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Advances in Brief

p21 Is Necessary for the p53-mediated G_1 Arrest in Human Cancer Cells[1]

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Abstract

DNA-damaging agents induce a p53-dependent G_1 arrest that may be critical for p53-mediated tumor suppression. It has been suggested that $p21^{WAF1/CIP1}$, a cdk inhibitory protein transcriptionally regulated by p53, is an effector of this arrest. To test this hypothesis, an isogenic set of human colon adenocarcinoma cell lines differing only in their p21 status was created. The parental cell line underwent the expected cell cycle changes upon induction of p53 expression by DNA damage, but the G_1 arrest was completely abrogated in p21-deficient cells. These results unambiguously establish p21 as a critical mediator of one well-documented p53 function and have important implications for understanding cell cycle checkpoints and the mechanism(s) through which p53 inhibits human neoplasia.

Introduction

Inactivation of the *p53* gene is common to a diverse array of tumor types. However, the mechanism by which *p53* functions to suppress the growth of tumors in which it has not yet been inactivated is much less clear. The *p53* gene encodes a transcription factor that binds to defined DNA consensus sequence and activates expression of adjacent genes (reviewed in Ref. 1). This observation stimulated efforts to identify those genes that are regulated by p53 in the hope that this would shed light on the mechanisms by which p53 suppresses tumor growth. The potential promise of this approach was highlighted when the *p21* gene was independently and simultaneously identified as a gene that inhibited cyclin/cyclin-dependent kinase complexes (CIP1), was induced by p53 (WAF1), and was differentially expressed during cellular senescense (SDI1) (2–6). The *p21* gene thus linked p53 expression and inhibition of cell cycle progression. The stature of p21 as a downstream mediator of tumor suppression was enhanced when it was discovered that p21 was the prototype for a family of small cyclin dependent kinase-inhibiting proteins. The latter included p16, implicated in the pathogenesis of familial melanomas and many other tumor types (reviewed in Refs. 7–9).

To test hypotheses about the importance of p21 in the p53-mediated growth suppression of tumor cells, we used homologous recombination to create a homozygous deletion of p21 in a colon cancer cell line. The analysis of these human tumor lines, as well as that of murine fibroblasts by Deng *et al.* (10), unambiguously establish p21 as a critical mediator of the p53-dependent G_1 arrest associated with DNA damage.

Materials and Methods

Cell Culture. HCT116 cells were obtained from the American Type Culture Collection and propagated in McCoy's 5A media (GIBCO-BRL, Bethesda, MD) supplemented with 10% fetal calf serum (Hyclone, Logan, UT) and penicillin/streptomycin (GIBCO-BRL). For routine passage, cells were split 1:6 when they reached confluence, generally every 3 days.

Targeting Constructs. A 10.4-kb fragment derived from a P1 clone of the human *p21* gene (6) was subcloned into pBluescript (Stratagene, La Jolla, CA) and mapped; then critical regions were sequenced. For the promoterless constructs, a 1.8-kb *BbsI/SfiI* restriction fragment encompassing 59 nucleotides of 3′ intron I, exon II, intron II, and 96 nucleotides of 5′ exon III was replaced with PCR-generated *NEO* or *HYG* gene cassettes containing polyadenylation signals. The constructs were engineered so that all sequences upstream of the translation initiation signals of *NEO* or *HYG* were identical to those in the endogenous *p21* gene. For the targeting constructs in which *NEO* was driven by a heterologous PGK[3] promoter, the same 1.8-kb *BbsI/SfiI* restriction fragment was replaced with the *NEO* cassette downstream of the PGK promoter. The vector backbones contained a thymidine kinase cassette driven by a PGK promoter; however, no negative drug selection was used in the experiments yielding homologous recombinants. The vectors were linearized at a unique *SalI* site in the vector backbone and used for transfection.

Transfection. Cells were transfected at 70% confluence with 3 μg of linearized cesium chloride-banded DNA using lipofectamine (GIBCO-BRL). Cells were washed twice in serum-free media (McCoy's 5A), then incubated in 3 ml Optimem (GIBCO-BRL). The DNA/liposome complexes were added to the flasks, mixed gently by rocking, and incubated for 3 h at 37°C. The Optimem was then replaced with serum-containing media, and the flasks were further incubated for 24 h at 37°C. Cells were trypsinized and distributed at various dilutions to the wells of 96-well plates and selected in either 0.4 mg/ml G418 (GIBCO-BRL) or 0.1 mg/ml Hygromycin (Calbiochem, La Jolla, CA) for 14 days to obtain clones by limiting dilution.

Southern Blot. DNA was prepared from cultured cells using standard techniques. Briefly, cells were harvested by trypsinization, resuspended in SDS/proteinase K solution at 60°C overnight, extracted with phenol/chloroform and precipitated with ethanol. Five μg of DNA were digested with *Bgl*II, separated on a 0.8% agarose gel, and then transferred to a Zeta Probe membrane (Bio-Rad, Hercules, CA; Fig. 1). A gel purified restriction fragment cloned from the promoter of p21 (Fig. 1) was radiolabeled by random-priming and used as probe. The blot was then washed and imaged using phosphorimager analysis (Molecular Dynamics, Sunnyvale, CA).

Cell Treatments. Cells were seeded 24 h prior to treatment and were 50–70% confluent at the time of treatment. Radiation (6 Gy) was delivered by a ^{137}Cs gamma irradiator at approximately 1 Gy/min. Cells were treated with Adriamycin (doxorubicin) at a concentration of 0.2 μg/ml.

Western Blot. Samples of protein from equivalent numbers of cells were separated by electrophoresis on a 4–20% gradient gel (Jule, Inc., New Haven, CT), transferred to Immobilon P membrane (Millipore, Bedford, MA), and blotted with either p53-specific monoclonal antibody 1801 or p21-specific monoclonal antibody EA10 (Oncogene Science, Cambridge, MA). After application of a horseradish peroxidase-coupled secondary antibody, reactive proteins were visualized with ECL (Amersham, Arlington Heights, IL).

FACS Analysis. H33258 staining and PI/BrdUrd double-staining were used to analyze the cycling characteristics of cell populations. For H33258 staining, cells were trypsinized, washed twice in HBSS (GIBCO-BRL), and resuspended in 40 μl HBSS. The cells were then added to 360 μl of fix/staining solution containing 0.7% NP40 (USB, Cleveland, OH), 4.7% formaldehyde (J. T. Baker, Phillipsburg, NJ), and 11 μg/ml H33258 in PBS. These

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[3] The abbreviations used are: PGK, phosphoglycerate kinase; BrdUrd, bromodeoxyuridine; PI, propidium iodide; MMR, mismatch repair; FACS, fluorescence-activated cell sorter.

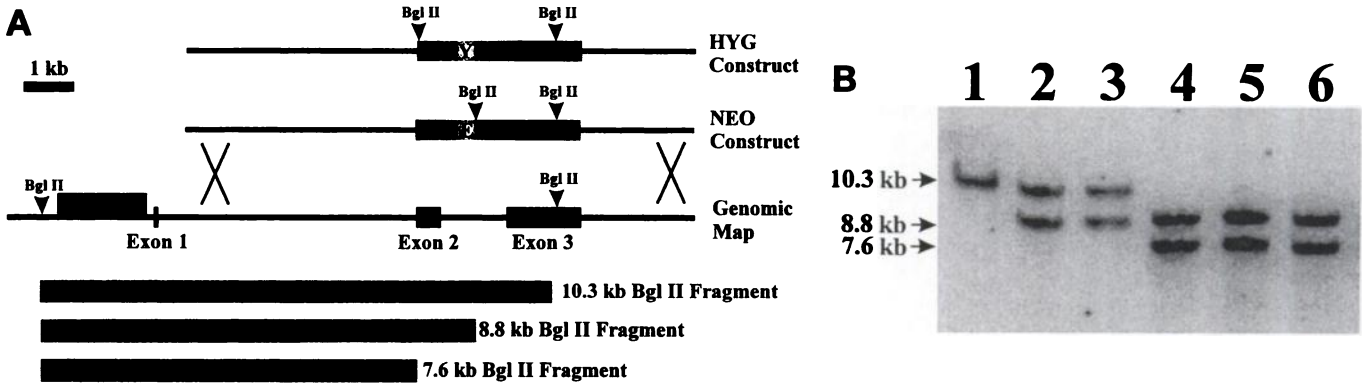

Fig. 1. Targeting p21 by homologous recombination. *A*, map of the genomic region encompassing the *p21* gene is shown, along with a diagram of the constructs used for gene targeting. The sizes of the *Bgl*II fragments expected from the wild-type and targeted alleles are also indicated. *B*, Southern blots of *Bgl*II-digested DNA from drug-selected clones are shown. Only the wild-type 10.3-kb allele is present in HCT116 cells *(Lane 1)*, while the wild-type plus an 8.8-kb *NEO*-targeted allele is present in the clones shown in *Lanes 2* and *3*, and the 8.8-kb *NEO* and 7.6-kb *HYG*-targeted alleles and no wild-type allele are present in the clones shown in *Lanes 4–6*.

fixed and stained cells were then used directly for FACS analysis. For PI/BrdU double staining, growing cells were pulsed with 10 μM BrdU (Sigma Chemical Co., St. Louis, MO) for 1 h. The cells were then harvested and fixed in 70% ethanol at −20°. After digestion with pepsin, the resultant nuclei were incubated with a mouse anti-BrdUrd monoclonal antibody (Pharmingen, San Diego, CA), followed by a flourescein isothiocyanate conjugated goat anti-mouse secondary antibody. The cells were then stained with PI and analyzed by flow cytometry for both DNA content and BrdUrd incorporation. The means and SDs shown were computed from analyses of at least three cultures for each time point using two $p21^{+/-}$ and two $p21^{-/-}$ clones.

Results and Discussion

We chose to perform these experiments in the human colorectal cancer cell line HCT116 for several reasons. These cells contain a wild-type *p53* gene, respond normally to DNA-damaging agents with respect to the induction of p53 and the associated cell cycle arrest, are derived from colorectal epithelial cells, are near diploid with two *p21* alleles,[4] and are MMR deficient (11). Though deleting chromosomal genes via homologous recombination is by now almost routine in murine embryonic stem cells (12), this is often difficult in other cell types. We chose MMR-deficient cells for this experiment largely because a deficiency in this repair system has been correlated with an increased capacity for homologous recombination in rodent cells as well as in unicellular organisms (13, 14). Nevertheless, using standard technology for gene targeting in murine cells, we were unable to successfully target p21 in HCT116 cells, either with replacement or insertion vectors (zero recombinants among 232 G418-resistant clones tested). We, therefore, resorted to a strategy shown to be more effective in somatic cells (15, 16), using a promoterless targeting vector in which the coding region of the G418 resistance gene was precisely substituted for the coding region of p21 (Fig. 1). Of 100 G418-resistant clones isolated following transfection of this vector, 37 proved to be homologous recombinants as assessed by Southern blot analysis (Fig. 1*B*, *lanes 2–3*). A similar, promoterless targeting vector was then generated, with a hygromycin-resistance gene substituted for the G418-resistance gene in the original vector (Fig. 1*A*). Of 20 hygromycin-resistant clones tested under normal culture conditions, 5 failed to produce p21 protein, and all of these proved to have both alleles deleted by homologous recombination (examples in Fig. 1*B*, *lanes 4–6*).

Parental HCT116 cells, two subclones with one *p21* allele deleted, and two subclones with deletions of both *p21* alleles were selected for further analysis. The five lines had identical morphology, growth rates, and cell cycle distributions under normal culture conditions, demonstrating that p21 is not required for cellular viability and had little, if any, effect on cell growth under normal culture conditions. To test the influence of p21 deletion on p53 responses, cells were irradiated under conditions shown previously to induce a p53-dependent growth arrest and induction of p21 (17–21). The cells were harvested at various times following irradiation, fixed, and stained with the DNA binding dye Hoechst 33258; then cells were analyzed with flow cytometry and Western blots. In HCT116 parental cells ($p21^{+/+}$) and heterozygotes ($p21^{+/-}$), the expected responses were observed: induction of p53 and p21 (Fig. 2), a decreased fraction of S-phase cells resulting from a G_1 arrest, and an increased fraction of cells in G_2 due to a G_2 arrest (Fig. 3 and data not shown). The fact that relatively few cells were in S phase throughout the period of measurement (12–24 h following irradiation) confirmed that this decrease was due to a cell cycle block. In p21-deficient ($p21^{-/-}$) cells, however, a high fraction of cells continued to be present in S phase following irradiation (Fig. 3). To demonstrate that the apparent persistence of S phase in the $p21^{-/-}$ cells was not a result of abnormal DNA content induced by irradiation, a pulse of BrdUrd was given to the cells 1 h before harvest, and they were subsequently assessed by simultaneous analysis of DNA synthesis (BrdUrd incorporation) and DNA content (PI staining). The results confirmed those derived from the analyses in Fig. 3. For example, 24 h following irradiation, the fraction of S phase cells varied between 12 and 18% in $p21^{+/+}$ and $p21^{+/-}$ cells and 40–52% in $p21^{-/-}$ cells when

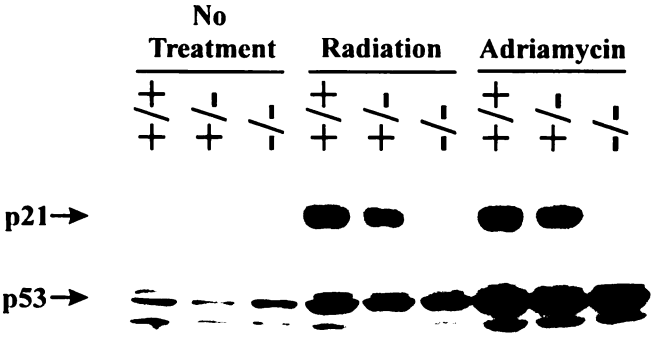

Fig. 2. Expression of p21 and p53 proteins in representative clones. Cultures of $p21^{+/+}$, $p21^{+/-}$, and $p21^{-/-}$ cells were harvested after no treatment, or 24 h after exposure to 6 Gy gamma-irradiation, or after continuous exposure to 0.2 μg/ml Adriamycin for 24 h, as indicated. Equal amounts of protein were separated by electrophoresis and subjected to Western blot analysis with p21- or p53-specific monoclonal antibodies (35). No p21 protein was present in the $p21^{-/-}$ cells, and a somewhat reduced amount of p21 protein was present in the heterozygote $p21^{+/-}$ cells.

[4] C. Lengauer and T. Waldman, unpublished results.

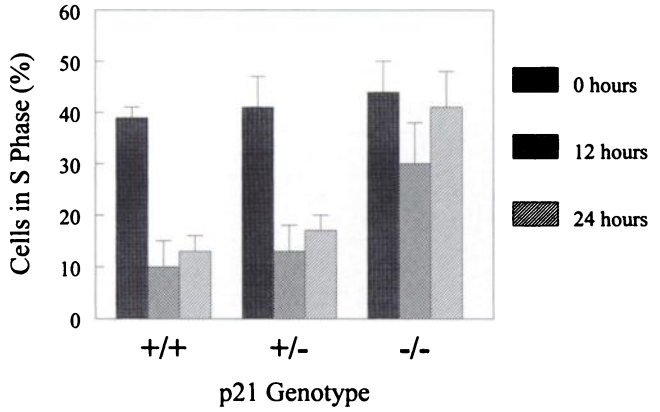

Fig. 3. S-phase changes following gamma irradiation. Cells were harvested at the indicated times following irradiation, fixed, and stained with the DNA-binding dye Hoechst 33258 and analyzed by flow cytometry as described (17). In each experiment, 10,000 cells were analyzed, and the S-phase population was quantitated using the Multicycle software package. The means and SDs were computed from analysis of at least three cultures from each time point, using two $p21^{+/-}$ and two $p21^{-/-}$ clones.

assessed by either staining for DNA content (H33258 or PI) or BrdUrd incorporation (range of three different experiments).

These results were consistent with a model in which p21 deficiency abrogated the G_1 checkpoint following irradiation. However, the interpretation of the experiments recorded in Fig. 3 was complicated by the acute nature of the DNA damage induced by gamma irradiation; the cells analyzed represented a mixture at various phases of radiation-induced block and recovery. To resolve this ambiguity, we treated cells with another DNA-damaging agent, Adriamycin, that has been shown previously to induce p53, p21, and cell cycle arrest in a fashion similar to that induced by gamma irradiation (Fig. 3; Refs. 19 and 22). Because the Adriamycin treatment was continuous, however, there was no opportunity for cells to recover, and the differences between p21-deficient and proficient cells was striking. Prominent blocks in both G_1 and G_2 were observed after 24 h of Adriamycin treatment in the $p21^{+/+}$ and $p21^{+/-}$ cells, with the result that virtually all cells in these populations were in either G_1 or G_2 (Fig. 4). In $p21^{-/-}$ cells, there was no apparent G_1 block, and all G_1 cells passed through S phase within 24 h of Adriamycin treatment. This resulted in a striking cell cycle distribution, with a nearly pure population of G_2-arrested cells (Fig. 4).

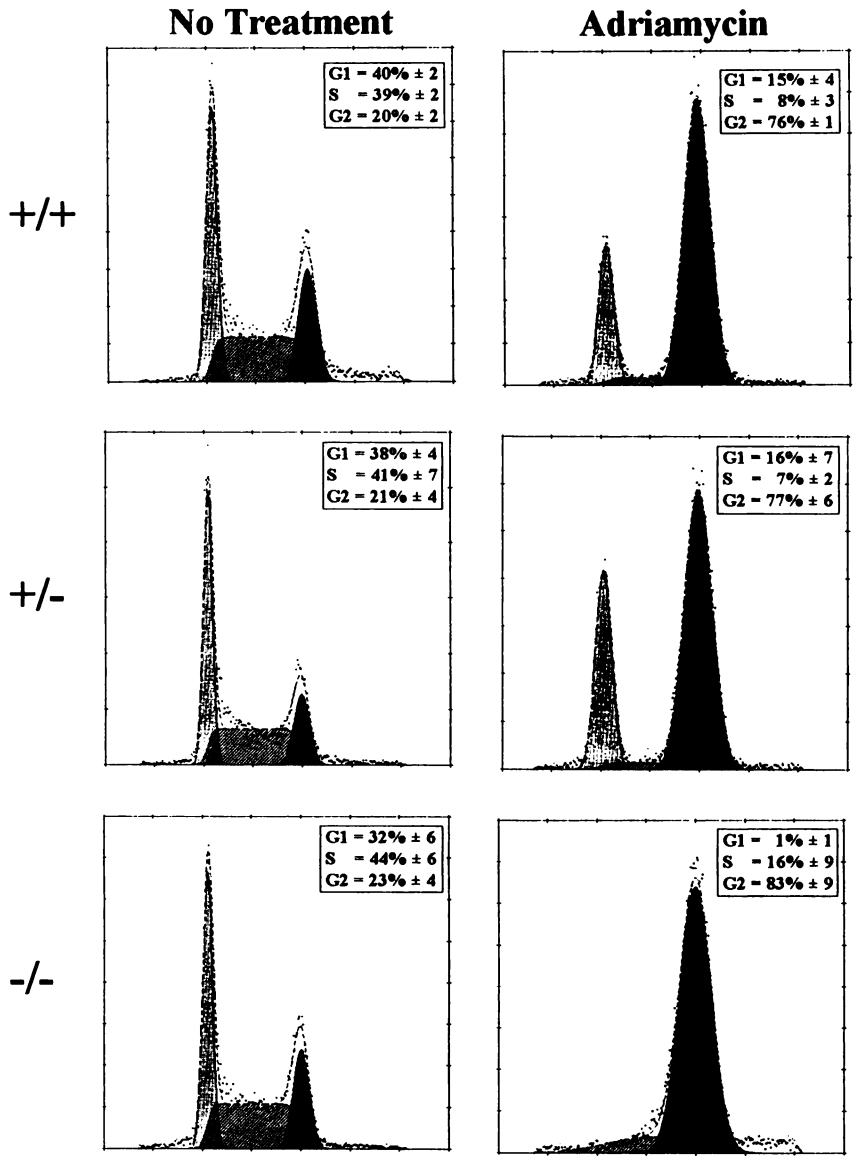

Fig. 4. Cell cycle analysis following Adriamycin treatment. Cells were harvested following Adriamycin treatment (see legend to Fig. 2) and analyzed by flow cytometry as described in "Materials and Methods." The *No Treatment* cells were harvested from identical cultures grown in the absence of Adriamycin. The patterns shown represent 24 h of Adriamycin treatment; similar patterns were observed after 48 h of treatment. Means and SDs shown in the *boxes* were computed as described in Fig. 3.

These data unambiguously demonstrate that p21 is required for the p53-dependent G_1 checkpoint that follows genomic damage by irradiation or DNA-damaging agents. Previous studies have demonstrated consistently that p53 is responsible for the radiation-induced G_1 block, but its role in G_2 is unresolved (17, 20, 23, 24, 36). Taken together, the results implicate p21 as a major mediator of p53 action in G_1, required for its cell cycle inhibitory properties following stimulation of its expression.

The cells we used for analysis were MMR-deficient, and this could conceivably affect responses to DNA-damaging drugs. However, in p21-containing HCT116 cells, these responses were entirely normal, and MMR-deficient cells were originally used to identify the p53-dependence of the G_1 checkpoint following DNA damage (18). Many questions, however, remain. For example, p53 has been shown to result in either cell cycle arrest or apoptosis, depending on the cell type and experimental conditions (25–29). HCT116 cells, like other wild-type p53-containing human colon cancer cells (18), undergo growth arrest but not apoptosis following p53 induction; therefore, the effect of p21 on apoptosis could not be determined in our experiments. Additionally, the effects of p53 on cell cycle control and on tumorigenesis are cell type and species dependent. Thus, mice with p53 mutations do not develop the same tumor spectrum as do humans (30–33), and cells of various origin do not respond identically with respect to cell cycle inhibitors when presented with the same insult (for an example, see Ref. 34). These species and cell type determinants may explain the differences observed between the $p21^{-/-}$ human colon cancer cells studied here and the $p21^{-/-}$ mouse fibroblasts described by Deng *et al.* (10). Although p21 deletion had an effect on the G_1 checkpoint in the murine cells, relief of the block induced by DNA damage was only partial, suggesting that a second mechanism existed, capable of enforcing an attenuated G_1 arrest, even in the absence of p21 (10). The experiments described here suggest that, at least in human colon cancer cells, no such second mechanism contributes independently to the arrest (Fig. 4). Moreover, p21-deficient mouse fibroblasts proliferated abnormally, especially after continued passage (10), while p21-deficient HCT116 cells grew indistinguishably from their parental cells in the absence of DNA damage. Because p53 is important in so many forms of neoplasia, it will be important to analyze the effect of p21 deletion in other normal and transformed cells and to determine which of the numerous biological effects of p53 are dependent on p21 in relevant cell types.

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