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p53 controls both the G_2/M and the G_1 cell cycle checkpoints and mediates reversible growth arrest in human fibroblasts

(p21/WAF1/Li-Fraumeni cells/tetracycline/mimosine/cyclin–cyclin-dependent kinase)

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ABSTRACT **Increased expression of wild-type p53 in response to DNA damage arrests cells late in the G_1 stage of the cell cycle by stimulating the synthesis of inhibitors of cyclin-dependent kinases, such as p21/WAF1. To study the effects of p53 without the complication of DNA damage, we used tetracycline to regulate its expression in MDAH041 human fibroblasts that lack endogenous p53. When p53 is expressed at a level comparable to that induced by DNA damage in other cells, most MDAH041 cells arrested in G_1, but a significant fraction also arrested in G_2/M. Cells released from a mimosine block early in S phase stopped predominantly in G_2/M in the presence of p53, confirming that p53 can mediate arrest at this stage, as well as in G_1. In these cells, there was appreciable induction of p21/WAF1. MDAH041 cells arrested by tetracycline-regulated p53 for as long as 20 days resumed growth when the p53 level was lowered, in striking contrast to the irreversible arrest mediated by DNA damage. Therefore, irreversible arrest must involve processes other than or in addition to the interaction of p53-induced p21/WAF1 with G_1 and G_2 cyclin-dependent kinases.**

The tumor suppressor gene p53 is important in the etiology of cancer and is mutated, deleted, or rearranged in more than half of all human tumors (1, 2). p53 mediates either apoptosis or cell cycle arrest in response to DNA damage, thus acting as a molecular "guardian of the genome" (3). Kastan *et al.* (4) reported that human ML-1 leukemia cells exposed to ionizing radiation are transiently arrested in G_1, supporting the idea that p53-dependent G_1 arrest following DNA damage allows cells time to repair the damage. In contrast, the more recent experiments of Di Leonardo *et al.* (5) with primary human fibroblasts have shown that, although radiation treatment does induce G_1 arrest, the arrest is irreversible, leading the authors to suggest that it is more important to stop cells containing damaged DNA from proliferating than to facilitate repair of their damaged DNA in advance of S phase. The basis for the different responses of these two different types of cells is not clear but may be related to the observation that lymphocytes usually undergo apoptosis in response to DNA damage, whereas normal fibroblasts do not show this response (6).

The ability of p53 to arrest the cell cycle in rodent cells has been studied with a temperature-sensitive p53 protein (7). When rat embryo fibroblast (REF) cells harboring this protein were grown at a permissive temperature, the protein acted like wild-type p53, and the cells did not enter S phase. Similarly, utilizing dexamethasone-regulated p53, Lin *et al.* (8) showed that human glioblastoma cells arrested mainly in late G_1. p53 is a transcriptional activator of several genes, through which it can regulate events such as transformation and DNA synthesis (9). An important target of p53 is p21/WAF1 (10, 11), an inhibitor of most of the cyclin-dependent kinases (12) which help to regulate the cell cycle (13). Since p21/WAF1 inhibits both the cyclin-dependent G_1 kinases and the G_2/M-specific cdc2 kinase, p53 may be capable of controlling both the G_1 and the G_2/M checkpoints. To investigate this possibility and to help clarify the role of p53 in transient or permanent cell cycle arrest, we have established a system for regulated expression of p53 without the need for DNA damage or a temperature shift, which may lead to unwanted complications. For this purpose, we used the tetracycline-regulated transactivator and operator plasmids developed by Gossen and Bujard (14). Regulated expression of wild-type p53 in p53-null human fibroblasts causes growth arrest in both G_1 and G_2/M. The arrest is associated with high levels of p21/WAF1 and is reversible.

MATERIALS AND METHODS

The MDAH041 postcrisis cell line was derived from the fibroblasts of a patient with Li-Fraumeni syndrome (15). There is a frameshift mutation of one p53 allele at codon 184 and the normal p53 allele has been lost (16). The cells, kindly provided by M. Tainsky (17), were grown in an atmosphere of 10% CO_2/90% air in Dulbecco's modified Eagle's medium, supplemented with 10% (vol/vol) fetal bovine serum. When employed, tetracycline (final concentration 1 μg/ml) was added directly to the medium. Plasmids pUHD 10-3, with the tetracycline operator; pUHD 151-1, with the tetracycline activator; and pUHC13-3, with a luciferase reporter gene, were kind gifts from H. Bujard (14). Plasmid pHSG-p53wt, containing wild-type human p53 cDNA, was the kind gift of Peter Chumakov (Engelhardt Institute, Moscow). For cell cycle experiments, trypsinized cells were stained with propidium iodide by using the Cycletest kit (Becton Dickinson) and analyzed for DNA content by using the FACScan (Becton Dickinson). Cell cycle distribution was determined using CELL FIT Software (HP340 Series 9000 Workstation). Dead cells were gated out by using pulse processing. In some experiments, the cells were treated with 200 μM mimosine (Sigma) for 48 h to synchronize them in early S phase (18). For Western blot analyses, total cellular protein was isolated by lysing the cells in 20 mM Tris·HCl, pH 7.5/2% (wt/vol) SDS/2 mM benzamidine/0.2 mM phenylmethanesulfonyl fluoride. Protein concentrations were determined by the Bradford method. Total proteins (25 μg) were separated by SDS/10% PAGE and electroblotted to polyvinylidene difluoride membranes (Stratagene) (19). After transfer was completed, the gels were stained with Coomassie blue to verify equal sample loading. Membranes were probed with antibodies DO-1 and C19 (Santa Cruz Biotech) directed against p53 and p21/WAF1, respectively. The bound antibodies were detected by enhanced chemiluminescence (Amersham). For analysis of proliferation, cells were grown on coverslips in the presence of tetracycline. After withdrawing tetracycline, the cells were incubated in 10

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Abbreviations: PALA, *N*-(phosphonacetyl)-L-aspartate; REF, rat embryo fibroblasts.

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μM BrdUrd for 2 h and fixed in methanol/acetic acid/water (90:5:5, vol/vol) for 30 min. The incorporation of BrdUrd into DNA was determined by immunostaining by using antibodies directed against BrdUrd (Amersham).

RESULTS

Regulated Expression of p53. To achieve tetracycline-regulated expression, human wild-type p53 cDNA was inserted into pUHD 10-3 (14), together with a neomycin-resistance gene, to yield pTO.p53.neo (Fig. 1). A sequence providing hygromycin resistance was inserted into pUHD 151-1 (14), which carries the tetracycline-responsive transactivator, to yield pTA.hygro (Fig. 1).

In the p53-null fibroblast cell line MDAH041, no p53 protein could be detected by Western blot analysis using the DO-1 antibody, which recognizes both wild-type and mutant proteins (data not shown). However, MDAH041 cells behave very much like normal cells when functional p53 is restored under control of the normal p53 promoter, suggesting that most or all downstream functions are intact. For example, these cells do not yield resistant colonies upon selection with *N*-(phosphonacetyl)-L-aspartate (PALA), in contrast with parental MDAH041 cells, which do yield such colonies readily (M.L.A., A.A., and G.R.S., unpublished data). This phenotype depends on a normal p53 pathway (16, 20).

MDAH041 cells were transfected with pTA.hygro, which contains the tetracycline-regulated transactivator (Fig. 1). Several hygromycin-resistant, stable clones were analyzed by transfecting them transiently with pTO.luc, which contains a luciferase reporter gene driven by the cis-acting element recognized by the tetracycline-regulated transactivator. The clone chosen for further use showed a 13-fold increase in luciferase activity after withdrawal of tetracycline. After transfection with pTO.p53.neo, several derivative clones resistant to both neomycin and hygromycin were isolated and cultured in the presence of tetracycline. A representative clone, TR9-7, which arrested after withdrawal of tetracycline for 3–4 days, was used for all subsequent experiments. When TR9-7 cells are grown in the presence of 1 μg of tetracycline per ml, p53 protein is expressed at a very low level, which is insufficient to induce p21/WAF1 or cell cycle arrest (Fig. 2*A*). Decreasing the concentration of tetracycline leads to an incremental increase in both p53 protein and function, as determined by induction of p21/WAF1 (Fig. 2*A*). Increased expression of p53 can be detected 2 h after removing tetracycline, and its expression continues to increase with time, reaching a peak at

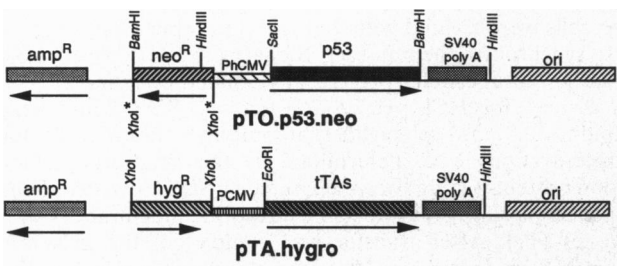

FIG. 1. A tetracycline-regulated system for expression of wild-type p53. The plasmids pTO.p53.neo and pTA.hygro are based on constructs described previously (14). pTO.p53.neo contains the wild-type p53 coding sequence under control of a derivative of the human cytomegalovirus promoter without the enhancer region (PhCMV). This minimal promoter is fused to a heptad of tetracycline operators at position −53. pTA.hygro contains the tetracycline-regulated transactivator (tTAs), driven by the entire cytomegalovirus promoter, including the enhancer region (PCMV). amp^R, ampicillin resistance gene; neo^R, neomycin resistance gene; hyg^R, hygromycin resistance gene; ori, origin of replication. *, Inactivated restriction site.

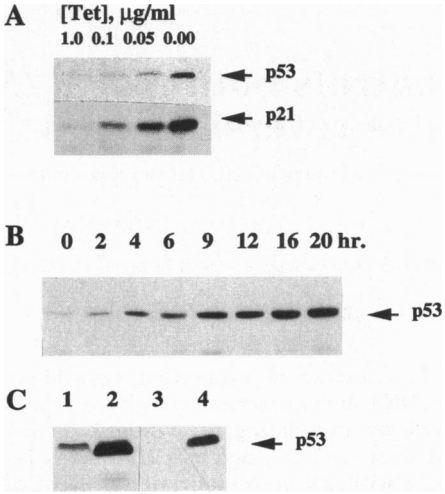

FIG. 2. Regulated expression of p53 in TR9-7 cells. (*A*) Analysis of p53 and p21 proteins in cells cultured in various concentrations of tetracycline (Tet) for 24 h. After cell lysis, the proteins, separated by SDS/PAGE, were transferred to a polyvinylidene difluoride membrane and probed with antibodies recognizing either p53 or p21. (*B*) Time course of p53 protein expression after withdrawal of tetracycline. (*C*) Levels of p53 protein induced by DNA damage in HT1080 cells or by tetracycline withdrawal in TR9-7 cells. Lane 1, untreated HT1080 cells; lane 2, HT1080 cells treated with 0.2 μg of adriamycin per ml for 24 h; lane 3, TR9-7 cells grown for 24 h in the presence of tetracycline; and lane 4, TR9-7 cells 24 h after withdrawal of tetracycline. Equal amounts of protein were loaded in each lane.

16–20 h (Fig. 2*B*). The p53 protein remains at a high level as long as tetracycline is absent.

p53-Mediated Reversible Growth Arrest in Human Cells. To study the effects of p53 expression in a nearly normal human cell line without DNA damage, tetracycline was withdrawn from TR9-7 cells. Four days later, the cells had stopped growing and were enlarged and flattened compared with cells grown in the presence of tetracycline. By day 10, no rounded mitotic cells were observed (Fig. 3). To study the time course

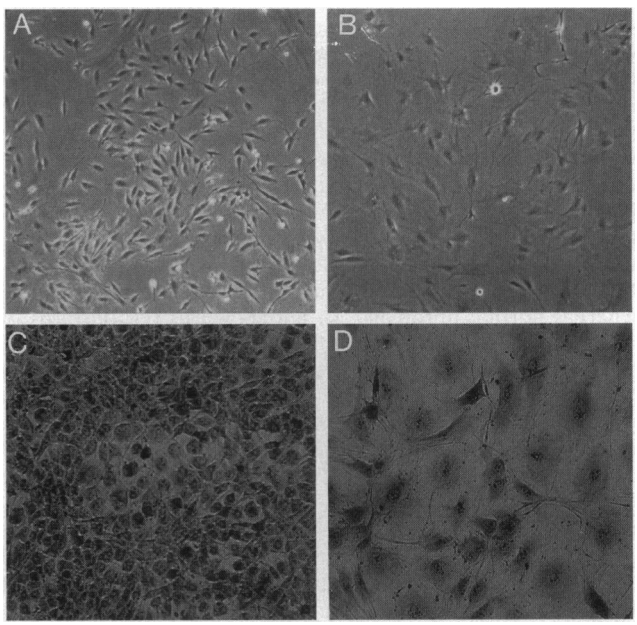

FIG. 3. Growth arrest of MDAH041 fibroblasts by regulated expression of wild-type p53 protein. Cells with tetracycline-regulated p53 were seeded at equal density and incubated for 4 days (*A* and *B*) or 10 days (*C* and *D*) in the presence (*A* and *C*) or absence (*B* and *D*) of tetracycline. (×57.)

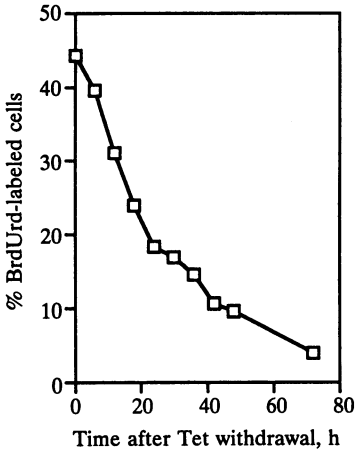

FIG. 4. Time course of BrdUrd uptake after withdrawal of tetracycline from TR9-7 cells. BrdUrd-positive nuclei were counted in fixed cells that were pulse labeled with BrdUrd for 2 h.

of the arrest of DNA synthesis, TR9-7 cells were pulse-labeled with BrdUrd for 2 h at various times after removing tetracycline (Fig. 4). The number of BrdUrd-positive cells decreased by as soon as 4 h, and by 72 h the majority of the cells did not incorporate BrdUrd. After 4 days, there was no BrdUrd incorporation (Fig. 5 *A* and *B*). Although the growth of TR9-7 cells ceased in the absence of tetracycline, the cells remained attached to the tissue-culture plates. When tetracycline was restored, the cells began to grow again, accompanied by incorporation of BrdUrd and a return of normal cell morphology (Fig. 5*C*). To measure the fraction of arrested TR9-7 cells that could resume growth upon removal of the p53 block, 500 dispersed cells were replated in the absence of tetracycline. After 5 days, tetracycline was restored, and colonies were counted 10 days later. The great majority of the cells (86%) were capable of forming colonies in this experiment. Cells arrested for up to 20 days in the absence of tetracycline were able to resume growth when tetracycline was restored.

p53 Controls Both G_1 and G_2/M Checkpoints. We determined cell-cycle distributions after withdrawing tetracycline from TR9-7 cells by FACScan analysis after staining with propidium iodide. There was a striking decrease in the number of cells in S phase after 48–72 h accompanied by an increase in the percentage of cells in both G_1 and G_2/M (Table 1). Similarly, analysis of the incorporation of BrdUrd after withdrawing tetracycline revealed a substantial decrease in DNA synthesis (Fig. 4). The percentage of cells in S phase determined by BrdUrd incorporation was higher than that determined by analysis of DNA content. This difference may be due to the inclusion of very early and very late S-phase cells in the G_1 and G_2/M compartments when analyzing propidium iodide-stained nuclei.

To investigate further the induction of G_2/M arrest by p53 in human fibroblasts in the absence of DNA damage, we synchronized the cells by treating them with 200 μM mimosine for 48 h, which arrests them reversibly at or near the start of S phase (18) (Fig. 6 *A* and *B*). Mimosine was then withdrawn and half of the cells were incubated with or without tetracycline, respectively (Fig. 6*C*). After 15 h, there was a considerable increase in the number of S-phase cells, in either the presence or absence of tetracycline. By 45 h, the cells with tetracycline (p53-minus) showed a nearly normal cell cycle distribution. However, those without tetracycline (p53-plus) showed a large increase in the number of cells in G_2/M, which was still maintained after 72 h. Thus, when p53 is expressed without DNA damage after the G_1/S checkpoint, the majority of cells arrest at the next available checkpoint, G_2/M. The level of p53 expression in TR9-7 cells withdrawn from tetracycline is comparable with the level induced in HT1080 cells in response to DNA damage (Fig. 2*C*), showing that the G_2/M arrest is achieved at physiological levels of p53 protein.

p53-Mediated G_2/M Arrest Is Associated with High Levels of p21/WAF1. Lysates of cells collected at various times after release from the mimosine block in the presence or absence of tetracycline were analyzed for levels of p53 and p21/WAF1 proteins (Fig. 7). p53 is induced after withdrawal of tetracycline from cell released from the mimosine block. p21/WAF1 expression is induced with similar kinetics only in cells grown in the absence of tetracycline after release from the block and is maximal after 45 h.

DISCUSSION

The p53 tumor suppressor is a potent inhibitor of cellular proliferation. In previous studies, the temperature-sensitive mutant $p53^{Val135}$ was used to analyze how cells respond to high levels of wild-type p53. REF cell lines transformed by activated human Ras and $p53^{Val135}$ arrest in G_1 and also in G_2/M and S phase when shifted to a temperature at which the p53 protein is wild type (7). In contrast, a different study with similar cells showed that expression of wild-type p53 led to arrest in G_1 only (21). A more recent study has provided evidence that p53 does hinder G_2/M progression in the rat cell line REF52 transfected with $p53^{Val135}$ (22). Expression of wild-type p53 in a human ovarian cancer cell line by using $p53^{Val135}$ led to arrest in G_2/M but not in G_1 (23). These apparently conflicting results might be explained if the point of cell cycle arrest effected by p53 were cell-type specific, were affected variably by the expression of dominant oncogenes, or were affected by mutations in other cell cycle control proteins.

To determine the effect of wild-type p53 on human fibroblasts with a normal phenotype and in the absence of DNA

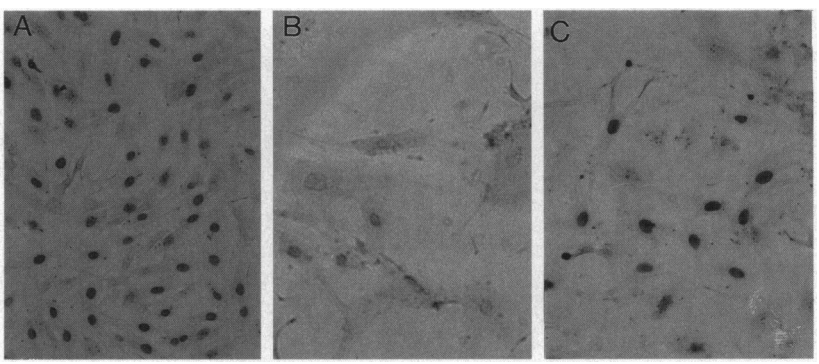

FIG. 5. Reversible growth arrest of human fibroblasts by p53. TR9-7 cells were grown on coverslips in the presence of tetracycline continuously (*A*), for 4 days without tetracycline (*B*), or without tetracycline for 4 days and with tetracycline for 4 days more (*C*). Cells were pulse labeled with BrdUrd for 2 h, fixed, stained, and photographed. (×57.)

Table 1. Cell cycle distribution of TR9-7 cells after withdrawal of tetracycline

Hours after withdrawal	Cell cycle distribution, %		
	G_0/G_1	S	G_2/M
0	59	28	13
3	59	25	16
12	61	22	17
20	61	21	18
40	69	10	21
48	70	7	23

damage, we used a well-controlled system to regulate p53 expression with tetracycline, which has little or no effect on mammalian cells (14). Induction of p53 within a physiological range upon removal of tetracycline led to dramatic but reversible arrest. Cell cycle analysis after induction of p53 revealed a marked reduction in S phase and an increase in both G_1 and G_2/M, suggesting that p53 can regulate both the G_2/M and G_1/S transitions. To determine whether p53 can mediate efficient arrest of human fibroblasts in G_2/M, we analyzed the effect of wild-type p53 in cells synchronized after the p53-dependent G_1 checkpoint and found that p53 indeed does block the cycle efficiently in G_2/M in the absence of DNA

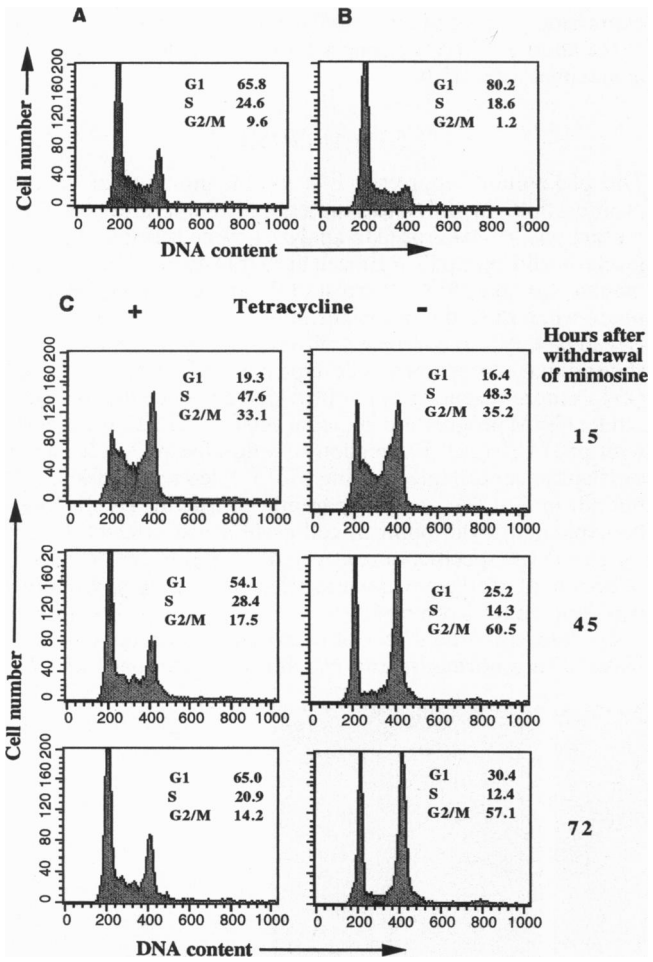

FIG. 6. Induction of G_2/M arrest by p53. Propidium iodide-stained cells were analyzed by the FACScan to determine the cell cycle distribution. (*A*) Logarithmically growing cells. (*B*) Cells synchronized by treatment with mimosine for 48 h. (*C*) Cells released from the mimosine block in the presence (+) or absence (−) of tetracycline. The percentage of cells in each phase of the cell cycle is shown in each panel.

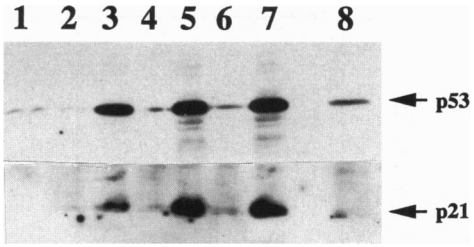

FIG. 7. p21/WAF1 expression after p53-induced G_2/M arrest. Cells released from a mimosine block in the absence or presence of tetracycline were lysed, and the proteins were analyzed by Western blotting. The times of sample collection were similar to those of Fig. 6. Lane 1, logarithmically growing cells; and lane 2, cells synchronized with mimosine. Cells were grown in the absence (lanes 3, 5, and 7) or presence (lanes 4, 6, and 8) of tetracycline after being released from the mimosine block for 13 h (lanes 3 and 4), 36 h (lanes 5 and 6), or 72 h (lanes 7 and 8).

damage. We also tested p53 function in cells arrested in G_2/M and found high levels of p21/WAF1.

DNA damage induced by nitrogen mustard leads to arrest of Burkitt lymphoma cells in G_2/M. These cells exhibit low levels of cdc2 activity, and it has been proposed that an inhibitor of the cdc25-catalyzed dephosphorylation of cdc2 is induced by DNA damage (24). It is not known if this effect is p53 dependent. We find it intriguing that the G_2/M arrest induced by p53 in the absence of DNA damage is associated with high levels of the cyclin-dependent kinase inhibitor p21/WAF1. There is also evidence that the G_2/M arrest induced by DNA damage can occur independently of p53 (4), and we have observed significant accumulation in G_2/M after treatment of p53-null MDAH041 fibroblasts with ionizing radiation (our unpublished data). Interestingly, recent studies have shown that p21/WAF1 can be induced by DNA damage through both p53-dependent and p53-independent mechanisms (25, 26). However, a role for p21/WAF1 in G_2/M arrest induced by either DNA damage or p53 alone remains to be elucidated.

The role of p53 in the cellular response to DNA damage has been the subject of a number of recent studies. Experiments performed in embryonic fibroblasts from knock-out mice suggest that p53 may have a role in cell cycle progression in response to ionizing radiation (4, 27). The results of experiments in human leukemic cells, which normally undergo apoptotic cell death in response to a variety of DNA-damaging agents, including ionizing radiation, have also been interpreted to suggest that ionizing radiation causes a p53-dependent transient arrest which might allow cells to repair damaged DNA (4). However, recently, Di Leonardo *et al.* (5) reported a p53-dependent permanent G_1 arrest in response to ionizing radiation by comparing normal human cell strains (with wild-type p53) and variant HT1080 cells (with mutant p53). These authors suggested that the role of p53 might be to ensure that cells containing damaged DNA are eliminated, avoiding the potential problems caused by inaccurate repair.

Several experiments have been performed to correlate p53 status with cellular survival following radiation. Colorectal cell lines containing either normal or nonfunctional p53 exhibit no difference in radiosensitivity. Neither do embryonic fibroblasts from normal or p53 knock-out mice (28). In contrast, loss of p53 function in REF cell lines containing activated Ras and the papillomavirus E7 protein is associated with decreased radiosensitivity (29). It is not known if the presence of E7 also contributes to the radioresistance of these cell lines. MDAH041 cells and the same cells in which we reinstated p53 expression under its own promoter show a similar decrease in survival after irradiation, regardless of p53 status (our unpublished data), suggesting that there may be one or more p53-independent pathways that stop human cells with

damaged DNA from growing. That is, the response of human fibroblasts to ionizing radiation may involve both p53-independent and p53-dependent irreversible growth arrest. Although growth arrest induced by DNA damage is irreversible, the arrest induced by p53 alone can be reversed. Similarly, agents that block DNA synthesis, such as PALA, cause long-term, reversible, p53-dependent arrest in normal cells. It is not likely that such treatment causes DNA damage in normal cells, and there is evidence to support this idea (30). Therefore, in normal cells, p53 may be involved in different pathways that are initiated by DNA damage on the one hand or inhibition of DNA synthesis on the other.

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