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p53-independent death and p53-induced protection against apoptosis in fibroblasts treated with chemotherapeutic drugs

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Summary Many recent studies have implicated p53 in the cellular response to injury and induction of cell death by apoptosis. In a rat embryonal fibroblast cell line transformed with c-Ha-*ras* and a mutant temperature-sensitive p53 (val135), cells were G_1 arrested at the permissive temperature of 32°C when overexpressed p53 was in wild-type conformation. In this state cells were resistant to apoptosis induced by etoposide (at up to 50 μM) or bleomycin (15 μU ml^{-1}). Cells at 37°C with overexpressed p53 in mutant conformation were freed from this growth arrest, continued proliferating and showed dose-dependent increases in apoptosis. This death is independent of wild-type p53 function. Control cells containing a non-temperature-sensitive mutant p53 (phe132) were sensitive to both etoposide and bleomycin after 24 h at 32°C and 37°C, indicating that the results are not simply due to temperature effects on pharmacokinetics or DNA damage. Our data show that induction of a stable p53-mediated growth arrest renders these cells much less likely to undergo apoptosis in response to certain anti-cancer drugs, and we conclude that the regulatory role of p53 in apoptosis is influenced by the particular cellular context in which this gene is expressed.

Keywords: p53; *ras*; apoptosis; etoposide; bleomycin; cell cycle; fibroblasts

Many tumours are resistant to chemotherapy, either intrinsically or following an initial partial response. A number of pharmacokinetic explanations may account for this, including overexpression of the multidrug resistance gene *mdr*1, overexpression of drug detoxication enzymes, or alteration of the drug target, for example topoisomerase II isoform. However despite intensive study of drug–target interactions, and drug metabolism, it is clear that in many instances drug resistance is associated with a failure of induction of apoptosis, even after an appropriate triggering event. Since many anti-cancer drugs and ionising radiation damage DNA, the response of the cell in recognising injury and proceeding to repair or apoptosis is of paramount importance (Hickman, 1992; Harrison, 1995).

Entry to apoptosis is regulated by a number of genes (see Bellamy *et al.*, 1995 for general review), each of which may show abnormal expression or function in cancer. In Rat-1 fibroblasts cell cycle arrest or serum deprivation in the presence of constitutive expression of the c-*myc* oncogene can cause apoptosis (Evan *et al.*, 1992). By contrast, overexpression of *bcl*-2 directly inhibits apoptosis in both normal and neoplastic cells (Hockenberry *et al.*, 1990; Sentman *et al.*, 1991; Miyashita and Reed, 1992, 1993; Veis *et al.*, 1993) and prevents c-*myc*-driven apoptosis (Wagner *et al.*, 1993). More recently evidence has accumulated implicating the tumour-suppressor gene p53 in an injury-response pathway leading to apoptosis. Thymocytes and myeloid progenitor cells from p53 knockout mice, fail to undergo *induced* apoptosis in the absence of a wild-type p53 allele following etoposide or ionising radiation treatment but not apoptosis associated with ageing *in vitro* or non-clastogenic insults such as dexamethasone treatment. (Clarke *et al.*, 1993; Lotem and Sachs, 1993; Lowe *et al.*, 1993*a*). Furthermore overexpression of wild-type p53 in a variety of cancer-derived cell lines such as M1 myeloid leukaemia (Yonish-Rouach *et al.*, 1991), murine erythroleukaemia (Ryan *et al.*, 1993) and HT29 colon carcinoma (Shaw *et al.*, 1992) resulted in an increase in *spontaneous* apoptosis.

By contrast, studies of p53 null fibroblasts grown in primary culture have failed to detect alteration in cell survival characteristics after DNA damage as compared with normal primary fibroblasts (Slichenmeyer *et al.*, 1993). In the latter experiments, cells were isogenic apart from p53 status. This suggests that other factors, including cell lineage and expression of oncogenes may modulate the effects of p53 on cellular physiology. In both experimental and human tumorigenesis p53 inactivation is believed to be a late event and is therefore superimposed on a series of progressive genetic abnormalities, such as activation of *ras* oncogenes (Fearon and Vogelstein, 1990).

In this study we have used a rat embryonal fibroblast line (Clone 6) transformed with activated Ha-*ras* and a temperature-sensitive p53 mutant as a model of the role of p53 in anti-cancer drug therapy in the presence of other genetic alterations. We report that induction by wild-type p53 of a G_1 arrest protects Clone 6 cells from apoptosis caused by the anti-cancer drugs etoposide and bleomycin. Our data imply that wild-type p53 provides a mechanism of resistance of cells to chemotherapy but by allowing continued proliferation p53 mutations may nonetheless contribute to the development of drug resistance.

Materials and methods

Clone 6 cells and RcGphe132.4 cells

Clone 6 cells are rat embryonic fibroblasts constitutively expressing a human mutationally activated c-Ha-*ras*1 gene and a murine, temperature-sensitive p53 mutant, p53val135. At the permissive temperature of 32°C the p53 protein is found predominantly in wild-type configuration but at 37.5°C it adopts mutant conformation and function (Michalovitz *et al.*, 1990). RcGphe132.4 cells contain a temperature-stable p53phe132 mutation in addition to activated c-Ha-*ras*1. The level of p53 expression in these two cell lines is similar. All manipulation of cell lines and counting was performed at the selected temperature to minimise the risk of inadvertent p53 conformational shifts.

Cell culture

Cells were plated in duplicate flasks at a density of 2×10^4 cells cm^{-2} in Glasgow modified Eagle's medium (GMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics.

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Quantitation of cell number and apoptosis

Reference points (three per flask) were used to count directly the number of cells in × 100 field using a 10 × 10 graticule. This permitted sequential counts at 20, 28 and 42 h after plating at 32°C or 37°C. Apoptotic cells adherent to the monolayer were counted at each time point as well as cell number. The apoptotic cells were recognised by virtue of their spherical, highly refractile appearance under phase contrast. These cells showed the classical appearances of apoptosis and were confirmed by electron microscopy and acridine orange fluorescence microscopy (Arends and Harrison, 1994).

Effects of bleomycin and etoposide

Twenty four hours after plating at 37°C cells were either moved to a 32°C incubator or maintained at 37°C for a further 16 h. Etoposide (10, 50 μM) or bleomycin sulphate (15 μU ml^{-1}: 1 U = 1 mg bleomycin A2) were added for 1 h and then washed with phosphate buffered saline (PBS). Controls were performed using equal concentrations of dimethyl sulphoxide (DMSO) or PBS vehicles.

The number of viable and apoptotic cells was counted at intervals up to 50 h following drug treatment. The mean number of apoptotic bodies per field was expressed as a percentage of the mean adherent cell number ('percentage apoptosis'). RcGphe132.4 cells were counted 24 h after drug treatment.

Cell cycle analysis

Nuclei were isolated and stained with propidium iodide (Vindelov *et al.*, 1983), and 1 × 10^4 cells were analysed on an EPICS CS flow cytometry (Coulter). Histogram analysis was performed using the Easy 2 Software. No doublets were seen. Bromodeoxyuridine incorporation analysis was carried out using the Amersham Cell Proliferation Kit (cat no. RPN20).

Results

Clone 6 cells are growth arrested at 32°C

Exponentially growing cells were shifted to a 32°C incubator. In three independent experiments cells ceased to show increase in cell number at 32°C (Figure 1). There was no increase in apoptosis in the presence of p53 with wild-type configuration (see Figure 2 controls). At 37°C, with mutant conformation p53 there was a 3-fold increase in cell number over the same period, confirming the original observations of Michalovitz *et al.* (1990). DNA flow cytometry showed both diploid and tetraploid peaks at permissive and non-permissive temperatures. At 32°C there was in increase in the diploid $G_{0/1}$ peak (Figure 3), and cells did not take up bromodeoxyuridine consistent with this state (data not shown). This growth arrest was reversible by transferring cells to 37°C, even after 2 weeks, or more. By contrast RcGphe132.4 cells continued to grow at a slightly reduced rate at 32°C in keeping with the previous observations of Michalovitz *et al.* (1990).

Clone 6 cells with wild type p53 are resistant to both etoposide and bleomycin

At 37°C, in the presence of mutant conformation p53, there was a progressive increase in apoptosis starting 6–10 h after pulsing with drug (Figure 2). The increase was dose dependent: etoposide at 10 μM induced a maximum of 6% apoptosis whereas at 50 μM the maximum was greater than 30% apoptosis (Figure 4). By contrast, cells maintained at 32°C with p53 in the wild-type conformation showed no increase in percentage apoptosis, nor in cell number (Figures 2 and 4). Treatment with bleomycin showed similar effects (Figure 5).

RcGphe132.4 cells are sensitive to apoptosis induced by etoposide and bleomycin at 32°C and 37°C. We considered the possibility that these differences in cell proliferation and apoptosis in response to DNA damage might be due simply to altered pharmacokinetics at the different temperatures. The RcGphe132.4 cell line was derived from the same parental stock as Clone 6, but contains a temperature-insensitive mutant p53; hence in this cell line wild-type p53 is excluded from function at both 32°C and 37°C. At 37°C Clone 6 and RcGphe132.4 cells show closely similar entry into apoptosis: 24 h after treatment with 50 μM etoposide the incidences were 18.0% and 19.4% respectively. In contrast, at 32°C the incidence of apoptosis in RcGphe132.4 cells was 9.5%, but had fallen to less than 2% in Clone 6 cells. Very similar results were obtained following treatment with bleomycin. At 37°C incidence of apoptosis in Clone 6 cells was 19.8%, but fell to less than 3% at 32°C. In contrast, RcGphe132.4 cells

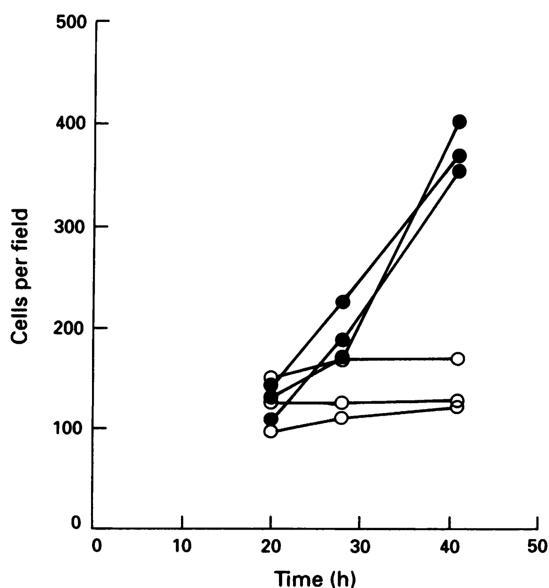

Figure 1 Growth properties of Clone 6 cells at 37°C (●) and at 32°C (○). Each point represents the mean number of cells per field ($n = 3$) in one flask at each time point. Note that the cells incubated at 32°C do not increase in number consistent with a wt p53-induced growth arrest.

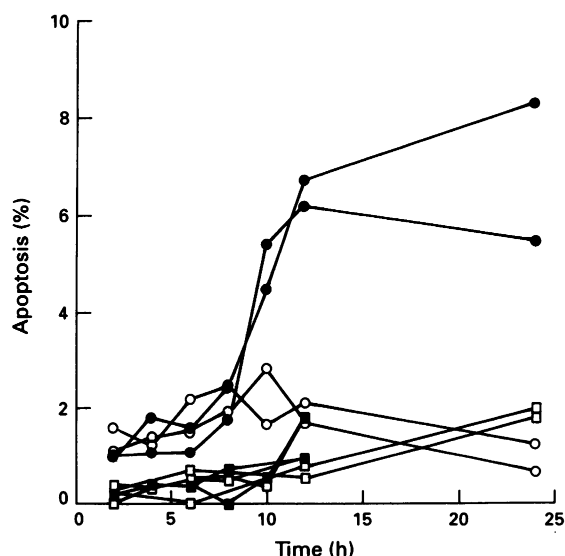

Figure 2 Treatment of Clone 6 cells with 10 μM etoposide at 37°C (●) for 1 h results in substantial apoptosis, where as treated cells at 32°C (■) and untreated controls (unfilled symbols) do not show this increase. Note the latent period during induction of apoptosis at 37°C. Each line represents a separate experiment (performed in triplicate and expressed as a mean, for low values the range was less than 0.6% and for higher value the range was up to 2%).

showed 10.3% apoptosis at 37°C and 8.9% at 32°C. Thus the profound inhibition of apoptosis in Clone 6 cells at 32°C is dependent upon the altered configuration of p53 to wild-type and is not explicable solely on the basis of temperature effects on pharmacokinetics.

Discussion

Expression of wt p53 has been shown to induce apoptosis in some cell types (Yonish-Rouach *et al.*, 1991; Shaw *et al.*, 1992; Ryan *et al.*, 1993), G_1 arrest and survival in others (Baker *et al.*, 1990; Diller *et al.*, 1990; Mercer *et al.*, 1990; Michalovitz *et al.*, 1990; Kastan *et al.*, 1991; Kuerbitz *et al.*, 1992). In addition, wt p53 has been shown to be an essential intermediate in a signal transduction pathway between the effects of DNA damaging agents (DNA strand breaks) and either apoptosis or G_1 arrest (Kastan *et al.*, 1992; Kuerbitz *et al.*, 1992; Clarke *et al.*, 1993; Lowe *et al.*, 1993*a*). In this way p53 seems to play a critical role in deleting certain cell types that have sustained DNA damage e.g. thymocytes (Clarke *et al.*, 1993), lymphocytes (Gottlieb *et al.*, 1994; Howie *et al.*,

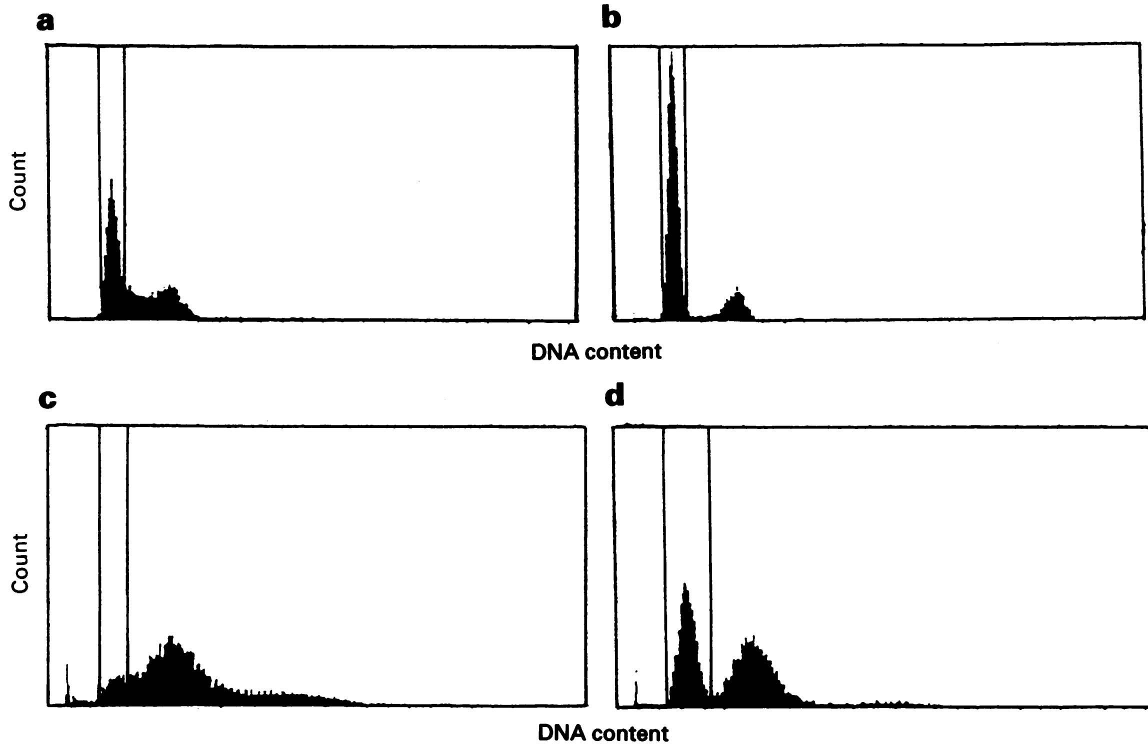

Figure 3 Cell cycle analysis of Clone 6 cells. (**a**) Exponentially growing cells at 37°C, untreated (G_0/G_1 fraction: 46.56%). (**b**) Following incubation at 32°C for 24 h, the G_0/G_1 peak is enlarged (72.51%) and there is a marked decrease in the proportion of cells between the G_0/G_1 and G_2/M peaks (S-phase). (**c**) At 37°C, 24 h after etoposide treatment (50 μM) cells accumulated in G_2/M with only 10.18% of cells occupying the G_0/G_1 position. (**d**) At 32°C, 24 h after treatment with 50 μM etoposide (G_0/G_1 fraction: 38.51%). Abcissa; DNA content (propidium iodide fluorescence).

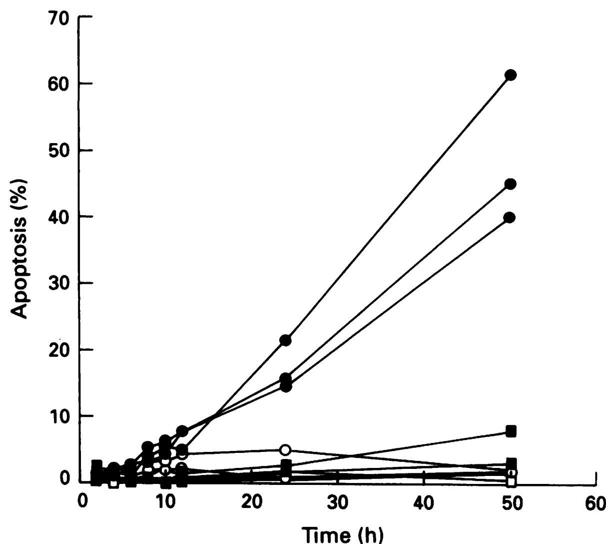

Figure 4 Treatment of Clone 6 cells with 50 μM etoposide for 1 h induces substantial apoptosis when cells are incubated at 37°C (●). At 32°C treated cells (■) and in control cells treated with an equivalent volume of DMSO vehicle (unfilled symbols) do not show an increase in percentage apoptosis. Each line represents a separate experiment (performed in triplicate and expressed as a mean. For low values range was 2% and for high values was up to 22%).

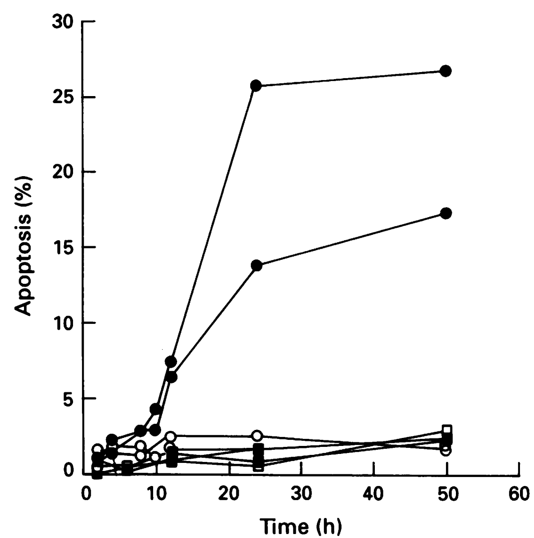

Figure 5 Clone 6 cells treated with 15 μU ml^{-1} bleomycin sulphate for 1 h at 37°C (●) and at 32°C (■). Note that treated cells incubated at 37°C undergo substantial apoptosis whereas treated cells at 32°C and untreated controls (open symbols) do not shown an increase in percentage apoptosis. Each line represents a separate experiment (performed in triplicate and expressed as a mean. For low values range was 2% and for high values range was up to 12%).

1994; Griffiths *et al.*, 1995) and myeloid progenitor cells (Lotem and Sachs, 1993) or in establishing a state of G_1 arrest, possibly permitting DNA repair (Lane, 1993; Bakalkin *et al.*, 1994). Clearly the cellular context in which p53 is expressed is important. Murine fibroblasts or primary rat kidney cells can be induced to undergo apoptosis by p53 in response to disruption of growth control by coexpression of c-*myc* (Wagner *et al.*, 1994) or adenovirus E1A (Debbas and White, 1993) respectively.

The finding that p53 function is lost in many authentic human and experimentally induced animal tumours has led to the assumption that p53 loss of function is causally associated with resistance to anti-cancer therapy (Lowe *et al.*, 1993*b*). In this study we have addressed the importance of p53 status on the sensitivity of cells to apoptosis induced by two anti-cancer drugs.

We have shown here, in a fibroblast cell line transformed with activated Ha-*ras* and temperature-sensitive p53 transgenes that wild-type p53 leads to G_1 arrest and at the same time resistance to the DNA damaging agents bleomycin and etoposide. By contrast, in the presence of mutant conformation p53, cells underwent apoptosis associated with a relative accumulation in G_2/M, a common response to DNA injury in yeast and mammalian cells (Hartwell and Weinert, 1989). We were unable to produce a G_0/G_1 arrest in Clone 6 cells at 37°C by either mimosine treatment or serum starvation as these treatments caused the death of the cultures. We were thus unable to show directly that a growth arrest in G_0/G_1, independent of p53, was protective against DNA damage.

Our findings apparently contrast with published work in which temperature-sensitive p53 was expressed in the M1 myeloid leukaemic (M1; Yonish-Rouach *et al.*,1993) and murine erythroleukaemic (MEL; Ryan *et al.*, 1993) cell lines induced apoptosis upon incubation at 32°C (i.e. with wild-type p53). MEL cells underwent G_1 arrest before undergoing apoptosis, but in M1 cells, no growth arrest could be observed at any position in the cell cycle. In addition, other cell types (including rat fibroblasts) have been shown to undergo G_1 arrest but not apoptosis in response to wild-type p53 induction (Diller *et al.*, 1990; Mercer *et al.*, 1990; Michalovitz *et al.*, 1990; Kastan *et al.*, 1992). While bleomycin and etoposide maximally kill cells in S-phase, where replication forks are forced to negotiate either cleaved complex / double strand breaks (etoposide; Bae *et al.*, 1988) or double-strand breaks resulting from free-radical attack (bleomycin; Kuo, 1981), they can damage and kill cells in G_0/G_1 (Roy *et al.*, 1992; Clarke *et al.*, 1993; Evans *et al.*, 1994). In cell lines derived from clinically sensitive human tumours, DNA injury-induced wild-type p53 was held to be responsible for decreased clonogenicity following ionising radiation and this effect could be reversed by transfection of a dominant negative mutant p53. (McIlwrath *et al.*, 1994). The simplest explanation of *our* data is that the G_1 arrest mediated by p53 facilitates survival of *ras*-transformed fibroblasts by allowing effective DNA repair and prevents entry into S-phase, a stage when cells are often most susceptible to DNA damage.

Depending upon the cell system chosen, induction of p53 can cause either G_1 arrest, apoptosis or both apoptosis and G_1 arrest (Michalovitz *et al.*, 1990; Debbas and White, 1993; Ryan *et al.*, 1993; Yonish-Rouach *et al.*, 1993; Wu and Levine, 1994). The mechanisms by which decisions are taken that favour any of these end points are poorly defined but these decisions can be affected by specific growth factors (Yonish-Rouach *et al.*, 1991; Gottleib *et al.*, 1994; Canman *et al.*, 1995). In particular, it is not known how p53 can mediate apoptosis in the thymocyte but not in the fibroblast. The recognition of DNA damage (possibly involving the ataxia telangiectasia gene products; Kastan *et al.*, 1992) leads, via p53, to the control of the cell cycle at the G_1 checkpoint. We have shown this pathway to be protective in fibroblasts. Our results complement those of Lowe *et al.* (1993*b*) who showed that p53-normal fibroblasts were susceptible to anti-cancer treatment as a result of abrogation of the p53-mediated G_1 arrest by adenovirus EIA expression. Further, interleukin 6 (IL6) protects M1 cells from undergoing p53-mediated cell death (Yonish-Rouach *et al.*, 1991, 1993) and this protection also correlates with the induction of a G_0/G_1 arrest. (Levy *et al.*, 1993).

Wafl (Cipl / sidl, p21), a gene product which is induced by wt p53, has potent inhibitory activity on cyclin E / cdk2 complexes in cells undergoing radiation-induced G_1 arrest (El-Deiry *et al.*, 1993, 1994; Dulic *et al.*, 1994). Wafl is therefore a major regulator of cell cycle progression at the G_1/S interface. The expression of Wafl in cell types that undergo apoptosis following activation of the p53 pathway suggests that it may be active in both arrest and death mechanisms. The decision of a cell to die may therefore be determined by other lineage-dependent messages or growth factors (Canman *et al.*, 1995), although the activity of *Wafl* as an apoptosis-inducing gene has not yet been directly tested. One such determinant may be the level of activity of the transcriptional regulator E2F-1. When constitutively overexpressed in the presence of wild-type p53 this triggers death in fibroblasts (Wu and Levine, 1994).

Using a different mutated p53 (proline substituted at residue 193) under its physiological promoter, Bristow *et al.* (1994) have recently shown that co-transfection of activated Ha-*ras* and mutated p53 into a primary rat embryonal fibroblast cell line resulted in enhanced clonogenicity *in vitro* and tumorigenicity in severe combined immunodeficient (SCID) mice after irradiation compared with cell lines containing *ras* alone. This effect was dependent on the level of mutant p53 expression, presumably as a result of competition with endogenous wild-type p53. However they did not directly assess the proportion of cells undergoing proliferation, growth arrest or cell death. We could not carry out experiments similar to those of Bristow *et al.* (1994) with ionising radiation sources as we found that reproducibility of results could not be maintained if there were fluctuations in temperature of Clone 6 cells before or during experiments. Indeed, clonogenicity of Clone 6 at 32°C is negligible.

Our *in vitro* experiments with DNA-damaging drugs (including the radiomimetic bleomycin) show that, under certain circumstances, overexpression of wild-type p53 can protect a cell which has suffered DNA injury against death rather than kill it, by causing cell growth to arrest in G_1. The corollary *in vivo* is that wild-type p53 in an appropriate cellular context could confer a state of increased drug resistance. The significance of mutated p53 oncosuppressor gene in clinical drug resistance is likely to be both complex and variable depending on the existence of other pathways of cell cycle activity control and response to injury. We show here that death of fibroblasts induced by etoposide and bleomycin occurs independently of wild-type p53 function. This confirms work by Strasser *et al.* (1994) which showed that thymic lymphoma cells from p53 – / – mice underwent apoptosis by p53-independent mechanisms following irradiation. Tumours which contain cells with mutated p53 initially may be more susceptible to cell death caused by therapy. However in the absence of G_1 arrest caused by wild-type p53, and therefore in the presence of continuing cell cycle activity, combined with karyotype instability (Livingstone *et al.*, 1992; Yin *et al.*, 1992), clones resistant to therapy may appear thus conferring a clinical state of 'drug-resistant' disease.

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