Oncogene (1999) 18, 5403–5412

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p53-dependent apoptosis or growth arrest induced by different forms of radiation in U2OS cells: p21$^{WAF1/CIP1}$ repression in UV induced apoptosis

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Following exposure to DNA damaging agents the p53 tumour-suppressor gene induces either a growth arrest (primarily in G1) or apoptosis. The factors governing which response a cell undertakes, however, are unclear. We find that the osteosarcoma cell line, U2OS, (wild-type for p53) is capable of undergoing either p53 dependent apoptosis or cell cycle arrest in response to distinct forms of radiation. Following exposure to UVC, the majority of U2OS cells were apoptotic within 2 days and cells continued to cycle even as viability was being lost. In contrast, after X-ray treatment, U2OS cells exhibited a cell cycle arrest. Western analysis showed that p53 protein was stabilized to a greater extent by UVC than X-ray. Treatment with X-rays induced p21$^{WAF1/CIP1}$ whereas p21$^{WAF1/CIP1}$ expression was specifically repressed at the post-transcriptional level after exposure to UVC. Ectopic expression of high levels of p21$^{WAF1/CIP1}$, which arrested U2OS cells in G1 and G2, initially conferred considerable protection against UVC-induced apoptosis. Ultimately, however, cells underwent apoptosis indicating that a high level of p21$^{WAF1/CIP1}$ delays but does not block apoptosis. Taken together, these results show that cell cycle arrest and apoptosis can occur in the same cell type in response to different forms of radiation and that the repression of p21$^{WAF1/CIP1}$ after UVC may contribute to the efficient induction of apoptosis in response to this particular insult.

Keywords: p53; p21$^{WAF1/CIP1}$; UV induced apoptosis; X-ray induced growth arrest; U2OS cells; radiation

Introduction

The p53 gene is believed to exert its tumour suppressor function, at least in part, in response to DNA damage. Following exposure to DNA damaging agents such as UV light or γ-radiation, wild-type p53 protein is stabilized through post-transcriptional mechanisms (Maltzman and Czyzyk, 1984; Kastan *et al*., 1991). This induces normal cells to undergo either cell cycle arrest, predominantly in G1 (Kastan *et al*., 1991), or apoptosis (Clarke *et al*., 1993; Lowe *et al*., 1993). Thus, p53 activation may have two distinct consequences for a particular cell. Although several factors, including the amount of DNA damage incurred (Lane, 1992), the cellular environment (Gottlieb *et al*., 1994; Canman *et al*., 1995) and cell type (Lowe *et al*., 1993; El-Deiry *et al*., 1994; Attardi *et al*., 1996) have been proposed to influence cell fate after DNA damage, the processes governing the decision between growth arrest and apoptosis remain largely obscure.

A variety of DNA damaging agents has been shown to result in p53-dependent growth arrest or apoptosis in many diverse cell types. For example, γ-irradiation induces growth arrest in normal diploid fibroblasts, the myeloid leukaemia cell line, ML-1, and the colorectal carcinoma cell line, RKO (Kastan *et al*., 1992; Kuerbitz *et al*., 1992). Conversely, γ-irradiation is associated with apoptosis in thymocytes (Clarke *et al*., 1993; Lowe *et al*., 1993) and in the spleen and small intestine (Komarova *et al*., 1997; Merritt *et al*., 1997). These studies are consistent with the DNA damage response being determined by cell type and, indeed, growth arrest and apoptosis have been rarely reported in the same cell type. Furthermore, although the p53 response has been shown to be modulated by inactivation of genes associated with G1 arrest, for example p21$^{WAF1/CIP1}$ (Polyak *et al*., 1996) or pRb (Morgenbesser *et al*., 1994), or by over-expression of E2F-1 which promotes cell cycle progression (Qin *et al*., 1994; Wu and Levine, 1994), or ectopic expression of Bcl-2, a gene known to inhibit apoptosis (Guillouf *et al*., 1995), little is known about the consequences of p53 activation by different forms of radiation in the same cell type.

The p53 protein is a transcription factor which induces the expression of a number of target genes, including p21$^{WAF1/CIP1}$ (El-Deiry *et al*., 1993; Harper *et al*., 1993; Xiong *et al*., 1993), *MDM2* (Barak *et al*., 1993; Wu *et al*., 1993), *BAX* (Miyashita and Reed, 1995) and insulin-like growth factor binding protein 3 (*IGF-BP3*) (Buckbinder *et al*., 1995). It is well established that transactivation is required for the efficient execution of p53-mediated growth arrest (El-Deiry *et al*., 1994; Waldman *et al*., 1995), yet its role in apoptosis is equivocal. Indeed, apoptosis has been shown to occur in the presence of transcriptional and translational inhibitors (Caelles *et al*., 1994; Wagner *et al*., 1994). In addition, p53 mutants which lack sequence-specific DNA-binding activity or transactivation activity, were able to induce apoptosis in Hela cells, albeit with reduced efficiency (Haupt *et al*., 1995). However, these mutants failed to induce apoptosis in BRK (Sabbatini *et al*., 1995), HEP3B (Roemer and Mueller-Lantzsch, 1996) or H1299 cells (Haupt *et al*., 1996). Thus, p53 transactivation activity appears to be required for apoptosis in some cells while, in others, apoptosis may occur independently of transcription.

Specific differences in p53 transactivational activity have been observed between cells undergoing growth

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Received 11 January 1999; revised 22 April 1999; accepted 22 April 1999

arrest and apoptosis. The p53-mediated G1 arrest is believed to occur primarily through induction of p21$^{WAF1/CIP1}$, a general inhibitor of cyclin dependent kinases whose functions are required for cell cycle progression (El-Deiry *et al*., 1993; Harper *et al*., 1993; Xiong *et al*., 1993). In contrast, although increases in p21$^{WAF1/CIP1}$ have been reported to be associated with apoptosis in some cases (El-Deiry *et al*., 1994; Vater *et al*., 1996; Mukhopadhyay and Roth, 1997), p21$^{WAF1/CIP1}$ activity appears to be dispensable for apoptosis, since p21$^{WAF1/CIP1}$ null cells retain a normal apoptotic response following γ-irradiation (Brugarolas *et al*., 1995; Deng *et al*., 1995). Further complexity has been observed with several p53 mutants which exhibit selective loss of transactivation of the *BAX* or *IGF-BP3* promoters but which retain the ability to induce expression of p21$^{WAF1/CIP1}$. These mutants cause cells to growth arrest, whereas wild-type p53 induces apoptosis (Friedlander *et al*., 1996; Ludwig *et al*., 1996). Taken together, these results suggest that the decision between growth arrest and apoptosis following activation of p53 may be determined by the selective upregulation of a specific subset of p53-responsive genes.

Clearly, many factors may contribute to the decision between growth arrest and apoptosis in response to DNA damage. To understand further the processes and p53-dependent functions involved, we have studied the effect of different types of radiation (X-ray and UVC) on the growth and survival of the osteosarcoma cell line, U2OS. In addition, we have compared the expression profiles of p53 and several p53-responsive genes following exposure of U2OS cells to X-ray and UVC. Finally, we have assessed the effect of ectopic p21$^{WAF1/CIP1}$ expression on the ability of U2OS cells to undergo apoptosis. Our results show that U2OS cells are capable of differential responses to distinct forms of radiation, indicating that U2OS cell should be a useful resource for investigating the processes of cell cycle arrest and apoptosis in a single cell line. Furthermore, ectopic p21$^{WAF1/CIP1}$ expression delayed but did not abrogate apoptosis, suggesting that the apoptotic pathway can be dominant over the p21$^{WAF1/CIP1}$ -mediated growth arrest response. Finally, analysis of p21$^{WAF1/CIP1}$ levels after UVC irradiation suggests that specific repression of p21$^{WAF1/CIP1}$ protein may promote the efficient induction of apoptosis by UVC in U2OS cells.

Results

X-irradiation induces p53-dependent growth arrest in U2OS cells

U2OS is a human osteosarcoma cell line of epithelial morphology which is wild-type for both p53 and Rb (Diller *et al*., 1990; Isfort *et al*., 1995). Thus, it was of interest to determine the DNA-damage response of U2OS cells to different forms of radiation. Initially, the effect of X-irradiation on cell cycle distribution was assessed by flow cytometry. It was observed that 24 h after exposure to 12 Gy X-ray, the S phase fraction decreased to negligible levels and cells accumulated in G1 and G2 (Figure 1a). Cell cycle arrest after X-ray was transient since cells started to cycle again by 72 h post irradiation.

To compare the effect of radiation other than X-ray, U2OS cells were exposed to UVC (15 J/m^2). Surprisingly, UVC did not elicit either a G1 or G2 arrest (Figure 1a). A decrease in BrdU incorporation by the majority of cells with S phase DNA content was observed, probably reflecting a stalling of DNA replication at bulky cyclobutane pyrimidine dimers which is the major lesion generated by UVC radiation (Donahue *et al*., 1994). Furthermore, U2OS cells failed to arrest in either G1 or G2 after irradiation with a lower UVC dose (5 J/m^2) when BrdU incorporation was not inhibited significantly (Figure 1a). Thus, the growth arrest response induced in U2OS cells after X-irradiation is absent following exposure to UVC.

We next analysed the response to X-irradiation of U2.9927.5 cells which are a clone of U2OS cells expressing high levels of a dominant negative p53 (A175H) protein. Following X-irradiation, the G1 arrest observed in the parental U2OS cells was completely abolished, although the G2 arrest was retained (Figure 1b). Similar results were found for four other independently derived U2.9927 clones containing high levels of dominant negative p53. These results indicate that, although the G2 block may be independent of wild-type p53, the G1 arrest in U2OS cells constitutes a p53-dependent response.

UVC-irradiation induces p53-dependent apoptosis in U2OS cells

U2OS cells were assayed for the induction of apoptosis after treatment with X-rays or UVC using TUNEL analysis and flow cytometry. Subconfluent cells were exposed to 12 Gy X-ray or 15 J/m^2 UVC and analysed 48 h post irradiation. After X-irradiation (up to 20 Gy) less than 1.5% of the U2OS cells underwent apoptosis as measured by staining by TUNEL (Figure 2a and c), similar to levels observed in non-irradiated control cells (Figure 2a and c). Furthermore, cells exposed to X-ray were very flat and slightly enlarged with very few metaphases being evident until 3 days post irradiation, in agreement with our observation of X-rays inducing a growth arrest in U2OS cells.

In contrast, UVC exposure resulted in substantial death with an average of 78.5% of cells undergoing apoptosis at 48 h (Figure 2a and c). This cytotoxic effect was dose-dependent with increasing levels of apoptosis being observed with 5–30 J/m^2 of treatment. Furthermore, UVC-treated cells exhibited shrinkage and membrane blebbing, characteristic of cells undergoing apoptosis (Wyllie *et al*., 1980), and the majority of cells detached from the plate. Following UVC-irradiation only 6.5% (mean, $n=3$) of U2.9927.5 cells, which contain a dominant negative p53, were apoptotic (Figure 2b and c). Similar results were obtained with four other p53-dominant negative clones, indicating that UVC-induced apoptosis in U2OS cells is dependent on functional, wild-type p53.

p53 expression and transactivation activity in response to X-ray and UVC

Following DNA damage, levels of p53 protein increase mainly as a result of protein stabilization rather than an increase in transcription (Maltzman and Czyzyk, 1984; Kastan *et al*., 1992). However, differences in the

kinetics and the extent of the p53 response to different forms of DNA damage have been observed (Lu and Lane, 1993). Therefore, we assessed the effect of UVC (15 J/m^2) and X-ray (12 Gy) on p53 levels in U2OS cells to ascertain whether any differences may be associated with the alternate cellular responses

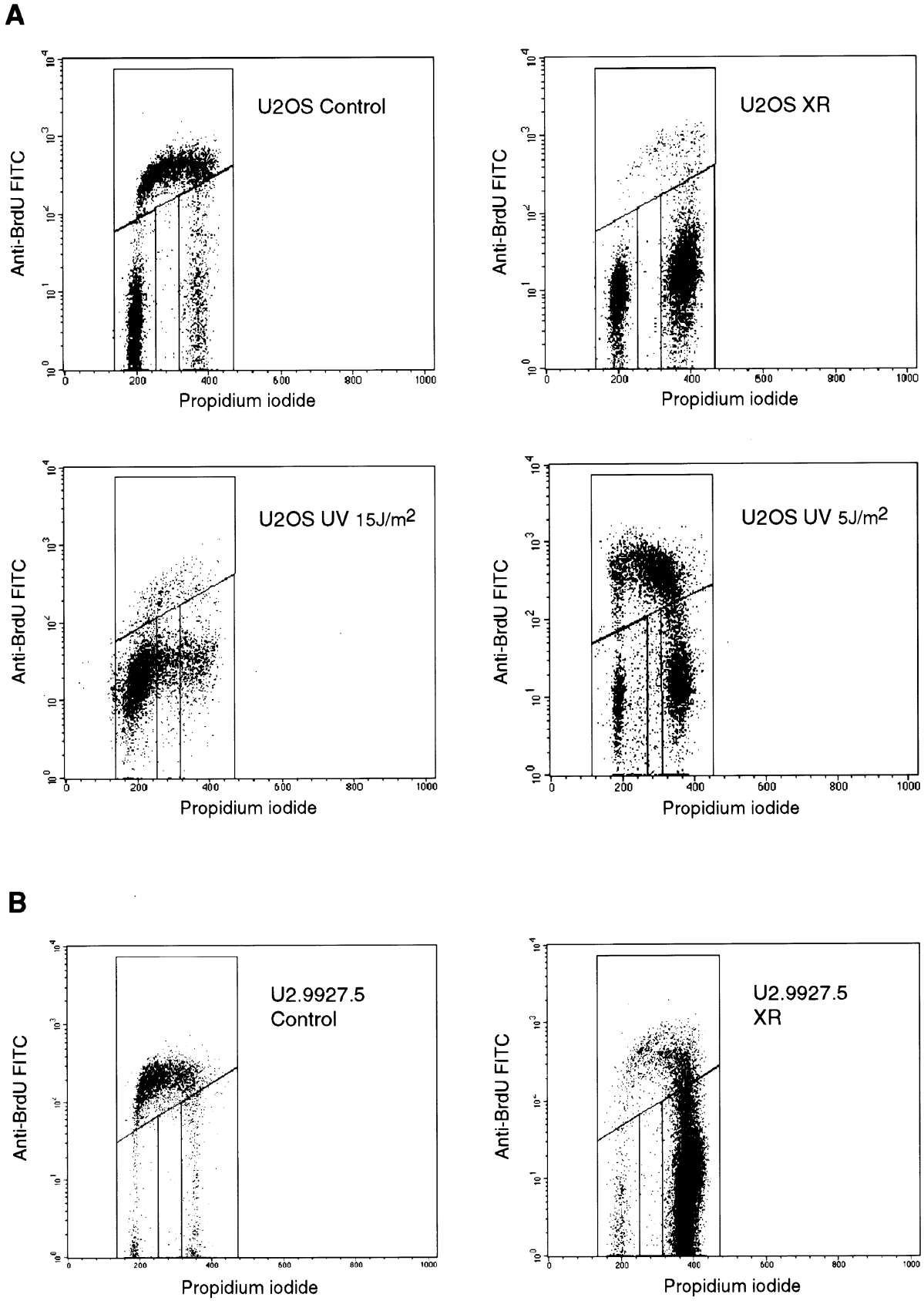
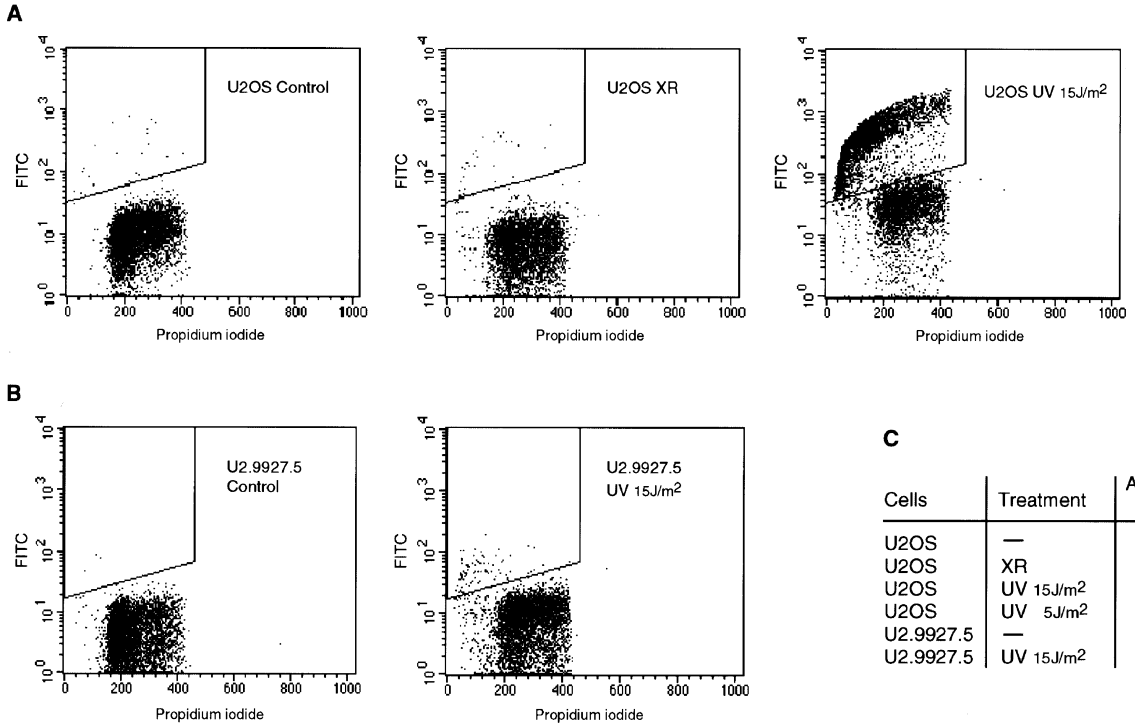


Figure 1 Effect of radiation on cell cycle progression in U2OS and U2.9927.5 (U2OS expressing dominant negative p53, A175H). (**a**) U2OS were exposed to 12 Gy X-ray (XR) or UVC (15 J/m^2 or 5 J/m^2) and analysed for incorporation of BrdU 24 h after irradiation. (**b**) Absence of G1 arrest in U2.9927.5 cells (dominant negative p53 clone) 24 h after 12 Gy X-irradiation (XR)

observed after the two forms of irradiation. Stabilization of p53 protein was observed 2 h after both treatments but continued to increase only after irradiation with UVC (Figure 3a). Similar results were obtained when cells were irradiated with only 5 J/m^2 UVC (not shown). Therefore, although the initial



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Cells	Treatment	Apoptosis (%)
U2OS	—	0.4
U2OS	XR	1.2
U2OS	UV 15J/m^2	78.5
U2OS	UV 5J/m^2	12.7
U2.9927.5	—	0.2
U2.9927.5	UV 15J/m^2	6.5

Figure 2 Analysis of apoptosis after irradiation. (**a**) U2OS were exposed to 12 Gy X-ray (XR) or 15 J/m^2 UVC and assayed for apoptosis 48 h later by TUNEL analysis and flow cytometry. (**b**) Absence of apoptosis in the p53 dominant negative clone, U2.9927.5, after UVC-irradiation revealed that this response is p53 dependent in U2OS. (**c**) Quantitiation of apoptosis 48 h after irradiation in U2OS and U2.9927.5 cells (represents the mean of three experiments)

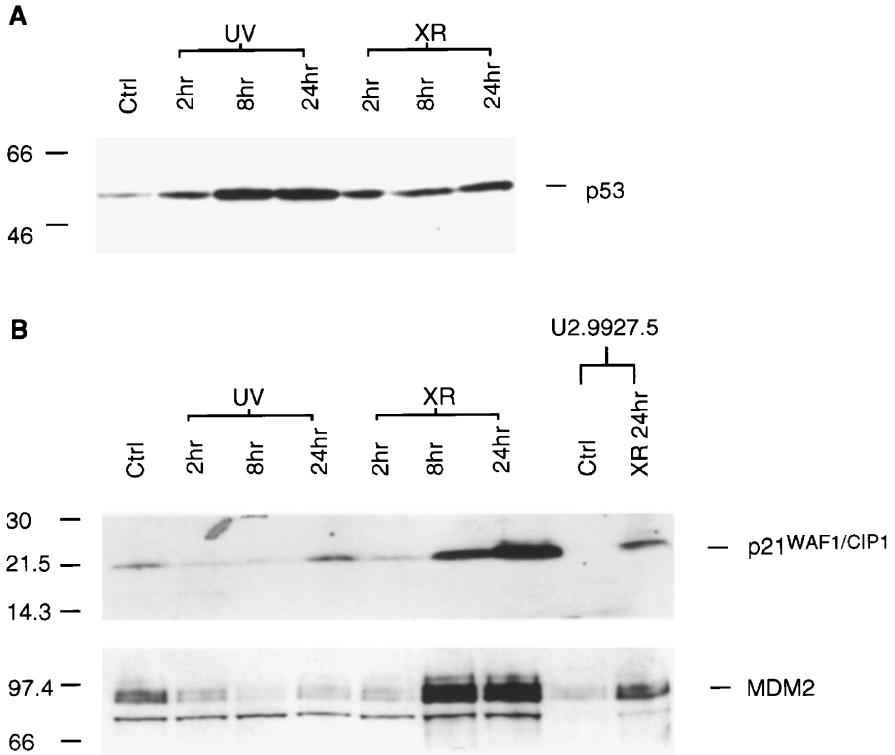

Figure 3 Western analysis of p53, p21$^{WAF1/CIP1}$ and MDM2 protein profiles following irradiation. U2OS cells were treated with either 15 J/m^2 UVC or 12 Gy X-ray and protein lysates prepared at times indicated. (**a**) p53 protein levels increased to a greater extent after UVC treatment than after X-ray (XR). (**b**) p21$^{WAF1/CIP1}$ and MDM2 proteins decrease initially after UVC but both are induced after X-ray (XR) treatment. Expression of dominant negative p53 mutant (U2.9927.5) represses the X-ray response

increase in p53 expression is similar following both treatments, ultimately, higher p53 levels are associated with UVC irradiation and apoptosis.

Stabilization of p53 protein is generally associated with an increase in its ability to transactivate a number of genes, but the situation may be more complex. Indeed, in some instances, an inverse correlation exists between levels of p53 protein stabilization and p53 transactivation activity (Weinberg *et al*., 1995; Lu *et al*., 1996) and it has been demonstrated that p53 protein may be expressed in both active and latent forms (Hupp and Lane, 1995). Therefore, we sought to determine if the differences in p53 protein levels observed after X-ray and UVC irradiation were reflected in the expression profiles of the p53-responsive genes, p21$^{WAF1/CIP1}$ and MDM2. Despite the induction of highly elevated levels of p53 protein after treatment with UVC the levels of p21$^{WAF1/CIP1}$ protein decreased to almost undetectable levels. By 24 h the p21$^{WAF1/CIP1}$ protein level had increased again to that of the control (Figure 3b). In contrast, the p21$^{WAF1/CIP1}$ protein was induced by X-rays in a p53 dependent manner since this response was considerably reduced in U2.9927.5 cells (Figure 3b). These results are consistent with the observation of a cell cycle block only after X-ray treatment (see above). We observed that the levels of MDM2 protein also decreased after UVC and remained below that of the control, even at 24 h (Figure 3b). After X-ray, however, MDM2 protein expression increased to a maximum at 8–24 h and required functional p53 as indicated by the attenuated induction of MDM2 in U2.9927.5 cells after X-irradiation (Figure 3b).

These results endorse previous findings of an inverse relationship between the degree of p53 protein stabilization and p53 transactivation activity. However, UVC-induced pyrimidine dimers are known to impede DNA and RNA polymerases (Perry *et al*., 1993; Donahue *et al*., 1994). Thus, we investigated the expression profiles of p21$^{WAF1/CIP1}$ and MDM2 after only 5 J/m^2 UVC-irradiation. As described previously, cell cycle analysis confirmed that this dose does not cause significant inhibition of replicative DNA synthesis since S phase cells continued to incorporate BrdU (Figure 1a). However, 5 J/m^2 UVC-irradiation is sufficient to induce apoptosis in U2OS, albeit with slower kinetics, and stabilizes p53 protein to the same extent as 15 J/m^2. After 5 J/m^2 UVC, p21$^{WAF1/CIP1}$ expression was still considerably reduced until 24 h when there was a slight induction (Figure 4a). However, MDM2 levels decreased only slightly before exhibiting an induction at 24 h (Figure 4a). Clearly, expression of p21$^{WAF1/CIP1}$ is more sensitive to the effects of UVC radiation. It is unlikely that this is the result of transcriptional inhibition by radiation-induced pyrimidine dimers since the p21$^{WAF1/CIP1}$ gene is predicted to be approximately three times smaller than the MDM2 gene (McKay *et al*., 1998), yielding a much smaller RNA (2.2 kb) and protein product (21 kD) than its MDM2 counterpart (5.5 kb and 90 kD, respectively) suggesting that it should contain fewer lesions and be repaired more quickly than the larger MDM2 gene. Furthermore, no differences were detected in the expression of either Bax or Bcl-2 proteins after either X-ray or UVC treatment (data not shown).

To investigate this further, the effect of UVC irradiation on p21$^{WAF1/CIP1}$ and MDM2 RNA levels was assessed. As shown in Figure 4b, MDM2 RNA levels reflect the protein expression pattern after different doses of UVC (compare with Figure 4a). Notably, p21$^{WAF1/CIP1}$ RNA levels exhibited considerable induction by 8 h after 5 J/m^2 UVC (Figure 4b), earlier than observed for MDM2 and in marked contrast to p21$^{WAF1/CIP1}$ protein levels (Figure 4a). Thus, for apoptosis to occur in U2OS after UVC, there may be a requirement for the specific down-regulation of p21$^{WAF1/CIP1}$ protein.

Ectopic expression of p21$^{WAF1/CIP1}$ delays apoptosis

The apparent specific suppression of p21$^{WAF1/CIP1}$ in U2OS cells undergoing apoptosis led us to question whether p21$^{WAF1/CIP1}$ might be influencing the decision between the cell cycle block or apoptosis responses in U2OS cells. To determine whether p21$^{WAF1/CIP1}$ expression could protect against p53-induced apoptosis, stable U2OS cell clones (U2.Hp21) were generated in which ectopic expression of p21$^{WAF1/CIP1}$ could be regulated by Ponasterone A (PonA) (Figure 5a). The effect of ectopic p21$^{WAF1/CIP1}$ expression on the cell cycle was analysed by flow cytometry, 30 h after the addition of the inducer. Prior to induction, U2.Hp21.A2 cells exhibited a cell cycle distribution comparable to parental U2OS cells (compare Figures 5b and 1a). Addition of Pon A resulted in a cell cycle block in both

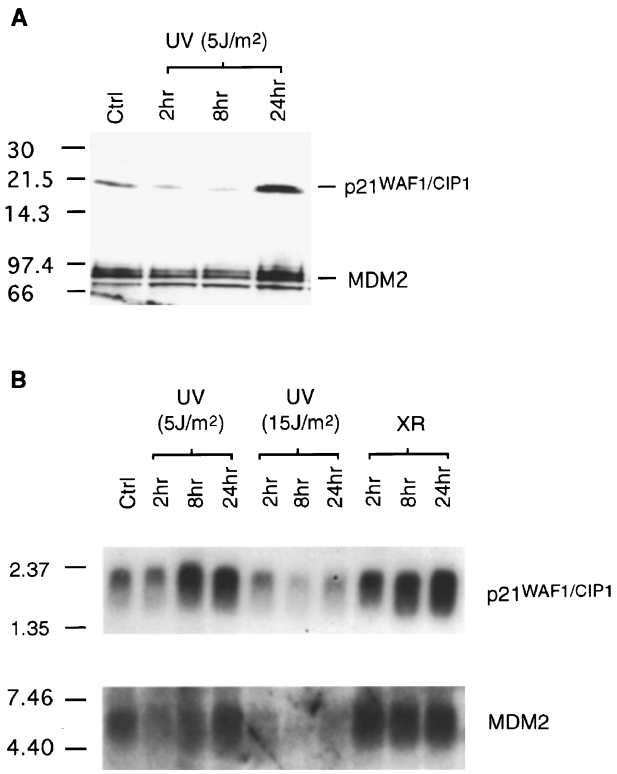

Figure 4 Effect of low UVC dose on p21$^{WAF1/CIP1}$ and MDM2 expression. (**a**) Western analysis of p21$^{WAF1/CIP1}$ and MDM2 proteins in U2OS at 2, 8 and 24 h after treatment with 5 J/m^2 UVC. (**b**) Comparison of p21$^{WAF1/CIP1}$ and MDM2 RNA levels by Northern analysis after treatment with 5 or 15 J/m^2 UVC, or 12 Gy X-ray (XR). Total RNA was extracted at times indicated. The blot was probed for p21$^{WAF1/CIP1}$, stripped and re-probed for MDM2

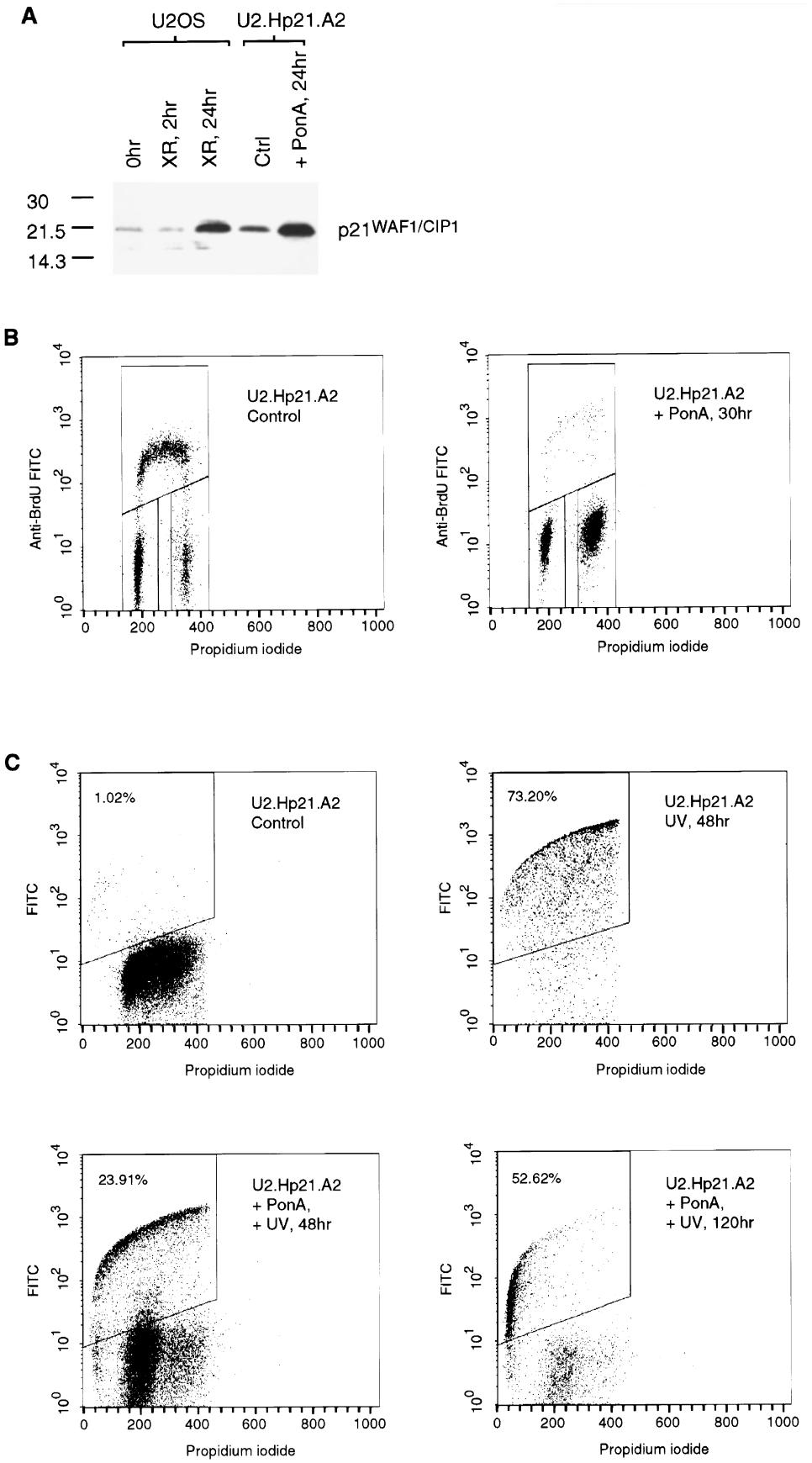

Figure 5 Ectopic p21$^{WAF1/CIP1}$ expression and its effect on the cell cycle and UVC-induced apoptosis in U2OS. (**a**) Western analysis of induction of exogenous p21$^{WAF1/CIP1}$ protein in U2.Hp21.A2 by Ponasterone A (PonA) at 24 h. Protein samples from parental U2OS at 0, 2 and 24 h after exposure to 12 Gy X-ray are included for comparison (first three lanes). (**b**) Flow cytometric dot plot of BrdU incorporation showing U2.Hp21.A2 growth arrested in G1 and G2 at 30 h after induction of p21$^{WAF1/CIP1}$ expression by PonA. (**c**) TUNEL analysis of apoptosis in U2.Hp21.A2 48 h after UVC irradiation and in U2.Hp21.A2 cells growth arrested by PonA at 48 and 120 h after UVC treatment. The percentage of apoptosis given represents the mean of two experiments

G1 and G2 with a concomitant reduction of the S phase fraction to negligible levels (Figure 5b). Similar results were obtained with another independently isolated U2.Hp21 clone. Growth arrest was dependent upon continued p21$^{WAF1/CIP1}$ expression since cells recommenced cycling after culture in Pon A-free medium.

Having established that induction of high levels of p21$^{WAF1/CIP1}$ results in substantial growth arrest, we assessed the effect of ectopic p21$^{WAF1/CIP1}$ expression on the ability of UVC to induce apoptosis in U2.Hp21.A2 cells growth arrested by the addition of Pon A 30 h prior to treatment with 15 J/m^2 UVC. TUNEL analysis was carried out 48 h after irradiation. As shown in Figure 5c, over-expression of p21 resulted in considerable protection against apoptosis after UVC irradiation with an average of only 23.9% apoptosis compared to 73.2% in cells without p21 induction. However, by extending the analysis it was found that levels of apoptosis increased to 52.6% by 5 days post-irradiation (Figure 5c), despite continued expression of high levels of exogenous p21$^{WAF1/CIP1}$. These results show that while elevated levels of p21$^{WAF1/CIP1}$ substantially delay apoptosis, ultimately the apoptotic response to UVC irradiation is dominant over the growth arrest induced by p21$^{WAF1/CIP1}$ in U2OS cells.

Discussion

We are interested in elucidating the factors that regulate the decision between p53-induced growth arrest and apoptosis following DNA damage. We find that U2OS cells undergo either growth arrest or apoptosis in response to two distinct forms of radiation. Exposure to X-rays causes an arrest in the G1 and G2 phases of the cell cycle (only the G1 block is p53-dependent). This growth arrest was temporary and cells re-entered the cell cycle 48 – 72 h after treatment. U2OS cells survived even high amounts of X-rays (20 Gy) with no cytotoxicity. Conversely, although replicative DNA synthesis was inhibited by $\geqslant$15 J/m^2, UVC failed to elicit a G1 and G2 growth arrest and the proportion of S phase cells remained largely unchanged when compared to unirradiated control cells. Increasing exposure to UVC resulted in increasing amounts of apoptosis. Thus, the response elicited by U2OS cells is dictated by the type of radiation exposure.

Since both growth arrest and apoptotic responses in U2OS cells were shown to be dependent on p53, we assessed the effect of the two forms of radiation on p53 levels. UVC was found to increase p53 levels to a far greater extent than X-rays. This is in agreement with previous findings (Lu and Lane, 1993) and may reflect differences in the nature of DNA lesion caused by the two different forms of radiation and/or the processes involved in their repair. Whereas the double-stranded DNA breaks caused by X-irradiation are repaired rapidly, UVC-induced cylcobutane pyrimidine dimers are repaired primarily by the lengthy process of nucleotide excision repair. Although we observed no initial differences in the kinetics of p53 stabilization between the two treatments, p53 levels continued to increase only in UVC treated cells, indicating that the augmented p53 response after UVC may result from the extended length of time taken for repair.

UVC, but not γ-radiation, may be associated with an inhibition of p53 degradation by the ubiquitin pathway indicating that γ-radiation may utilize a different pathway to stabilize p53 (Maki and Howley, 1997). Interestingly, our results reveal a correlation between the degree of p53 stabilization and cellular response such that high p53 levels result in apoptosis while a moderate increase is associated with growth arrest. Similar results have been reported previously in cells expressing different levels of exogenous p53, although the apoptotic response in high expressers was lost during extended time in culture (Chen *et al*., 1996). However, in U2OS cells, ectopic p53 expression at levels similar to, or greater than, those induced by UVC did not result in apoptosis, but induced a partial growth arrest accompanied by induction of p21$^{WAF1/CIP1}$ and MDM2 proteins (unpublished observations). Thus, differences in p53 levels *per se* do not appear to account for the alternate responses in U2OS cells.

These results raise the question of whether X-ray and UVC modulate p53 activity in discrete ways. One possibility is that UVC and X-radiation may have distinct effects on p53 transactivation activity. It is interesting that a correlation has been observed between the potential of different p53 mutants to upregulate specific target genes and their ability to induce growth arrest or apoptosis (Friedlander *et al*., 1996; Ludwig *et al*., 1996). To address this possibility, we analysed the expression of two p53-responsive genes, p21$^{WAF1/CIP1}$ and MDM2, after radiation. As expected, X-ray induced both p21$^{WAF1/CIP1}$ and MDM2 while UVC caused inhibition of MDM2 protein at a moderate dose and delayed induction at the lower dose (consistent with observed MDM2 RNA levels). In contrast, even a low amount of UVC (5 J/m^2) caused a significant decrease in p21$^{WAF1/CIP1}$ protein, despite a high induction at the RNA level. Given that UVC causes apoptosis in U2OS cells, these results suggest that the specific repression of p21$^{WAF1/CIP1}$ expression may be required for apoptosis to proceed efficiently. Importantly, the expression of both BAX, a gene reported to be induced in some instances during p53-dependent apoptosis (Selvakumaran *et al*., 1994; Zhan *et al*., 1994), and Bcl-2 which can inhibit apoptosis (Selvakumaran *et al*., 1994) and whose transcription may be repressed by p53 (Miyashita *et al*., 1994), remained constant after both X-ray and UVC treatment. These findings support the specificity of decreased p21$^{WAF1/CIP1}$ expression during UVC-induced apoptosis in U2OS cells. Post-transcriptional mechanisms which could account for this decrease in p21$^{WAF1/CIP1}$ protein, include translational repression and the specific destruction of the p21$^{WAF1/CIP1}$ protein product (Levkau *et al*., 1998).

Our results suggest that suppression of p21$^{WAF1/CIP1}$ expression may direct cells towards apoptosis following DNA damage. Therefore, induction of p21$^{WAF1/CIP1}$ may inhibit apoptosis, and, although increased p21$^{WAF1/CIP1}$ expression correlates with apoptosis in some instances (El-Deiry *et al*., 1994; Vater *et al*., 1996; Mukhopadhyay and Roth, 1997), most recent evidence points towards a protective effect (Poluha *et al*., 1996; Polyak *et al*., 1996; Gorospe *et al*., 1997; Bissonnette and Hunting, 1998; Canman and Kastan, 1998). To investigate this further, we assessed the effect of ectopic p21$^{WAF1/CIP1}$ expression on the ability of UVC to induce apoptosis in U2OS cells. As predicted, high

levels of p21WAF1/CIP1, which growth arrested the cells in both G1 and G2, afforded significant protection against apoptosis. However, by extending our analysis, we found that most cells had undergone apoptosis by 5 days after UVC treatment, despite continued induction of p21WAF1/CIP1 expression. This suggests that, although ectopic expression was able to delay cell death, the apoptotic signals generated by UVC-irradiation ultimately dominate the growth arrest response induced by p21WAF1/CIP1. The apoptosis pathway was also observed to be dominant over the growth arrest pathway in a different system (Polyak *et al*., 1996). In this work inactivation of the p21WAF1/CIP1 gene converted the growth arrest response to an apoptotic response implying a protective effect of p21WAF1/CIP1 against apoptosis. However, fusion of two cell lines showed that apoptotic cell-death signals can overcome the p21WAF1/CIP1-mediated growth arrest response.

Thus, suppression of p21WAF1/CIP1 may be required for U2OS cells to initiate an efficient apoptotic response to UVC radiation. Precisely what functions of p21WAF1/CIP1 would need to be inhibited for apoptosis to proceed remains unclear. It is well established that p21WAF1/CIP1 binds to and inhibits the activity of most cyclin-cdk complexes, thereby preventing phosphorylation of downstream substrates such as Rb (Harper *et al*., 1993; Xiong *et al*., 1993). However, p21WAF1/CIP1 also inhibits the ability of casein kinase II to phosphorylate p53 at serine 392 (Guerra *et al*., 1997). Phosphorylation of this site after UV treatment is associated with increased DNA binding by p53 (Kapoor and Lozano, 1998; Lu *et al*., 1998). Such inhibition of phosphorylation by p21WAF1/CIP1 has been proposed as a mechanism by which it can protect against apoptosis (Bissonnette and Hunting, 1998).

Our results show that even in the presence of consistently high levels of p21WAF1/CIP1, apoptosis is merely delayed. This implies the existence of a threshold of p21WAF1/CIP1 inhibition which apoptotic signals must overcome for cell death to occur. It is tempting to speculate that repression of p21WAF1/CIP1 after UVC may result in the upregulation of apoptotic-specific genes by p53 and/or the interaction of p53 with other proteins to form effectors of the apoptotic response. Clearly, further studies will be required to understand how different forms of radiation specifically activate p53 and how this influences the decision between growth arrest and apoptosis. Our results suggest that modulation of p21WAF1/CIP1 expression contributes to this decision. In addition, since U2OS cells harbour endogenous wild-type p53, the consequences of exposure to DNA damaging agents likely represent legitimate, physiological responses. Thus U2OS should be a useful tool in the attempt to understand the processes which regulate the decision between growth arrest and apoptosis.

Materials and methods

Cell culture, plasmids and transfection

U2OS, a human osteosarcoma cell line (also known as HTB96), was obtained from the American Type Culture Collection. For all experiments, cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% foetal calf serum (DMEM; 10%FCS), 100 μg/ml penicillin and 100 μg/ml streptomycin. U2.9927 clones were established by transfection with a p53 dominant negative mutant construct, pCMV-9927, which contained an Arg→His mutation at codon 175 (gift from C Midgley), and selection in G418 (800 μg/ml). To obtain stable transfectants with regulated, exogenous p21WAF1/CIP1 expression, the Ecdysone-Inducible Expression Kit (Invitrogen) was used: pVG/RXR was co-transfected with pIND-Hp21, containing p21WAF1/CIP1 cDNA subcloned from DM81 (gift from D Mann), and single clones (U2.Hp21) were selected in G418 (800 μg/ml) and Zeocin (500 μg/ml). p21WAF1/CIP1 expression was induced by addition of Ponasterone A (PonA, 5 μM) (Invitrogen) and maintained by supplementing with fresh PonA every 24 h. Cell cycle analysis or exposure to UVC for analysis of apoptosis was carried out 30 h after addition of PonA. All transfections were carried out in 5 cm dishes using 2 μg CsCl-purified plasmid DNA and 15 μl Superfect in DMEM; 10% FCS according to the manufacturer's instructions (Qiagen). Expression of transfected genes was confirmed by Western analysis.

Radiation treatment

Cells grown to approximately 70% confluence were exposed to either X-ray (12 Gy, Pantax HS320) or UVC (15 J/m^2) radiation. For UVC treatment, medium was removed and replaced with PBS after washing twice with PBS. After irradiation, cells were cultured in DMEM; 10% FCS for 24 h prior to cell cycle analysis, for 48 h prior to analysis of apoptosis, or for indicated times for protein analysis.

Cell cycle analysis

Cell cycle distribution was analysed by flow cytometry. Twenty-four hours after irradiation, or 30 h after induction of p21WAF1/CIP1 expression by the addition of PonA, cells were incubated for 30 min with Bromodeoxyuridine (BrdU, 10 μM), harvested, washed twice with PBS and fixed in 70% ethanol. Subsequently, cells were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-BrdU antibody and with propidium iodide (PI). DNA synthesis (FITC) and DNA content (PI) were analysed using a fluorescent activated cell analyser (FACSCalibur, Becton-Dickinson).

Analysis of apoptosis

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labelling (TUNEL) was used to assay for apoptosis. TUNEL preferentially labels DNA double-strand breaks which occur during the nuclear fragmentation which is characteristic of apoptosis (Gavrieli *et al*., 1992). Cells were harvested, washed twice with PBS and fixed in 1% paraformaldehyde 48 h after irradiation, unless stated otherwise. TUNEL analysis was carried out using the APO-BRDU kit according to the manufacturer's protocol (Pharmingen). Cells were counterstained with PI and analysed by flow cytometry (FACSCalibur, Becton-Dickinson).

Western analysis of protein expression

Cells were lysed directly in protein loading buffer (50 mM Tris (pH 6.8), 2% SDS, 10% glycerol). Protein concentration was measured using the DC Protein Assay (Biorad). 2-mercaptoethanol (10%) was added to 50 μg protein samples prior to loading on 8% or 15% SDS-polyacrylamide gels. After electrophoresis, proteins were transferred to ECL nitrocellulose paper (Amersham) by semi-dry transfer, and blocked in 5% milk powder in PBS at 4°C overnight or at room temperature for 1 h. Blots were probed for p53 (NCL-p53-CM1, 1 : 2500, Novocastra), p21WAF1/CIP1 (DCS-60.2, 0.5 μg/ml) and Bcl-2 (100/D5, 0.5 μg/ml) (both NeoMar-

kers), MDM2 (2A10, 1 : 50, tissue culture supernatant, gift from A Levine) and Bax (N-20, 0.1 μg/ml) (Santa Cruz). Antibody incubations were carried out in 5% milk powder at room temperature for 1–2 h followed by 1×15 min and 2×5 min washes with PBS; Tween (0.2%). Blots were then incubated at room temperature for 1 h with the appropriate anti-rabbit or anti-mouse horse radish peroxidase-conjugated antibody (Amersham) and washed as before. Detection was performed using ECL according to the manufacturer's instructions (Amersham).

Northern analysis of RNA

Total RNA was prepared using 1 ml Trizol reagent/2×10^6 cells according to the manufacturer's instructions (Gibco–BRL). RNA samples (25 μg) were electrophoresed on a 1% formaldehyde agarose gel and transferred onto Hybond N^+ nitrocellulose membrane (Amersham) with $20 \times$ SSC overnight. RNA was fixed to the membrane by baking at 80°C for 2 h. The random priming method (Feinberg and Vogelstein, 1984) was used to label 25 ng probe DNA. The membrane was prehybridized at 65°C for 1 h using RapidHyb buffer (Amersham) prior to addition of probe directly to the prehybridization solution. Hybridization was carried out at 65°C for 2 h prior to washing once in $2 \times$ SSC; 0.1% SDS at room temperature for 20 min followed by two washes in 0.2 SSC; 0.1% SDS, at 65°C for 20 min. Autoradiography was carried out at −70°C for 6–96 h.

Acknowledgements
The authors would like to thank Carol Midgley and David Mann for the pCMV9927 and DM81 plasmids respectively and Arnold Levine for the MDM2 antibody. We would also like to thank Gordon Peters and Hartmut Land for their help in the preparation of this manuscript and Derek Davies and Aaron Rae for the FACS analysis.

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