted, however, that while irradiation produced no changes in bax protein levels in our LBC, it did induce a 2.6-fold increase in bax mRNA levels. Whether this discrepancy between the mRNA and protein data reflects post-translational mechanisms which control the levels of bax, as recently found in the case of cells over-expressing bcl-2 (Miyashita *et al.*, 1995b), remains to be investigated. These findings stand in contrast to other reports where γ -irradiation was shown to induce increases in Bax protein levels in lymphoid cells *in vivo* (Kitada *et al.*, 1996). Thus, we cannot currently exclude that the process of EBV-mediated immortalization, which does not neutralize the function of p53 with regard to p21^{WAF1} and MDM2 gene regulation (Allday *et al.*, 1995a), may have altered the downstream effects of p53 on Bax and other genes, compared to normal lymphocytes.

It has been recently shown that apoptosis induced by DNA-damaging agents proceeds from G₁/S in cells with normal p53, but from G₂/M in cells with mutant p53 (Allday *et al.*, 1995b). To verify if similar events occurred in LBC cells, we have employed a direct

method based on multiparameter flow cytometric analysis and verification by cell sorting. In contrast to the data of Allday *et al.*, our data show that radiation-induced apoptosis proceeds from all phases of the cell cycle, both in p53 wt/wt and wt/mut LBC, albeit in the latter cells the onset of apoptosis is delayed by more than 24 h.

Accumulation of wt p53 in irradiated cells causes cell cycle arrest at the G₁/S boundary, through the transactivation of its target gene p21^{WAF1}, which inhibits cyclin-dependent G₁ kinases (Harper *et al.*, 1993). The role of p21^{WAF1} in this process is emphasized by the fact that fibroblasts from mice null for p21^{WAF1} are deficient in their ability to arrest in G₁ in response to γ -radiation (Deng *et al.*, 1995). By gene transfer experiments it was recently shown that p53 plays also a role in G₂/M block (Stewart *et al.*, 1995; Agarwal *et al.*, 1995). To directly demonstrate the cell-cycle expression of p53 and of p21^{WAF1}, we have performed immunoblot analysis on G₁, S and G₂ fractions of unirradiated and irradiated LBC. By 3 h after irradiation, the amounts of p53 and of p21^{WAF1} proteins in LBC-N markedly increase in the three phases of the cell cycle. Similar increases of p53 and p21^{WAF1} are also observed in LBC286, although at a later time, as expected. Interestingly, the levels of p21^{WAF1} in irradiated cells are lower in S phase than in G₁ or G₂/M. This result is consistent with data by Li *et al.* (1994) showing lowest levels of p21^{WAF1} mRNA during S phase. Taken together, the expression of p53 and p21^{WAF1} in G₁, S and G₂/M suggests a possible role for these proteins throughout the cell cycle. The functional analysis of these proteins directly isolated from G₁, S and G₂/M will eventually clarify their exact role in cell cycle.

Premature activation of the serine-threonine kinase p34^{CDC2} has been reported to precede apoptotic cell death (Shi *et al.*, 1994). This finding, along with the observation that the disassembly of the nuclear lamins by p34^{CDC2} activation in cells entering mitosis causes similar morphological changes as those found in cells undergoing apoptosis (King and Cidlowski, 1995 for a review), has led to the suggestion that activation of this protein may also represent a general phenomenon of commitment to apoptosis. Our analysis has shown that p34^{CDC2-Tyr15} protein, the inactive form of p34^{CDC2}, in addition to being highly expressed in S and G₂/M but not in G₁ cells, is markedly reduced 24 h post irradiation in LBC-N but not in LBC286 or LBC282, at a time-point when apoptosis occurs in the former but not in the latter cells. Interestingly, the reduction of inactive p34^{CDC2-Tyr15} in irradiated LBC-N correlated with enhanced p34^{CDC2} histone H1 kinase activation. Although this result further supports the role of p34^{CDC2} activation in the apoptotic process, nonetheless the involvement of this protein kinase in apoptotic cell death remains controversial, as no activation of p34^{CDC2} has been detected in certain cell types undergoing apoptosis (Norbury *et al.*, 1994; Martin *et al.*, 1995), even accompanied by lamins degradation (Oberhammer *et al.*, 1994). Whether the cell type and/or the nature of the apoptotic stimulus influence the phosphorylation and activity of p34^{CDC2}, remains to be seen.

In conclusion, we have demonstrated that LBC heterozygous for p53 mutation at codon 282 have,

unlike those with p53 mutation at codon 286, a normal G₁ checkpoint, most likely associated with a relatively functional p53/DNA binding activity and a normal p21^{WAF1} transactivation response. We have shown that radiation-induced apoptosis in LBC cells is cell cycle-phase unrelated and appears not to involve bcl-2, bax, bcl-x or mcl-1. Further, we have demonstrated that basal and radiation-induced levels of p53 and p21^{WAF1} are found in G₁, S and G₂/M cells, but functional studies are required to establish their precise role throughout the cell cycle. Finally, we have provided evidence suggesting that p34^{CDC2} plays a part in the process of radiation-induced cell death.

Materials and methods

Production of lymphoblastoid cells, irradiation

Lymphoblastoid B-cells (LBC) were obtained by incubation of Ficoll-Hypaque separated peripheral blood lymphocytes from patients with Li-Fraumeni syndrome or from normal individuals with the supernatant of the EBV-producing cell line B95-8, as described (Pelloquin *et al.*, 1986). The cell lines were grown as polyclonal populations in RPMI 1640 (Bio-Wittaker, Walkersville, MD) plus 15% heat-inactivated fetal calf serum (Irvine Scientific, Santa Ana, CA), 100 U/ml penicillin, 100 μ g/ml streptomycin and 2 mmol/l glutamine, and grown at 37°C in 5% CO₂. Irradiations were performed using an IBL437CO instrument (Oris Industries, France) equipped with a ¹³⁷Cesium source providing 825 cGy/min. Control cultures were held at room temperature for the duration of irradiation.

Fluorescent dUTP labelling, flow cytometry and cell sorting

Apoptotic cells containing fragmented nuclear DNA were detected by *in situ* fluorescent dUTP-labelling of the 3'OH end of DNA by the enzyme TdT (Delia *et al.*, 1993) using reagents from Boehringer-Mannheim, Germany, and quantified by flow cytometry using a FACSVantage instrument (Becton-Dickinson, San José, CA). Cell viability was determined on samples stained (30 min at 37°C with 5 μ g/ml of the DNA-specific supravital fluorescent dye Hoechst 33342 (Calbiochem, San Diego, CA) and counted on a hemocytometer by fluorescence microscopy. For the simultaneous analysis of apoptosis and cell cycle phase, samples were fixed in 2% paraformaldehyde (10 min at 4°C), washed twice in Tris-buffered saline (TBS), treated for 20 min at 37°C with 10 μ g/ml RNase (Sigma, St Louis, MO), stained with 1 μ g/ml phalloidin-FITC (Sigma) and 25 μ g/ml propidium iodide (Sigma) and examined by flow cytometry as described (Endresen *et al.*, 1995). Live G₁, S and G₂/M fractions (purity >95%) were obtained from samples stained with 10 μ g/ml Hoechst 33342 (40 min at 37°C), and sorted using a FACSVantage instrument equipped with a 5 W argon-ion laser emitting 20 mW of light in the u.v. (352 nm), and with a cooling system for the sample and collection holders.

SDS-PAGE, immunoblot analysis, histone H1 kinase assay

Cell pellets from total or sorted samples were lysed in Lysis buffer (0.125 M Tris-HCl, pH 6.8, 5% SDS) containing protease plus phosphatase inhibitors, 1 mM PMSF, 10 μ g/ml pepstatin, 100 KIU/ml aprotinin, 10 μ g/ml leupeptin (all from Calbiochem) and 1 mM sodium orthovanadate (Sigma). The lysates were boiled for 2 min, sonicated for 20 s, and quantitated for protein content by the micro-

BCA method (Pierce, Rockford, IL). Aliquotes containing 20 µg/ml of protein plus 5% beta-mercaptoethanol were loaded on 12% or 7% SDS-PAGE, size-fractionated and electroblotted overnight onto PVDF membranes (Millipore, Bedford, MA). After blocking with 5% non-fat dried milk in PBS plus 0.1% Tween (Sigma), the membranes were incubated overnight with appropriate dilutions (1:800 to 1:1500) of the primary antibody. After extensive washing, the blots were incubated for 1 h with 1:2000 dilution of the peroxidase-labelled secondary antibody, washed again and revealed by enhanced chemiluminescence (ECL) using a kit from Amersham (Amersham, UK) and exposures to autoradiographic films. The reagents used were monoclonal antibodies to human bcl-2 (clone 100, Dako), p53 (clone DO7, Dako), p21^{WAF1} (Cat 15091A, Pharmingen, San Diego, CA), Rb (Cat 14001A, Pharmingen), MDM2 (Cat OP46, Oncogene Science), and the rabbit antibodies to Bax (Krajewski *et al.*, 1995a) and Mcl-1 (Krajewski *et al.*, 1995b). The rabbit antibody specific for p34^{cdc2} phosphorylated on Tyr-15 was from New England Biolabs (Beverly, MA), whereas the monoclonal antibody to total p34^{cdc2} (Cat SC054) was from Santa Cruz (Santa Cruz, CA). To normalise for protein content, all blots were re-probed with rabbit antibodies to HAP1 (Walker *et al.*, 1994) (kindly provided by Prof. Adrian Harris, ICRF, Oxford) or to β-actin (Sigma). The relative intensity of bands on the resulting films was quantified by scanning densitometry using an IBAS II instrument (Kontron, Italy).

Histone H1 kinase assay was performed as described (Donaldson *et al.*, 1994). Cell pellets were solubilised in NP40 lysis buffer (50 mM Tris-HCl, pH 7.5, 1% NP40, 2 mM EDTA, 2 mM EGTA, 1 mM PMSF, 5 mM sodium orthovanadate, 100× protease inhibitors) for 30 min on ice. After centrifugation at 13 000 g, the supernatants were recovered and quantitated for protein content. One hundred µg of this lysate was mixed with 30 µl of mouse anti-p34^{cdc2} (Clone A17.1.1, Neomarkers, Fremont, CA) for 1 h at 4°C, then 50 µl of protein A/G-Sepharose (Santa Cruz) was added for 3 h. Sepharose-bound immunocomplexes were washed 4× in NP40 lysis buffer and once in 50 mM Tris-HCl, thereafter incubated for 20 min at 37°C with 45 µL kinase buffer containing Tris-HCl pH 8.0, 10 mM MgCl₂, 1 mM dithiothreitol, 5 µCi[γ-³²P]ATP (Amersham) and 15 µg/ml histone H1 type IV (Sigma). After adding 30 µl Laemmli buffer and boiling, the products were resolved on 12% SDS-PAGE. Undried gels were autoradiographed to visualise the phosphorylation of histone H1, and then Western blotted onto PVDF membrane (as above) and probed with the mouse anti-p34^{cdc2} and ECL detection, to verify that equal amount of p34 protein were loaded per lane.

Mutation determinations, Northern blots, probes, gel shift assays

Heterozygous mutations of p53 were detected by SSCP analysis (Gaidano *et al.*, 1991) and confirmed by direct sequencing. The presence of wild type and mutated p53 alleles in each cell line was determined by direct sequencing of p53 RT-PCR products (Wada *et al.*, 1994) using the p53-specific sense oligonucleotide 5'-GCGTGTGGAG-TATTTGGATG-3' and the antisense oligonucleotide 5'-

TATTCTCCATCCAGTGGTTT-3'. The transactivation activity of the separated p53 alleles in yeast was analysed as reported (Flaman *et al.*, 1995). Northern blots were performed on total RNA isolated by RNAzol (Biotex, Houston, TX), size-fractionated (10 µg/lane) on agarose gels and blotted onto membranes. Membranes were hybridized in the presence of ExpressHyb solution (Clontech, Palo Alto, CA) to human DNA probes radiolabelled by a multiprime labelling kit (Amersham) to a specific activity of 1–2 × 10⁹ c.p.m./µg with [³²P]. Binding of probes was detected by autoradiography or by Fuji BAS2000 instrument (Japan). Levels of bcl-2, bax and mcl-1 were analysed with cDNA probes previously described (Tsujiimoto and Croce, 1986; Miyashita *et al.*, 1995b, Bodrug *et al.*, 1995), while bcl-x was analysed using a cDNA probe generated in our laboratory by RT-PCR and confirmed by sequencing. All membranes were re-probed with a β2-microglobulin cDNA probe also generated by one of us (SM), to normalise for RNA content. For the gel retardation assays nuclear extracts were obtained as described by Schreiber *et al.* (1989) from cells 3 h after exposure to 400 rads. Total protein concentration in these extracts was determined by micro-BCA method (Pierce), while p53 protein content was determined by immuno dot blot using the mouse anti-p53 antibody DO7 and ECL detection, followed by densitometric analysis. The p53 consensus oligonucleotide was 5'-TAGACATGCCTAGACATGCCTA-3' (El Deiry *et al.*, 1992) and its reverse (HPLC purified, Perkin-Elmer, Milano, Italy). The oligonucleotide 5'-GGACAGTCGGCCGACTGTCC-3' (and its reverse), containing mismatches in the p53-recognition site, was used as a negative control. Annealed oligonucleotides were radiolabelled in the presence of [γ-³²P]ATP (Amersham) using the T4 polynucleotide kinase (New England Biolabs, Beverly, MA) passed through a push column to remove free ATP. For the gel shift assay, the binding reactions were performed essentially as described by Funk *et al.* (1992), except that appropriate amounts of nuclear extracts were employed (between 8 and 12 µg) to normalise for p53 protein content. Whenever indicated, 2 µl of Pab421 hybridoma supernatant, which supershifts and stabilizes p53-DNA complexes (Funk *et al.*, 1992), or 50 ng of competing double strand oligonucleotides were added to the reaction mixture. After 20 min incubation at room temperature, the samples were electrophoresed at 120 V at 4°C on native polyacrylamide gel (4.5% in Tris-Borate-EDTA). Gels were dried and autoradiographed.

Acknowledgements

The work was financially supported by the Italian Association for Cancer Research (AIRC) and Foundation for Cancer Research (FIRC); by a Grant-in-Aid for Paediatric Research (6-5) from the Ministry of Health, Japan; by a Grant-in-Aid for Cancer Research from the Ministry of Health and Welfare, Japan; by grants of the NCI (CA 60181) and of the University of California Breast Cancer Research Program. The artwork was by Mario Azzini.

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