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Irradiation induces G2/M cell cycle arrest and apoptosis in p53-deficient lymphoblastic leukemia cells without affecting Bcl-2 and Bax expression

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Abstract

The tumor suppressor p53 has been implicated in gamma irradiation-induced apoptosis. To investigate possible consequences of wild-type p53 loss in leukemia, we studied the effect of a single dose of gamma irradiation upon p53-deficient human T-ALL (acute lymphoblastic leukemia) CCRF–CEM cells. Exposure to 3–96 Gy caused p53-independent cell death in a dose and time-dependent fashion. By electron microscopic and other criteria, this cell death was classified as apoptosis. At low to intermediate levels of irradiation, apoptosis was preceded by accumulation of cells in the G2/M phase of the cell division cycle. Expression of Bcl-2 and Bax were not detectably altered after irradiation. Expression of the temperature sensitive mouse p53 V135 mutant induced apoptosis on its own but only slightly increased the sensitivity of CCRF–CEM cells to gamma irradiation. Thus, in these, and perhaps other leukemia cells, a p53- and Bcl-2/Bax-independent mechanism is operative that efficiently senses irradiation effects and translates this signal into arrest in the G2/M phase of the cell cycle and subsequent apoptosis.

Keywords: p53; apoptosis; CCRF–CEM; human leukemia; cell cycle; gamma irradiation: Bcl-2; Bax; p21/WAF1

Abbreviations: ALL, acute lymphoblastic leukemia; FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; ts, temperature sensitive; PBS, phosphate buffered saline; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling

Introduction

Gamma irradiation induces apoptosis in many cell types and this phenomenon has been considered to critically depend upon the product of the tumor suppressor gene p53 (Lowe *et al*, 1993; Clarke *et al*, 1993; Komarova *et al*, 1997) whose main biological functions are control of cell proliferation and induction of apoptosis (Lane, 1992; Canman *et al*, 1994; White *et al*, 1994; White, 1995). Gamma irradiation induces DNA damage which is a known trigger of p53 activity (Vogelstein and Kinzler, 1992). The concept that irradiation causes cell death through p53-mediated apoptosis has been corroborated by studies with p53-deficient mice that showed markedly reduced sensitivity to irradiation-induced cell death in various cell types (Lowe *et al*, 1993; Clarke *et al*, 1993). Since over 50% of human cancers are deficient in p53 (Prokocimer and Rotter, 1994; Imamura *et al*, 1994), the proposed dependence of irradiation-induced apoptosis upon functional p53 has considerable consequences for cancer therapy. More recently, however, evidence has been provided suggesting that gamma irradiation may also cause apoptosis through p53-independent mechanisms (Strasser *et al*, 1994; Bracey *et al*, 1995; Allday *et al*, 1995; MacFarlane *et al*, 1996). Which cells might be equipped with such mechanisms and how these two types of cell death relate to each other on a mechanistic level, is not well understood. In the present study, we investigated the effect of gamma irradiation upon the widely used human acute lymphoblastic T-cell leukemia cell line CCRF-CEM (Norman and Thompson, 1977). CEM cells are compound heterozygous for the p53 mutations R175H and R248Q (Cheng and Haas, 1990) that render p53 deficient in the ability to transactivate typical p53 response genes and to mediate apoptosis (Geley *et al*, 1997a). Hence this cell line, and stably transfected subclones thereof expressing a p53 temperature sensitive mutant, are suitable substrates for the comparison of p53-dependent and p53-independent irradiation-induced cell death forms.

Results and Discussion

Gamma irradiation induces G2/M arrest and cell death in p53-deficient CCRF–CEM leukemia cells

To determine how p53-deficient CEM human leukemia cells respond to ionizing radiation, we exposed CEM–C7H2 cells to various doses of gamma irradiation. Subsequently, the cells were cultured for up to 96 h and subjected to determination of apoptosis by fluorescence activated cell sorter (FACS) analyses of propidium iodide-stained nuclei. As shown in Figure 1, gamma irradiation

caused cell death in these cells in a time and dose-dependent fashion. Thus, 0.75–1.5 Gy failed to induce significant apoptosis, 3–12 Gy led to about half-maximal to maximal apoptosis between 64–96 h while 24–96 Gy had killed significant numbers of cells already after 18 h and reached maximal levels of cell death soon thereafter.

In p53 wild type cells, irradiation causes arrest in the G1 phase of the cell cycle through transcriptional induction of the cyclin-dependent kinase inhibitor p21/WAF1 (El-Deiry *et al*, 1993; Dulic *et al*, 1994) and other mechanisms (Smith *et al*, 1994). To assess a possible effect of irradiation on cell cycle progression in the absence of functional p53, we subjected the gamma irradiated CEM–C7H2 cells shown in Figure 1 to FACS cell cycle analysis. At 1.5–6 Gy, the cells arrested in the G2/M phase of the cell cycle about 12–18 h after irradiation, with percentages directly proportional to the intensity of irradiation (Figure 2). The G2/M arrest did not appear to be irreversible as part of the cells re-entered the cell cycle as suggested by the reappearance of a G1 peak within the subsequent 12–24 h (exemplified in Figure 3). Nevertheless, another 24–48 h later, the majority of cells treated with 3–6 Gy succumbed to death (Figure 1). In cells treated with 24–96 Gy, significant cell death occurred already after 12 h. Since it takes these unsynchronized cells 12–18 h to accumulate in a particular phase of the cell division cycle, possible effects on the cell cycle were obscured by the early cell death. Cells treated with 12 Gy showed an intermediate phenotype, i.e., after 18 h some cells had already undergone apoptosis (Figure 1) while about 50% had arrested in the G2/M phase (not shown).

Gamma irradiation induced cell death in p53-deficient CCRF–CEM cells reveals typical features of apoptosis

To ascertain that the observed cell death reflected typical apoptosis, irradiated CEM–C7H2 cells were investigated by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL), Annexin V-staining and electron microscopy. As exemplified in Figure 4, TUNEL gave similar results as propidium iodide-staining, supporting the notion that cell death observed by the latter method was indeed apoptosis. This conclusion was further corroborated by the Annexin V method that, as expected, detected apoptosis several hours earlier than the propidium iodide-staining protocol (see last Results paragraph, Figure 9). Electron microscopy further revealed ultrastructural changes of the irradiated cells characteristic for apoptosis (Figure 5).

Expression of Bcl-2 and Bax are not detectably altered during irradiation-induced apoptosis of CEM–C7H2 cells

Bcl-2 protects various cell types from cell death induced by different apoptosis inducers including irradiation (Sentman *et al*, 1991; Strasser *et al*, 1991; Yin *et al*, 1994; Cory, 1995; Reed *et al*, 1996), possibly by regulating release of cytochrome c and apoptosis inducing factor from mitochondria (Kluck *et al*, 1997; Kroemer, 1997). To determine whether irradiation-induced apoptosis in our system is associated with, and perhaps caused by, reduction in Bcl-2 expression, we used Western blot analysis (Figure 6) to determine Bcl-2

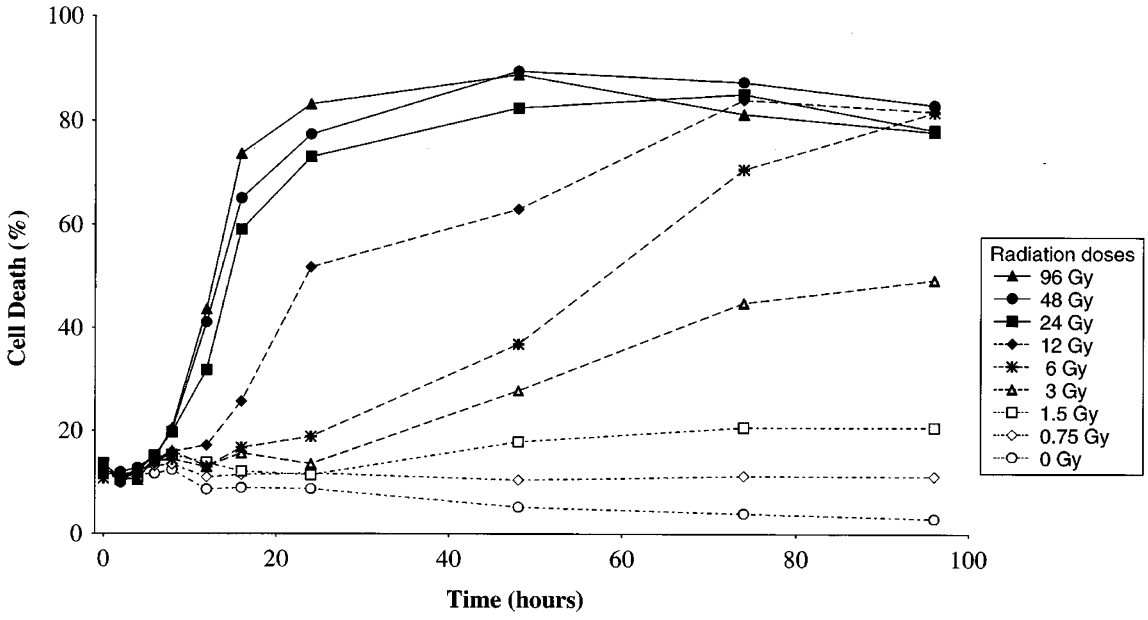

Figure 1 Radiation-induced cell death. CEM–C7H2 cells were irradiated with 0–96 Gy as indicated by the various symbols, cultured for up to 96 h, and apoptosis determined by propidium iodide-staining and FACS analysis

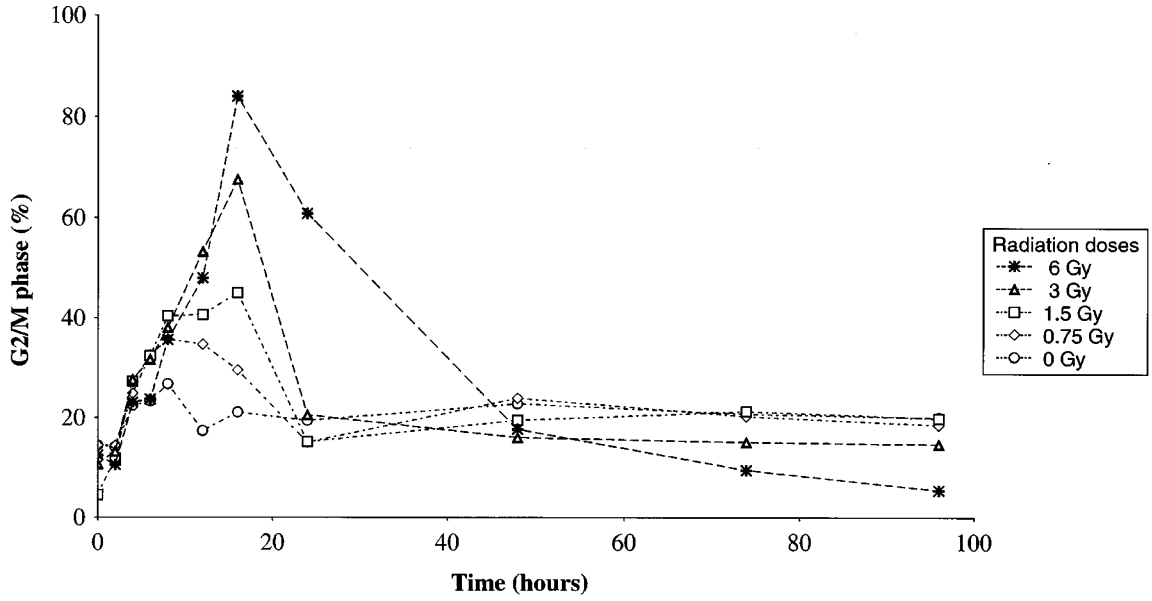

Figure 2 Radiation-induced G2/M cell cycle arrest. CEM–C7H2 cells were irradiated with 0–6 Gy as indicated by the various symbols, cultured for up to 96 h and the percentage of cells in the G2/M phase of the cell division cycle determined by propidium iodide-staining and FACS analysis

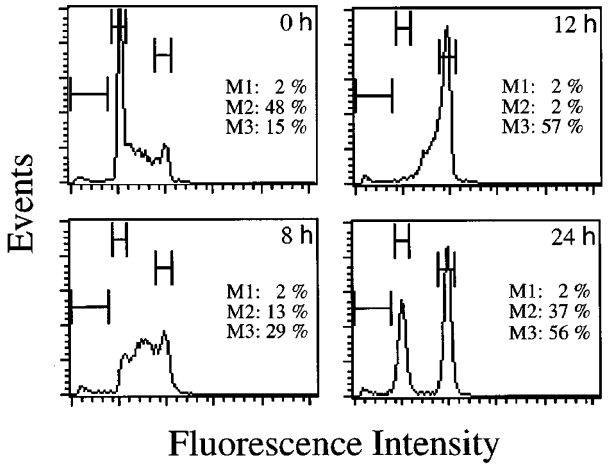

Figure 3 Example of a cell cycle analysis during gamma irradiation. CEM–C7H2 cells were irradiated with 6 Gy and subjected to cell cycle determination by propidium iodide-staining and FACS analysis after the time points indicated. The percentages of nuclei corresponding to apoptotic cells (marker window M1, sub-G1 peak) and cells in the G1 (marker window M2) or G2/M (marker window M3) phase of the cell division cycle are indicated in each panel

levels in CEM–C7H2 leukemia cells exposed to apoptosis-inducing levels of gamma irradiation. However, no reduction in Bcl-2 expression was detected. Since overexpression of the Bcl-2 antagonist Bax might equally account for cell death (Oltvai and Korsmeyer, 1994), we investigated expression of its mRNA in irradiated CEM–C7H2 cells. As shown in Figure 7, Bax mRNA was, however, not detectably increased in irradiated CEM–C7H2 cells. Thus, as far as the two investigated members of the Bcl-2 family were concerned, disturbance of the 'Bcl-2 rheostat' did not seem to be responsible for irradiation-induced cell death in this system.

Functional p53 only marginally increases sensitivity of CCRF–CEM cells to gamma irradiation-induced apoptosis

To investigate whether functional p53 affects the radiosensitivity of CEM cells, we employed three CEM–C7H2 subclones (C7H2-4G5, 3E12, 3H3) that constitutively express a temperature-sensitive p53 mutant (p53ts) (Geley *et al*, 1997a). When cultured at the permissive temperature (32°C), these cells expressed significant levels of the p53-regulated p21/WAF1 protein (Figure 8), supporting the functionality of the transfected p53. Although p53 induces apoptosis in these cells (Geley *et al*, 1997a; and Figure 9), Bcl-2 was not detectably altered (Figure 8), similar to the situation in irradiation-induced apoptosis in the absence of functional p53 (see previous paragraph, Figure 6). Parental C7H2 and the p53ts-transfected subclones were irradiated, cultured at 37°C or 32°C, and subsequently analyzed for development of apoptosis by the Annexin V and propidium iodide methods (Figure 9). All cells were about equally sensitive to irradiation at 37°C and as expected from our previous time course studies (Geley *et al*, 1997a), up to 14 h at the permissive temperature (32°C) did not yet entail significant levels of apoptosis in the non-irradiated p53ts-transfected C7H2 cells. However, when p53ts-transfected C7H2 subclones were irradiated and cultured at 32°C, they underwent higher degrees of apoptosis than non-irradiated cells at 32°C or irradiated cells cultured at 37°C suggesting that functional p53 increased the sensitivity of these cells to gamma irradiation (exemplified in Figure 9). However, this increase, although consistently detectable, was sometimes only a few percent and never exceeded 50% by much supporting the concept that the p53-independent pathway to irradiation-induced cell death functions quite efficiently in these leukemia cells.

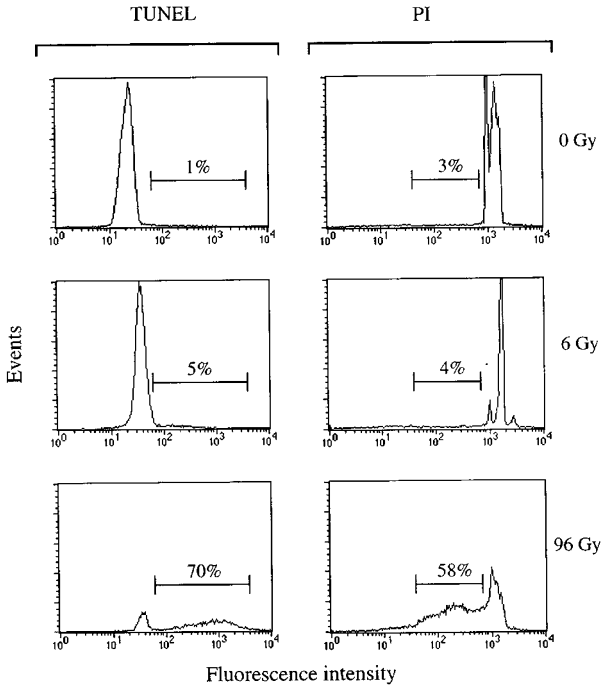

Figure 4 TUNEL and propidium iodide (PI) methods detect similar levels of apoptosis. CEM–C7H2 cells were exposed to 0, 6 or 96 Gy, cultured for 24 h and subjected to determination of apoptotic cells by the TUNEL and PI methods. The percentages of apoptotic cells are indicated above the marker windows

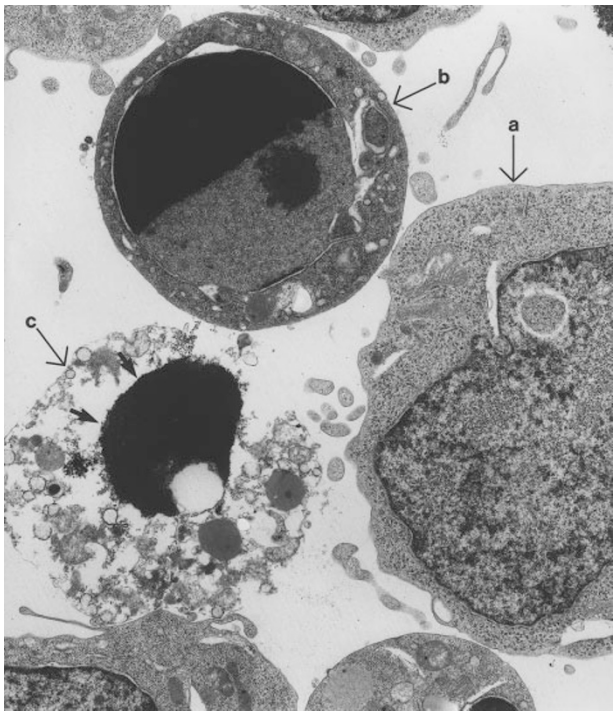

Figure 5 Electron microscopic appearence of irradiated CEM–C7H2 cells. CEM–C7H2 cells were exposed to 96 Gy, cultured for 8 h and subjected to transmission electron microscopy. Cell 'a' resembles a normal lymphocytes of the control samples. Cell 'b' exhibits typical features of apoptosis (condensed chromatin in a semilunar arrangement within the nuclear space; intact cytoplasmic membrane). Lymphocyte 'c' reveals typical apoptotic features (condensed chromatin, arrows) and in addition a massive destruction of all cellular components, typical for late apoptotic cells. Magnification × 11.220

The present study shows that CCRF–CEM cells, like many other cell types (Strasser *et al*, 1994; Bracey *et al*, 1995; Allday *et al*, 1995; MacFarlane *et al*, 1996), are equipped with a p53-independent gamma irradiation-triggered cell death pathway. How this pathway relates to the p53-dependent death pathway on a mechanistic and molecular level is not well understood. While irradiation in p53 wild type cells often causes G1 arrest (Dulic *et al*, 1994; Allday *et al*, 1995; Pellegata *et al*, 1996), we observed that, in the absence of functional p53, this treatment leads to increased numbers of cells in the G2/M phase of the cell division cycle. Similar observations were made by us in CCRF-CEM cells treated with the DNA-damaging compound doxorubicin (Geley *et al*, 1997a) and by others in other systems (Allday *et al*, 1995; Kan *et al*, 1995) supporting the notion that DNA-damage in the absence of functional p53 activates a checkpoint at the G2/M transition. Regarding the molecules transmitting the death signals, activation of p53 by irradiation causes transcriptional repression of bcl-2 and induction of bax gene expression (Reed *et al*, 1996). Bcl-2 mRNA downregulation has also been observed during irradiation-induced death of U937 and HL-60 human leukemia cells (Chen *et al*, 1995) that are p53-deficient (Danova *et al*, 1990; Dou *et al*, 1995). CCRF–CEM cells behaved differently in this respect, because Bcl-2 levels were unaltered during irradiation-triggered, p53-independent apoptosis (Figure 6). However, Bcl-2 was also not regulated during apoptosis induced by functional p53 (Figure 8), hence, the unresponsiveness of Bcl-2 levels to these

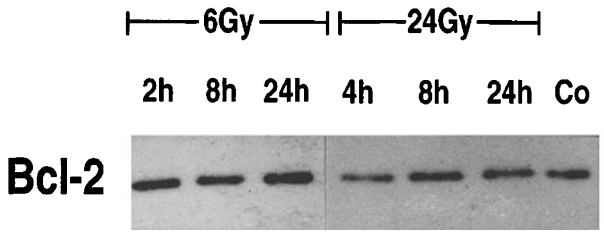

Figure 6 Irradiated CEM–C7H2 cells do not downregulate Bcl-2 expression. CEM–C7H2 cells were exposed to 0, 6 or 24 Gy, cultured for the indicated time, and subjected to Western blot analysis using a monoclonal antibody to Bcl-2

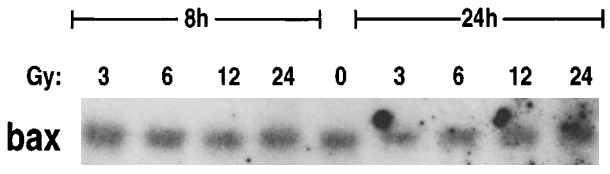

Figure 7 Irradiated CEM–C7H2 cells do not upregulate Bax mRNA expression. CEM–C7H2 cells were exposed to 0, 3, 6, 12 or 24 Gy, cultured for 8 h or 24 h and subsequently subjected to Northern blot analysis using a ^{32}P-labeled cDNA probe for mouse bax

apoptosis inducing stimuli may be specific for T-cell leukemia or for this particular cell line. Bax mRNA expression was also not detectably altered in the course of p53-independent irradiation-induced apoptosis (Figure 7), although we previously noted a weak induction of bax mRNA in association with p53-mediated apoptosis in p53ts expressing CCRF–CEM cells (Geley *et al*, 1997a) suggesting a possible difference in the pathways to p53-dependent and independent cell death. Whether other members of the growing Bcl-2 family are involved in p53-independent irradiation-induced apoptosis remains to be analyzed.

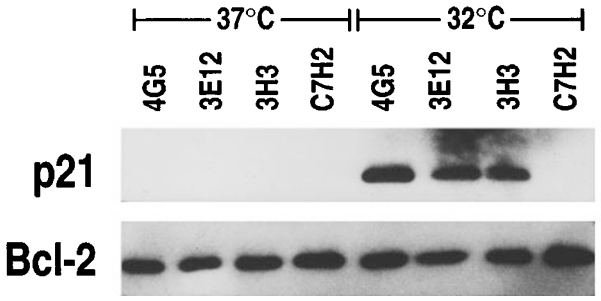

Figure 8 p53 induces p21/WAF1 but has no effect upon Bcl-2 expression in p53ts expressing CEM–C7H2 subclones. Parental CEM–C7H2 and p53ts-transfected subclones 4G5, 3E12 and 3H3 were cultured at 37°C or at 32°C (the permissive temperature for p53ts) for 14 h and subjected to Western blot analysis using a monoclonal antibody to p21/WAF1

Material and Methods

Cell lines and gamma irradiation

Cell culture conditions, the CEM–C7H2 cell line (Strasser-Wozak *et al*, 1995), and the C7H2 subclones 4G5, 3E12 and 3H3 (Geley *et al*, 1997a) that are stably transfected with plasmid pLTRp53V135 (Yonish-Rouach *et al*, 1991) have been described previously. Proliferating CEM cells (1×10^6/ml) were irradiated (0.75–96 Gy, 8.7 Gy/min) in one dose with a gamma source (IBL 437C, ORIS Industry, GIF sur Yvette, France) and subsequently cultured for various time points as indicated in the Results section.

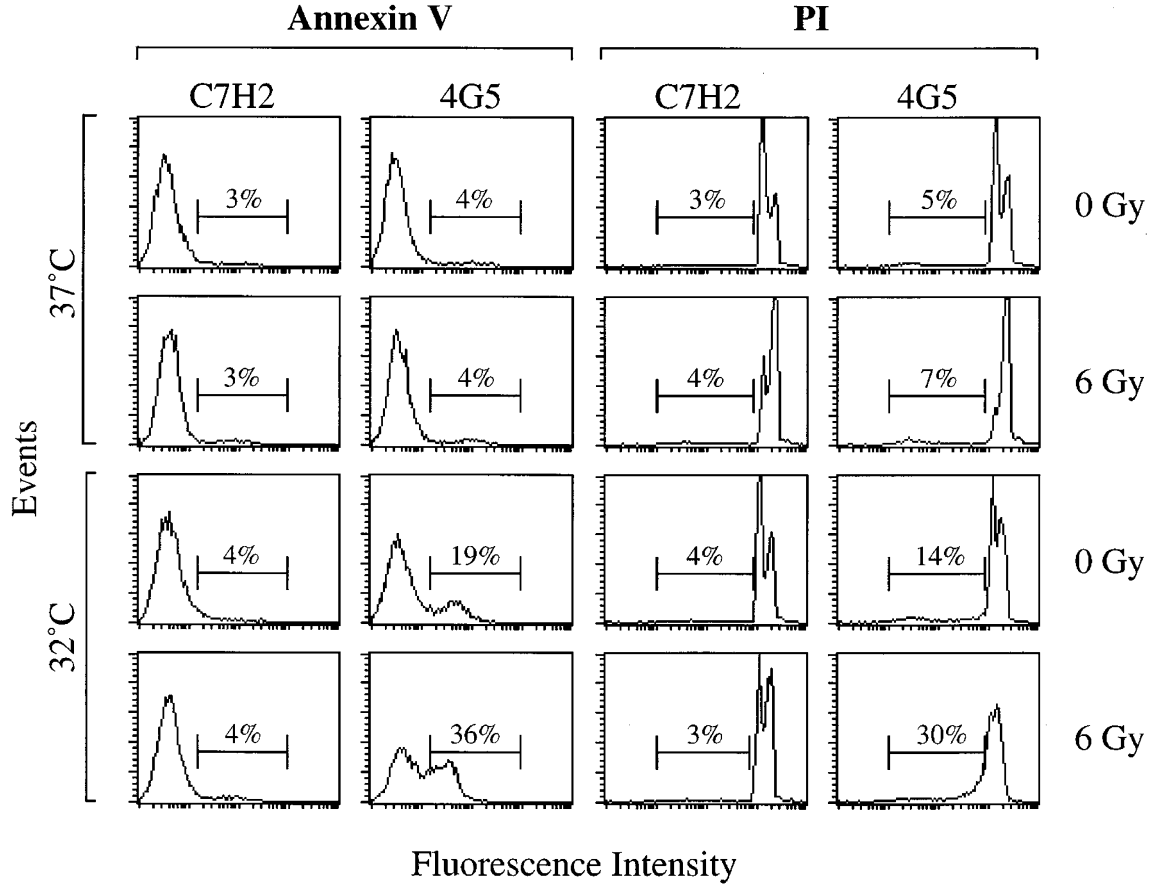

Figure 9 Functional p53 cooperates with gamma irradiation to induce apoptosis in CEM–C7H2 cells. CEM–C7H2 subclones stably transfected with a temperature sensitive p53 mutant (p53ts) and the parental CEM–C7H2 line were exposed to 0 or 6 Gy and cultured for 8 h (cells subjected to Annexin V analysis) or 14 h (cells analyzed by the propidium iodide/PI method) either at 37°C, the temperature at which the transfected p53ts mutant is inactive, or at 32°C, the permissive temperature for p53ts. Subsequently the cells were subjected to apoptosis analysis by the Annexin V or the PI method. The percentages of apoptotic cells are indicated above the marker windows. Shown are the results obtained with subclone 4G5. Additional experiments with this and the subclones 3H3 and 3E12 gave similar results although the cooperative effect was sometimes less apparent than in the example shown

Determination of apoptosis

For detection and quantification of apoptosis, four distinct methods were used. DNA-staining with propidium iodide and the TUNEL method have been detailed previously (Geley *et al*, 1997a). For electron microscopic examination, fixation of the cells was performed in 2.5% glutaraldehyde buffered to pH 7.2 in 0.1 M sodium cacodylate for 2 h at 4°C. After an overnight rinse in buffer, the cells were postfixed in 1% acetate-buffered OsO_4 for 2 h, dehydrated in graded series of ethanol and embedded in Epon. Ultrathin sections stained in alcoholic uranyl acetate and alkaline lead citrate were examined in a Philips CM 120. Annexin V binding (Martin *et al*, 1996) was determined using the TACS Annexin V - FITC kit (TREVIGEN, Gaithersburg, MD) as described by the manufacturer. Briefly, approximately 2.5×10^5 cells were incubated with FITC-labeled Annexin V and propidium iodide, washed and analyzed on a FACScan as above (forward/sideward scatter, red and green fluorescence).

Cell cycle analyses

For cell cycle analyses, the propidium iodide method of Nicoletti *et al* (1991) was used. To gate out cell doublets, dot plot analysis was additionally conducted in FL3-Width/FL3-Area and fluorescence intensity was plotted on a linear rather than a logarithmic scale.

Western and Northern blot analyses

Bcl-2 and p21/WAF1 Westerns were performed similar as previously described (Geley *et al*, 1997b) using mouse monoclonal antibodies against human Bcl-2 (Clone 124, Genosys, London, UK) or human p21/WAF1 (Clone 70, Transduction Laboratories, Lexington, KY) followed by horseradish peroxidase-labeled anti-mouse IgG antibody (Southern Biotechnology Association, Birmingham, AL), and developed using the enhanced chemiluminescence technique (Amersham, Buckinghamshire, UK). Northern analyses were performed as previously detailed (Geley *et al*, 1996) using heat-denatured ^{32}P-labeled mouse bax or humna α-tubulin cDNA probes, respectively (kindly provided by Drs S.J. Korsmeyer and A. Helmberg, respectively).

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