Oncogene (1997) 14, 929–935

p21$^{Waf1/Cip1}$ protects against p53-mediated apoptosis of human melanoma cells

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The tumor suppressive effect of p53 is believed to be rooted in its two primary functions: the implementation of cellular growth arrest and the execution of apoptotic cell death. While p53-regulated expression of the cyclin-dependent kinase inhibitor p21$^{Waf1/Cip1}$ appears to be central for the implementation of G_1 arrest, the participation of p21$^{Waf1/Cip1}$ in p53-triggered cell death remains controversial. In the present study, overexpression of p53 in human melanoma SK-MEL-110 cells through use of an adenoviral expression vector (AdCMV.p53) was found to result in apoptosis, while similar infection of primary vascular smooth muscle cells (VSMC) instead resulted in a moderate inhibition of growth. Expression of p21$^{Waf1/Cip1}$ was strongly elevated in VSMC, but showed little change in SK-MEL-110 cells, although expression of another p53-regulated gene (GADD45) was comparable in both AdCMV.p53-infected cell types. Evidence that p21$^{Waf1/Cip1}$ expression may be required for surviving p53-induced cell death was further supported by the finding that p53 overexpression was highly toxic for p21-deficient mouse embryonal fibroblasts (p21$^{-/-}$ MEFs). In both SK-MEL-110 and p21$^{-/-}$ MEFs, adenovirus-driven ectopic expression of p21$^{Waf1/Cip1}$ resulted in a substantial protection against p53-induced apoptosis, indicating that p21$^{Waf1/Cip1}$ rescued cells from a path of programmed cell death to one of enhanced survival.

Keywords: melanoma; p53; p21; apoptosis

Introduction

The tumor suppressor protein and transcription factor p53 plays an important role in the response of normal mammalian cells to DNA damage and other stresses (Waldman *et al*., 1996; Di Leonardo *et al*., 1994; Kastan *et al*., 1992, 1995; Waldman *et al*., 1995). Two prominent functions of p53 have thus far been identified: implementation of growth arrest and execution of apoptotic cell death (Kastan *et al*., 1995). It is widely believed that the p53-regulated gene p21$^{Waf1/Cip1}$ is required for effecting the p53-mediated G_1 arrest, functioning to inhibit cyclin-dependent kinases (cdk), the primary drivers of cell cycle progression (Xiong *et al*., 1993; Morgan *et al*., 1995; Deng *et al*., 1995; El-Deiry *et al*., 1993). Although recent evidence indicates that p53-driven transcriptional activation may be dispensable for p53-mediated apoptosis (Attardi *et al*., 1996; Rowan *et al*., 1996), the involvement of p53-effector genes in this process remains controversial. Thus, GADD45, MDM2, BAX, PCNA and p21$^{Waf1/Cip1}$ have all been proposed to participate in p53-induced apoptosis (Chen *et al*., 1995; Shivakumar *et al*., 1995; Oliner *et al*., 1993; Barak *et al*., 1993; Morris *et al*., 1996; Waldman *et al*., 1995; Gorospe *et al*., 1996c).

In a variety of systems where exposure to stressful stimuli results in p53-independent cell death, p21$^{Waf1/Cip1}$ has been implicated in the survival response. Inhibition of p21$^{Waf1/Cip1}$ expression through transfection of p21$^{Waf1/Cip1}$ antisense oligonucleotides was recently demonstrated to block growth factor-induced differentiation of SH-SY5Y neuroblastoma cells and result in their death (Poluha *et al*., 1996). Similarly, we have demonstrated that suppression of p21$^{Waf1/Cip1}$ expression in MCF-7 cells through expression of antisense p21$^{Waf1/Cip1}$ transcripts attenuates the growth arrest and p21$^{Waf1/Cip1}$ induction that normally follows treatment with the cyclopentenone prostaglandin A_2 (PGA_2), and promotes death (Gorospe *et al*., 1996a,b). Conversely, in RKO cells, which normally undergo apoptosis in response to PGA_2 and show low expression of p21$^{Waf1/Cip1}$, ectopic expression of p21$^{Waf1/Cip1}$ was found to confer protection against PGA_2-mediated cell death (Gorospe *et al*., 1996d).

From the above studies it appears that, at least in p53-independent models of stress, failure to elevate p21$^{Waf1/Cip1}$ expression is associated with low cell survival, while the presence of p21$^{Waf1/Cip1}$ confers a survival advantage. In this study, we sought to extend this general hypothesis to a model of p53-dependent cell death. SK-MEL-110 human melanoma cells ectopically overexpressing p53 (through infection with an adenovirus expressing p53) underwent apoptotic cell death associated with low p21$^{Waf1/Cip1}$ expression, consistent with a recent report demonstrating that inactivation of p21$^{Waf1/Cip1}$ sensitizes colorectal cancer cells to apoptosis by p53 (Polyak *et al*., 1996). Supporting the notion that failure to induce p21$^{Waf1/Cip1}$ expression contributed to this effect, overexpression of p53 in embryonal fibroblasts derived from p21-knockout mice (p21$^{-/-}$ MEFs) likewise resulted in extensive cytotoxicity. In both SK-MEL-110 cells and p21$^{-/-}$ MEFs, ectopic p21 expression conferred protection against a subsequent

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Received 17 July 1996; revised 21 October 1996; accepted 22 October 1996

p53 challenge, providing direct proof that p21$^{Waf1/Cip1}$ could rescue cells from a path leading to cell death to one of enhanced survival.

Results

p53 overexpression leads to apoptosis of SK-MEL-110 cells, but not of VSMC

The effect of p53 overexpression in a primary culture of VSMC was compared with that of p53 overexpression in melanoma SK-MEL-110 cells. p53 overexpression was achieved by infection with an adenovirus expressing p53 under the cytomegalovirus (CMV) promoter (AdCMV.p53). A virus dose of 100 p.f.u./cell was sufficient to achieve infection of >95% of cells, as determined using a AdCMV.NLSβgal adenovirus (Table 1 and data not shown). As indicated in Figure 1a, infection of VSMC with 100 p.f.u./cell AdCMV.p53 resulted in only a slight decrease in cell number relative to infection with 100 p.f.u./cell of an 'empty' (Ad.null) adenovirus. By contrast, infection of SK-MEL-110 cells was associated with a marked loss in cell viability, with only approximately 20% of the cells remaining 48 h after AdCMV.p53 infection relative to Ad.null-infected cells (Figure 1a). In keeping with our prior studies in related cell lines (Cirielli *et al*., 1995), AdCMV.p53-infected SK-MEL-110 cells exhibited morphological features indicative of apoptotic cell death (not shown) associated with genomic DNA fragmentation that was detectable within 36 h of AdCMV.p53 infection (Figure 1b). In contrast, neither Ad.null- nor AdCMV.p53-infected VSMC exhibited any loss of DNA integrity.

Expression of p53-regulated genes in VSMC and in SK-MEL-110 cells

Infection of both VSMC and SK-MEL-110 cells with AdCMV.p53 resulted in high amounts of p53 expression evidenced at both the mRNA and protein levels (Figure 2a and b). It also resulted in elevated expression of the p53-regulated gene GADD45 in both cell lines (Figure 2b). In contrast, p21$^{Waf1/Cip1}$ was differentially expressed in the two cell types following p53 overexpression. While infection of SK-MEL-110 cells resulted in little or no p21$^{Waf1/Cip1}$ mRNA induction, infected VSMC cells showed high levels of p21$^{Waf1/Cip1}$ mRNA (Figure 2b).

p21$^{Waf1/Cip1}$ expression prior to infection with AdCMV.p53 largely prevents p53-induced death of SK-MEL-110 cells

The correlation between p21$^{Waf1/Cip1}$ expression and cell survival in AdCMV.p53-infected VSMC and SK-MEL-110 cells prompted us to investigate whether p21$^{Waf1/Cip1}$ could protect SK-MEL-110 cells from p53-mediated apoptosis. To this end, an adenovirus expressing p21$^{Waf1/Cip1}$ (AdWAF1) was utilized (Katayose *et al*., 1995a,b). Infection of SK-MEL-110 cells with Ad-WAF1 (100 p.f.u./cell) alone resulted in a 30- to 35-fold increase in p21$^{Waf1/Cip1}$ protein expression (Figure 3a). A sequential infection strategy was utilized whereby Ad.null- or AdWAF1-infected cells were cultured for 2 days prior to a second infection with Ad.null or AdCMV.p53 adenoviruses. The relative cell numbers in each infection group (null/null, null/p53, p21/null and p21/p53) were determined 48 h following the second infection by direct cell counts in the presence of trypan blue dye (Figure 3b). DAPI staining provided independent indication that attached cells were not undergoing apoptosis as they failed to exhibit nuclear condensation or fractionation (not shown). As demonstrated in Figure 3b, p21$^{Waf1/Cip1}$-overexpressing cells exhibited enhanced survival following a second infection with AdCMV.p53 compared to their Ad.null-infected counterparts. While the number of viable cells remaining 48 h post-infection in the null/p53 treatment group was only 10–20% of that in the null/null population, viability of the p21/p53 cells was greater than 50% of that seen in the p21/null population (Figure 3b). Time-dependent changes in the actual cell numbers for the four infection groups over the 48 h time period following the second infection are shown in Figure 3c. The absolute number of cells in the null/p53 population was found to be less than that in either the p21/null or p21/p53 treatment groups (Figure 3c). To investigate the possibility that AdWAF1-mediated protection might result from decreased ability of already-infected SK-MEL-110 cells to be infected by the AdCMV.p53 adenovirus, we sought to directly assess the efficiency of sequential infections. To this end, we first infected with AdWAF1 (or Ad.null, not shown) and 48 h later we infected with AdCMV.NLSβgal. Quantitation of β-galactosidase activity in all infection groups (Table 1) indicated that infection with one adenovirus does not alter the susceptibility of the cell to a second adenoviral infection, further supporting the hypothesis that

Table 1 β-galactosidase activity following secondary infection of SK-MEL-110 cells and p21$^{-/-}$ MEFs with AdCMV.NLSβgal

Cell type	*Primary infection AdWAF1* (p.f.u./cell)	*Secondary infection AdCMV.NLS*βgal (p.f.u./cell)	*% β-galactosidase positive cells*
SK-MEL-110	0 (mock)	100	>95
	50	100	>95
	100	100	>95
p21$^{-/-}$ MEFs	0 (mock)	90	>95
	45	90	>95
	90	90	>95

To determine if infection of SK-MEL-110 or p21$^{-/-}$ MEFs with AdWAF1 (or mock infection) alters the susceptibility of cells to infection by a second virus, SK-MEL-110 cells or p21$^{-/-}$ MEFs were sequentially infected with AdWAF1 and AdCMV.NLSβgal and percent β-galactosidase positive cells were scored 48 h after infection with AdCMV.NLSβgal

protection was directly engendered by p21Waf1/Cip1 overexpression. The cell cycle distribution of SK-MEL-110 cells in each of the four treatment groups is shown in Figure 3d. AdWAF1 infection alone resulted in a marked enrichment in G_1-phase cells (from 50% G_1-phase cells in asynchronously growing cultures -null/null- to 75–80% G_1-phase cells in p21-overexpressing cultures -21/null-) (Figure 3d). AdCMV.p53 infection alone (null/p53), on the other hand, resulted in the emergence of a sub-G_1 apoptotic peak (indicated by the arrowhead) in keeping with the reduced cell viability and DNA fragmentation noted above (Figure 1b and Figure 3b and c). A marked reduction in this apoptotic population was observed in the p21-overexpressing AdCMV.p53-infected cultures (p21/p53). Taken together, these results indicate that ectopic p21Waf1/Cip1 expression conferred substantial protection against p53-mediated cytotoxicity.

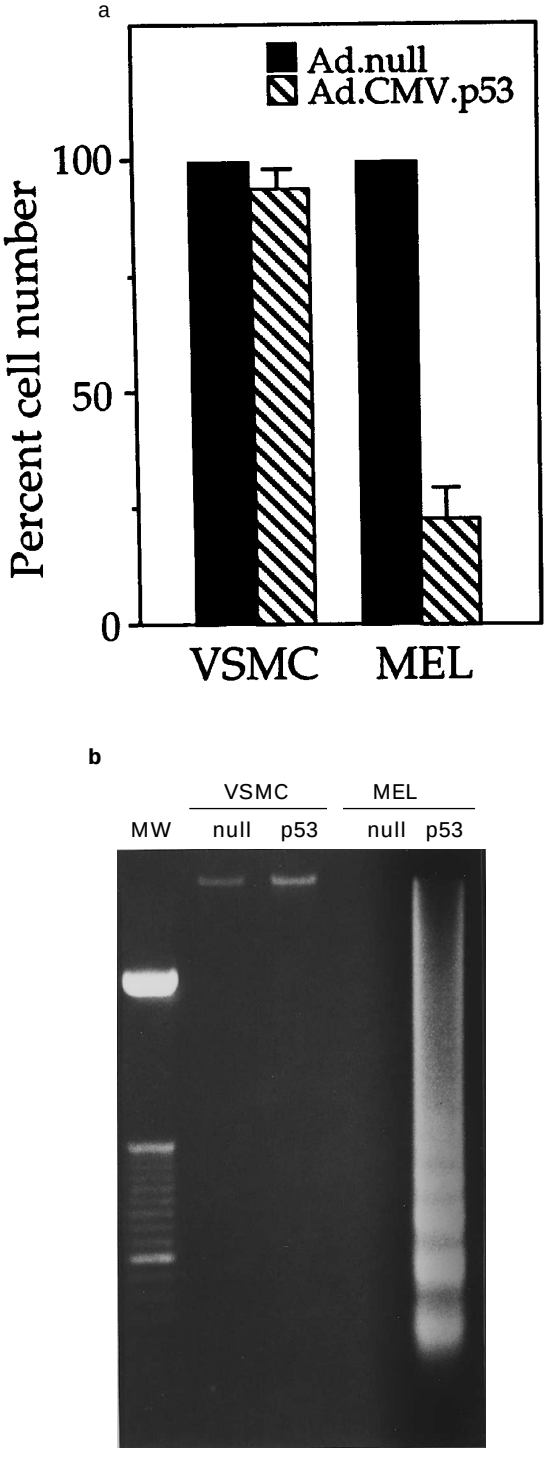

Figure 1 p53 overexpression induces apoptosis of SK-MEL-110 (MEL) cells. (**a**) Effect of Ad.null or AdCMV.p53 infections in SK-MEL-110 cells and VSMC. Infected cells (100 p.f.u./cell in all experiments) were analysed for relative changes in cell number (counted using a hemocytometer) 48 h after infection with the indicated viruses (solid bars, Ad.null; hatched bars, AdCMV.p53). Values represent mean ± SEM of three independent experiments. (**b**) Apoptotic cell death of SK-MEL-110 cells infected with AdCMV.p53. Thirty-six hours after infection of VSMC and SK-MEL-110 cells with 100 p.f.u./cell Ad.null (*null*) or AdCMV.p53 (*p53*), genomic DNA was extracted and assayed from fragmentation. MW: Molecular weight marker

p53-mediated cytotoxicity in p21$^{-/-}$ MEFs is prevented by prior ectopic p21$^{Waf1/Cip1}$ expression

In support of our view that p21Waf1/Cip1 serves a protective function during stress, we reasoned that cells lacking p21Waf1/Cip1 should exhibit sensitivity to the cytotoxic effects of p53. Further, we should be able to alter this susceptibility through restoration of p21Waf1/Cip1 expression. To address this issue, we examined the effect of p53 overexpression in embryonic fibroblasts (MEFs) derived from a mouse carrying targeted inactivation of both p21Waf1/Cip1 alleles (Deng *et al*., 1995) (Figure 4). p53 expression was extremely toxic for p21$^{-/-}$ MEFs, with less than 15% of the cells remaining viable 48 h post-infection with 120 p.f.u./cell AdCMV.p53. In contrast, the Ad.null-infected group showed greater than >90% viability at the same time point. Cells exhibited morphological changes characteristic of programmed cell death, associated with genomic DNA condensation and fragmentation, as visualized by DAPI staining (Figure 4c). Thus, p53-overexpression results in apoptotic cell death of p21$^{-/-}$ MEFs, similar to SK-MEL-110 cells (Figure 2). Although p21$^{-/-}$ MEFs did not tolerate high levels of p21Waf1/Cip1 expression (greater than 150 p.f.u./cell AdWAF1 was itself cytotoxic), prior expression of p21Waf1/Cip1 was achieved with low-level AdWAF1 infection (90 p.f.u./cell) (Figure 3c). Figure 3d shows the results of two independent experiments wherein the resulting p21Waf1/Cip1 overexpression *via* AdWAF1 infection was protective, as evidenced by a marked reduction in cell loss following AdCMV.p53 infection. Direct comparison of the effects of p53 overexpression between p21$^{-/-}$ MEFs and their wild-type counterparts (p21$^{+/+}$ MEFs) could not be carried out due to the extremely low infection rates seen in wild-type MEFs (5000 p.f.u./cell resulted in infection of only 1% of cells, not shown). That the efficiency of adenoviral infection of p21$^{-/-}$ MEFs is unaffected by prior infection by another virus is demonstrated in Table 1, indicating that protection does not arise from a lessened susceptibility of the cell to infection by AdCMV.p53, but rather through p21 overexpression.

Discussion

In this report we have provided several lines of evidence that support a role for p21Waf1/Cip1 in protecting cells against p53-mediated apoptosis. First,

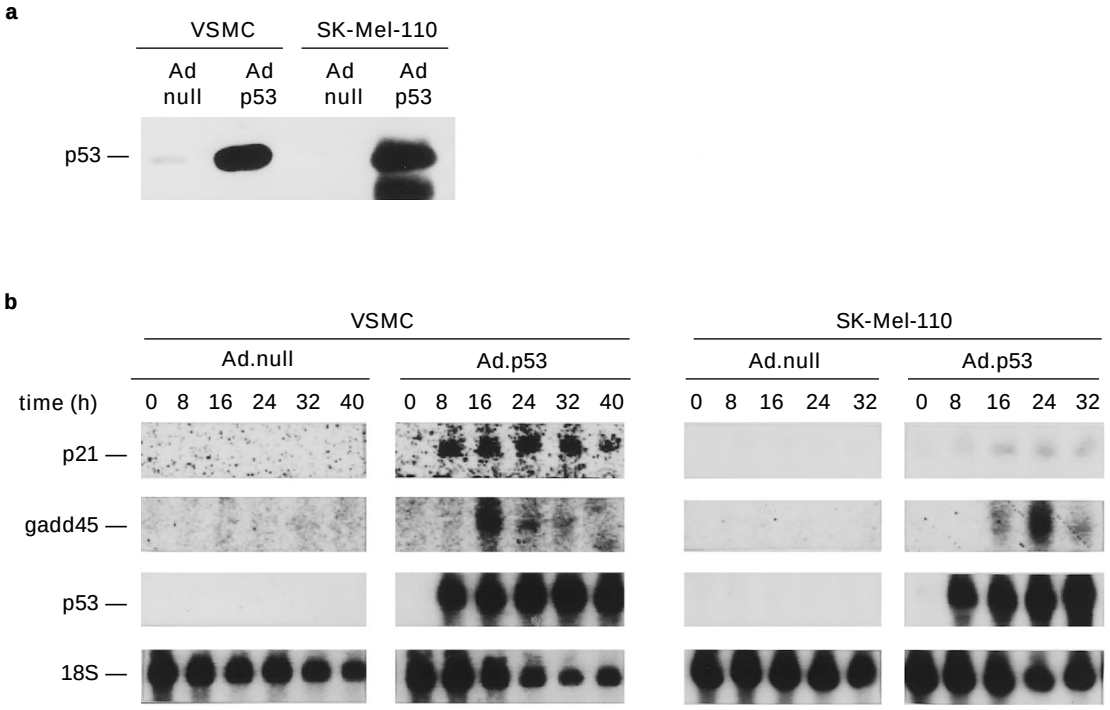

Figure 2 (**a**) p53 expression in VSMC and SK-MEL-110 cells. Cells were infected with 100 p.f.u./cell AdCMV.p53 and p53 expression was monitored 24 h later by Western blot analysis. (**b**) Northern blot analysis of $p21^{Waf1/Cip1}$, GADD45 and p53 mRNA expression in Ad.null- or AdCMV.p53-infected VSMC and SK-MEL-110 cells. Expression was monitored at the times indicated following infection. Loading and transfer differences among samples were normalized using an oligomer recognizing 18S rRNA

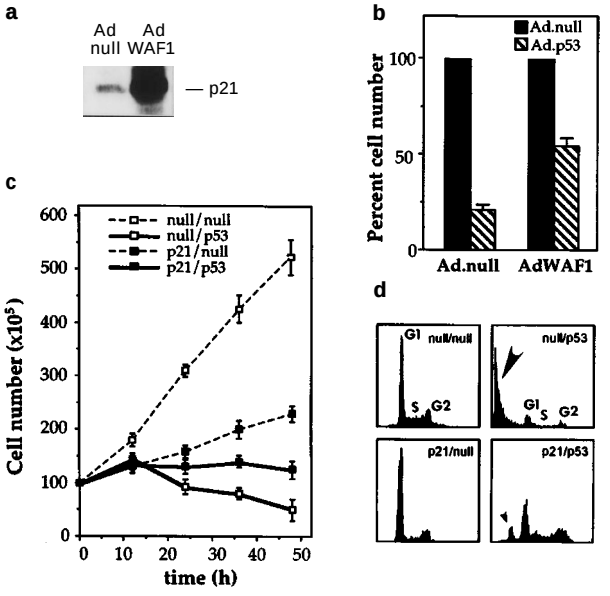

Figure 3 (**a**) $p21^{Waf1/Cip1}$ protein expression in SK-MEL-110 cells 24 h after infection with the indicated viruses. (**b**) Loss in cell viability of control SK-MEL-110 cultures infected with AdCMV.p53 relative to that of $p21^{Waf1/Cip1}$-overexpressing cells infected with AdCMV.p53. Abscissa, first infection with Ad.null (left), or AdWAF1 (right); second infection: solid bars (Ad.null), hatched bars (AdCMV.p53). Values represent mean ± SEM of at least three independent experiments. (**c**) Time-dependent changes in cell number of SK-MEL-110 cultures that had been infected with Ad.null (*null*) or AdWAF1 (*p21*) 48 h prior to a subsequent infection with either Ad.null or AdCMV.p53 (*p53*). Infection groups: null/null (- -□- -), null/p53 (–□–), p21/null (- -■- -) and p21/p53 (–■–). Values represent mean ± SEM of at least three independent experiments. (**d**) FACS analysis of SK-MEL-110 cultures after sequential infections with Ad.null (*null*) or AdWAF1 (*p21*) for 48 h (first infection) followed by Ad.null (*null*) or AdCMV.p53 (*p53*) for the following 48 h (second infection). Note the apoptotic peak (arrowhead), readily visible in the *null*/*p53* treatment group

modulation of endogenous $p21^{Waf1/Cip1}$ levels directly correlated with survival in several cell lines infected with a p53-expressing adenoviral vector: SK-MEL-110 cells, where overexpression of p53 led to apoptotic cell death with no induction of $p21^{Waf1/Cip1}$, and primary VSMC, where p53 overexpression had little effect on cell viability and $p21^{Waf1/Cip1}$ was markedly elevated. Second, overexpression of p53 was highly toxic for MEFs derived from a p21-knockout mouse ($p21^{-/-}$). And third, ectopic $p21^{Waf1/Cip1}$ expression in both SK-MEL-110 and $p21^{-/-}$ MEFs was found to confer protection against subsequent p53 overexpression. These findings, implicating $p21^{Waf1/Cip1}$ as a survival factor against p53-mediated apoptosis, are in keeping with our previous studies demonstrating that $p21^{Waf1/Cip1}$ overexpression correlated with survival of various cell types in response to treatment with an alternative antiproliferative agent, prostaglandin A_2 (PGA_2). In this system, PGA_2 treatment led to growth arrest of MCF-7 cells associated with enhanced $p21^{Waf1/Cip1}$ expression. Constitutive expression of antisense $p21^{Waf1/Cip1}$ transcripts, which reduced endogenous $p21^{Waf1/Cip1}$ levels, resulted in an attenuation in growth arrest and enhanced toxicity. In contrast to MCF-7 cells, RKO cells failed to respond to PGA_2 treatment with growth arrest or $p21^{Waf1/Cip1}$ induction, but instead underwent rapid cell death, regardless of p53 status. However, ectopic $p21^{Waf1/Cip1}$ expression resulted in marked protection against a subsequent PGA_2 challenge. Thus, based on these observations coupled with our current findings, it appears likely that $p21^{Waf1/Cip1}$ plays a more generalized role important for survival during various conditions of stress.

The study presented here highlights the existence of fundamental differences between the responsiveness of VSMC and SK-MEL-110 cells to p53 overexpression,

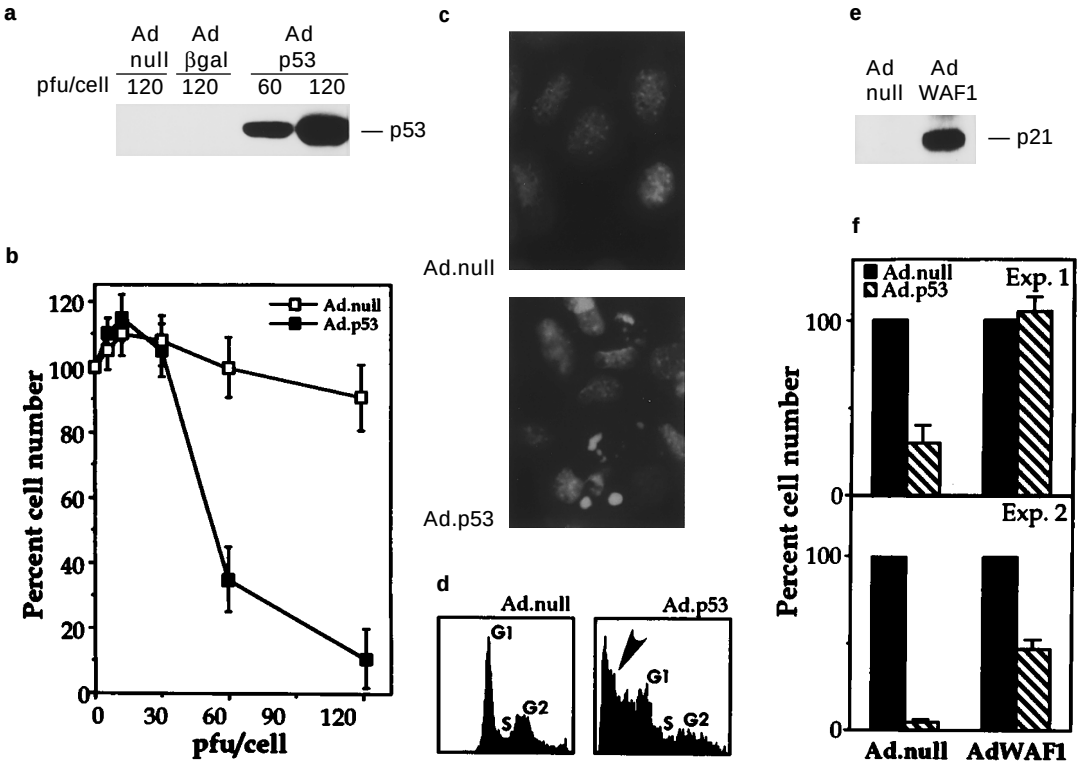

Figure 4 p53-mediated cytotoxicity in mouse embryonal fibroblasts lacking the $p21^{Waf1/Cip1}$ gene ($p21^{-/-}$ MEFs) is prevented by prior expression of $p21^{Waf1/Cip1}$. (**a**) Western blot analysis of p53 expression in $p21^{-/-}$ MEFs following infection with either Ad.null AdCMV.NLSβgal or AdCMV.p53 adenoviruses at the indicated p.f.u./cell. (**b**) Cytotoxic effect of p53 overexpression in $p21^{-/-}$ MEFs. 90 p.f.u./cell was sufficient to achieve infection of >95% of the cells as determined by monitoring β-galactosidase activity following infection with AdCMV.NLSβgal (Table 1). Cell viability, confirmed independently by trypan blue dye exclusion and DAPI staining (not shown), was quantitated by crystal violet staining 48 h after infection with 90 p.f.u./cell Ad.null (□) or AdCMV.p53 (■) adenoviruses. Values represent mean ± SEM of at least four independent experiments. (**c**) p53-overexpressing $p21^{-/-}$ MEFs exhibit nuclear condensation. Genomic DNA condensation was visualized 36 h after infection of $p21^{-/-}$ MEFs with either Ad.null (*Ad.null*) or AdCMV.p53 (*Ad.p53*) by DAPI staining. (**d**) FACS distribution of $p21^{-/-}$ MEFs 48 h after infection with either Ad.null (*Ad.null*) or AdCMV.p53 (*Ad.p53*) viruses. Note the appearance of a sub-G_1 cell population, consistent with an apoptotic process. (**e**) Western blot analysis of $p21^{Waf1/Cip1}$ expression in $p21^{-/-}$ MEFs 48 h after infection with 90 p.f.u./cell Ad.null or AdWAF1 (arrowhead). (**f**) $p21^{Waf1/Cip1}$ expression protects against p53-mediated cytotoxicity. Forty-eight hours after infection (90 p.f.u./cell) with either Ad.null or AdWAF1, $p21^{-/-}$ MEFs were infected with either Ad.null (solid bars) or AdCMV.p53 (hatched bars). Viable cells remaining in each treatment group (null/null, null/p53, p21/null, p21/p53) were counted at the end of the treatment period. Two representative experiments are shown

which are reflected in their opposing fates. These differences may be attributed, at least in part, to the differential expression of $p21^{Waf1/Cip1}$. Similar opposing effects of p53 overexpression have been described in other systems where rapidly growing cancerous cells generally undergo death in response to chemotherapeutic agents, while normal (untransformed) cells survive the treatment (Hainaut, 1995; Drazan *et al*., 1994; Clayman *et al*., 1995). Important questions remain concerning why elevated p53 results in increased $p21^{Waf1/Cip1}$ expression in certain cell types, while failing to do so in others. Perhaps cells that do not survive p53 overexpression become committed to apoptotic cell death very early on, precluding other physiological functions such as expression of $p21^{Waf1/Cip1}$. While the issue regarding whether p53-mediated apoptosis requires active gene expression remains unresolved, our results indicate that p53-driven $p21^{Waf1/Cip1}$ expression is not involved (at least in the cell types systems examined). Interestingly, however, p53-mediated transcription is not abrogated indiscriminately, since another p53-regulated gene, GADD45, is effectively upregulated in AdCMV.p53-infected cells, regardless of the outcome (Figure 2b).

Precisely what $p21^{Waf1/Cip1}$ functions are important for its protective influence remain to be determined. The recent observation that $p21^{Waf1/Cip1}$-deficient colorectal carcinoma cells undergo aberrant DNA replication without mitosis and are highly susceptible to the cytotoxic effects of a variety of chemotherapeutic drugs led Waldman *et al*. (1996) to propose that $p21^{Waf1/Cip1}$ operates in the orchestration of events leading from S phase to the onset of mitosis. Further, interference with $p21^{Waf1/Cip1}$ expression results in the disruption of this series of events, rendering the cell more susceptible to cytotoxic treatments. The findings of Polyak *et al*. (1996) that $p21^{Waf1/Cip1}$-deficient carcinoma cells are more susceptible to p53-mediated apoptosis are consistent with this hypothesis. In this regard, the enhanced susceptibility to killing by chemotherapeutics that is observed in some cancer cells deficient in p53 function may be partly due to their lower $p21^{Waf1/Cip1}$ expression, since p53 status (mutant *versus* normal) influences both basal and inducible $p21^{Waf1/Cip1}$ levels. Alternatively, Shim *et al*. (1996) have recently reported an inhibitory effect of $p21^{Waf1/Cip1}$ on c-Jun N-terminal kinase (JNK), a key regulatory protein in various stress-activated pathways. As JNK has recently been implicated in the implementation of programmed cell death (Xia *et al*., 1995; Shim *et al*., 1996; Verkeij *et al*., 1996; Chen *et al*.,

1996), its interaction with p21$^{Waf1/Cip1}$ raises the intriguing possibility that p21$^{Waf1/Cip1}$-mediated inhibition of apoptosis may be directly linked to its inhibitory effect on JNK.

In summary, our current findings and previous observations support the view that, in situations of stress, p21$^{Waf1/Cip1}$ plays a fundamental role in the decision fork between cell death and survival, with enhanced p21$^{Waf1/Cip1}$ expression contributing to the ability of the cell to endure stress, and unchanged p21$^{Waf1/Cip1}$ expression contributing to cellular demise. These findings have important implications for the design of chemotherapeutic approaches for cancer treatment: since enhanced p21$^{Waf1/Cip1}$ expression exerts a protective function, therapies aimed at preventing its expression may prove most successful for the elimination of cancer cells.

Materials and methods

Cell culture and virus preparation, titer and infection

The human melanoma SK-MEL-110 cell line, vascular smooth muscle cells (isolated from rats and prepared using standard techniques) and mouse embryonal fibroblasts (MEFs) derived from a p21-knockout mouse (Deng *et al*., 1995), were maintained in Dulbecco's modified essential medium (Gibco BRL, Gaithersburg, MD), supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and 50 μl/ml gentamicin (Gibco BRL). Adenoviruses lacking an insert (Ad.null), or expressing either p53 (AdCMV.p53), nuclear-localized β-galactosidase (AdCMV.NLSβgal) or p21$^{Waf1/Cip1}$ (AdWAF1), were amplified and titered in 293 packaging cells containing the genes for viral replication (Cirielli *et al*., 1995; Yang *et al*., 1995). Infections were carried out in serum-free DMEM for 1 h. For determination of infection efficiency in different cell lines, either from single or sequential infections, VSMC, SK-MEL-110, and p21$^{-/-}$ MEF cultures were infected with AdCMV.NLSβgal at various plaque-forming units (p.f.u.)/cell and cultured for 48 h. Following fixation and incubation with 1 μg/ml X-gal for 1–4 h to detect β-galactosidase activity, the percentage of positive (blue) cells was determined (Table 1 and data not shown).

Northern blot analysis

Total RNA was isolated by the STAT-60 method (Tel-Test 'B', Friendswood, TX) following the manufacturer's specifications. Twenty μg RNA samples were denatured, size-fractionated by electrophoresis in 1.2% agarose/formaldehyde gels and transferred onto GeneScreen Plus nylon membranes (DuPont/NEN, Boston, MA). For the detection of GADD45 and p53 mRNA in VSMC and SK-MEL-100 cells and the detection of p21$^{Waf1/Cip1}$ in SK-MEL-110 cells, cDNAs for GADD45 (excised from the pCMV45 plasmid, kindly provided by Dr AJ Fornace Jr), p53 (Oncogene Science) and p21$^{Waf1/Cip1}$ (excised from the pCEP-WAF1 plasmid (El-Deiry *et al*., 1993), were labeled with [α-^{32}P]dCTP (3000 Ci/mmol) with a random primer labeling kit (Boehringer Mannheim, Indianapolis, IN). An oligomer complementary to the mouse p21$^{Waf1/Cip1}$ mRNA (5′-CTCCGTGACGAAGTCAAAGTTCCACCG-TTCTCGGGCCTCCTGGAGACAGCC-3′) was utilized to detect p21$^{Waf1/Cip1}$ mRNA in VSMC, and an oligomer complementary to the 18S rRNA (5′-ACGGTATCTGAT-CGTCTTCGAACC-3′) (Integrated DNA Technologies, Coralville, IA), was used to normalize for loading and transfer of samples. The oligonucleotides were 3′ end-labeled with [α-^{32}P]dATP (3000 Ci/mmol) by terminal deoxynucleotidyl transferase (Life Technology Laboratories, Gaithersburg, MD). Hybridization and washes were performed according to the method of Church and Gilbert (1984).

Cell counts and flow cytometric cell cycle analysis

Cell number was assessed by direct cell counts using a hemocytometer. Trypan blue dye exclusion and DAPI staining were used as indicators of cell viability. Indirect cell number measurements were determined by crystal violet staining (Gorospe *et al*., 1996a). Cell cycle distribution was analysed using flow cytometry as described (Kim *et al*., 1992). Briefly, $2-5\times10^6$ cells were trypsinized, washed once with PBS and fixed in 70% ethanol. Fixed cells were washed with PBS, incubated with 1 μg/ml RNase A for 30 min at 37°C and stained with propidium iodide (Boehringer Mannheim). The stained cells were analysed on a FACscan flow cytometer for relative DNA content. The percentage of the cells in the various cell cycle compartments were determined using the MULTICYCLE software program (Phoenix Flow Systems, San Diego, CA).

Western blot analysis

Fifty-μg samples of total cell lysates were size-fractionated by SDS–PAGE and transferred onto PVDF membranes using standard techniques (Harlow and Lane, 1988). p53 and p21$^{Waf1/Cip1}$ proteins were detected with the ECL system (Amersham, Arlington Heights, IL) following incubation with the monoclonal anti-human p53 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit polyclonal anti-p21 antibody (Oncogene Science).

Abbreviations

MEFs, mouse embryonal fibroblasts; cdk, cyclin-dependent kinase; VSMC, vascular smooth muscle cells; PGA_2, prostaglandin A_2; JNK, C-Jun N-terminal kinase.

Acknowledgements

We thank Dr I Kovesdi for providing the Ad.null adenovirus, Dr RG Crystal for the AdCMV.NLSβgal adenovirus, Dr B Vogelstein for the pCEP-WAF1 plasmid, and AJ Fornace Jr for the pCMV45 plasmid. We are grateful to FJ Chrest and R Pyle for the flow cytometry analysis, Drs K Inyaku and T Riccioni for technical assistance, and Dr DL Longo for critical review of this work and helpful suggestions.

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