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Anti-apoptotic activity of low levels of wild-type p53

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Induction of apoptosis is a function of both an external stimulus and the physiology of the cell, which includes the expression of multiple oncogenes and tumor suppressors. Here we have studied the apoptotic response of immortalized mouse fibroblasts to serum withdrawal. We show that, in addition to the p53-independent apoptosis observed in p53$^-$ cells, overexpression of wild-type p53 tumor suppressor results in a high rate of programmed cell death. However, physiological range, low levels of the p53 protein protect fibroblasts from induction of apoptosis. Our results indicate that, as a function of its dose, the wild-type p53 can either protect from death or promote apoptosis. This new, anti-apoptotic, activity of p53 may have implications for the understanding of the role played by p53 in embryonic development as well as in initial stages of oncogenesis.

Keywords: apoptosis/cell cycle/oncogenesis/p53

Introduction

Mammalian cells undergo apoptosis in the absence of cytokines, which act as either growth or survival factors (Raff, 1992). However, the kinetics of serum withdrawal-induced apoptosis vary greatly for different cell types. Deregulated expression of oncogenes can influence the cellular response to cytokine starvation, for example bcl-2 expression will delay, while elevated c-myc levels will accelerate, the apoptotic response of serum-starved fibroblasts in culture (Bissonnette *et al.*, 1992; Evan *et al.*, 1992; Fanidi *et al.*, 1992; Wagner *et al.*, 1993; Harrington *et al.*, 1994; Hermeking and Eick, 1994). The effect of c-myc overexpression on apoptosis induction has been reported to be mediated by the wild-type p53 tumor suppressor (Hermeking and Eick, 1994; Wagner *et al.*, 1994).

There is ample evidence linking p53 to apoptosis control: DNA damage can result in p53-dependent apoptosis (Clarke *et al.*, 1993; Lowe *et al.*, 1993a; Caelles *et al.*, 1994; Canman *et al.*, 1995), while forced expression of wild-type p53 in transformed cells leads to cell death even in the absence of any identified co-stimulus (Yonish-Rouach *et al.*, 1991; Shaw *et al.*, 1992; Fujiwara *et al.*, 1993; Yonish-Rouach *et al.*, 1994). A p53 null mutation leads to tumorigenesis in mice (Donehower *et al.*, 1992; Jacks *et al.*, 1994; Symonds *et al.*, 1994), while epidemiological studies have clearly linked loss of wild-type p53 function with tumor progression in humans (Hollstein *et al.*, 1991; Harris, 1993). It would appear that the increased resistance to apoptosis is a major effect of p53 mutations in transformed cells (Lotem and Sachs, 1993; Lowe *et al.*, 1993b; Graeber *et al.*, 1996).

We report here that the apoptotic response of immortalized fibroblasts subjected to serum depletion is a quantitative function of p53 expression. Overexpression of wild-type p53 leads to cell cycle arrest in the presence of growth factors and to a strong apoptotic response in their absence, confirming the reported behavior of hematopoietic cells (Canman *et al.*, 1995). However, in our experimental system, low, close to physiological, levels of the wild-type form of p53 significantly promote cell survival, thus indicating that, among its many functions, p53 also gives rise to an anti-apoptotic phenotype.

Results

Death of immortalized fibroblasts induced by serum withdrawal is apoptotic

Balb/c 3T3 and 10.1 cell lines are both spontaneously immortalized Balb/c mouse embryonic fibroblasts. The 3T3 cells express low levels of endogenous wild-type p53, while 10.1 cells have suffered deletions in both p53 alleles and are p53$^-$ (Harvey and Levine, 1991; Wu *et al.*, 1993 and data not shown). Val5 is a clonal cell line derived from the 10.1 cells by stable transfection of the temperature-sensitive p53 mutant (*val135*) driven by the Harvey sarcoma virus long terminal repeat (Michalovitz *et al.*, 1990), resulting in a high level of expression of p53^{val135}. The three cell lines cultured in 10% fetal calf serum (FCS) grow well at 37 and 38.5°C; however, at 32°C, the permissive temperature for the *val135* mutation, val5 cells arrest growth while retaining viability. The 3T3 and 10.1 cells continue proliferation at 32°C. Removal of serum from the culture medium at either temperature drives all three cell lines into the G_0–G_1 arrest within 24 h (data not shown). The arrest is accompanied by significant cell death of both 10.1 and val5 cells, while the 3T3 cells suffer no loss of viability. As shown in Figure 1, the observed cell death is accompanied by morphological and biochemical changes typical of apoptosis, namely chromatin condensation visualized by Hoescht dye staining and DNA fragmentation revealed by the TUNEL reaction (Gavrieli *et al.*, 1992). Little or no apoptosis is observed in serum-starved 3T3 cells at either 32, 37 or 38.5°C throughout the 48 h duration of the experiment (Figure 1 and data not shown).

Kinetics of apoptosis induction is a function of the expression level of wild-type p53

Dead and dying fibroblasts tend to detach from the culture plate, making it difficult to count the apoptotic cells. In

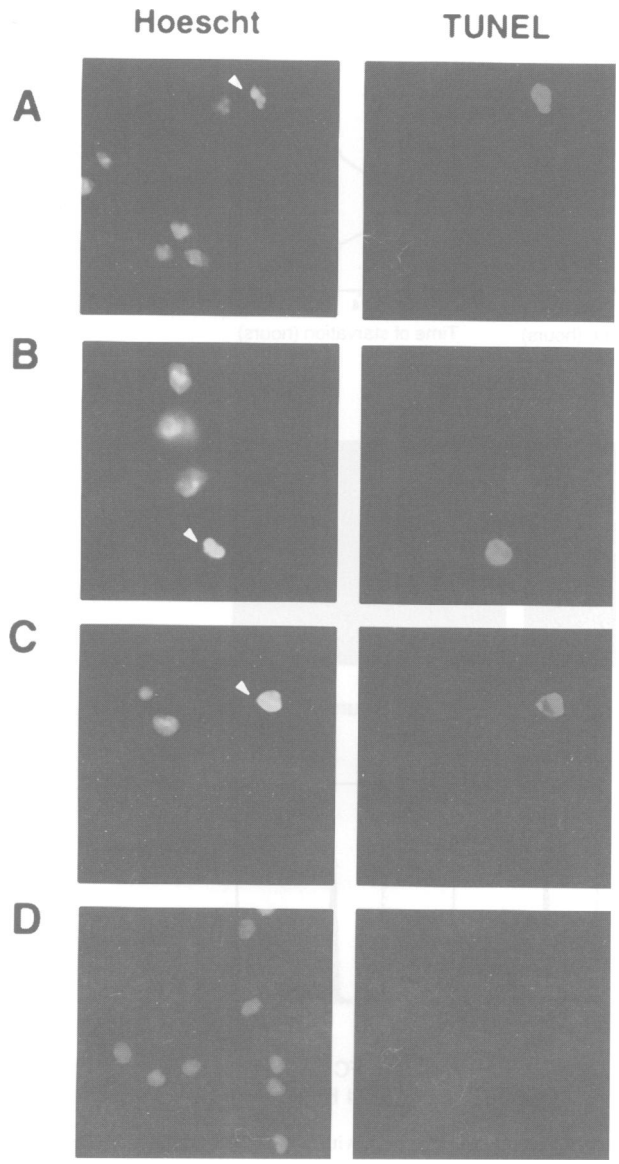

Fig. 1. Apoptosis of serum-starved fibroblasts is a function of p53. Cells were cultured in medium suplemented with 10% FCS at 38.5 or 32°C and subjected to serum withdrawal for 20 h. The nuclear morphology was revealed by staining with Hoescht 33258 dye. The DNA degradation was assayed by the TUNEL method and revealed by the Texas red fluorochrome, as described in Materials and methods. The same field analyzed for the two fluorochromes is shown for each cell line. (**A**) 10.1 cells, 32°C culture; (**B**) val5 cells, 32°C culture; (**C**) val5 cells, 38.5°C culture; (**D**) 3T3 cells, 32°C culture. Apoptotic cells (indicated by arrowheads) have a condensed nuclear morphology, revealed by the Hoescht staining and fragmented DNA, giving rise to a positive TUNEL reaction.

order to quantitate apoptosis induced by serum depletion, we pooled the floating and attached cells, collected at different times after serum removal, and quantitated viable cells using the vital dye exclusion test. Our count of apoptotic cells is certainly an underestimate, since early apoptotic cells maintain their membrane integrity, while very late apoptotic cells would escape notice if they had already undergone secondary necrosis and lysis. This limitation notwithstanding, our experiments demonstrated that there was no significant loss of viability in serum-starved 3T3 ($p53^+$) cells at 32, 38.5 or 37°C, with >90% of cells remaining viable for 48 h (Figure 2A and data not shown). The $p53^-$ 10.1 cells were significantly more sensitive to serum withdrawal at both temperatures (Figure 2B). Overexpression of $p53^{val135}$ gave rise to an unexpected phenotype: while the val5 cells grown at the restrictive temperature (mutant p53 conformation) were highly sensitive to apoptosis upon serum depletion, the same treatment performed at the permissive temperature (wild-type p53 conformation) led to an initially high rate of cell death followed by a recovery of viability (Figure 2C). When apoptosis was assayed by changes in nuclear morphology rather than vital dye exclusion (Figure 2D), high numbers of apoptotic cells were observed at 12 h but not at 24 h after serum withdrawal. Since changes in nuclear morphology precede loss of membrane integrity in an apoptotic cell, the kinetics of death assayed by vital dye exclusion were slower than apoptosis estimated by nuclear labeling (Figure 2C and D). The observed recovery of cell viability was not due to an artefactual, selective loss of apoptotic cells from val5 maintained at 32°C in the absence of serum, since under these conditions the absolute number of viable cells increased after ~20 h culture (not shown). This increase was due to a wave of re-entry of the starved cells into the cell cycle (Figure 2E), a behavior not observed for either 3T3 or 10.1 nor for val5 at 38.5°C.

The paradox of re-entry into a division cycle of cells first arrested in G_1 by p53 overexpression and then subjected to growth factor withdrawal was resolved by analyzing the effects of serum depletion on p53 expression in these cells. Indeed, many promoters, including retroviral ones, are sensitive to culture conditions, and we hypothesized that the val5 cells, which strongly overexpress $p53^{val135}$ when cultured in 10% FCS, altered the expression of the transgene upon removal of serum. Figure 3A and B shows that this was indeed the case: Western blot analysis of the p53 content in val5 cells demonstrated high levels of p53 protein prior to serum withdrawal and a gradual decrease of p53 content as a function of time during the period when the cells were maintained in the absence of serum. The level of cellular proteins remained constant, as shown by equivalent levels of GAPDH in all samples. The loss of $p53^{val135}$ took place at both the restrictive and permissive temperatures (Figure 3) and reflected diminished p53 mRNA levels (not shown). Long exposures of the Western blots confirmed that there was no detectable p53 protein in the 10.1 cells and a very low level of p53 in the 3T3 cells, which appeared to be independent of the presence of serum in the culture medium (results not shown). This is not unexpected, since, in contrast to the $p53^{val135}$ expression in the val5 cells, its expression was driven from the endogenous cellular p53 promoter.

It cannot be formally excluded that val5 cells are a heterogenous population with respect to the $p53^{val135}$ expression levels and that the serum depletion leads to a selective loss of cells with a high p53 content. We consider it unlikely, since no differences in the p53 immunostaining of val5 cells were detected in studies performed prior to and after serum withdrawal (data not shown). Moreover, the re-entry of starved cells into the cell cycle (Figure 2D) argues against the continuous presence of cells with a low p53 content.

The results shown in Figures 2 and 3 defined a threshhold level of the wild-type conformation of $p53^{val135}$

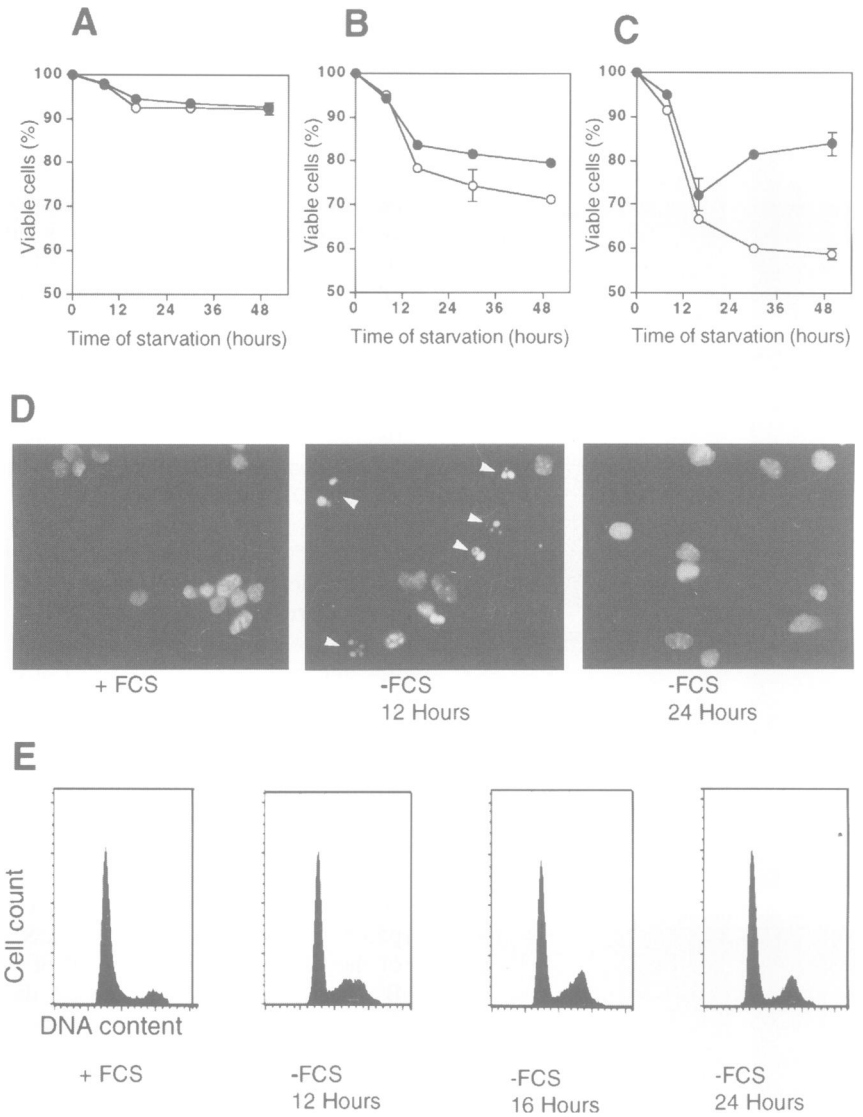

Fig. 2. Kinetics of serum withdrawal-induced apoptosis. Cells were grown in culture medium supplemented with 10% FCS at 38.5°C. Where indicated (closed symbols), subconfluent cells were left at 32°C for 24 h. Serum withdrawal was initiated by rinsing cells with serum-free medium and culturing without serum at 32 or 38.5°C. At the indicated times, cells were collected and the adherent and floating populations pooled. The viable and dead cells were counted by Trypan blue exclusion. The viability was calculated as the ratio of cells impermeable to the vital dye over the total cell number. (**A**) 3T3 cells (endogenous wild-type p53); (**B**) 10.1 cells ($p53^-$); (**C**) val5 cells (exogenous $p53^{val135}$ thermosensitive mutant overexpression). Open symbols: 38.5°C culture, closed symbols: 32°C culture for 24 h in 10% FCS, followed by serum withdrawal at 32°C. Each point is the mean ± S.D. of triplicate determinations. The survival curves shown were measured in a single experiment and are representative of at least three experiments performed with each cell line. (**D**) Nuclear morphology analysis of val5 cells cultured at 32°C and subjected to serum withdrawal for 0, 12 and 24 h. The cells were collected and the nuclei visualized by Hoescht dye labeling, as described in the legend to Figure 1. The apoptotic morphology is indicated by arrowheads. (**E**) Cell cycle analysis by flow cytometry of viable val5 cells at 32°C. The cells were stained with propidium iodide and analyzed in the Becton and Dickinson FacsScan flow cytometer. In the presence of 10% FCS, the cell cycle was arrested due to p53 overexpression (Michalovitz *et al.*, 1990). Serum withdrawal from arrested cells led to a wave of cell cycle entry, visible at 12 and 16 h of treatment. At the 24 h point, the cell cycle was again arrested.

below which the G_1 arrest was not maintained in val5 cells. After a single wave of passage through the cycle, the cells were again arrested in G_0–G_1 (Figure 2D), this time presumably due to lack of serum in the culture medium, suggesting that serum removal blocks cells at an earlier stage of G_1 than the p53 arrest. The pattern of expression of the $p53^{val135}$ transgene in val5 cells points to a correlation between resistance to apoptosis and the level of the wild-type form of p53. Fibroblasts devoid of p53 show a rapid apoptosis induction in the absence of serum (Figure 2B); the expression of the mutant form of $p53^{val135}$ or a very high level of expression of the wild-type form of the protein have no major effect on this phenotype (Figure 2C). In contrast, the presence of low to moderate levels of the wild-type form of p53 protects cells from serum withdrawal-induced apoptosis (Figure 2A and C).

The anti-apoptotic and anti-proliferative activities of p53 can be dissociated

To confirm these unexpected results, we have analyzed two additional cell lines, val3 and val4 (a kind gift of Dr A.Levine), which have the same genetic background as the val5 cells ($p53^-$ Balb/c mouse embryonic fibroblasts)

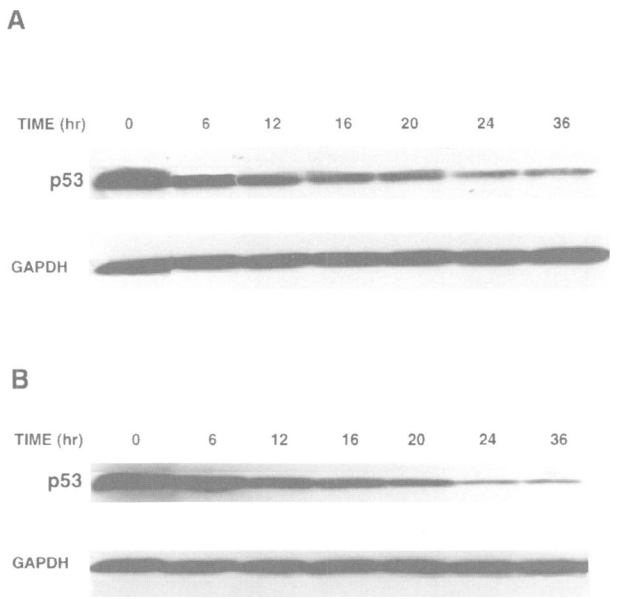

Fig. 3. The level of $p53^{val135}$ protein expression in val5 cells decreases in the absence of serum. Western blot analysis was performed on 60 mg of protein of total cell extracts of val5 cells submitted to serum withdrawal at 32°C (**A**) or 38.5°C (**B**) for the indicated times. The proteins were separated on a 10% SDS–PAGE gel, transferred to nitrocellulose and probed with the X77 anti-p53 monoclonal antibody (a gift of Dr T.Soussi). The results were normalized by probing the blot with an anti-GAPDH polyclonal antibody (a gift of J.-M.Blanchard).

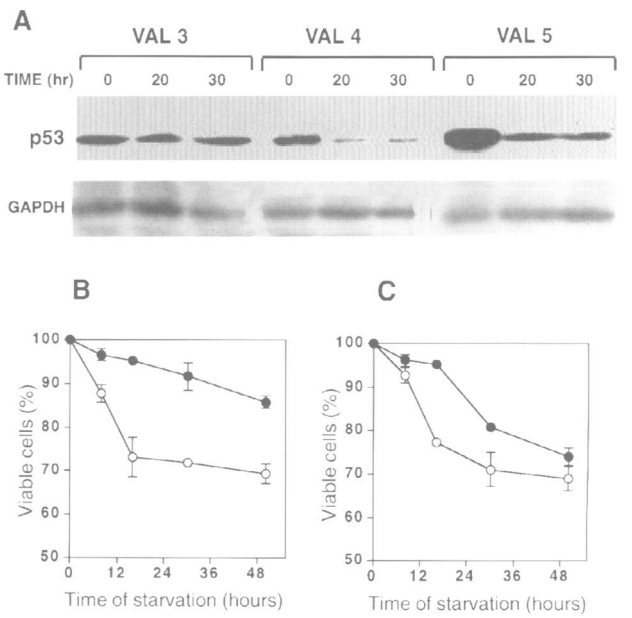

Fig. 4. Wild-type form of $p53^{val135}$ promotes survival of serum-starved val3 and val4 cells. (**A**) Val3 and val4 express the $p53^{val135}$ temperature-sensitive mutant at a lower level than val5 cells. The transgene expression is insensitive to the absence of serum in the val3 line, whereas p53 expression in val4 and val5 cells is decreased upon serum withdrawal. Western blot analysis of 80 mg of total protein extracts was performed as described in the legend to Figure 3. (**B**) Effect of serum withdrawal on the viability of val3 cells. (**C**) Effect of serum withdrawal on the viability of val4 cells, open symbols: 38.5°C culture, closed symbols: 32°C culture. The experimental conditions are as described in the legend to Figure 2.

and have been transfected with the same $p53^{val135}$ construct as val5 cells. Both val3 and val4 cell lines constitutively express $p53^{val135}$ at moderate levels, considerably lower than those observed in the val5 cells (Figure 4A). Serum withdrawal leads to a decreased level of p53 in the val4 cells but, presumably due to a positional effect of the integration site, has no effect on p53 levels in the val3 cells (Figure 4A). Consistent with the results described above, withdrawal of serum does not significantly affect the val3 viability at 32°C (Figure 4B), while the val4 cells are resistant to apoptosis at the beginning of the experiment and then begin to die (Figure 4C). The entry into apoptosis is concomitant with the fall in the p53 expression level. We interpret this to represent a fall below the threshhold of $p53^{val135}$ expression needed to protect against apoptosis.

Prior to serum withdrawal, both val3 and val4 cells express easily detectable amounts of $p53^{val135}$ (Figure 4A), which assumes the wild-type conformation at 32°C. However, in contrast to the val5 cell line, neither val3 nor val4 stop proliferating when transferred to 32°C. The analysis of bromodeoxyuridine (BrdU) incorporation indicates that whereas the three cell lines grow indistinguishably at the restrictive temperature (38.5°C), the temperature shift to 32°C for 18 h leads to a complete inhibition of entry into S phase in the val5 line, while the same treatment has little effect on proliferation of the val3 and val4 cells (Figure 5). These results, together with those shown in Figure 2C and D, indicate that the anti-apoptotic activity of p53 is detected at levels of p53 insufficient to cause the cell cycle arrest, thus dissociating the anti-apoptotic and anti-proliferative activities of the tumor suppressor.

Inducible expression of wild-type p53 confirms the anti-apoptotic phenotype of p53

We have observed a protective effect of endogenous wild-type p53 on apoptosis in 3T3 cells. Additionally, the moderate concentrations of $p53^{val135}$, which assumes a wild-type conformation at 32°C, promote survival of the val3, val4 and val5 cell lines. However, it could be argued that even though the $p53^{val135}$ protein causes cell cycle arrest at the permissive temperature in the val5 cells, it is not strictly a wild-type protein, as indeed has been described for this mutant in different cell types (Sehgal and Margulies, 1993). Moreover, although we have not analyzed it in detail, higher levels of $p53^{val135}$ at the permissive temperature, compared with the wild-type p53, are required both for cell cycle arrest and protection from apoptosis. We have, therefore, turned to study the effects of the genuine wild-type p53 on serum withdrawal-induced apoptosis in 10.1 cells. We have isolated a subclone of the 10.1 cells harboring the chimeric tetracycline repressor, which allows inducible expression of genes cloned under the control of a tetracycline-repressible promoter (Gossen and Bujard, 1992). A transient expression of a luciferase reporter gene, under the control of the same tetracycline-sensitive promoter, is regulated by a factor of 60 by tetracycline in these cells, with a gradual increase of expression observed at the intermediate tetracycline concentrations (Figure 6A). It should be noted that, even with maximal repression of the promoter, there is a significant background expression of the protein. The apoptotic response was assayed in cells co-transfected with a constitutively expressed *lacZ* gene and wild-type p53 driven

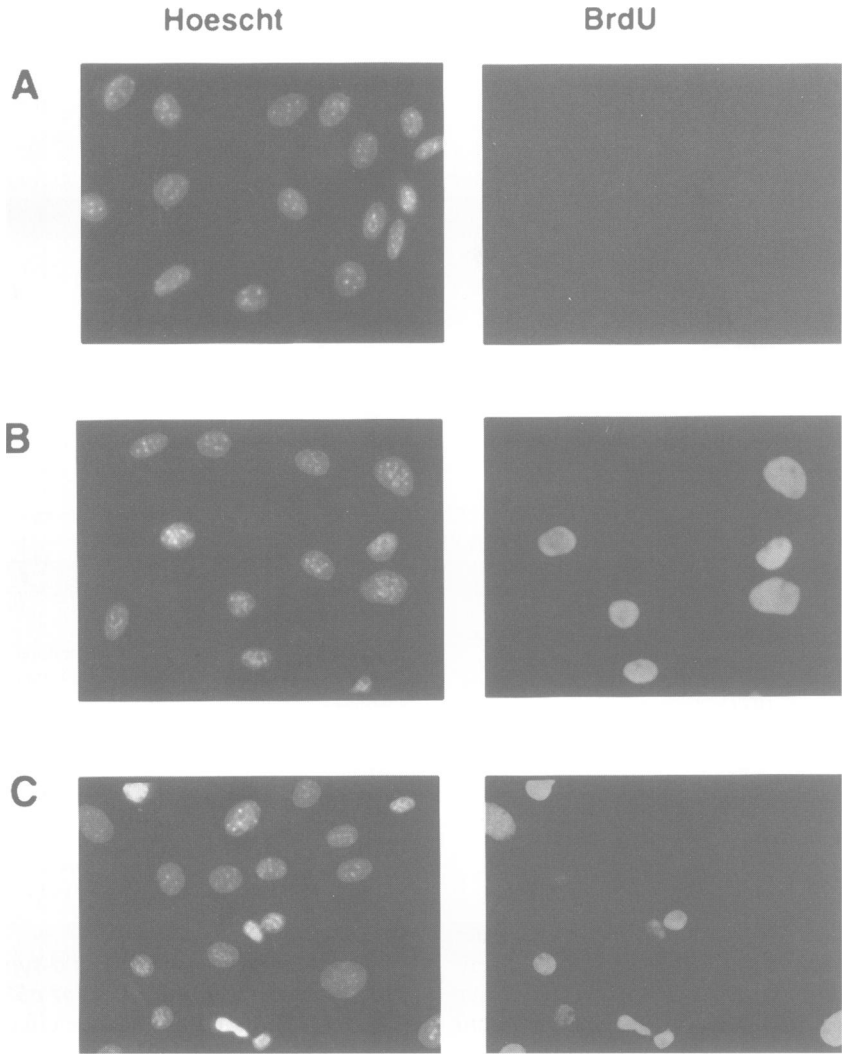

Fig. 5. Val3 and val4 cells continue to proliferate at the $p53^{val135}$ permissive temperature. Cells were grown at 38.5°C and transferred to 32°C for 18 h prior to labeling with BrdU for an additional 36 h at 32°C. The nuclei were visualized by Hoescht staining and the cells entering the S phase by BrdU incorporation. (**A**) val5 cells, (**B**) val3 cells, (**C**) val4 cells. Val5 cells have totally arrested their proliferation at 32°C, while ~50% of both val3 and val4 cells have entered S phase during the labeling period.

by the tetracycline-sensitive promoter. The cells were cultured in different tetracycline concentrations and subjected to serum withdrawal for 8 h. The transfected cells were visualized by X-gal staining, and viable cells were distinguished from apoptotic cells by their flat or shrunken morphology, respectively. Neither the expression of p53 nor the presence of tetracycline in the medium influenced the viability of cells cultured in 10% FCS (Figure 6D). As shown in Figure 6B, cells co-transfected with *lacZ* and an empty tetracycline control vector underwent apoptosis upon serum removal. The number of apoptotic cells was 10.4 ± 0.2–11.7 ± 1.1% and, as expected, was independent of the tetracycline concentration in the medium. In contrast, the apoptotic response in cells co-transfected with *lacZ* and p53 and subjected to serum withdrawal was a function of the tetracycline concentration in the medium (Figure 6C). Thus, under conditions where the promoter was repressed, allowing a background level of transcription (see Figure 6A), there was a low percentage of apoptotic cells (3.7 ± 0.7%), while increasing the p53 level by gradually lowering the tetracycline concentration led to a higher percentage of dead cells, culminating in 17 ± 1.5% apoptotic cells in the culture with no tetracycline. The regression analysis indicated that the inverse correlation between the tetracycline concentration in the medium and the number of dead cells was highly significant ($P<0.0005$). These results indicate that the dual effect of p53 on the induction of apoptosis, i.e. aggravation of the apoptotic response at high expression levels and protection against apoptosis at low to moderate levels, is a function of wild-type p53 and not limited to the $p53^{val135}$ thermosensitive mutant.

Discussion

We have described a hitherto unknown function of the p53 tumor suppressor, namely a protective effect of low or moderate p53 protein levels against serum withdrawal-induced apoptosis. In contrast to the effect of p53 on apoptosis induced by genotoxic drugs (Malcomson *et al.*, 1995), the anti-apoptotic effect of p53 is dissociated from its anti-proliferative function in the experimental system used here. Therefore, the improved survival of the $p53^{+}$ cells that we have described here is not a trivial con-

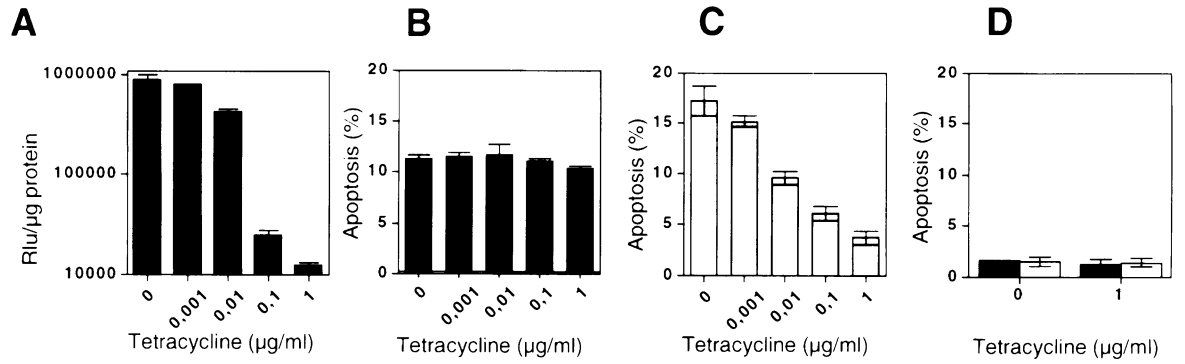

Fig. 6. Effect of inducible expression of wild-type p53 on apoptosis induced by serum withdrawal. The 10.1 ($p53^-$) cell line was stably transfected with the pUHD 15-1 vector, giving rise to a 10.10 line constitutively expressing the chimeric tetR–VP16 protein (Gossen and Bujard, 1992). (**A**) Transient transfection of the firefly luciferase gene under the control of the tetracycline-repressible promoter leads to a low level of luciferase expression in 10.10 cells cultured in 1 mg/ml tetracycline and is induced by a factor of 60 in the tetracycline-free medium. (**B**) 10.10 cells were co-transfected with the pCH110 LacZ vector (Pharmacia) constitutively expressing β-galactosidase and the empty pUHD 10-3 vector (Gossen and Bujard, 1992) containing the tetracycline-repressible promoter. Following transfection, cells were cultured in medium supplemented with 10% FCS and the indicated tetracycline concentrations for 16 h at 37°C, then rinsed once and incubated in a serum-free medium with the indicated concentrations of tetracycline for an additional 8 h. The transfected cells were identified by staining with X-gal to reveal the β-galactosidase activity. The apoptotic cells among the transfectants were identified by their shrunken morphology compared with a flat, adherent phenotype of viable cells. (**C**) 10.10 cells were co-transfected with pCH110 LacZ and the murine wild-type p53 cloned in the pUHD 10-3 vector and treated as described in (B). The transfected, viable and apoptotic cells were identified as in (B). (**D**) The viability of the 10.10 cells is not altered by p53 or by tetracycline in the presence of 10% FCS. The cells were treated as in (B) or (C), but the serum withdrawal step was omitted. The mean values and the range of variation from two independent experiments are shown.

sequence of different sensitivities to apoptotic stimuli of cycling and quiescent cells.

Our results are in apparent contradiction to abundant epidemiological and experimental data showing a selective advantage acquired by a tumor cell through the loss of wild-type p53 protein expression (Hollstein *et al.*, 1991; Harris, 1993; Metz *et al.*, 1995). We suggest that previous studies may not have assessed the effects of physiological levels of p53 expression in the cellular context described here: while p53 expression may be deleterious to an established tumor cell, our results suggest that low levels of p53 favor the survival of immortalized, non-transformed fibroblasts. Additionally, it would appear that sub-obtimal growth conditions used in this work might select for a different p53 phenotype than culture in rich medium, where it has been reported that murine fibroblasts tend to lose wild-type p53 expression upon immortalization (Harvey and Levine, 1991). We believe that the results reported here may be physiologically relevant, as limiting cytokine concentrations would seem to be a major determinant of the regulation of apoptosis *in vivo* (Raff, 1992). It remains to be determined whether our conclusions can be extended to other cell types at the early stage of an oncogenic process as well as to other inducers of apoptosis. That this may indeed be the case is suggested by the increased sensitivity to apoptosis induction by taxol, a tubulin depolymerization inhibitor, of fibroblasts lacking p53 (Wahl *et al.*, 1996).

Recently, it has become apparent that p53 may have a role to play in the absence of genotoxic stress. Even though the lack of p53 does not interfere with embryonic development (Donehower *et al.*, 1992) (see, however, Sah *et al.*, 1995), recent results from MDM-2 knock-outs and MDM-2-p53 double knock-out mice strongly hint at a role for p53 in normal embryogenesis. MDM-2 nullizygous embryos die at a stage of few cells; however, they are rescued by the simultaneous inactivation of the p53 gene (Jones *et al.*, 1995; Montes de Oca Luna *et al.*, 1995), strongly suggesting that p53 activity must be down-regulated for development to proceed. This in turn implies that p53 is active even in the absence of DNA damage. High expression levels of p53 mRNA in several tissues during development are compatible with this hypothesis (Schmid *et al.*, 1991). One possibility is that activation of p53 during a normal cell cycle is part of its function as a checkpoint protein: in the absence of DNA damage, MDM-2 would inactivate p53 allowing progression through the cell cycle (Olson *et al.*, 1993). Our results show that p53 can also protect cells against apoptosis, suggesting that one possible role for p53 in development could be the regulation of the sensitivity to apoptotic signals.

Materials and methods

Cell culture and serum withdrawal

Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS in 5% CO_2 at 38.5 or 32°C. Sub-confluent cells were rinsed once in serum-free medium and culture continued in serum-free medium for the indicated times. Cells were then collected and the adherent and floating populations pooled. The viable and dead cells were counted by Trypan blue exclusion. The viability was calculated as the ratio of cells impermeable to the vital dye over the total cell number.

Nuclear morphology and the TUNEL assay

Cells were fixed in formaldehyde 2%, glutaraldehyde 0.2% and adsorbed on polylysine-coated microscope slides. The nuclear morphology was revealed by staining with 10 mM Hoescht 33258 dye at room temperature for 10 min followed by extensive washing in phosphate-buffered saline (PBS) and distilled water. DNA fragmentation was assayed on the same cells by the TUNEL reaction (Gavrieli *et al.*, 1992). Briefly, after permeabilization of cells attached to the microscope slide with 0.1% Triton X-100, 0.1% sodium citrate at 0°C for 2 min and washing with PBS and H_2O, the terminal transferase reactions were performed in 15 µl in the buffer supplied by the manufacturer (Boehringer) at 37°C for 1 h in a humid atmosphere. The reaction mixture contained 6 µM biotin-dUTP, 60 µM dATP and 7.5 U of deoxynucleotide terminal transferase. Biotinylated dUTP incorporated into DNA was revealed by incubation with Texas red-conjugated streptavidin (Amersham). The fluorescence was observed at 400× magnification with a Zeiss Axiophot fluorescence microscope.

Western blot analysis

The Western analysis was performed as previously described (Leroy-Viard *et al.*, 1995).The antibody used for detection of the p53 protein was a monoclonal antibody X77, kindly provided by Dr Thierry Soussi.

BrdU incorporation assay

Cells were grown to <50% confluence at 38.5°C and transferred to 32°C for 18 h. BrdU (100 μM, Sigma) was then added to the culture and the incubation continued at 32°C for 36 h. The cells were then fixed in 2% formaldehyde, 0.2% glutaraldehyde and permeabilized in acetone at –20°C for 30 s. DNA was denatured by treatment with 4 M HCl for 10 min at room temperature and neutralized by abundant washes in PBS and distilled water. The incorporated BrdU was visualized by incubation with a monoclonal anti-BrdU antibody (Dako) followed by the FITC-conjugated goat polyclonal anti-mouse Ig antibody (Immunotech). The nuclei were stained with the Hoescht dye, as described above.

Transient transfections

The 10.1 ($p53^-$) cell line was stably transfected with the pUHD 15-1 vector, giving rise to a 10.10 line constitutively expressing the chimeric tetR–VP16 protein (Gossen and Bujard, 1992). 10.10 cells were co-transfected with the pCH110 LacZ vector (Pharmacia) constitutively expressing β-galactosidase and the pUHD 10-3 vector (Gossen and Bujard, 1992) containing the human wild-type p53 cDNA cloned under the control of the tetracycline-repressible promoter. Following transfection, cells were cultured in medium supplemented with 10% FCS and the indicated tetracycline concentrations for 16 h at 37°C, then rinsed once and incubated in serum-free medium with the indicated concentrations of tetracycline for an additional 8 h. The transfected cells were identified by staining with X-gal to reveal the β-galactosidase activity. The apoptotic cells among the transfectants were identified by their shrunken morphology compared with a flat, adherent phenotype of viable cells. At least 150 cells were counted for each experimental point.

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