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Advances in Brief

Loss of p21$^{Waf1/Cip1}$ Sensitizes Tumors to Radiation by an Apoptosis-independent Mechanism[1]

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Abstract

Cellular checkpoints are important mediators of the response of normal cells following genotoxic damage, and interruption of these checkpoints is a common feature of many solid tumors. Although the effects of loss in checkpoint function in tumor cells are well understood in terms of cell cycle control, there is little information on their role in determining treatment efficacy *in vivo*. We have examined both the *in vitro* and *in vivo* responses of isogenic lines differing only in the p53-transactivated checkpoint gene, *p21*$^{Waf1/Cip1}$. When assayed *in vitro*, loss of p21 in human colon tumor cells results in a selective induction of apoptosis [Waldman, T., *et al.*, Nature (Lond.), *381:* 713–716, 1996.] but no difference in the clonogenic survival. However, when grown as xenografts and irradiated *in situ*, p21-deficient tumors were significantly more sensitive to radiation as assessed both by clonogenic survival and by regrowth of the tumors following treatment. These data indicate that loss of p21 results in increased sensitivity to killing by ionizing radiation that is independent of the induction of apoptosis and cell cycle arrest but that is specific to cells when they are grown as a solid tumor. These results have important implications for assessing both the genetic determinants of sensitivity to anticancer agents and efficacy of anticancer agents.

Introduction

Apoptosis and cell cycle checkpoints play crucial roles in maintaining tissue homeostasis, and the loss of these processes is often an important event in the development of the malignant phenotype (1, 2). This has been elucidated in part by identification of tumor suppressor genes, including *p53*, which act under normal conditions to elicit growth arrest or apoptosis in response to various cellular stresses (3). Functionally active p53 sensitizes cells to apoptosis in response to radiation and to many of the genotoxic drugs that are currently used in cancer treatment (4). Loss of the p53 activated checkpoint gene *p21*$^{Waf1/Cip1}$, which is responsible for G_1-S arrest following DNA damage, also results in sensitization to apoptosis following treatment with common anticancer therapies (5). These studies have contributed to a widely held paradigm that anticancer drugs kill cells through apoptosis and that cells resistant to apoptotic cell killing will be less responsive to treatment (6). However, it is unclear whether or not the sensitization determined by these *in vitro* short-term viability assays necessarily corresponds to sensitization of tumor cells *in vivo* due to differences in the tumor microenvironment and cell-cell contact effects. To address this question, the inherent sensitivities of the wild-type p53 HCT116 human colorectal cancer cell line and the derivative that is ablated in the cyclin-dependent kinase inhibitor gene *p21*$^{Waf1/Cip1}$ were tested when grown both as monolayers *in vitro* and as xenografts *in vivo*.

Materials and Methods

Cell Lines and Cell Culture. HCT116 human colon carcinoma cells and a derivative in which both *p21*$^{Waf1/Cip1}$ alleles have been deleted through homologous recombination were kindly provided by Dr. Bert Vogelstein (Johns Hopkins Oncology Center, Baltimore, MD). The cell lines were maintained in monolayer culture in McCoy's 5A modified medium supplemented with 10% fetal bovine serum. For *in vitro* experiments, cells were seeded 2 days prior to exposure at densities that ensured exponentially growing populations at the time of exposure.

Apoptosis Assays. Cells growing in 60-mm Petri dishes were exposed to 10 Gy irradiation using a Cs^{137} source with a dose rate of ~1.6 Gy/min or to 5 μg/ml of etoposide. At different time points after exposure, adherent cells were trypsinized and pooled with detached cells in the overlying medium. Following centrifugation at 100 × *g*, the cell pellet was resuspended in PBS containing 10% FCS, 5 μg/ml PI,[3] and 5 μg/ml of bisbenzimide (Hoechst 33342). The percentage of apoptotic cells was then determined by microscopic visualization as the sum of cells that had visible apoptotic bodies with intact membranes (early apoptosis) plus those that had lost membrane integrity and thus stained positive for PI (late apoptosis). Apoptosis was confirmed by terminal deoxynucleotide transferase-mediated end labeling staining (data not shown). For each sample, a minimum of 500 cells were counted. In each experiment and for each time point unexposed cells served as controls.

Clonogenicity Assays. Exponentially growing cells were trypsinized and exposed to either various doses of radiation with a ^{137}Cs source at ~1.5 Gy/min or to different doses of etoposide for 1 h. Following exposure, cells were centrifuged, resuspended in growth medium, and plated for colony survival. After 14 days of growth, Petri dishes were stained with crystal violet, and colonies containing more than 50 cells were scored as survivors. The surviving fraction at each dose was determined as the ratio of survivors in the treated cell populations relative to untreated controls.

Cell Cycle Response. Cells were grown as monolayers in 60-mm Petri dishes and exposed to 10 Gy as described for the apoptosis assay. Cells were harvested at different time points and fixed in 70% ethanol. Fixed samples were washed, resuspended in PBS containing 10 μg/ml PI, and then incubated for an additional 30 min with 200 units of RNase A. Stained samples were then analyzed on a Becton Dickinson FACScan flow cytometer.

***In Vivo* Clonogenic and Regrowth Assays.** HCT116 $p21^{+/+}$ and $p21^{-/-}$ cells were injected intradermally in the midline of the backs of SCID mice and allowed to grow until tumors had reached a mean size of 100 mm^3. Mice with a similar range in tumor sizes (62–181 mm^3 for $p21^{-/-}$ and 71–167 mm^3 for $p21^{+/+}$) were chosen and divided among different irradiation groups such that the mean tumor size was the same. Local irradiation of the tumor was carried out using a 250-kVp X-ray source at a dose rate of ~1.5 Gy/min; mice were placed within individual lead boxes containing a cutout portion at the rear of each box through which the tumor protruded. In some cases, tumors had their blood supply occluded using a metal clamp, thereby rendering all of the cells uniformly deficient in oxygen. For the *in vivo* clonogenic survival experiment, tumors were excised 24 h after irradiation and dissociated with an enzyme mixture consisting of DNase (0.02%), Pronase (0.05%), and collagenate (0.02%). The number of viable cells obtained from the tumor was determined

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[3] The abbreviations used are: PI, propidium iodide; SCID, severe combined immunodeficient.

by counting trypan blue excluding cells. Cells were then plated for colony survival in the same manner as for the *in vitro* assay. Clonogenic survival for each dose point was determined from the average of three separate tumor-bearing mice. For regrowth delay, the average volume of five tumors for each irradiation group was determined by measuring each tumor in three orthogonal directions. Volume was estimated by the formula $(d_1 \cdot d_2 \cdot d_3)\pi/6$.

Results and Discussion

The sensitivity of HCT116 human colon cancer cells with and without p21 to DNA-damaging agents was first determined *in vitro*. As shown in Fig. 1, exposure of these cells *in vitro* to etoposide or to radiation produces selective cell death by apoptosis in the $p21^{-/-}$ cell line after 3–4 days. However, despite the selective apoptotic cell killing in the p21 knockout cell line, no difference in the viability of the two cell types was found when they were assayed by clonogenic survival. The responses of $p21^{+/+}$ and $p21^{-/-}$ cells to etoposide were identical, and the responses to radiation indicated a small protection in the p21 knockout cell line.

The failure of the apoptotic fraction to correlate with the clonogenic fraction can be explained by the cell cycle responses of these cell lines (Fig. 2). The parental $p21^{+/+}$ cells behave similarly to normal human untransformed cells and undergo both G_1 and G_2 blocks that are of extended duration in most cells (7). In the $p21^{-/-}$ cell line, cells are transiently blocked in G_2 but are not blocked in G_1, consistent with a lack of p21 function. Some of the cells complete proper division and enter subsequent G_1 with a DNA content of 2 N, whereas a significant fraction fail to complete mitosis and enter S phase with a 4 N DNA content. Confirmation of cells actively synthesizing DNA with polyploid DNA contents was made by examining the incorporation of the thymidine analogue 5-bromodeoxyuridine (data not shown). By 48 h, substantial numbers of cells can be observed with DNA contents

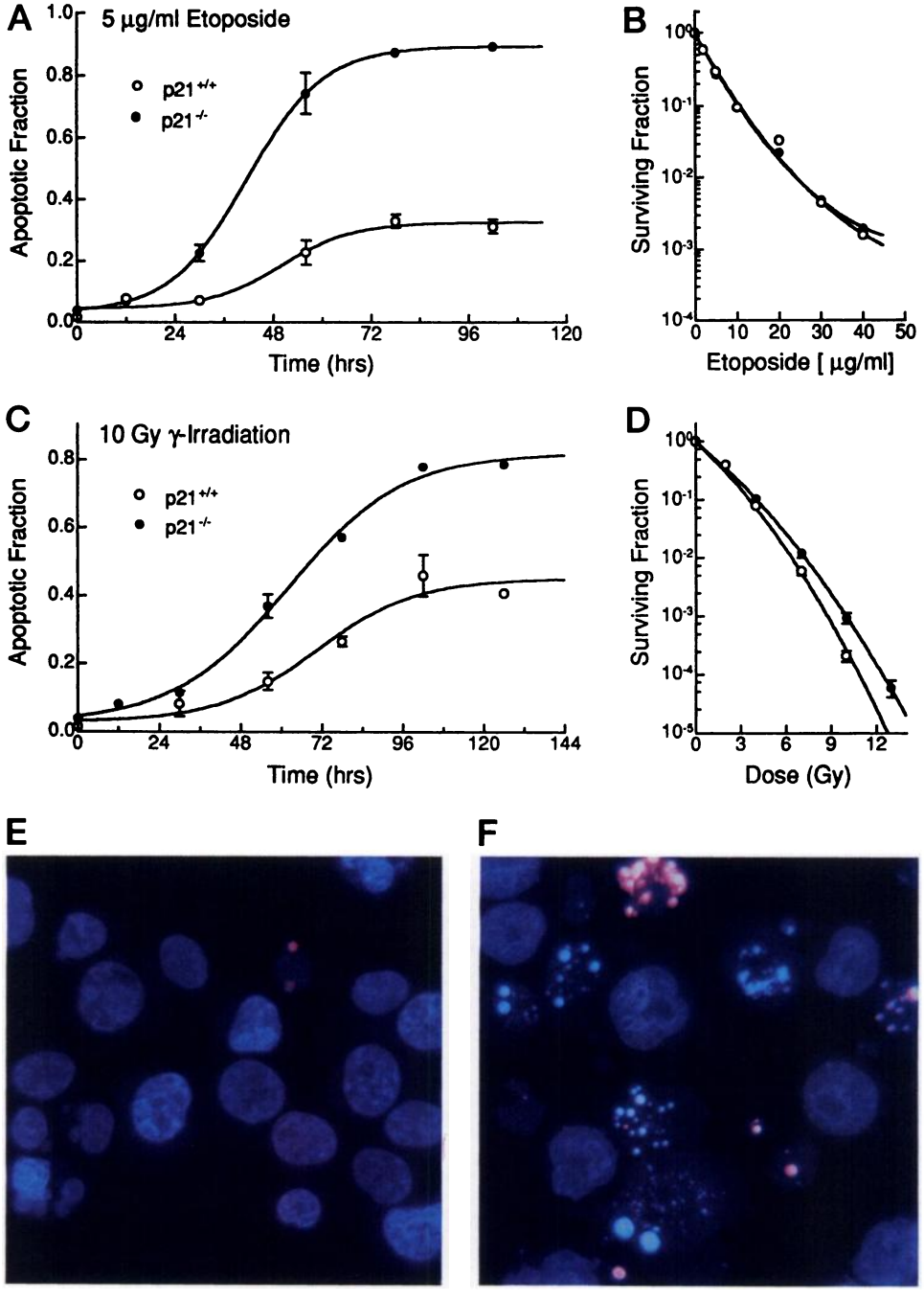

Fig. 1. Analysis of apoptosis and clonogenic survival in exponentially growing HCT116 cells isogenic for p21 exposed to radiation (*A* and *B*) or etoposide (*C* and *D*) *in vitro*. The p21 knockout cells (●) demonstrated significantly higher levels of apoptosis to these two treatments as compared with $p21^{+/+}$ wild type cells (*A* and *C*, ○). However, the $p21^{-/-}$ cells were no more sensitive than the $p21^{+/+}$ cells to either radiation or etoposide when assessed by clonogenic survival (*B* and *D*). Apoptosis assays are the average of at least three independent experiments. Clonogenic assays are the average of two independent experiments. Examples of $p21^{+/+}$ (*D*) and $p21^{-/-}$ cells (*E*) 54 h after treatment with etoposide. Cells were stained with Hoechst 33342 and PI as described in "Materials and Methods." The $p21^{-/-}$ cells illustrate nuclear condensation and formation of membrane bound nuclear bodies characteristic of apoptosis.

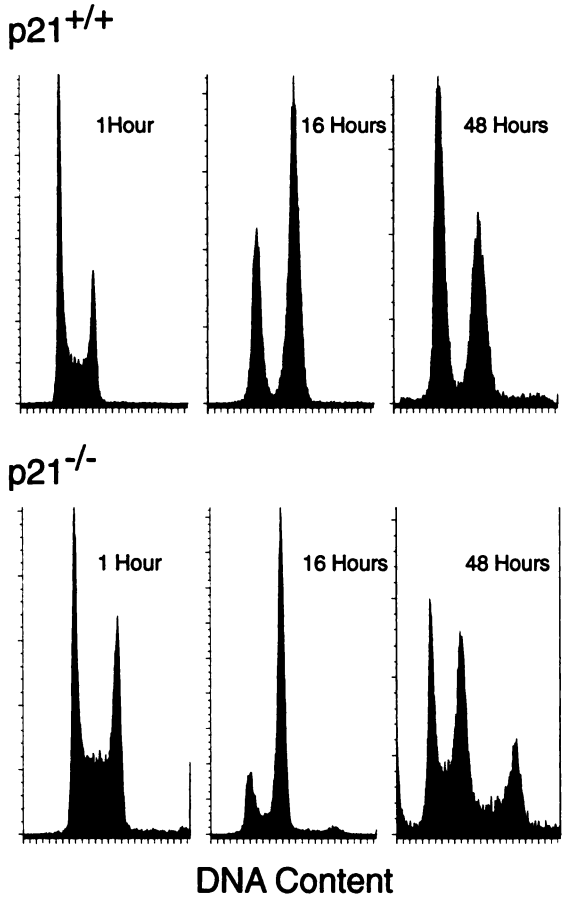

Fig. 2. Cell cycle response of HCT116 cells isogenic for p21 following irradiation. Exponentially growing cells were irradiated with 10 Gy, and the DNA distribution was determined at various times afterward by staining with PI. The $p21^{+/+}$ cells were arrested in G_1 and G_2. The $p21^{-/-}$ cells show a transient G_2 block but are not blocked in the initial G_1 phase. At later time points, $p21^{-/-}$ cells were released from arrest, and some entered S phase without completing mitosis, resulting in the formation of polyploid cells.

of 4, 8, and 16 N. These genetically unstable polyploid cells are subsequently removed from the cell population by apoptosis. The differences in the nature of cell death in these two cell lines was also observable in the clonogenic assay. At the time of colony formation assessment (14 days after treatment), Petri dishes from the $p21^{+/+}$ cell line contained a "lawn" of attached but clonogenically dead cells, whereas the $p21^{-/-}$ cell line did not, indicating they had died and detached from the dish. Because the clonogenic assay measures the ability of a cell to propagate into a colony starting from a single cell, it encompasses permanent cell cycle arrest, as well as cell death by apoptosis, and is thus regarded as a measure of the overall cell survival. It should be noted that the similar radiosensitivity of these two cell lines cannot be explained by a recently reported cell cycle arrest that is specific to cells irradiated and left attached to plastic Petri dishes (8), because cells were plated for colony formation after irradiation. These data thus reveal no role for the selective apoptosis observed in the $p21^{-/-}$ cell line in contributing to the overall cellular sensitivity to radiation or etoposide *in vitro*.

Loss of p21 checkpoint control was also investigated in tumors. Isogenic HCT116 cell lines were grown as xenografts in SCID mice, and when the tumors reached a mean size of 100 mm^3, their responses to radiation were determined both by clonogenic survival and by regrowth delay. Fig. 3*a* shows the radiation survival responses to 7.5 and 15 Gy of $p21^{+/+}$ and $p21^{-/-}$ tumors irradiated *in vivo*, and then, 24 h later, removed, dissociated, assayed for viable cells with trypan blue, and plated for colony formation. This figure indicates that when irradiated *in vivo*, the loss of p21 results in sensitization as assessed by the clonogenic assay. This *in vivo* result is in contrast to the situation *in vitro*, in which these cells have a similar, if not more resistant, response as compared with the $p21^{+/+}$ cells (Fig. 1).

The sensitization caused by loss of p21 is also evident when tumor response is assessed by regrowth delay (Fig. 3, *b* and *c*). Irradiation of the $p21^{+/+}$ tumors with 5 or 10 Gy resulted in only modest delays in regrowth. Irradiation with 15 Gy produced a delay of approximately 2

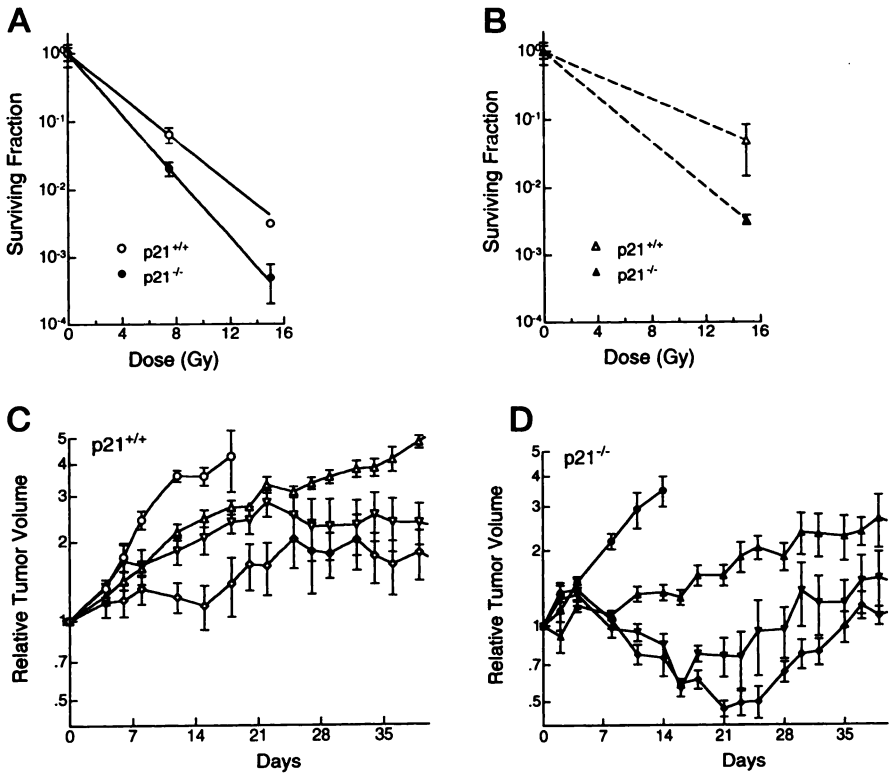

Fig. 3. *In vivo* sensitization of cells lacking p21. HCT116 cells isogenic for p21 were grown as xenografts in SCID mice to a mean diameter of 100 mm^3 and then irradiated. Tumor sensitivity was assessed in terms of the average clonogenic survival 24 h after irradiation (*A* and *B*) or by tumor regrowth following irradiation (*C* and *D*). *A*, tumors were irradiated with 7.5 or 15 Gy under standard conditions; *B*, tumors were clamped to deprive them uniformly of oxygen just prior to treatment with 15 Gy. The *in vivo* clonogenic survival in both cases indicated a significant radiosensitization of tumors lacking p21 (● and ▲). Irradiation with 5 (△ and ▲), 10 (▽, ▼), or 15 (◇, ◆) Gy also resulted in significantly more tumor shrinkage and an increased growth delay relative to controls (○, ●) in tumors derived from the $p21^{-/-}$ cells (*D*) as compared with those from $p21^{+/+}$ cells (*C*).

Table 1 *Total number of viable[a] cells ($\times 10^6$) recovered from the tumors 24 h after treatment*

Treatment	$p21^{+/+}$	$p21^{-/-}$
No treatment	2.5 ± 0.5	3.7 ± 0.9
7.5 Gy unclamped	2.5 ± 0.9	5.0 ± 2.5
15 Gy clamped	2.2 ± 0.4	2.1 ± 0.4
15 Gy unclamped	2.2 ± 0.7	3.1 ± 1.0

[a] Viable cells defined as those excluding trypan blue. Values are means (±1 SE) of three tumors per group. The values for different treatments in each tumor are not statistically different ($P > 0.1$).

weeks without significant tumor shrinkage. In contrast, $p21^{-/-}$ tumors irradiated with 5 Gy had a delay that was intermediate between the 10- and 15-Gy responses of the $p21^{+/+}$ tumors. When treated with 10 or 15 Gy, the $p21^{-/-}$ tumors exhibited substantial shrinkage and did not begin to increase in size until 3 weeks after irradiation. The similar findings from both the clonogenic survival and regrowth delay assays show that loss of p21 provides a sensitization to radiation treatment that is specific to cells grown as tumors and irradiated *in vivo*.

To rule out the possibility that differences in the tumor oxygenation of these two cell types were responsible for the different *in vivo* sensitivities to radiation, we examined the responses of tumors when they were irradiated under hypoxic conditions. The absence of adequate oxygenation results in resistance to cell killing by ionizing radiation and is a common feature of both rodent and human tumors (9, 10). Differences in oxygenation between the $p21^{+/+}$ and $p21^{-/-}$ tumors could therefore potentially explain their different radiation sensitivities *in vivo*. This possibility was examined by clamping a second set of tumors to uniformly deprive them of oxygen prior to irradiation. Fig. 3*b* shows that clamping produced a similar degree of protection for both the $p21^{+/+}$ and $p21^{-/-}$ tumors as compared with the unclamped responses (Fig. 3*a*), indicating that these tumors contained a similar fraction of resistant (hypoxic) cells. Because the differential sensitivity between the two cell lines is maintained under oxygen-deprived conditions, differences in tumor oxygenation cannot account for the *in vivo* sensitivity of tumors deficient in p21.

A second possibility is that the *in vivo* sensitization is related to the propensity of the $p21^{-/-}$ cells to undergo apoptosis as opposed to a permanent or prolonged arrest following irradiation. This could more rapidly eliminate $p21^{-/-}$ tumor cells, thereby possibly removing a "feeder layer" effect that might promote the survival of less damaged cells. However, we can rule out this possibility because there were no significant differences in the total number of intact cells between the $p21^{+/+}$ and $p21^{-/-}$ tumors at the time that the tumors were explanted, disaggregated, and assayed for cell survival 24 h after irradiation (Table 1). This lack of cell death by apoptosis at 24 h postirradiation is consistent with the kinetics of apoptosis observed following *in vitro* exposure of these cells to radiation (Fig. 1). Because the *in vivo* survival curve indicated a significant sensitization of $p21^{-/-}$ tumors when assayed at this time, differences in apoptosis cannot account for the contrasting *in vitro* and *in vivo* results.

A more likely explanation is that in addition to its role in controlling cell cycle arrest following DNA damage through inhibition of cyclin/CDK complexes, p21 plays a role in mediating the acquired resistance of cells when grown as a three-dimensional mass. This phenomenon, known as the "contact effect," describes the acquired resistance of cells when grown either *in vitro* as spheroids or *in vivo* as tumors, compared with cells grown as a monolayer in a tissue culture dish (11, 12). Up-regulation of p21 has been reported to occur through mechanisms independent of p53 transactivation, including cell starvation, cell-cell contact, reactive-oxygen species, and differentiation factors (13–16). Furthermore, in addition to its role in blocking cell entry into S phase, p21 inhibits proliferating cell nuclear antigen (17) and can stimulate nuclear factor κB-dependent gene expression by inhibiting p300-associated cyclin E-Cdk2 activity (18). Interestingly, the inhibition of nuclear factor κB has recently been shown to provide a protective effect against radiation-induced apoptosis (19). It is therefore possible that signals to p21 from the tumor environment, independent of p53 or DNA damage, modify the intrinsic sensitivity of the tumor to radiation through p21-mediated downstream events. This possibility is supported by the recent finding that the p21-related cyclin-dependent kinase inhibitor gene, $p27^{Kip1}$, is up-regulated in *in vitro* three-dimensional cell cultures and contributes to the resistance of cells exposed to chemotherapeutic agents when grown in this manner (20). In this study, p21 was also found to be up-regulated in a similar manner in at least one other human colon cell line, SW480, although up-regulation was not observed in all cell lines. If p21 is acting by a similar mechanism in these human colon cells when they are grown as tumors in mice, the loss of p21 would be expected to reduce the resistance afforded *in vivo* while having no effect *in vitro*, as we have observed. These results demonstrate the requirement for *in vivo* assessment of the genetic determinants of sensitivity to various anticancer agents. In addition, the specific *in vivo* radiosensitization resulting from loss of p21 in human colon carcinoma demonstrates a potential mechanism for exploitation in cancer therapy.

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