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p21-induced cycle arrest in G_1 protects cells from apoptosis induced by UV-irradiation or RNA polymerase II blockage

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Cells expressing the R273H mutant of p53, which lacks sequence specific DNA binding capacity, do not undergo cell cycle arrest in G_1 following exposure to ionizing or UV radiation because of their inability to induce $p21^{Waf1/Cip1}$, a cyclin-dependent kinase inhibitor and downstream mediator of p53-dependent DNA damage-induced growth arrest. Following UV-irradiation or treatment with an inhibitor of RNA pol II, we observed a rapid induction of the apoptotic process, as evidenced by DNA fragmentation and the proteolytic cleavage of poly(ADP-ribose) polymerase. Using mimosine, a $p21^{Waf1/Cip1}$ inducer that bypasses the requirement for transcriptional transactivation by p53, we demonstrated that a G_1 cell cycle arrest can prevent apoptosis following UV-irradiation or treatment with an RNA polymerase II inhibitor. Serum starvation, which also synchronized cells in G_1 but did not induce $p21^{Waf1/Cip1}$, did not protect cells from apoptosis. These results demonstrate that restoring a late G_1 checkpoint by inducing $p21^{Waf1/Cip1}$ expression can protect cells from DNA damage induced apoptosis. Our results suggest that $p21^{Waf1/Cip1}$ can interrupt the apoptotic process at a point downstream from p53 accumulation but upstream from caspase-3 activation.

Keywords: DNA; $p21^{Waf1/Cip1}$; PARP; RNA polymerase II; apoptosis; p53

Introduction

Factors which determine whether eukaryotic cells continue to proliferate or cease dividing and differentiate appear to operate mainly during the G_1 phase of the cell cycle (Pardee, 1989). Key transitions in the cell cycle are regulated by a family of serine/threonine protein kinases termed cyclin-dependent kinases (CDKs) that consist of a catalytic subunit, CDK and a regulatory subunit, cyclin. The enzymatic activity of a CDK is regulated at different levels: cyclin activation, subunit phosphorylation and dephosphorylation, and association with members of a group of heterologous small regulatory proteins, belonging to two families of CDK inhibitor proteins. One CDK inhibitor (CKI) family includes $p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$ and $p19^{INK4D}$, which target the CDK4 and CDK6 kinases and prevent their interaction with cyclin D (Sherr and Roberts, 1995). The other CKI family includes $p21^{WAF1/CIP1}$, $p27^{KIP1}$ and $p57^{KIP2}$. $p21^{Waf1/Cip1}$ binds to cyclin/CDK complexes and either inhibits their kinase activities (Brugarolas *et al*., 1995; Sherr and Roberts, 1995) or prevents their activation by cyclin activating kinase (CAK) *in vivo* (Kato *et al*., 1994) and *in vitro* (Aprelikova *et al*., 1995).

$p21^{Waf1/Cip1}$ was the first CKI to be identified, as a mediator of p53-induced growth arrest in response to DNA damage (El-Deiry *et al*., 1993; Gu *et al*., 1993; Harper *et al*., 1993; Xiong *et al*., 1993). The signals for p53 activation include DNA damage (DiLeonardo *et al*., 1994; Kastan *et al*., 1992; Kastan 1996; Maltzman and Czyzyk, 1984), hypoxia (Graeber *et al*., 1994, 1996), and perturbations of the ribonucleotide pools (Linke *et al*., 1996). Wild-type p53 is required for arrest in G_1, mediated by $p21^{Waf1/Cip1}$ (Brugarolas *et al*., 1995), in response to genotoxic agents, such as ionizing radiation, and to perturbations of nucleotide pools (Kastan *et al*., 1991, 1992; Kuerbitz *et al*., 1992; Yin *et al*., 1992; Khanna *et al*., 1995; Gorospe *et al*., 1997; Guillot *et al*., 1997).

Induction of p53 can lead to either cell cycle arrest or apoptosis. p53 transactivates a family of genes, including *p21/WAF1/CIP1*, *GADD45*, *MDM2* and *BAX*, containing the p53 response element in their promoters. The question of whether p53 requires its transcriptional transactivation activity to induce apoptosis is still controversial. Some authors have provided evidence that p53 may have a transcription-independent function in apoptosis (Haupt *et al*., 1995; Caelles *et al*., 1994; Chen *et al*., 1996; Bissonnette *et al*., 1997).

The participation of $p21^{Waf1/Cip1}$ in p53-triggered cell death is still not clear. Some authors have shown that induction of apoptosis is associated with up-regulation of endogenous $p21^{Waf1/Cip1}$ (Duttaroy *et al*., 1997) and Bax protein levels (Miyashita and Reed, 1995), whereas others have shown that the p21 gene is not essential for apoptosis (Attardi *et al*., 1996). Using a tetracycline-inducible system, several authors found that induction of $p21^{Waf1/Cip1}$ neither sensitized to nor protected from apoptosis (Chen *et al*., 1996; Sheikh *et al*., 1997). In some cells, a high level of $p21^{Waf1/Cip1}$ inhibits apoptosis rather than promotes it (Canman *et al*., 1995; Gorospe and Holbrooke, 1996; Wang and Walsh, 1996), whereas in others, p53 expression overcomes $p21^{Waf1/Cip1}$-mediated G_1 arrest and induces apoptosis (Kagawa *et al*., 1997). Inactivation of *p21/WAF1/CIP1* sensitizes colorectal cancer cells to apoptosis by p53 (Gorospe *et al*., 1996; Polyak *et al*., 1996) and overexpression of p53 in embryonal fibroblasts derived from p21-knockout mice ($p21^{-/-}$ MEFs) likewise resulted in extensive cytotoxicity whereas ectopic p21 expression conferred protection against a subsequent p53 induction (Gorospe *et al*., 1997).

$p21^{Waf1/Cip1}$ has been found to inhibit the protein kinase activation of G_1 cyclin/CDK complexes, thereby preventing phosphorylation of the retinoblastoma (Rb)

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protein (Slebos *et al*., 1994) required for G_1/S transition (Deng *et al*., 1995; Tarunina *et al*., 1996). It is well established that $p21^{Waf1/Cip1}$ is involved in p53-dependent growth suppression (Del Sal *et al*., 1996) and confers a survival advantage (Gorospe *et al*., 1997). However, it still remains to be established whether $p21^{Waf1/Cip1}$ protects cells from apoptosis induced by DNA damage and/or a stalled RNA pol II, thus providing a delay, such as a G_1 arrest and consequently the time to mend the cellular damage. In this study, we have investigated the apoptotic suppressor activity of the $p21^{Waf1/Cip1}$ protein, elevated through a p53-independent pathway by mimosine, an amino acid found in plants (Lalande, 1990; Mosca *et al*., 1992; Alpan and Pardee, 1996). Our results provide additional and direct evidence that a p53-dependent pathway can protect cells from apoptosis induced by DNA damage and/or a stalled RNA pol II.

Results

UVB radiation induces apoptosis in human epidermal carcinoma A431 cells in a dose-dependent manner

A431 cells were irradiated with different doses of UVB radiation and the extent of apoptosis was determined (Figure 1). Total cellular DNA was extracted from keratinocytes 6 h after UVB irradiation and separated by gel electrophoresis to detect the nucleosomal DNA ladders that are a hallmark of apoptosis. Control unirradiated and low dose (200–400 J/m^2) UVB-irradiated A431 cells contained only high-molecular-weight DNA (Figure 1). At a dose approximately 600 J/m^2, apoptotic cell death was triggered, as seen by the typical nucleosomal DNA ladder. At higher doses (e.g. 2000 J/m^2), the apoptotic process was faster, as seen by the appearance of the nucleosomal ladder as early as 2 h post-irradiation (data not shown).

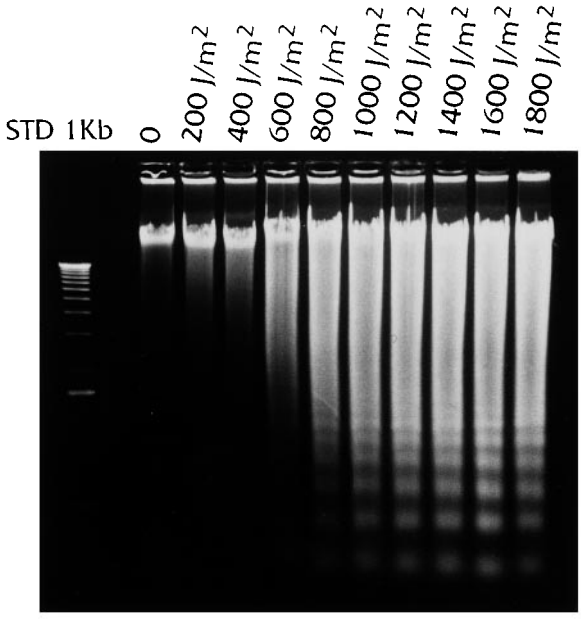

Figure 1 UVB dose-dependent induction of apoptosis in keratinocyte carcinoma cells (A431). Cells were treated with UVB at the indicated dose and incubated for 6 h prior to harvest. DNA extraction is described in Materials and methods

Inhibition of RNA polymerase II leads to apoptosis

$p21^{Waf1/Cip1}$ protein expression in MCF-7 cells is induced following DNA damage (Figure 2), since these cells express wild-type p53. Accumulation of the $p21^{Waf1/Cip1}$ protein requires transcription (El-Deiry *et al*., 1993; Harper *et al*., 1993). We therefore used p21 protein accumulation as an indirect measure of p53-dependent transcriptional activity following DNA damage by UV radiation. A direct correlation was observed between the dose of UVB required to inhibit transcription of *p21/WAF1/CIP1* (Figure 2) and the initiation of the apoptotic process (Figure 1). At doses of UVB which impaired the induction of $p21^{Waf1/Cip1}$ protein expression (approximately 500 J/m^2 and over), apoptosis was induced. It has been shown that UV radiation transciently inhibits both DNA and RNA synthesis *in vivo* (Mayne and Lehmann, 1982) and completely blocks RNA pol II transcription *in vitro* (Donahue *et al*., 1994; Selby *et al*., 1997). Furthermore, it has been suggested previously that blockage of RNA polymerase may trigger UV-induced apoptosis (Ljungman and Zhang, 1996). To investigate the possibility that stalled RNA pol II complexes may initiate this apoptotic process observed in A431 and MCF-7 cells, we used an RNA pol II inhibitor, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), to completely block transcriptional activity (Yankulov *et al*., 1995). As with UVB-irradiation (Figure 1), DRB induced apoptosis as seen by the nucleosomal DNA ladder (Figure 10a) but also by the cleavage of poly(ADP-ribose) polymerase (PARP) to its 85 kDa fragment (Figure 3B), the proteolytic hallmark of the apoptotic cell death process (Lazebnik *et al*., 1994). PARP cleavage was also dependent on UVB dose, since a higher dose (2000 J/m^2 *versus* 500 J/m^2) accelerated the appearance of the cleaved 85 kDa fragment (Figure 3a).

$p21^{Waf1/Cip1}$ can be induced by a p53-independent pathway using mimosine

Mimosine, an amino acid present in plants, has been found to synchronize cells at the G_1/S border by inducing accumulation of $p21^{Waf1/Cip1}$ (Lalande, 1990; Mosca *et al*., 1992; Alpan and Pardee, 1996). To confirm that p53 is not required for induction of $p21^{Waf1/Cip1}$ in primary cultures of fibroblasts from normal Balb C (p53+/+) and p53(−/−) mice. $p21^{Waf1/Cip1}$ protein expression was induced following

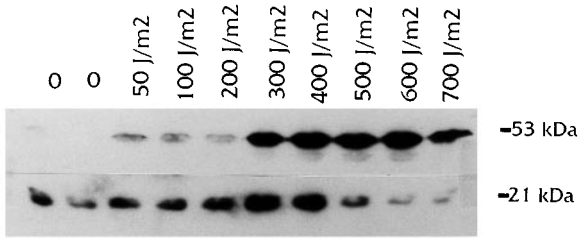

Figure 2 Expression of $p21^{Waf1/Cip1}$ in MCF-7 cells following UVB irradiation. Total cellular protein was extracted 7 h following irradiation at the indicated dose of UVB, as described in Materials and methods

DNA damage in p53+/+ fibroblasts whereas no induction was seen in p53-null fibroblasts (Figure 4) at any dose or at any time following irradiation. Mimosine can induce the $p21^{Waf1/Cip1}$ protein by a p53-independent pathway (Alpan and Pardee, 1996), and we confirmed this using p53-null primary fibroblasts (Figure 4, right panel). The difference in the p21 protein expression in the p53+/+ *versus* the p53−/− cells is primarily the result of differences in exposure of the films during the Western analysis. Nevertheless, it is possible that the induction of *p21/WAF1/CIP1* by mimosine is slightly enhanced by the presence of a wtp53 protein.

$p21^{Waf1/Cip1}$ protein expression is not induced in A431 cells following DNA damage

No $p21^{Waf1/Cip1}$ induction was seen following irradiation of A431 cells with UVB or γ-rays (Figure 5a and b respectively). Interestingly, a decrease in the basal level of $p21^{Waf1/Cip1}$ was observed following UVB irradiation with a dose response corresponding to that required for the induction of apoptosis in A431 cells (Figure 1) and also corresponding to the dose required to inhibit the induction of $p21^{Waf1/Cip1}$ expression in MCF-7 cells (Figure 2). The tumor-derived line of transformed human keratinocytes (A431) contains only one allele of p53, mutated in its central core at residue 273 (ArgHis) (Harlow *et al.*, 1985). This mutant p53 lacks transcriptional transactivation activity (Wang *et al.*, 1996). We confirmed the presence of this mutation (R273H) by pcr using primers that amplified exon 8 of the endogenous gene (data not shown, details given in Materials and methods).

Exponentially growing A431 cells were incubated with mimosine, which induces *p21/WAF1/CIP1* by a mechanism independent of p53. Treatment with 500 μM mimosine increased the level of the $p21^{Waf1/Cip1}$

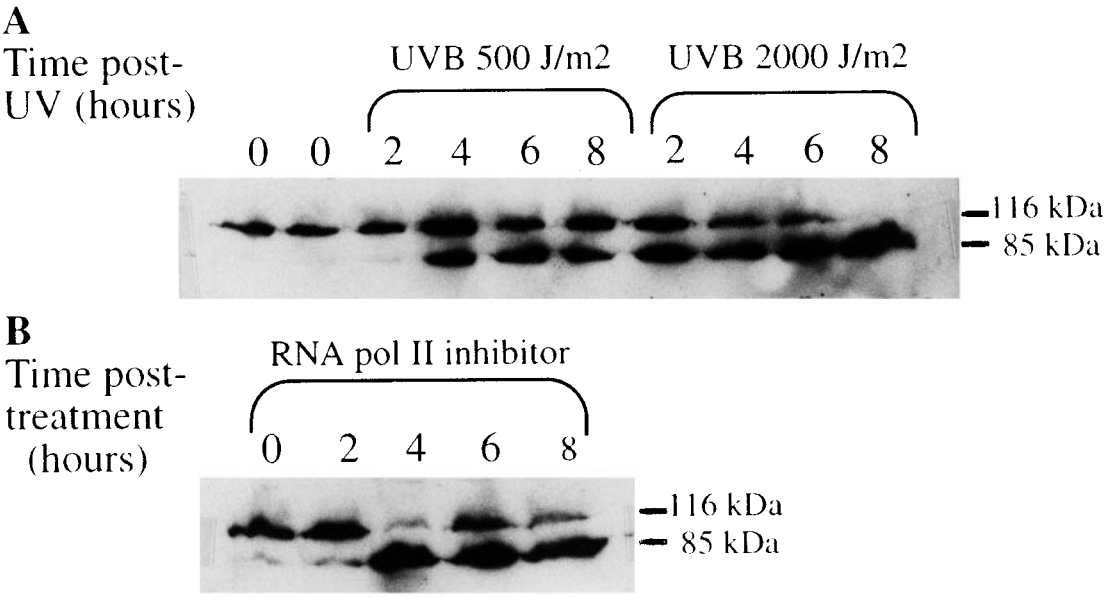

Figure 3 Proteolytic cleavage of poly(ADP-ribose) polymerase (PARP) in A431 cells following treatment with UVB (**A**) or an inhibitor of RNA pol II (DRB: 50 μg/ml) (**B**). Total cellular protein was extracted as described in Materials and methods

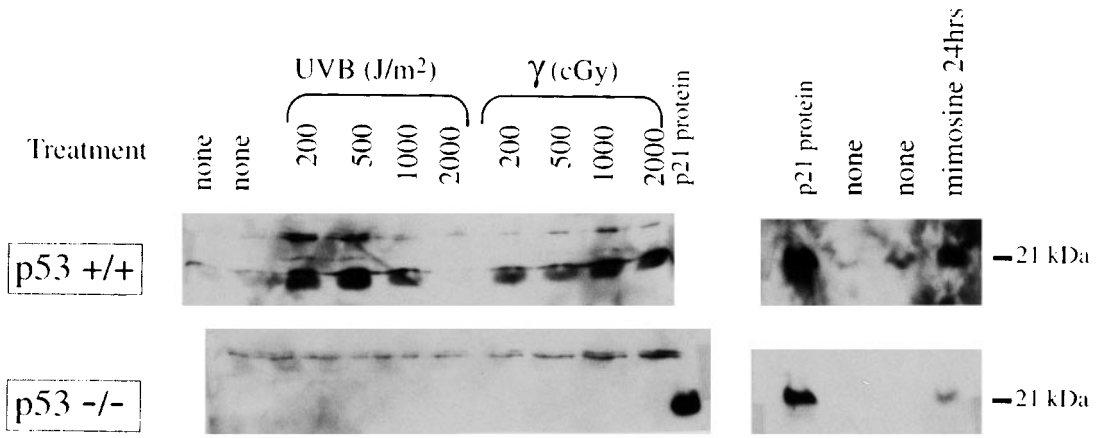

Figure 4 Induction of $p21^{Waf1/Cip1}$ by mimosine or DNA damage in primary fibroblasts from knockout (p53−/−) and normal (p53+/+) mice. Total protein was extracted 7 h following irradiation (UV or γ-rays) or following 24 h of mimosine (500 μM) treatment, as described in Materials and methods

protein (Figure 6) and induced an accumulation of cells in G_1 (Figure 7d). As expected, serum starvation for 24 h also synchronized cells in G_1 (Figure 7d).

p21-dependent cell cycle arrest in late G_1 protects A431 cells from apoptosis

We tested the hypothesis that a G_1 cell cycle arrest can protect cells from UVB-radiation induced apoptosis. A431 cells were arrested in G_1 either by induction of the $p21^{Waf1/Cip1}$ protein following a treatment with mimosine (500 μM) or by serum starvation for 24 h (Figure 7d). In most cells, DNA damage results in the induction of *p21/WAF1/CIP1* by a p53 dependent pathway. Since this could confound the interpretation of the results, we used A431 cells which express a transcriptionally inactive mutant of the p53 protein. Thus, UVB or gamma irradiation of A431 cells did not result in any accumulation in G_1 (Figure 7a, b or c). An accumulation in G_2 was observed following a high dose of γ-irradiation (Figure 7b) whereas an accumulation of cells with a sub-G_1 content of DNA was seen following high dose of UVB-irradiation, an effect usually associated with apoptosis (Haupt *et al*., 1996). Following a 12 h incubation with 500 μM mimosine, $p21^{Waf1/Cip1}$ protein levels were elevated (Figure 6, Figure

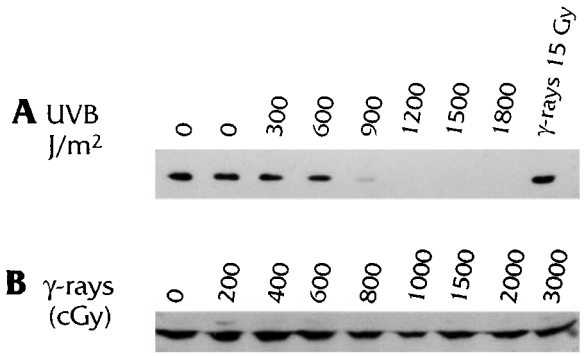

Figure 5 Expression of $p21^{Waf1/Cip1}$ in A431 cells following DNA damage. $p21^{Waf1/Cip1}$ protein levels following UVB (**A**) or γ-rays (**B**). Total protein was extracted after the indicated time as described in Materials and methods

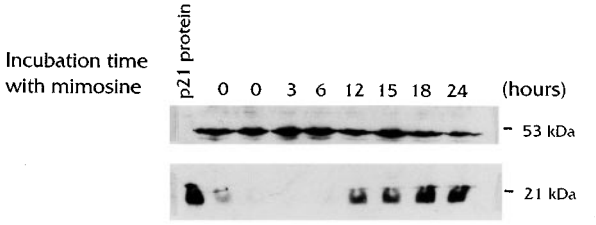

Figure 6 Induction of $p21^{Waf1/Cip1}$ expression by mimosine (500 μM) in A431 cells. Total protein was extracted following the indicated incubation period, as described in Materials and methods

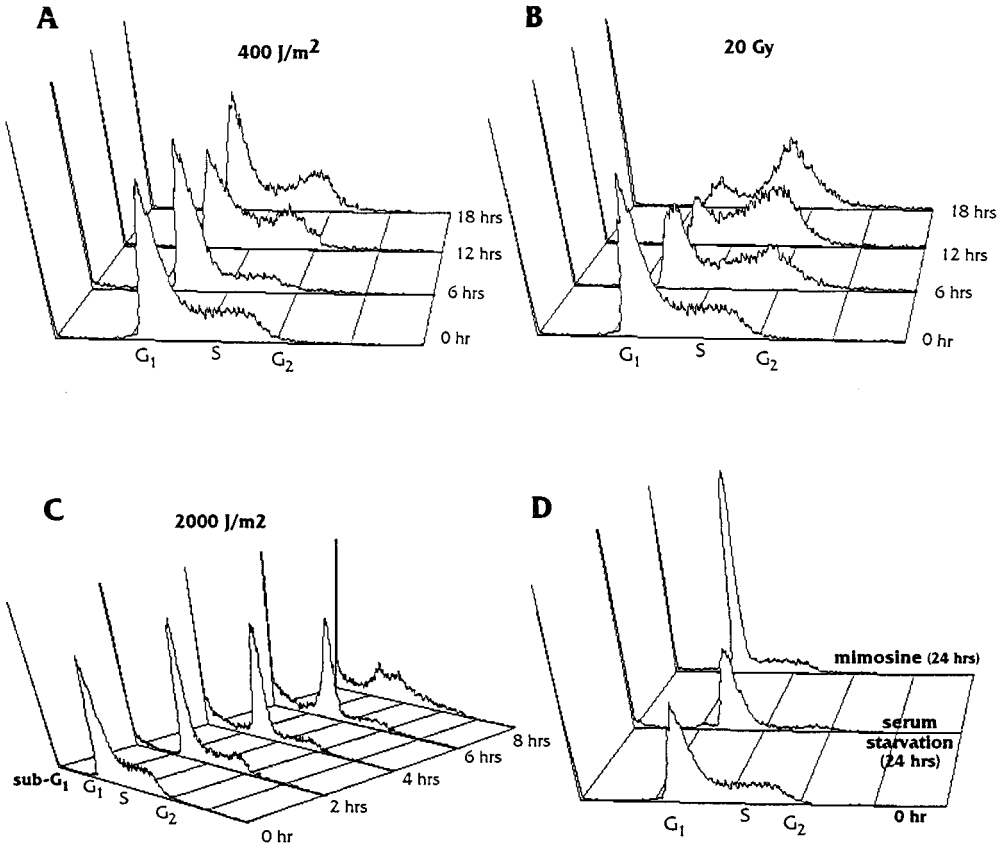

Figure 7 Cell cycle distribution of A431 cells following treatment. Cells were irradiated with 400 J/m^2 or 2000 J/m^2 UVB radiation (panel **A** and **C**, respectively) or with γ-irradiation with a dose of 20 Gy (**C**), following incubation for the period indicated prior to harvest. (**D**) Cells were treated with mimosine (500 μM) or serum starvation for 24 h prior to harvest. The corresponding phase of the cell cycle is indicated at the bottom of each graph. Results are representative of several experiments. Details of the cell treatments and the cell cycle analysis are described in Materials and methods

9, bottom panel) but UVB-irradiation caused the p21 protein level to decrease and apoptosis was induced, as seen by the appearance of a nucleosomal DNA ladder (Figure 8) and the cleavage of PARP (Figure 9). We observed that a 24 h incubation with mimosine was required to prevent the appearance of the nucleosomal DNA ladder following irradiation with UVB (Figure 8). Under these conditions, $p21^{Waf1/Cip1}$ protein levels remained elevated following UVB-irradiation and PARP cleavage was almost completely inhibited (Figure 9, last lane). The fact that some PARP cleavage was observed suggests that a small fraction of the cells were not protected from apoptosis. The apoptotic protection was not restricted to UVB-irradiation since a 24 h treatment with mimosine fully protected A431 cells (Figure 10a) or MCF-7 cells (results not shown) from apoptosis induced by DRB, an inhibitor RNA pol II. Serum starvation for 12 or 24 h did not induce $p21^{Waf1/Cip1}$ and did not afford any protection against apoptosis following UVB irradiation (Figure 10b) or treatment with DRB (data not shown), even though the A431 cells were synchronized in G_1 (Figure 7d). Similar results were obtained with MCF-7 cells (results not shown).

Cell cycle analysis of cells synchronized in G_1 by a mimosine treatment revealed that the population of cells in the G_1 phase of the cell cycle were fully protected from apoptosis induced by UVB and were resistant to a sub-G_1 shift, indicative of apoptosis (Figure 11). This protective effect lasted for up to 20 h following removal of mimosine and prevented the loss cell viability following irradiation with UVB (data not shown).

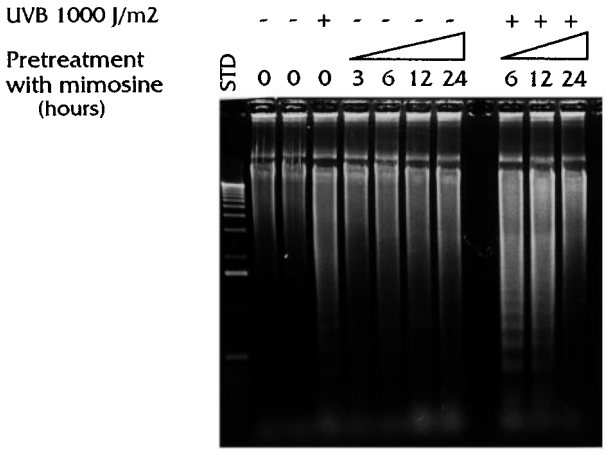

Figure 8 Protection by mimosine from UVB-induced apoptosis, as measured by the inhibition of the formation of a nucleosomal ladder in A431 cells. Cells were preincubated with 500 μM mimosine for the indicated time and then harvested or irradiated with 1000 J/m^2 UVB. Cellular DNA was extracted following the mimosine treatment and/or 6 h following irradiation, as described in Materials and methods

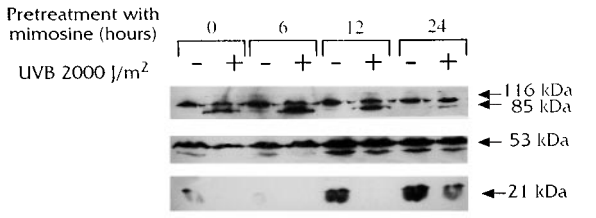

Figure 9 Protection by mimosine from UVB-induced apoptosis, as measured by the inhibition of PARP cleavage in A431 cells. Cells were incubated mimosine (500 μM) for the indicated time and irradiated with 2000 J/m^2 UVB. Total protein was extracted following mimosine treatment and/or 6 h following irradiation, as described in Materials and methods. The same blot was used for hybridization with the CII-10 (anti-PARP), DO-1 (anti-p53) and anti-p21 antibodies

Discussion

p53 dependent and independent induction of p21$^{Waf1/Cip1}$

We have studied the effects of $p21^{Waf1/Cip1}$ on the apoptotic responses of two human cell lines: MCF-7 cells, which express functional p53 protein, and A431 cells in which express only a mutant form of p53. The mutant p53 (R273H) present in A431 cells has impaired sequence specific binding capacity (Vogelstein and Kinzler, 1992) and thus lacks transcriptional transactivation activity (Wang *et al*., 1996). Induction of $p21^{Waf1/Cip1}$ expression following DNA damage requires functional p53 protein as seen in normal (p53+/+) primary fibroblasts compared with cells from knockout mice (p53−/−) (Figure 4), in agreement with published results (Khanna *et al*., 1995; Waldman *et al*., 1995; Guillot *et al*., 1997). A431 cells constitutively express very low levels of $p21^{Waf1/Cip1}$ and are considered functionally equivalent to p21−/− cells with respect to their response to DNA damage (Waldman *et al*., 1996). No $p21^{Waf1/Cip1}$ induction was observed following DNA damage (Figure 5). The basal $p21^{Waf1/Cip1}$ expression is largely independent of p53 (Parker *et al*., 1995; Macleod *et al*., 1995) and this can account for the basal

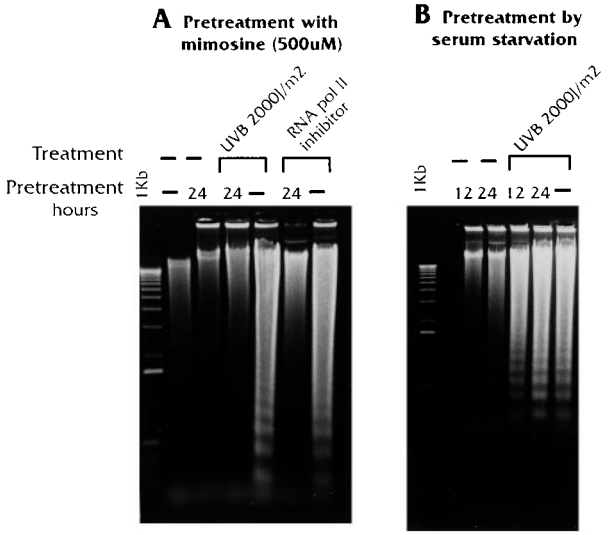

Figure 10 Protection from apoptosis, as measured by intrancucleosomal cleavage, by $p21^{Waf1/Cip1}$ expression in A431 cells. A431 cells were irradiated with 2000 J/m^2 UVB, following either: no pretreatment or preincubated with 500 μM mimosine for 24 h (**A**); or serum starvation for 12 or 24 h (**B**). DNA was extracted as described in Materials and methods

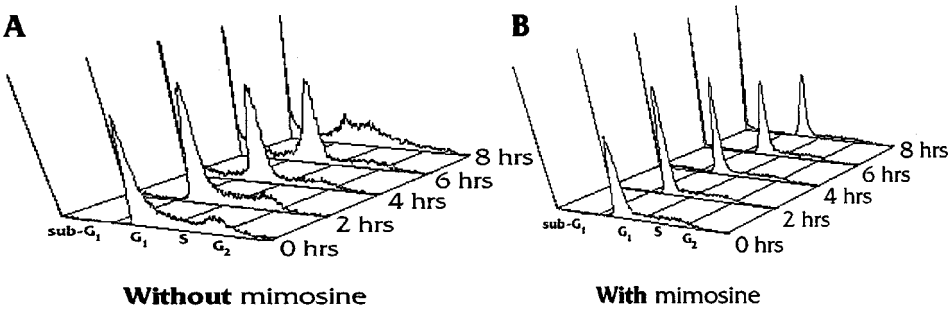

Figure 11 Cell cycle analysis of A431 cells following irradiation with 2000 J/m^2 UVB. Cells were irradiated without pretreatment (**a**) or preincubated 24 h with 500 μM mimosine (**b**) and harvested following incubation for the period indicated, as described in Materials and methods

expression seen in A431 cells (Figure 5). Furthermore, p21$^{Waf1/Cip1}$ can be induced by a p53-independent pathway possibly involving perturbation of ribonucleotide pools. The drug mimosine is a non-protein amino acid which has been hypothesized to interact indirectly with ribonucleotide reductase by complexing iron ions and thereby blocking DNA synthesis by decreasing cellular dNTP concentrations (Gilbert *et al*., 1995). Cell cycle analysis of A431 cells treated with hydroxyurea, which depletes deoxyribonucleotide pools by specifically inhibiting ribonucleotide reductase (Elledge and Davis, 1990), showed an arrest in S-phase that differs from the G_1 arrest induced by mimosine (Figure 7d). Furthermore, mimosine does not induce wp53 in MCF-7 cells, even though p21$^{Waf1/Cip1}$ was induced (results not shown). However, the mimosine effect cannot be accounted by the direct effect of its ion chelating capacity since it does not inhibit DNA synthesis in *p21/WAF1/CIP1*-null mice fibroblasts (Alpan and Pardee, 1996; Missero *et al*., 1996), suggesting an indirect effect through *p21/WAF1/CIP1* induction. This drug is a potent late G_1 blocker and induces p21$^{Waf1/Cip1}$ by a p53-independent pathway (Alpan and Pardee, 1996). Mimosine induces both p21 expression (Figure 6) and G_1 phase cell cycle accumulation in A431 cells (Figure 7d). Similar results were obtained in knockout (p53−/−) primary fibroblasts (Figure 4 and unpublished data), confirming that p21$^{Waf1/Cip1}$ can be induced in a p53-independent manner (Alpan and Pardee, 1996).

DNA damage-induced G_1/S arrest involves p21$^{Waf1/Cip1}$ expression mediated by p53

Of the known transcriptional targets of p53, p21$^{Waf1/Cip1}$ is the most likely to control G_1 cell cycle arrest following DNA damage. Cells lacking *p21/WAF1/CIP1* or haboring a transcriptionally inactive mutant p53 are defective in G_1 checkpoint control but have normal G_2 control (Deng *et al*., 1995; Haupt *et al*., 1995). Thus, it is not surprising that epidermal carcinoma A431 cells do not undergo a cell cycle arrest in G_1 following DNA damage (Figure 7) given that they express a transcriptionally inactive mutant p53. After irradiation with UVB or γ-rays, no p21$^{Waf1/Cip1}$ induction was seen (Figure 5). Furthermore, γ-irradiation induced a G_2 block, but no G_1 block in A431 cells (Figure 7).

The basal level of p21$^{Waf1/Cip1}$ decreased in a dose and time dependent manner following UVB-irradiation (Figure 5a) but not γ-irradiation (Figure 5b). This can be accounted for by a blockage of transcription induced by DNA-damage, since cyclobutyl pyrimidine dimers are excellent inhibitors of RNA polymerase II dependent transcription (Selby *et al*., 1997), and by the limited half-life of the p21$^{Waf1/Cip1}$ mRNA and protein.

p21$^{Waf1/Cip1}$-dependent G_1 cell cycle arrest protects cells from apoptosis induced by DNA damage or an RNA polymerase II inhibitor

Several studies have demonstrated that G_1/S cycle blockers and inhibitors of CDKs prevent cell death (Gorospe *et al*., 1997; Park *et al*., 1997). Our results show that mimosine, a potent p21 inducer, can protect A431 cells from apoptosis induced by DNA damage. Protection from apoptosis is afforded by a 24 h incubation with mimosine prior to irradiation (Figures 8 and 9), but not by a 6 or 12 h incubation, as demonstrated by a proteolytic cleavage of PARP (Figure 9) or the formation of a nucleosomal ladder (Figure 8). There is an excellent correlation between the continued presence of the p21$^{Waf1/Cip1}$ protein following UV-irradiation and protection against apoptosis. For example, 6 and 12 h incubations with mimosine elevate p21$^{Waf1/Cip1}$ levels but the levels decline after UV-irradiation (Figure 9); however, a 24 h incubation with mimosine results in the maintenance of high levels of p21$^{Waf1/Cip1}$ following irradiation. These post-irradiation differences in p21$^{Waf1/Cip1}$ levels must reflect differences in the relative rates of translation and proteolysis since transcription is blocked at the dose of UVB used (2000 J/m^2) (Mayne and Lehmann, 1982; Donahue *et al*., 1994; Selby *et al*., 1997). Both p21$^{Waf1/Cip1}$ and p53 protein degradation proceed via ubiquitination (Maki *et al*., 1996; Maki and Howley, 1997) and p53 stabilization involves reduced ubiquitination (Maki and Howley, 1997). Thus, it is possible that reduced p21$^{Waf1/Cip1}$ ubiquitination may account for the stability of p21$^{Waf1/Cip1}$ following a 24 h pre-incubation with mimosine.

p21$^{Waf1/Cip1}$ acts upstream to caspase-3 to inhibit apoptosis

One of the hallmarks of apoptosis is the proteolytic cleavage of poly(ADP-ribose) polymerase (PARP) by caspase-3, a homologue of the interleukin 1-β-converting enzyme/*ced-3* family of proteases involved in the early phase of apoptosis (Lazebnik *et al*., 1994). Cleavage of PARP to a fragment of 85 kDa is an early

event, and can be observed 2 h after irradiation of A431 cells (Figure 3a). Interestingly, PARP cleavage was also seen following treatment with an inhibitor of RNA pol II, DRB, a competitive inhibitor for both ATP and GTP (Zandomeni, 1989) and an efficient inhibitor of CAK, which can associate with the transcription factor IIH (Yankulov *et al*., 1995), in the absence of any obvious DNA damage (Figure 3b). However, the $p21^{Waf1/Cip1}$ dependent cell cycle arrest blocked PARP cleavage and protected cells from apoptosis. Thus, the inhibition of apoptosis by $p21^{Waf1/Cip1}$ involves a step upstream to the activation of the caspase-3.

Serum depletion synchronizes cells in G_1 but does not protect against apoptosis

Although serum starvation synchronized A431 cells in G_1, there was no induction of $p21^{Waf1/Cip1}$ protein expression (data not shown) nor any protection from apoptosis (Figure 10b). The absence of $p21^{Waf1/Cip1}$ induction is not surprising because serum starvation does not induce $p21^{Waf1/Cip1}$ in p53 wt cells (Alpan and Pardee, 1996), as we observed in primary human fibroblasts and MCF-7 cells (data not shown). The CKI which is the more relevant candidate for the serum starvation G_0/G_1 cell cycle arrest is $p27^{Kip1}$. It accumulates in serum-starved and density-arrested cells and its overexpression causes cell cycle arrest in G_1 phase (Polyak *et al*., 1994; Coats *et al*., 1996). Interestingly, in contrast to the $p21^{Waf1/Cip1}$ dependent G_1 arrest, the population of cells synchronized in the G_0/G_1 phase of the cell cycle by serum starvation are not protected from apoptosis induced by DNA damage (Figure 10b) or by an inhibition of RNA pol II using DRB (data not shown).

The antiapoptotic action of p21$^{Waf1/Cip1}$

It is surprising that $p21^{Waf1/Cip1}$ but not $p27^{Kip1}$ inhibits apoptosis given the similarities in the modes of action of these two CKI's. Both CKI's inhibit the interaction of cyclin activating kinase (CAK) with cyclins (Aprelikova *et al*., 1995) and both result in a G_1 cell cycle arrest. It is clear from our results that G_1 arrest *per se* is not sufficient to afford protection from DNA damage-induced apoptosis. This suggests that some action of $p21^{Waf1/Cip1}$, in addition to blocking the activation of G_1 cyclins by CAK, inhibits apoptosis. This effect is clearly downstream from p53 activation since the high levels of $p21^{Waf1/Cip1}$ induced by mimosine have no effect on p53 levels (data not shown). $p21^{Waf1/Cip1}$ may participate directly in DNA repair (McDonald *et al*., 1996) and has been found to increase survival after UV-irradiation (Figure 11 and results not shown) (Sheikh *et al*., 1997). However, since p21 acts downstream from p53, it is unlikely that improved repair efficiency could block the induction of apoptosis by high levels of p53. Furthermore, the increase in survival conferred by p21 probably results from its inhibition of apoptosis rather than any effects on DNA repair.

The literature concerning the role of $p21^{Waf1/Cip1}$ in apoptosis is confusing, as described in the introduction. While some of the divergence in conclusions concerning $p21^{Waf1/Cip1}$ may result from the use of different cell systems, it is more likely a reflection of other variables, such as the apoptotic pathway involved, the method used to induce $p21^{Waf1/Cip1}$ as well as the levels of $p21^{Waf1/Cip1}$ prior to the stimulation of apoptosis. We have attempted to define the role of $p21^{Waf1/Cip1}$ by studying only the DNA damage-induced apoptotic pathway, by using cells which express a transcriptionally inactive but apoptotically comptetent mutant form of p53 and by inducing $p21^{Waf1/Cip1}$ expression by a p53-independent mechanism, using mimosine. It is clear from our results that $p21^{Waf1/Cip1}$ can block apoptosis induced by DNA damage. However, since the signal for the induction of apoptosis by DNA damage involves inhibition of transcription and since $p21^{Waf1/Cip1}$ accumulation proceeds by activation of transcription, the $p21^{Waf1/Cip1}$ must be present at high levels prior to the induction of DNA damage.

It has recently been shown that salicylate, an inhibitor of phosphorylation, can prevent apoptosis following UVC, even in the presence of Bax (Chernov *et al*., 1997). We hypothesize that the mechanism by which $p21^{Waf1/Cip1}$ confers apoptotic protection results from the inhibition of phosphorylation of key apoptotic proteins, such as p53. $p21^{Waf1/Cip1}$ is a universal cdk inhibitor (Xiong *et al*., 1993) but is also an inhibitor of casein kinase II, an ubiquitous Ser/Thr protein kinase which phosphorylates the C-terminal p53 domain (Meek *et al*., 1990). $p21^{Waf1/Cip1}$ interacts directly with protein kinase CKII and inhibits the regulatoryβ-subunit responsible for p53 phosphorylation (Götz *et al*., 1997). Although the phosphorylation state of the CKII site of p53 does not influence its wild-type transcriptional transactivation activity (Fiscella *et al*., 1994; Hall *et al*., 1996), it may affect its apoptotic activity. CKII functions are required for progression in the cell cycle since cells will not progress if they are microinjected with anti-CKII antibodies (Pepperkok *et al*., 1994) or if the expression of CKII is blocked with antisense oligonucleotides (Pepperkok *et al*., 1991). Another potential candidate for the anti-apoptotic effects of p21 is $p34^{cdc2}$ which is known to phosphorylate p53 at serine 315 (Addison *et al*., 1990; Bischoff *et al*., 1990; Sturzbecher *et al*., 1990; Price *et al*., 1995) and is inhibited by $p21^{Waf1/Cip1}$ (reviewed in Pines, 1995). Furthermore, the phosphorylation of Thr161/160 of $p34^{cdc2}$ by CAK is necessary for the activity of this key cell cycle regulatory kinase (see review in Morgan, 1997). $p34^{cdc2}$ kinase activity increases during apoptosis (Schröter *et al*., 1996) and this is consistent with the observations of Gorospe and Holbrook (1996) who observed a correlation between a reduction in endogenous $p21^{Waf1/Cip1}$, an enhanced $p34^{cdc2}$ activity and apoptosis. Thus, the apoptotic protection afforded by increased levels of $p21^{Waf1/Cip1}$ may result from the inhibition of $p34^{cdc2}$.

In conclusion, our results demonstrate that the p53-independent induction of $p21^{Waf1/Cip1}$ by mimosine protects cells from apoptosis induced by DNA damage and/or inhibition of RNA polymerase II. However, G_1 arrest, in the absence of $p21^{Waf1/Cip1}$ induction, is not sufficient to protect cells from apoptosis. Finally, the anti-apoptotic effect of $p21^{Waf1/Cip1}$ occurs downstream from p53 accumulation and upstream from caspase-3 activation.

Materials and methods

Cell culture

Primary mouse fibroblasts were established from the skin of p53 null BALBc mice (−/−) or wild-type BALBc mice (+/+) and kept in culture for four passages. The status of both p53 alleles was determined by PCR analysis of DNA extracted from mouse skin using three primers in a single reaction. The common primer was specific for exon 7 (GTACTTGTAGTGGATGGTG) while the second primer was either for exon 6 (CGTGGTGGTACCTTATGAG) or for the neo gene (CCTCGTGCTTTACGGTATC). Normal human primary fibroblast cells (Coriell Institute for Medical research: AG01519A) and epidermal carcinoma (A431) cells (American Type Culture Collection: CRL1555) were grown as monolayers in DMEM (Dulbecco's modified Eagle's medium; Life Technologies, Inc., Grand Island, NY) supplemented with 9% FCS (fetal bovine serum) at 37°C in a humidified atmosphere of 7% CO_2. MCF-7 cells (American Type Culture Collection; HTB-22) were grown under the same conditions except in a 5% humidified atmosphere.

Sequencing of exon 8 of the TP53 *gene*

Exon 8 of *TP53* was amplified by PCR using the two primers ACCTGATTTCCTTACTGCCTCTTG and TTGCTTACCTCGCTTAGTGCTCC and was sequenced by the University Core DNA sequencing services from the University of Calgary, Calgary, Alberta, T2N 4N1, Canada.

Irradiation and cellular lysate preparation

Cells were irradiated with a ^{60}Co source at 15 cGy/s (gamma radiation) or with UVB (302 nm) at a dose rate of 20 Watts/s. Cells were washed and scraped in buffered saline. Total cellular protein was extracted with lysis buffer containing 25 mM HEPES (pH 7.8), 2 mM EDTA, 1% NP40, 15% glycerol, 1 mg/ml phenylmethanesulfonyl fluoride, 10 g/ml of peptide inhibitor cocktail (leupeptin, chymostatin and pepstatin A) and 2 mM dithiothreitol.

Electrophoresis and immunoblotting

SDS–PAGE was performed in minigels using a 5% stacking gel and a 12% separating gel. Equal amounts of protein from different samples were placed in boiling water for 4 min in the presence of SDS gel sample buffer (125 mM Tris-HCl (pH 6.8), 4% SDS, 0.01% bromophenol blue, 10% 2-mercaptoethanol and 15% glycerol) and electrophoresed for 90 min at 160 Volts. After the transfer onto nitrocellulose, the membrane was first blocked with 8% powdered skimmed milk in TBS (10 mM Tris-HCl (pH 8.0) and 150 mM NaCl) for 1–2 h and incubated with the appropriate first antibody overnight. Visualization of the second antibody was performed with a chemiluminescence detection procedure (PIERCE) according to the manufacturer's protocol (Chromatographic Specialities Inc.).

Antibodies

The monoclonal antibody against p53 (DO-1), was provided by Dr Bohdan Wasylyk (IGBMC Illkirch, France); antibody against p21 (Ab-1) was purchased from Oncogene Science (Cambridge, MA) and anti-PARP antibody was kindly provided by Dr Guy Poirier (CHUL Research Centre, Québec, Canada).

Characterization of apoptosis

Cells (100 mm petri dish) were incubated for 20 min in 700 μl lysis buffer (10 mM EDTA and 0.6% SDS) and NaCl solution was added to give a final concentration of 1 M. After an overnight incubation at 4°C, the lysate was centrifuged in a microcentrifuge at 4°C for 30 min. The supernatant was incubated at 50°C for 2 h with 100 μg/ml proteinase K and brought to 1.3 M NaCl prior to isopropanol precipitation. Pellets were resuspended in TE (10 mM Tris (pH 7.5) and 2 mM EDTA) and incubated for 3 h at 37°C with 80 μg/ml of RNase A. The DNA samples were electrophoresed through a 1.2% agarose gel.

Cell cycle analysis

For cell cycle analysis, 2.5×10^5 cells were seeded per 90 mm plate and grown for 48 h prior to treatment. At the indicated times, cells were trypsinized and fixed with 1 ml of 70% ethanol for at least 12 h. The fixed cells were centrifuged and resuspended in 500 μl of PBS solution containing 50 μg/ml of propidium iodide (PI) (Sigma) and hydrated for at least 8 h. The stained cells were analysed in a fluorescence-activated cell analyser (FACSCaliber, Becton Dickinson). The percentage of cells in various cell cycle phases was determined by using the WinMDI program developed by Joe Trotter and the cell cycle analysis program developed by Terry Hoy (based on Watson *et al*., 1987).

Abbreviations

RNA pol II: RNA polymerase II; PARP, poly(ADP-ribose) polymerase; TFIIH: transcription factor IIH; CDK: cyclin dependent kinase; DRB: 5,6-chloro-1-β-D-ribofuranosylbenzimidazole.

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