Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 10199–10204, September 1997
Cell Biology

Effects of *p53* mutations on apoptosis in mouse intestinal and human colonic adenomas

(colon cancer/genomic instability)

AMIN FAZELI*†, ROBERT G. STEEN†‡, STEPHANIE L. DICKINSON*, DOLORES BAUTISTA§, WILLIAM F. DIETRICH‡, RODERICK T. BRONSON¶, ROBERT S. BRESALIER‖, ERIC S. LANDER‡, JOSE COSTA§, AND ROBERT A. WEINBERG*,**

*Whitehead Institute for Biomedical Research and Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02142; ‡Whitehead Institute/MIT Center for Genome Research, Whitehead Institute for Biomedical Research, One Kendall Square, Cambridge, MA 02139; §Department of Pathology, Yale University School of Medicine, New Haven, CT 06510; ¶Department of Pathology, Tufts University Schools of Medicine and Veterinary Medicine, Boston, MA 02111; and ‖Gastrointestinal Oncology, Henry Ford Hospital, 2799 West Grand Boulevard, Detroit, MI 48202

Contributed by Robert A. Weinberg, June 2, 1997

ABSTRACT **We have examined the effects of inactivation of the *p53* tumor suppressor gene on the incidence of apoptotic cell death in two stages of the adenoma-to-carcinoma progression in the intestine: in early adenomas where *p53* mutations are rare and in highly dysplastic adenomas where loss of *p53* occurs frequently. Homozygosity for an inactivating germline mutation of *p53* had no effect on the incidence or the rate of progression of $Apc^{Min/+}$-induced adenomas in mice and also did not affect the frequency of apoptosis in the cells of these adenomas. To examine the effect of *p53* loss on apoptosis in late-stage adenomas, we compared the incidence of apoptotic cell death before and after the appearance of highly dysplastic cells in human colonic adenomas. The appearance of highly dysplastic cells, which usually coincides during colon tumor progression with loss of heterozygosity at the *p53* locus, did not correlate with a reduction in the incidence of apoptosis. These studies suggest that *p53* is only one of the genes that determine the incidence of apoptotic in colon carcinomas and that wild-type *p53* retards the progression of many benign colonic adenoma to malignant carcinomas by mechanism(s) other than the promotion of apoptosis.**

Studies using cultured cells have ascribed several distinct cell physiologic functions to the *p53* tumor suppressor gene. Its product, the p53 protein, may respond to DNA damage by triggering either growth arrest during G_1 (1) or G_2 (2, 3) phase of the cell cycle or programmed cell death (1). In this manner, p53 may protect the normal cell from proceeding to replicate damaged DNA (1). Conversely, mutation of the *p53* gene may render a cell's genome more mutable, thereby accelerating the accumulation of mutations that represent rate-limiting steps in tumor progression.

In other circumstances, p53 plays a role in mediating cellular senescence, which is hypothesized to be triggered by telomeric collapse (4, 5) or by the activity of the *ras* oncogenes (6). This senescence prevents cells from progressing to an immortalized state. Thus, the inactivation of p53 function during tumor progression may allow evolving premalignant cell populations to circumvent senescence, thereby facilitating progression to a state of immortality (4, 7–10).

Yet, other studies have demonstrated that oncogenes such as *myc* and E1A trigger an apoptotic response in a variety of cultured cells (11–14) that represents an obstacle to the further clonal expansion of these oncogene-bearing cells. In some cells, this apoptotic response has been shown to be p53-dependent (13–19). Hence, an inactivating mutation of *p53* may enable cell populations carrying an oncogenic mutation to avoid attrition through apoptosis and thus allow them to benefit from the growth impetus provided by the mutant oncogene.

Finally, p53-dependent apoptosis is also observed in cells that are anoxic, as might occur in tumors that are inadequately perfused by vasculature (20). Conversely, wild-type p53 may limit tumor growth by up-regulating the expression of angiogenic inhibitors (21). Together, these reports indicate that p53 is an important regulator that enables normal cells to halt growth or to enter apoptosis in response to a variety of physiologic stresses.

We focus here on the contribution of *p53* inactivation to colon cancer. In many human colon carcinomas, loss of *p53* heterozygosity (LOH) occurs as the last in a defined succession of mutations affecting the adenomatous polyposis coli gene (*APC*), the N- or Ki-*ras* protooncogene, a presumed tumor suppressor gene on chromosome 18q, and mutation of one of the two alleles of *p53* gene (22, 23).

This loss of p53 function during a defined step in colonic tumor progression must be rationalized in terms of the known biochemical and physiologic functions of the wild-type *p53* gene and protein. For example, if p53 prevented colonic cells from replicating damaged DNA, one would predict that *p53* inactivation would occur relatively early in tumor progression, thereby increasing the overall rate of mutation and accelerating the advance of colonic cell clones through subsequent stages of this process.

However, loss of p53 at a relatively late stage in colon cancer progression would need to be rationalized by invoking other known biological properties of wild-type p53. For instance, the role played by normal p53 in colonic adenomas could be to induce apoptosis in response to the cumulative effects of mutations affecting the *APC*, *ras*, and other genes. If this were the case, then inactivation of *p53* would benefit tumor progression only at a late stage. Alternatively, p53 could be responsible for inducing senescence following extensive cell division or *ras* oncogene activation. With these models in mind, we examined the effects of *p53* inactivation on an early and an intermediate stage of colonic tumor progression and tested the effects of *p53* inactivation on the incidence of apoptosis in intestinal adenomas.

MATERIALS AND METHODS

Polyp Analysis and Genotyping. C57BL/6 $Apc^{Min/+}$ mice were purchased from The Jackson Laboratory. Min progenies

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: *p53*, *p53* tumor suppressor gene; LOH, loss of heterozygosity; TUNEL, terminal deoxynucleotidyltransferase-mediated UTP end labeling; *Apc*, murine *Adenomatous polyposis coli* gene.

†A.F. and R.G.S. contributed equally to this study.

**To whom reprint requests should be addressed.

were genotyped by PCR as described before (29). 129 $p53^{+/-}$ mice and the DNA probe used for LOH analysis at the mouse *p53* locus were gifts of Tyler Jacks (Massachusetts Institute of Technology, Cambridge, MA). Mice were genotyped for the *p53* locus by PCR using the *p53*×6S (5′-TTATGAGCCACCCGAGGT-3′) or the $p53^{-}$-specific primer Neo 18.5 (5′-TCCTCGTGCTTTACGGTATC3-′) and the downstream primer *p53*×7AS (5′-TATACTCAGAGCCGGCCT3-′). Polyp count and histological analyses were performed as described previously (53). For loss of heterozygosity LOH analysis, adenomas were removed surgically and DNA was prepared from them using standard methods. Southern blot analysis was performed as described previously (27). For γ-irradiation experiments, mice were subjected to 8 grays of γ-irradiation using a cesium source. Eight hours later the mice were euthanized and their intestines were fixed in formalin and prepared for cell death analysis.

Statistical Analysis. Polyp multiplicities were compared using a modification of the Wilcoxon's Sum of Ranks Test as described by H. Mann and D. Whitney (54). The incidence of apoptotic cells in adenomas was compared using the Student's *t* test.

Quantifying the Apoptotic Cells. Terminal deoxynucleotidyltransferase-mediated UTP end labeling (TUNEL) assay was performed on 5- to 6-μm paraffin-embedded sections of adenomas or normal intestines. To do this, the sections were deparaffinized in Xylenes (Sigma) (2 × 8 min) and dehydrated through sequential steps of 100% ethanol (2 × 3 min), 90% ethanol (3 min), 70% ethanol (3 min), and distilled H_2O (3 min). Each tissue section was then treated with 100 μl of pepsin (Sigma) (0.5% V/W in H_2O), incubated at 37°C (60 min in humid air), and washed in PBS^{-} (3 × 3 min). The samples were then treated with Saponin (0.055% V/W in H_2O, 30 min) at room temperature and washed with PBS^{-} (3 × 3 min). Then, the samples were incubated in 3% H_2O_2 at room temperature for 60 min and washed in PBS^{-} (3 × 3 min). The samples were treated with the terminal transferase mix [terminal deoxytransferase enzyme (TdT, GIBCO/BRL), TdT buffer (GIBCO/BRL), biotin-16-dUTP (Boehringer Mannheim), and H_2O] and incubated at 37°C (60 min in humid air). The reactions were terminated by adding the TdT reaction termination buffer (15 min) and washing the samples in PBS^{-} (3 × 3 min). The nuclei that had incorporated the biotinylated dUTP were detected using DAB (SK4100, Vector Laboratories) and ABC Kits (PK4000, Vector Laboratories). The samples were washed in PBS^{-} (2 × 3 min), dehydrated through sequential steps of 70 and 100% ethanol (3 min each), dried, and mounted.

Counts of the apoptotic cells were performed using a light microscope at ×1,000 magnification. Only the TUNEL-positive epithelial cells were counted; the mesenchymal cells were excluded from our analyses. The fractions of the sloughed cells that remained in the tissue preparation varied from one preparation to the next and were therefore excluded from the counts. Only the apoptotic epithelial cells that remained a part of the solid tissue were counted. The TUNEL-positive cells had the appearance of apoptotic cells described previously (33). The apoptotic cells appeared shrunken and fragmented and often had pyknotic nuclei. A "halo" was often seen around the TUNEL-positive cells.

Human Colonic Adenomas. Adenomas in Table 2 were obtained from sequential lists of resected tumors from the Ann Arbor Veterans Affairs Medical Center and the Henry Ford Hospital (Detroit) and fixed in formalin soon after removal. Microallelotyping of these adenomas using markers on chromosome 17p were reported (42). (These tumors and the detailed clinical and histopathological data regarding these tumors are available upon request from R.S.B.)

RESULTS

p53 and Initiation of Mouse Intestinal Adenomas. To examine the effect of *p53* inactivation on colonic epithelial cells that are still at a relatively early stage in tumor progression, we used mice of the Min strain (24), in which the first of the mutational steps involved in colonic tumor progression—mutation of the *Apc* gene—is already present in all colonic epithelial cells. Because of the germ line Apc^{Min} mutation carried by these mice (25), these mice are strongly predisposed to intestinal adenomas (polyps) (24, 26). To determine the effects of mutations of *p53* on the initiation and development of adenomas in these mice, we bred a mutant allele of the *p53* gene (27) into the germ line of $Apc^{Min/+}$ mice.

Thus, 129 $Apc^{+/+}$ $P53^{+/-}$ (27) mice were crossed to C57BL/6 $Apc^{Min/+}$ $p53^{+/+}$ (24) mice to generate 129/B6 F1 $Apc^{Min/+}$ $p53^{+/-}$ progeny. F2 and F3 progeny were generated by breeding F1 or F2 siblings, respectively. Because there was no statistically significant difference ($P > 0.2$) between the average number of adenomas among the F1, F2, and F3 $Apc^{Min/+}$ $p53^{+/+}$ mice (Fig. 1), the effect of *p53* dosage reduction on polyp incidence was gauged in mice of a mixed 129/B6 genetic background.

The entire length of the gastrointestinal tract of these mice was examined, registering both the size and the number of polyps. The great majority of the adenomas in these $Apc^{Min/+}$ mice were found to be localized to the small intestine regardless of the genotype of these mice at the *p53* locus (data not shown). Min mice in this colony rarely developed colonic or gastric adenomas.

Homozygosity for a germ line inactivating mutation of *p53* did not enhance polyp initiation in the small intestines of the Min mice (Fig. 1), confirming previous reports (28). Statistical analysis indicated that the number of adenomas did not increase after 80 days of age in any of the three Min groups in which the Apc^{Min} mutation was present in a $p53^{+/+}$, $p53^{+/-}$, or $p53^{-/-}$ background. Thus, we counted polyps at various ages beyond 80 days and assumed that these numbers accurately reflected the cumulative number of polyps arising in the mice. The $Apc^{Min/+}$ $P53^{-/-}$ mice showed a mean number of adenomas per mouse (94 adenomas, $n = 11$) similar to the $Apc^{Min/+}$ $p53^{+/-}$ (83 adenomas, $n = 26$) and $Apc^{Min/+}$ $p53^{+/+}$ mice (86 adenomas, $n = 29$) (Fig. 1). The difference between the $Apc^{Min/+}$ $P53^{-/-}$ mice and other Min cohorts was not statistically significant ($P = 0.2$). The number of adenomas varied

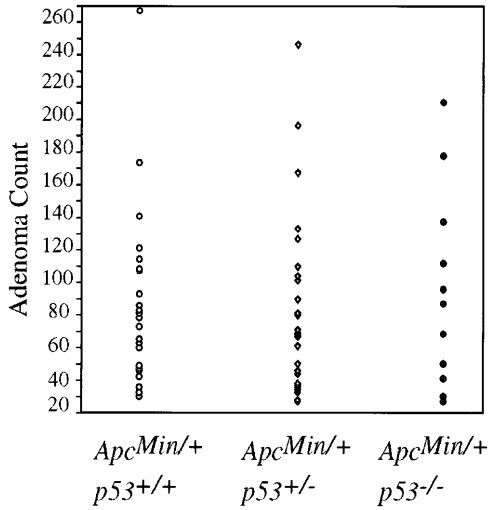

FIG. 1. Effect of *p53* dosage reduction on polyp initiation in the Min mice. The ordinate shows the number of adenomas, and the abscissa shows the genotype of the mice. Each data point represents the number of adenomas observed along the entire length of intestine of a single mouse of 80 days of age or older.

from 30 to 267 in the $Apc^{Min/+}$ $p53^{+/+}$, from 27 to 197 in $Apc^{Min/+}$ $p53^{+/-}$ cohort, and from 27 to 211 in the $Apc^{Min/+}$ $p53^{-/-}$ cohort.

Approximately two-thirds of the $Apc^{+/+}$ $p53^{-/-}$ progeny also developed a single adenoma at the ileocecal junction, but none of the $Apc^{+/+}$ $p53^{+/+}$ or $Apc^{+/+}$ $p53^{+/-}$ mice developed such adenomas (data not shown). We did not further study this effect of *p53* inactivation. Moreover, possible cooperative effects of Apc^{Min} allele as well as the *p53* mutation on tumor initiation in other organs were not examined. $p53^{-/-}$ mice examined were between 80 and 132 days, and the $P53^{+/+}$ and $P53^{+/-}$ groups examined were between 80 and 210 days of age.

We determined the extent of progression of the adenomas that did appear by examining their size and histological and morphological appearance (22). Significantly, the absence of a functional *p53* gene had no effect on polyp progression. We detected no difference in the size of the $p53^{-/-}$, $p53^{+/-}$, or $p53^{+/+}$ adenomas in age-controlled Min mice upon macroscopic examination. Furthermore, histological analysis showed that all adenomas in the Min mice were benign, similar in morphology, and characterized by a similar degree of dysplasia regardless of their genotype at the *p53* locus (data not shown).

The above experiments suggested that elimination of p53 function had little effect either on the initiation of adenomas or their subsequent progression from early- to intermediate-stage polyps. This notion was reinforced by Southern blot analysis performed on DNA samples that were prepared from 20 adenomas of $Apc^{Min/+}$ $p53^{+/-}$ mice. This analysis showed that the adenomas in $Apc^{Min/+}$ $p53^{+/-}$ mice retained both the mutant and the wild-type allele of *p53* even up to 150 days of age (Fig. 2). This retention of the wild-type *p53* allele contrasts with previous analysis of the fate of the wild-type *Apc* allele in adenomas of $Apc^{Min/+}$ mice, which showed that the wild-type *Apc* allele is invariably discarded as adenomas progress (29–31). Hence, elimination of the surviving wild-type *p53* allele conferred no growth advantage on adenomatous cells, whereas loss of the wild-type *Apc* function was indeed advantageous.

We concluded that any effects that loss of p53 function may have on the growth properties of adenomatous cells were not sufficient to influence either the size or morphology of adenomas in the early stages of intestinal tumor progression in the mouse.

p53-Dependent Apoptosis in Apc^{Min}-Induced Adenomas. Although *p53* inactivation had no apparent effect on the overall rate of progression of adenomatous polyps in the Min mice, it remained possible that *p53* loss influenced adenoma development in more subtle ways by affecting the rate of apoptosis of the cells in the polyps. For this reason, we examined the incidence of apoptotic cell death in the *p53*-wild-type and *p53*-deficient benign mouse adenomas.

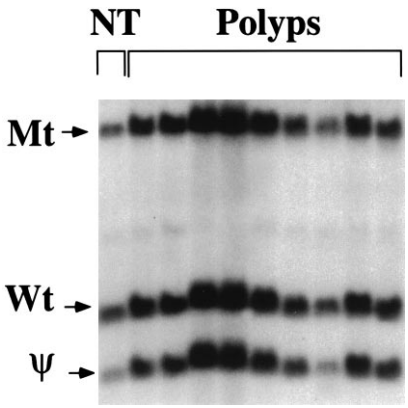

FIG. 2. LOH analysis of the *p53* locus in adenomas from $Apc^{Min/+}$ $p53^{+/-}$ mice. The first lane from the left is the constitutional DNA from the normal tissue (NT) of a $Apc^{Min/+}$ $p53^{+/-}$ mouse. Lanes 2–10 are adenomas from 5-month-old $Apc^{Min/+}$ $p53^{+/-}$ mice. The top arrow points to the mutant (Mt) 3.0-kb band, and the middle arrow indicates the 1.3-kb wild-type (Wt) band. The lowest arrow marks the 1.0-kb fragment that appears due to hybridization of the probe to DNA corresponding to a *p53* pseudogene.

In the normal intestine, the proliferative zone is found in the crypts and in the base of the villi and the apoptotic zone is largely in the tips of the villi (32). In addition, a small fraction of epithelial cells in the proliferative zone is also seen to be in the process of undergoing apoptotic cell death (33–35). These apoptotic cells of the proliferative zone, to the extent that they can be observed, are largely confined to the crypts of the intestinal epithelium (33–35). The presence of the apoptotic cells of intestine can be demonstrated on thin paraffin-embedded sections of this tissue using the TUNEL assay (36).

We first tested the sensitivity of the TUNEL assay, as used by us, in two circumstances in which apoptosis is a frequent occurrence. Macrophages that have just engulfed apoptotic cells can be readily identified upon microscopic examination of the Peyer's patches of the mouse intestine (37). All nuclei of the apoptotic cells that were engulfed by macrophages registered positive in our TUNEL assay in several formalin-fixed sections of Peyer's patches (data not shown).

We also verified the sensitivity of our assay by examining the apoptotic cells in irradiated intestines. Gamma-irradiation of $p53^{+/+}$ intestines is known to result in the appearance of many cells with pyknotic nuclei in the normal crypts (34, 35). In several experiments, all of the intestinal epithelial cells having pyknotic nuclei following 8 grays of ionizing radiation registered positively in our TUNEL assay (Fig. 3). We also extended the reports of others (34, 35) that this γ-irradiation-induced apoptosis is p53-dependent, in that both the crypts and the adenomas of $Apc^{Min/+}$ $p53^{-/-}$ intestines displayed a low rate of apoptosis similar to that of nonirradiated controls (Table 1; Fig. 3).

We then used the TUNEL assay, as validated above, to gauge apoptotic rates in $p53^{+/+}$ and $p53^{-/-}$ adenomas. Our analyses showed that on average, 1.5% of the epithelial cells of adenomas were undergoing the process of apoptotic cell death at any time (Table 1). In contrast to the effects of p53 on radiation-induced apoptosis, the presence or absence of p53 had no obvious effect on the incidence of apoptotic cells in the nonirradiated early adenomas ($P = 0.3$, 1.5% in $p53^{+/+}$ vs. 1.6% in $p53^{-/-}$, Table 1 and Fig. 3*B*). Thus, *p53* loss did not affect development of early adenomas through its ability to suppress apoptosis. This was in consonance with our earlier observations indicating that *p53* loss had no effect on the growth rate or histopathology of these early adenomas.

***p53* Inactivation and Apoptosis in Late-Stage Human Colonic Adenomas.** In human colonic adenomas, LOH at the *p53* gene coincides with the appearance of highly dysplastic cells within adenomas. These highly dysplastic nodules are often found to arise within large, less dysplastic, intermediate-grade adenomas. This observation has suggested that *p53* mutation plays an important role in a relatively late step of colon cancer progression by enabling intermediate-grade adenomas to progress to those having a highly dysplastic phenotype. The highly dysplastic regions of polyps, which are presumably the immediate precursors of colon carcinomas, are rarely encountered in mice. For this reason, we turned to human biopsy samples and compared highly dysplastic nodules with closely apposed, less dysplastic precursors, hoping to gauge the influence, if any, of *p53* loss on this progression.

Loss of p53 function in colonic tumors involves two genetic steps. In the first of these, p53 function is partially lost through the appearance of a mutant, dominant-negative allele (38, 39); the protein product of such an allele has lost its ability to mediate wild-type p53 signaling but gained the ability to compromise most but not all of the functioning of the wild-type protein, which continues to be expressed in heterozygous cells. Residual wild-type p53 function can then be totally eliminated

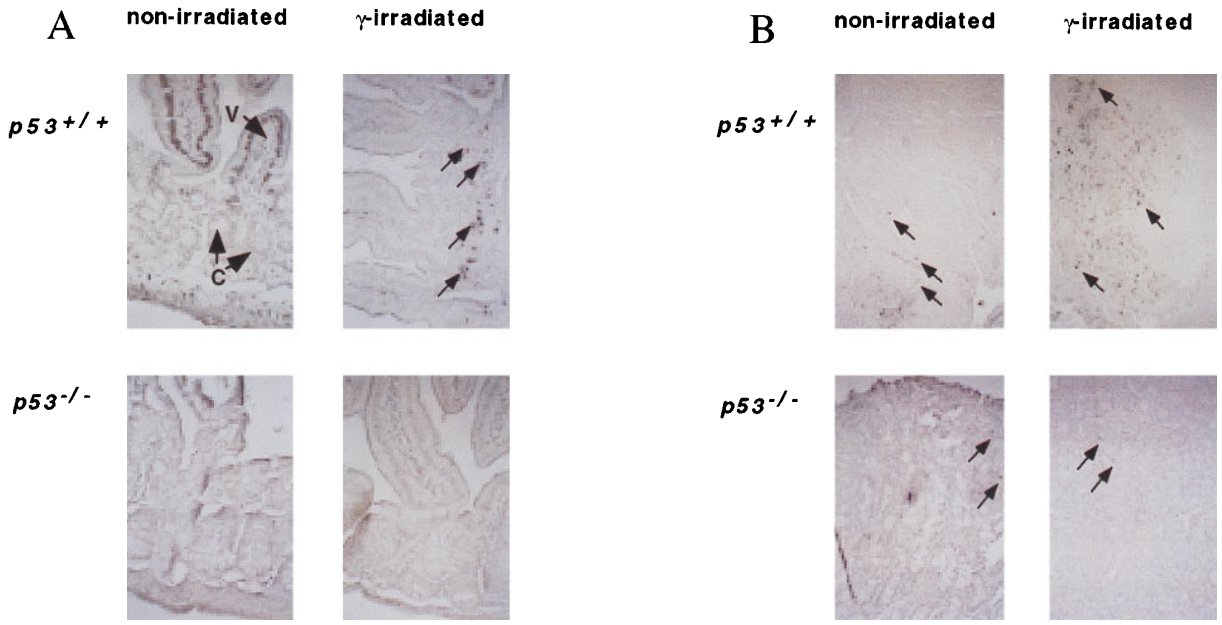

FIG. 3. Effects of *p53* inactivation and γ-irradiation on apoptosis in intestine of the Min mice. (*A*) Normal small intestine (×400 magnification). (*B*) Small intestinal adenomas (×200 magnification). Intestines were prepared and the apoptotic cells were identified using the TUNEL assay. C and V refer to the crypt and villus, respectively. Arrows mark some of the apoptotic cells.

through the loss of the surviving wild-type allele and duplication of the already mutant allele. This second step is characterized by the LOH of chromosome 17p on which the *p53* gene is located (38, 40, 41).

LOH at the *p53* locus is detectable in the highly dysplastic regions of about 40% of human colonic adenomas (42). By the time the subsequent conversion to carcinoma is complete, LOH of the *p53* gene is detectable in an additional 20% of colonic growths (42). We focused our attentions on the subset of colonic growths in which LOH of *p53* gene accompanied the transition to a highly dysplastic phenotype and asked whether *p53* inactivation was accompanied by a change in apoptotic rates.

We measured the incidence of apoptotic cell death in a panel of eight adenomas having distinct regions within them that displayed high grades of dysplasia (Table 2). Previously published microallelotyping analyses (42) had demonstrated that in this panel of eight adenomas, LOH of markers on chromosome 17p (including the *p53* gene) had occurred in the regions with high grades of dysplasia, whereas the regions with low grades of dysplasia were still heterozygous for loci on chromosome 17p.

TUNEL analysis showed no statistically significant difference between the incidence of apoptotic cell death before and after the LOH at chromosome 17p in these adenomas ($P = 0.14$, Table 2). Thus, two of these adenomas showed an increase, two showed no change, and four showed a decrease in the incidence of apoptosis following the loss of *p53*.

As mentioned above, LOH of the *p53* gene is detectable in only 40% of the adenomas during the transition to the highly dysplastic phenotype. Analysis of a panel of six adenomas from the group with no detectable *p53* LOH revealed that the incidence of apoptosis decreased in five of these adenomas during the transition to a highly dysplastic phenotype even though the *p53* LOH could not be detected during this transition (Table 2). Indeed, the degree of reduction of apoptosis was comparable to that seen in the adenomas that did suffer LOH. In sum, the transition of adenomas to a highly dysplastic phenotype is often associated with a decrease in the incidence of apoptosis, but this change could not be correlated with LOH of the *p53* gene.

DISCUSSION

Mice predisposed to adenomatous polyposis through mutation of the *Apc* gene sustain multiple intestinal adenomas in the first 3 months of life (24). Our initial studies attempted to gauge the effects, if any, of inactivation of p53 function on the appearance and subsequent development of these adenomatous polyps. Our results indicate that polyp number and growth were not affected by the absence of functional alleles of the *p53* gene in the 4-month life span of the $p53^{-/-}$ mice. We concluded that, to the extent that *p53* mutation plays a role in colon cancer progression, such a role becomes important only in later stages.

Table 1. Rate of apoptosis in both normal crypts and adenomas of γ-irradiated and nonirradiated mice

	Normal crypts		Adenomas	
P53	Nonirradiated	γ-Irradiated	Nonirradiated	γ-Irradiated
+/+	1.4%* (n = 10)†	11.3% (n = 10)	1.5% (n = 9)	17% (n = 11)
−/−	0.5% (n = 17)	0.9% (n = 8)	1.6% (n = 25)	2.7% (n = 12)

*Numeric values represent mean percentage of epithelial cells that were apoptotic as determined by a combination of the TUNEL assay and histological analysis. Percentage of apoptotic cells was measured for each segment of normal intestine or each adenoma by examining five independent, nonoverlapping regions.

†*n* is the number of adenomas or number of segments of the normal intestine where apoptotic cells were measured.

Table 2. Effect of loss of *p53* on the incidence of apoptotic cell death in human colonic carcinomas

Tumor name in Boland *et al.* (42)	17p LOH	Apoptotic cells† LGD	Apoptotic cells† HGD
2, Right	+	0.60%	0.51%
4, Right	+	0.85%	3.35%
11, Left	+	0.53%	0.63%
8, Left	+	1.20%	0.51%
13, Left	+	0.46%	0.82%
17, Left	+	1.38%	0.38%
22, Left	+	0.95%	0.57%
29, Left	+	1.70%	0.57%
3, Right	−	2.35%	1.30%
15, Left	−	0.91%	0.87%
10, Left	−	2.17%	1.11%
28, Left	−	4.48%	0.35%
20, Left	−	1.30%	0.97%
21, Left	−	2.04%	1.52%

*+ indicates that LOH of loci on chromosome 17p, including the *p53* gene, was detected in the highly dysplastic regions (HGD or high-grade dysplasia) of adenoma but not in the neighboring regions of the same adenomas with a lower degree of dysplasia (LGD or low-grade dysplasia) in a previously reported study (42). − indicates that LOH of chromosome 17p was not detectable in regions with either high or low grades of dysplasia.

†The numeric values represent the mean percentage of the epithelial cells that were apoptotic. Percentage of apoptotic cell was determined for 10 independent, nonoverlapping segments of the adenoma or the carcinoma regions using a combination of TUNEL and histological characterization performed on two tissue sections of each tumor.

P53 mutations rarely occur in cells of human colonic adenomas having a low to intermediate degree of dysplasia but frequently occur in adenomas as they progress to a high grade of dysplasia (42). This inactivation of *p53* at a relatively late stage of tumor progression might be explained as a necessary response to mutations sustained previously in other growth-regulating genes, including protooncogenes and tumor suppressor genes. Thus, earlier mutations in these growth-regulating genes might provoke apoptosis in colonic epithelial cells, as has been shown for other types of cells (13–15, 17, 18, 43–47), which may then be countered by the inactivation of their *p53* gene.

The results of the present study argue against such a model. We have gathered evidence from human adenomas that causes us to propose that avoidance of apoptosis, as mediated by *p53* mutation, is not the primary effect of the loss of *p53* in intestinal adenomas. Our data show that the transition of intermediary adenomas to late-stage adenomas often correlates with suppression of apoptosis, but this reduced incidence of cell death occurs independently of LOH of the *p53* gene, and in some tumors loss of *p53* correlates with an increase in the incidence of apoptosis. These data suggest that *p53* status is not the sole determinant of apoptosis in intestinal adenomas.

The conclusion that *p53* may be only one of several genes that determine the incidence of apoptosis in colonic tumors is supported by the studies of Polyak *et al.* (48) that showed that the status of other genes, especially the P21 gene, determines the effect of p53 on apoptosis in colon carcinomas. Another gene that is likely to affect apoptosis in intestinal adenomatous cells and colonic carcinomas is the BAX gene, which is mutated in 50 percent of colon carcinomas with the microsatellite mutator phenotype (49). One study showed that even colon cancer cell lines with BAX mutations can be assigned to different apoptosis complementation groups (49). This further supports the idea that apoptosis in colon cancer is determined by genes of several different pathways (50).

Together with the data in the present study, these observations redirect attention to other functions ascribed to normal p53, including, notably, p53's ability to mediate cellular senescence. One attractive possibility is that the mutation of *ras* or other growth-regulating genes that occurs early in colon tumor progression might provoke a senescence in adenomatous cells that may be countered by the subsequent inactivation of the *p53* gene (50). Such *p53* inactivation may then confer other benefits. Thus, the *p53*-dependent senescence associated with the excessive generational doublings may be circumvented, permitting cells to proceed to crisis and immortalization (6). Subsequently, *p53* inactivation may allow other consequences of *p53* loss such as increased angiogenic potential (21), an uncoupling of DNA synthesis from mitosis (51), and increased genomic instability (42, 52) to conspire to accelerate the progression that leads to malignant colonic tumors.

We thank Drs. C. Richard Boland and Andrew Feinberg for the gift of tumors and for helpful discussions. R.A.W. is a Research Professor of the American Cancer Society and receives support from National Cancer Institute OIG Grant R35 CA39826.

1. Lane, D. P. (1992) *Nature (London)* **358,** 15–16.
2. Agarwal, M. L., Agarwal, A., Taylor, W. R. & Stark, G. R. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 8493–8497.
3. Stewart, N., Hicks, G. G., Paraskevas, F. & Mowat, M. (1995) *Oncogene* **10,** 109–115.
4. Bond, J. A., Wyllie, F. S. & Wynford-Thomas, D. (1994) *Oncogene* **9,** 1885–1889.
5. Bond, J., Haughton, M., Blaydes, J., Gire, V., Wynford-Thomas, D. & Wyllie, F. (1996) *Oncogene* **13,** 2097–2104.
6. Shay, J. W. & Wright, W. E. (1996) *Trends Genet.* **12,** 129–131.
7. Harvey, D. M. & Levine, A. J. (1991) *Genes Dev.* **5,** 2375–2385.
8. Harvey, M., Sands, A. T., Weiss, R. S., Hegi, M. E., Wiseman, R. W., Pantazis, P., Giovanella, B. C., Tainsky, M. A., Bradley, A. & Donehower, L. A. (1993) *Oncogene* **8,** 2457–2467.
9. Shay, J. W., Tomlinson, G., Piatyszek, M. A. & Gollahon, L. S. (1995) *Mol. Cell. Biol.* **15,** 425–432.
10. Metz, T., Harris, A. W. & Adams, J. (1995) *Cell* **82,** 29–36.
11. Evan, G. I., Wyllie, A. H., Gilbert, C. S., Littlewood, T. D., Land, H., Brooks, M., Waters, C. M., Penn, L. Z. & Hancock, D. C. (1992) *Cell* **69,** 119–128.
12. Rao, L., Debbas, M., Sabbatini, P., Hockenbery, D., Korsmeyer, S. & White, E. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 7742–7746.
13. Qin, X. Q., Livingston, D. M., Kaelin, W., Jr. & Adams, P. D. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 10918–10922.
14. Wu, X. & Levine, A. J. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 3602–3606.
15. White, E., Sabbatini, P., Debbas, M., Wold, W. S., Kusher, D. I. & Gooding, L. R. (1992) *Mol. Cell. Biol.* **12,** 2570–2580.
16. Lowe, S. W., Ruley, H. E., Jacks, T. & Housman, D. E. (1993) *Cell* **74,** 957–967.
17. Lowe, S. W., Jacks, T., Housman, D. E. & Ruley, H. E. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 2026–2030.
18. Hermeking, H. & Eick, D. (1994) *Science* **265,** 2091–2093.
19. White, E. (1994) *Nature (London)* **371,** 21–22.
20. Graeber, T. G., Osmanian, C., Jacks, T., Housman, D. E., Koch, C. J., Lowe, S. W. & Giaccia, A. J. (1996) *Nature (London)* **379,** 88–91.
21. Dameron, K. M., Volpert, O. V., Tainsky, M. A. & Bouck, N. (1994) *Science* **265,** 1582–1584.
22. Vogelstein, B., Fearon, E. R., Hamilton, S. R., Kern, S. E., Preisinger, A. C., Leppert, M., Nakamura, Y., White, R., Smits, A. M. & Bos, J. L. (1988) *N. Engl. J. Med.* **319,** 525–532.
23. Fearon, E. R. & Vogelstein, B. (1990) *Cell* **61,** 759–767.
24. Moser, A. R., Pitot, H. C. & Dove, W. F. (1990) *Science* **247,** 322–324.
25. Su, L. K., Kinzler, K. W., Vogelstein, B., Preisinger, A. C., Moser, A. R., Luongo, C., Gould, K. A. & Dove, W. F. (1992) *Science* **256,** 668–670.
26. Moser, A. R., Dove, W. F., Roth, K. A. & Gordon, J. I. (1992) *J. Cell Biol.* **116,** 1517–1526.
27. Jacks, T., Remington, L., Williams, B. O., Schmitt, E. M., Halachmi, S., Bronson, R. T. & Weinberg, R. A. (1994) *Curr. Biol.* **4,** 1–7.
28. Clarke, A. R., Cummings, M. C. & Harrison, D. J. (1995) *Oncogene* **11,** 1913–1920.

29. Luongo, C., Moser, A. R., Gledhill, S. & Dove, W. F. (1994) *Cancer Res.* **54,** 5947–5952.
30. Levy, D. B., Smith, K. J., Beazer-Barclay, Y., Hamilton, S. R., Vogelstein, B. & Kinzler, K. W. (1994) *Cancer Res.* **54,** 5953–5958.
31. Laird, P. W., Jackson-Grusby, L., Fazeli, A., Dickinson, S. L., Jung, W. E., Li, E., Weinberg, R. A. & Jaenisch, R. (1995) *Cell* **81,** 197–205.
32. Gordon, J. I. & Hermiston, M. L. (1994) *Curr. Opin. Cell. Biol.* **6,** 795–803.
33. Anilkumar, T. V., Sarraf, C. E., Hunt, T. & Alison, M. R. (1992) *Br. J. Cancer* **65,** 552–558.
34. Clarke, A. R., Gledhill, S., Hooper, M. L., Bird, C. C. & Wyllie, A. H. (1994) *Oncogene* **9,** 1767–1773.
35. Merritt, A. J., Potten, C. S., Kemp, C. J., Hickman, J. A., Balmain, A., Lane, D. P. & Hall, P. A. (1994) *Cancer Res.* **54,** 614–617.
36. Gavrieli, Y., Sherman, Y. & Ben-Sasson, S. A. (1992) *J. Cell Biol.* **119,** 493–501.
37. Earnshaw, W. C. (1995) *Curr. Opin. Cell. Biol.* **7,** 337–343.
38. Nigro, J. M., Baker, S. J., Preisinger, A. C., Jessup, J. M., Hostetter, R., Cleary, K., Bigner, S. H., Davidson, N., Baylin, S., Devilee, P., White, R. & Vogelstein, B. (1989) *Nature (London)* **342,** 705–708.
39. Hollstein, M., Sidransky, D., Vogelstein, B. & Harris, C. C. (1991) *Science* **253,** 49–53.
40. Baker, S. J., Fearon, E. R., Nigro, J. M., Hamilton, S. R., Preisinger, A. C., Jessup, J. M., van Tuinen, P., Ledbetter, D. H., Barker, D. F., Nakamura, Y., Glover, T., Collins, F. S., Weston, A., Modali, R., Harris, C. C. & Vogelstein, B. (1989) *Science* **244,** 217–21.
41. Baker, S. J., Preisinger, A. C., Jessup, J. M., Paraskeva, C., Markowitz, S., Willson, J. K., Hamilton, S. & Vogelstein, B. (1990) *Cancer Res.* **50,** 7717–7722.
42. Boland, C. R., Sato, J., Appelman, H. D., Bresalier, R. S. & Feinberg, A. P. (1995) *Nat. Med.* **1,** 902–909.
43. Lowe, S. W. & Ruley, H. E. (1993) *Genes Dev.* **7,** 535–545.
44. Kim, S. H., Roth, K. A., Moser, A. R. & Gordon, J. I. (1993) *J. Cell Biol.* **123,** 877–893.
45. Morgenbesser, S. D., Williams, B. O., Jacks, T. & De Pinho, R. A. (1994) *Nature (London)* **371,** 72–74.
46. Symonds, H., Krall, L., Remington, L., Saenz-Robles, M., Lowe, S., Jacks, T. & Van Dyke, T. (1994) *Cell* **78,** 703–711.
47. Howes, K. A., Ransom, N., Papermaster, D. S., Lasudry, J. G., Albert, D. M. & Windle, J. J. (1994) *Genes Dev.* **8,** 1300–1310.
48. Polyak, K., Waldman, T., He, T. C., Kinzler, K. W. & Vogelstein, B. (1996) *Genes Dev.* **10,** 1945–1952.
49. Rampino, N., Yamamoto, H., Ionov, Y., Li, Y., Sawai, H., Reed, J. C. & Perucho, M. (1997) *Science* **275,** 967–969.
50. Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D. & Lowe, S. W. (1997) *Cell* **88,** 593–602.
51. Waldman, T., Lengauer, C., Kinzler, K. W. & Vogelstein, B. (1996) *Nature (London)* **381,** 713–716.
52. Offerhaus, G. J., De Feyter, E. P., Cornelisse, C. J., Tersmette, K. W., Floyd, J., Kern, S. E., Vogelstein, B. & Hamilton, S. R. (1992) *Gastroenterology* **102,** 1612–1619.
53. Fazeli, A., Dickinson, S. L., Hermiston, M. L., Tighe, R. V., Steen, R. G., Small, C. G., Stoeckli, E. T., KeinoMasu, K., Masu, M., Rayburn, H., Simons, J., Bronson, R. T., Gordon, J. I., Tessier-Lavigne, M. & Weinberg, R. A. (1997) *Nature (London)* **386,** 796–804.
54. Zar, J. H. (1974) *Biostatistical Analysis* (Prentice–Hall, Englewood Cliffs, NJ).