Role of the Alternating Reading Frame (P19)-p53 Pathway in an in Vivo Murine Colon Tumor Model

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ABSTRACT

Considering the importance of the oncogene checkpoint function of the alternating reading frame (ARF)-p53 pathway, studies were undertaken to evaluate the status of this pathway in azoxymethane (AOM)-induced mouse colon tumors. A PCR-based analysis of ARF and p53 cDNAs in normal colon tissues and AOM-induced colon tumors failed to detect mutations in either of these two critical tumor suppressor genes. In addition, laser capture microdissection of tumors followed by PCR-based sequencing of exons 5-7 of genomic p53 showed that even the most pleomorphic cancer cells were p53 normal. A marked increase in ARF mRNA and protein levels was observed in colon tumors, indicating activation of the ARF-p53 pathway in these tumors. High levels of ARF protein stabilized p53 protein in the tumors, but the p53 protein showed little biochemical activity. Compared with a mouse colonocyte cell line that expresses high levels of wild-type p53 (YAMC), the p53 protein in tumors showed no detectable DNA binding activity or did it activate p21 expression. In fact, p21 levels were lower in tumor tissue relative to normal mucosa, even though p53 levels were ~30-fold higher in tumors relative to control. Within the A/J tumors, we also used a cDNA microarray approach to screen a panel of genes that are transcriptionally up- or down-regulated by functional p53. The expression patterns of these p53-regulated genes were consistent with a lack of functional p53. This work demonstrates that the ARF-p53 oncogene checkpoint can be overcome without p53 mutations and that the mechanism used to overcome this checkpoint involves the suppression of p53 transcriptional activating activity. The AOM colon cancer model may be well suited for studying tumor promotion events that precede p53 disruption.

INTRODUCTION

Colorectal cancer is the second leading cause of cancer death in the United States (1). Repetitive treatment with the organotropic colon carcinogen AOM produces tumors in rodents that share a number of features with human colon cancer. AOM-induced mouse tumors are localized primarily to the distal colon and are histologically similar to sporadic forms of human colon cancer (2-5). In addition, a number of changes associated with human colorectal cancer have been identified in AOM-induced rodent colon tumors, including alterations in Ki-ras, adenomatous polyposis coli (APC), cyclin D1, and CDK4 (6-13). Thus, the mouse model of colon carcinogenesis provides a useful experimental system for studying molecular and pathological changes associated with human colorectal cancer.

The INK4/ARF locus is the second most commonly disrupted genetic locus in human cancer (14). This locus has the unusual capacity to encode two distinct proteins: a cyclin-dependent kinase inhibitor, p16, and ARF (known as p19ARF in mouse), a potent growth suppressor that stabilizes and activates p53 by its actions upstream of p53 (14, 15). ARF mechanistically prevents MDM2-mediated degradation of p53 by physically interacting with MDM2, and blocking MDM2-induced nuclear export and subsequent p53 degradation. Hyperproliferative signals induced by c-myc, E1A, Ras, and E2F-1 induce ARF expression, which leads to increased p53 stability and enhancement of p53-related functions, such as transactivation of growth inhibitory genes and apoptosis (16-20). This ARF-mediated stabilization of p53, which is independent of DNA damage, provides the cell with an “oncogene checkpoint function” (20). The ability of ARF to sense hyperproliferative stimuli and its importance in tumor surveillance is supported by data generated in mouse knockouts and in vitro models, wherein ARF loss strongly predisposes mice to spontaneous tumor development and accelerates tumor induction by irradiation or carcinogens (16, 20-24). Thus, ARF forms a crucial link between potentially harmful growth stimuli and the activation of the growth inhibitory p53 pathway.

Although the ARF-p53 pathway is effective at limiting the proliferation and survival of oncogenically transformed cells, this checkpoint is overcome during the carcinogenic process. Therefore, understanding the mechanisms used by cancer cells to survive the ARF-p53 checkpoint is a critical issue in cancer biology. Because AOM activates a similar panel of oncogenes in the mouse as those found in human colon tumors (6-13), we determined whether the oncogene-ARF-p53 pathway was activated in the AOM model and whether its activation was overcome in tumors. A number of possible mechanisms were considered, including direct mutation of the p53 and ARF genes. Interestingly, our data indicate that both ARF and p53 are sequence normal in AOM-induced colon tumors but that the ability of p53 to activate or repress transcription is suppressed. Similarly, non-genetic inhibition of p53 may play a critical role in the early stages of human colon carcinogenesis and in p53 sequence normal tumors (25-27).

MATERIALS AND METHODS

Animals and Treatment. Five week-old male A/J and AKR/J mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and housed in a temperature-controlled environment (23°C) with a 12-h light/dark cycle. Mice were provided with Purina laboratory rodent chow (St. Louis, MO) and water ad libitum. After an acclimation period of 1 week, mice were injected with AOM dissolved in 0.9% NaCl by i.p. injection at a dose of 10 mg/kg body weight once per week for a total of 6 weeks. A separate group of mice received 0.9% NaCl by i.p. injection at a dose of 10 mg/kg body weight once per week for a total of 6 weeks. A separate group of mice received 0.9% NaCl and served as vehicle controls.

Tissue Sample Preparation, RNA Isolation, and Reverse Transcription. Mice were sacrificed 24 weeks after the last injection of AOM. Tumor incidence and multiplicity are described in Table 1. Ten colon tumors from AOM-treated A/J mice, 5 AOM-treated AKR/J colons, and 3 normal colon tissues from the vehicle-treated controls from either strain were divided into three macroscopically similar portions. One portion was immediately frozen in liquid nitrogen, total cellular RNA was isolated, and reverse transcription was performed as described earlier (10). The second portion was embedded in cryoprotectant OCT medium and frozen at –80°C for subsequent cryosectioning. The third portion was fixed in 10% neutral buffered formalin for 12 h and
then paraffin-embedded for subsequent histological evaluation and laser-capture microdissection.

**RPA.** mRNA levels for selected genes were quantified using the RiboQuant Multiprobe RPA system from PharMingen (San Diego, CA). Because ARF, mdm2, p21, and HPRT probes were not commercially available, we cloned cDNA fragments (127–548 bp) using the PGEM-T Easy vector system from Promega (Madison, WI). The probes were synthesized according to the manufacturer’s protocol. Total RNA samples (5 μg) were incubated with 32P-labeled probes overnight at 56°C. The samples were then digested by RNase and resolved on 7% denaturing gels. Gels were dried and exposed to X-ray film at −70°C. Image densitometry was performed using the NIH image software package.

**Immunostaining.** Frozen colon sections (5 μm) embedded in OCT from A/J mice were incubated at 4°C overnight with rabbit polyclonal ARF antibody (Novus Biologicals, Littleton, CO) at a dilution of 1:50. For 53 immunohistochemistry, formalin-fixed, paraffin-embedded colon sections from A/J mice were incubated with rabbit polyclonal p53 CM5 antibody (Novacstra, Newcastle-upon-Tyne, United Kingdom) at 1:500 dilution. Sections were subsequently processed as described earlier (10). As a negative control, duplicate sections were immunostained with rabbit IgG instead of the primary ARF and p53 antibody.

**Laser Capture Microdissection for DNA Sequencing and RNA Expression Analyses.** Entire colons were fixed in 10% formalin for 4 h, and multiple sections with appropriate representations of neoplastic and abutting normal tissue from three A/J mice were sectioned and paraffin-embedded. Serial 0.5-μm sections were stained with H&E, and slides were stored in a desiccator until used for laser capture microdissection. Pure populations of cells from colon tumors and morphologically normal colon crypts adjacent to tumors were laser-captured using a 75 mW, 850 nm laser-capture microdissection system (Arcturus, Madison, WI). The probe was synthesized according to the manufacturer’s protocol. Total RNA samples (5 μg) were incubated with 32P-labeled probes overnight at 56°C. The samples were then digested by RNase and resolved on 7% denaturing gels. Gels were dried and exposed to X-ray film at −70°C. Image densitometry was performed using the NIH image software package.

**RNA Extraction, Linear Amplification of Laser-captured Cells, and cDNA Microarray from Microdissected Samples.** Total RNA from laser-captured tumor cells and vehicle-treated control colons were extracted using the Picopure RNA isolation kit (Arcturus) according to the manufacturer’s specifications. The extracted RNA was subjected to two rounds of linear amplification using the RiboAmp RNA amplification kit (Arcturus). A two-round linear amplification of RNA from each of the two samples, were combined and hybridized to a 15 K mouse cDNA array, obtained from the Kek Core Facilities (Yale University, New Haven, CT). The glass slides were scanned using a model 418 dual wavelength scanner (Affymetrix, Inc., Santa Clara, CA), and two grayscale images corresponding to the Cy5 or Cy3 signal were generated for each array. The TIFF images were imported into the Glems 3.0 software (Nutech Sciences, Stafford, TX) and analyzed for expression. The level of red versus green fluorescence at each of the arrayed spots directly reflected the mRNA levels for the particular gene within the tumor relative to the normal colon tissue and is denoted by a ratio as shown in Table 2.

**Sequencing Analysis of p53 and ARF.** Ten A/J tumors and 3 vehicle-treated controls were used for the p53 and ARF sequence analyses. The primers used for amplification of genomic segments of the p53 gene from laser-captured cells were as follows: (a) sense, 5'-TACCTCTCCTCCCT-CAA-3' and antisense, 5'-GGCTCATAAGGTACCACCCAC-3' for a 370-bp product of exon 5, intron 5, and exon 6; and (b) sense, 5'-GGCTCTCGAG-TATACCACCAT-3' and antisense, 5'-GGAGTCTTCCAGTGTTGATGA for a 110-bp product of exon 7 (28). The PCR conditions consisted of 1 cycle at 94°C for 3 min; 34 cycles at 94°C for 1 min; 55°C for 1 min; and 72°C for 2 min. PCR products were generated in a volume of 25 μl containing 10 μl of the template DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl2, 0.2 μM each of dATP, dGTP, dCTP, and dTTP, 0.25 μM each of forward and reverse primers, and 1.25 units of Taq DNA polymerase (Life Technologies, Inc., Gaithersburg, MD). Two overlapping fragments spanning the coding sequence of the p53 gene (nucleotides 106–929 and 459-1377) including all 11 exons of the gene, were PCR-amplified using sense 5'-ggcctccatctcctcc-3' and antisense 5'-ccctccagaggttcctc-3'; sense 5'-tgctccacggtgcctc-3' and antisense 5'-tacgcctgtagataagca-3' primers were designed, respectively, using the Primer 3 program (Massachusetts Institute of Technology, Boston, MA). For ARF, one fragment spanning the entire coding sequence (nucleotides 56-624) was PCR amplified from cDNA of A/J mouse colon tumors using primers as described previously (29). PCR products were generated in a volume of 50 μl containing 4 μl of the template cDNA, 43.5 μl of Taq PCR Master Mix kit (Qiagen Inc., Valencia, CA), containing Taq DNA polymerase, 2× Qiagen PCR buffer, 3 mM MgCl2, 400 μM each dATP, dGTP, dCTP, and dTTP, and 0.25 μM each of forward and reverse primers. The PCR conditions consisted of 1 cycle at 95°C for 5 min; 30 cycles at 95°C for 30 s; 62°C for 1 min; and 72°C for 2 min, followed by a final extension step at 72°C for 5 min. All of the PCR products (ARF and p53 products) were electrophoresed through a 2% agarose gel in 0.5× Tris-borate EDTA buffer. The PCR-amplified bands were excised and purified with Qiaquick Gel Extraction kit (Qiagen Inc.), and a direct sequencing reaction was carried out using the antisense primers for PCR-amplified p53 and ARF, and the Big Dye Terminator sequencing reaction kit. Sequencing products were purified and run on an ABI prism 377 sequencer (Applied Biosystems, Foster City, CA). Sequences from control and tumor samples were aligned and compared with their respective reference sequence from GenBank using Sequencer software (Gene Codes Corp., Ann Arbor, MI). The sensitivity of our sequence analysis is supported by the fact that we have reported previously the detection of K-ras mutations within ~20% of the AOM-induced A/J tumors using the same method (12).

**Immunoblotting.** Tissue samples from AOM- and vehicle-treated mice were isolated and rinsed with cold HBSS-10 mM DTT and incubated with...
HBSS-10 mM EDTA on ice for 1 h with constant agitation. Colon sections were centrifuged at 5000 rpm for 10 min, and resultant pellets were collected after washing twice with cold PBS. The cells were then lysed by incubation at 4°C for 8 min with Buffer A (cytoplasmic extract) and Buffer C (nuclear extract) as described previously (30). Protein concentrations of nuclear and cytoplasmic extracts were measured by the Bio-Rad assay (Bio-Rad Laboratories, Hercules, CA) and stored at −80°C. Nuclear protein (30 μg) was denatured under reducing conditions, resolved on 12% SDS polyacrylamide gels, and transferred to nitrocellulose by voltage gradient transfer. Specific proteins were detected with appropriate antibodies using enhanced chemiluminescence for detection following the manufacturer’s recommended protocol (Santa Cruz Biotechnology, Santa Cruz, CA). The p53 monoclonal antibody AB-1 (Oncogene Research Products, San Diego, CA) that recognizes amino acid residues 371–380 of the COOH-terminal domain of p53 was used at a dilution of 1:1000. Recombinant human p53 protein with an arginine 273 mutation was used as a positive control for immunoblotting (Oncogene Research Products).

**YAMC Cell Culture.** The conditionally immortalized YAMC was cultured in RPMI 1640 with Glutamax (Life Technologies, Inc.) supplemented with 5% fetal bovine serum and 1% insulin-transferrin-selenium-linoleic acid. The medium was supplemented with 5000 units/liter of recombinant IFN-γ because the temperature-sensitive mutant SV40 large T-antigen gene (tsA58) is under the control of an IFN-inducible promoter, the mouse H-2K class 1 gene. Cells were cultured under permissive growth conditions (33°C plus IFN-γ) to maintain activity and expression of the large T antigen or nonpermissive growth conditions (37°C without IFN-γ) to inactivate large T-antigen activity (31).

**Electromobility Shift Assay.** Nuclear extracts were prepared from YAMC cells and tissues for subsequent DNA binding reactions following a protocol reported previously (30, 32, 33). A double-stranded p53 DNA oligonucleotide (Santa Cruz Biotechnology) was end-labeled with [32P]dCTP (3000 Ci/mmol at 10 mCi/ml) using T4 polynucleotide kinase. Binding reactions were performed by mixing 7.5 μg of nuclear extract (in 7.5 μl) with 2.5 μg poly(dI-dC) and 1 μg BSA to give a final volume of 14 μl. After a 15-min incubation on ice, 40 fmol of labeled oligonucleotide (1 μl) was added to each reaction. Reactions were transferred to room temperature for an additional 15 min and then separated on a 4% polyacrylamide/Tris-borate EDTA gel. For supershift experiments, 0.75 μl of antibody was preincubated with cellular extract on ice for 30 min. After this preincubation, the binding reaction was performed as described above. AB-1 p53 monoclonal antibody from Oncogene Science and the p65 C-20 antibody from Santa Cruz Biotechnology were used as the antibodies for supershifting. For DNA competition experiments, a 50-fold molar excess of unlabeled oligonucleotide was added to the extract, which was then incubated on ice for 30 min. After this incubation, the DNA binding reaction was performed as described above. The oligonucleotides used were as follows: a consensus and mutated p53 binding site (Santa Cruz Biotechnology) or an NFeB consensus oligonucleotide (Promega).

**Statistical Analysis.** The generalized linear model using SAS software was used to determine the overall treatment (AOM) effect across the strains. Significant differences in the expression levels of the genes within each strain were determined by the PDIFF between the means. A P < 0.05 was considered statistically significant. For the microarray analyses, ratios >1.5 and <0.5 were considered significant.

**RESULTS**

**Histopathologic Examination.** H&E-stained sections were examined microscopically to confirm the histology of each type of lesion. The AOM-induced tumors that formed in A/J colons were mostly noninvasive, exophytic adenomas and *in situ* adenocarcinomas (Fig. 1). The number of tumors observed within the A/J distal colons are shown under Table 1. Colonic mucosa from untreated control A/J mice and colonic mucosa from untreated control A/J mice were histologically normal. We also analyzed colon tissue from a mouse strain resistant to AOM-induced carcinogenesis, the AKR/J strain. No tumors appeared in either control or AOM-treated AKR/J mice (data not shown). The AOM-treated AKR/J tissue is used as a tumor-free, AOM-treated control to determine which tissue changes are directly related to tumorigenesis.
expression levels would suggest a concomitant increase in p53 protein levels. Therefore, we evaluated the levels of p53 in tumor cells by immunoblot and immunohistochemical analyses. As shown in Fig. 3A, p53 protein was highly expressed in nuclear extracts prepared from A/J tumors, relative to vehicle-treated control colons from A/J mice. In addition, Fig. 3B demonstrates increased accumulation of p53 protein within the tumor cell nuclei. Minimal levels of p53 protein were found in the resistant AKR/J colons (with or without AOM treatment; Fig. 3A). Similar lack of staining was observed within the normal-appearing crypts abutting the tumor crypts within the A/J colon (Fig. 3B), indicating that ARF expression and p53 stabilization occur almost exclusively in transformed tumor cells.

Because a high level of p53 protein in tumors is generally associated with mutational inactivation (34–36), sequence analysis of the entire coding region of the p53 gene (exons 1–11, 107-1377 nucleotides) from 10 A/J tumors was successfully performed using direct PCR-based sequencing. No mutational alterations were observed in the tumors isolated from A/J mice. In addition, laser-capture microdissection of tumors followed by PCR-based sequencing of exons 5–7 of genomic p53 was performed to determine whether subpopulations of tumor cells acquired p53 mutations (Fig. 4, A and B). No carcinogen-induced alterations were found, even in the most pleomorphic cells within the tumor mass. The mechanism of p53 stabilization in these tumors is not clear, but it does not appear to involve the loss of mdm2 expression. In fact, we observed an increase in mdm2 mRNA levels within A/J tumors (Fig. 5A).

Functional Analysis of p53 in AOM-induced A/J Tumors. To additionally evaluate the transactivation function of the p53 protein found in A/J tumors, we measured the expression levels of p21, a cyclin-dependent kinase inhibitor of which the transcriptional activation is dependent on functional p53 (37–40). As shown in Fig. 5B, p21 mRNA levels in A/J tumors, assessed by RPA, were significantly lower (40% of control; *P* < 0.05) than in vehicle-treated controls. No such carcinogen-induced alterations were found in AKR/J colons. Down-regulation of p21 mRNA associated with elevated levels of p53 suggests that the p53 expressed in the A/J tumors may not be functional in transcriptional activation. This finding can be contrasted with results obtained with YAMC cells, which have a functional p53 protein that can be activated by increasing their growth temperature from 33°C to 37°C or higher (which inactivates a temperature-sensitive SV40 T antigen expressed in these cells; Ref. 31). YAMCs show robust activation of p21 and mdm2 expression resulting from the disruption of the SV40 large T antigen-p53 interaction (Fig. 6). In addition, we indirectly evaluated the functional status of the p53

Fig. 2. ARF status in A/J mouse colon epithelium. A, RPA-based expression analysis of ARF mRNA in AOM and saline-treated A/J and AKR/J mice. HPRT was used as a loading control for normalization. Statistical analysis was performed using the generalized linear model procedure followed by PDIFF post-hoc analysis for comparing the means. Each column represents the mean of each treatment group; bars, ± SE, * indicates a significant difference (P < 0.05) in the mRNA levels between control and tumors within A/J colons. B, immunohistochemical analysis of ARF expression in AOM-induced A/J colon tumor (×1000). ARF staining can be visualized as dark staining discrete bodies within the nucleus of the tumor crypt. C, immunohistochemical analysis of ARF expression in the colon from a vehicle-treated A/J mouse (×1000).

Fig. 3. p53 status in A/J mouse colon epithelium. A, immunoblot analysis of p53 protein levels in mouse colon epithelium. Nuclear extract prepared from AOM and vehicle-control mice sacrificed 24 weeks after the last treatment was separated with 12% SDS-PAGE and immunoblotted with anti-p53 monoclonal antibody. P (positive control), recombinant human p53 protein with codon 273 missense mutation. B, immunohistochemical analysis of p53 protein in AOM-induced A/J colon tumor (×200). Positive staining nuclei are seen within the tumor crypts (arrow) whereas the abutting normal appearing colon crypts do not show staining (arrowhead).
protein in tumors by analyzing the expression of a panel of genes that are transcriptionally regulated by p53, including GADD45, 14-3-3, IGFBP3, cyclin G, and Siah1a (41–43) using a cDNA array approach. As described in Table 2, the levels of expression of each of these genes were decreased in tumors relative to the controls. Apart from its transactivation function, p53 has also been shown to transcriptionally repress multiple genes, including MAP4, Stathmin, - and -tubulin, presenilin-1, DNA topoisomerase IIα, Prothymosin α, and HMG-17 (44). We measured the levels of expression of each of these genes by cDNA array and found no decrease in mRNA levels, underscoring the lack of biochemical function of the accumulated p53 within the tumor cells.

To analyze p53 activity in more detail, the electrophoretic mobility shift assay was used to measure the DNA binding activity of p53. For this analysis, the conditionally transformed YAMC cells were used as a control. As reported for other cell types, incubation of nuclear extracts prepared from YAMC cells (cultured at 37°C) with an antibody that binds the p53 COOH terminus stimulates the sequence-specific p53 DNA binding activity in the extracts (Fig. 7A; Ref. 45). The p53-DNA complexes indicated in Fig. 7A were considered to be specific because they could be competed with a 50-fold molar excess of unlabeled p53 oligonucleotide but not by a mutated p53 oligonucleotide or a NFkB-binding oligonucleotide. In addition, the antibody supershift complexes were observed specifically for the p53 antibody and were not formed with an antibody to an NFkB subunit (Fig. 7A). Using these same DNA binding conditions, we analyzed nuclear extracts isolated from control and tumor tissue for p53 binding activity (Fig. 7B). In contrast to YAMC cells, extracts from A/J tumors lacked detectable p53 DNA binding activity, regardless of whether the binding reaction was performed in the presence or absence of p53 antibody (Fig. 7B). The low level of p53 binding activity in tumors was observed even though the p53 levels as assessed by immunoblotting were comparable in YAMC and A/J tumor extracts (Fig. 7C).

To address the possibility that tumor cell extracts may contain an inhibitory factor that prevented p53-DNA binding, we mixed YAMC with tumor protein extracts (1:1) to determine whether the tumor extract inhibited p53 binding activity in the YAMC extract. As shown in Fig. 7D, the tumor extract did not inhibit but marginally stimulated p53-DNA binding activity. This finding indicated that the tumor extract inhibited p53 binding activity in the YAMC extract. As shown in Fig. 7D, the tumor extract did not inhibit but marginally stimulated p53-DNA binding activity. This finding indicated that the tumor extract inhibited p53 binding activity in the YAMC extract.
extracts do not possess a factor that inhibits p53 DNA binding. As with most biochemical analyses, the possibility that cellular extraction is influencing p53 activity cannot be completely ruled out. Nonetheless, all of our current data are consistent with a model in which the suppression of p53 DNA binding activity minimizes its ability to regulate cell proliferation and death in A/J tumors.

**DISCUSSION**

ARF expression is regulated by the activation of hyperproliferative stimuli, including oncogenes such as c-myc, K-ras, and ELA (16–19). Because ARF functions by stabilizing p53 through its inhibition of mdm2-mediated degradation, induction of ARF in response to oncogenic insults results in growth arrest via p53-dependent mechanisms. Thus, dysregulation of any of the components of the ARF-p53 pathway can potentially result in inactivation of its critical oncogene checkpoint. Most of the studies on ARF-p53 pathway have been performed on in vitro cell culture systems (46–48). We wished to determine whether the ARF-p53 checkpoint was deployed and overcome in the more complex environment of an intact, functioning tissue. Therefore, we used the mouse AOM model of colon tumorigenesis. This model provides an excellent experimental system because AOM targets tumor development to the distal colon, a site that is typically affected in humans (4, 5). Moreover, AOM activates oncogenes that are also activated in human colon tumors (7–12). We obtained evidence that the oncogene activation and hyperproliferation associated with AOM-induced tumorigenesis does activate the ARF-p53 pathway, but this checkpoint may be overcome in tumors by the suppression of p53 activity.

ARF expression can be induced by several distinct mechanisms, including the presence of hyperproliferative stimuli or increased degradation of negative regulators of ARF, such as BMI, TWIST, or TBX2 (16–19). In fact, BMI-1, TWIST, or TBX2 have been identified recently as potential oncogenes playing an important role in primary human lymphomas, rhabdomyosarcomas, and breast carcinoma, respectively (51). More importantly (in relation to our observations), altered ARF expression may be directly linked to p53 function (23, 51, 52). It has been shown that p53 activity can suppress ARF expression and loss of functional p53 in mouse embryonic fibroblasts is associated with ARF overexpression (20, 53). The failure to induce p53 in mouse embryonic fibroblasts can even occur in the absence of wild-type p53. Thus, we considered the possibility that in AOM-induced tumors, overexpression of ARF may be a direct consequence of loss of p53 function. As an initial step to address this possibility, we measured p53 status in A/J tumors and found very high protein levels by immunoblot analysis and immunohistochemistry (Fig. 3). High levels of p53 are often associated with mutational inactivation of the p53 protein, resulting in its increased half-life (34–36). However, we found no evidence for mutational alterations in the protein coding sequences of the p53 gene. Although AOM-induced A/J tumors are classified as in situ carcinomas and rarely infiltrate the muscularis mucosa or undergo metastasis, cells within the tumor are markedly pleomorphic with anisokaryosis and anisocytosis (Fig. 1). However, sequencing of genomic p53 from the highly anaplastic laser-captured cells revealed the absence of mutations within exons 5–7. The lack of p53 sequence alterations in these behaviorally benign tumors with many cellular features of late-stage tumors (i.e., anisokaryosis and anisocytosis) was a surprising observation.
With the observed high levels of p53 protein in A/J tumors, we would expect a concomitant increase in the expression of all of the direct transcriptional targets of p53, including p21, GADD45, cyclin G, 14-3-3, IGFBP3, and Siah1a. However, our results indicated a significant decrease (40% of controls; *P < 0.05) of p21, as well as down-regulation of GADD45, cyclin G, 14-3-3, IGFBP3, and Siah1a within tumors. These data additionally indicate that the p53 expressed in the tumors may not be a functional transcriptional activator. We also evaluated the expression levels of a panel of genes on a cDNA microarray that are normally repressed by functional p53, including MAP4, Stat3, α- and β-tubulin, presenilin-1, DNA topoisomerase IIa, Prothymosin α, and HMGI-α, and found no decrease in the mRNA levels. The mRNA expression levels for MAP4 and stat3 were also confirmed by reverse transcription-PCR (data not shown).

Markedly reduced biochemical function of p53 protein within A/J tumors is additionally supported by our finding that p53 DNA binding activity is low in tumors relative to YAMC cells, even though similar levels of protein are present in both cell populations (Fig. 7). One possible explanation for the suppression of wild-type p53 activity in AOM-induced tumors may be differences in post-translational modification of p53. This possibility is supported by recent observations that there is a differential pattern of phosphorylation in human tumors and tumor-derived cell lines harboring wild-type p53 relative to the adjacent normal tissue (26). It has also been observed that the aberrant phosphorylation of the COOH terminus leads to defective transcriptional activation by wild-type p53 within radioresistant human melanoma cells (25). Furthermore, recent studies by Gu and Roeder (27) have shown that acetylation of p53 via its coactivator, p300, dramatically enhances its biochemical function. Additional analyses are required to determine whether post-translational modifications are indeed involved in suppressing p53 activity in AOM-induced tumors. For example, as shown in Fig. 7C, p53 protein from AOM-induced colon tumors was found to migrate more slowly on an SDS gel relative to the active form of p53 found in YAMC cells.

Alterations within the INK4a locus that encodes both ARF as well as the p16 gene (alternatively spliced products) occur in a wide spectrum of human cancers (54). Framed microdeletions/insertions and point mutations within exon 2, shared by both p16 and ARF, have been described in a variety of human tumors (54). Recently, Zhang and Xiong (55) have shown that mutations within exon 2 of ARF can impede its function by altering the nucleocytoplasmic shuttling of mdm2. To our knowledge, no spontaneous mutations have been described within exon 1β that encodes the NH2-terminal moiety of ARF, a region shown to be sufficient to induce cell cycle arrest (20, 56). In the present study, no mutational alterations within the cDNA encoding the entire ARF gene were found in A/J tumors. Furthermore, the high levels of wild-type p53 in AOM-induced tumors is consistent with the presence of functional ARF.

Whereas the p53/ARF circuit is clearly induced in A/J tumors, albeit with only limited functional activity, a fundamental question remains concerning the status of ARF-p53 in the colons of tumor-resistant AKR/J mice. We have reported previously that AKR/J mice sustain Ki-ras activation at early time points in response to carcinogen exposure (12). However, as noted in Figs. 2 and 3, ARF mRNA and p53 protein were below the level of detection in AKR/J colons 24 weeks after AOM treatment. It is possible that the lack of progression of initiated colon cells in AKR/J mice reflects a transient response to an intact ARF-p53 circuit, whereby a growth inhibitory response is activated during early stages of tumorigenesis.

In conclusion, our results describe the dysregulation of a potential oncogene-checkpoint pathway in an in vivo model of colon carcinogenesis. These studies raise some interesting questions regarding the status of the ARF-p53 pathway in AOM-induced tumorigenesis. It is possible that the increased stability of p53 protein in the absence of sequence alterations may be a result of aberrant alterations in the acetylation and/or phosphorylation status of p53 that suppress its binding to DNA consensus sequences. It is also possible that alternative mechanisms may inhibit the transactivation and repression function of p53 protein. Thus, the AOM model provides an in vivo experimental system to additionally elucidate potential mechanisms through which the ARF-p53 circuit is regulated during colon tumorigenesis.

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